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A BOX-BEHNKEN EXPERIMENTAL DESIGN IN THE DEVELOPMENT OF OPTIMIZED MEDIUM FOR *STREPTOCOCCUS THERMOPHILUS*

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ABSTRACT

Streptococcus thermophilus had been wildly used in food industries especially in milk productions. The objective of present work was to study the combine effects of soybean peptone, casein hydrolysate and glutamate on the viable counts of *Streptococcus thermophilus* in the medium and realize medium was cultured with high activity and density for bacteria. A Box-Behnken design was applied to perform the experiments and regression analysis. The viable counts of *Streptococcus thermophilus* can reach high at $(1.91\pm0.07)\times10^9$cfu/mL at the the optimum medium with soybean peptone 3%, casein hydrolysate 1%, glutamate 0.0015%, glucose 1%, K_2HPO_4 0.2%, Tomato juice 10%, Tween-80 0.05% and pH 6.8, respectively, which is consistent with the predicted values of the regression model. The results showed that the model used is feasible and adequate and it could provide theoretical basis for milk productions.

1. Introduction

Probiotics are a type of beneficial microorganisms and they had multiple functions like treat atopic dermatitis, balance intestinal environment, food allergy, cancer preventive, lactose intolerance, acute gastroenteritis, crohn's disease, change microbial community structure, et al. (Marco, et al., 2006; Million and Raoult, 2012; Tatsuya, et al., 2015). Probiotic bacteria had been widely applied in various industries like food, shrimp farms, and health production especially for dairy productions (Paulraj, et al., 2013). *Streptococcus thermophilus* (S. *thermophilus*) and *Lactobacillus bulgaricus* are the most common probiotics that used as yogurt starter in the dairy productions. They have a proto-cooperation interaction in yogurt and which combine metabolism with positive effects on the dairy product and other fermented products. (Pette and Lolkema, 1950; Angelov, et al., 2009). Furthermore,

Streptococcus thermophilus can significantly reduce the amount of serum total cholesterol and low density lipoprotein cholesterol (Akalin, et al., 1997). In addition, *Streptococcus thermophilus* can relieve the lactose intolerance and has the ability to inhibit tumor. In general, yogurt consumption was mainly lied on probiotic effects especially for these effects of number of live bacteria (Shihata and Shah, 2000). So the aim of the present study was to obtain the medium with the cultivation of high activity and high density for bacteria.

In our previous work, *Streptococcus thermophilus* which was suitable for the fermentation of goat milk have been screened out from commercial milk (Chen, et al. 2010). The effects of inoculum and temperature on the

fermentation of goat yogurt by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* have been studied (Shu, et al., 2014). And optimum nitrogen sources, carbon sources/prebiotics and amino acids in the medium for *Streptococcus thermophilus* had been screened using Plackett-Burman design (Chen, et al., 2012; Chen, et al 2013). All of the research above provided a good theoretical and practical basis for the study of the present work. The objective of the present work was to apply a Box-Behnken design to study the effects of soybean peptone, casein hydrolysate and glutamate on the viable counts of *Streptococcus thermophilus* and determine the best composition of the medium.

2. Materials and methods

2.1. Materials

The strain used in this work, *Streptococcus thermophilus* was kindly provided by School of Food and Biological Engineering, Shaanxi University of Science and Technology. Soybean peptone, casein hydrolysate and glutamate were purchased from Sigma Chemical Co., USA. The chemicals used in the experiments were of analytical grade. The medium used for the cultivation for s*treptococcus thermophilus* were: glutamate 1g, peptone 0.75g, yeast 0.75g, potassium dihydrogen phosphate 0.2g, Tomato Juice 10mL, Twain-80 0.05mL and distilled water 90mL, and cultured at 118℃ for 15 min.

2.2. Preparation of starter

3-5% activated *Streptococcus thermophilus* power was inoculated into M17 medium and cultured at 42℃for 24 h. Microscopic judgment was conducted to make sure the viability of bacteria is stable until repeating experiments several times. It should pay attention that the bacteria be reactivated to maintain a high activity if it was not used after 7 days.

