Influence of culture conditions and preconditioning on survival of *Lactobacillus delbrueckii* subspecies *bulgaricus* ND02 during lyophilization

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ABSTRACT

The cryotolerance of *Lactobacillus delbrueckii* ssp. *bulgaricus* is weak during vacuum freeze-drying. Many factors affect cryoresistance of these bacteria, such as cryoprotectant composition, the lyophilization technology used, and the intrinsic characteristics of the bacteria. In this research, we explored the fermentation technology and other preconditioning treatments of cells in improving the cryoresistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* strains during lyophilization. The addition of yeast extract in the propagation medium exerted a negative effect on the cryotolerance of these bacteria and decreased survival during lyophilization. The count of the freeze-dried cells from medium containing a high level (4%) of yeast extract was only 4.1×10^9 cfu/g, indicating a death rate as high as 88%, compared with the culture medium without yeast extract, with a lower death rate of 44.7%. When *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02 was propagated in yeast extract-free de Man, Rogosa, and Sharpe broth at a set pH value of 5.1, the cells showed unexpectedly higher survival after freeze-drying. Viable counts of the lyophilized cell of strain ND02 cultivated at pH 5.1 could reach 1.05×10^{11} cfu/g and survival of the freeze-drying process was 68.3%, whereas at pH 5.7, survival was only 51.2%. We also examined the effects of pretreatment of cells on survival of the bacteria after vacuum freeze-drying. By analyzing the effect of pretreatment conditions on the expression of cold- and heat-shock genes, we established 2 pretreatments that improved survival of cells after lyophilization. Optimal fermentation conditions and pretreatment of the cell-cryoprotectant mixture at 10°C for 2 h or 37°C for 30 min improved the cryoresistance of 4 strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* to varying degrees. Cells of IMAU20269 and IMAU20291 that were pretreated showed enhanced survival of 16.06 and 16.82%, respectively, after lyophilization. Expres-

sion of cold- and heat-shock genes for pretreated strains ND02, IMAU80423, IMAU20269, and IMAU20291 was analyzed by using quantitative PCR. From the expression of 2 cold shock-induced genes (*cspA* and *cspB*) and 6 heat shock-induced genes (*groES*, *hsp*, *hsp20*, *hsp40*, *hsp60*, and *hsp70*), strain ND02 showed a higher relative quantity of gene expression and displayed superior resistance to cold-induced stress during the freeze-drying process.

Key words: *Lactobacillus delbrueckii* ssp. *bulgaricus* ND02, cryoresistance, cryotolerance

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* (hereafter referred to as *Lactobacillus bulgaricus*) is one of the most important bacteria in dairy fermentations. It is used as a component starter in the production of yogurt and Italian and Swiss variety cheeses. Yogurt is gaining popularity in China, and the demand for yogurt will continue to grow rapidly. More starter culture strains are needed in dairy industry for the manufacture of yogurt. Lyophilized starter cultures have several advantages over frozen cultures, such as economy in storage space, convenience in shipping, and lower energy cost for storage either at normal refrigeration temperatures or at ambient temperatures. In our laboratory, we are engaged in developing suitable technology for growing and preserving selected strains of lactic acid bacteria isolated from indigenous fermented dairy products for use in yogurt production. In industrial production of yogurt starters, *Lb. bulgaricus* strains are subjected to cold shock by preservative techniques such as frozen storage and vacuum freeze-drying. These techniques influence the quality and functionality of starter cultures and the fermented products made using them. Losses in viability and functionality have to be minimized to ensure efficient and successful manufacture of yogurt.

The survival and low temperature stability of starters obtained by vacuum freeze-drying are governed by several factors. They include the strain characteristic; fermentation conditions such as the composition of

