Studii şi Cercetări Ştiinţifice 2020, **21** (2), pp. 227 – 242
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

STATISTICAL OPTIMIZATION OF CULTIVATION CONDITIONS FOR *SACCHAROMYCES BOULARDII VIA* CENTRAL COMPOSITE DESIGN

Guowei Shu[1], Xin Yang[1*], Li Chen[2*], Dan Huang[3], Zhangteng Lei[1], He Chen[1]

[1]*Shaanxi University of Science & Technology, School of Food and Biological Engineering, Xi'an, China*
[2] *Shaanxi Normal University, College of Food Engineering and Nutritional Science, Xi'an, PR China*
[3]*Department of Research and Development, Shaanxi Pucheng Shiyang Feed Co. Ltd., Xi'an, China*

*Corresponding author: Yangxin906@163.com, chenlisp@snnu.edu.cn

Received: May, 10, 2019
Accepted: May, 27, 2020

Abstract: *Saccharomyces boulardii* (*S. boulardii*) is used in many countries as both a preventive and therapeutic agent for diarrhea and other gastrointestinal disorders caused by the administration of antimicrobial agents. However, there are few reports on its culture conditions. Therefore, the aim of this study was to optimize the cultivation conditions of *S. boulardii* by single factor experiment and central composite design of Response Surface Methodology (RSM). The effects of temperature (28, 31, 34, 37, 41 °C), initial *p*H value (2, 4, 6, 8 and 10), liquid loading (25, 50, 75, 100 and 125 mL in 250 mL triangular flask), inoculum size (1, 3, 5, 7 and 9 %), and shaking speed (140, 160, 180, 200, 220 rot·min^{-1}) were investigated. Among these variables, the combination of 37 °C temperature, 6.4 the initial *p*H value, 250 mL triangular flask with 52 mL medium volume, 3 % inoculum size, and 181 rot·min^{-1} shaking speed were found to be the most suitable cultivation conditions. Under the optimized conditions, the maximal yeast count was (1.81±0.06)×10^{8} CFU·mL^{-1}, which was in a good agreement with the predicted value of the model. This study has provided useful information on how to improve the viable cells of yeast cells for *S. boulardii* culture.

Keywords: *cultivation conditions, optimization, Saccharomyces boulardii, viable counts*

INTRODUCTION

Regardless of its etiology, infectious diarrhea is associated with a modification of the bowel's complex ecosystem leading to colonization by pathogenic bacteria. In recent years, more and more research evidence suggests that the use of ingested living microorganisms may play a role in the prevention or the treatment of intestinal infections. These organisms may have direct effects against enteric pathogens and/or indirect effects through modulation of the endogenous flora or the immune system. Suggest mechanisms of action include the production of antimicrobial substances, inhibition of pathogen adhesion, stimulation of secretory IgA production, change into toxins or toxin receptors, competition for nutrients [1].

The terms "*probiotics*" or "*biotherapeutic agent*" have been used to describe such beneficial microorganisms. The term "*probiotic*" has been defined by Fuller as a "live non-pathogenic microbial feed or the food supplement which beneficially affects the host by improving its intestinal microbial balance" [2]. This definition has been since broadened and extended to human health as "non-pathogenic microorganisms that, when ingested, exert a positive influence on the health or the physiology of the host" [3]. Probiotics are similar to the microorganisms that are found in the human gut, and also known as "friendly bacteria" or "good bacteria," help to maintain the natural balance of organisms (microflora) in the intestines [4]. In addition to improving gut health, probiotics can play a beneficial role in several medical conditions, including lactose intolerance, cancer, hepatic disease, allergies, *Helicobacter pylori* infections, urinary tract infections, irritable bowel syndrome, hyperlipidaemia and diabetes mellitus [5]. Therefore, they have been widely used in healthy food, especially in fermented dairy products, such as cheese, ice cream and milk slice [6]. Although the most probiotics are bacteria, one strain of yeast, *Saccharomyces boulardii*, has been found to be an effective probiotic in double-blind clinical studies [7].

In 1923, the French microbiologist Henry Boulard isolated a yeast strain (later named after him) after observing natives of Indochina affected by digestive disorders to chew litchi and mangosteen skins. It was said (but never proven) that these people could even protect themselves thereby against outbreaks of cholera. Ever since, there has been an increasing body of medical reports addressing the beneficial properties of *S. boulardii* as a probiotic to treat cases of diarrhea, reconstituting the gut flora after antibiotic treatment and even in the treatment of patients suffering from Helicobacter pylori that can cause stomach ulcers [8]. *S. boulardii* is common yeast, such as the prevention and treatment of infectious enteritis, *Clostridium difficile*-associated enterocolitis, ulcerative colitis and Crohn's disease [9]. This yeast is used in many countries as both a preventive and therapeutic agent for diarrhoea and other GI disorders caused by the administration of antimicrobial agents. Although *S. boulardii* is a subspecies of *S. cerevisiae*, this strain is different from *S. cerevisiae* in several taxonomic, metabolic and genetic properties [10, 11]. *S. boulardii* is resistant to the acidity, and to all antibacterial antibiotics [12, 13]. *S. boulardii* possesses many properties that make it a potential probiotic agent, i.e. it survives transit through the gastrointestinal tract, its temperature optimum is 37 °C, it inhibits the growth of a number of microbial pathogens, both in vitro and in vivo. In addition, yeast cells are rich in protein, vitamins, various enzymes and other nutrients and synergistic factors, to supplement various nutrients and growth factors for animals to promote their healthy growth [14]. It is expected that *S. boulardii* as a safe feed

additive will have a place in promoting the use of feed antibiotics, and the prospects are good. The industry is just beginning to use, the improvement of strain and fermentation optimization has great potential.
At present, *S. boulardii* has been given recognition by many countries, including China, the European Union countries and so on [15, 16]. Cultivation conditions are essential for the growth and reproduction of *S. boulardii*. Based on the previous experimental, screening of carbon sources, nitrogen sources and salts for *S. boulardii* used a two-level factorial design and using Box-Behnken design (BBD) to optimize the medium compositions for *S. boulardi* [17, 18]. The aim of the present study is to optimize cultivation conditions for the growth of *S. boulardii*. On the basis of Yeast Extract Peptone Dextrose Medium (YPD), the effects of temperature, initial *p*H, liquid loading, inoculum size, and shaking speed on the growth of *S. boulardii* were investigated by single factor test. Then the Central Composite Design and response surface analysis were carried out to finally obtain the optimal cultivation conditions. Provide reference data for the production and preparation of living bacteria preparation.

