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OPTIMIZATION OF FREEZE-DRIED STARTER FOR YOGURT BY FULL FACTORIAL EXPERIMENTAL DESIGN

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Abstract: With the rapidly development of fermented milk product, it is significant for enhancing the performance of starter culture. This paper not only investigated the influence of anti-freeze factors and freeze-drying protective agents on viable count, freeze-drying survival rate and yield of *Lactobacillus bulgaricus* (LB) and *Streptococcus thermophilus* (ST), but also optimized the bacteria proportion of freeze-dried starter culture for yogurt by full factorial experimental design. The results showed as following: the freeze-drying protective agents or anti-freeze factors could enhanced survival rate of LB and ST; the freeze-dried LB and ST powders containing both of anti-freeze factors and freeze-drying protective agents had higher viable count and freeze-drying survival rate that were 84.7% and 79.7% respectively; In terms of fermentation performance, the best group of freeze-dried starter for yogurt was the compound of LB3 and ST2.

Keywords: *Lactobacillus bulgaricus, Streptococcus thermophilus,* full factorial experimental design, fermentation performance, yogurt

INTRODUCTION

Probiotics is benefit to the health of human and animals (Hoover, 1991; O'Sullivan *et al.,* 1992). Probiotics could be control of intestinal infections, reduce lactose intolerance, lower serum cholesterol levels and increase anticarcinogenic activity et al. Yogurt in which probiotics outnumbers 10^6 cfu/mL should be consumed more than 100g per day (Rybka *et al.,* 1995). Yoghurt (Analie *et al.,* 2001) is a kind of traditional dairy product which is fermented by probiotics. It not only has rich nutrition and good flavor, but also

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is benefit for lactose intolerance and digestion. So it is deeply loved by consumers.

Starter cultures (Surono *et al.,* 2002) are important for fermented foods, which are the `heart' of fermented products. Therefore, highly active starter culture is one of the most crucial factors in yogurt production process.

Freeze drying (Fernandes *et al.,* 2013) is applied to the production of probiotics powders and sourdough starters for wheat and rye bread (Denkova *et al.,* 2014 a) on a large scale. However it will exposes probiotics to extreme environment, which makes strains difficult to survive (Meng *et al.,* 2008). Protective agents, anti-freeze factors and microencapsulation technology can increase the survive rate of probiotics during freeze-drying ((Denkova *et al.,* 2014 b).

Full factorial design of experiment is a multi-factor cross-group design. It is used to study the main effects among various levels of each factor and the interaction effects between two factors (Yi *et al.*, 2011). The result of the interaction between the factors is very precise by full factorial experimental design.

As far as can be ascertained, the present literatures mainly contain the preparation and the application of freeze-dried probiotics powders by vacuum freeze-drying (Wang *et al.,* 2007). This paper is aimed at not only studying the condition of culturing and freeze-drying for LB and ST, but also determining the best group of freeze-dried starter culture by fermentation performance. It would provide a scientific basis for researching and application of the high concentrated freeze-dried starter culture.

MATERIALS AND METHODS

Materials

The bacteria strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were all provided by School of Food and Biological Engineering, Shaanxi University of Science and Technology. MRS broth and M17 broth came from Qingdao Hope Bio-Technology Co., Ltd. The raw milk was skim milk powder from Anchor.

Anti-freeze factors of LB are lactose, soy peptone, oligofructose, yeast extract powder and leucine. Their percentage respectively are 2.1%, 1.0%, 10%, 0.4%, and 0.0012%. Anti-freeze factors of ST are NaCl, mannitol and betaine. Their percentage respectively is 0.5%, 0.1% and 0.05%. (The percentage is the mass ratio of anti-freeze factor and medium)

Freeze-drying protective agents of LB are lactose, xanthan gum, PVP, oligofructose, xylo-oligosaccharides, galacto-oligosaccharide and FeSO4. Their percentage respectively are 16.4%, 1.1%, 2.3%, 10%, 10%, 10% and 0.5%. Freeze-drying protective agents of ST are glucos, soluble starch and Vc.