2.3. Analysis method

The pH of medium was directly measured through a pH-meter (pHS-3C Shanghai Precision Scientific Instrument Co., Ltd, Shanghai) at the room temperature and OD value was evaluated by ultraviolet spectrophotometer with version SP-756PC at the condition of 600nm. The viable counts of *Streptococcus thermophilus* were determined by plate coating method. Bacteria growth was determined by inoculating 2% bacteria into the optimum medium and cultured for 24h under anaerobic conditions. The pH and viable counts of samples were determined each 2h and each point were conducted in triplicate and average values were used.

2.4. Optimization of process parameters using response surface method

Three operational parameters (soybean peptone, casein hydrolysate and glutamate) important in the medium were researched using Box-Behnken design (Box and Behnken, 1960) with three levels as shown in Table 1, coded -1, 0 and 0 for low, middle and high concentrations (or values), respectively.

Table 1. The factors levels of Box-Behnken experimental design

Factors	Coded variable levels		
	-1	0	1
X_1 Soybean peptone (%)	2.4	3.0	3.4
X_2 Casein hydrolysate (%)	0.8	1.0	1.2
X_3 Glutamate (mg/L)	12	15	18

In order to obtain the optimal point, the relationship between independent variables and response value was fitted the second-order polynomial model and the polynomial equation represents in the following form:

$$Y=\beta_0+ \beta_1X_1+ \beta_2X_2+ \beta_3X_3+ \beta_{11}X_1^2+ \beta_{22}X_2^2+ \beta_{33}X_3^2 + \beta_{12}X_1X_2+ \beta_{13}X_1X_3+ \beta_{23}X_2X_3 \quad (1)$$

Where Y is the response value (viable counts of Streptococcus *thermophilus*), X_1, X_2 and X_3 are the independent variables corresponding to the concentration of soybean peptone, casein hydrolysate and glutamate, respectively. β_0 is the constant coefficient. β_1, β_2 and β_3 are liner coefficients. β_{11}, β_{22} and β_{33} are the quadratic coefficients. β_{12}, β_{13} and β_{23} are the interaction terms coefficients.

Statistical software SAS was used for the regression analysis of the experimental data

obtained. Analysis of variance (ANOVA) was employed to test the statistical significance of the regression equation coefficients by Box-Behnken design. The fitting of the second-order model equations was determined by the coefficient of determination (R^2) and a $p<0.05$ was considered statistically significant for all analysis.

3. Results and discussion

3.1. The results and analysis of the Box-Behnken design

The fifteen experimental results of the Box-Behnken design consisting of 12 trials plus 3-center points is summarized and listed in Table 2. X_1, X_2 and X_3 represent soybean peptone, casein hydrolysate and glutamate, respectively. The number of viable counts of *Streptococcus thermophilus* was represented by Y ($\times 10^9$ cfu/mL). The response values are OD, pH and Y, respectively. There is not a significant difference between OD and pH value as shown in Table 2. Hence, the major influence of medium was measured by Y.

The regression equation obtained according to the results of Box-Behnken experimental design and applying multiple regression analysis could be gotten as follows: $Y_1=1.953-0.031*X_1+0.016*X_2+0.072*X_3-0.230*(X_1)^2+0.042*X_1*X_2+0.295*X_1*X_3-0.115*(X_2)^2-0.125*X_2*X_3-0.178*(X_3)^2$ (2)

