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ORIGINAL RESEARCH PAPER

SCREENING OF MEDIUM COMPOUNDS USING A TWO-LEVEL FACTORIAL DESIGN FOR *SACCHAROMYCES BOULARDII*

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Abstract: Even if the probiotic effect of *Saccharomyces boulardii* is has been reported, this yeast is rarely used in medium composition. Based on single factor experiment, two-level factorial design was employed to evaluate the effect of carbon sources (sucrose, glucose), nitrogen sources (soy peptone, beef extract, yeast extract, calf serum, malt extract) and salts (K_2HPO_4, KH_2PO_4, $MgSO_4$, Na_2HPO_4, NaH_2PO_4, $CaCl_2$, sodium citrate, sodium glutamate) on the growth of *S. boulardii*. At the same time, the optical density (OD) in the medium was measured at 560 nm after 36 h of incubation. The result of two-level factorial design experiment showed that calf serum (p = 0.0214) and sodium citrate (p = 0.0045) are the significant growth factors of *S. boulardii*, sucrose (p = 0.0861) and malt extract (p = 0.0763) are important factors. In addition, sucrose and sodium citrate showed positive effect on the growth of *S. boulardii*. However, calf serum and malt extract showed negative effect on the growth. And we determined that the optimum medium composition for *S. boulardii* was as follow: 37.5 $g \cdot L^{-1}$ sucrose, 6 $g \cdot L^{-1}$ calf serum, 6 $g \cdot L^{-1}$ malt extract, 5 $g \cdot L^{-1}$ sodium citrate.

Keywords: *carbon sources, factorial design, nitrogen sources, optical density, Saccharomyces boulardii*

INTRODUCTION

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [1, 2]. Probiotics can modify the balance of intestinal micro-ecology in the host (animal or human) [3], enhance human immunity and promote the absorption of nutrients [4].

Most probiotics are bacteria, *Saccharomyces boulardii* is the only yeast whose effect has been proved in double-blind studies [5]. *S. boulardii* was isolated from litchi fruit in 1923 by French scientist Henri Boulardii in Indonesia [6], it has many specific properties, i.e. its optimal growth temperature is 37 °C (physiological temperature of the host), both in vitro and vivo [7, 8], it can inhibit the growth of most pathogenic bacteria [9, 10]. And the biological characteristics of *S. boulardii* are different from common yeast [11]. Research indicated that, the survival rate of *S. boulardii* was significantly higher than that of common yeast under the condition of gastric acid [8]. Therefore, the survival ability of *S. boulardii* in the intestinal tract is obviously higher than that of common yeast, which is favorable to the probiotic effects of *S. boulardii*.

In several clinical studies, *S. boulardii* has been used in prevention and treatment of various diarrhea and inflammatory bowel disease [12], for instance, traveller's diarrhea [13, 14], antibiotic associated diarrhoea [15 – 18] and recurrent *Clostridium difficile* disease [19, 20]. Furthermore this is a part of action mechanism that has been illuminated [21, 22]. According to reports, *Clostridium difficile*, *Vibrio cholerae* and *Escherichia coli* were the main pathogenic bacteria which can cause severe diarrhea in human and animal [13, 23]. In case of the pathogenic bacteria infection, the micro-ecological balance of the host would be destroyed in the intestine. While *S. boulardii* could be effective in the treatment of diarrhea caused by the above pathogenic bacteria, and has achieved good therapeutic effect in clinic [24 – 26]. The host intestinal microflora balance once destroyed, *C. difficile* easily invade the intestine and releases two toxins, i.e. Toxin A (308 kDa) and toxin B (270 kDa) [19]. Toxin A can cause inflammation of the small intestine and damage colon cells, and toxin B can promote the host to release inflammatory cytokines which makes colitis aggravated [27]. Meanwhile, *S. boulardii* can secrete a 54 kDa protease that acted by proteolysis of both the toxin A and B, thereby reducing the content of toxins in the host to effectively prevent pathogenic enteritis [12, 28]. Moreover, *S. boulardii* could also prevent diarrhea that infected by *E. coli* [7].

To achieve the probiotic benefits, selected live yeast cell need to reach the sufficient amount of cell. Therefore, this paper studied the effect of carbon sources, nitrogen sources and salts on the growth of *S. boulardii*, and initially evaluated the significant factors of carbon sources, nitrogen sources and salts in the medium for *S. boulardii*.

