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ORIGINAL RESEARCH PAPER

EFFECT OF AMINO ACIDS ADDED TO CULTURE MEDIUM ON THE GROWTH AND SURVIVAL OF *LACTOBACILLUS BULGARICUS LB6* DURING FREEZE-DRYING

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Plackett–Burman design was applied to evaluate the effects of amino acids on the growth in medium and survival during freeze-drying of *Lactobacillus bulgaricus* LB6. Moreover, in consideration of the optimal amino acids for the growth and survival of *Lactobacillus bulgaricus* LB6 before and after freeze-drying, viable counts and survival rate were monitored in the medium containing selected amino acids (Glutamate, Alanine, Glycine, Leucine, Serine, Arginine, Lysine and Hydroxyproline). The results indicated that Leucine (p=0.0117 for viable counts, p=0.0121 for survival rate) and Arginine (p=0.0120 for viable counts, p=0.0043 for survival rate) out of the investigated amino acids can significantly affect both the growth and survival rate of *Lactobacillus bulgaricus LB*6, and have a positive effect.

Keywords: *Lactobacillus bulgaricus*, promoting-growth, freeze-drying, amino acid

Introduction

Dairy starter cultures are of industrial importance and commercial significance for fermented foods, and have been well recognized worldwide (Carvalho *et al*., 2004b; Santivarangkna *et al*., 2008). The *Lactobacillus bulgaricus*, a lactic acid bacterium,

is able to produce lactic acid in the production of yogurt, cheese and other fermented products (Guchte *et al.*, 2006), and is of vital importance to the fermented food in combination with *Streptococcus thermophilus*.

The efficacy of *L. bulgaricus* as starter cultures for the dairy industry depends strongly on the number of viable and active cells (Chen *et al.*, 2014). Thus, during the preparation of the starter cultures, the production and maintenance techniques that maximize the viability and activity of the bacterial cells must be established. The lactic acid bacterium had been preserved and distributed in several forms, such as liquid, spray-dried and lyophilised. The lyophilized or freeze-drying is the most convenient and successful method of preserving bacteria (Berny and Hennebert, 1991), and has been widely used in microbiology for many decades to stabilize and store cultures (Morgan and Vesey, 2009). However, not all cells survived this treatment and a survival rate as low as 0.1% has been reported (Abadias *et al.*, 2001). The major causes of cell viability loss during freeze-drying are related to ice crystal formation, membrane damage from high osmolarity due to high concentrations of internal solutes, maromolecule denaturation, and the removal of water, which affects properties of many hydrophilic macromolecules in cells (Fonseca *et al.*, 2000; Fowler and Toner, 2005; Brennan *et al.*, 1986; Allison *et* al., 1999). Thus, to protect the viability of probiotics during dehydration, people have added varieties of protective agents to the drying media before freeze-drying (Hubalek, 2003). The carbohydrates that have protective effects for probiotic bacteria during freeze-drying were well documented, for instance, sorbitol (Linders *et al.*, 1997a; Foerst *et al.*, 2012), mannitol (Efiuvwevwere *et al.*, 1999), sucrose (Carvalho *et al.*, 2003a), lactose (Higl *et al.*, 2007), and mannose (Carvalho *et al.*, 2004a), inulin and fructo-oligosaccharides (Clarissa et al., 2007). Amino acids, including phenylalanine, arginine, glycine (Mattern *et al.*, 1999) and sodium glutamate (Font *et al.*, 1983; Teixeira *et al.*, 1995) were employed to protect the cells. Some salt buffers, such as NaCl or KCl (Carvalho *et al.*, 2003a), sodium citrate (Kets *et al.*, 2004; Lone *et al.*, 2009), phosphate (Ohtake, 2004), calcium carbonate and manganese sulphate 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 varies depending on the growth medium, and the composition of the growth media as a contributing factor to the survival rate of probiotic cultures during drying has been demonstrated (Meng *et al.*, 2008). The presence of sugars, such as lactose, sucrose, trehalose, mannose, fructose, glucose, fructose etc. in the growth media has an impact on the survival rate of probiotic cultures during drying (Ferreira *et al.*, 2005;

Carvalho *et al*., 2003b; Carvalho *et al*., 2004a). Other additives that can affect the viability or survival rate used in growth media were NaCl (Linders *et al*., 1997b), manganese sulphate, Tween 80 and ascorbic acid (Carvalho *et al*., 2003b), carnitine and betaine (Kets and de Bont, 1994; Åsa *et al*., 2012). There is still lack of studies on the influence of growth media on the subsequent survival of cells during freeze-drying, in particularly the insights into the amino acids that can improve the proliferation and survival when added in the growth medium are few. The effects of sugar alcohol and proteins on the survival of *Lactobacillus bulgaricus* LB6 during freeze-drying were also studied (Chen *et al*., 2015).