MATERIALS AND METHODS

Microorganisms and Chemicals

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).
The main reagents contained lactose, peptone and agar (Beijing Aobox Biotechnology Co., Ltd), glucose (Tianjin Zhiyuan Chemical Reagent Co., Ltd).

Culture conditions

The YPD (Yeast Extract Peptone Dextrose Medium) broth (*p*H = 5.6 ± 0.1) containing 1 % (w/v) yeast powder, 2 % (w/v) glucose, and 2 % (w/v) peptone, was used for the activation and cultivation of *S. boulardii*. YPD agar medium was obtained by the supplementation of YPD broth with 2 % agar and then sterilized at 118 °C for 15 min. The cells were cultured on YPD agar medium and the cell viability was determined.

Determination of viable counts

Plate counting method was used to measure the viable counts of *S. boulardii*. Under aseptic condition, take 1 mL of *S. boulardii* yeast culture solution in 9 mL of sterile physiological saline, diluted to 10^{-1} dilution, then increased it by 10 times according to this method, diluted to a suitable gradient. 0.1 mL diluent was evenly coated on sterilized YPD agar medium, each sample was made two dilution, each dilution was 3 parallel, then placed in 37 °C constant temperature incubator for 48 h. Selected the number of colonies between 30 and 300, and then calculated the number of viable cells per unit ($CFU \cdot mL^{-1}$).

Statistical Analysis

Data from three replicated trials for each treatment are presented as the format of means with standard deviation (Mean±SD). Design of Expert (DOE Version 8.0.6, Stat-Ease, Inc, Minneapolis, MN, USA) was used for the experiment design, graphs construction and results analysis. The optimum value of variables was obtained by the calculation of regression equation and analysis of contour plots and 3D surface plots. The Origin 9 software package (Origin Lab Inc., Alexandria, VA, USA) was used for describing the viable counts.

RESULTS AND DISCUSSION

Effect of temperature

Temperature affects all the physiological activities in a living cell. It is an important environmental factor to control the growth, microbial activities, and normal functioning of the cellular enzymes. The primary role of temperature is to change rates of biochemical reactions necessary for the cell growth [19]. When the temperature rises, the growth and metabolism of the microorganism accelerates, the yeast begins to reproduce in large numbers, but the heat generated by its metabolism will make the temperature of the whole fermentation system higher, speed up the aging and death of the yeast. Nevertheless, the temperature is too low, causing the cells grows slowly. Therefore, it is necessary to choose the appropriate fermentation temperature.
In this study, the *S. boulardii* was inoculated in YPD medium according to the 2 % inoculum size, the liquid loading was 35 mL / 250 mL, and it was placed in a 180 rot·min^{-1} shaker for 24 h, and compared the viable counts at different temperatures (28, 31, 34, 37, 41 °C) to determine the optimal fermentation temperature of the cells.

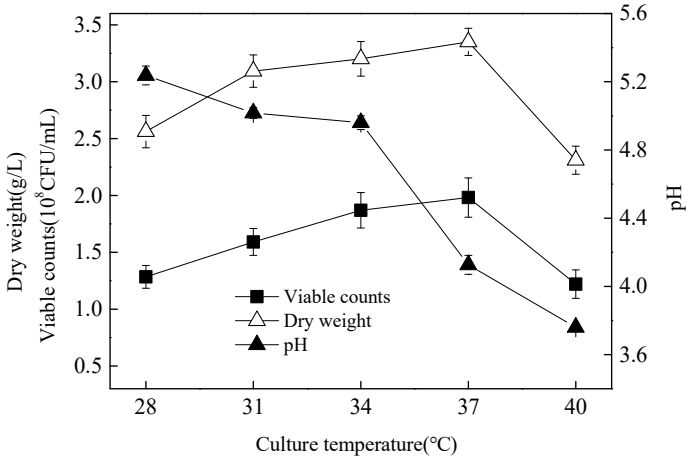


Figure 1. *Effect of cultivation temperature on the growth of Saccharomyces boulardii*

In Figure 1, it is observed that the viable counts slightly ascend to a maximum value of 1.98×10^8 CFU·mL^{-1} when the temperature is increased from 28 to 37 °C. However,

when the temperature further increases in 41 °C, the viable counts remarkably decline and exhibit a continuous reduction as the increase in the temperature. The dry weight performs the identical tendency with the viable counts, which significantly enlarges to the best-performance point of 3.3556 g·L^{-1} when the temperature increases to be 37 °C and sharply drops with the increasing of the temperature. Besides, culture temperature plays an important role in the *p*H value of the culture solution. With the temperature increased, the *p*H value decreased continuously. It may be due to an increase in temperature, the yeast metabolic rate is accelerated, and the *p*H value of the culture solution is decreased by acid production during the metabolic process. At 37 °C, the *p*H value of the culture solution is 4.1, so the optimum culture temperature of *S. boulardii* is 37 °C.

Effect of initial *p*H value of fermentation medium

The *p*H of the culture medium affects the absorption of nutrients, the secretion of metabolites, and also affects the decomposition of some nutrients in the medium. Therefore, *p*H is an important environmental factor that affects the growth of microorganisms. The initial *p*H value of the medium can change the charge of the cell membrane and affect the activity of intracellular enzymes, thus affecting the absorption of nutrients by cell in the growth environment. Eventually lead to imbalance of cellular metabolism. Each microorganism has its best growth *p*H range, and the amount of the cell is the highest at the optimum growth *p*H.
The initial *p*H of the medium was adjusted with 1 mol·L^{-1} of HCl or NaOH, so that the initial *p*H values were 2, 4, 6, 8, 10, liquid loading was 35 mL / 250 mL, adding *S. boulardii* to YPD medium with 2 % inoculum. At the optimum fermentation temperature, the cells were cultured in a 180 rot·min^{-1} shaker for 24 h, and the amount of the culture solution in different *p*H values was determined, thereby determining the optimum initial *p*H value of the medium.

