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Research Article

Application of Plackett-Burman Design in Screening of Carbohydrate, Prebiotics/Alcohols and Protein/Amino Acid for Cryoprotectants of *Streptococcus thermophilus*

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Abstract: The carbohydrate, prebiotics/alcohols as well as protein/amino acid have shown important influence on the cryoprotectants for *Streptococcus thermophiles*, this study aimed to screen the main effective factors for cryoprotectants of Streptococcus thermophiles using Plackett-Burman design. Viable counts with cryoprotectants were studied containing eight carbohydrate (glucose, fructose, sucrose, lactose, galactose maltose, trehalose, soluble starch), eleven prebiotics/alcohols (xylooligosaccharide, fructo-oligosaccharide, galactooligosaccharide, isomaltooligosaccharide, stachyose, synanthrin, dextran sulfate, glycerin, sorbitol, mannitol, inositol) and eight protein/amino acid (peptone, yeast powder, casein hydrolysate, glutamic acid, methionine, cysteine, alanine, Vc). The results indicated that sucrose and soluble starch, dextran sulfate, casein hydrolysate among the carbohydrate, prebiotics/alcohols and protein/amino acid affected the cryoprotectants for *Streptococcus thermophiles* markly, which should be appropriately increased in the subsequent experiments.

Keywords: Carbohydrate, cryoprotectants, prebiotics/alcohols, protein/amino acid, *Streptococcus thermophiles*

INTRODUCTION

Probiotics are microorganisms that are beneficial to human with adequate amounts (FAO/WHO, 2002; Guarner and Schaafsma, 1998). In recent years, many probiotics are used in fermented milks, yogurts, ice creams and some pharmaceutical products (Oliveira *et al*., 2012). *Streptococcus thermophiles*, a thermophilic Gram-positive microorganism, is universally applicable to fermented milks and yogurts with *lactobacillus bulgaricus* (Gezginc *et al*., 2013; Wu *et al*., 2003; Iyer *et al*., 2010). Freeze-drying is a common way for a long-term preservation of microorganism, however, not all microorganisms could adapt. Thus, it is crucial to add cryoprotectants to ensure the level of cell viability (Yang *et al*., 2012; Schoug *et al*., 2006; Zdenek, 2003; Capela *et al*., 2006). The key to most bacteria freeze-drying success lies in the appropriate and effective use of cryoprotectants. According to the nature, cryoprotectors are classified into sugar, alcohols, amino acid, protein and peptide, vitamins, salts, etc. Currently, soluble substances cryoprotector is commonly used, such as non-reducing disaccharides, oligosaccharides, sugar alcohol, protein, amino acid and other complex mixtures (Tian *et al*., 2012; Zhang *et al*., 2006b).

Plackett-Burman statistical method is based on non-completely counterbalance principle, efficiently picking out several significantly affected factors from mass, estimating as precisely as possible with minimum experiments. It is generally used to preliminary optimization of experimental process and confirmsation of major factors that affect experimental process. The influence of experimental process due to too much factors or some insignificantly factors in the later stage is avoided (Chauhan *et al*., 2007; Naveena *et al*., 2005). Hence it is an excellent filter method without volume experiments. So time and unnecessary labor, physical and financial resources are saved (Srinivas *et al*., 1994).

In our previous work, the carbon sourses, prebiotics and amino acids for growth of *Streptococcus thermophiles* had been screened by using Plackett-Burman design (Chen *et al*., 2012). The aim of this study was to investigate the effects of various factors on cryoprotectants for *Streptococcus thermophiles* during the process of freeze-drying, which contributed to the further optimization of cryoprotectants formula, as well as provided reference for enhancing bacterial freeze-drying viability.

MATERIALS AND METHODS

Microorganisms and culture conditions: *Streptococcus thermophiles* (ST) were separated and screened preliminarily from College of Life Science and Engineering, Shaanxi University of Science and Technology that are suitable for goat milk fermentation.