Their percentage respectively is 17%, 17% and 0.4%. (The percentage is the mass ratio of freeze-drying protective agent and the collected bacteria after centrifuge)

Preparation of lyophilized powder

Centrifuge bacterium culture solution at 9000r/min for 15min by high-speed centrifuge (TG16A-WS, Hunan Shaite Xiangyi Co., Ltd, Hunan, China); Collect bacteria; Mix the collected bacteria and protective agents together into Freeze-drying tube; Standing for 30min; Pre-freeze at -35□ for 18h; Freeze-drying at -55□ and 4-6pa for 24h by using a vacuum freeze drier (FD-1D-50, Beijing Boyikang Instruments Co., Ltd., Beijing, China). The freeze-dried LB and ST powders were respectively divided into the following three classes:

LB: (1) LB1 represented a freeze-dried powder cultivated in MRS broth medium and containing freeze-drying protective agents in the process of freeze-drying; (2) LB2 represented a freeze-dried powder cultivated in MRS broth medium to which added anti-freeze factors and containing freeze-drying protective agents in the process of freeze-drying; (3) LB3 represented a freeze-dried powder cultivated in MRS broth medium.

ST: (1) ST1 represented a freeze-dried powder cultivated in M17 broth medium and containing freeze-drying protective agents in the process of freeze-drying; (2) ST2 represented a freeze-dried powder cultivated in M17 broth medium to which added anti-freeze factors and containing freeze-drying protective agents in the process of freeze-drying; (3) ST3 represented a freeze-dried powder cultivated in M17 broth medium.

Determination of viable bacterial count

Weigh respectively 0.1g freeze-dried LB and ST powders. Dilute to suitable concentration with 0.9%NaCl. Take 1mL of the dilution into MRS and M17 agar medium. Cultivate them at 37ºC for 48h. Select the number of colony between 30 and 300 and then determine the viable count per milliliter (cfu/mL).

The survival rate (%) is the ratio of the viable count after freeze-dry and the viable count before freeze-dry in the same volume.

Determination of pH

According to the ratio of freeze-dried powder and the reconstituted milk is 1:1000, inoculum starter culture in milk, ferment for 6 and 8h, measure pH by a pH-meter (pHS-3C, Shanghai Precision Scientific Instrument Co., Ltd, Shanghai, China). The measurement was replicated three times.

Determination of yogurt acidity

Titrate sample containing phenolphthalein with 0.1mol/L NaOH solution until

the colour of sample solution was slightly red. Volume of NaOH consumed was used to determine the acidity of sample. The unite of fermentation acidity was ^{o}T. Consuming 1mL 0.1mol/L NaOH solution consumed 1^{o}T.

Determine of OD of yogurt

Mix 1mL fermented milk and 9mL 0.2% EDTA whose pH was 11 to 12 on micro vortex mixer (WH-2, Shanghai Analytical Instrument Factory, Shanghai, China). Then test the value of OD at 640nm by using the spectrophotometer (VIS-722, Shanghai Phenix Optical Scientific Instrument Co., Ltd, Shanghai, China), and the control group was reconstituted milk.

RESULTS AND DISCUSSIONS

The effect of different freeze-drying protective agents on LB and ST freeze-drying survival rate, viable bacterial count and yield of freeze-dried LB and ST powders are showed in Table 1.

Table 1. Freeze-drying survival rate, viable bacterial count and yield of freeze-drying LB and ST powders

Type	Survival rate (%)	Viable bacterial count ($\times10^{10}$cfu/g)	Yield (g/L)
LB1	78.5	15.15	14.67
LB2	84.7	5.02	17.67
LB3	76.3	2.19	15.00
ST1	75.1	10.8	17.67
ST2	79.7	3.73	15.00
ST3	53.6	2.35	14.00

From Table 1, we found that LB2 had the highest survival rate which was 84.7% among LB1, LB2 and LB3; and the survival rate of ST2 was 79.7%, which was the highest among ST1, ST2 and ST3. The survival rate of LB1 or LB2 was higher than LB3 and the survival rate of ST1 or ST2 was higher than ST3. It indicated freeze-drying protective agents or anti-freeze factors could enhance survival rate of LB and ST. The viable bacterial counts of LB1 and ST1 respectively were higher than the other, which were 1.15×10^{11} cfu/g and 1.08×10^{11} cfu/g. The yield of LB2 and ST1 respectively were higher than the others.

Optimization of freeze-dried LB and ST powders by full factorial experimental design. The pH, OD, acidity and the sensory evaluation were determined, the results were shown in Table 2. According to these data, we gained the results which were presented in Table 3-6 and figure1-4 by experiment design.

