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Full Length Research Paper

Fermentation optimization and antioxidant activities of mycelia polysaccharides from *Morchella esculenta* using soybean residues

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The mycelia polysaccharides from *Morchella esculenta* are active ingredients in a number of medicines that play important roles in immunity improvement and tumor growth inhibition. So far, the production of polysaccharides from *M. esculenta* mycelia has not been commercialized. The aims of this work were to screen and optimize the fermentation conditions to produce mycelia polysaccharides from using soybean residues as basic substrates in the composition of the medium, and to evaluate the antioxidant activities of mycelia polysaccharides from *M. esculenta*. Our results demonstrate that *M. esculenta* mycelia made good use of soybean residues. The optimal media contained the following components (g/l): soybean residue, 22.2; glucose, 20.1; KH_2PO_4 2.0 and $MgSO_4 \cdot 7H_2O$ 1.5. The optimum parameters of liquid culture were identified as the following: initial fermentation pH 7.0, inoculation volume 10%, temperature 28°C, and fermentation time 56 h. Under these optimized conditions, the values of dry cell weight (DCW) and the production rates of mycelia polysaccharides were 36.22 g/l and 68.23 mg/g, respectively. Furthermore, the mycelia polysaccharides from *M. esculenta* demonstrated strong antioxidant activities, suggesting their potential therapeutic use as antioxidants. Our study provides insights to the commercial production of mycelia polysaccharides from *M. esculenta*. Our study also provides a new route for utilization of soybean residues.

Key words: *Morchella esculenta*, liquid fermentation, mycelia polysaccharides, soybean residues, antioxidant activity.

INTRODUCTION

Morchella esculenta belongs to *Ascomycotina, Discomycetes, Pezizales, Morchellaceae, Morchella. M. esculenta* was named due to its rough surfaces and similarity to lamb tripe is one of the famous edible and medicinal fungi with many bioactive materials such as protein, fat, minerals and carbohydrates (Litchfteld et al., 2006). The polysaccharides isolated from sporophores and mycelia from *M. esculenta* can be used as major ingredients for many medicines. Besides the anti-viral activity, the polysaccharides also display antioxidative and anti-cancer properties that can resist fatigue (Nitha and Janardhanan, 2008; Elmastas et al., 2006; Meng et al., 2010). Studies have shown that polysaccharides from *M. esculenta* may possess radical scavenging properties (Nitha et al., 2010; Meng et al., 2010). Cellular damage caused by reactive oxygen species (ROS) has been implicated in several diseases and antioxidants are known to protect body from this damage.

The production of *M. esculenta* polysaccharides has not been commercialized yet. The submerged fermentation of *M. esculenta* has advantages such as low cost, easily controlled by fermentation efficiency and unaffected by the surrounding environment. Based on these advantages, *M. esculenta* fermentation is ideal for industrialized production of polysaccharides. Most

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importantly, the fermented sporophores and mycelia of *M. esculenta* have similar properties in flavor, chemical composition and physiological function (Zhang et al., 2010; Wang et al., 2007). Therefore, the polysaccharides isolated from fermented sporophores and mycelia of *M. esculenta* are the major sources for production. Zhao and Ma (2002) studied the components required for the liquid media for fermentation. The spore germination of the wild *M. esculenta* was studied by Li et al. (2004), and the optimal germination and growth factors of the *M. esculenta* were investigated. Strains of *M. esculenta* suitable for liquid fermentation were identified; and the optimal conditions were identified (Xing et al., 2004). Sun and Wu (2000) determined the optimal conditions for *M. esculenta* fermentation and growth. While most studies were focused on the production of polysaccharides using cultural conditions of mycelia in *M. esculenta* (Xu et al., 2008; Meng et al., 2010), fewer studies were focused on using submerged fermentation to obtain polysaccharides from *M. esculenta.*

Soybean residues are the by-products of soybean food processing after the soluble proteins have been removed. Soybean residues received less attention due to their low calories and poor oral taste (Xue et al., 2009). However, soybean residues contain rich nutritional values with 50% fibers, 20% proteins and 10% fats. Soybean residues are mainly used for animal feeding, or used as protein feeds after microbiological fermentation (Mo, 2007). Although the extraction of polysaccharides from *M. esculenta* has been optimized, there is no study on polysaccharide optimization in *M. esculenta* mycelia through fermentation using soybean residues. In this paper, we applied the response surface methodology to optimize the fermentation media and conditions for polysaccharides production in mycelia *M. esculenta* using soybean residues. We further studied the antioxidant activities of the raw polysaccharides isolated from mycelia of *M. esculenta.* Our results provide a reference for large-scale polysaccharide extraction from mycelia of *M.* esculenta by industrial fermentation using soybean residues.

MATERIALS AND METHODS

The strains and media was purchased from Edible Fungi Research Institute in Chaoyang City and maintained on synthetic potato dextrose agar (PDA). The components for slant and agar media (PDA media) were as follows: 200 g potato, 20 g glucose, 20 g agar, and 1000 ml water. PDA media without agar were used as seeding media. Soybean residues were used as the base for fermentation medium, with 20 g glucose, 1.5 g $MgSO_4$, 1g KH_2PO_4 and 1000 ml of water. Soybean residues, provided by the food courts in Dalian Nationality University, were powdered and baked at 60°C until they were used.

