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10.1515/aucft-2017-0001

# OPTIMIZATION OF CULTURE MEDIUM FOR *LACTOBACILLUS BULGARICUS* USING BOX-BEHNKEN DESIGN

– Research paper –

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**Abstract:** *Lactobacillus bulgaricus* is a common yogurt starter in dairy production. But the viable counts of the bacteria in the productions are relatively low during free-drying and storage which is not good for its commercial production. In order to obtain a medium with high activity and high density for bacterial cultured, the experiments and regression analysis were conducted by.Box-Behnken design in this study, and a model was established to predict the influence of glucose (9-11 $g \cdot L^{-1}$), casein hydrolysate (15-17 $g \cdot L^{-1}$) and glutamate (6.5-7.5 $mg \cdot L^{-1}$) on viable counts of *L. bulgaricus*and. The results showed that the glucose, 9.5 $g \cdot L^{-1}$; casein hydrolysate, 15.5 $g \cdot L^{-1}$; glutamate, 7.0$mg \cdot L^{-1}$, the number of viable bacteria of *L. bulgaricus* could reach (2.95±0.07) $\times 10^9$, which was very similar to the predicted value of the model of $3.00 \times 10^9$ $cfu \cdot mL^{-1}$, indicating that the optimized conditions and models used were feasible and effective. The optimized medium components can improve the viable counts of bacteria which are useful from its application in industrial production.

**Key words:** casein hydrolysate, probiotics, glutamate, *Lactobacillus bulgaricus*, yogurt

## INTRODUCTION

Probiotics had been widely used in food industries for it is a type of beneficial microorganisms that can improve the host's micro-ecological balance (Million and Raoult 2012).Most of the lactic acid bacteria are indispensable in the human body and has important physiological function, which is widely found in the human intestinal tract. And which can increase intestinal beneficial bacteria, balance intestinal environment, improve immunity, and protect human health, etc. (An et al, 2010; Maragkoudakis et al, 2010).

The key to the development of the *Lactobacillus bulgaricus* starter is to obtain high density and high activity culture medium. And by adding proliferation factors in medium can increase the bacteria survival rate and maintain the biological activity. Where the glucose can provide an additional carbon source for bacterial growth when lactose was insufficient, but the concentration of glucose is too high or too low all not well for the growth of bacteria (Meng et al, 2009). The Casein hydrolysate has a function of promoting the proliferation and metabolism of the bacteria. Glutamate is important for protection of cells during freeze-drying (Chen et al, 2013). The amino acids can induce cellular synthesis of anti-freeze proteins. Anti-freeze protein can make the freezing point of the solution inconsistent, inhibiting occurrence of eutectic and recrystallization phenomenon, so as to improve the cell survival rate in the process of freeze-drying (Miao et al, 2016).

In the process of freeze-drying and storage, the growth medium had a great influence on the survival rate of the cells. For example, in comparison to MRS broth medium, the optimal medium containing glusose, sodium pyruvate, meat extract, potassium phophate, sodium acetate, and ammonium citrate for *Lactobacillus*

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*rhamnosus* PEN showed better growth activity (Magdalena et al 2010).

The preparation of suitable enrichment medium is the basis of the cultivation of bacteria with high density of cells. The ideal enrichment medium should meet the requirements of high cell production, easy to concentrate and separate cells, raw materials and low price. However, the production cost of the traditional MRS medium is higher than optimal medium. While the lactic acid bacteria grew well in the culture medium of the matrix and whey, but there were problems that viscosity was large and the bacteria were not easy to be separated (Paulo et al, 2012). So the composition and concentration of enrichment medium will have a great influence on the number of viable counts of bacteria and lay the foundation for the development of yogurt fermented products.

In our previous study, The *Lactobacillus bulgaricus* which is suitable for goat milk fermentation was screened from commercial yogurt (Chen et al, 2010). Plackett-Burmann design was used to screen multiplication medium of *Lactobacillus bulgaricus,* glucose, casein hydrolysate and glutamate was well for the growth of *Lactobacillus bulgaricus* (Chen et al, 2012; Chen et al, 2014). In our present research, the effect of medium composition on the number of viable counts for *L. bulgaricus* is studied. Response surface method is used to perform and analyse the experiments. The optimum medium could provide the technical basis for the development and industrialization of goat yogurt production.