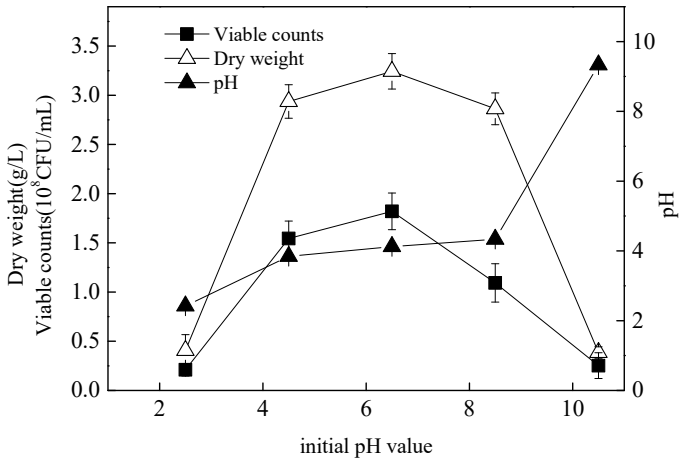


Figure 2. *The effect of initial pH value of fermentation medium on the growth of Saccharomyces boulardii*

It can be observed from Figure 2 that the initial *p*H value of the medium has a large effect on the viable counts and dry weight of the cells. With the initial pH increased, the viable counts increased first and then decreased. Similarly, the dry weight performs the identical tendency with the viable counts. The viable counts and dry weight reached the maximum value of 1.82×10^8 CFU·mL^{-1} and 3.2444 g·L^{-1} at *p*H 6. The *p*H value of the culture solution increases with the increasing of the initial pH value. When the initial *p*H is 6, the *p*H of the culture solution is 4.12. When the *p*H in the range 4 - 8, the *p*H in the culture solution do not change much because the production of acid by yeast in the culture process, resulting in the decrease of *p*H in the culture solution. In this case, the initial *p*H of 6 is selected to reach highest biomass.

Effect of liquid loading

Adequate nutrients and oxygen can initiate the strain growth. In a 250 mL triangular flask, set the liquid loading with 25, 50, 75, 100 and 125 mL, and inoculated 2 %. Adjusted the initial *p*H of the medium to 6.0 and placed at 37 °C. After incubating for 24 h at 180 rot·min^{-1} shaker, the amount of cells in different liquid loading was measured to select the most suitable liquid loading for the growth of the *S. boulardii*.

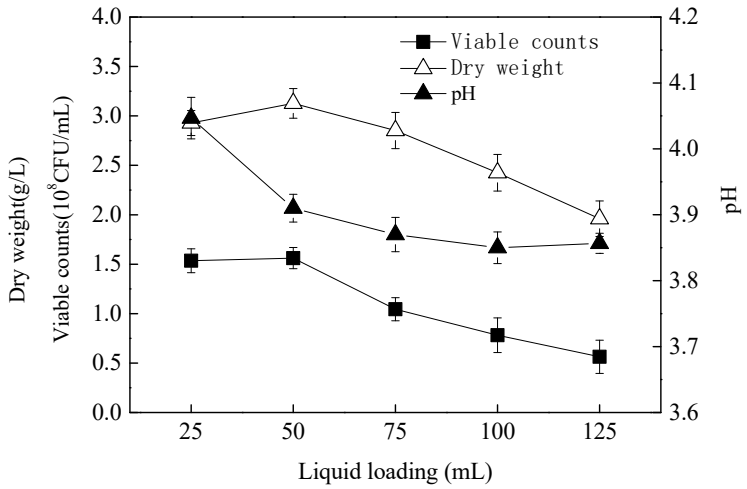


Figure 3. *The effect of different liquid loading on the growth of Saccharomyces boulardii*

As shown in Figure 3, it is found that the viable counts slightly ascend to a maximum value of 1.56×10^8 CFU·mL^{-1} when the liquid loading is increased from 25 to 50 mL/250 mL. However, when the liquid loading is further added to over 50 mL/250 mL, the viable counts remarkably decline and exhibit a continuous reduction as the increase in the liquid loading. Correspondingly, the dry weight performs the identical tendency with the viable counts, which significantly enlarges to the best-performance point of 3.1333 g·L^{-1} when the liquid loading increases to be 50 mL/250 mL and sharply drops with the increasing of the liquid loading. For the liquid loading less than 50 mL/250 mL, the observed results may be interpreted that the dissolved oxygen coefficient is improved and the growing ability of yeast is too strong, leading to the reduction of the cell number after 24-hour culture. While the liquid loading over than 50 mL/250 mL, it

is may be the fact that the decrease in the dissolved oxygen coefficient causes the deficiency of the dissolved oxygen, inhibiting the growth of *S. boulardii*. Notably, the liquid loading plays a certain role in the *p*H value of the medium. The *p*H value generally decreases along with the increasing of liquid loading. For the liquid loading of 50 mL / 250 mL, the *p*H value of the medium is 3.91. Therefore, the subsequent test selected the 250 mL triangular flask with 52 mL medium volume to ensure enough dissolved oxygen.
Dissolved oxygen is one of the important factors affecting cell growth. When the culture solution in the flask is oscillated on the shaker, gas-liquid mixing and dispersion are generated so that the oxygen in the air can be effectively dissolved. Therefore, the amount of liquid loading will directly affect the dissolved oxygen level, thereby affecting the growth of the cells.

Effect of inoculum size

The synthesis of useful bio-products by microorganisms is dependent on nutrient consumption which in turn is largely dependent on the density of bacteria in the finite volume of culture medium. This requires that inoculum size should be controlled to ensure optimum nutrient uptake that conduces in a high product synthesis [20]. The inoculum size will affect the culture period of the cell. Generally, the inoculum size is large, the lag period is short whereas this trend is reversed at the small inoculum size. Nevertheless, the inoculum size is too large, which may cause dissolved oxygen deficiency or nutrient deficiency, thus affecting the growth of cell. So controlling the inoculum size is an important factor of ensuring normal culture.
For this purpose, 1, 3, 5, 7 and 9 % were selected as inoculum size added to the YPD medium, the liquid loading was 50 mL / 250 mL, and the initial *p*H of the medium was adjusted to 6.0, placed on the shaking table at 37 °C, 180 rot·min^{-1} for 24 h.