Table 2. The experimental design and results of Box-Behnken

Number	X_1	X_2	X_3	OD	pH	$Y/10^9$cfu/mL
1	-1	-1	0	0.920	4.09	1.61
2	-1	1	0	0.946	4.13	1.63
3	1	-1	0	0.946	4.17	1.50
4	1	1	0	0.943	4.17	1.69
5	0	-1	-1	0.929	4.15	1.50
6	0	-1	1	0.938	4.17	1.86
7	0	1	-1	0.943	4.16	1.71
8	0	1	1	0.947	4.17	1.57
9	-1	0	-1	0.889	4.13	1.80
10	1	0	-1	0.927	4.22	1.11
11	-1	0	1	0.021	4.16	1.39
12	1	0	1	0.918	4.22	1.88
13	0	0	0	0.894	4.25	2.04
14	0	0	0	0.913	4.27	1.93
15	0	0	0	0.899	4.25	1.89

ANOVA of the regression model was performed to see significance of the main effects and interaction effects of independent variables on viable counts of *Streptococcus thermophilus*. The liner coefficients of the regression equation for parameters X_1, X_3 are larger, which indicates that there is not a simple linear relationship between soybean peptone, glutamate on the viable counts of *Streptococcus thermophilus.* The negative effects of liner and quadratic coefficients of X_1 in the regression equation indicate that a decrease of viable counts occurs at a low level of parameter X_1. The interaction coefficient of X_1X_3 is bigger than others' showing that the interaction of soybean peptone and glutamate had a great influence on the growth of bacteria. It is also evident that the interaction effects of X_1X_3 is extremely significant with probability value (p=0.001) as shown in Table 3.

The influence of various factors on the response of the *Streptococcus thermophilus* was determined by F-test. The regression model was found to be very significant, as is

evident for F-test with a very low probability

The probability value for the lack-of-fit was found to be non-significant with high failure of a model to obtained data in the experimental value (p=0.004<0.01), shown in Table 3. The range at points which not containing in the regression was evaluated by Lack-of-fit test.

Table 3. The ANOVA of regression equation of Streptococcus *thermophilus*

Source	DF	SS	MS	F	Pr > F	Sig
X1	1	0.009	0.009	1.403	0.289	
X2	1	0.002	0.002	0.379	0.565	
X3	1	0.042	0.042	7.552	0.040	*
X1*X1	1	0.196	0.196	35.205	0.002	**
X1*X2	1	0.007	0.007	1.298	0.306	
X1*X3	1	0.348	0.348	62.514	0.001	***
X2*X2	1	0.049	0.049	8.833	0.031	*
X2*X3	1	0.063	0.063	11.224	0.020	*
X3*X3	1	0.117	0.117	20.990	0.006	**
model	9	0.789	0.088	15.742	0.004	**
Liner	3	0.052	0.017	3.111	0.127	
Quadratic	3	0.319	0.106	19.103	0.004	**
Cross	3	0.418	0.139	25.012	0.002	**
Error	5	0.028	0.006			
Lack of fit	3	0.016	0.005	0.872	0.574	
Pure error	2	0.012	0.006			
Total	14	0.817				

Note: *** P<0.001, extremely significant, ** P<0.01, very significant; * P<0.05, significant.
DF degree of freedom, SS sum of squares, MS mean square, F and Pr means F and P values, respectively.

Probability value was 0.574, which further tested that the quadratic model was statistical effective and feasible. Therefore, the optimum ratio of three factors can be obtained by using the regression equation. The determination coefficient R^2 and multiple correlation coefficients R^2 could measure the goodness of the regression model. The value of R^2 (0.9659) obtained from the present model indicating that the experimental and predicted values of the response had a good correlation. The adjusted R-squared can evaluate the amount of variation around the mean explained by the model adjusted for the number of terms. The value for adjusted R-squared (R^2_{adj}=90.46%) suggested the fitting degree of the regression equation and the experimental data is 90.46%, and the reliability is high. Furthermore, the F-value for first-order X_3 and quadratic X_1^2, X_3^2 are all relatively big which suggesting the there is not a simple liner relationship between the parameters X_1, X_3 and the response value. The effects of different independent variables on the viable counts of *Streptococcus thermophilus* at different levels are described in figure 1. The quadratic items of X_1^2 (P=0.002), X_2^2 (P=0.031) and X_3^2 (P=0.006) all are very significant. The viable counts of *Streptococcus thermophilus* increased first and then decreased sharply with the increase of soybean peptone

concentration, while the effects of casein hydrolysate on the viable counts of bacteria are relatively mild. When the concentration of casein hydrolysate was at a low level, the dependent variable increased slowly. The response value of Y decreased gradually when it reached its maximum value. Similarly, the response value of Y increased greatly and then decreased with the increase of parameter of glutamate. The rule was found out that the maximum value of Y occurs at inflection point of the three factors.