## MATERIALS AND METHODS

**Microorganism and medium preparation:** A strainof *L. bulgaricus* was a gift from the School of Food and Biological Engineering, Shaanxi University of Science and Technology. MRS (Lactose) medium and MRS agar medium was used for strain activation and the determination of the viable counts, respectively. Among them, MRS (Lactose) medium and MRS agar medium were purchased from a local retail market (Hopebio, Qindao, China). The reagents used in the test were all of analytical grade.

**Strain activation and cultivation:** MRS medium was sterilized at 118℃ for 15min, and inoculating with 2% *Lactobacillus bulgaricus* to MRS medium to culture at 37℃for 24h under sterile conditions.Then according to the results of microscopic analysis, repeated the experiments until the vitality of bacteria remained stable. When the strain was not used for more than 7 days, it needed to be reactivated.

**Measurement of pH, OD value and viable counts**: The pH was measured at room temperaturebythe pH-meter (pHS-3C) and OD value was measured through UV-visible spectrophotometer SP-756PC (Dario and Gines, 2005) at 600nm.Viable counts were determined by plate counting method (Shu et al, 2014).

**Design of experiments:** The SAS software was used to evaluate responses of the independent variables by model analysis of the gained data, and for experimental design. The response surface method was used to show the statistical significance of the composition of glucose (carbon source), casein hydrolysates (nitrogen source) and glutamate (amino acids) on the viable counts of *Lactobacillus bulgaricus*. To determine the effect of glucose, casein hydrolysate and glutamate on the number of cfu per ml of surface, Box-Behnken design (Box and Behnken 1960) was used. The lowest and the highest levels of the above parameters were given in Table1.

Table 1. The experimental design levels of selected variables

| Variable parameter | Levels | | |
|---|---|---|---|
| | **-1** | **0** | **1** |
| $X_1$ Glucose [$g \cdot L^{-1}$] | 9 | 10 | 11 |
| $X_2$ Casein hydrolysate [$g \cdot L^{-1}$] | 15 | 16 | 17 |
| $X_3$ Glutamate [$mg \cdot L^{-1}$] | 6.5 | 7.0 | 7.5 |

An approximate polynomial relationship for response value of viable counts of *Lactobacillus bulgaricus* based on the experimental results was obtained. A second-order polynomial model which includes all interaction terms was defined to fit the response:

$$Y_1=\beta_o+\sum \beta_i X_i+\sum \beta_{ii} X_i^2+\sum \beta_{ij} X_i X_j \quad (1)$$

where $Y_1$ represents the predicted viable counts

of *L. bulgaricus*, $ß_o$ represents the constant, $ß_i$ represents the linear terms, $ß_{ii}$ is quadratic terms and $ß_{ij}$ is interaction terms; $X_i$ and $X_j$ represent the independent variables.

**Statistical analysis of the data:** The responses were evaluated by model analysis of the test data. The coefficient of determination ($R^2$) was used to assess the fitting of second-order regression equations, and the statistical significance was described by analysis of variance and F test ($p$<0.05) for all analysis.

## RESULTS AND DISCUSSIONS

### Effects of casein hydrolyse, glutamate and glucose on bacteria viable counts

Table 2 shows that the experimental values obtained for the viable counts of *L. bulgaricus* under the various conditions. Theglucose, casein hydrolysate, glutamate and the viable counts were represented by $X_1$, $X_2$ , $X_3$and $Y_1$ ($\times 10^9$cfu·mL$^{-1}$), respectively. The response values are pH and $Y_1$, respectively.

### Regression analysis of the data

The experimental results obtained from the Table 2 were analyzed by variance analysis. The variance analysis of regression equation was analyzed using response surface methodology which is an empirical modeling technique, and it could evaluate the relationship between the experimental factors and obtained results. Besides, a prior knowledge of the process is required to get a statistical model. The second-order regression equation provided the levels of viable counts of *Lactobacillus bulgaricus* as a function of glucose, casein hydrolysate and glutamate can be described by the following equation:

$$Y_1=2.98+0.167X_1-0.15X_2-0.11X_3-0.42X_1^2+0.30X_1X_2+0.01X_1X_3+0.40X_2^2+0.10X_2X_3+0.43X_3^2 \quad (2)$$

where $X_1$, $X_2$, $X_3$ and $Y_1$ are represents independent variables of glucose, casein hydrolysate, glutamate and the numbers *L. bulgaricus,* respectively. Coefficients of linear and quadratic for parameter $X_1$, $X_2$ and $X_3$both are very big indicating that the effects of three independent variables on response value $Y_1$ are not a simple linear relationship. The regression coefficient of the interaction terms $X_1$*$X_2$ is also larger as compared with other parameters, indicating that they have significant effects on the growth of *L. bulgaricus*.