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Table 1: Carbohydrate at different levels in Plackett-Burman design

Variables	Factors	Lower level (g/100 mL)	Higher level (g/100 mL)
X1	Glucose	5	10
X2	Fructose	5	10
X3	Saccharose	5	10
X4	Mycose	5	10
X5	Galactose	5	10
X6	Maltose	5	10
X7	Soluble starch	5	10
X8	Lactose	5	10

Table 2: Prebiotics/alcohols at different levels in Plackett-Burman design

Variables	Factors	Lower level (g/100 mL)	Higher level (g/100 mL)
X1	Xylooligosaccharide	5	10
X2	Fructooligosaccharide	5	10
X3	Galactooligosaccharide	5	10
X4	Isomaltooligosaccharide	5	10
X5	Stachyose	5	10
X6	Synanthrin	5	10
X7	Synanthrin	5	10
X8	Glycerin	5	10
X9	Sorbitol	5	10
X10	Mannitol	5	10
X11	Inositol	0.2	0.4

Table 3: Protein/amino acid at different levels in Plackett-Burman design

Variables	Factors	Lower level (g/100 mL)	Higher level (g/100 mL)
X1	Peptone	2	1
X2	Yeast powder	2	1
X4	Casein hydrolysate	2	1
X5	Glutamic acid	1.5	0.75
X7	Methionine	1.5	0.75
X8	Cysteine	1.5	0.75
X10	Vc	1.0	0.5
X11	Alanine	1.5	0.75

Medium preparation: The M17 medium was used in the study, containing casein peptone (Beijing Abxing Bio-tech Co., Ltd.) 5.0 g, fish peptone (Beijing Abxing Bio-tech Co., Ltd.) 5.0 g, beef extract (Beijing Abxing Bio-tech Co., Ltd.) 5.0 g, yeast extract (Beijing Abxing Bio-tech Co., Ltd.) 2.5 g, Vc (Tianjin Fuchen Chemical Reagent Factory) 0.5 g, magnesium sulfate heptahydrate (Xi'an Chemical Reagent Factory) 0.25 g, Glycerin (Tianjin Fuchen Chemical Reagent Factory) 10 g, lctose(Beijing Abxing Bio-tech Co., Ltd.) 5.0 g, dipotassium hydrogen phosphate(Xi'an Chemical Reagent Factory) 5.0 g, agar powder (Beijing Abxing Bio-tech Co., Ltd.) 10.0 g, 1000 mL water. All the media were autoclaved at 118°C for 15 min.

ST fermentation culture medium contained glucose 1 g, peptone 0.75 g, dipotassium hydrogen phosphate 0.2 g, tomato juice(was purchased and made from a local store and kept at 4°C prior to use) 10 mL, Tween-80 0.05 mL, distilled water 90 mL. All the media were autoclaved at 118°C for 15 min.

Cryoprotectant preparation: Sugars and oligosaccharides were moist-heat autoclaved at 115°C for 15 min, amino acid and Vc concentrated solution were autoclaved with 0.22 μm filter membrane at 121°C for 20 min, as well as other concentrated solution were autoclaved at 108°C for 15 min. Each cryoprotectant solution was proposed five concentrations with distilled water.

Screening of composite cryoprotectants using Plackett-Burman design: Plackett-Burman design that comprised 8 factors spanning over 12 runs with each factor fixed at two levels (namely a lower level and a higher level, represent by the +1 and -1, respectively) was used. The ingredients tested are showed in Table 1 to 3. Along with their actual levels which were screened for their significance in viable counts.

Culture conditions: ST was activated in triplicate and cultivated at 42°C for 11 h, centrifuged for 10 min at 8000 r/min. Then cryoprotectants and phosphate buffer that kept equivalent were added to the remaining bacteria, which were freeze-dried after 4-24 h prefreezation. The experiment was completed in triplicate and the survival rate was calculated after freeze-drying.

Analysis method: The viable counts were determined according to plate coating method. The viable counts of freeze-drying bacterial powder should be switch into the original number of viable bacteria per unit volume. Formula: original number of viable bacteria per mL = the average of colonies in three plates at same dilutability×dilution ratio×10.

The survival of cells as well as the viable counts was calculated using the following equation (Huang *et al*., 2006):

$$\text{Survival (\%)} = \frac{\text{viable cells after freeze-drying(cfu/ml)}}{\text{viable cells before freeze-drying(cfu/ml)}} \times 100\%$$

$$\text{Viable counts (cfu/g)} = \frac{\text{viable counts after freeze-drying} \times \text{volume of cell suspension}}{\text{mass of fungus powder before freeze-drying}}$$

Statistical analysis of the data: The PB experiment design, statistical analysis and model found performed by the SAS (Version, 9.1) and Origin (Version, 7.0) to identify the significant factors which were subsequently taken the steepest grade test and the response surface methodology. Then the experiment results were obtained.