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the culture medium, temperature, and pH control; and the neutralizer used. Other factors also affect the cryotolerance and cryoresistance of the bacteria. Streit et al. (2010) investigated the steps affecting the cryotolerance of *Lb. bulgaricus* CFL1. They found that the speed and duration of centrifugation affected the cryotolerance of the cell concentrate and that optimal conditions during centrifugation improved the cryostability of the cells. Other studies found that harvesting conditions, the acidity of the fermentation, postharvest handling of the concentrate such the rate of cooling, and washing of cell concentrate with different reagents all affected the cryostability of cultures (Fonseca et al., 2001; Saarela et al., 2005; Wang et al., 2005b; Streit et al., 2007). Additionally, the technology of lyophilization is also critical in ensuring cryostability, because vacuum freeze-drying is a complicated process. It is not just freezing the material being dried and carrying out sublimation under vacuum. The temperature of the frozen material should be increased carefully through a gradient over time, such that the eutectic barrier of the frozen substrate is not breached. The aim is to obtain a highly efficient sublimation aided by vacuum to obtain maximum cell survival. In regard to survival to freezing, Bâati et al. (2000) found that a gradual step-wise decrease in temperature through a range of 37°C, 20°C, -4°C, -20°C, and -80°C yielded greater survival of a strain of *Lactobacillus acidophilus* compared with direct one-step freezing at -80°C. Recent work has revealed that the cooling-thawing rate and preconditioning of the cells, which affect cryostress, could be optimized through an analysis of cold- and heat-shock proteins and expression of related genes. Several studies have investigated the cold- and heat-shock response and adaptation in *Escherichia coli* and *Bacillus subtilis* (Hecker and Völker, 1990; Jones et al., 1992; Willmsky et al., 1992; Bukau, 1993; Storz and Hengge-Aronis, 2000), but not much is known about such responses in *Lb. bulgaricus*. The family of cold-shock protein (*csp*) genes, designated *cspA*, *cspB*, *cspC*, *cspD*, and *cspE*, that encode very similar cold-shock proteins has been described in the lactic acid bacterium *Lactococcus lactis* MG1363 (Wouters et al., 1998). Komatsu et al. (1990) demonstrated that the enhanced induction of such genes and gene products improved the cryoresistance of bacteria. Hence, a better understanding of the responses and allied mechanisms in *Lb. bulgaricus* strains might contribute to greater cryostability of these industrially important bacteria during freezing and subsequent lyophilization. Analysis of different pretreatments of *Lb. bulgaricus* and the expression of cold- and heat-shock genes and gene products could provide a means to develop efficient cryostability of these bacteria.

In this study, we examined the effects of several factors on the cryostability of *Lb. bulgaricus* strains; namely, the nutrient composition of the propagation medium, the pH set point during propagation in a fermentor, and preconditioning of the cell crop at different time-temperature treatments and resultant induction and expression of *csp* and heat-shock protein (*hsp*) genes and gene products. Based on the results, we propose a set of optimized and reliable protocols for freezing, frozen storage, and lyophilization of *Lb. bulgaricus*. These protocols could be adapted to preserve other cryosensitive bacteria. The observations made in this study could be extended to developing procedures for successful industrial production of starter cultures for dairy and food industries.

MATERIALS AND METHODS

Bacterial Strains, Culture Media, and Growth Conditions

Lactobacillus bulgaricus strains ND02, IMAU80423, IMAU20269, and IMAU20291 used in this study were isolated from traditional naturally fermented milks made by Chinese minority groups living in different geographical areas in China. These strains were obtained from the culture collection at the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, China. Previous study in our laboratory has shown that strains ND02 and IMAU80423 have relatively higher cryostability during freeze-drying compared with strains IMAU20269 and IMAU20291 (Bao, 2012).

Strain ND02 was of special interest to us. This strain was isolated from naturally fermented yak milk in Qinghai province and was the predominant strain in the sample (Sun et al., 2010). Yogurt made with this strain was judged to be of excellent quality, displaying moderate acidity, high viscosity, and good water-holding capacity. Additionally, the whole-genome sequencing of this strain was completed in 2011. It had been adapted for industrial production for use as yogurt starter by the largest dairy group in China, the Inner Mongolia Yili Industrial Group (Sun et al., 2011a).

Routine Propagation of Strains

Lactobacillus bulgaricus strains were activated by growing in sterilized 10% (wt/vol) skim milk (121°C for 7 min). The activated cultures were propagated in de Man, Rogosa, and Sharpe (MRS) broth (sterilized at 121°C for 15 min) using a 1 to 2% inoculum and incubation at 37°C for 12 h.