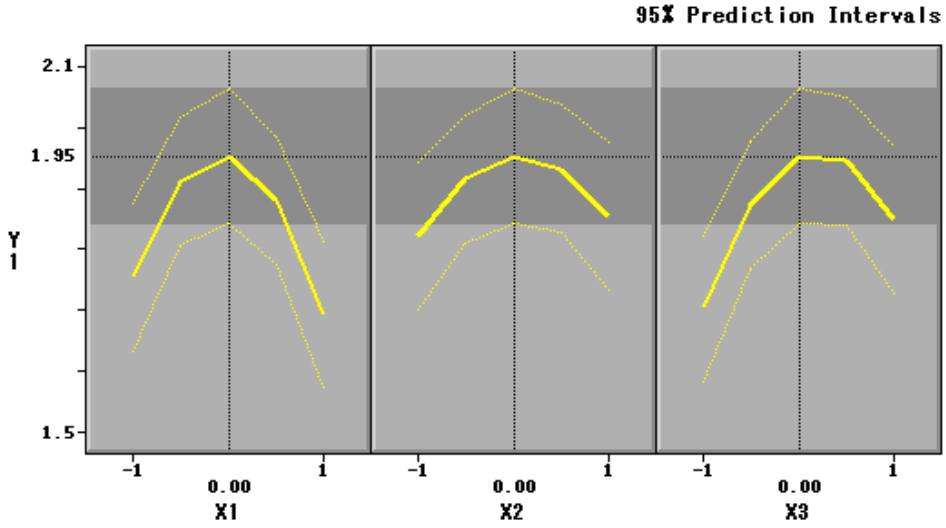

Figure 1. The trends of response value Y with factors

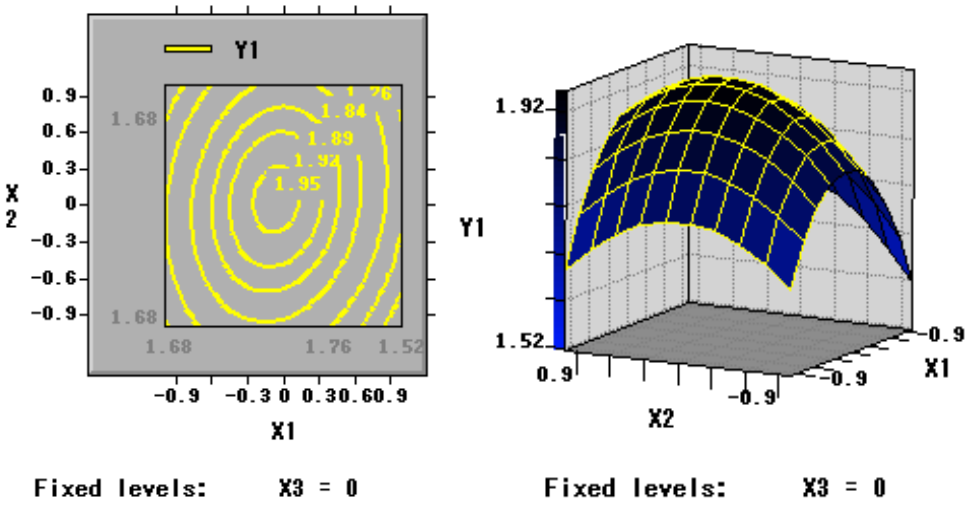

Figure 2. Response surface and contour plots of *S. thermophilus* as a function of X_1 and X_2