Table 2. The test design and results of viable counts of *L. bulgaricus*

| Number | $X_1$ | $X_2$ | $X_3$ | $Y_1$($\times 10^9$cfu·mL$^{-1}$) | pH |
|---|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 1.95 | 4.55 |
| 2 | -1 | 1 | 0 | 2.16 | 4.54 |
| 3 | 1 | -1 | 0 | 2.74 | 4.41 |
| 4 | 1 | 1 | 0 | 1.77 | 4.59 |
| 5 | 0 | -1 | -1 | 2.33 | 4.44 |
| 6 | 0 | -1 | 1 | 2.19 | 4.45 |
| 7 | 0 | 1 | -1 | 2.30 | 4.71 |
| 8 | 0 | 1 | 1 | 1.78 | 4.71 |
| 9 | -1 | 0 | -1 | 1.94 | 4.49 |
| 10 | 1 | 0 | -1 | 2.43 | 4.50 |
| 11 | -1 | 0 | 1 | 1.86 | 4.36 |
| 12 | 1 | 0 | 1 | 2.30 | 4.40 |
| 13 | 0 | 0 | 0 | 2.96 | 4.46 |
| 14 | 0 | 0 | 0 | 2.93 | 4.47 |
| 15 | 0 | 0 | 0 | 3.04 | 4.47 |

Table 3 lists the results of variance analysis of regression model of *L. bulgaricus* and tests the significance of the coefficient. Table 3 shows the low Pr>F value for *Lactobacillus bulgaricus*

($p$=0.003<0.01) which hasF-value (17.696) demonstrated the regression model was extremely significant, and the lack of fit ($p$>0.05) is insignificant showing the regression model is feasible. Furthermore, the probability value of liner, quadratic and cross interaction terms for the model are 0.014, 0.001 and 0.023, respectively, indicating that regression model is reliable and feasible. Moreover, all factors examined that significantly affected the viable counts for *Lactobacillus bulgaricus* except $X_3$ , $X_1*X_3$ and $X_2*X_3$, and the model equation as expressed in Eq. (2) is confirmed to be suitable to describe the response value of $Y_1$. Besides, the coefficient of determination $R^2$ was 0.9696, indicating that 96.96% of variability could be explained by the model.Adjusted R-squared ($R^2_{adj}$) can be explained by the number of model adjustments to evaluate the average variation.The experimental data with $R^2_{adj}$=91.48% and the fitting degree of regression equation was very well and the reliability is high.

Table 3. Variance analysis of regression model for viable counts of *L. bulgaricus*

| Source | Degrees of freedom | Sum of squares | Mean square | Fvalue | **Pr > F** | **Sig** |
|---|---|---|---|---|---|---|
| $X_1$ | 1 | 0.221 | 0.221 | 13.873 | 0.014 | * |
| $X_2$ | 1 | 0.180 | 0.180 | 11.294 | 0.020 | * |
| $X_3$ | 1 | 0.095 | 0.095 | 5.936 | 0.059 | |
| $X_1^2$ | 1 | 0.650 | 0.650 | 40.784 | 0.001 | ** |
| $X_1X_2$ | 1 | 0.348 | 0.348 | 21.840 | 0.005 | ** |
| $X_1X_3$ | 1 | 0.001 | 0.001 | 0.039 | 0.851 | |
| $X_2^2$ | 1 | 0.597 | 0.597 | 37.453 | 0.002 | ** |
| $X_2X_3$ | 1 | 0.036 | 0.036 | 2.265 | 0.193 | |
| $X_3^2$ | 1 | 0.666 | 0.666 | 41.762 | 0.001 | ** |
| Model | 9 | 2.538 | 0.282 | 17.696 | 0.003 | ** |
| Liner | 3 | 0.496 | 0.165 | 10.368 | 0.014 | * |
| Quadratic | 3 | 1.658 | 0.553 | 34.671 | 0.001 | *** |
| Cross interaction | 3 | 0.385 | 0.128 | 8.048 | 0.023 | * |
| Error | 5 | 0.073 | 0.016 | | | |
| Lack of fit | 3 | 0.073 | 0.024 | 7.549 | 0.119 | |
| Pure error | 2 | 0.006 | 0.003 | | | |
| Total | 14 | 2.618 | | | | |

Note: * $P$<0.05; ** $P$<0.01; *** $P$<0.001

Figure 1 shows that the effects of three independent variables on thenumber of viable bacteria at different levels. The low probability value for quadratic terms $X_1^2$, $X_2^2$ and $X_3^2$ indicating their effects on response value $Y_1$ were very significant.

