Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(6):894-899

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Effects of carbon sources and prebiotics added to growth media on proliferation and survival of *Lactobacillus bulgaricus LB*6 during freeze-drying

[1]He Chen, [1]Shiwei Chen, [2]Hongli Chen, [2]Yanyan Wu and [1]Guowei Shu*

[1]College of Life Science & Engineering, Shaanxi University of Science & Technology, Xi'an, China
[2]Xinjiang Shihezi Garden Dairy Co. Ltd., China

ABSTRACT

Plackett–Burman design employed to evaluate the effect of carbon sources and prebiotics both on proliferation and survival of Lactobacillus bulgaricus LB6 during freeze-drying. Out of consideration of the optimal carbon sources and prebiotics for proliferation and survival of Lactobacillus bulgaricus LB6 before and after freeze-drying, viable counts and survival rate were detected in the medium containing various carbon sources and prebiotics (Glucose, lactose, maltose, sucrose, inulin, trehalose, mannitol, xylooligosaccharides, fructooligosaccharides, galactooligosaccharides). The results indicated that Trehalose out of the investigated carbon sources and prebiotics could both affect the growth (negative) and survival rate (positive) of Lactobacillus bulgaricusLB6 significantly. In addition, Lactose and Galactooligosaccharides have markedly effect on proliferation and survival (all negative), respectively.

Keywords: *Lactobacillus bulgaricus*; growth media; freeze-drying; carbon sources; prebiotics

---

INTRODUCTION

Dairy starter cultures are of industrial importance and commercial significance for fermented foods, and have been well recognized worldwide [1, 2]. The bacterium *Lactobacillus bulgaricus*, which is a member of lactic acid bacterium, have been used as a probiotic culture [3] and is of vital importance to the fermented food in combination with *Streptococcus thermophilus*.

The efficacy of *Lactobacillus bulgaricus* as starter cultures for the dairy industry depends strongly on the number of viable and active cells. Lyophilized or freeze-drying is the most convenient and successful method of preserving bacteria [4], and it has been widely used in microbiology for many decades to stabilize and store cultures [5]. However, not all cells were survived in this treatment, the survival rate as low as 0.1% has been reported [6]. Thus, to protect the viability of probiotics during dehydration, people have added varieties of protective agents to the drying media before freeze-drying [7]. For example, the carbohydrates that have protective effects for probiotic bacteria during freeze-drying were well documented, sorbitol [8, 9], mannitol [10], sucrose[11], lactose[12], and mannose [13], inulin and fructo-oligosaccharides[14]. Amino acids, including phenylalanine, arginine, glycine [15] and sodium glutamate [16] were employed to protect the cells. Some salt buffers, such as NaCl or KCl [11], sodium citrate [17, 18], phosphate [19], calcium carbonate and manganese sulfate can help to protect cells during freeze-drying together with other protectants. On the other hand, it is well known that the growth of bacterial cultures vary depending on the growth medium, and the composition of the growth media is a contributing factor to the survival rate of probiotic cultures during drying has been demonstrated [20]. The present of sugars, such as lactose, sucrose, trehalose, mannose, fructose, glucose, fructose etc. in the growth media have contribution to the survival rate of probiotic cultures during drying [13, 21-23]. There is still lack of studies on the influence of growth

media on subsequent survival of the cells during freeze-drying.

In the previous work, we screened 4 *Lactobacillus* strains that have high angiotensin converting enzyme (ACE) inhibitory activity from 28 probiotic strains. In which, the *Lactobacillus reuteri*, *Lactobacillus bulgaricus* (*LB*6), *Lactobacillus rhamnosu* and *Lactobacillus helveticu* showed high ACE inhibitory activity with 95.92%, 84.61%, 82.79% and 78.57%, respectively [24]. The aim of the present study was to investigate the carbon sources and prebiotics that can potentially improve both survival rate and the number of viable cells of *Lactobacillus bulgaricus LB6* when added into the growth medium.