Influence of Yeast Extract in MRS Broth Used to Grow Strain ND02 in the Fermentor

To study the effect of different concentrations of yeast extract in MRS broth on cryostability, *Lb. bulgaricus* ND02 was grown in MRS broths containing 0, 2, or 4% yeast extract. Sterile MRS broths containing variable levels of yeast extract were pumped into three 5-L fermentors (Eastbio, GUJS-5, Zhenjiang East Bio-tech Equipment and Technology Co. Ltd., Zhenjiang, China) installed in parallel. The initial pH was adjusted to 6.5, pH during fermentation was set at 5.7, and the fermentation temperature was controlled at 37°C. The fermentation parameters were electronically controlled and recorded in a computer attached to the fermentors. At the end of fermentation (determined by the cessation of neutralizer-25% ammonia water addition), samples were removed from individual fermentors to determine cell morphology using a photon microscope (DM4000B, Leica, Wetzlar, Germany). The remaining broths were individually harvested by centrifugation ($5,116 \times g$ for 10 min at 4°C). The sedimented cells were washed twice using buffer solution (0.8% NaCl, 0.02% KH_2PO_4 , and 0.115% Na_2PO_4 , wt/vol). The washed cells were mixed with a cryoprotective agent consisting of 10% skim milk fortified with 1% sodium glutamate in the ratio of 1:6. Cell counts of the cell-cryoprotective mixture were determined by plate count method. The mixtures were then frozen at -40°C and held for 2.5 h before freeze-drying. Bacterial counts on dried powders were made by plate counts (viable count; VC) and the survival of cells from the individual fermentations was calculated according to the following formula:

$$\text{Survival (\%)} = \frac{\text{VC of lyophilized powder} \times \text{Wt of lyophilized powder}}{\text{VC of cell protectant} \times \text{Wt of cell protectant}}$$

Relationship Between pH and Cryotolerance of Lyophilized *Lb. bulgaricus* ND02

Lactobacillus bulgaricus ND02 was activated as described earlier, and 2% of the activated culture was inoculated into sterile MRS broth and incubated at 37°C for 12 h. The fermentors (5-L capacity) installed in parallel, were designated as F1 and F2 and were filled with yeast extract-free MRS broth (3 L each). Each of the fermentors was inoculated with 30 mL of aforementioned MRS broth culture. The initial pH of both fermentors was adjusted to 6.5. The pH for F1 was set at 5.7 and for F2 at 5.1. The fermentation temperature was set at 37°C. The fermentation parameters were continuously monitored and recorded by the computer connected to the fermentors. The end of fermentation

was determined as described in the previous section. At the end of fermentation, samples from F1 and F2 were taken to examine the morphology of the cells under light microscope. The image of the bacteria was captured by software LAS V3.7 (Leica Microsystems Ltd., Heerbrugg, Switzerland) and the measurement of length of the cells was carried out using Leica QWin V3. The postfermentation operations (harvesting cells, washing cells, addition of cryoprotectants, sampling for plate counts, freezing, and lyophilization) were carried out as outlined in the previous section. The percentage survival of cells after freeze-drying was calculated using the same formula described in the previous section.

Assaying the Functionality of the Lyophilized Powders

To determine the functionality of the freeze-dried powders, acid-producing activity of the dried cultures in milk at standard conditions was measured. The powders were reconstituted in 0.8% (wt/vol) NaCl so that 1.0×10^7 cfu/mL could be added to sterile 10% skim milk. After incubating the inoculated milks at 42°C for 4 and 6 h, the pH and titratable acidity of the milks were recorded (Zhang and Zhang, 2007).

Cold- and Heat-Shock Pretreatment

Lactobacillus bulgaricus strains ND02, IMAU80423, IMAU20269, and IMAU20291 were activated as described earlier and grown in MRS broth until the cells reached the exponential growth phase. The cells were then harvested by centrifugation ($6,000 \times g$ at 4°C for 10 min). Harvested cells were washed thrice in buffer solution mentioned above. One-gram portions of the washed cells from each of the 4 strains were transferred into separate Eppendorf tubes in triplicate. The cell-loaded Eppendorf tubes were incubated at 4, 7, and 10°C for 2 h. After incubation, the samples were tested using real-time quantitative PCR (qPCR) for the determination of cold-shock induction of the *cspA* and *cspB* genes. The experiment was repeated twice.

To determine the heat-shock responses, 1.0 g of cells prepared as described earlier were transferred in triplicate into Eppendorf tubes and incubated for 30 min at 30, 37, and 45°C. The gene expression of *groES*, *hsp*, *hsp20*, *hsp40*, *hsp60*, and *hsp70* was evaluated by qPCR.