As is shown in Figure 1, the viable counts of *Lactobacillus bulgaricus* improve significantly then reduced with glucose concentration increasing. The effects of glutamate and casein hydrolysate on the response value were the same. In total, the maximum values of response for the three factors were appeared in the inflection point. The contour plots and three-dimensional response surface plots were obtained by using the software SAS 9.1.3 basing on the regression equation (Figure 2-4). The plots can analyze the interaction of each two factors on the growth of *Lactobacillus bulgaricus.* The shape of contour plots reflects the intensity of the interaction between the factors to a certain extent. The interaction between the factors is significant when the contour plot is elliptical, while the circle means no interaction between factors. Thus, the range levels of three optimum factors can be determined.

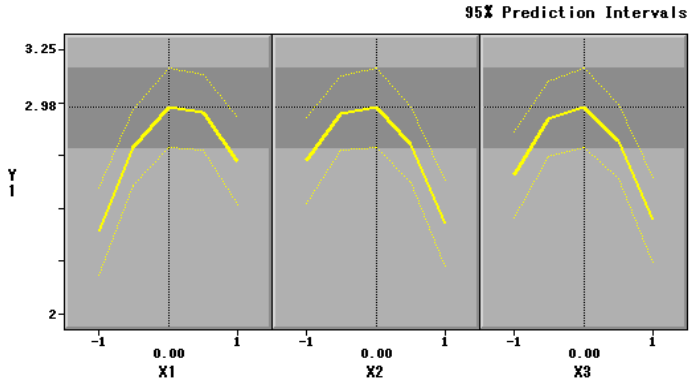


Figure 1. The changes of viable counts of *L. bulgaricus* with the selected variables

Figure 2 shows that the viable counts of *Lactobacillus bulgaricus* showed a tendency to increase first and then decrease, and the increase is due to the constant amount of glutamate, and the decrease is due to the increase of casein hydrolysate and glucose, which not only provide energy for the cell, but also will inhibit cell growth when the glucose is too high. Furthermore, the contour plot of $X_1$ and $X_2$ to $Y_1$ is oval indicating that the interaction between them is relatively significant.

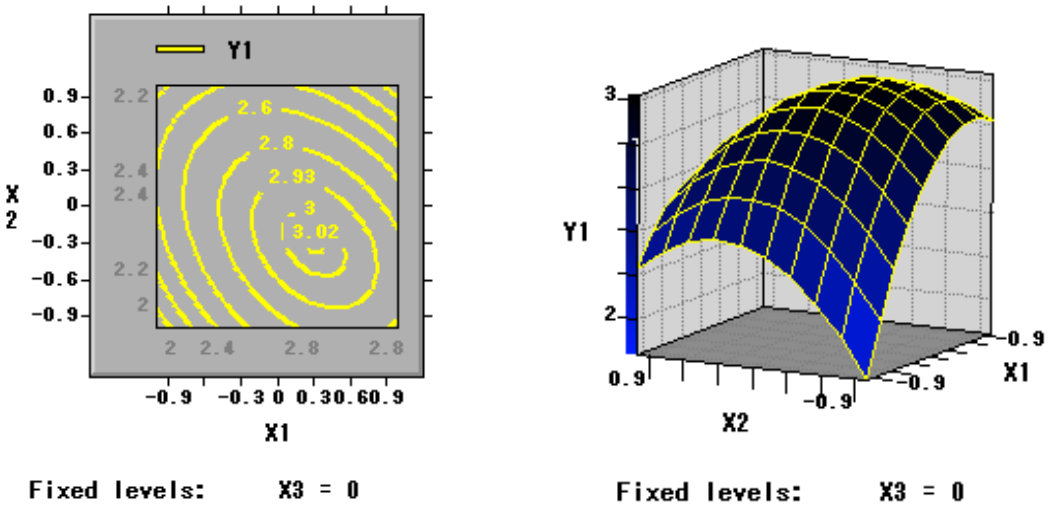


Figure 2. 2D and 3D plots of glucose and casein hydrolysate to viable counts of *L. bulgaricus*

In Figure 3, the viable counts of *Lactobacillus bulgaricus* was increased by no further change in the amount of casein hydrolysate, and then decreased along with the adding of glucose and glutamate. The reason for this phenomenon was due to the excessive addition of glutamic acid and glucose in the culture medium. Meanwhile, the contour maps were similar to the circle shown that the interaction between them is weak.