EXPERIMENTAL SECTION

Microorganism and Media preparation

*Lactobacillus bulgaricus LB*6 were obtained from College of Life Science & Engineering, Shaanxi University of Science & Technology and inoculated three successive times with the basal LAB growth medium at 37 ^{o}C for 24h until the viability of bacteria stays stable. The basal LAB growth medium contains 20g of glucose, 4g yeast extract powder, 10 g soya peptone, 1000mL water. All the media were autoclaved at 121 ºC for 15min.3% active culture was added to each the basal LAB growth media that were autoclaved after cooling to 50 ^{o}C, incubated at 37 ^{o}C, and then viable counts at optional incubation time.

Vacuum freeze-drying

After incubation, *LB*6 culture was centrifuged at 10000 ×g for 15min and the supernatant was discarded to harvest *LB*6 cells. The cells were prefrozen at -40 ^{o}C for 12-24h after protective agents (phosphate buffer) were added, and then frozen at -55 ^{o}C, 6.93pa for 24h using a vacuum freeze dryer.

Determination of cell counts

After a serial dilution on sterile saline solution (NaCl, 0.9% w/v), the diluted bacterial suspension 0.1mL with a syringe and dropped into count plate before coated uniformly, and the plates were carried out at 37 ^{o}C for 48h, the viable cells of *LB*6 were conducted in triplicates by plating on the plate. The freeze-dried powder were reconstituted to their original pre-freeze dried volumes by adding sterile saline solution and number of viable cells counted as above.

Calculation of survival

Survival rate (%) = (CFU/mL after freeze-drying / CFU/mL before freeze-drying) ×100%

Screning of carbon sources and prebiotics using Plackett–Burman design

The Plackett–Burman design was used to identify the selected carbon sources and prebiotics (Glucose, lactose, maltose, sucrose, inulin, trehalose, mannitol, xylooligosaccharides, fructooligosaccharides, galactooligosaccharides) in which have significant effect on both viable counts and survival rate before and after freeze-drying. According to Plackett–Burman design, all 10 factors were tested at a lower and a higher level coded as (+1) and (-1) (Table 1), respectively. The design matrix is shown in Table 2 where it can be seen the effect of 11 variables (including one error terms: X8, in order to estimate the standard deviation) was investigated in 12 independent experimental runs.

Tab. 1 Carbon sources and prebiotics at different levels in Plackett–Burman design

Variables	Medium components	Lower level (%)	Higher level (%)
X1	Glucose	1	1.5
X2	Lactose	1	1.5
X3	Maltose	1	1.5
X4	Sucrose	1	1.5
X5	Inulin	0.2	0.3
X6	Trehalose	0.2	0.3
X7	Mannitol	0.2	0.3
X9	Xylooligosaccharides	0.2	0.3
X10	Fructooligosaccharides	0.2	0.3
X11	Galactooligosaccharides	0.2	0.3

Statistical analysis of the data

The statistical analysis performed by the Design-Expert (Version, 8.0.6) to identify the significant variables and their corresponding coefficients, so that the levels of various managed to obtain a desired output. Hence, F-value, sum of squares, p-value and confidence interval (CI) analyzed using the experimental results of the viable bacteria and survival rate. The experimental results (response function, Y) were fitted to first order multiple regression equations (Eq. (1)) using coded level (-1 or +1) of the variables (Xi):

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \varepsilon \quad (1)$$

RESULTS AND DISCUSSION

The experimental design and results

In the present study, the experimental design and results showed in Table 2. The value Y1 represent for viable counts in the fermentation broth (the unit 10^9 CFU/mL) and Y2 (%) for survival rate after freeze-drying.