Strain activation, seed incubation and fermentation

The strain (*M. esculenta)* was streaked onto agar slant and incubated at 25°C for seven days. The strain inoculated in PDA liquid media were incubated at 28°C and shaken on a rotary shaker (Fengmeng Inc., Shanghai, China) at 150 rpm/min for five to seven days. 10% of the seeds from PDA liquid media were placed into fermentation media, incubated at 28°C and shaken at 150 r/min for five to seven days. After centrifugation at 5000 rpm for 20 min, the mycelial pellets were washed with distilled water three times. The washed mycelia were baked at 60°C prior to use.

Mycelia polysaccharides isolation and determination of dry weights

The polysaccharides from mycelia were isolated using the hot-water method. The dried mycelia and water were placed at 1:20 (W/V ratio) at 60°C for 3 h. After filtration, three volumes of ethanol were added to the filtrates and the resulting mixture was incubated at 4°C for 12 h. After centrifugation at 5000 rpm for 15 min, the precipitates were washed with 75% ethanol twice to eliminate reduced sugars. The precipitates were dissolved in distilled water for determination of polysaccharides. The polysaccharides were determined by the method of phenol-sulfuric acid using glucose as the standard (Chaplin and Kennedy, 1994). Then, 10 ml of fermented mycelia were spun down at 4000 rpm for 15 min. The precipitates were washed with distilled water three times and were baked at 60°C followed by measurements for dry weights of mycelia polysaccharides. All experiments were performed three times and the average values were presented.

Hydroxyl radical scavenging activity

The Fenton Reaction was used to determine the hydroxyl activity. H_2O_2 and Fe^{2+} were mixed to produce $OH^{\cdot}$. Iron 2^+ was reacted with o-phenanthroline to form a colored complex ion. The intensity of the absorbance was measured using UV-2000 spectrophotometer (UNICO Shanghai Instruments Co., Ltd, P. R, China). The following solutions were added in turn to seven tubes: 1 ml Tris-HCl at pH 8.2, 0.3 mL ammonium ferrous sulfate, and 7.5 mmol/L o-phenanthroline. Number 1 was used as the background blank. 1 ml solution of mycelia polysaccharide from *M. esculenta* was added to tubes number three to seven. 1 ml 7.5 mmol/L H_2O_2 was added to tubes number two to seven. After the reactions were run at 37°C for 1 h, the absorbance at 510 nm was measured and the scavenging rate for $OH^{\cdot}$ was calculated as follows:

$$\text{Scavenging rate (\%)} = \frac{(A_3 - A_2)}{(A_1 - A_2)} \times 100$$

Where, A_1 and A_2 are the absorbance with and without water and A_3 is the absorbance after the sample solution was added.

Superoxide anion [$O_2^{-}\cdot$] radical scavenging activity

Pyrogallol is able to form the intermediate product O_2^{-} with colored substance by oxidation under basic conditions. This free radical can stimulate the self-oxidation of Pyrogallol. The scavenging activities of O_2^{-} were measured by determining the inhibition to the self-oxidation of pyrogallol. Tris-HCl (4.5ml 0.05mol/L) buffer was added to 5 separate tubes with incubation at 25°C water bath for 25 min, 0.1 ml mycelia polysaccharide solution and 0.4 ml 0.5 mmol/L Pyrogallol solution were added to each tube and mixed at 25°C for 4 min. Two drops of 8 mol/ L HCl were added to each tube to stop the reaction. The absorbance (Ai) at 320nm was measured using the UV-2000 spectrophotometer (UNICO Shanghai Instruments Co., Ltd, P. R, China). The same volume of water was used as

Table 1. The factors and levels of response surface analysis.

Code	Soybean residue content (%) X_1	Glucose content (%) X_2	KH_2PO_4 content (%) X_3	$MgSO_4$ content (%) X_4
-1	1.0	1.0	0.1	0.1
0	2.0	2.0	0.2	0.15
1	3.0	3.0	0.3	0.2

Soybean residue content (%)ξ corresponds to independent variable X_1; glucose content (%)ξ_2 corresponds to independent variable X_2; KH_2PO_4 content (%)ξ_3 corresponds to independent variable X_3; $MgSO_4 \cdot 7H_2O$ content (%)ξ_4 corresponds to independent variable; X_4. Code variation: $X_1 = (\xi_1\text{-}2.0)/1.0$; $X_2 = (\xi_2\text{-}2.0)/1.0$; $X_3 = (\xi_3\text{-}0.2)/0.1$; $X_4 = (\xi_4\text{-}0.15)/0.05$.

control. Vc was used as a positive control. The following equation was used to calculate the scavenging rate of superoxide anion [$O_2^{-}\cdot$]:

$$\text{Scavenging rate (\%)} = \frac{(A_0 - A_i)}{A_0} \times 100$$

Where, A_0 is the absorbance of blank and Ai is the absorbance of samples.