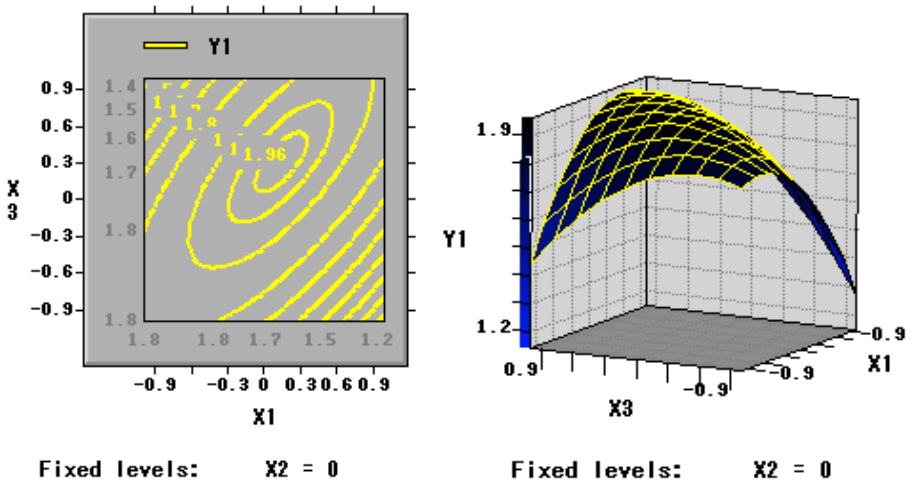

Figure 3. Response surface and contour plots of *S. thermophilus* as a function of X_1 and X_3

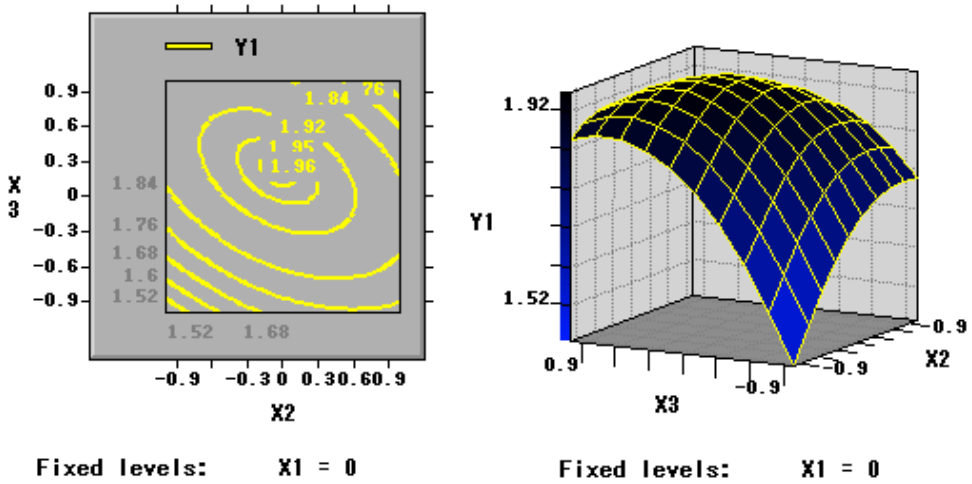

Figure 4. Response surface and contour plots of *S. thermophilus* as a function of X_2 and X_3

The aim of the present study was to track efficiently for the optimum medium for the growth of *Streptococcus thermophilus* which is the best and maximal for the bacteria. The best response range can be calculated by analyzing the plots according to the Box-Behnken design results. Response surface and contour plots was used to measure viable counts of bacteria for a pair of interactive variables including soybean peptone, casein hydrolysate and glutamate (Figures 2-4). The interaction effects of the factors on the response value are larger when the shape of the plots is oval, while the circular indicates that the interaction effects are light. One parameter is fixed to zero level, and other two parameters are investigated to study their interaction effects on the growth of *Streptococcus thermophilus.* As is shown in Fig.2, when the amount of glutamate was constant, the viable counts of *Streptococcus thermophilus* increased consistantly until reached it's maximal value and then decreased with the increase of the concentration of soybean peptone and casein hydrolysate. This is due to the high concentration of nitrogen source will inhibit the bacteria growth. The contour plots is not like oval indicating the effects of factors X_1 and X_2 is not significant, which further verified the analysis of the regression model.

