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ORIGINAL RESEARCH PAPER

OPTIMIZATION OF MEDIUM COMPOSITIONS FOR *SACCHAROMYCES BOULARDII* BY BOX-BEHNKEN DESIGN

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Abstract: Based on the previous experimental results, the Box-Behnken design (BBD) was employed to study the individual and interactive effects of sucrose, malt extract, calf serum and sodium citrate on the growth of *Saccharomyces boulardii*, and the optimum medium compositions were obtained. Meanwhile, the optical density (OD) in the fermentation suspension was measured at 560 nm after 36 h of incubation. It is shown that the optimized medium compositions were 36.28 $g \cdot L^{-1}$ sucrose, 6.38 $g \cdot L^{-1}$ malt extract, 5.69 $g \cdot L^{-1}$ calf serum and 5.3 $g \cdot L^{-1}$ sodium citrate. The result indicated that the growth of *S. boulardii* could increase significantly in the optimized medium, and the OD_{560nm} value reached 1.397 ± 0.013 after 36 h, which increased 18.59 % compared with that of pre-optimized medium. In addition, the OD_{560nm} value 1.397 ± 0.013 in the optimized medium was very closely to the expected value 1.394. This result suggested that optimization of medium compositions for *S. boulardii* by BBD in this study was reliable and effective.

Keywords: *Box-Behnken design, calf serum, malt extract, optical density, Saccharomyces boulardii, sodium citrate, sucrose*

INTRODUCTION

Saccharomyces boulardii (*S. boulardii*) is a thermophilic, non-pathogenic yeast used as probiotic agent to prevent or treat a variety of human gastrointestinal dysfunctions, for instance, antibiotic associated diarrhea and recurrent *Clostridium difficile* disease [1, 2]. A recent report has also indicated that *S. boulardii* may be useful in the preventing clinical relapse of Crohn's disease [3 – 5]. In addition, based on the results of the study of experiment animals, several putative action mechanisms for the protective effect of *S. boulardii* have been devised [6 – 8]. Some studies suggest that *S. boulardii* can exert its beneficial effect through multiple mechanisms, for example, competition with pathogenic for nutrients, inhibit the adhesion of pathogenic bacteria and neutralization of bacterial virulence factors [9 – 11]. *S. boulardii* has been widely used in the prevention of diarrhea and other disturbances caused by the use of antibiotics [12 – 15]. This yeast remains viable in the gastrointestinal tract and has been proved to inhibit the growth of the number of pathogenic bacteria [16 – 19]. Double-blind-controlled trails have proved the efficacy of *S. boulardii* against antibiotic-associated diarrhea in humans [20, 21].

Although *S. boulardii* is a variety of *S. cerevisiae*, it differs from *S. cerevisiae* in taxonomic, metabolic and genetic properties [22 – 24]. Its optimal growth temperature is 37 ºC (physiologycal temperature of the host), and the yeast is resistant to acidity. *S. boulardii* has the unique biological activity, it can inhibit the growth of some pathogenic microorganisms [13, 22, 25]. This yeast has been used in many countries as lyophilized product for the treatment of diarrhea in children and adult [26 – 28]. Pharmacokinetic studies have shown that, after continuous oral, *S. boulardii* can exist in the colon stability about 3 days, and after the cessation of the drug, it will be excreted in feces, not be colonized in the intestine [14, 29]. *S. boulardii* belongs to fungi preparation, no degradation by antibiotics, which can be used in conjunction with antibiotics, and it can effectively prevent the use of antibiotics with dysbacteriosis [30].

Response surface method (RSM) had been employed in many research areas, it could analyze the significance of interaction factors in the medium and carry out the optimal levels of variables [31, 32]. The advantage of RSM is that it can analyze the various levels of experimental factors in the process of optimizing the experimental conditions, which overcomes the shortcomings of orthogonal experiment, that is, the orthogonal experiment can only analyze the isolated test point and can not presents an intuitive graphical [33]. Therefore, the RSM is widely used in the research of experimental design and process optimization. The RSM mainly include Central Composite design (CCD), Box-Behnken design (BBD), D-optimal design, User Defined design and Historical Data design. Among them, the most commonly used is CCD and BBD.