Tab. 2 The Plackette-Burman experimental design matrix and results for evaluating data

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Y1(×10^9CFU/mL)	Y2 (%)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	15	3.33
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	10.8	0.93
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	5.2	1.92
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	5.7	1.75
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	5.9	1.69
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	1.5	6.67
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	8.4	3.57
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	6.5	3.08
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	9.3	3.23
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	6.3	19.05
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	1.6	6.25
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	11.1	4.5

Effect of the various on growth of *L. bulgaricus*

Analysis of variance (ANOVA) was performed to estimate the effect on growth of each factor (Table 3). In the ANOVA, "p-value" less than 0.1000 indicate that the terms are significant. In this case, Lactose (X2) (p=0.0650), Trehalose (X6) (p=0.0614) and Xylooligosaccharides (X9) (p=0.0587) are most significant in all various, and according to this assumption the above three were found to be significant factors for growth of *LB*6. The p-value greater than 0.1000 indicate the model terms are not significant. Furthermore, the positive or negative of coefficients in Final Equation in Terms of actual factors means that all the selected various have positive or negative effect on viable counts (Y1), the equation have been shown as follow(Determination coefficient R^2= 0.9978):

Viable counts =7.2750+0.2583 * X1-1.7083 * X2-0.2250 * X3+0.4917 * X4 -1.4917 * X5 -1.8083 * X6+0.0083 * X7+1.8917 * X9-1.2583 * X10-0.1583 * X11

Tab.3 Result of ANOVA of various for Y1 (Viable counts)

Source	SS	DF	MS	F-Value	p-value
A-X1	167.5150	10	16.7515	45.5823	0.3790
B-X2	0.8008	1	0.8008	2.1791	0.0650
C-X3	35.0208	1	35.0208	95.2948	0.4208
D-X4	0.6075	1	0.6075	1.6531	0.2177
E-X5	2.9008	1	2.9008	7.8934	0.0743
F-X6	26.7008	1	26.7008	72.6553	0.0614
G-X7	39.2408	1	39.2408	106.7778	0.9697
J-X9	0.0008	1	0.0008	0.0023	0.0587
K-X10	42.9408	1	42.9408	116.8458	0.0880
L-X11	19.0008	1	19.0008	51.7029	0.5318
Residual	0.3675	1	0.3675		
Cor Total	167.8825	11			

SS: Sum of Squares; MS: Mean Square; DF: Degree of Freedom.

Effect of the various on survival of *L. bulgaricus*

The Table 4 showed the ANOVA of the ingredients for survival rate of *L. bulgaricus LB6*. The model presented a high determination coefficient (R^2= 0.9741). The relative importance of the variables was as follows: X4> X6 > X11 > X7 > X3 > X5 > X2 > X1 > X10 >X9.Out of the above factors, Sucrose (X4) (p=0.2014), Trehalose (X6) (p=0.2192) and Galactooligosaccharides (X11) (p=0.2734) can affect the survival rate of *LB*6. The linear regression equation was as follows:

Survival rate= 4.6642+ 0.9058 * X1-1.1592 * X3- 1.2775 * X3- 2.2892 * X4+ 1.2758 * X5+ 2.0892 * X6+1.4975 * X7+ 0.6742 * X9+ 0.8325 * X10- 1.6358 * X11

Tab. 4 Result of ANOVA of various for Y2 (Survival rate)

Source	SS	DF	MS	F-Value	p-value
A-X1	9.8464	1	9.8464	1.4620	0.4399
B-X2	16.1240	1	16.1240	2.3941	0.3653
C-X3	19.5841	1	19.5841	2.9078	0.3377
D-X4	62.8834	1	62.8834	9.3368	0.2014
E-X5	19.5330	1	19.5330	2.9002	0.3380
F-X6	52.3754	1	52.3754	7.7766	0.2192
G-X7	26.9101	1	26.9101	3.9956	0.2953
J-X9	5.4540	1	5.4540	0.8098	0.5335
K-X10	8.3167	1	8.3167	1.2348	0.4665
L-X11	32.1114	1	32.1114	4.7678	0.2734
Residual	6.7350	1	6.7350		
Cor Total	259.8735	11			

SS: Sum of Squares; MS: Mean Square; DF: Degree of Freedom.