Figures 3 showed that when the casein hydrolysate is constant, the viable counts of *Streptococcus thermophilus* increased first and then showed a downward trend with the

increase of soybean peptone and glutamate. This is because excess soybean peptone and glutamate are not conducive to the growth of bacteria. At the same time, the contour plots of *Streptococcus thermophilus* as a function of X_1 and X_3 have oval-shaped meaning that the two factors had a strong interaction effects on the viable counts of bacteria. This is consistent with the results of analysis of variance of the regression equation. Figures 4 described when the soybean peptone was at 3 g/100mL, the viable counts of bacteria was the same trend with the figure 3 described. Similarly, the interaction effects of casein hydrolysate and glutamate on the response value was relatively strong as it is shown in the contour plots of *Streptococcus thermophilus* as a function of X_2 and X_3, and the contour plots for it is oval.

Statistical software SAS was used to analysis the regression model and take the derivative for independent variables of X_1, X_2 and X_3 to obtain the maximal value of viable counts. The corresponding point of the code (0, 0, 0) for maximal value represent the level of the actual value, soybean peptone 3g/100mL, casein hydrolysis 1g/100mL and glutamate 1.5mg/100mL, respectively. The model predicts the viable counts of *Streptococcus thermophilus* was 1.95×10^9 cfu/mL under these optimum conditions. Three repeating experiments were conducted to test the predicted values in the optimum medium. The mean value of the three groups was $(1.91\pm0.07)\times10^9$ cfu/mL, which was very close to the predicted values. The results show that the mathematical model obtained by the response surface method can fit well with the experimental data.

3.2. Analysis of growth curve for Streptococcus thermophilus

The growth curve for *Streptococcus thermophilus* was determined to study the ability of growth, reproduction and acid producing for bacteria in the medium. The growth curve of *Streptococcus thermophilus* could evaluate if there is a difference in the medium optimized or not. Taking time as a horizontal coordinate, and pH, viable counts of bacteria as longitudinal coordinate, the growth curve plot of *Streptococcus thermophilus* was drawn as shown in Figures 5.

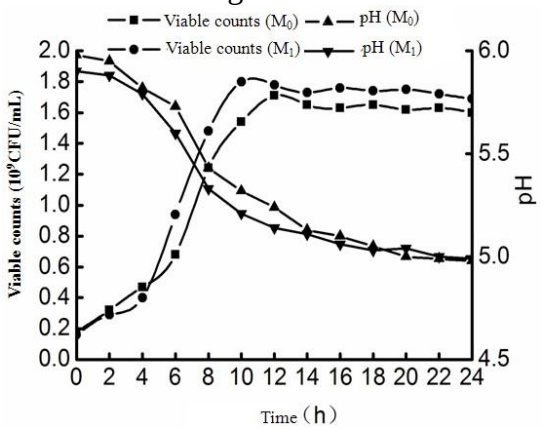

Figure 5. The growth curve and pH of Streptococcus thermophilus in the medium

As it shown in Figures 5, *Streptococcus thermophilus* began to enter the exponential growth period after the adjustment of beginning 4h. The bacteria started to grow, propagate and divide; the culture fluid became turbid; the number of viable bacteria increased and the pH value decreased significantly in this period. The bacteria entered a period of stability at 14-20h where the newly bred cells were roughly equal to the dying cells. While after 20 h, the cell began to enter a period of decline and the cell death rate is greater than the propagation rate which suggesting that environment is not conducive to bacterial growth. Besides, the whole cell populations showed a negative growth and viable counts of bacteria decreased significantly. At the same time, it can be seen that the viable counts of bacteria may reach 1.61×10^9 cfu/mL, 1.95×10^9 cfu/mL before and after the medium was optimized at 12h. The optimum medium for the bacteria is about 1.2 times higher than that of not optimized. The response surface method using to optimize the medium for *Streptococcus thermophilus* proved to be feasible and reliable for improving the viable counts.