S. boulardii as probiotics, to ensure its biological effect, the intestine must achieve a sufficient number of viable cell count. However, there are scarce reports involved in the medium composition of *S. boulardii* [34]. In our previous study, the two-level factorial design was employed to screen the main influence factors on the growth of *S. boulardii.* And the sucrose, malt extract, calf serum and sodium citrate were selected for further research [35]. In the present study, the Box-Behnken design was used to optimize medium compositions for *S. boulardii*.

MATERIALS AND METHODS

Microorganism

The strain used in this study was *Saccharomycess boulardii*, which was provided by School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China). Malt extract was purchased from Kingbee Biotechnology Co., Ltd (Shanghai, China). Calf serum was purchased from Tianhang Biological Technology Co., Ltd (Zhejiang, China). All chemicals used in this experiment were of analytical grade.

Culture conditions

S. boulardii was grown and activated in YPD medium containing 10 g of yeast powder, 20 g glucose, 20 g peptone, and then was dissolved in 1000 mL distilled water, and autoclaved at 118 ºC for 15 min.
S. boulardii was inoculated with 2 % (v/v) inoculum in 250 mL flask containing 35 mL medium, and then incubated at 37 ºC for 36 h in the shaker at 180 rpm. Using microscope to confirm that there were no miscellaneous bacteria in the culture medium, then activated three successive times and incubated under the same condition. The activation and inoculation was operated on the super clean workable.

Growth measurement

The growth of strain was monitored by measuring the optical density at 560 nm (OD_{560}) through a spectrophotometer (Sp-756pC, Shanghai Spectrum Instruments Co., Ltd., Shanghai, China) after 36 h of incubation. The OD value of each experiment was measured in triplicate and the mean was calculated for each treatment group.

Experiment design

Based on the determined key factors, a Box-Behnken design (BBD) with four variables was used to study the response pattern and to determine the optimum medium compositions of *S. boulardii*. Each factor with three variation levels was coded as - 1, 0, 1 (Table 1). The effect of independent variables X_1 (sucrose), X_2 (malt extract), X_3 (calf serum) and X_4 (sodium citrate), at three variation levels (Table 1) on the growth of *S. boulardii* is shown in Table 2. The experiment order is completely randomized. This design was applied to determine the maximum response value and evaluation of the main effects, interaction effects and quadratic effects. And the mathematical relationship connecting the variables and the response was described by the quadratic polynomial equation:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4 \quad (1)$$

Where Y is predicate response, b_0 is constant coefficient. The regression coefficients (b_1, b_2, b_3 and b_4), (b_{11}, b_{22}, b_{33} and b_{44}) and (b_{11}, b_{12}, b_{13} and b_{14}) respectively represent linear, quadratic and interaction effects of the model, estimated by multiple regression

analysis. X_1 (sucrose), X_2 (malt extract), X_3 (calf serum) and X_4 (sodium citrate) were coded variables ranging from - 1 to 1.

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

Factors	coded levels		
	-1	**0**	**1**
X_1 sucrose [g·L^{-1}]	35	37.5	40
X_2 malt extract [g·L^{-1}]	5.5	6	6.5
X_3 calf serum [g·L^{-1}]	5.5	6	6.5
X_4 sodium citrate [g·L^{-1}]	4.5	5	5.5

Statistical analysis of the data

The Design-Expert (Version 8.0.6) software was used for the experiment design and regression analysis of the experimental data to estimate the response of the independent variables. Three dimensional response surface plots and contour plots were also drawn using Design-Expert. The fitting of the second-order model equations was determined by the coefficient of determination (R^2).

RESULTS AND DISCUSSION

The experimental design and results of Box-Behnken

To determine the optimal concentration of the medium compositions of S. *boulardii*. The significant factors: X_1 (sucrose), X_2 (malt extract), X_3 (calf serum) and X_4 (sodium citrate) were further optimized using a four-factor, three-level Box-Behnken design (BBD) in the present study. The design matrix of BBD and results of response (Y) were shown in Table 2. The response value Y represented OD value in the fermentation suspension.