RESULTS AND DISCUSSION

Screening of carbohydrate cryoprotectants: In the present study, experiment design and results are shown in Table 4. Detailed experimental results are shown in Table 5. Y_1 that representing survival (%) and Y_2 that

Table 4: The experimental design and results of carbohydrate Plackett-Burman

Number	X1	X2	X3	X4	X5	X6	X7	X8	Survival Y1 (%)	Viable counts of fungus powder Y2 ($\times 10^{11}$ cfu/g)
1	1	-1	1	-1	-1	-1	1	1	64.09	1.93
2	1	1	-1	1	-1	-1	-1	1	45.56	1.04
3	-1	1	1	-1	1	-1	-1	-1	51.55	1.35
4	1	-1	1	1	-1	1	-1	-1	50.56	1.26
5	1	1	-1	1	1	-1	1	-1	40.27	0.93
6	1	1	1	-1	1	1	-1	1	54.36	1.62
7	-1	1	1	1	-1	1	1	-1	72.48	2.13
8	-1	-1	1	1	1	-1	1	1	46.30	0.98
9	-1	-1	-1	1	1	1	-1	1	34.68	0.86
10	1	-1	-1	-1	1	1	1	-1	42.18	0.91
11	-1	1	-1	-1	-1	1	1	1	60.06	1.74
12	-1	-1	-1	-1	-1	-1	-1	-1	52.68	1.48

Table 5: The ANOVA of carbohydrate Plackett-Burman

Variables	Factors	Low level	High level	T-test	Pr> |t|	Importance ranking
X1	Glucose	5	10	-1.365	0.266	6
X2	Fructose	5	10	2.225	0.113	5
X3	Saccharose	5	10	4.208	0.025	2
X4	Mycose	5	10	-2.309	0.104	4
X5	Galactose	5	10	-5.011	0.015	1
X6	Maltose	5	10	0.913	0.428	7
X7	Soluble starch	5	10	2.370	0.099	3
X8	Lactose	5	10	-0.308	0.779	8