Effect of the various on growth combine with survival of *L. bulgaricus*

According to the Analysis of Variance for viable counts (Y1) and survival rate (Y2), only Trehalose (X6) showed significant effect on both viability and survival. In addition, the Lactose(X2) showed can effect the proliferation of the cell markedly; Galactooligosaccharides(X11) have impact in the survival, so these two various would be selected for further research. In addition, the positive or negative of these three various showed at Figure1, 2 and 3.

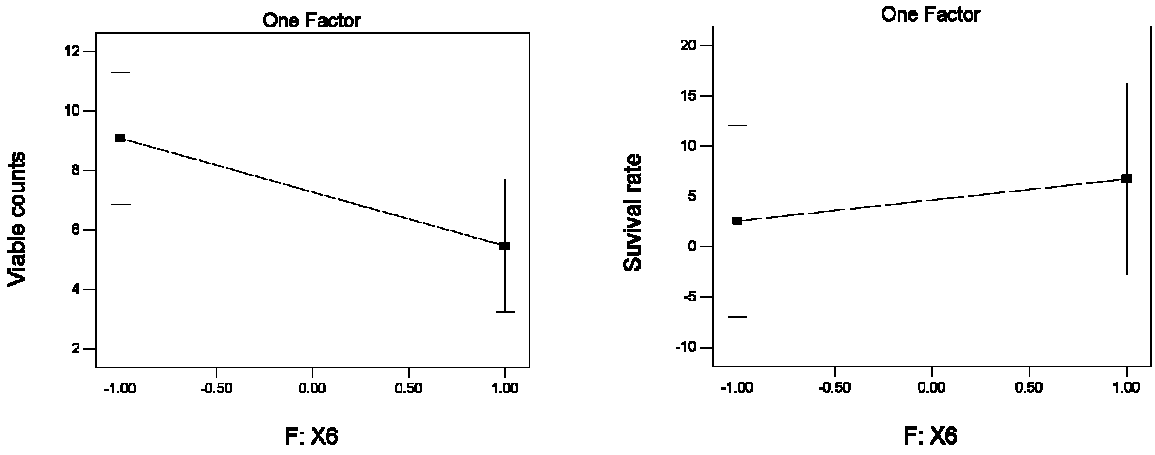

Fig. 1 The 95% confidence interval of Trehalose (X6)

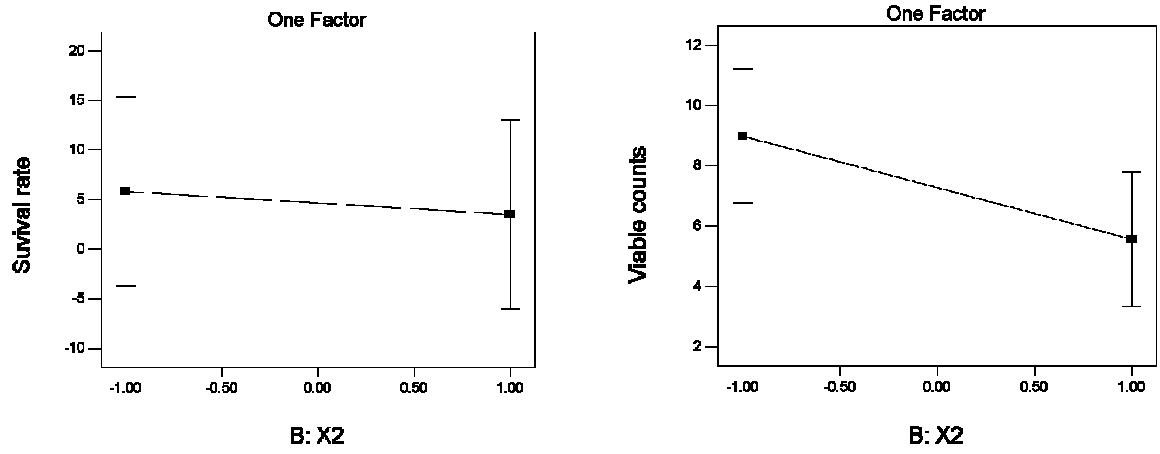

Fig. 2 The 95% confidence interval of Lactose (X2)