3.3. Discussions

The articles about the effects of the composition and conditions of medium on the survival rate of the bacteria had been reported a

lot. Such as, Carvalho (Carvalho, et al., 2002) reported that the sugar, amino acids and sugar alcohols and other substances could improve the survival rate of *Lactobacillus bulgaricus* and *Lactobacillus plantarum* for the reason that small molecules had advantages of containing more than one hydrogen bond and ionizable groups, which can stop bacterial exposure to the medium by linking to the surface of the bacteria. And at pressure conditions such as low temperature, low pH and low water activity, the bacteria will easily died. While Beney (Beney and Gervais, 2001) found a different conclusion, the bacteria could form tolerance and accumulate the permeability of protective substances under pressure conditions, which can give a help of bacteria to establish a new balance of osmotic equilibrium and improve the viable counts of the bacteria. The strains used in the above research were different may caused the difference of the two conclusions. Furthermore, Carvalho (Carvalho, et al., 2003) study found that sugar (such as glucose, fructose, lactose, mannose, sucrose, et al.), sugar alcohols (sorbitol, inositol, et al.), non-reducing sugars (trehalose) was added in the medium, and it can play a protective role for bacteria during the freeze-drying process. Gao's (Gao, et al., 2008) study of enrichment medium for *Lacabacillus casei* Zhang had reached a conclusion that the optimum composition of the medium were: glucose 20.9g/L, soybean peptone 10.45g/L, yeast extract 10.45g/L, K_2HPO_4 3.5g/L, sodium citrate 2.35g/L, sodium acetate 14.6g/L, $MnSO_4 \cdot 5H_2O$ 54mg/L, $MgSO_4 \cdot 7H_2O$ 1.0g/L, $CuSO_4 \cdot 5H_2O$ 10mg/L, tween 80 1.0g/L, and the viable counts of *Lacabacillus casei* Zhang reached 4.78×10^9 cfu/mL, which was 10 times higher than MRS. Similarly, Zhang (Zhang and Yu, 2007) had screened and studied the culture medium for *Lactobacillus acidophilus*, and the viable counts could reach high at 2.2×10^9 cfu/mL. In our present study, soybean peptone, casein hydrolysate and glutamate were chosen as the enrichment factors, and the viable counts of *Streptococcus thermophilus* can reach at 1.95×10^9cfu/mL, which was 1.2 times than that of not been optimized (1.61×10^9cfu/mL).

4. Conclusions

An optimization of medium for improving the viable counts of *Streptococcus thermophilus* was conducted using Box-Behnken design experiment. ANOVA was used to test the predicted model. The viable counts of *Streptococcus thermophilus* can reach high at $(1.91\pm0.07)\times10^9$ cfu/mL at the the optimum conditions with soybean peptone 3%, casein hydrolysate 1%, glutamate 0.0015%, glucose 1%, K_2HPO_4 0.2% Tomato juice 10%, Twain-80 0.05% and pH 6.8, respectively. The predicted value (1.95×10^9 cfu/mL) is very close to the verification value confirming that regression model used for the experiments is useful. Moreover, the growth curve of the *Streptococcus thermophilus* had been analyzed in 24h before and after the medium optimized. And the viable counts of *Streptococcus thermophilus* can reach at 1.95×10^9 cfu/mL after it cultured for 12 h at the optimum medium which was 1.2 times than that values without optimization, besides, stable period of the bacteria take in advance. The optimum medium has obvious effects on the growth of bacteria, so the optimum medium for *Streptococcus thermophilus* using response surface methodology is proved to be feasible and effective.

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