The aim of the present study was to investigate the potential of different amino acids, added into the culture medium, to act as growth promoting substances for *L. bulgaricus LB6*, and protective agents for freeze-drying applications.

Materials and methods

Microorganisms

L. bulgaricus LB6 was obtained from School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China), and activated for 24 h at 37 °C with basal LAB growth medium which was repeated three times until the viable counts were stable. The basal LAB growth medium contained 20g of glucose, 4g yeast extract powder, 10g soya peptone, 1000mL water, which was obtained from Beijing Land Bridge Technology Co., Ltd. MRS medium. The amino acids were added to the basal LAB growth media that were autoclaved and cooled to 50 ºC, and 3% active culture was inoculated into the medium and incubated at 37 ºC, and then viable counts at 18-20h were performed. All the amino acids were sterilized using 0.22 μm membrane filtration before added into the autoclaved medium.

Vacuum freeze-drying

After incubation, *L. bulgaricus LB6* culture was centrifuged at 10000×g for 15min and the supernatant was discarded to harvest *L. bulgaricus LB6* cells. The cells were pre-frozen at -80 ºC for 6-12h after protective agents (phosphate buffer) were added, and then frozen at -55 ºC, 6.93pa for 24h using a vacuum freeze dryer LGJ-22D (Beijing Four-Ring Science Instrument Plant Co., Ltd., Beijing, China).

Determination of cell counts

After a serial dilution on sterile saline solution (NaCl, 0.9% w/v), the diluted bacterial suspension was aliquoted into 0.1mL doses with Hamilton syringe and dropped into a count plate, then spread uniformly. The count plates were incubated

at 37°C anaerobically for 36-48h and then the viable *L. bulgaricus* cells were counted (Shu *et al*., 2014). The freeze-dried powders were reconstituted to their original pre-freeze dried volumes by adding sterile saline solution and the number of viable cells counted as above.

Calculation of survival

Survival percentage was calculated as the number of viable cells after drying/number of viable cells before drying×100%.

Experimental Design of Amino Acid Screening

The goal of applying Plackett–Burman design was to identify which factors of the selected amino acids (Glutamate, Alanine, Glycine, Leucine, Serine, Arginine, Lysine and Hydroxyproline) have significant effect on both viable counts and survival rate before and after freeze-drying. According to Plackett–Burman design, all eight 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 can be seen the effect of the 11 variables (including three error terms: Lactose (X2), Fructooligosaccharides (X10) and Galactooligosaccharides (X11), in order to estimate the standard deviation) as resulted after running 12 independent experimental tests.

Table 1. Amino acids tested in a Plackett-Burman survey for their efficacy in increasing the viability and cell survival of *Lactobacillus bulgaricus* during freeze-drying

Variables	Medium components	Lower level (mg/L)	Higher level (mg/L)
X1	Glutamate	4	6
X3	Alanine	4	6
X4	Glycine	4	6
X5	Leucine	4	6
X6	Serine	4	6
X7	Arginine	4	6
X8	Lysine	4	6
X9	Hydroxyproline	4	6

The statistical analysis was performed by the Design-Expert (Version, 8.0.6) to identify the significant variables and their corresponding coefficients, so that the levels of various can be managed to obtain a desired output. Hence, F-value, sum of squares, p-value and confidence interval (CI) were 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 the coded level (-1 or +1) of the variables (Xi):

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

Results

The experimental design and results

In the present study, the experimental design and results showed in Table 2 are followed by the Plackett–Burman design. The value Y1 stands for viable counts before centrifuged (the unit 10^9 CFU/mL) and Y2 (%) for survival rate after freeze-drying.

Table 2. The Plackett-Burman experimental design matrix and results for the evaluated data

Ru	X1	X2	X3	X	X	X	X	X	X	X1	X	Y1/10^9CFU/	Y2
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	1.70	7.35
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	1.20	23.00
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	1.66	8.07
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	1.74	15.11
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	9.45	1.66
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	1.93	6.94
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	1.06	10.85
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	1.38	4.96
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	1.59	13.08
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	13.5	1.17
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	2.33	2.68
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.42	9.51
The control												0.97	0.77