The 2D and 3D plots of casein hydrolysate and Glutamate on dependent variable $Y_1$ are described in Figure 4. The viable counts of bacteria were increased by no further change in the amount of glucose until it not changed. While the adding substrate reached a certain extent, the viable counts of bacteria showed a downward trend. Furthermore, the contour plots are near to the circle which indicated that the interaction for $X_2$ and $X_3$ is weak.

The maximum value were obtained by analyzing the regression equation and the partial derivative of X1, X2 and X3 through the software SAS 9.1.3, where the corresponding actual value are: glucose 9.5g·L$^{-1}$ (code value=-0.5), casein hydrolysate 15.5g·L$^{-1}$ (code value=-0.5) and glutamate 7.0mg·L$^{-1}$ (code value=0), respectively.

The viable counts of *Lactobacillus bulgaricus* can reach $3.00\times10^9$ cfu·mL$^{-1}$ which was predicted by the establishing model at these optimum conditions. Under the optimum conditions, the three parallel experiments were carried out to verify the model predictive value. The average verified results are (2.95±0.07) $\times10^9$ cfu/mL, which is very near the predictive value. The optimum parameters of culture medium that obtained by the response surface method are reliable.

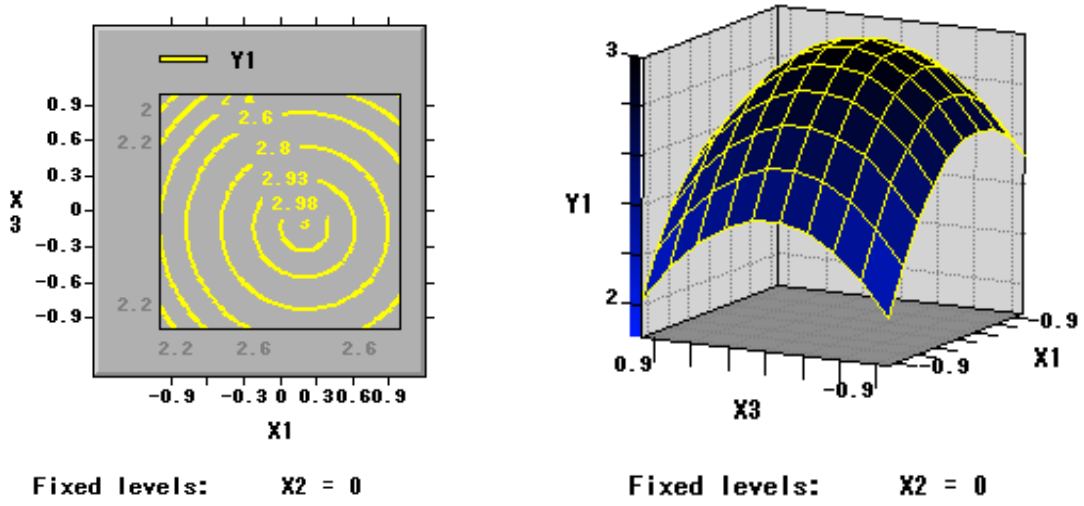


Figure 3. 2D and 3D plots of glucose and glutamate to viable counts of *L. bulgaricus*

