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Research Paper

NaCl stress impact on the key enzymes in glycolysis from *Lactobacillus bulgaricus* during freeze-drying

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Abstract

The viability of *Lactobacillus bulgaricus* in freeze-drying is of significant commercial interest to dairy industries. In the study, *L. bulgaricus* demonstrated a significantly improved ($p < 0.05$) survival rate during freeze-drying when subjected to a pre-stressed period under the conditions of 2% (w/v) NaCl for 2 h in the late growth phase. The main energy source for the life activity of lactic acid bacteria is related to the glycolytic pathway. To investigate the phenomenon of this stress-related viability improvement in *L. bulgaricus*, the activities and corresponding genes of key enzymes in glycolysis during 2% NaCl stress were studied. NaCl stress significantly enhanced ($p < 0.05$) glucose utilization. The activities of glycolytic enzymes (phosphofructokinase, pyruvate kinase, and lactate dehydrogenase) decreased during freeze-drying, and NaCl stress were found to improve activities of these enzymes before and after freeze-drying. However, a transcriptional analysis of the corresponding genes suggested that the effect of NaCl stress on the expression of the *pfk2* gene was not obvious. The increased survival of freeze-dried cells of *L. bulgaricus* under NaCl stress might be due to changes in only the activity or translation level of these enzymes in different environmental conditions but have no relation to their mRNA transcription level.

Key words: *Lactobacillus bulgaricus*, freeze-drying, NaCl stress, semi-quantitative RT-PCR, glycolysis.

Introduction

Compared with traditional liquid cultures, freeze-dried cultures have many advantages, including their low volume, convenience for transportation and storage, and ease of use. However, freeze-drying can cause many types of damage to cells, including a loss of viability, reduction of metabolic activity, and changes in cell morphology, which can affect the physiology and characterization of lactic acid bacteria (LAB), fermentation efficiency and useful functions (Castro *et al.*, 1997; Conrad *et al.*, 2000; Giulio *et al.*, 2005; Martos *et al.*, 2007). Previous studies found that sub-lethal stress can improve the survival rate and activity of freeze-dried LAB (Prasad *et al.*, 2003; Wang *et al.*, 2005b). It has also been shown that by adding an electrolyte (NaCl) to the medium, the survival rate of *Lactobacillus bulgaricus* was significantly different in a dry state, and supplementing the growth medium with NaCl markedly increased viability of dried cells (Carvalho *et al.*, 2003). The use of 2.5% (w/v) NaCl significantly enhanced ($p < 0.05$) the survival of freeze-dried *L. delbrueckii* subsp. *lactis*, and cultures grown with NaCl at 2.5% exhibited high yields of viable cells in broths before and after freeze-drying (Koch *et al.*, 2007). In the changing environment, LAB could enhance their resistance to external conditions through changes to their metabolism. *L. bulgaricus* participates in homofermentation, which mainly occurs via the glycolytic pathway (Fraenkel, 1992; Kandler, 1983). The glycolytic pathway is the main energy source for the biological activity of lactic acid bacteria. The activity of key metabolic enzymes is an important indicator for evaluating the rate of glycolysis. During sugar glycolysis, the key enzymes that are regulated include the following: phosphofructokinase (PFK, EC 2.7.1.11), pyruvate kinase (PK, EC 2.7.1.40),

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lactate dehydrogenase (LDH, EC 1.1.1.28) and the proteases annotated by *las* operon genes (Viana *et al.*, 2005).

In this study, we used the stress caused by the presence of 2% NaCl to improve the viability of freeze-dried *L. delbrueckii* subsp. *bulgaricus* ATCC 11842. The effect of this NaCl-induced stress on the glucose metabolism activity of *L. bulgaricus* during freeze-drying was mainly investigated by assessing the intracellular PFK, PK and LDH activity and the mRNA expression level of these enzymes, before and after freeze-drying.

Materials and Methods

Bacterial strain and culture conditions

L. bulgaricus ATCC 11842 was obtained from the American Type Culture Collection (ATCC), subcultured three times in de Man, Rogosa and Sharpe medium (MRS) and then maintained as frozen stock in 40% (v/v) glycerol at -80 °C. For use in experiments, the strain was cultured in MRS broth at 37 °C for 14 h, up to 10^8 cfu mL^{-1}.