Effect of amino acids on the growth of L. bulgaricus

The analysis of variance (ANOVA) was performed to estimate the effect on the growth of each factor (Table 3). The Model F-value of 19.22 implies the model is significant. The p-value of linear model was 0.0168, which demonstrated that the fit of the linear model was satisfied. Values of "Prob > F" less than 0.0500 indicate

model terms are significant; therefore, the sum of squares confirmed the significances of each amino acid. In this case, Glutamate (X1) (p=0.0116), Alanine (X3) (p=0.0117), Leucine (X5) (p=0.0117), Arginine (X7) (p=0.0120) and Lysine (X8) (p=0.0142) are significant model terms and according to this assumption these five amino acids were found to be significant factors for the growth of *L. bulgaricus*. Values 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 mean that all the selected various have positive or negative effect on Y1; the equations have been shown as follows (R^2= 0.9809):

Viable counts = 3.2467+1.6733* X1-1.6683* X3-0.5100* X4+1.6717* X5+0.4450* X6+1.6567* X7-1.5583* X8+0.2050* X9

Table 3. Result of ANOVA on the effect of various factors on viable counts

Source	SS	DF	MS	F-Value	Prob>F(p-value)	
Model	168.6114	8	21.0764	19.2157	0.0168	significant
X1	33.6005	1	33.6005	30.6341	0.0116	
X3	33.4000	1	33.4000	30.4513	0.0117	
X4	3.1212	1	3.1212	2.8456	0.1902	
X5	33.5336	1	33.5336	30.5731	0.0117	
X6	2.3763	1	2.3763	2.1665	0.2374	
X7	32.9345	1	32.9345	30.0269	0.0120	
X8	29.1408	1	29.1408	26.5682	0.0142	
X9	0.5043	1	0.5043	0.4598	0.5463	
Residual	3.2905	3	1.0968			
Total	171.9019	11				

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

Effect of amino acids on the survival of L. bulgaricus

Table 4 shows the ANOVA of the ingredients for the survival rate of *L. bulgaricus*. The model presented a high determination coefficient (R^2= 0.9793). The Model F-value of 17.73 and p-value of 0.0188 revealed that the model is significant, there is only a 1.88% chance that a "Model F-Value" this large could occur due to noise. The relative importance of the variables was found as follows: X7> X4 > X5 > X9 > X8 > X1 > X6 > X3. Among the factors above, Arginine (X7) (p=0.0043), Glycine(X4) (p=0.0118), Leucine (X5) (p=0.0121) and Hydroxyproline (X9) (p=0.0323) can significantly affect the survival rate of *L. bulgaricus*. The linear regression equation was as follows:

Survival rate=8.6983+0.5067* X1+0.1817* X3+2.7450* X4-2.7183* X5-0.3933 * X6-3.9200* X7+0.9700* X8+1.8883* X9

Table 4. Result of ANOVA on the effect of various factors on survival rate

Source	SS	DF	MS	F-Value	Prob>F(p-value)	
Model	422.9027	8	52.8628	17.7275	0.0188	significant
X1	3.0805	1	3.0805	1.0331	0.3843	
X3	0.3960	1	0.3960	0.1328	0.7397	
X4	90.4203	1	90.4203	30.3224	0.0118	
X5	88.6720	1	88.6720	29.7361	0.0121	
X6	1.8565	1	1.8565	0.6226	0.4877	
X7	184.3968	1	184.3968	61.8373	0.0043	
X8	11.2908	1	11.2908	3.7864	0.1469	
X9	42.7896	1	42.7896	14.3495	0.0323	
Residual	8.9459	3	2.9820			
Total	431.8486	11				

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

Effect of amino acids on the growth in medium and survival during the freeze-drying of *L. bulgaricus*

The above-mentioned Analysis of Variance for viable counts (Y1) and survival rate (Y2) suggested that only Leucine (X5) and Arginine (X7) showed significant effect on both viability and survival. The coefficients of these two variables in linear regression equation mean that Leucine (X5) and Arginine (X7) have a positive effect on the proliferation of the cell and negative effect on the survival. Figure 1 and 2 can indicate this as the trend of the line.

Discussion

The growth medium is a critical parameter, which is more likely to play a role in survival following freeze-drying, and the results already indicated the importance of the growth and drying medium on survival during the storage of freeze-dried *L. bulgaricus* (Carvalho *et al*., 2004a). The effects of 16 kinds of amino acids on enriching the anti-freezing ability of *L. acidophilus* were investigated and it was found that the L-glutamic acid, L-arginine, L-leucine, L-lysine, L-methionine, L-proline, L-phenylalanine and L-threonine could promote the growth of *L. acidophilus*. L-alanine, L-isoleucine, L-cysteine, L-serine, L-phenylalanine, L-aspartic acid, glycine and L-proline could increase the anti-freezing ability of *L.*

acidophilus (Wang *et al*., 2011).