DPPH· free radicals scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radicals are stable with purple color in ethanol solution, with a strong absorbance at 517 nm. The scavenging rate to DPPH could be measured by determining the removal of DPPH from the unknown substances. Different amounts of Tris-HCl buffer (2, 1.98, 1.98, 1.98, 2.98 mL, pH 7; 40 and 50mmol/L) were added to 5 tubes (number 1 to 5) separately. 1 ml of 0.1mmol/L DPPH ethanol solution was added to number 1 to 4 tubes. 0.02 ml mycelia polysaccharide solutions of different concentrations were added to tubes number 2 to 5. The tubes were mixed well and incubated at room temperature for 30 min. The absorbance at 517 nm was measured with the UV-2000 spectrophotometer (UNICO Shanghai Instruments Co., Ltd, P. R, China). Vc was used as a positive control. Inhibition of free radical by DPPH in percent (I%) was calculated using the following equation:

$$\text{Scavenging rate (\%)} = [1 - \frac{(A_2 - A_0)}{A_1} \times 100\ ]$$

Where, A_0 and A_1 are the absorbance with and without DPPH, and A_2 is the absorbance after the sample solution was added.

RESULTS AND DISCUSSION

Optimization of the fermentation media for mycelia polysaccharides in *M. esculenta* using soybean residues

The response surface analysis was used to optimize the components of the soybean residue fermentation media of mycelia polysaccharides. Table 1 lists the factors and levels by response surface analysis. The mycelia polysaccharide production rate (mg polysaccharides/g dried mycelia) was used as the response value; the results are listed in Table 2. The regression model simulator and variance analysis were used to analyze the results. The results were also analyzed by SAS software. Table 3 lists the regression coefficients of polynomial model. The variance analysis of the experimental results is shown in Table 4. The equation from the second regression simulator was:

$$Y=66.38-9.94X_1+0.31X_2+0.40X_3+0.86X_4-22.87X_1^2+0.36X_1X_2-10.52X_2^2+1.50X_1X_3-1.79X_2X_3-14.00X_3^2+1.50X_1X_4-1.42X_2X_4+1.65X_3X_4-6.89X_4^2.$$

The R-square of the regression equation equal 0.9120, indicating better agreement with the fitted equation. 91.20% of the response value variations came from the selected variable. The F- and P- values were used to examine the variance and interaction strength between independent variables. The factors will be more significant if the absolute F-value becomes larger and the P-value becomes smaller (Elibol, 2004). The F-value of the regression model was very significant ($P<0.01$) (Table 4) from the variance analysis, indicating the good agreement with the quadratic regression equation. This model can explain 91.2% variation of the dependent variable Y. The coefficient of Y was 13.9116. Therefore, the regression equation reflects the genuine relationship between various factors and response values. The optimal components of the fermentation media can be determined by the regression equation. The linear F test of regression equation was obvious ($P<0.01$), the quadratic F test was obvious and the cross product was not obvious. This indicates that the dormant effect of the selected four variables was obvious and the cross effect of variables were less obvious. X_1 (soybean residue contents) and X_3 (KH_2PO_4 content) were very obvious to the response values ($P<0.01$), respectively whereas X_2 (glucose content) and X_4 ($MgSO_4 \cdot 7H_2O$ content) were not very obvious to the response values, respectively (Table 4).

3-D response surfaces plots were generated according to the regression model (Figures 1 to 6). Each curve shows the effect of two independent variables varying within the experimental range of polysaccharides production. The 3-D graphs of response surfaces and different factors (X_1 and X_2, X_1 and X_3, X_1 and X_4, X_2 and X_3, X_2 and X_4, X_3 and X_4) are shown in Figures 1 to 6. The

Table 2. The experimental design and results of response surface analysis.

Experiment number	X_1	X_2	X_3	X_4	Response number Y
1	-1	-1	0	0	45.08
2	-1	1	0	0	40.74
3	1	-1	0	0	17.83
4	1	1	0	0	14.91
5	0	0	-1	-1	46.16
6	0	0	-1	1	41.41
7	0	0	1	-1	39.58
8	0	0	1	1	41.41
9	-1	0	0	-1	41.24
10	-1	0	0	1	40.83
11	1	0	0	-1	26.24
12	1	0	0	1	31.83
13	0	-1	-1	0	35.41
14	0	-1	1	0	43.99
15	0	1	-1	0	40.16
16	0	1	1	0	41.58
17	-1	0	-1	0	46.16
18	-1	0	1	0	43.83
19	1	0	-1	0	22.08
20	1	0	1	0	25.74
21	0	-1	0	-1	48.33
22	0	-1	0	1	55.16
23	0	1	0	-1	55.49
24	0	1	0	1	56.66
25	0	0	0	0	62.49
26	0	0	0	0	65.08
27	0	0	0	0	68.16
28	0	0	0	0	68.90
29	0	0	0	0	67.28

Table 3. The regression coefficients of polynomial model.