Establish regression model

According to the experimental results in Table 2, Design-Expert software was used to analyze the data of BBD, and the quadratic regression equation about OD value of the fermentation suspension was obtained at eqn 2:

$$Y = 1.39 + 0.049 X_1 + 0.0002 X_2 - 0.0162 X_3 - 0.001 X_4 + 0.003 X_1 X_2 + 0.0065 X_1 X_3 - 0.0228 X_1 X_4 + 0.0038 X_2 X_3 + 0.038 X_2 X_4 - 0.0348 X_3 X_4 - 0.011 X_1^2 - 0.0125 X_2^2 - 0.0301 X_3^2 - 0.0501 X_4^2 \quad (2)$$

where Y is predicted value of OD value in the fermentation broth, X_1, X_2, X_3, X_4 are the coded values of the test variables sucrose, malt extract, calf serum and sodium citrate, respectively.

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

Run	X_1	X_2	X_3	X_4	Y
1	0	0	-1	1	1.372
2	-1	0	1	0	1.335
3	0	0	0	0	1.402
4	0	0	1	1	1.272
5	0	1	0	-1	1.303
6	0	0	0	0	1.382
7	1	0	0	1	1.295
8	0	-1	0	-1	1.376
9	1	0	1	0	1.356
10	0	0	0	0	1.388
11	1	0	0	-1	1.355
12	0	-1	-1	0	1.373
13	0	0	0	0	1.394
14	-1	0	-1	0	1.366
15	1	-1	0	0	1.373
16	1	1	0	0	1.381
17	-1	0	0	-1	1.306
18	-1	-1	0	0	1.361
19	0	0	0	0	1.386
20	-1	0	0	1	1.337
21	0	0	1	-1	1.32
22	0	1	0	1	1.366
23	0	-1	1	0	1.317
24	0	1	-1	0	1.359
25	0	0	-1	-1	1.281
26	0	1	1	0	1.318
27	-1	1	0	0	1.357
28	0	-1	0	1	1.287
29	1	0	-1	0	1.361

Variance analysis of the data

The significance of each coefficient was determined using analysis of variance (ANOVA). The effect of corresponding variables will be more significant if the p-value becomes smaller. The coefficient of determination R^2 represents the variability in the actual values which could be explained by the factors in the model and their interactions. The closer the R^2 value is to 1, the higher the predictive ability of the model. The ANOVA of the regression equation is shown in Table 3.

Table 3. *Analysis of variance (ANOVA) for response surface quadratic model*

Sources	Sum of squares	df	Mean squares	F-value	p-value prob>F	significance
Model	0.036	14	2.54E-03	15.96	< 0.0001	***
X_1	2.90E-04	1	2.90E-04	1.82	0.1982	
X_2	7.50E-07	1	7.50E-07	4.72E-03	0.9462	
X_3	3.14E-03	1	3.14E-03	19.73	0.0006	***
X_4	1.20E-05	1	1.20E-05	0.075	0.7875	
X_1*X_2	3.60E-05	1	3.60E-05	0.23	0.6415	
X_1*X_3	1.69E-04	1	1.69E-04	1.06	0.32	
X_1*X_4	2.07E-03	1	2.07E-03	13.02	0.0028	**
X_2*X_3	5.63E-05	1	5.63E-05	0.35	0.5615	
X_2*X_4	5.78E-03	1	5.78E-03	36.33	< 0.0001	***
X_3*X_4	4.83E-03	1	4.83E-03	30.38	< 0.0001	***
X_1*X_1	7.78E-04	1	7.78E-04	4.89	0.0441	*
X_2*X_2	1.01E-03	1	1.01E-03	6.32	0.0248	*
X_3*X_3	5.87E-03	1	5.87E-03	36.9	< 0.0001	***
X_4*X_4	0.016	1	0.016	102.3	< 0.0001	***
Residual	2.23E-03	14	1.59E-04			
Lack of fit	1.98E-03	10	1.98E-04	3.26	0.133	
Pure Error	2.43E-04	4	6.08E-05			
Correlation total	0.038	28				