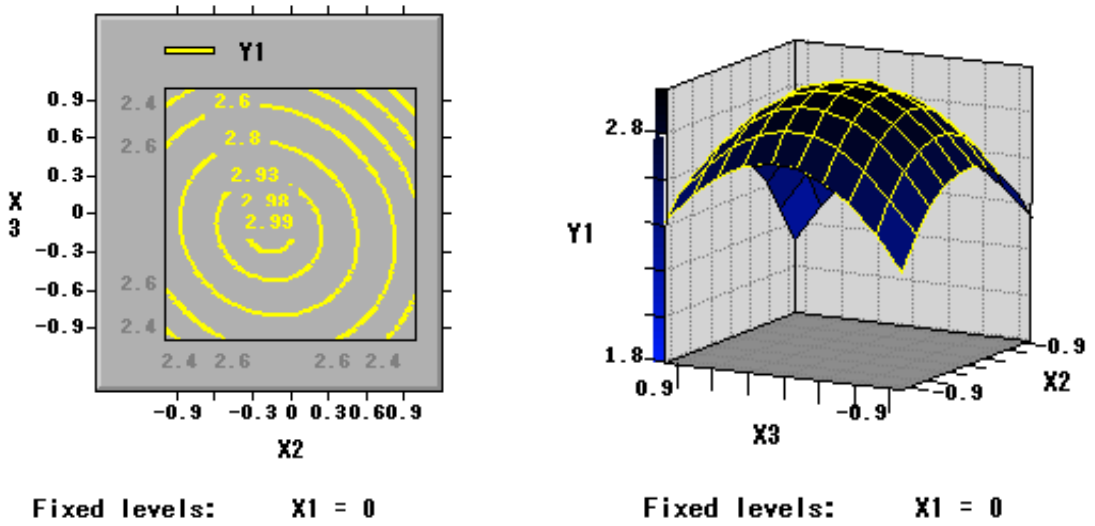


Figure 4. 2D and 3D plots of casein hydrolysate and glutamate to viable counts of *L. bulgaricus*

***Determination of growth curve of Lactobacillus bulgaricus***

The determination of the growth curve is of great significance in studying the acid producing, growth and reproduction of bacteria in culture medium.To evaluate the effect of optimum medium on the growth of bacteria, the growth curve of *L. bulgaricus* was determined separately before and after the optimization of medium. Figure 5 showed the growth curve plotted of *L. bulgaricus* with time as the abscissa, pH and viable counts as the ordinate.At the beginning 4 h, the cell growth is relatively slow because the cell has just entered a new environment and need a period of time to adapt it. The exponential growth period for bacteria is at 4-16h. In this period, the viable counts of bacteria grow faster, the propagation rate was the highest, the culture became turbid, and the pH value decreased with the increase of the

metabolites of lactic acid and other organic acids. The bacteria entered a period of stability and viable counts bacteria remained stable at 16-22 h. While after 22 h, the cell began to decline and autolysis.For change of the environment and consumption of nutrient, the whole cell population showed negative growth. Meanwhile, the number of viable bacteria was closed to $2.04\times10^{9}$cfu·mL$^{-1}$in $M_0$ medium at 20h, while the viable counts of bacteria can reach $2.76\times10^{9}$cfu·mL$^{-1}$in $M_1$ medium, which is about 1.35 times higher than the $M_0$ medium.

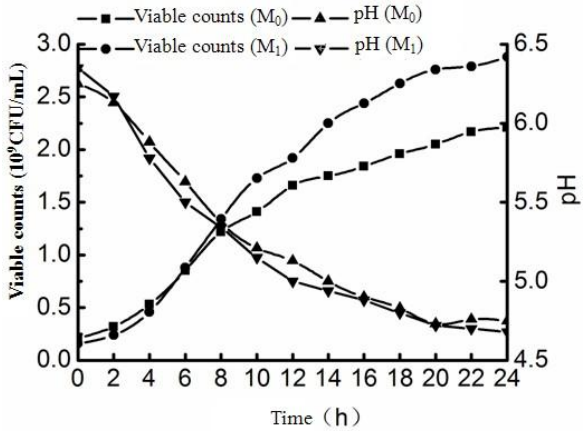


Figure 5. The growth curve of *Lactobacillus bulgaricus* in $M_0$ and $M_1$ medium ($M_0$: the medium before optimization; $M_1$: the medium after optimization)

## CONCLUSIONS

The Box-Behnken design was used to optimize the production of enrichment medium of *L. bulgaricus* with varying levels of glucose, casein hydrolysate and glutamate, which based on regression model of the experimental data.When the optimal conditions were glucose 9.5 g·L$^{-1}$, casein hydrolysate15.5 g·L$^{-1}$ and glutamate 7.0mg·L$^{-1}$, the viable counts of *L. bulgaricus* reached (2.95±0.07) $\times10^{9}$cfu·mL$^{-1}$.Verification value was similar to the predicted value, which confirmed that the regression model was useful for experiments. In addition, *L. bulgaricus* cultured under optimal conditions for 20 hours can reach at $2.76\times10^{9}$cfu·mL$^{-1}$ which is 1.35 times the unoptimized. The optimum medium components stimulating the bacterial growth and protecting against freeze-drying can be used together, which can be useful from economic and technological point of view.


## ACKNOWLEDGEMENTS

The work was partly supported by the science and technology project of Xi'an city [No.XJR1506-(10)], the science and technology project of Baqiao district of Xi'an city [No.2016-(7)].


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