NaCl stress

Sterilized, saturated NaCl solution was slowly added to the late growth phase (13.5 h) of 5 L MRS cultures of *L. bulgaricus* at 37 °C with stirring at 100 rpm to achieve final concentrations of 2.0% (w/v). *L. bulgaricus* was further incubated for 2 h at the same temperature. The control culture was incubated for 15.5 h without NaCl stress at the same growth conditions.

Cell preparation for freeze-drying

The culture was harvested by centrifugation at 11,000 *g* for 15 min at 4 °C. Next, the pellet was washed twice with distilled water and then was suspended in cryoprotective agent of 3-fold volume. The composition of the cryoprotective agent was 12% (w/v) skim milk, 5% (w/v) sucrose and 5% (v/v) glycerol. The cryoprotective agent was sterilized at 115 °C for 15 min. The mixture was pre-frozen for 12 h in a -80 °C refrigerator. Freeze-drying was performed in a freeze-drier (CHRIST, Alpha 1-2/LD plus, Osterode, Germany). The sample was pre-frozen at -80 °C for 12 h before lyophilization. Initially, freezing was performed at a rate of 5 °C min^{-1} until reaching -40 °C. After freezing, the vacuum was reduced to 13.3 Pa, and then the shelf temperature was raised to -20 °C. Secondary drying was performed step-wise to 30 °C for a total of 16 h. Subsequently, the vials were sealed at 13.3 Pa and then analyzed on the same day.

Rehydration was performed within 2 h after freeze-drying by adding membrane filtered water at ambient temperature to the same volume as before freeze-drying. The cell viability was determined by ten-fold serial dilutions and by plating of 10 µL onto MRS agar plates. The plates were incubated at 37 °C for 48 h, and 10-30 colony forming units (CFU) in three replicates were counted. Survival is reported as the ratio between cell counts before freeze-drying and after freeze-drying and given as percentage values.

Glucose assay

The treatment sample and the control sample were centrifuged at 11,000 *g* for 15 min at 4. An aliquot of 10 mL supernatant of each sample was removed and mixed with 0.5 mL lead acetate solution, followed by adding water to the 20 mL level. The mixture was placed for 10 min at room temperature and then filtered to remove protein by ultrafiltration membrane (UEOS.503, Tianjin motian membrane, China); when its retention molecular weight reached 6 kDa, the filtrate was used for further analysis. The utilization rate of glucose was measured by GOD-POD kit (Shanghai, China). A volume of 0.4 mL of glucose standard solution or sample filtrate was added to 3 mL of mycophenolate mixture and then placed into a water bath for 15 min at 37 °C; subsequently, the optical density was measured at 505 nm.

Protein extraction

A volume of 40 mL of *L. bulgaricus* cells before freeze-drying was collected at 12,000 *g* for 15 min at 4 °C, and then the pellet was washed twice with PBS. 1 mL of cell lysate suspension (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 0.5% TritonX-100, pH 8.5~9.0, 100 µg mL^{-1} lysozyme, 1 µL mL^{-1} PMSF) was added into the pellet and 0.1 g of freeze-dried *L. bulgaricus*. Cell-free extracts were obtained by ultrasonic agitation for 15 min at 300 W (working time: interval time = 1:3) using an ultrasonic cell disruption apparatus (JY92-IIDN, China) in an ice bath, followed by centrifugation at 12,000 *g* for 10 min at 4 °C. The protein concentration was measured using a BCA protein assay kit (Beyotime, China) according to the manufacturer's instructions. The final concentration of 0.5 mg/mL protein standard was prepared and then added to 96-well plates at the volumes of 0, 1, 2, 4, 8, 12, 16, and 20 µL. The total volume of the samples was brought up to 20 µL with PBS. Then, 10 µL of dilute samples with PBS were added to 96-well plates and brought up to 20 µL with PBS. Finally, 200 µL BCA working liquid was added to each well, and the mixture was incubated at 37 °C for 30 min before the absorbance was measured at 562 nm by a Model 680 microplate reader (BIO-RAD, Japan). The protein concentration of the sample was calculated according to the standard curve.