MATERIALS AND METHODS

Microorganism

The strain used in this study, *Saccharomycess boulardii* was obtained from School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China). Soy peptone, beef extract and yeast extract were purchased from Aoboxing

Biotechnology Co., Ltd (Beijing, 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

The YPD media was used in this study, containing 10 g of yeast powder, 20 g glucose, 20 g peptone, 1000 mL water, and autoclaved at 118 °C for 15 min. *S. boulardii* was inoculated in YPD medium and activated three successive times at 37 °C for 36 h.

Factorial design

In order to evaluate the significant factors of carbon sources, nitrogen sources and salts in the medium for *S. boulardii*, the design used was 2-level factorial design. Each factor was tested at two levels, high level (+1) and low level (-1). The range of variables was determined by single factor experiment. The independent variables of carbon sources were sucrose (X1) and glucose (X2), and nitrogen sources were soy peptone (X3), beef extract (X4), yeast extract (X5), calf serum (X6) and malt extract (X7). Those 7 ingredients tested are given in Table 1 along with their actual levels. While the independent variables of salts were K_2HPO_4 (Y1), KH_2PO_4 (Y2), $MgSO_4$ (Y3), Na_2HPO_4 (Y4), NaH_2PO_4 (Y5), $CaCl_2$ (Y6), sodium citrate (Y7) and sodium glutamate (Y8), which listed in Table 4.

Growth measurement

The growth of strain was monitored by measuring the optical density at 560 nm (OD_{560}) through a spectrophotometer (Sp-756 pC, 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.

Statistical analysis of the data

The Design-Expert (Version 8.0.6) software was used for the experiment design and analysis of the experiment data to identify the significant factors and their corresponding coefficients. Therefore, sum of squares, F-value, p-value and confidence intervals (CI) were evaluated to analyze the OD value of each experiment.

RESULTS AND DISCUSSION

Factorial design screening of carbon sources and nitrogen sources for *S. boulardii*

Using factorial design ($N = 2^{7-3}$) to screen the significant effects of carbon sources and nitrogen sources for *S. boulardii*. The factorial design comprised 7 factors spanning over 16 runs with each factor fixed at two levels (namely a low level and a high level). The levels of 7 ingredients were given in Table 1.

Table 1. *The factors levels for carbon sources and nitrogen sources of factorial design*

Factor level [g·L^{-1}]	X1 (sucrose)	X2 (glucose)	X3 (soy peptone)	X4 (beef extract)	X5 (yeast extract)	X6 (calf serum)	X7 (malt extract)
-1	30	40	8	8	8	6	6
1	37.5	50	10	10	10	7.5	7.5

Test design and results of factorial design were shown in Table 2. According to the test design in Table 2, activated *S. boulardii* was inoculated with 2 % (v/v) inoculum in 250 mL flask containing 35 mL medium, and then incubated at 37 °C for 36 h in the shaker at 180 rpm. The response value R1 represented OD value in the fermentation suspension.

Table 2. *The experimental design and results for carbon sources and nitrogen sources of factorial design*

Run	X1	X2	X3	X4	X5	X6	X7	R1
1	1	1	1	-1	1	-1	-1	0.961
2	1	1	1	1	1	1	1	0.937
3	-1	-1	1	1	1	-1	-1	1.02
4	1	-1	1	-1	-1	1	-1	0.955
5	-1	-1	-1	-1	-1	-1	-1	1.011
6	1	-1	1	1	-1	-1	1	1.005
7	1	1	-1	-1	-1	1	1	0.985
8	-1	-1	1	-1	1	1	1	0.907
9	1	-1	-1	-1	1	-1	1	1.017
10	-1	1	-1	-1	1	1	-1	0.937
11	-1	1	1	-1	-1	-1	1	0.919
12	-1	1	1	1	-1	1	-1	0.98
13	1	1	-1	1	-1	-1	-1	1.029
14	-1	1	-1	1	1	-1	1	0.963
15	1	-1	-1	1	1	1	-1	0.99
16	-1	-1	-1	1	-1	1	1	0.941

According to the Table 3 analysis of variance, the model p-value of 0.0481 implied the model was significant. In the ANOVA, the p-value greater than 0.1000 indicated the model terms are not significant. In Table 3, the effect of each factor on the growth of *S. boulardii* as follows: calf serum (X6) > malt extract (X7) > sucrose (X1) > soy peptone (X3) > beef extract (X4) > glucose (X2) > yeast extract (X5).