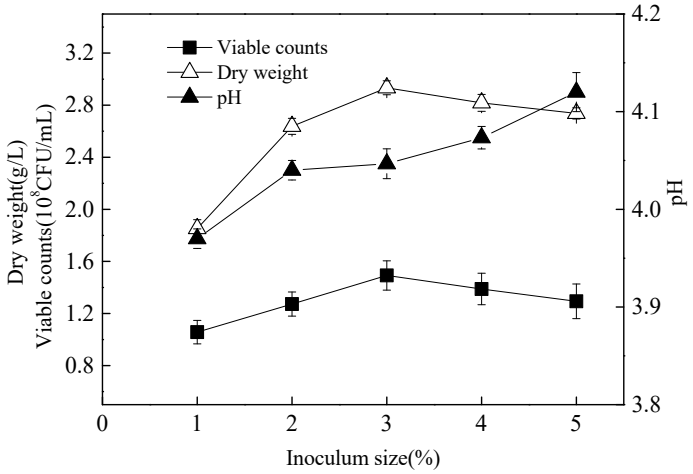


Figure 4. *The effect of inoculum size on the growth of Saccharomyces boulardii*

The viable counts and the dry weight increased first and then decreased with the inoculum size increased (Figure 4). For the inoculum size is 3 %, the viable counts and dry weight reached the maximum value of 1.49×10^8 CFU·mL^{-1} and 2.9411 g·L^{-1},

respectively. For the inoculum size less than 3 %, the inoculum size is too small, the cell lag phase is prolonged, culture time is increasing, leading slow growth of the cells; For the inoculum size over than 3 %, the results may be explicated the inoculum size is too large, causing dissolved oxygen deficiency or lack of nutrients in the medium, and the culture solution is thick in appearance, thereby inhibiting the value-added of *S. boulardii*. Further, the *p*H value of the culture solution increased steadily with increase in inoculum size, and when the inoculum size is 3 %, the *p*H of the culture solution is 4.0. So we will chose 3 % inoculum size as the optimal inoculum size.

Effect of shaking speed

Culture shaking speed significantly affected cell biomass. Higher rotation rates gave lower cell biomass yields. When aerobic microorganisms are cultured, the oxygen in the culture solution is mainly used, and too low or too high dissolved oxygen affect the growth of microorganisms. During shake flask culture, the dissolved oxygen rate is varied by selecting the appropriate liquid loading and shaking speed. When the liquid loading is a certain value, increase the shaking speed of the shaker, and the rate of dissolved oxygen will increase. In this experiment, *S. boulardii* was inoculated into YPD medium with 2 % inoculum size, the liquid loading was 50 mL / 250 mL, the initial *p*H value of the medium was adjusted to 6.0, and the culture was shaken at 37 °C for 24 h. Shaking speeds of 140, 160, 180, 200, 220 rot·min^{-1} was studied.

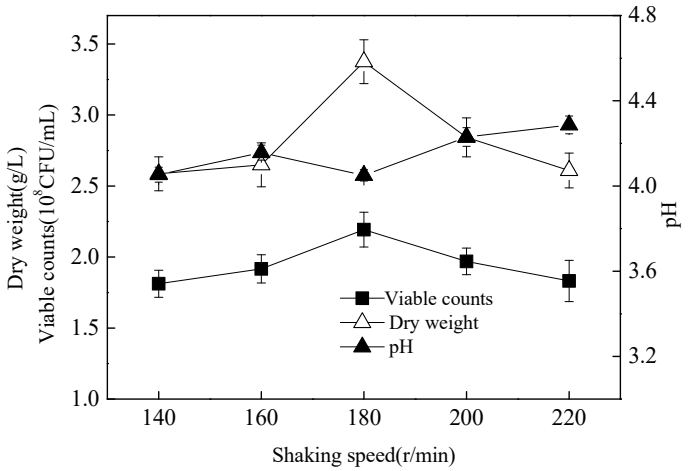


Figure 5. *The effect of shaking speed on the growth of Saccharomyces boulardii*

As shown in Figure 5, the number of viable cells and the dry weight increased first and then decreased with the shaking speed of the shaker increased. When the shaking speed of the shaker is 180 rot·min^{-1}, the number of viable counts and the dry cell weight reach the maximum values of 2.19x10^{8} CFU·mL^{-1} and 3.3752 g·L^{-1}, respectively. The *p*H value of the culture solution decreased first and then increased with the increasing of shaking speed. For the shaking speed of 180 rot·min^{-1}, the *p*H value of the medium is 4.05. So choose the optimal speed of the shaker is 180 rot·min^{-1}.

Central Composite design results and response surface analysis

According to the results of single factor test, the viable counts R1 of *S. boulardii* were the response value, and the Central Composite Design (CCD) was used to carry out the four-factor and five-level response surface test of N = 30 for the cultivation conditions. The Factor level coding table was shown in Table 1, test design results were shown in Table 2.

Table 1. *The factors level coding table of Central Composite Deign for culture conditions of S. boulardii*

Factor	**A**	**B**	**C**	**D**
Level	Liquid loading [mL/250mL]	Shaking speed [rot·min^{-1}]	Inoculum size [%]	*p*H
-2	30	160	2.0	4.5
-1	40	170	2.5	5.5
0	50	180	3.0	6.5
1	60	190	3.5	7.5
2	70	200	4.0	8.5

Table 2. *The experimental design and results of Central Composite Design for culture conditions of S. boulardii*

Runs	**A**	**B**	**C**	**D**	**R1 ×10^8 [CFU·mL^{-1}]**	**Runs**	**A**	**B**	**C**	**D**	**R1 ×10^8 [CFU·mL^{-1}]**
1	1	1	-1	-1	1.24	16	1	-1	1	1	1.12
2	1	-1	-1	1	1.03	17	0	-2	0	0	1.02
3	0	0	0	0	1.87	18	0	0	0	0	1.58
4	1	-1	-1	-1	1.23	19	0	0	0	2	0.72
5	0	0	2	0	1.32	20	1	1	1	1	1.29
6	-1	-1	1	1	0.81	21	-1	-1	1	-1	1.04
7	1	1	1	-1	1.35	22	1	1	-1	1	1.14
8	-1	1	1	1	0.85	23	-1	-1	-1	1	1.16
9	0	0	0	0	1.83	24	1	-1	1	-1	1.26
10	-2	0	0	0	0.89	25	0	0	-2	0	1.38
11	0	0	0	-2	0.73	26	2	0	0	0	1.02
12	0	0	0	0	1.81	27	0	0	0	0	1.78
13	0	0	0	0	1.62	28	-1	1	1	-1	1.1
14	-1	-1	-1	-1	0.86	29	-1	1	-1	1	0.98
15	0	2	0	0	1.19	30	-1	1	-1	-1	1.13

Visualizing the predicted model equation can be obtained by the response surface and contour plot. The response surface plot is a theoretical three-dimensional plot that shows the relationship between the response and independent variables. The two-dimensional display of the surface plot is called contour plot. In the contour plot, lines of the constant response are drawn in the plane of the independent variables. The contour plots help to visualize the shape of a response surface. These plots give useful information about the model fitted, but they may not represent the true behavior of the

system. One must not forget the contours (or surfaces) represent contours of estimated response and the general nature of the system arises as a result of a fitted model, not the true structure [21].