Enzyme activity analysis

The activity of PFK was assayed by the modified method by Sudo *et al.* (2000) using a chromatographic column of 5 µm x 4.6 mm x 150 mm (Waters 2695, America) (Sudo *et al.*, 2000). The chromatographic column conditions were as follows: the mobile phase was 50 mM potassium phosphate buffer (pH 6.5), flow rate, 0.7 mL min^{-1}

column temperature, room temperature; detection wavelength, 254 nm; and injection volume, 20 μL. For the enzyme activity assay solution, 1 mL contained 50 mM Tris-HCl, (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM fructose-6-phosphate (F-6-P), and 0.2 mM ATP. A solution of 0.5 mL of cell-free extracts was added as a start reaction reagent. Samples were placed in a water bath for reaction for 20 min at 30 °C and then treated for 3 min in boiling water bath to terminate the reaction. One unit of activity is defined as the amount of enzyme required to generate 1 μg ADP per min at 30 °C.

PK was measured as described by Bourniquel *et al.* (2005), with minimal modifications. A solution of 0.5 mL of cell-free extracts was added to the mixture, including 0.1 mM 1,6-diphosphate, 0.1 mM phosphoenolpyruvate, 0.1 mM ADP, 2 mM NADH and 4 U of lactate dehydrogenase. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol NADH per minute at 37 °C. The NADH concentration was determined by measuring the optical density at 340 nm.

LDH was determined by the modified method based on that of Engstrom *et al.* (2007). One milliliter of buffer solution included 50 mM Tris, 1 mM EDTA, 1 mM NADH and 1.2 mM sodium pyruvate (pH 7.4). A solution of 0.5 mL cell-free extracts was added as the starting reaction reagent. The change of the reaction was monitored via the absorbance at 340 nm (A) at 25 °C, and the reduction in the value of A per minute (ΔA) was calculated. One unit of activity is defined as the amount of enzyme required to oxidize 1 μmol NADH per minute at 25 °C.

Transcription assay

According to the relevant GenBank sequence (NC_008054.1), PCR primers were designed by Primer Premier 5.0 and were synthesized by Beijing sunbiotech Co., Ltd (primers shown in Table 1).

An aliquot of 1 mL of *L. bulgaricus* samples was reacted with 100 μL 2% (w/v) lysozyme for 20 min and then briefly centrifuged to collect the cell pellet. The total RNA was extracted from *L. bulgaricus* cells cultured in MRS using the RNeasy Midi Kit (Qiagen) and then treated with DNase. The RNA was stored at -70 °C. Reverse transcription analysis was performed using the Reverse Transcription Kit Quantscript RT Kit (TIANGEN). The reaction system was composed of 2 μL of 10 x RT mix, 2 μL of the dNTP mixture (2.5 mM), 2 μL of Oligo-dT15 (10 μM), 1 μL of Quant Reverse Transcriptase, 3 μL of RNase-free water, and 10 μL of template RNA. The mixture was treated for 60 min at 37 °C. The ± were stored at 20 °C. The thermal cycling conditions of PCR amplification were designated as follows: initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 30 s, and then annealing at 57 °C for 30 s, followed by 30 cycles at 72 °C for 30 s, with a final extension at 72 °C for 10 min. The PCR products were stored at -20 °C.

Results and discussion

Effect of NaCl stress on freeze-drying survival

The growth status of *L. bulgaricus* in MRS medium was determined without adding NaCl at 600 nm. *L. bulgaricus* enters the logarithmic growth phase at 5 h, the logarithmic growth stage lasts for approximately 11 h, and then the stable phase appears at approximately 16 h. Due to the differences of metabolic activity and protein synthesis, different growth stages of cells could exhibit different resistances to NaCl stress. In this study, culturing for 13.5 h was selected to ensure that *L. bulgaricus* entered the late growth phase before adding exogenous NaCl.

Applying 2% NaCl stress for 2 h significantly enhanced ($p < 0.05$) the survival of freeze-dried *L. bulgaricus*. The survival rate of *L. bulgaricus* increased 1.45-fold compared with the control.

Glucose analysis

Table 2 indicates that glucose utilization significantly increased by NaCl stress in *L. bulgaricus*. The glucose utilization for the control sample and the treatment sample was 57.32% and 62.27%, respectively ($p < 0.01$). The differ-

Table 1 - Oligonucleotides used in PCR amplifications.