*Note: * p < 0.05, ** p < 0.01, *** p < 0.001*

According to the ANOVA of Table 3, the model p-value < 0.0001, which implied the regression model, was extremely significant, and the lack of fit p-value of 0.133 indicated the lack of fit was not significant relative to the pure error. Thus, the model equation as expressed in Equation. (2) provided a suitable model to describe the response value in the fermentation suspension. In addition, the coefficient of determination R^2 was 94.1 %, which means that only 5.9 % of the total response variation remained unexplained by the model. So the fit is good between the quadratic model and the experimental data. Meanwhile, the adjusted R^2 (R^2_{adj} = 88.21 %) was also calculated, which was close to the R^2 value. There is thus good agreement between the experimental and predicted values. In this model, the X_3 was significant, and all the quadratic term was significant, which implied that there was not a simple linear correlation between response value and variables. The F-value of $X_1 \cdot X_4$, $X_2 \cdot X_4$ and $X_3 \cdot X_4$ were higher, which demonstrated that they had a significant interaction between them. However, $X_1 \cdot X_2$, $X_1 \cdot X_3$ and $X_2 \cdot X_3$ had a smaller F-value, which indicated that there was weak mutual interaction between them.

Interaction between variables on the OD value in the fermentation broth

The regression model was analyzed using Design-Expert (Version 8.0.6) software, the two-dimensional contour plots and three-dimensional response surface plots were shown in Figure 1 to Figure 6.

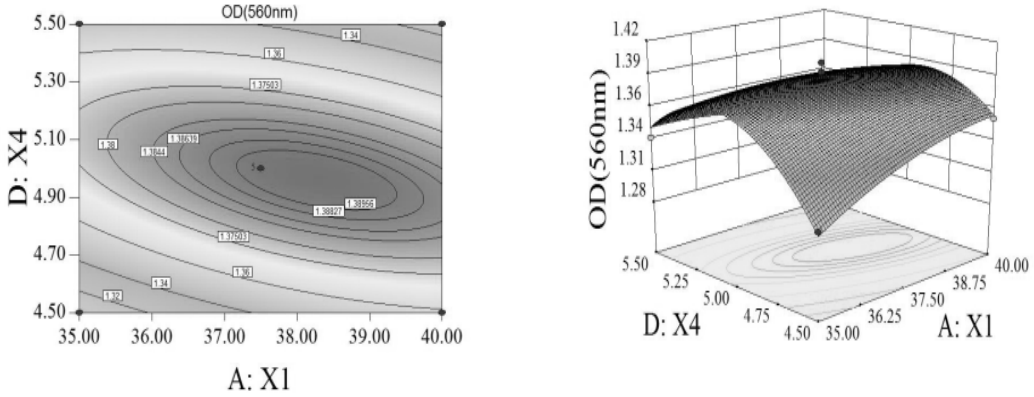

Figure 1. *Contour plot and response surface plots of sucrose (X_1) and sodium citrate (X_4) on the OD value*

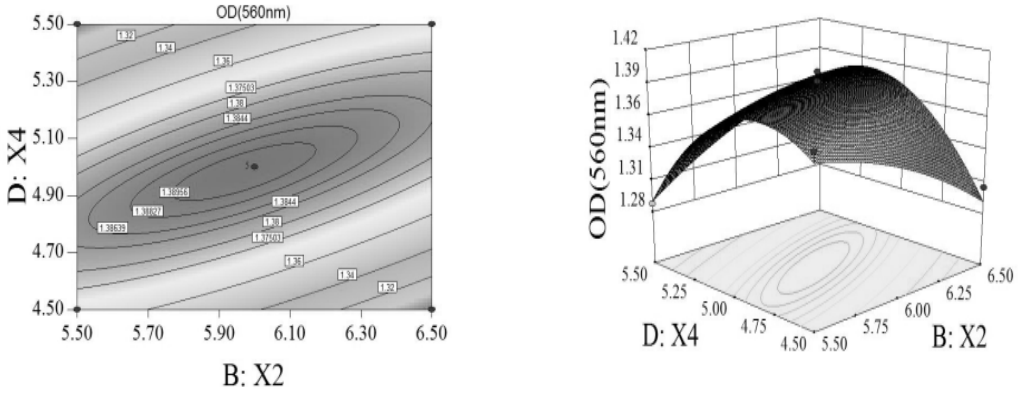

Figure 2. *Contour plot and response surface plot of malt extract (X_2) and sodium citrate (X_4) on the OD value*