RNA Extraction and cDNA Synthesis

Total cellular RNA was extracted from cells treated at different conditions using RNAiso Plus (TaKaRa, Dalian, China) according to the manufacturer's instructions. Genomic DNA elimination was performed

Table 1. Primer information for cold- and heat-induced genes evaluated in the study

Primer ¹	Primer sequence (5' → 3')	Tm ² (°C)	Length (bp)	Gene function
<i>cspA</i> -F	GCTTTGGGTTTATCAC	41.0	16	Cold-induced genes
<i>cspA</i> -R	ATTAGTCGCTTGAGGT	42.8	16	
<i>cspB</i> -F	GCGAACCGGACTTGTA	47.8	16	
<i>cspB</i> -R	AGCCCTCCTCCTTGAT	46.8	16	
<i>groES</i> -F	AACTTCATCGCCCTCT	45.7	16	Heat-induced genes
<i>groES</i> -R	CAAACCTGTTGGTGGTAT	43.0	17	
<i>hsp</i> -F	TATCCCGCCTGGTCAA	47.9	16	
<i>hsp</i> -R	TACATCGCCGACAACC	47.9	16	
<i>hsp20</i> -F	TTGGCGCTAATGCCTTTC	50.4	18	
<i>hsp20</i> -R	GATCACATGGGCGACTGG	52.2	18	
<i>hsp40</i> -F	GGAATCACGACCTCAACCG	52.6	19	
<i>hsp40</i> -R	AGACCCAGAAGCAGACCCT	53.8	19	
<i>hsp60</i> -F	AGCGATGATTAGGAGTGAGC	51.4	20	
<i>hsp60</i> -R	TCGGCAAGGACGGTGTTA	52.8	18	
<i>hsp70</i> -F	GATGAAGACGACGAAACTG	48.4	19	Reference gene
<i>hsp70</i> -R	GGAGATGAAAGGAAGGGAG	48.4	19	
<i>gyrB</i> -F	TGCCAGCCAGATTCAA	46.8	16	
<i>gyrB</i> -R	ATGGTCCCGTGTTCACT	49.3	17	

¹F = forward, R = reverse.²Melting temperature.

in a 10-μL DNA elimination reaction solution containing 2 μL of 5 × gDNA Eraser Buffer (TaKaRa), 1 μL of gDNA Eraser (TaKaRa), 1 μL of total RNA (500 ng of total RNA), and 6 μL of RNase-free distilled H₂O. The DNA present in the total RNA was eliminated by heating the DNA elimination reaction solution at 42°C for 2 min. The purified RNA from each sample was reverse-transcribed with PrimeScript RT reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. Reverse transcriptions were performed in a 20-μL reverse transcription mixture containing 10 μL of total RNA (1 μg of total RNA, DNase-treated), 4 μL of 5 × PrimeScript Buffer 2 (TaKaRa), 1 μL of PrimeScript RT Enzyme Mix I (TaKaRa), 1 μL of RTPrimer Mix, and 4 μL of RNase-free distilled H₂O. Reverse transcription reaction conditions were 37°C for 15 min followed by 85°C for 5 s. The total RNA and cDNA concentrations were quantified by measuring the optical density at 260 nm (OD₂₆₀) and 280 nm (OD₂₈₀), respectively, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Quantitative PCR

The primer pairs for the qPCR were designed based on the whole genome of *Lb. bulgaricus* ND02 using the Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA; Table 1). Primers were synthesized by Sangni Biosciences Corporation (Shanghai, China).

All qPCR assays were carried out in a 48-well plate using StepOnePlus Real-Time PCR System (Applied

Biosystems, Carlsbad, CA) in accordance with the manufacturer's instructions. The qPCR reactions were carried out using SYBR Premix Ex Taq II (TaKaRa) and samples were tested in triplicate. The reactions were done in a 20-μL volume containing 10 μL of 2 × SYBR Premix Ex Taq, 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), 0.4 μL of 50 × ROX Reference Dye II, 2 μL of cDNA, and 6 μL of distilled H₂O. The cDNA concentrations were adjusted to approximately 100 ng/μL by adding distilled H₂O. Sterile ultrapure water was substituted for DNA in the negative control for detecting possible contamination. Relative normalized fluorescence change (ΔRn) was plotted against time (PCR cycle number) to facilitate real-time assessment of each PCR. For each sample, the amount of cold-shock induced genes *cspA* and *cspB* mRNA was quantified by real-time qPCR. The calculated cycle threshold (Ct) provides an arbitrary cut-off point at which a Ct value is assigned for the individual samples.