Protein	gene	Primer	Product (bp)
GAPDH	*gap*	F: 5'-TATGTTGGCTCACCTGTT-3'	432
		R: 5'-GCAGTGTAAGCGTGGAT-3'	
PK	*pyk*	F: 5'-ACACGGTTTCGTTAAGGATGG-3'	337
		R: 5'-AACAACTGGAATGCCGAGTGA-3'	
PFK-1	*pfk1*	F: 5'-GCTTCTAGCCACCACCG-3'	203
		R: 5'-ACGCCTTCAGCAACAACT-3'	
PFK-2	*pfk2*	F: 5'-GGCTTCACGGTCATTGC-3'	325
		R: 5'-CTTCCAGGGCGATAGAGTC-3'	

F: Forward oligonucleotide, R: Reverse oligonucleotide.

Table 2 - The glucose utilization rate of the control and the NaCl treated *L. bulgaricus*.

	Control	2% NaCl 2 h^{-1}
Optical density (A_{505})	0.1978	0.1751
Glucose concentration (mmol/L)	47.38 ± 1.03	41.88 ± 1.53
Glucose utilization (%)	57.32	62.27**

**The results are significantly different ($p < 0.01$).

ence may be caused by a difference in the glucose metabolism rate.

Enzyme activity analysis

The self-protection of LAB in adverse environments led to physiological changes in the cells: the activity levels of PK, phosphoglycerate kinase and LDH of *Lactococcus lactis* changed in the acid tolerance response (Budin-Vemeuil *et al.*, 2005). Viana indicated the key enzymes related to these physiological changes, including PFK, PK, LDH and protease annotated by *las* operon genes in glycolysis (Viana *et al.*, 2005). In this study, the changes in intracellular PFK, PK and LDH activities in *L. bulgaricus* caused by NaCl stress were studied before and after freeze-drying.

The intracellular PK, PFK, and LDH activities of the control sample after freeze-drying were lower compared with their relative values before freeze-drying (Table 3): PFK, PK and LDH activities were decreased relative to their previous levels by 68.71%, 55.81% and 70.19% after freeze-drying, respectively, indicating that the freeze-drying procedure affected the normal physiological metabolism of intracellular enzymes, reduced the enzyme activity, and damaged the physiological metabolism of cells. This effect of freeze-drying on the enzyme may be caused by cell membrane damage, which leads to leakage of the intracellular part of the enzymes into the extracellular part of the cell.

PFK is a rate-limiting enzyme of glycolysis (Viana *et al.*, 2005). The results from Table 3 indicate that the PFK activity before freeze-drying was 1.4095 U/mg for the control sample and that its activity increased by 37.37% for the treatment sample by NaCl stress ($p < 0.01$). The PFK activity after freeze-drying reduced to 0.9685 U/mg for the control, and its activity increased by 31.40% for the NaCl stress-treated sample ($p < 0.01$). PFK was found to directly affect the survival of cells during freezing, which is consistent with the research results of Marceau *et al.* (2004).

PK is the key enzyme to regulate carbon metabolism (Ramos *et al.*, 2004). Compared with the control, the PK activity of the cells increased by 165.4% as a result of the application of NaCl stress on the culture prior to performing freeze-drying of the cells. Wang *et al.* confirmed that in several *Lactobacillus* species, intracellular PK is a cold-induced protein (CIP) (Wang *et al.*, 2005a, 2005b). Thus, the increasing activity of PK has great significance for improving the survival rate after freeze-drying cells.

LDH is a key enzyme for lactic acid production during the metabolism of LAB. During homofermentation and heterofermentation, LDH catalyzes pyruvate, resulting in the final product of lactic acid. Therefore, monitoring the change of LDH activity is also important. The activity of LDH after freeze-drying increased by 1.178-fold due to NaCl stress compared with the control.

Some stress responses are characterized by the transient induction of general and specific proteins and by physiological changes that generally enhance an organism's ability to withstand more adverse environmental conditions (Ang *et al.*, 1991). In this study, the freeze-drying procedure decreased the activities of key enzymes in glucose metabolism, but NaCl stress could markedly prevent their downward trend. NaCl stress regulated the metabolic pathway of cells through altering of the activities of the key enzymes, thus improving the resistance to freeze-drying of *L. bulgaricus*. Some literature reports indicated that the survival rate for freeze-drying may be related to the accumulation of glycine-betaine, and they found this compatible solute in *L. plantarum* (Glaasker *et al.*, 1998), *L. rhamnosus* (Prasad *et al.*, 2003), *L. acidophilus* (Hutkins *et al.*, 1987), *L. casei* (Piuri *et al.*, 2005) and *L. lactis* (Van der Heide *et al.*, 2000); however, this solute was not detected in the case of NaCl stress in this study. Kets *et al.* (1994) and Hutkins *et al.* (1987) confirmed that *L. bulgaricus* cannot accumulate glycine-betaine, based on high performance liquid chromatography analysis.