Parameter	Degrees of freedom	Parameter estimate	Standard Error	TforH0: Parameter=0	Prob > |T|
INTERCEPT	1	66.38	2.73	24.29	0.0000
X1	1	-9.94	1.76	-5.63	0.0001
X2	1	0.31	1.76	0.18	0.8623
X3	1	0.40	1.76	0.22	0.8257
X4	1	0.86	1.76	0.49	0.6354
X1*X1	1	-22.87	2.40	-9.53	0.0000
X2*X1	1	0.36	3.06	0.12	0.9091
X2*X2	1	-10.52	2.40	-4.38	0.0006
X3*X1	1	1.50	3.06	0.49	0.6316
X3*X2	1	-1.79	3.06	-0.59	0.5673
X3*X3	1	-14.00	2.40	-5.83	0.0000
X4*X1	1	1.50	3.06	0.49	0.6311
X4*X2	1	-1.42	3.06	-0.46	0.6504
X4*X3	1	1.65	3.06	0.54	0.5987
X4*X4	1	-6.89	2.40	-2.87	0.0123

Table 4. The variance analysis of experimental results.

Regression	Degrees of freedom	Sum of square	R-square	F-value	Prob>F
X1	5	4596.49	919.30	24.62	0.0000
X2	5	739.98	148.00	3.96	0.0189
X3	5	1305.12	261.02	6.99	0.0018
X4	5	344.73	68.95	1.85	0.1682
Linear	4	1196.87	0.20	8.01	0.0014
Quadratic	4	4172.34	0.70	27.94	0.0000
Cross product	6	50.12	0.01	0.22	0.96
Total Regress	14	5419.32	0.91	10.37	0.0000

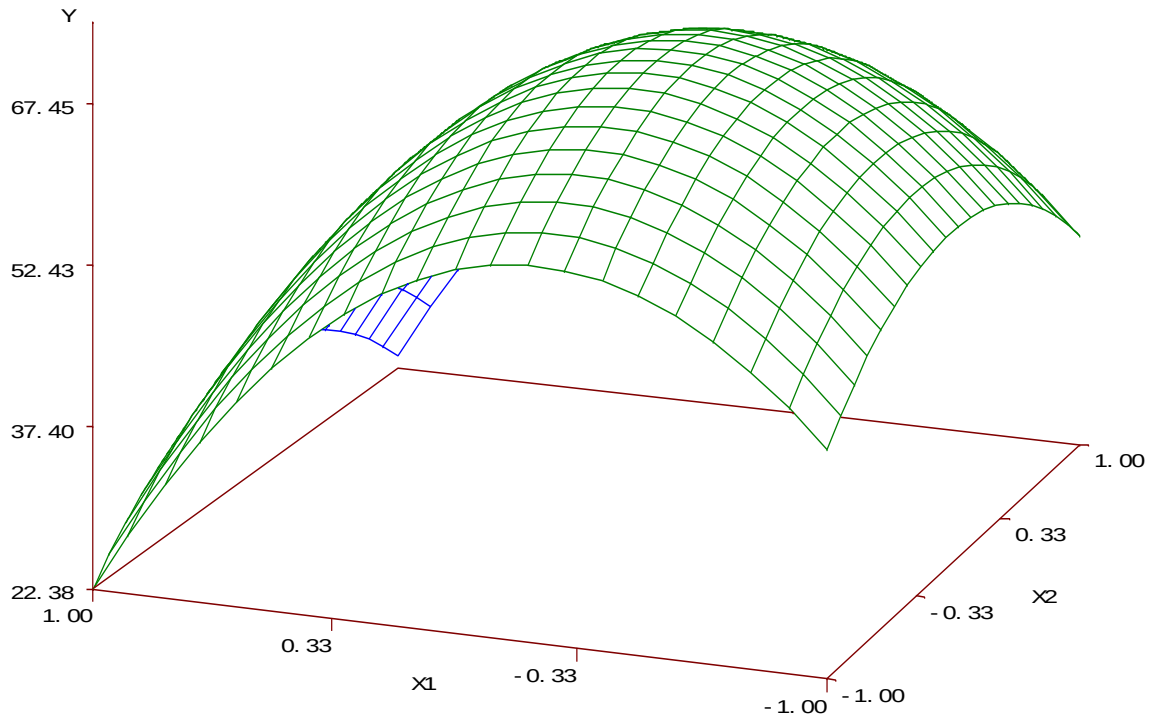

Figure 1. The effect of x1 (soybean residue) and x2 (glucose) on polysaccharides production.

graphic visualization allows the relationships between the experimental levels of each factor and the response to be investigated, and the type of interactions between the variables to be determined, which is necessary to establish the optimal composition of the fermentation medium. As can be seen from the shapes of coutour lines shown in Figures 1 to 6, the optimal values of the independent variables could be observed, and the interaction between each independent variable could be easily understood. It can been seen from the response surface curves and contour curves that the polysaccharide production rate existed at an extreme within the tested range. The highest point of the response surface plots was also the center of the smallest oval in the contour plots. According to the shape of fitted responding curve, the effect of X_1 to X_4 to the polysaccharide production rate was evaluated.