Table 2. Result of full factorial design for freeze-dried powders

No.	LB	ST	Time	OD	Sensory	Acidity	pH
1	LB2	ST3	8	0.424	74	91.4	5.44
2	LB3	ST2	6	0.049	84	72.4	5.78
3	LB1	ST3	6	0.251	76	70.4	5.84
4	LB3	ST1	6	0.255	83	70.4	5.97
5	LB1	ST1	8	0.469	80	76.2	5.02
6	LB3	ST3	8	1.145	75	102.6	5.32
7	LB2	ST2	6	0.136	70	55.0	6.19
8	LB1	ST2	6	0.446	72	64.4	5.70
9	LB2	ST1	6	0.082	70	37.7	6.18
10	LB3	ST2	8	0.728	85	87.2	5.43
11	LB2	ST1	8	0.258	73	51.6	5.80
12	LB2	ST2	8	0.544	78	111.2	5.37
13	LB1	ST3	8	0.395	78	91.2	5.17
14	LB1	ST2	8	1.224	77	114.6	5.31
15	LB2	ST3	6	0.134	72	53.0	6.23
16	LB3	ST3	6	0.323	83	81.8	5.74
17	LB3	ST1	8	1.217	76	91.4	5.34
18	LB1	ST1	6	0.202	72	41.2	5.60

Table 3. ANOVA of OD

Source	SS	DF	MS	F	p
Model	2.333	13	0.179	6.356	0.0438
A-LB	0.394	2	0.197	6.980	0.0496
B-ST	0.037	2	0.018	0.647	0.5709
C-Time	1.138	1	1.138	40.313	0.0032
AB	0.494	4	0.124	4.379	0.0908
AC	0.236	2	0.118	4.179	0.1048
BC	0.034	2	0.017	0.595	0.5940
Residual	0.113	4	0.028		

In statistics, the factor whose reliability is more than 95% ($0.01<p<0.05$) is a significant factor and the factor whose reliability is more than 90% ($0.05<p<0.1$) is an important factor. Analyzing Table 3, the model ($p=0.0438$) was significant, it could be used to analyze the experimental result. LB ($p=0.0496$) was a significant factor. The fermented time ($p<0.01$) was a very significant factor. The interaction of LB and ST ($p=0.0908$) was important, so the interaction of LB and ST influenced the value of OD.

Table 4 shows that the model of sensory evaluation ($p=0.0364$) is significant, it could be used to analyze the experimental result. LB ($p=0.0056$) is a very

significant factor and the interaction of LB and fermented time (p=0.0253) was significant, which could have an effect on the sensory evaluation.