In statistics, the confidence level was greater than 95 % ($0.01< p <0.05$), the factor was defined as a significant factor. The confidence level was greater than 90 % ($0.05 < p < 0.1$), the factor was defined as an important factor. It was shown that showed calf serum (X6) was a significant factor, malt extract (X7) and sucrose (X1) were important factors on growth of *S. boulardii*.

Table 3. *Analysis of variance (ANOVA) of carbon sources and nitrogen sources for R1 (OD value)*

Sources	Sum of squares	df	Mean squares	F value	p-value prob>F	Significance	Significance ordering
Model	0.016	7	0.0023	3.55	0.0481	*	
A-X1	0.0025	1	0.0025	3.83	0.0861		3
B-X2	0.0011	1	0.0011	1.73	0.2252		6
C-X3	0.0022	1	0.0022	3.39	0.1031		4
D-X4	0.0019	1	0.0019	2.84	0.1306		5
E-X5	0.0005	1	0.0005	0.82	0.3917		7
F-X6	0.0054	1	0.0054	8.14	0.0214		1
G-X7	0.0027	1	0.0027	4.14	0.0763		2
Residual	0.0053	8	0.0007				
Cor Total	0.022	15					

Moreover, the positive or negative effects of these three variables are presented in Figure 1.

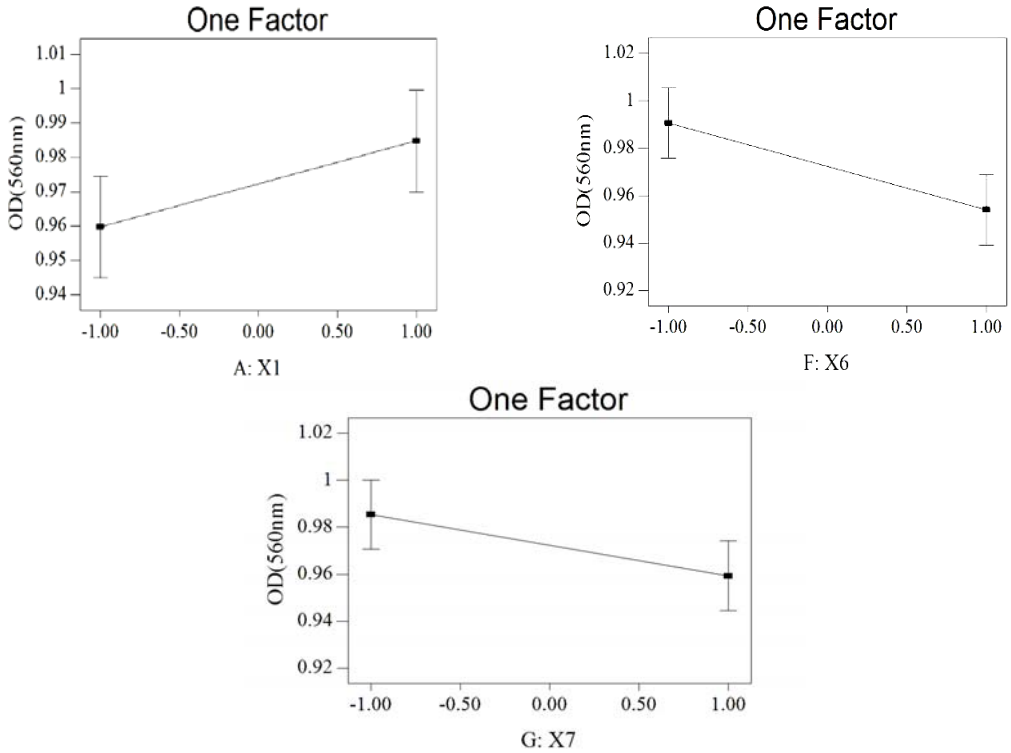

Figure 1. *The 95 % confidence interval of A, F and G (A-sucrose, F-calf serum, G-malt extract)*

From Figure 1, sucrose had a positive effect on the growth of *S. boulardii*, however, calf serum and malt extract showed negative effect on the growth. Namely, with the

increasing concentration of sucrose, the OD value increased, and raising the concentration of calf serum and malt extract resulted in the OD value decreased. Thus, the optimal concentration of sucrose, calf serum and malt extract were 37.5 $g \cdot L^{-1}$, 6 $g \cdot L^{-1}$, 6 $g \cdot L^{-1}$, respectively.