SAS was used to do the canonical analysis of regression model to find the optimal response areas. As shown in Table 5, the stationary point existed with the maximum value. According to the model, the maximum polysaccharide production estimate was 67.47 mg/g.

Optimization of fermentation conditions of mycelia polysaccharides in *M. esculenta*

The orthogonal experiments with three factors and three levels were used to optimize the fermentation conditions. The factors and levels are listed in Table 6 and the results are listed in Table 7. As Table 7 shows, the optimal fermentation conditions were A2B2C2 that

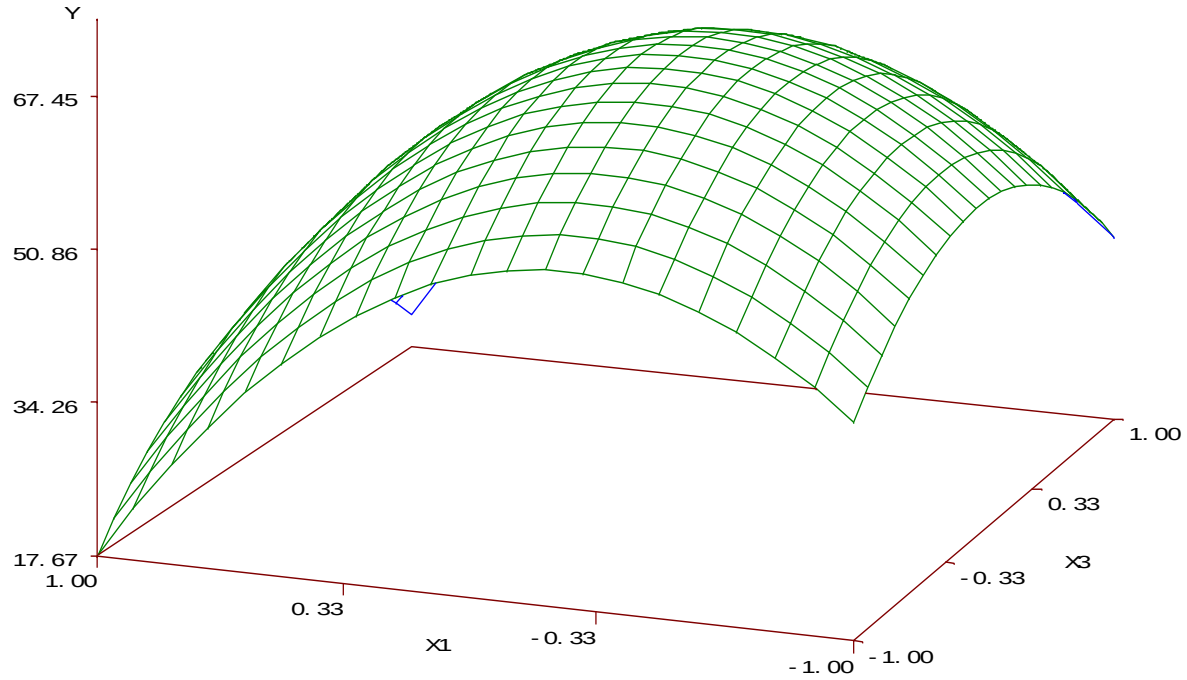

Figure 2. The effect of x1 (soybean residue) and x3 (KH_2PO_4) on polysaccharides production.