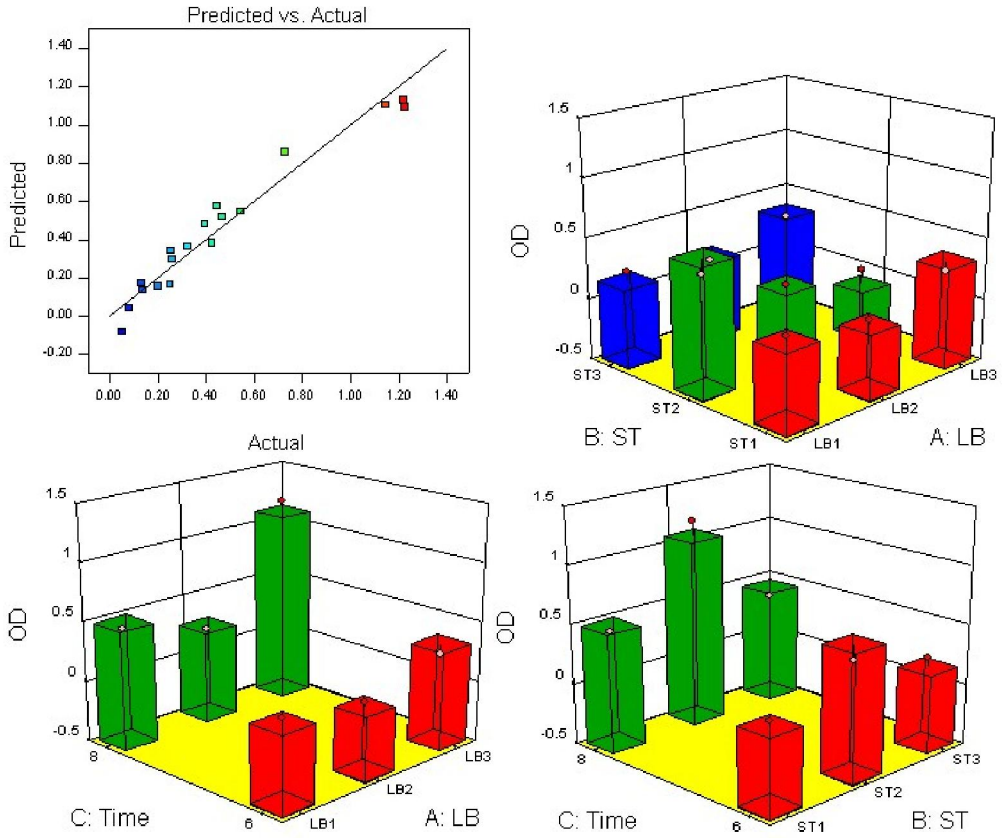

Figure 1. The predicted vs. the actualvalue and 3-D plots of OD

Table 4. ANOVA of sensory evaluation

Source	SS	DF	MS	F	p
Model	379.889	13	29.222	7.060	0.0364
A-LB	204.778	2	102.389	24.738	0.0056
B-ST	12.444	2	6.222	1.503	0.3259
C-Time	10.889	1	10.889	2.631	0.1801
AB	37.222	4	9.306	2.248	0.2260
AC	87.444	2	43.722	10.564	0.0253
BC	27.111	2	13.556	3.275	0.1437
Residual	16.556	4	4.139		

According to Table 5, the model of fermentation acidity (p=0.0497) is significant. LB (p=0.0998) is an important factor, ST (p=0.0354) is a significant factor and the fermented time (p<0.01) is a very significant factor on the fermentation acidity.

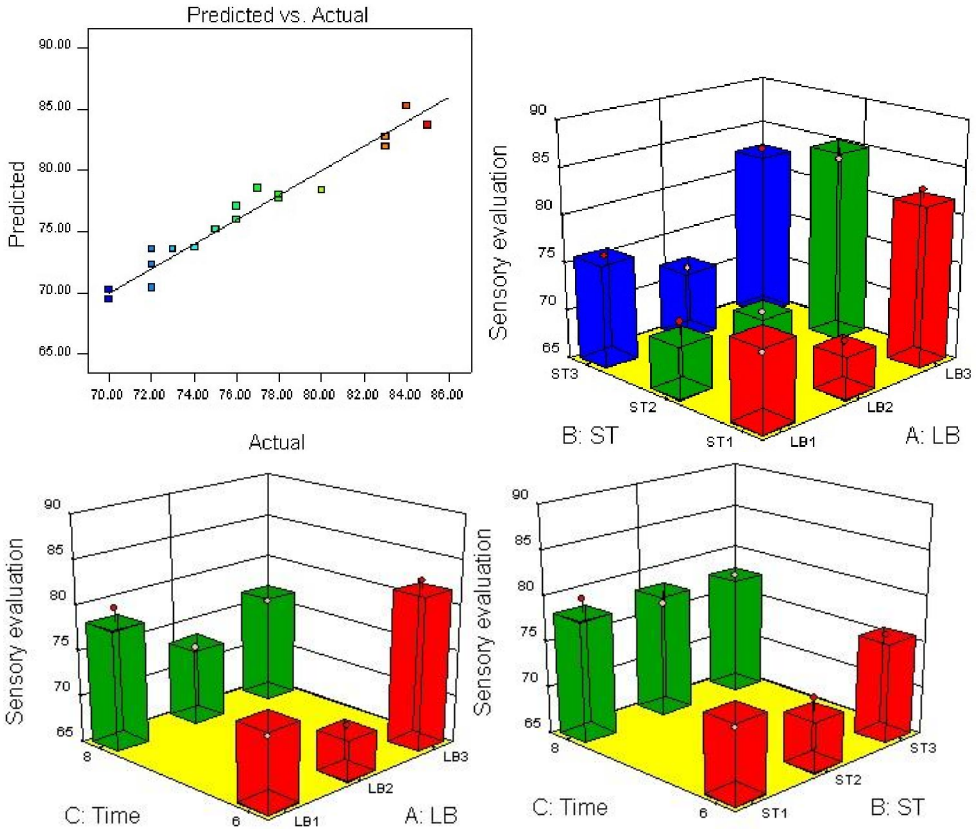

Figure 2. The predicted vs. the actualvalue and 3-D plots of sensoryevaluation

Table 5. ANOVA of fermentation acidity

Source	SS	DF	MS	F	p
Model	8322.082	13	640.160	5.912	0.0497
A-LB	937.915	2	468.958	4.331	0.0998
B-ST	1869.779	2	934.889	8.634	0.0354
C-Time	4083.730	1	4083.730	37.713	0.0036
AB	898.933	4	224.733	2.075	0.2484
AC	285.701	2	142.850	1.319	0.3631
BC	246.024	2	123.012	1.136	0.4067
Residual	433.136	4	108.284		