According to the test results from Table 2, quadratic regression analysis of Central Composite Design test results by Design-expert software. The influence of each factor on the response value R1 can be predicted by the regression model – equation (1), and the regression equation obtained as follows:

$$R1=1.75+0.083A+0.038B-0.003C-0.035D+0.012AB+0.044AC-0.011AD+0.009BC-0.018BD-0.033CD-0.19A^2-0.15B^2-0.089C^2-0.24D^2 \quad (1)$$

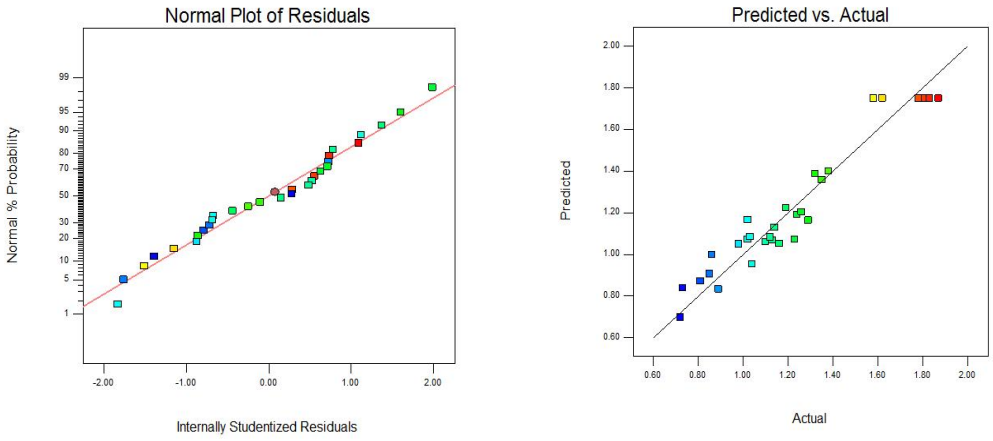
In the equation, R1 was the predicted value of the viable counts of the culture solution to *S. boulardii*, and A, B, C, and D represented the liquid loading, shaking speed, inoculum size, and *p*H value, respectively. In order to verify the validity of the regression model and the influence of various factors on the model, the obtained regression model was analyzed by the variance analysis and the significance test. The results were shown in Table 3.

Table 3. *The ANOVA of the results of Central Composite Design for culture conditions of S. boulardii*

Factor	**Sum of Squares**	**df**	**Mean Square**	**F value**	**P value prob>F**	**Significant**
Model	2.86	14	0.20	13.72	< 0.0001	***
A	0.17	1	0.17	11.07	0.0046	**
B	0.035	1	0.035	2.32	0.1489	
C	2.042E-004	1	2.042E-004	0.014	0.9084	
D	0.030	1	0.030	2.02	0.1756	
AB	2.256E-003	1	2.256E-003	0.15	0.7026	
AC	0.032	1	0.032	2.11	0.1665	
AD	1.806E-003	1	1.806E-003	0.12	0.7325	
BC	1.406E-003	1	1.406E-003	0.094	0.7629	
BD	5.256E-003	1	5.256E-003	0.35	0.5614	
CD	0.018	1	0.018	1.18	0.2948	
A^2	0.96	1	0.96	64.65	< 0.0001	***
B^2	0.62	1	0.62	41.36	< 0.0001	***
C^2	0.22	1	0.22	14.47	0.0017	**
D^2	1.65	1	1.65	110.41	< 0.0001	***
Residual	0.22	15	0.015			
Lack of Fit	0.15	10	0.015	1.07	0.5005	
Pure Error	0.071	5	0.014			
Cor Total	3.09	29				
Coefficient	R^2=0.9276	Adj R^2=0.8600	Pred R^2=0.6824	Adeq Precisior=12.170		

*** extremely significant (p<0.001), ** very significant (p<0.01), * significant (p<0.05)

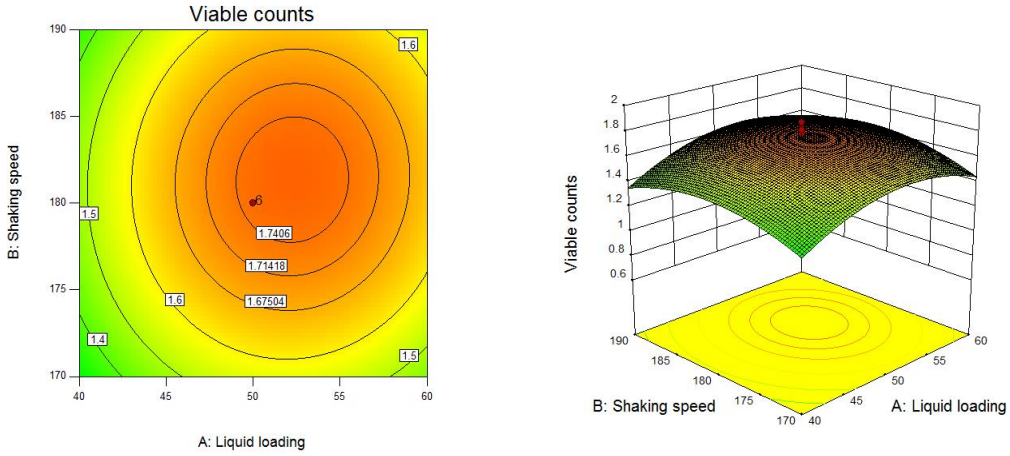
The analysis of variance in Table 3 exhibited the *p* value of the regression equation model was less than 0.001, indicating that the model was extremely significant and the test method was reliable. In terms of lack of fit value, the *p* value was 0.5005 (>0.05), which indicated it was not significant, and the difference between the obtained model and the test results was small. Also, the correlation coefficient of the model was 0.9276, which was close to 1, further indicated that the model for predicting the viable counts of *S. boulardii* was reliable, so the regression equation could be used to predict the response value R1 instead of the real point of the test. At the same time, it was found by *p* value test that the quadratic terms A^2, B^2 and D^2 were extremely significant ($p<0.001$) and contributed greatly to the model.
The testes residual of the response value R1 (viable counts) and the actual values of the test are compared with the predicted values (Figure 6).