The growth medium is a critical parameter, which is more likely to play a role upon survival following freeze-drying, and the results had already indicated the importance of the growth and drying medium on survival during storage of freeze-dried *L. bulgaricus* [13]. It has been reported that [9] trehalose is one of the most well known protective sugars for *Lactobacillus paracasei* cells, especially during storage. Panoff *et al.* [21] showed that cells of *L. delbrueckii* sub sp. *bulgaricus* can be adapted to freezing and thawing by an osmotic stress, when they are grown in the presence of sugars such as lactose, sucrose and trehalose. Similarly, in the study of Carvalho *et al*. [13]*L. bulgaricu*s clearly survived better during storage when cells had been grown in the presence of fructose, lactose or

mannose. Addition of trehalose to the growth media can enable cells to increase the amount of trehalose within the cytoplasm, which in turn stabilizes the cytoplasmic membrane during desiccation [25]. Carvalho *et al*. [1] also suggested that the mechanism for the protection of sugars in the growth media is likely that growth in the presence of various sugar substrates produces cells with distinct morphological and physiological traits, thus reflecting distinct resistances to the various stress treatments tested. The presence of carbohydrates plays an importance role in the survival rate of probiotic cultures, nevertheless, not all the carbohydrates showed growth effect on the *Lactobacillus bulgaricus*.

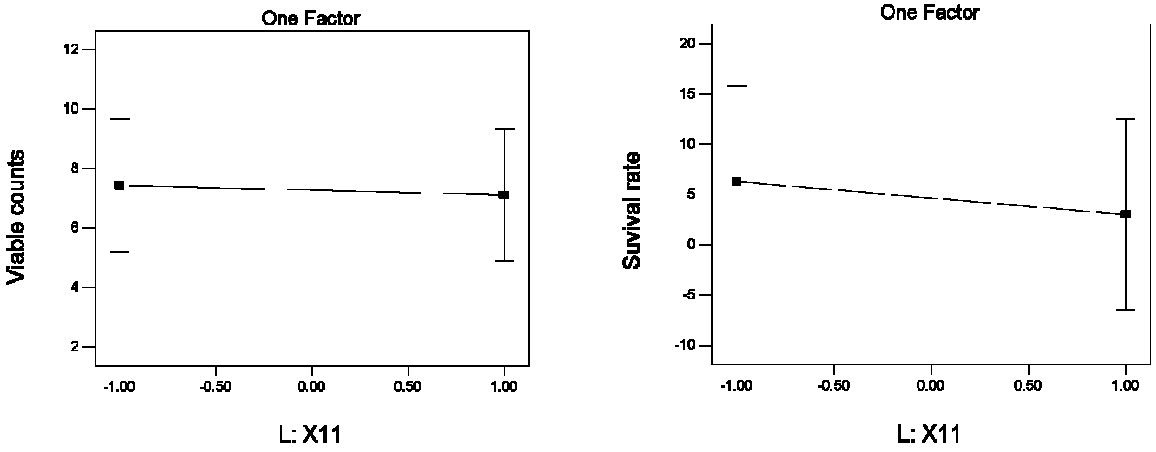

Fig. 3 The 95% confidence interval of Galactooligosaccharides(X11)

CONCLUSION

In conclusion, when added into the growth medium and, trehalose can both affect the growth (negative) and survival rate (positive) of *Lactobacillus bulgaricus LB*6 before and after freeze-drying. Besides, the lactose has markedly effect on the proliferation of the cell, galactooligosaccharides can influence the survival very well, and these two various showed all negative effect on both growth and survival rate. Thus, these three various would be selected for further research and application.