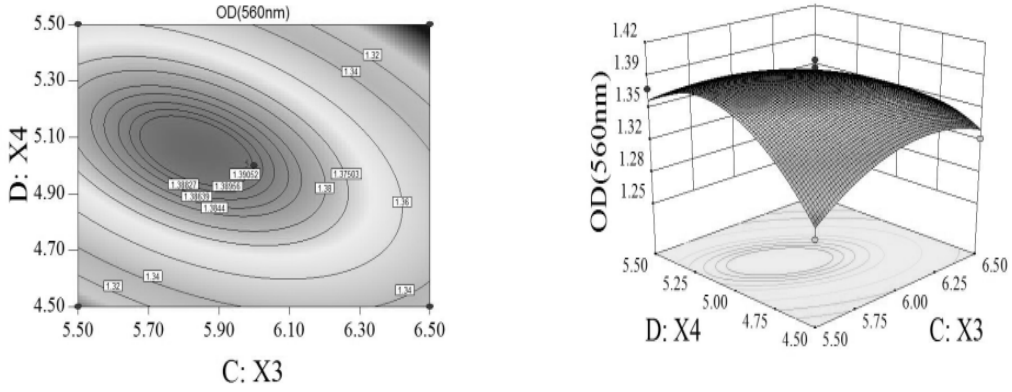

Figure 3. *Contour plot and response surface plot of calf serum (X_3) and sodium citrate (X_4) on the OD value*

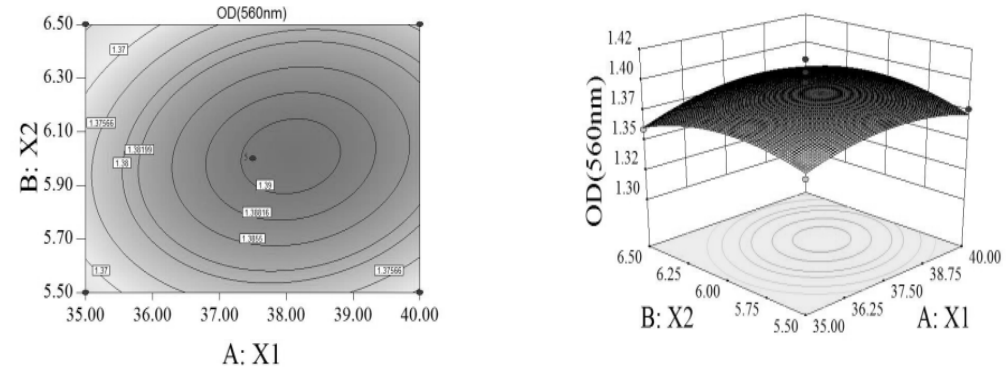

Figure 4. *Contour plot and response surface plot of sucrose (X_1) and malt extract (X_2) on the OD value*

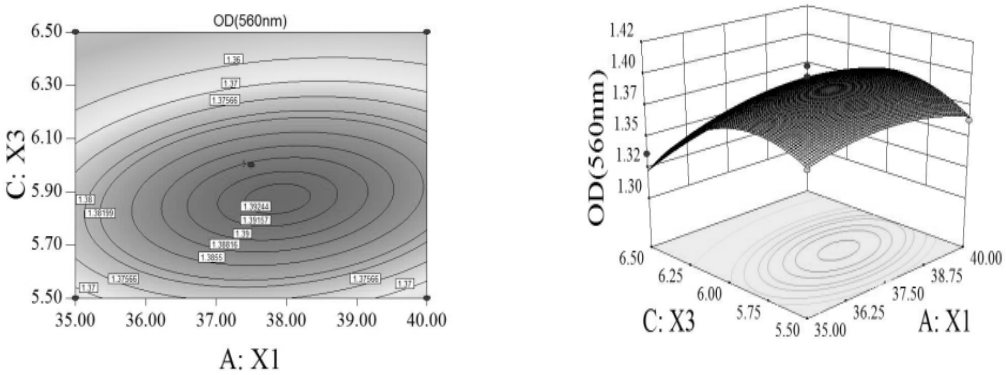

Figure 5. *Contour plot and response surface plot of sucrose (X_1) and calf serum (X_3) on the OD value*