(a) **(b)**

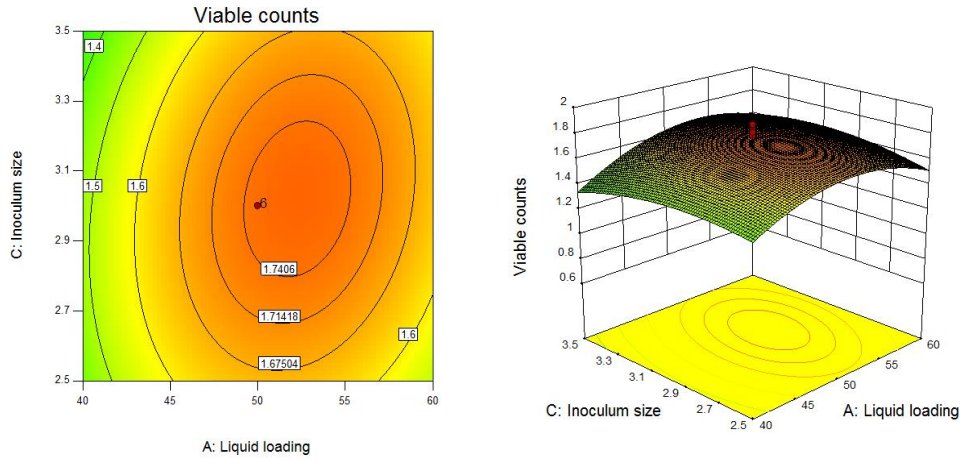
Figure 6. *(a) Control diagram of the internally studentized residuals; (b) The comparison diagram of actual and predicted values*

As shown in Figure 6, the test residuals were basically distributed on a straight line, and the actual value of the test was also around the predicted value of the model, indicating that the model was well fitted.
In order to determine the optimal conditions for each factor, the response surface analysis of the regression model was performed using Design-Expert 8.0.6 software, and the response surfaces were used to predict the effect of each factor on the response value R1 (viable counts), in which the contour plots and the response surface plots were shown from Figure 7 to 12.



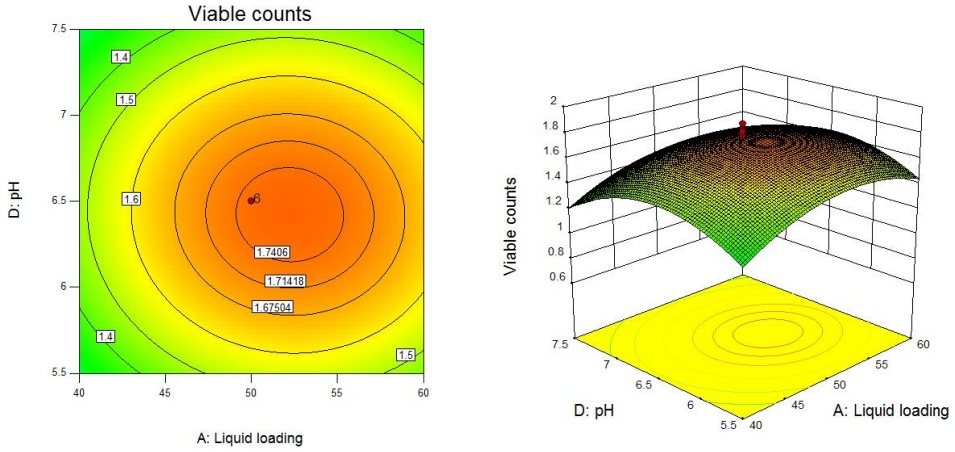
(**a**) (**b**)

Figure 7. *Effect of liquid loading and shaking speed on viable counts*



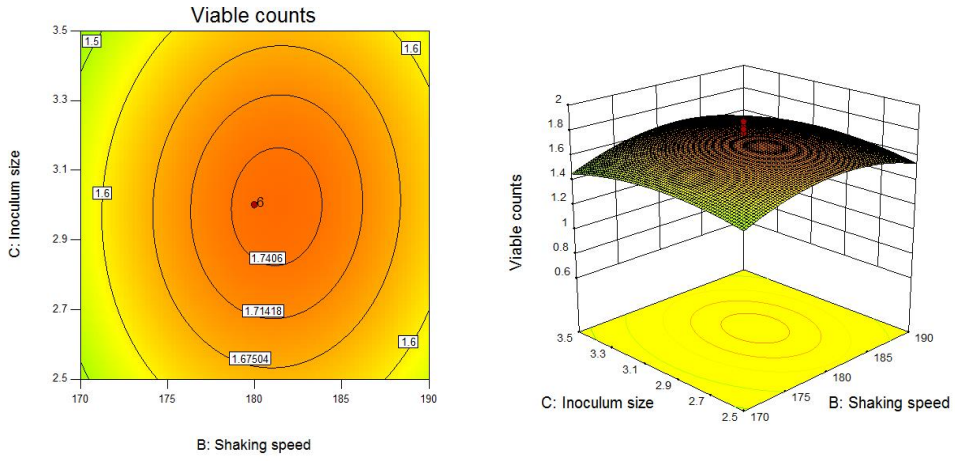
(**a**) (**b**)

Figure 8. *Effect of liquid loading and inoculum size on viable counts*



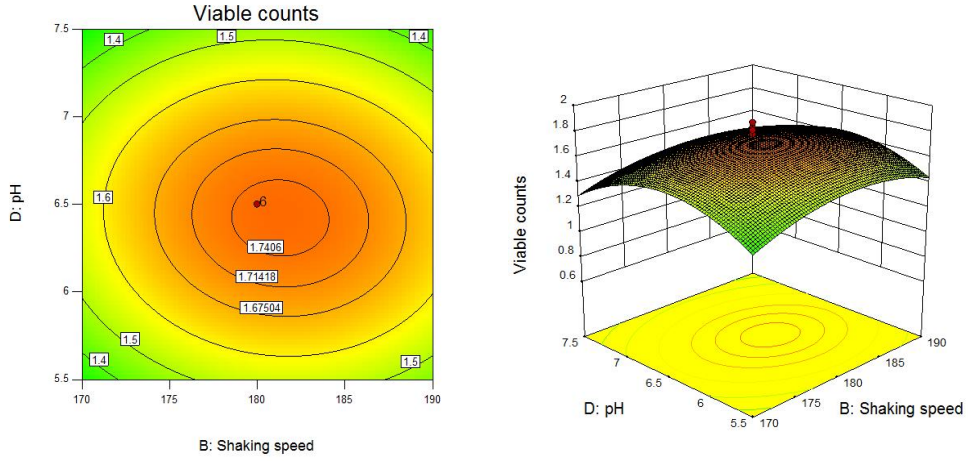
(**a**) (**b**)