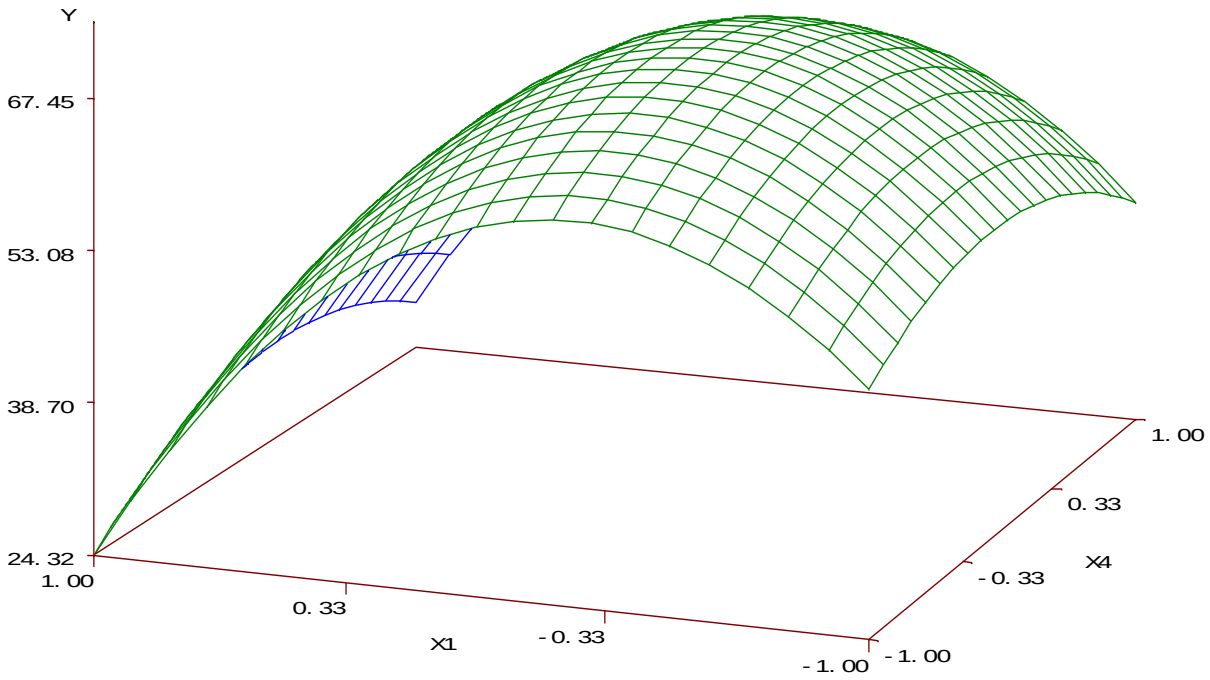

Figure 3. The effect of x1 (soybean residue) and x4 ($MgSO_4 \cdot 7H_2O$) on polysaccharides production.

correlated to pH 7, 10% inoculation and 28°C fermentation temperature. The factors that affect the polysaccharide production rate were in the following order: $R_B > R_A > Rc$. The variance analysis of experimental results in Table 7 indicates that the three factors had no obvious effect to experimental results.

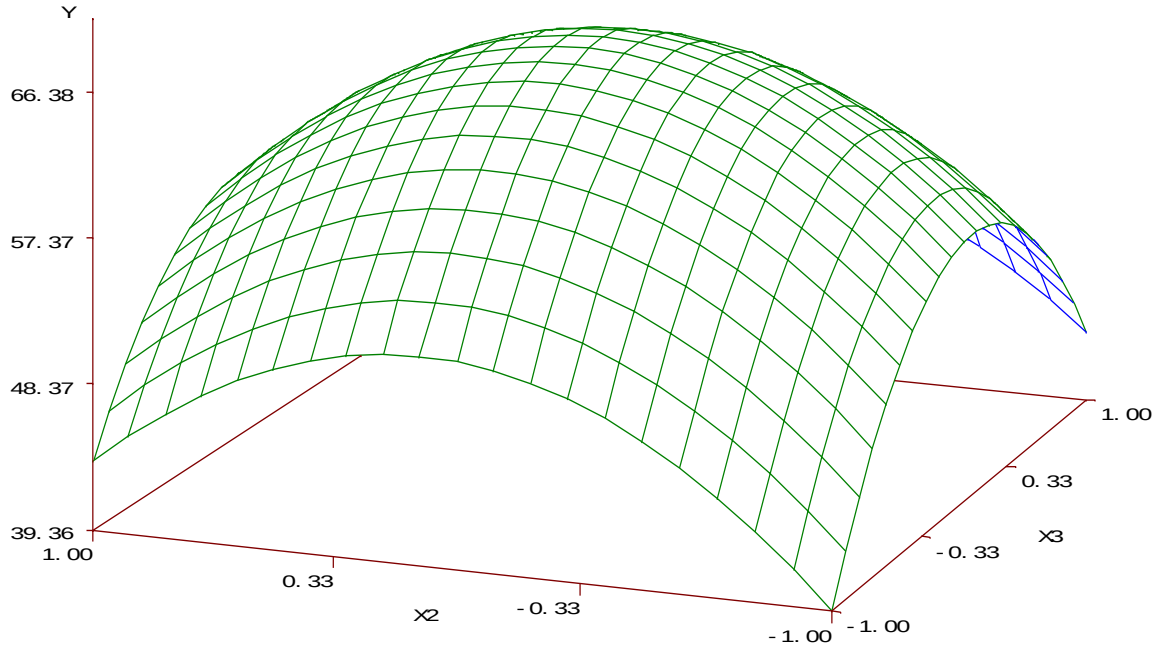

Figure 4. The effect of x2 (glucose) and x3 (KH_2PO_4) on polysaccharides production.