Statistical Analyses

Statistical analysis for the mean relative expression levels for the genes related to cold- and heat-shock genes was conducted using the StepOne software v2.2 (Applied Biosystems). Statistical analysis of the routine data was performed using Data Processing System (DPS) 6.55 (Zhejiang University and Hangzhou Rui Feng Information Technology Co. Ltd., Hangzhou, China). Figures presented in this paper were plotted using software GraphPad Prism 5.01 (GraphPad Inc., San Diego, CA). All results are expressed as means ± standard deviation (SD).

Table 2. Results of fermentations with pH controlled at 5.1 and 5.7

Item	pH 5.1	pH 5.7
Viable count of the fermented broth (cfu/mL)	4.3×10^9	8.9×10^9
Viable count of the lyophilized powder (cfu/g)	1.05×10^{11}	6.2×10^{10}
Survival of the freeze-drying process (%)	68.3	51.2
Consumption of neutralizer (mL)	80	110
Fermentation time (h)	11.5	7

RESULTS AND DISCUSSION

Influence of Yeast Extract on Survival of Cells During Vacuum Freeze-Drying

In this study, the higher level of yeast extract (4%) yielded a high viable count in the fermented broth (1.15×10^{10} cfu/mL) and a short fermentation time (6 h) for *Lactobacillus bulgaricus* ND02. However, the count of freeze-dried cells from medium containing a high level of yeast extract was only 4.1×10^9 cfu/g, indicating a death rate as high as 88%. In contrast, the culture medium without yeast extract yielded better survivability to freeze-drying. In spite of the lower viable count at the end of fermentation (7.95×10^9 cfu/mL), the viable count of cells in the freeze-dried powder was 5.7×10^{10} cfu/g, showing a higher survival of 55.3%. Additionally, the average length of *Lb. bulgaricus* ND02 cells was 2.04 μm (ranging from 1.26 to 2.94 μm) in yeast extract-free medium, whereas in medium containing yeast extract, cell length ranged from 3.5 to 6.22 μm , with an average of 4.54 μm .

Carvalho et al. (2004) noted that the nutrient composition of fermentation medium had a great effect on the survivability of lactic acid bacteria during freeze-drying. Wright and Klaenhammer (1981, 1983) showed that availability of Ca^{2+} played a significant role in the survivability of *Lb. acidophilus* and *Lb. bulgaricus* to freezing and freeze-drying. The authors also noted that lower pH facilitated the availability of Ca^{2+} . Furthermore, the presence of Mn^{2+} , sucrose, Tween-20, or Tween-80 had a positive effect on the cryostability of lactobacilli and other bacteria (Smittle et al., 1972; Fonseca et al., 2001; Carvalho et al., 2003; Li et al., 2012). The effect of lactose starvation on cryotolerance of *Lb. acidophilus* RD758 was investigated by Wang et al. (2011); they found greater cryotolerance in lactose-starved cells than cells grown with lactose.

Effect of Fermentation pH on the Cryotolerance of Lb. bulgaricus ND02

The summary of results of growing *Lactobacillus bulgaricus* ND02 at pH 5.7 and 5.1 is shown in Table 2. When the strain was grown in MRS broth in a fermenta-

tor set at a pH of 5.7, the viable count at the end of fermentation was 8.9×10^9 cfu/mL, twice the count obtained at a pH of 5.1. However, the survival of cells grown at pH 5.7 was lower than that of cells controlled at pH 5.1. Viable counts of the lyophilized powder of *Lb. bulgaricus* ND02 cultivated at pH 5.1 reached 1.05×10^{11} cfu/g, and the survival of the freeze-drying process was 68.3%, whereas at pH 5.7, survival was only 51.2%. This is significant in industrial production of *Lactobacillus bulgaricus* ND02, because greater survival will result in higher functionality (acid production in milk). Further, the amount of neutralizer consumed during fermentation was greater at pH 5.7. Probably because of the more acid environment encountered at pH 5.1, the growth of bacteria was slower and required more time to finish the fermentation.

Figures 1 and 2 depict micrographic images of *Lb. bulgaricus* ND02 cells grown at pH 5.1 and 5.7, respectively. Cells grown at the lower pH were shorter and more compact. Some cells displayed a curved and even semicircular and circular morphology. Occurrence of this phenomenon may be due to the over-acidic culture environment, which made the growth of bacteria react to the surrounding environmental conditions. Cells from the fermentation controlled at pH 5.7 were relatively longer and straighter. As observed in this experiment and in the comparison of cells grown in the absence of yeast extract versus those grown in the presence of yeast extract, we found a correlation between the length of the cells and cryostability: the shorter the cells, the greater their cryostability. We previously studied the response of the yogurt starter strain *Streptococcus thermophilus* ND03 to high-cell-density cultivation and vacuum freeze-drying. We found that the vacuum freeze-drying survival of *Streptococcus thermophilus* ND03 (Sun et al., 2011b) could reach 90 or 100% (Zhang, 2011). *Streptococcus thermophilus* is small and globular, a different morphology from the baculiform lactobacilli. Consequently, morphology is an influential factor for the survival of the bacteria.