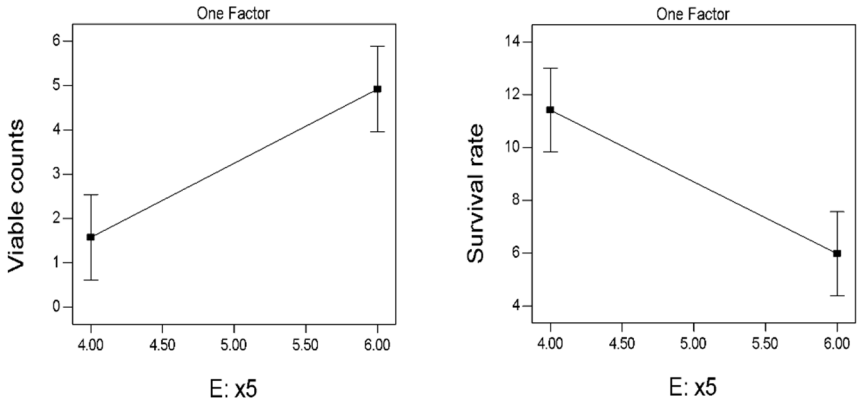

Figure 1. The 95% confidence interval for Leucine

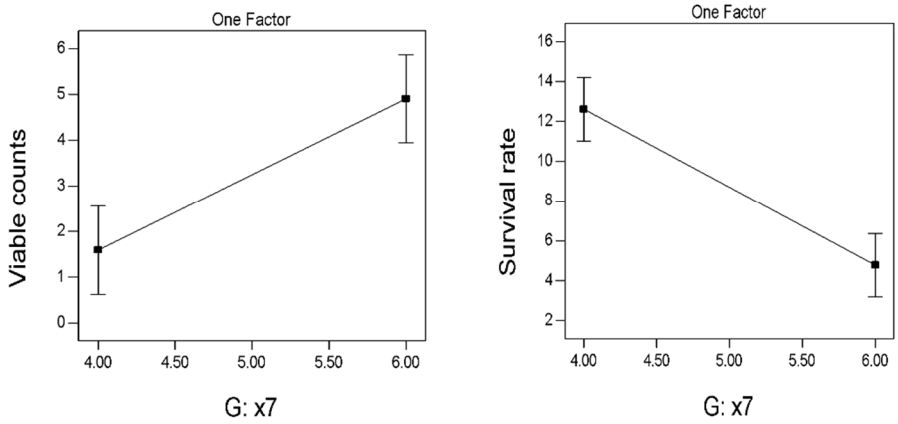

Figure 2. The 95% confidence interval for Arginine

The results of the present work showed that Glutamate, Alanine, Leucine, Arginine and Lysine could affect the growth of *L. bulgaricus LB6* when added into the growth medium. However, when taking into account the survival of the cultures during freeze-drying, only a few amino acids could contribute to the survival rate of lactic acid bacteria. For instance, there were no significant differences in survival during freeze-drying after addition of sorbitol or monosodium glutamate (Carvalho *et al*., 2003c). Kets and de Bont (1994) found that *Lactobacillus plantarum* grew significantly better in the presence of betaine under osmotically stressful conditions (0.6 M sodium chloride); however, only 11% of viable cells survived drying. Furthermore, the study has shown that glutamate, which remains inside the cell, may be responsible for the distinct survival behaviors during dehydration (Wisselink *et*

al., 2002). Nevertheless, the present work showed that two (Leucine, Arginine) out of eight selected amino acids could significantly affect the survival of *LB6*, but glutamate had no effect. A study by Mattern *et al*. (1999) showed that phenylalanine, arginine, and glycine could prevent denaturation during protein vacuum drying. This could explain the protection effects of Leucine, Arginine in the present work. Therefore, the effects of these two agents on both proliferation and survival of *L. bulgaricus* when added into the growth medium were unreported in the previous studies, and the mechanism of significant effect of the two amino acids is not clear and needs further examination.

Conclusions

In this study, 9 selected amino acids (Glutamate, Alanine, Glycine, Leucine, Serine, Arginine, Lysine and Hydroxyproline) were investigated as promoting-growth substances in media and protective agents during freeze-drying for *L. bulgaricus LB*. Both Leucine and Arginine out of the investigated amino acids have significant effect on the growth and survival rate of *Lactobacillus bulgaricus LB*6 ($p < 0.05$). Moreover, they both have a positive effect on the growth and a negative effect on the survival of *L. bulgaricus LB*6.

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