Figure 9. *Effect of liquid loading and pH value on viable counts*



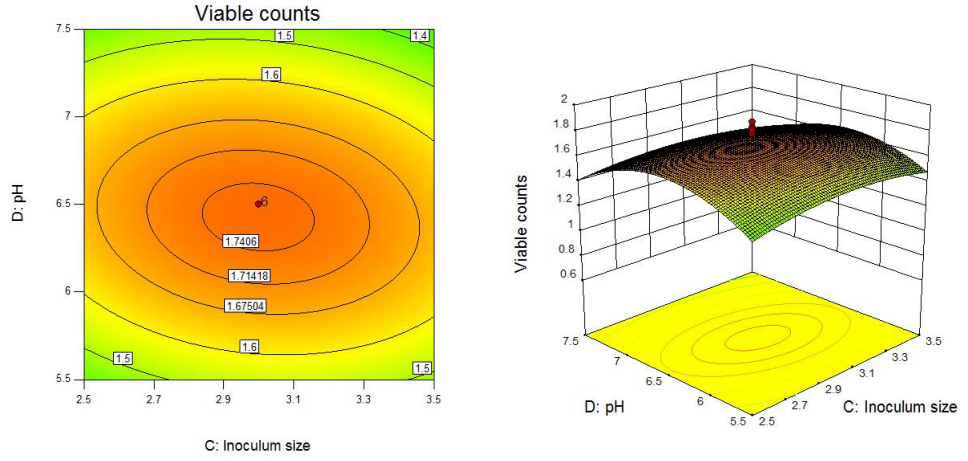
(a) **(b)**

Figure 10. *Effect of shaking speed and inoculum size on viable counts*



(a) **(b)**

Figure 11. *Effect of shaking speed and pH value on viable counts*



(a) **(b)**

Figure 12. *Effect of inoculum size and pH value on viable counts*

According to the results of the response surface plot, the effect of changes in various factors on the response value can be inferred. In addition, the contour plots can be used to determine whether there was an interaction between the factors. If the contour plot was an ellipse, the interaction between the factors was significant; if the contour plot was circular, the interaction between the factors was small.

Figures 7 to 12 showed contour plots and response surface plots could directly reflect the influence of each factor and its interaction on the viable counts. It can be seen from the figure, each contour plots were closed and elliptical, indicating that they had a significant interaction with each other. The contour plots were arched. When two of the four factors were fixed, the viable counts of the *S. boulardii* increased first and then decreased with the increase of the other two factors.

Through the Design-expert software, the variables in the above regression equation were separately guided. After the maximum point was obtained, the coded value was converted into the actual value: the initial *p*H of the medium 6.4, 250 mL triangular flask with 52 mL medium volume, the inoculum size 3 %, and the shaking speed 181 rot·min^{-1}. Under these conditions, the viable count was predicted to be 1.76×10^{8} CFU·mL^{-1}. Three repeated validation tests were performed under the optimized cultivation conditions, the cultivation conditions before optimization were used as the control group, and the viable count of the verification test was $(1.81\pm0.06)\times10^{8}$ CFU·mL^{-1}, before the optimization (1.63×10^{8} CFU·mL^{-1}) increased by 11 %, and the verification value was close to the predicted value of the model, indicating that optimizing the fermentation conditions on the response surface can increase the viable counts.

Du et al. used the response surface method to optimize the cultivation conditions of the *S. boulardii*, and the optimal fermentation conditions were: 50 mL/250 mL liquid loading, natural *p*H, 5 % inoculum size, 32 °C culture temperature, 160 rot·min^{-1} shaking speed [22]. Under this condition, the biomass of the cells was the highest. There are some differences between the cultivation conditions optimized in this experiment and Du Liping *et al.*, which may be due to differences in the composition of the medium. This experiment was based on the YPD medium to optimize the cultivation conditions of the *S. boulardii*. The medium composition of Du *et al.* in the cultivation of *S. boulardii* was 0.1 % (w/v) yeast extract, 1 % (w/v) peptone and glucose 2 % (w/v). It can be seen that the medium components of the same strain change and the corresponding cultivation conditions will also change. Similar associations include Agyei et al. showed that optimal conditions for proteinase production included an initial *p*H of 6.0, 45 °C incubation temperature, 2 % (v/v) inoculum size of OD_{560} = 1, 150 rpm agitation speed, and growth medium carbon/nitrogen ratio of 1.0 [23]. Maximum proteinase activity obtained from whole cells was 0.99 U·mL^{-1} after 8 h of incubation; Y. Paola Maturano et al. have shown that the lowest ethanol produced by *H. uvarum* BHu9/*S. cerevisiae* BSc114 coculture was obtained when *H. uvarum* BHu9 was inoculated 48 h 37 min before *S. cerevisiae* inoculation, at a fermentation temperature of 25 °C and at an initial inoculum size of 5×10^{6} cells/Ml [24]. The lowest alcohol production of *C. membranaefaciens* BCm71/*S. cerevisiae* BSc114 was observed when *C. membranaefaciens* BCm71 was inoculated 24 h 15 min before *S. cerevisiae* at a fermentation temperature of 24.94 °C and at an initial inoculum size of 2.72×10^{6} cells·mL^{-1}.

CONCLUSIONS

The demand for probiotic products is growing rapidly due to the increasing awareness of consumers about the effects of probiotics on health. The quality of probiotic products is evaluated by the number of viable bacteria because the health benefit of probiotic on the host works depends on their concentration. Thus, the key to increase the yield of the powder is to increase the viable counts of *S. boulardii* in the culture medium. In this study, the cultivation conditions for *S. boulardii* are comprehensively optimized to be temperature 37 °C, the initial *p*H value 6.4, 250 mL triangular flask with 52 mL medium volume, inoculum size 3 %, and shaking speed 181 rot·min^{-1}. Under these conditions, the viable counts in the culture solution is $(1.81\pm0.06)\times10^{8}$ CFU·mL^{-1}, which is increased 11 % than before optimization. Therefore, the results demonstrate that it is feasible to use the CCD to optimize the cultivation conditions of *S. boulardii*. It has practical significance for the application of probiotics *S. boulardii* in animal husbandry, medicine and other fields.