Factorial design screening of salts for *S. boulardii*

Screening the significant effects of salts for *S. boulardii* was used by factorial design ($N = 2^{8-4}$). In this experiment, the carbon source was 37.5 $g \cdot L^{-1}$ sucrose, and the nitrogen sources were 6 $g \cdot L^{-1}$ calf serum and 6 $g \cdot L^{-1}$ malt extract. The design consisted of 8 factors spanning over 16 runs with each factor fixed at two levels. The factorial experiment design and the levels of 8 factors were presented in Table 4.

Table 4. *The factors levels for salts of factorial design*

Factor level [$g \cdot L^{-1}$]	Y1 (K_2HPO_4)	Y2 (KH_2PO_4)	Y3 ($MgSO_4$)	Y4 (Na_2HPO_4)	Y5 (NaH_2PO_4)	Y6 ($CaCl_2$)	Y7 (sodium citrate)	Y8 (sodium glutamate)
-1	5	0	5	4	2	0	0	0
1	6.25	1	6.25	6	2.5	1	5	2.5

Test design and results of factorial design were shown in Table 5.

Table 5. *The experimental design and results for salts of factorial design*

Runs	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	R1
1	1	1	1	1	1	1	1	1	1.072
2	-1	1	1	-1	-1	-1	1	1	1.236
3	-1	1	1	1	-1	1	-1	-1	0.896
4	1	1	1	-1	1	-1	-1	-1	1.036
5	1	-1	1	1	-1	-1	1	-1	1.115
6	1	-1	1	-1	-1	1	-1	1	0.767
7	-1	-1	-1	1	-1	1	1	1	1.095
8	-1	-1	1	-1	1	1	1	-1	0.979
9	1	1	-1	-1	-1	1	1	-1	1.03
10	-1	1	-1	-1	1	1	-1	1	0.957
11	-1	-1	1	1	1	-1	-1	1	1.034
12	1	-1	-1	-1	1	-1	1	1	1.338
13	1	1	-1	1	-1	-1	-1	1	1.097
14	-1	1	-1	1	1	-1	1	-1	1.124
15	1	-1	-1	1	1	1	-1	-1	0.741
16	-1	-1	-1	-1	-1	-1	-1	-1	1.139

Based on the carbon source and nitrogen sources mentioned above and the test design in Table 5, activated *S. boulardii* was inoculated with 2 % (v/v) inoculum in 250 mL flask containing 35 mL medium, then incubated at 37 ^{o}C for 36 h in the shaker at 180 rpm. The response value R1 expressed the OD value in the fermentation suspension.
The variance (ANOVA) of salts (OD value) of *S. boulardii* is presented in Table 6.

Table 6. *Analysis of variance (ANOVA) of salts for R1 (OD value)*

Source	Sum of squares	df	Mean square	F value	p-value prob>F	Significance	Significance ordering
Model	0.31	8	0.0384	5.9689	0.0147	*	
A-Y1	0.0044	1	0.0044	0.6768	0.4378		6
B-Y2	0.0036	1	0.0036	0.5593	0.4789		7
C-Y3	0.0093	1	0.0093	1.4468	0.2681		4
D-Y4	0.0059	1	0.0059	0.9211	0.3691		5
E-Y5	0.0006	1	0.0006	0.0858	0.7781		8
F-Y6	0.1564	1	0.1564	24.3018	0.0017		1
G-Y7	0.1092	1	0.1092	16.9702	0.0045		2
H-Y8	0.0180	1	0.0180	2.7897	0.1388		3
Residual	0.0451	7	0.0064				
Cor Total	0.3524	15					

The model p-value of 0.0147 implied the model was significant. The relative importance of the variables was as follows: Y6 >Y7 > Y8 > Y3 > Y4 > Y1 > Y2 >Y5. The factors mentioned, $CaCl_2$ (Y6) (p = 0.0017) and sodium citrate (Y7) (p = 0.0045) were significant factors, which showed significant effect on the growth of *S. boulardii.* In addition, the positive or negative effects of these two various showed at Figure 2. As Figure 2 showed that sodium citrate had a positive effect on the growth of *S. boulardii*, however, $CaCl_2$ had a negative effect on the growth. Therefore, these two factors were determined as: the Y6 ($CaCl_2$) to -1 level, Y7 (sodium citrate) to 1 level, but we found the -1 level of Y6 ($CaCl_2$) was 0 g·L^{-1}, so only select Y7 (sodium citrate) as salt, and its optimal concentration was 5 g·L^{-1}.