Transcriptional analysis

In this study, NaCl stress affected significantly the enzyme activity, and expression of the genes encoding the

Table 3 - The changes in the activities of key enzymes in *L. bulgaricus* before and after freeze-drying.

Enzyme	Before freeze-drying (U mg^{-1}protein)		After freeze-drying (U mg^{-1}protein)	
	Control	2% NaCl	Control	2% NaCl
PFK	1.4095 ± 0.0155	1.9363 ± 0.0406**	0.9685 ± 0.0126	1.2726 ± 0.0259**
PK	0.0133 ± 0.0036	0.0353 ± 0.0034*	0.0074 ± 0.0001	0.0123 ± 0.0006**
LDH[b]	0.0104 ± 0.0002	0.0134 ± 0.0013*	0.0073 ± 0.0003	0.0086 ± 0.0003**

*The results are significantly different ($p < 0.05$). ** The results are significantly different ($p < 0.01$).

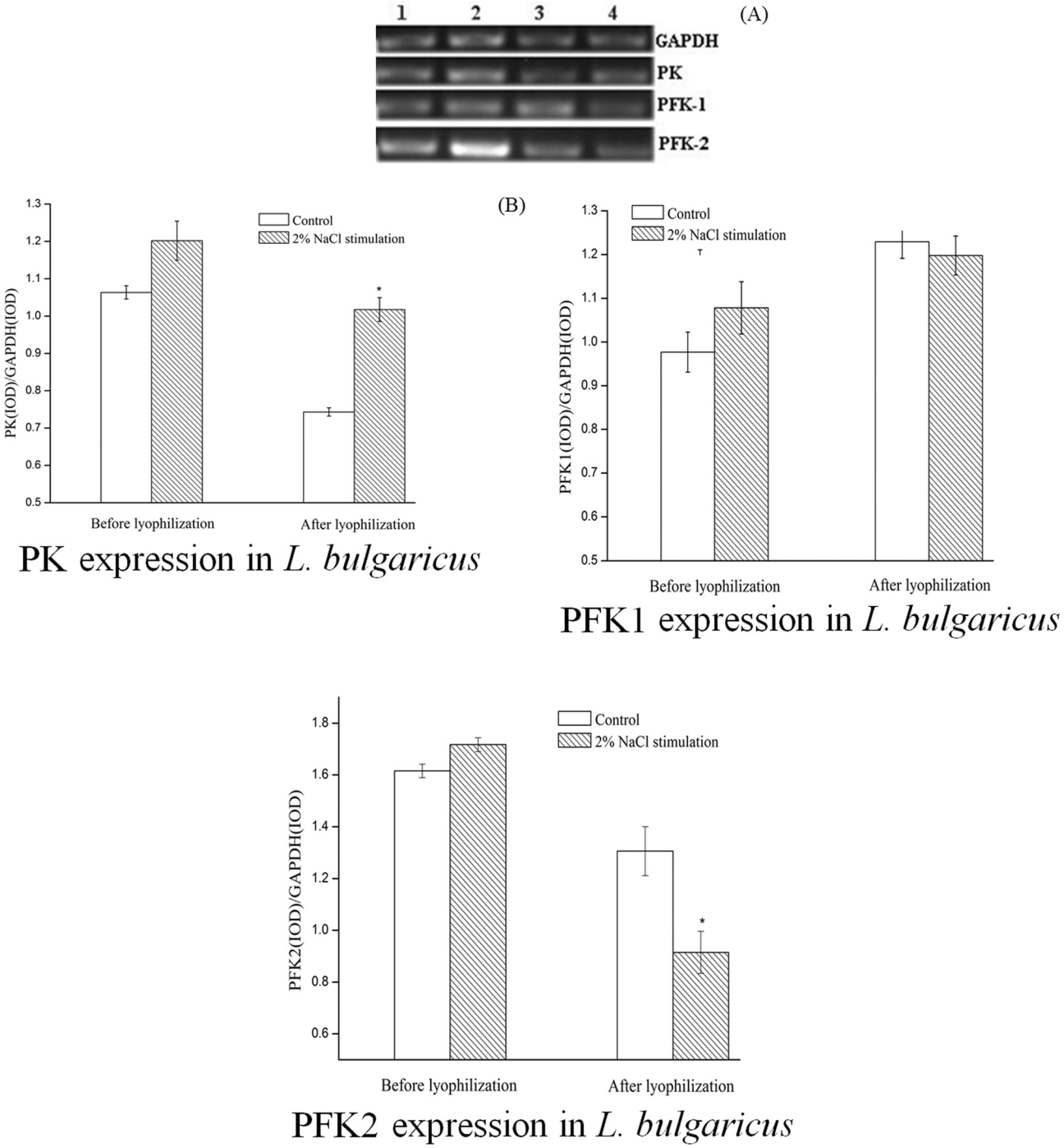

Figure 1 - (A) Electrophoregram of the RT-PCR products. Line 1, the control sample before freeze-drying. Line 2, 2% NaCl treatment sample before freeze-drying. Line 3, the control sample after freeze-drying. Line 4, 2% NaCl treatment sample after freeze-drying. (B) Gray analysis of electropherograms.

key enzyme in glycolysis (Figure 1). Compared with the control sample, NaCl stress increased the expression of *pyk* before and after freeze-drying, which was related to the function of PK encoded by *pyk*. PK is not only a key enzyme in the glycolytic pathway but also an important enzyme for physiology function regulation. Wang *et al.* (2005b) confirmed that PK of several *Lactobacillus* is a CIP protein and plays a regulation role for cold conditions during freeze-drying. Compared with the control group, the expression of *pyk* is 1.13-fold ($p > 0.05$) before freeze-drying and 1.37-fold ($p < 0.05$) after freeze-drying under NaCl stress. In many microorganisms, the glycolytic flux depends on the activities of 6-phosphofructokinase (*pfk*) and pyruvate kinase (*pyk*), and the genes of *pfk* and *pyk* are

clustered and cotranscribed in *L. bulgaricus* (Branny *et al.*, 1993; Viana *et al.*, 2005). The overexpression of *pyk* by NaCl stress caused high resistance for *L. bulgaricus* to the freeze-drying environment in this study. The results indicated a higher glycolytic flux under salt stress for *L. bulgaricus*, which can resist adverse environments by regulating the transcription level of mRNA. Carvalho *et al.