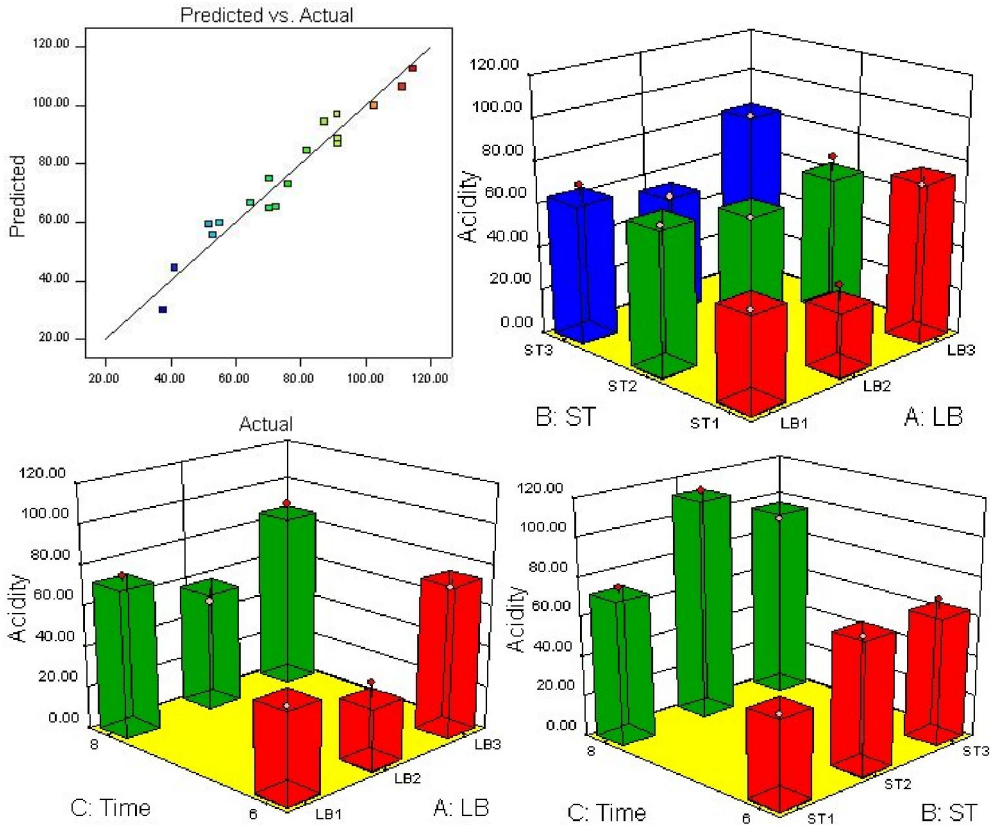

Figure 3. The predicted vs. the actualvalue and 3-D plots of acidity

The model of pH (p=0.0361) is significant, it could be used to analyze the experimental result. LB (p=0.0198) is a significant factor and the fermented time (p<0.01) is a very significant factor on the fermented pH, as showed in Table 6.

Table 6. ANOVA of pH

Source	SS	DF	MS	F	p
Model	2.124	13	0.163	7.096	0.0361
A-LB	0.563	2	0.281	12.222	0.0198
B-ST	0.003	2	0.001	0.058	0.9448
C-Time	1.407	1	1.407	61.108	0.0014
AB	0.112	4	0.028	1.213	0.4281
AC	0.030	2	0.015	0.642	0.5731
BC	0.010	2	0.005	0.224	0.8084
Error	0.092	4	0.023		
Total	2.124	13	0.163	7.096	0.0361