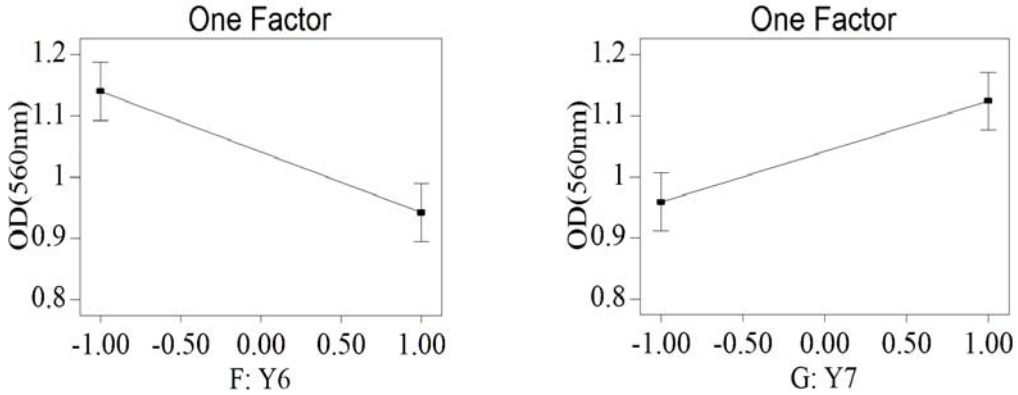

Figure 2. *The 95 % confidence interval of F and G (F-$CaCl_2$, G-sodium citrate)*

DISCUSSION

In this study, factorial design was employed to estimate the effect of carbon sources, nitrogen sources and salts on the growth of *S. boulardii*. The result indicated that 37.5 $g \cdot L^{-1}$ sucrose, 6 $g \cdot L^{-1}$ calf serum, 6 $g \cdot L^{-1}$ malt extract and 5 $g \cdot L^{-1}$ sodium citrate had a significant effect on the growth of *S. boulardii*. Especially, sucrose was the most efficient carbon source for the yeast growth. In general, yeast preferably consumes monosaccharides and next complex sugars. However, in this study, sucrose is a better source of carbon then glucose [29]. While there are some relevant reports, according to single factor experiment, Wei *et al* determined the optimal carbon source in the medium for *Saccharomyces uvarum* was 4 % sucrose [30]. Qi *et al* optimized the components of proliferation medium for *Candida utilis* by single factor experiment and response surface, the result showed that the optimal carbon source was 76.17 $g \cdot L^{-1}$ source [29]. Also the study of Zhong *et al*. [31], showed that *Candida carpophila* can grow very well in the presence of sucrose, and its optimal concentration was 3 %.
Sodium citrate can promote the growth of *S. boulardii* very well. Generally, the suitable *p*H for the growth of yeast is between 4 - 6. Because the medium acidity increased in the process of self-metabolism of yeast, it's not suitable for the growth of yeast. Sodium citrate is an acidity regulator, having a good *p*H regulation performance. Zhao *et al.* [32] added citric acid and sodium citrate buffer into the culture medium, which promoted the growth and metabolism of yeast.

CONCLUSION

In this study, screening of carbon sources, nitrogen sources and salts for *S. boulardii* used a two-level factorial design.
The result showed that the most important factors for the growth of *S. boulardii* are: calf serum, sodium citrate, sucrose and malt extract. From these four factors only, in the case of sucrose and sodium citrate a positive effect on the growth was observed. The optimum medium composition for *S. boulardii* was as follow: 37.5 $g \cdot L^{-1}$ sucrose, 6 $g \cdot L^{-1}$ calf serum, 6 $g \cdot L^{-1}$ malt extract and 5 $g \cdot L^{-1}$ sodium citrate.

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