Earlier reports have suggested that injury and lethality of cells during freezing could be brought about by ice crystal formation within cells, and formation of larger ice crystals could puncture the cell membrane, resulting in loss of semi-permeability and collapse of some the

Figure 1. Morphology of *Lactobacillus bulgaricus* ND02 cultivated at a constant pH of 5.1. Color version available in the online PDF.

cell membrane-associated proton-motive force (PMF) systems. It appears that long filamentous cells are more liable to such injury, whereas shorter, more compact cells are relatively resistant to such events. Several reports in the literature show that shorter, compact cells are relatively more resistant to freezing than longer, filamentous cells. Koch et al. (2008), working with a strain of *Lactobacillus delbrueckii* ssp. *lactis* FAM10991, which was sensitive to freezing and lyophilization, found that the strain grown at pH 5.0 exhibited 20- to 45-fold higher survival after freeze-drying than cells grown at pH 6.0. Palmfeldt and Hahn-Hägerdal (2000)

Figure 2. Morphology of *Lactobacillus bulgaricus* ND02 cultivated at a constant pH of 5.7. Color version available in the online PDF.

showed that cells of *Lactobacillus reuteri* ATCC 55730 grown at pH 5.0 had a cell dimension of 2 to 4 μm and frequently occurred in pairs, whereas cells grown at pH 6.0 were longer (4 to 15 μm long) and occurred as single cells. The same study reported that cells showing the shorter, more compact morphology (grown at pH 5.0), survived cryoprocessing much better than cells grown at pH 6.0, which were longer. Wang et al. (2005a) found that for good survival after freeze-drying, *Lb. acidophilus* RD758 had to be grown at 30°C and a pH of 5.0.

Other aspects of pH control have been investigated. Fonseca et al. (2001) reported that the use of NaOH as the neutralizer for constant pH control was preferable for a greater cryotolerance of lactic acid bacteria. In our research, we used NH_4OH as the neutralizer to validate its suitability.

Profiles of Cold Shock-Induced Genes *cspA* and *cspB*

Quantitative PCR was conducted to investigate the effect of the cold-shock pretreatment at different temperatures on the expression of *csp* genes of 4 different *Lb. bulgaricus* strains. The qPCR amplification plots are shown in Figure 3. The relative expression levels of *cspA* of *Lb. bulgaricus* ND02 after cold pretreatment at 10°C showed a 1.7-fold increment over that after 4°C treatment. The expression of *cspA* after 10°C pretreatment exhibited the greatest increase. However, at higher cold-shock treatment temperatures of 15 and 18°C, the relative quantity of gene expression (**RQ values**) decreased to 0.51221 and 0.46188, respectively (not shown in figure). All the other strains used in this study showed similar trends for pretreatment temperatures examined. Of the 4 strains tested, strains ND02 and IMAU80423 recorded higher RQ values. These 2 strains also gave higher cryostability in an earlier study (Bao, 2012) and in the present study.

Similar variations in the trends of the expression of *cspB* with cold-shock pretreatment were found for all 4 strains (Figure 4). All strains responded favorably to pretreatment at 10°C for 2 h. Observations made in these experiments revealed that for high expression of both *csp* genes, pretreatment at 10°C for 2 h is necessary.

Profiles of Heat Shock-Induced Genes

Expression of heat shock-induced genes for the 4 strains was examined using pretreatment at 30, 37, and 45°C for 30 min. The trends of RQ values for 4 strains of the 6 *hsp* genes (*groES*, *hsp*, *hsp20*, *hsp40*, *hsp60*, and *hsp70*) are depicted in Figure 5. The expression of *hsp* and *hsp60* genes showed similar patterns for all 4

Figure 3. Expression of cold-shock gene *cspA* in 4 *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, IMAU20291) with different pretreatment temperatures. RQ value = relative quantity of expression of cold-shock gene *cspA* in 4 *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, and IMAU20291) with different pretreatment temperatures. Color version available in the online PDF.