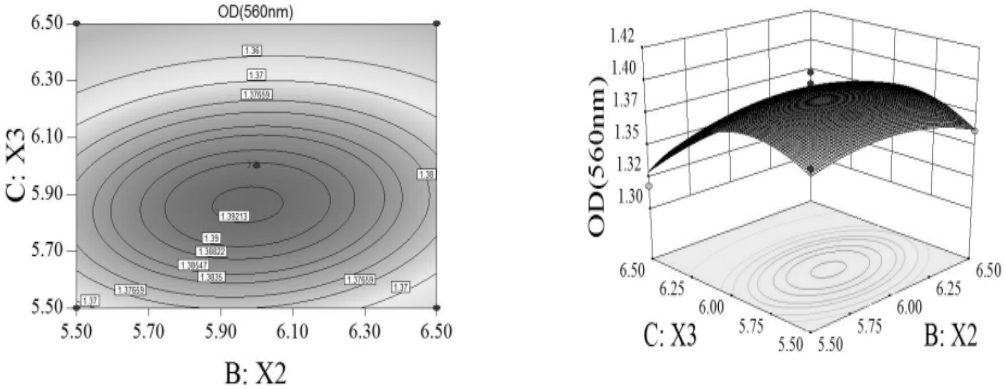

Figure 6. *Contour plot and response surface plot of malt extract (X_2) and calf serum (X_3) on the OD value*

In contour plots, the round contour lines represent that there are weak mutual interaction between variables, meanwhile, the oval in contour plots declare that the interaction of variables is significant. As shown in Figure 1, we can see that the two-dimensional plot of X_1 and X_4 was oval, which illustrated the mutual interaction of them was significant for the response Y. In the response surface plots, when the added amount of X_2 (malt extract) and X_3 (calf serum) was fixed (fixed levels: X_2 = 0, X_3 = 0), with the concentration of X_1 increasing, the response Y increased continuously; and the response

value increased first and then decreased following the increase of the concentration of X_4. According to the Figure 2, the oval in the contour plot of X_2 and X_4 illustrated that the interaction effect on the corresponding variables between X_2 and X_4 was significant. When the dosage of X_1 and X_3 was fixed (fixed levels: X_1 = 0, X_3 = 0), as the concentration of X_2 increasing, the response value decreased gradually; however, the response value increased first and decreased rapidly following the increase of the concentration of X_4. And the Figure 3 showed that the oval contour lines of X_3 and X_4, which represented the mutual interaction of them was significant. In addition, as the concentration of X_1 and X_2 was fixed (fixed levels: X_1 = 0, X_2 = 0), the response value increased gradually first and then decreased following the increase of the concentration of X_3 and X_4. Moreover, according to the Figure 4, Figure 5 and Figure 6, we can see that all the contour lines seemed to be a round, which indicated that the interaction effect on the response value Y between X_1 and X_2, X_1 and X_3, X_2 and X_3 were not significant. In addition, we can easily see that the above-mentioned six response surface plots are arched, which suggested that there was an optimal response value under the action of the four variables.

Furthermore, the quadratic regression equation for Y was analyzed using statistical software Design-Expert (Version 8.0.6), and the optimal medium compositions calculated were: 36.28 $g \cdot L^{-1}$ sucrose, 6.38 $g \cdot L^{-1}$ malt extract, 5.69 $g \cdot L^{-1}$ calf serum and 5.3 $g \cdot L^{-1}$ sodium citrate. The predicted OD value of fermentation suspension in this model was 1.394. In order to verify the accuracy of the predicted value, the OD value was 1.397 ± 0.013 on average by testing three times in the optimized medium after 36 h of incubation. This result suggested that the statistical method was successfully used to determine the optimal medium compositions for *S. boulardii*.