* (2013) also found that *pyk* was overexpressed more than 2-fold in cells grown under acid stress. However, He *et al.* (2012) researched the molecular mechanism involved in the tolerance and adaptation of ethanologenic strains to ethanol stress and found that *pyk* expression was downregulated. These results demonstrated that *pyk* from different strains exhibited different responses to different types of stress.

Although *pfk1* expression under NaCl stress increased 1.25-fold before the freeze-drying process, the expression of *pfk1* was not clear ($p > 0.05$) after freeze-drying was completed, compared with the control sample. The expression of *pfk2* under NaCl stress increased 1.06-fold before freeze-drying, but *pfk2* expression was significantly reduced for the NaCl-stressed sample after freeze drying ($p < 0.01$), compared with the control sample. Because PFK is a key enzyme during the glycolytic pathway and is related to the survival of the cells the upregulation of *pfk* expression is implicated in the viability of the cells to some extent during the freeze-drying process. Although the protein expression level of PK activity was similar to PFK in the presence of salt, there is a difference in the expressions of their coding genes. This result indicated that the two genes might not belong to the same operon, which is consistent with the report of Marceau *et al.* (2004).

NaCl stress increased the survival rate of freeze-dried *L. bulgaricus*, perhaps because the environmental conditions changed the glucose metabolism vitality of the key enzymes or their translation level or because the stress was not strongly related to the transcription level of enzymes.

Conclusions

In this study, 2% NaCl stress was found to improve the survival rate of freeze-dried *L. bulgaricus* and increase its glucose utilization and sugar metabolism rate. It is possible that NaCl affected the key metabolic enzymes and thus regulated cellular metabolism activity. The increasing survival rate due to NaCl stress during freeze-drying was unrelated to the transcriptional level of the key enzymes.

Acknowledgments

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