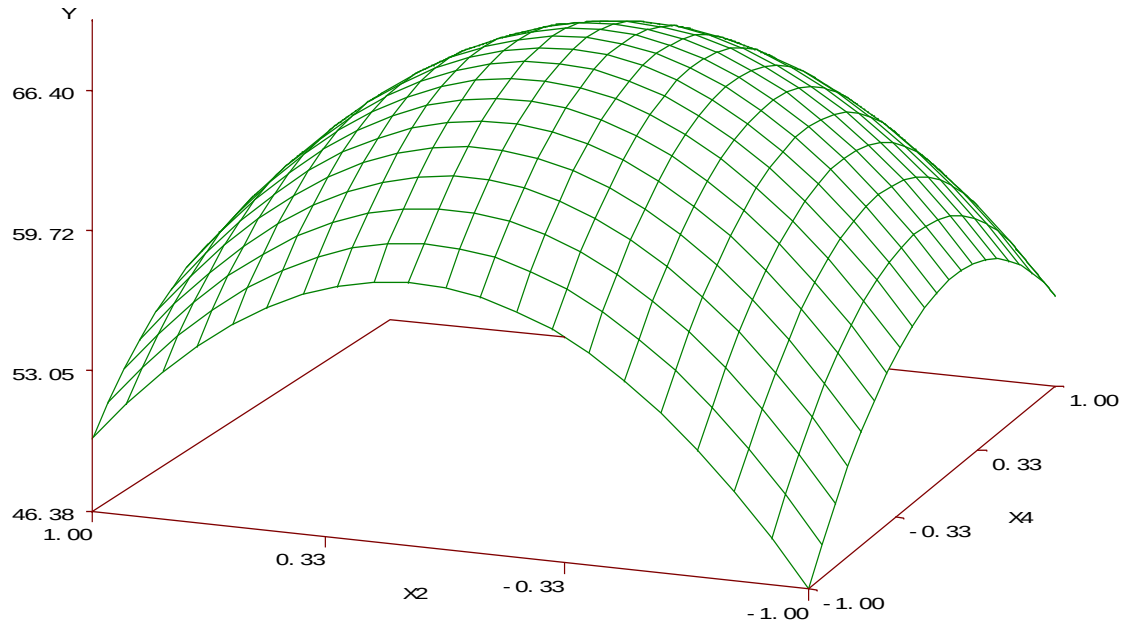

Figure 5. The effect of x2 (glucose) and x4 ($MgSO_4 \cdot 7H_2O$) on polysaccharides production.

Fermentation curves of mycelia polysaccharides from *M. esculenta*

The optimized conditions using soybean residues as base were used to ferment the *M. esculenta* mycelia. The growth of mycelia and the polysaccharides production were measured (Figure 7). The growth of mycelia was in lag-phase during 0 to 16 h, log-phase during 16 to 48 h and reached the stationary phase afterwards. At 56 h of fermentation, the maximum dry weights reached 36.22 g/L. The production of polysaccharides synchronized with the growth of mycelia. The rate of polysaccharide

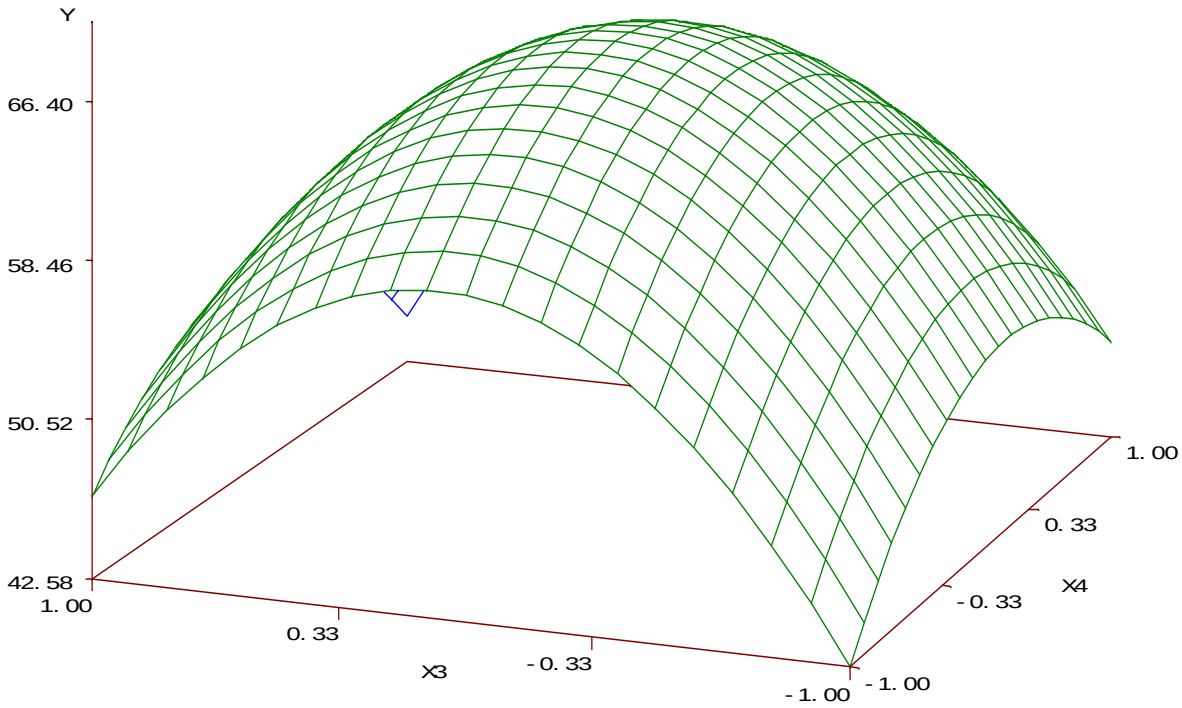

Figure 6. The effect of x3 (KH_2PO_4) and x4 ($MgSO_4 \cdot 7H_2O$) on polysaccharides production.