DISCUSSION

The composition of culture media is an important factor for growth in yeasts, especially nutrient contents such as carbon sources, nitrogen source, and inorganic ions [36]. The carbon source of yeast commonly used are glucose, sucrose, starch, fructose, maltose, etc; and the nitrogen source commonly used are peptone, yeast extract powder, urea, $(NH_4)_2SO_4$, KNO_3, etc. In addition, growth factors, microelements and so on are also essential to the growth of yeast [37].

In present study, using Box-Behnken design to optimized the medium compositions for *S. boulardi*. The result showed that the optimal medium compositions were 36.28 $g \cdot L^{-1}$ sucrose, 6.38 $g \cdot L^{-1}$ malt extract, 5.69 $g \cdot L^{-1}$ calf serum and 5.3 $g \cdot L^{-1}$ sodium citrate. Carbon source is a fundamental energy producer for yeast, Zhang *et al* optimized the medium components of *Saccharomyces cerevisiae* by the method combined single factor test with orthogonal test, the result indicated that the optimal carbon source was 40 $g \cdot L^{-1}$ sucrose [38]. Wang *et al* conducted the medium optimization for *Saccharomyces cerevisiae* FL-1 using BBD, and showed that the optimal medium compositions were 115.5 $g \cdot L^{-1}$ sucrose, 10 $g \cdot L^{-1}$ $(NH_4)_2SO_4$ and 25 $g \cdot L^{-1}$ yeast extract [39], It was proved that BBD is useful to optimize the medium. Sun *et al* optimized bread yeast seed culture by response surface method, the result showed that the optimal carbon source was 119.98 $g \cdot L^{-1}$ sucrose and the viable cells increased 5.2 times under the optimized conditions [40]. Sucrose is a nutritional agent of yeast, which can provide

energy for the fermentation process. Generally, yeast preferably consume monosaccharides (for example: glucose, fructose) and next complex sugars (for example: sucrose, maltose). However, in this study, sucrose is a better source of carbon than glucose. The possible reason is that sucrose is disaccharide, the degree of carbonization of sucrose is lower than that of glucose in the process of high temperature sterilization, and then there is less loss of sucrose compared to glucose [41]. In addition, sucrose can be decomposed into glucose and fructose by yeast.
Furthermore, nitrogen source is essential for the growth of yeast. Zhao *et al* optimized fermentation medium of *Candida rugosa*, and the result indicated that the optimal nitrogen sources were 0.3 % yeast extract, 0.5 % peptone and 0.3 % malt extract [42]. Malt extract is a natural nutrient that extracted from barley and malt through enzymatic hydrolysis, which contains maltose, fructose, amino acids, vitamins, microelements and various growth factors. Besides carbon and nitrogen source, the growth of yeast also requires P, S, Mg, Ca, K and other microelements. In addition, growth factors can regulate the metabolism of yeast, which are the indispensable micro-organic elements, including various amino acids, vitamins, purine, etc. Thus, malt extract can promote the growth of *S. boulardii* very well.
And sodium citrate had a significant effect on the growth of *S. boulardii*. Because the *p*H value of the medium increased in the process of growth and metabolism, it's not conductive to the growth of yeast. However, sodium citrate could regulate the *p*H of the medium [43], and promote the growth of yeast.

CONCLUSION

In this study, Box-Behnken design (BBD) was used to optimize the medium compositions for *S. boulardi*. The optimal medium compositions were 36.28 $g \cdot L^{-1}$ sucrose, 6.38 $g \cdot L^{-1}$ malt extract, 5.69 $g \cdot L^{-1}$ calf serum and 5.3 $g \cdot L^{-1}$ sodium citrate, those variables had a significant impact on the growth of *S. boulardi*. The OD value of the fermentation suspension reached 1.397 ± 0.013 after 36 h of incubation, which increased 18.59 % compared to that of the control. Moreover, there was no significant difference between predicted value and actual value, this indicated the optimization of medium compositions used BBD was reliable and effective.

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