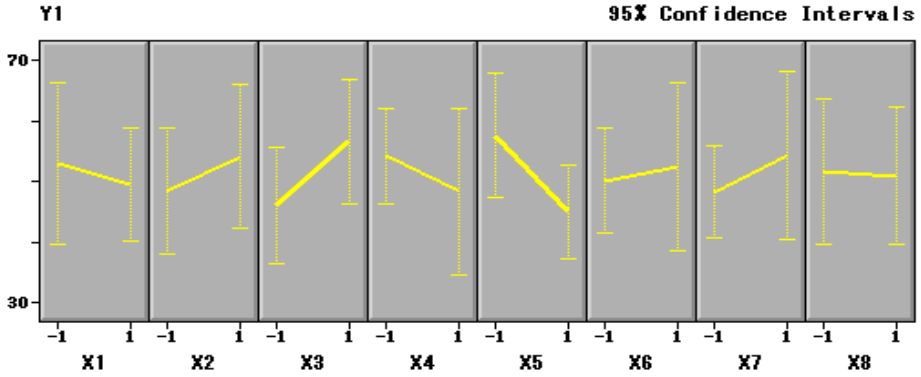

Fig. 1: The 95% confidence interval of carbohydrate

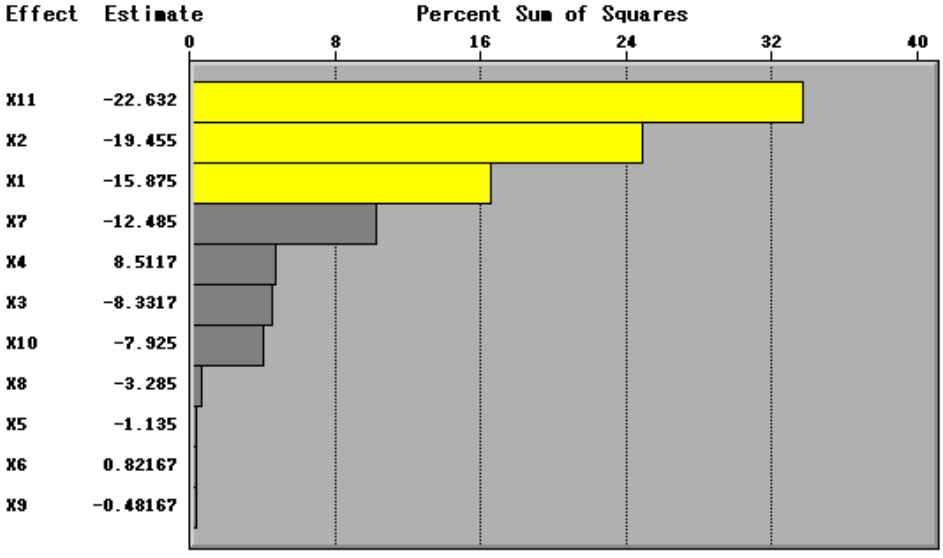

Fig. 2: Main factors figure of prebiotics/alcohols Plackett-Burm

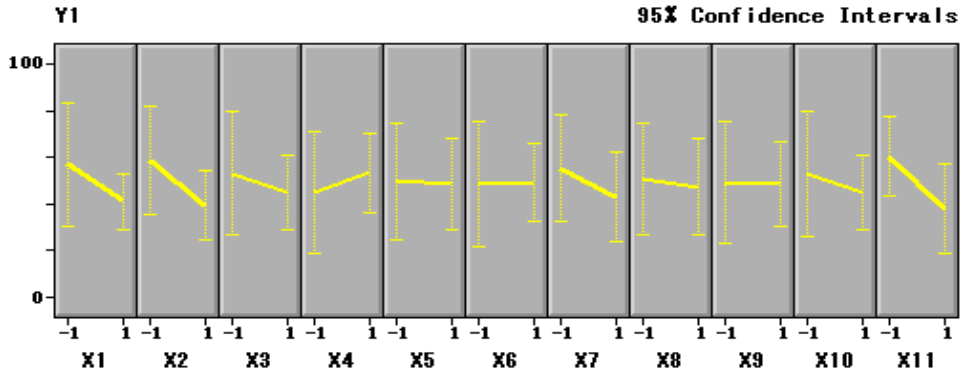

Fig. 3: The 95% confidence interval of prebiotics/alcohols

Table 6: The experimental design and results of prebiotics/alcohols plackett-burman

Number	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Survival Y1 (%)	Viable counts of fungus powder Y2 ($\times 10^{11}$cfu/g)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	27.12	0.83
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	51.70	1.62
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	30.25	1.85
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	44.78	1.20
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	27.14	1.4
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	42.95	1.03
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	58.79	1.93
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	65.56	2.50
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	72.01	1.32
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	53.13	2.44
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	25.25	0.89
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	90.21	2.77

Table 7: The experimental design and results of protein/amino acid Plackett-Burman

Number	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Survival Y1 (%)	Viable counts of fungus powder Y2 ($\times 10^{11}$cfu/g)
1	1	-1	1	-1	-1	-1	1	1	1	-1	1	43.67	1.22
2	1	1	-1	1	-1	-1	-1	1	1	1	-1	58.30	1.73
3	-1	1	1	-1	1	-1	-1	-1	1	1	1	42.50	1.05
4	1	-1	1	1	-1	1	-1	-1	-1	1	1	55.75	1.48
5	1	1	-1	1	1	-1	1	-1	-1	-1	1	60.70	2.25
6	1	1	1	-1	1	1	-1	1	-1	-1	-1	82.50	2.72
7	-1	1	1	1	-1	1	1	-1	1	-1	-1	62.70	2.10
8	-1	-1	1	1	1	-1	1	1	-1	1	-1	48.79	1.28
9	-1	-1	-1	1	1	1	-1	1	1	-1	1	76.50	2.45
10	1	-1	-1	-1	1	1	1	-1	1	1	-1	24.50	0.85
11	-1	1	-1	-1	-1	1	1	1	-1	1	1	33.00	0.91
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	70.00	2.32

representing viable counts of fungus powder (unit 10^{11}cfu/g) were used as response value (Table 5).

As the tables show, galactose ($p = 0.015$), saccharose ($p = 0.025$) and soluble starch ($p = 0.099$) are important factors affecting the survival rate of ST. According to the experimental analysis shown in Fig. 1, the 95% confidence interval of carbohydrate implied that saccharose and soluble starch showed positive effect while galactose showed negative effect, thus, the survial rate of ST could be enhanced when the two variables were used. Chen and Lv (1996) reported that the protection mechanism of carbohydrate may be the stabilization of cell membrane and lipid bilayer.

Screening of prebiotics/alcohols cryoprotectant factors: The experiment design and results are shown in Table 6.

As Fig. 2 shows, inositol, fructooligosaccharide, xylooligosaccharide and synanthrin formed higher percent sum of squares than other 7 factors, so these four factors had significant effects on the survival rate, while the 95% confidence interval of prebiotics/alcohols (Fig. 3) agreed with this result. However, inositol, fructooligosaccharide and xylooligosaccharide have negative effects on the response Y. Y1 and Y2 decreased with the increasing of concentration of these factors. Hence the additive amount of them should be appropriately decreased in the subsequent experiments.