ACKNOWLEDGMENT

The work was partly supported by the Key Research and Development Program of Shaanxi (Program No. 2019ZDLNY06-02 and 2020NY-185), Xi'an Science and Technology Plan Project (201806118YF06NC14(2)) and the Science and Technology Bureau of Weiyang District (No. 201941).

REFERENCES

1. Czerucka, D., Rampal, P.: Experimental effects of *Saccharomyces boulardii* on diarrheal pathogens, *Microbes and Infection*, **2002**, **4** (7), 73-739;
2. Fuller, R.: Probiotics in human medicine, *Gut*, **1991**, **32** (4), 439-442;
3. Marteau, P.: Protection from gastrointestinal diseases with the use of probiotics, *American Journal of Clinical Nutrition*, **2001**, **73** (2), 430-436;
4. Bondarenko, V.M., Chuprinina, R.O., Aladysheva, Z., Matsulevich, T.V.: Probiotics and mechanisms of their curative action, *Experimental & Clinical Gastroenterology*, **2004**, **105** (3);
5. Karaolis, C., Botsaris, G., Pantelides, I., Tsaltas, D.: Potential application of *Saccharomyces boulardii* as a probiotic in goat's yoghurt: survival and organoleptic effects, *International Journal of Food Science & Technology*, **2013**, **48** (7), 1445-1452;
6. Chen, H., Tian, M.Q., Chen, L., Cui, X.X., Meng, J.P., Shu, G.W.: Optimization of composite cryoprotectant for freeze-drying *Bifidobacterium bifidum* BB01 by response surface methodology, *Artificial Cells, Nanomedicine, and Biotechnology*, **2019**, **47** (1), 1559-1569;
7. Czerucka, D., Piche, T., Rampal, P.: Review article: yeast as probiotics -*Saccharomyces boulardii*, *Alimentary Pharmacology & Therapeuti*, **2007**, **26** (6), 767-778;
8. Mcfarland, L.V.: Systematic review and meta-analysis of *Saccharomyces boulardii* in adult patients, *World Journal of Gastroenterology*, **2010**, **16** (18), 2202-2222;
9. Hu, Y.L., Qin, H.H., Zhan, Z.C., Dun, Y.H., Zhou, Y., Peng, N., Ling, H.Y., Liang, Y.X., Zhao, S.: Optimization of *Saccharomyces boulardii* production in solid-state fermentation with response surface methodology, *Biotechnology & Biotechnological Equipment*, **2015**, **30** (1), 173-179;
10. Buts, J.P., De Keyser, N., Stilmant, C., Sokal, E., Marandi, S.: *Saccharomyces boulardii* enhances *n*-terminal peptide hydrolysis in suckling rat small intestine by endoluminal release of a zinc-binding metalloprotease, *Pediatric Research*, **2002**, **51** (4), 528-534;
11. Nomoto, K.: Prevention of infections by probiotics, *Journal of bioscience and bioengineering*,

2005, **100** (6), 583-592;
12. Sazawal, S., Hiremath, G.U., Malik, P., Deb, S., Black, R.E.: Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials, *Lancet Infectious Diseases*, **2006**, **6** (6), 374-382;
13. Guslandi, M., Giollo, P., Testoni, P.A.: A pilot trial of *Saccharomyces boulardii* in ulcerative colitis, *European Journal of Gastroenterology & Hepatology*, **2003**, **15** (6), 697-698;
14. Bontempo, V., Giancamillo, A.D., Savoini, G., Dell'Orto, V., Domeneghini, C.: Live yeast dietary supplementation acts upon intestinal morpho-functional aspects and growth in weanling piglets, *Animal Feed Science and Technology*, **2006**, **129** (3-4), 224-236;
15. Chu, J., Wang, F.: Research progress on the biological and therapeutic effects of *S.boulardii*, *Pharmaceutical Biotechnology*, **2006**, **13** (1), 71-73;
16. Chu, J., Zhang, D.: Adjustment on the disorder of intestinal flora of *S. boulardii*, *China Animal Husbandry & Veterinary Medicine*, **2008**, **35** (7), 87-89;
17. Shu, G.W., Lei, Z.T., Chen, H., Wang, P., Huang, D.: Screening of medium compounds using a two-level factorial design for *Saccharomyces boulardii*, *Scientific Study & Research-Chemistry & Chemical Engineering, Biotechnology, Food Industry*, **2016**, **17** (1), 45-54;
18. Lei, Z.T., Chen, H., Huang, D., Zhai, Y.L., Shu, G.W.: Optimization of medium compounds for *Saccharomyces boulardii* by Box-Behnken Design, *Scientific Study & Research-Chemistry & Chemical Engineering, Biotechnology, Food Industry*, **2016**, **17** (4), 405-416;
19. Panesar, P.S., Kennedy, J.F., Knill, C.J., Maria, K.: Production of L(+) lactic acid using *Lactobacillus casei* from whey. *Brazilian Archives of Biology and Technology*, **2010**, **53** (1), 219-226;
20. Abusham, A.R., Salleh, A.B., Basri, M.: Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain Rand, *Microbial Cell Factories*, **2009**, **8** (1), 20;
21. Sarlak, N.: Effects of electrospinning parameters on titanium dioxide nanofibers diameter and morphology: an investigation by box–wilson central composite design (ccd), *Chemical Engineering Journal*, **2012**, **210** (6), 410-416;
22. Du, L.P., Hao, R.X., Xiao, D.G, Guo, L., Gai, W.D.: Research on the characteristics and cultivation conditions of *Saccharomyces boulardii*, *Advanced Materials Research*, **2012**, **343-344**, 594-598;
23. Agyei, D., Potumarthi, R., Danquah, M.K.: Optimization of Batch Cultivation conditions for Cell-Envelope-Associated Proteinase Production from *Lactobacillus delbrueckii* subsp. lactis ATCC® 7830™, *Applied Biochemistry and Biotechnology*, **2012**, **168** (5), 1035-1050;
24. Maturano, Y.P., Mestre, M.V., Kuchen, B., Vazquez, F., Toroa, M.E., Mercado L.A., Combina, M.: Optimization of fermentation-relevant factors: A strategy to reduce ethanol in red wine by sequential culture of native yeasts, *International Journal of Food Microbiology*, **2018**, **289**, 40-48.