strains. No *hsp40* and *hsp70* genes were expressed by *Lb. bulgaricus* IMAU20269, and we observed no expression of *hsp70* gene by strain IMAU80423. In general, 37°C pretreatment yielded higher responses for the following genes: *hsp*, *hsp40*, *hsp60*, and *hsp70*. In strains ND02 and IMAU20291, the expression of *groES* was greater following 45°C pretreatment. The expression of *hsp20* by strain IMAU20269 was higher compared with other strains at 45°C. Of the 4 *Lb. bulgaricus* strains tested, strain ND02 exhibited relatively higher heat-shock gene expression with pretreatment at 37°C for 30 min. This was reflected in greater survivability of strain ND02 after freeze-drying (Tables 3 and 4). Broadbent and Lin (1999) reported that heat-shocking at 42°C for 25 min increased the resistance to freezing in *Lactococcus lactis* ssp. *lactis* and heat-shocking at 39°C for 25 min boosted the cryotolerance of *Lactococcus lactis* ssp. *cremoris*. An increase in the cryoresistance of the subspecies *lactis* and *cremoris* was achieved by cold-shock treatment at 10°C for 2 h. Fiocco et al. (2007) found that overproduction of heat-shock protein improved the growth of *Lactobacillus plantarum* at 37°C and 40°C.

Three species of *Lactobacillus* were investigated by characterizing their heat responses at 30°C and 37°C after an incubation of 20 min, and thermo-tolerance experiments showed that pretreatment improved the ability of 3 *Lactobacillus* strains to withstand a 20-min higher temperature (Broadbent et al., 1997).

Comparative Evaluation of Survival of Untreated and Pretreated Cells of 4 *Lb. bulgaricus* Strains to Freeze-Drying

Using the data obtained in this study for optimal propagation of the 4 *Lb. bulgaricus* strains (without yeast extract in the propagation medium and controlling pH at 5.1 during fermentation), cells pretreated at 10°C for 2 h and 37°C for 30 min (optimum treatments for the expression of *csp* and *hsp* genes) were subjected to freeze-drying. In parallel, cells similarly propagated but without any pretreatment were lyophilized. Using the formula for determining the percentage survival as described in the Methods section, we evaluated the survival of the 2 cell crops. The methodology is summa-

Figure 4. Expression of cold-shock gene *cspB* in 4 *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, IMAU20291) with different pretreatment temperatures. RQ value = relative quantity of expression of cold-shock gene *cspB* in 4 *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, and IMAU20291) with different pretreatment temperatures. Color version available in the online PDF.

Figure 5. Expression of heat-shock genes *groES*, *hsp*, *hsp20*, *hsp60*, *hsp40*, and *hsp70* (A to F, respectively) of 4 different *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, IMAU20291). RQ value = relative quantity of expression of heat-shock genes *groES*, *hsp*, *hsp20*, *hsp60*, *hsp40*, and *hsp70* (A to F, respectively) in 4 *Lactobacillus bulgaricus* strains (ND02, IMAU80423, IMAU20269, and IMAU20291) with different pretreatment temperatures. Color version available in the online PDF.

Table 3. Experimental protocol used to compare untreated versus treated cells

Technology	No pretreatment	10°C pretreatment	37°C pretreatment
Culture medium	de Man, Rogosa, and Sharpe without yeast extract		
Fermentation temperature		37°C	
Initial pH value		6.5	
Constant pH value		5.1	
Pretreatment	None	10°C for 2 h	37°C for 30 min
Freezing before lyophilization		−40°C for 2.5 h	

rized in Table 3 and the results of the survivability after lyophilization are shown in Table 4. From the results presented in Table 4, it can be seen that pretreatment of cells resulted in greater survival after freeze-drying across the board. However, the greatest increment in survival was noted with strains IMAU20269 and IMAU20291, with pretreatment at 10°C for 2 h and at 37°C for 30 min. The overall results indicated that pretreatment of cells before freeze-drying protected them from stress generated during the drying process.