Acknowledgements
The project was partly supported by the Science and technology assistance project of Xinjiang (No.2014AB033), the science and technology plan project of Xi'an city [No. NC1317 (1)], the Science and Technology Research Development plan project of Shaanxi Province, China (No. 2014K01-17-07).

REFERENCES

[1]AS Carvalho; J Silva; P Ho; P Teixeiraa; FX Malcata; P Gibbs, *Int. Dairy J*., **2004**, 14,835–847.
[2] C Santivarangkna; B Higl; P Foerst, *Food Microbiol*., **2008**, 25, 429–441.
[3] M van de Guchte; S Penaud; C Grimaldi; V Barbe; K Bryson; P Nicolas, et al., *PNAS*,**2006**, 103,9274–9279.
[4]JF Berny;GL Hennebert, *Mycologia*, **1991**, 83, 805–15.
[5] C Morgan;G Vesey, Freeze-Drying of Microorganisms Encyclopedia of Microbiology, 3rd Edition, **2009**, 162–173.
[6] M Abadias; A Benabarre; N Teixido; J Usall; I Vi, *Int. J. Food Microbiol*., **2001**, 65, 173–82.
[7]Z Hubalek., *Cryobiology*,**2003**, 46:205–229.
[8]LJM Linders;GIW de Jong; G Meerdink; K van't Riet, *J. Food Eng*., **1997**, 31, 237–250.
[9]P Foerst;U Kulozik; M Schmitt; S Bauer; C Santivarangkna, *Food Bioprod. Process*., **2012**, 90, 295–300.
[10]BJO Efiuvwevwere ; LGM Gorris ; EJ Smid ; EPW Kets, *Appl. Microbiol. Biot.*, **1999**, 51, 100–104.
[11] AS Carvalho; J Silva; P Ho; P Teixeiraa; FX Malcata; P Gibbs, *J. Food Sci*., **2003**, 68, 2538–2541.
[12] B Higl;L Kurtmann; CU Carlsen; J Ratjen; P Forst, *et al*., *Biotechnol. Progr*., **2007**, 23, 794–800.
[13] AS Carvalho;J Silva; P Ho; P Teixeiraa; FX Malcata; P Gibbs, *Biotechnol. Progr*., **2004**, 20, 248–254.
[14]C Schwab; R Vogel; MG Gänzle, *Cryobiology*, **2007**, 55(2), 108–114.
[15] M Mattern; G Winter; U Kohnert; G Lee, *Pharm. Dev. Technol*., **1999**, 4(2), 199-208.
[16]PC Teixeira; MH Castro; FX Malcata; RM Kirby, *J. Dairy Sci*., **1995**, 78, 1025–1031.
[17]EPW Kets;JAM de Bont, *FEMS Microbiol. Lett*., **1994**, 116, 251–256.
[18]L Kurtmann; CU Carlsen; J Risbo; LH Skibsted, *Cryobiology*, **2009**, 58(2), 175–180.
[19]Satoshi Ohtake, *Cryobiology*, **2004**, 48, 81-89.
[20]XC Meng; C Stanton; GF Fitzgerald; C Daly; RP Ross, *Food Chem*., **2008**, 106, 1406-1416.

[21] JM Panoff; B Thammavongs; M Gueguen, *Cryobiology*, **2000**, 40, 264–269.
[22]V Ferreira;V Soares; C Santos; J Silva; PA Gibbs; P Teixeira, *Biotechnol. Lett.*, **2005**, 27, 249–252.
[23] AS Carvalho; J Silva; P Ho; P Teixeiraa; FX Malcata; P Gibbs, *J. Appl. Microbiol*., **2003**, 94, 947–952.
[24]H Chen, Z Ji, GW Shu, HN Xing, *Adv. Mater. Res.*, **2012**, 531, 442-445.
[25] JG Streeter, *J. Appl. Microbiol*., **2003**, 95, 484–491.