Screening of protein/amino acid cryoprotectant factors and the significance: The experiment design and results are shown in Table 7.

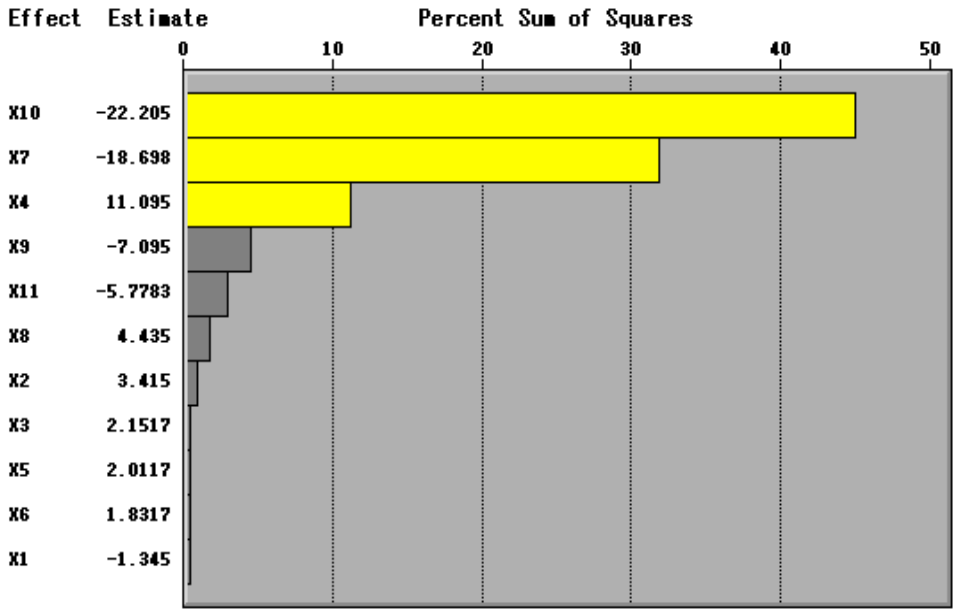

Fig. 4: Main factors figure of protein/amino acid Plackett-Burman

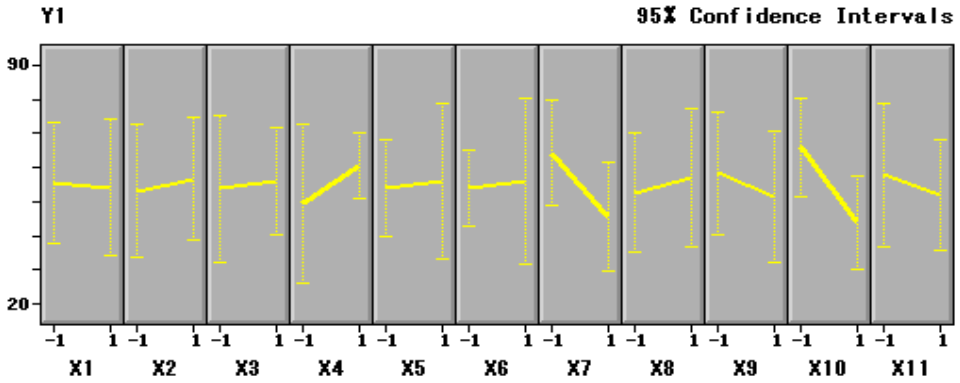

Fig. 5: The 95% confidence interval of protein/amino acid

Analysis of dates for the percent sum of squares revealed presence of Vc, methionine and casein hydrolysate accounted for a large proportion of it and it indicated that these three factors had a significant impact and Vc was the most significant factor (Fig. 4). The 95% confidence interval of protein/amino acid showed the same results (Fig. 5). In addition, casein hydrolysate had positive effects on response Y1 and Y2, which means response Y1 and Y2 increased with the increase of additive amount. On the contrary, While Vc and methionine showed negative trend. The study of Zhang *et al*. (2006a) also reported that casein hydrolysate could make *Lactobacillus* a higher survival rate.

CONCLUSION

With PB design as well as path steepest ascent and response surface methodology, the optimum cryoprotectants factors have been selected. Sucrose and soluble starch, dextran sulfates and casein hydrolysate are cryoprotectant significantly effective factors of carbohydrate, prebiotics/alcohols and protein/amino acid, respectively. Hence the dosage of each factors should be appropriately increased in the subsequent experiments. While the negative trend of galactose, inositol, fructooligosaccharide, xylooligosaccharide, Vc and methionine suggested that these substances must be limited.

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