Evaluating the Functionality of Pretreated *Lb. bulgaricus* Cells After Freeze-Drying

Merely obtaining better survival after freeze-drying of pretreated cells was not the goal in this study. Loss or impairment of functionality in freeze-dried pretreated cells would not be of much of a gain in developing this technology. Therefore, cells of the 4 *Lb. bulgaricus* strains lyophilized without pretreatment were evaluated against pretreated cells for acid production in milk under standard incubation conditions. The results are presented in Table 5. The acid-producing activity in milk was not decreased or impaired by pretreatment. We observed no significant differences between the functionality of untreated and treated freeze-dried cells. Additionally, we found no differences between the treated and untreated cells with respect to body (viscosity), texture (smoothness), water-holding capacity, or flavor (mildness) of the fermented product. Several similar studies were previously reported. *Lactobacillus*

plantarum strain WCFS1 was studied to detect the effect of heat shock on overproduction of the heat-shock proteins. After 2 heat treatments of 37°C and 40°C in a water bath, the functionality of the pretreated strains were almost unaffected and no significant differences were observed between the strains (Fiocco et al., 2007). Prasad et al. (2003) found that *Lactobacillus rhamnosus* HN001, when prestressed at 50°C, showed significant improvement in viability.

CONCLUSIONS

This research was designed to improve the survival of *Lactobacillus bulgaricus* strains after freeze-drying. The techniques developed will benefit commercial production of starter strains for large-scale manufacture of yogurt and high-cook cheese such as Swiss cheese varieties and Italian cheeses. With the growing popularity of yogurt in China, the need for reliable *Lb. bulgaricus*-containing starters will be important. The production of superior starter cultures consists of a series of complex steps, and it is necessary that every step in the production must be considered. The extension of these findings to other *Lb. bulgaricus* strains of differing phage susceptibilities and pairing with compatible *Streptococcus thermophilus* strains would provide a series of starters that could be used in rotation to manage phage-related problems in the yogurt and cheese industry. This paper has provided some guidelines in the production of high-quality starters and should stimulate further research in this area.

Table 4. Survival (% ± SD) of *Lactobacillus bulgaricus* after freeze-drying untreated and treated cells

Strain	No pretreatment	10°C pretreatment		37°C pretreatment	
		Survival	ΔR ¹	Survival	ΔR
ND02	71.70 ± 1.00 ^a	79.81 ± 0.81 ^a	8.11 ± 0.31 ^b	77.32 ± 0.44 ^a	5.62 ± 0.70 ^b
IMAU80423	60.50 ± 2.61 ^b	70.02 ± 1.64 ^b	9.52 ± 1.02 ^b	65.32 ± 2.18 ^b	4.82 ± 0.44 ^c
IMAU20269	45.83 ± 1.15 ^c	61.89 ± 0.82 ^c	16.06 ± 0.42 ^a	58.88 ± 0.15 ^c	13.05 ± 1.01 ^a
IMAU20291	46.72 ± 0.39 ^c	63.54 ± 1.32 ^c	16.82 ± 0.93 ^a	60.06 ± 0.24 ^c	13.48 ± 0.38 ^a

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

¹ΔR = difference of survival compared with no pretreatment.

Table 5. Functionality [pH and titratable acidity (TA; $\pm$ SD)] of *Lactobacillus bulgaricus* cells with and without pretreatment

Freeze-drying method	Strain	4 h		6 h	
		pH	TA ($^{\circ}$ T)	pH	TA ($^{\circ}$ T)
No pretreatment	ND02	5.16	46.89 $\pm$ 2.72	4.67	60.71 $\pm$ 1.60
	IMAU80423	5.22	44.10 $\pm$ 3.11	4.74	56.29 $\pm$ 0.78
	IMAU20269	5.32	41.76 $\pm$ 4.89	4.81	52.78 $\pm$ 3.54
	IMAU20291	5.38	40.23 $\pm$ 1.22	4.89	52.73 $\pm$ 4.83
10 $^{\circ}$ C pretreatment	ND02	5.14	47.76 $\pm$ 1.18	4.62	61.27 $\pm$ 3.01
	IMAU80423	5.23	43.95 $\pm$ 3.51	4.69	58.73 $\pm$ 2.16
	IMAU20269	5.33	43.10 $\pm$ 1.58	4.77	55.28 $\pm$ 1.00
	IMAU20291	5.4	40.46 $\pm$ 2.02	4.85	52.62 $\pm$ 3.39
37 $^{\circ}$ C pretreatment	ND02	5.1	48.01 $\pm$ 3.79	4.68	59.69 $\pm$ 0.94
	IMAU80423	5.15	46.51 $\pm$ 1.34	4.71	55.99 $\pm$ 1.26
	IMAU20269	5.21	44.55 $\pm$ 2.06	4.78	54.33 $\pm$ 1.00
	IMAU20291	5.34	42.32 $\pm$ 0.88	4.9	53.01 $\pm$ 3.56

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