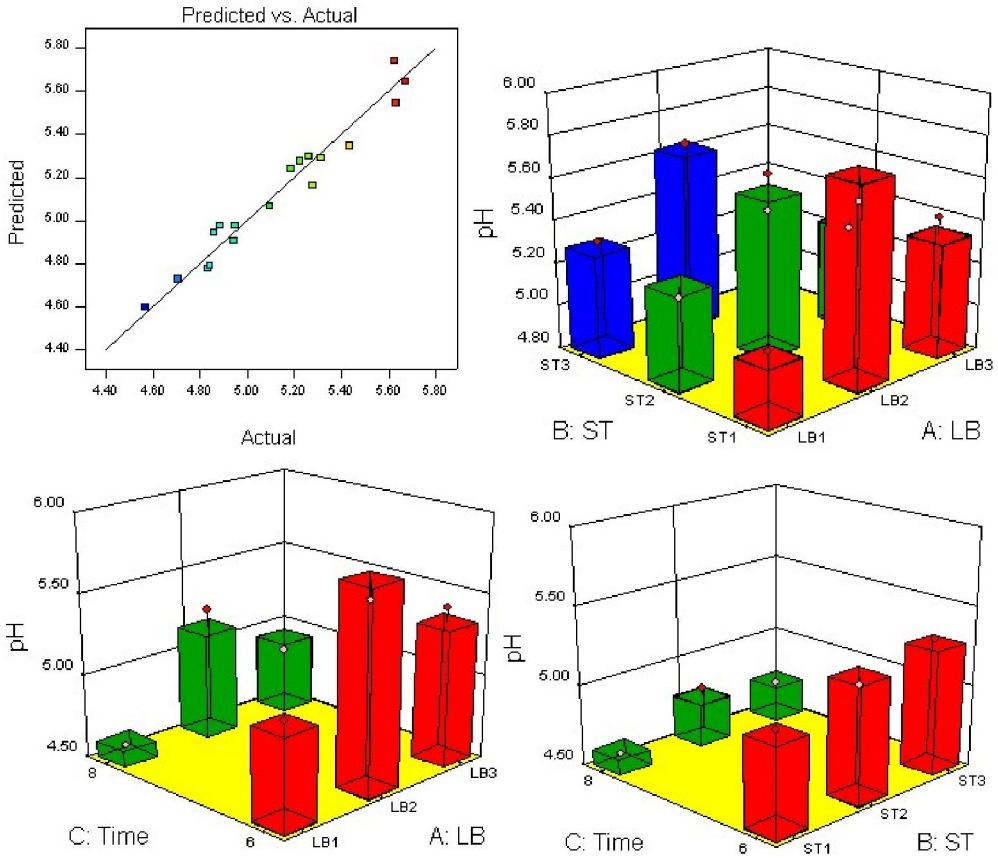

Figure 4. The predicted vs. the actualvalue and 3-D plots of pH

According to the result of Table 3-6 and optimized conditions (the OD value was maximal; sensory evaluation was up to 80%; fermentation acidity was 70ºT; pH 5.0 was better for fermentation), we gained that Figure 5 in which when combination was LB3 and ST2, the desirability is 0.672 that was the highest.

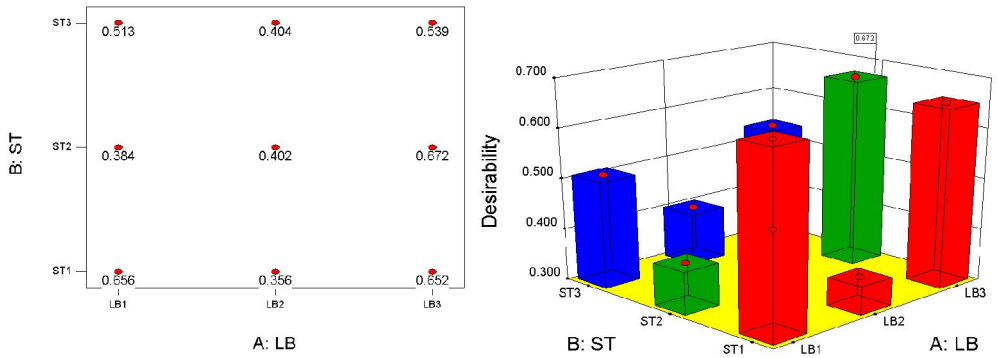

Figure 5. The result of the best group by full factorial experimental design

CONCLUSIONS

The results showed that the freeze-dried LB and ST powders containing both of anti-freeze factors and freeze-drying protective agents had high freeze-drying survival rate, which respectively were 84.7% and 79.7%. In terms of fermentation performance, the compound of LB3 and ST2 was the best group of freeze-dried starter culture for yogurt fermentation.

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