Table 5. The canonical analysis of response surface.

Factor	x1	x2	x3	x4	Estimate	Stationary point
Critical Value	-0.22	0.01	0.004	0.04	67.47	maximum

Table 6. The factors and levels of orthogonal experiment for the fermentation conditions of mycelia polysaccharides from *Morchella esculenta*

Level	Factor		
	A (pH)	B (inoculation %)	C (fermentation temperature)
1	6	5	26
2	7	10	28
3	8	15	30

production was maximized at 68.23 mg/g, which is in good agreement with the predicted value (67.47 mg/g) from the model, indicating that the model was adequate. Our polysaccharide production yielded were much high than a previous study where the extraction of polysaccharides were optimized without fermentation (Meng et al., 2010).

The antioxidant activities of mycelia polysaccharides from *M. esculenta*

To test the antioxidant potentials of mycelia polysaccharides from *M. esculenta*, the scavenging effects of mycelia polysaccharides to three free radicals (OH·, O_2^-·, and DPPH·) were investigated. Polysaccharides were isolated from the mycelia using the optimized media and fermentation conditions. 75.54% of crude polysaccharides was obtained and used to study the antioxidant activities. All experiments were performed in triplicates and the mean values were presented. As shown in Figure 8, the scavenging rate of mycelia polysaccharides to OH· was higher than Vc after its concentration was over 0.04 mg/ml. The savaging rate was 71.78% under the concentration of 0.1 mg/mL. The scavenging rate to O_2^- was higher than Vc after its

Table 7. The results of orthogonal experiment for the fermentation conditions of mycelia polysaccharides from *M. esculenta.*

Experiment number	Factor			Production rate of polysaccharides (mg/g)
	A	B	C	
1	1	1	1	57.82
2	1	2	2	65.38
3	1	3	3	41.58
4	2	1	2	60.43
5	2	2	3	68.61
6	2	3	1	59.42
7	3	1	3	53.21
8	3	2	1	61.36
9	3	3	2	58.75
K1	54.94	57.15	59.53	
K2	62.82	65.12	61.52	
K3	57.77	53.25	54.47	
R	7.89	11.87	7.053	

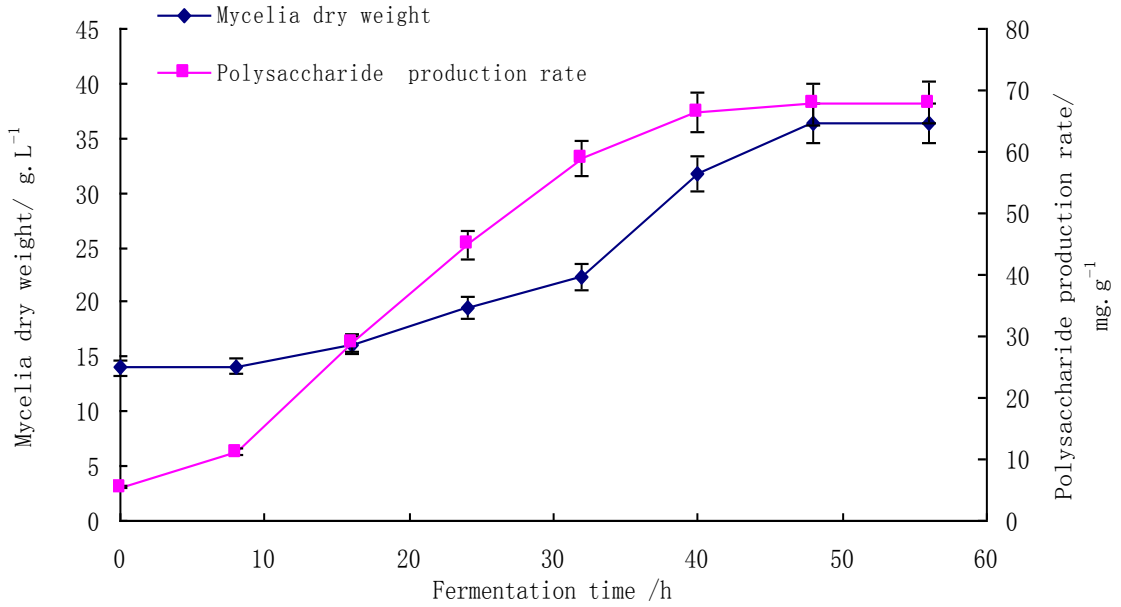

Figure 7. *Morchella esculenta* mycelia growth curve with polysaccharide production. Values presented are means ± S.D. of the three parallel experiments.

concentration was over 0.02 mg/ml. At the concentration of 0.04 mg/ml, the scavenging rate reached 79.34% (Figure 9). Since O_2^- is a free radical that was produced during metabolism, accumulation of this radical in the human body results in decrease in immunity. Mycelia polysaccharides can protect human by scavenging free radicals. The *M. esculenta* mycelia polysaccharides were also able to remove DPPH with a scavenging rate of 58.74 % at the concentration of 0.1 mg/ mL. The scavenging ability was greater than Vc when its concentration was greater than 0.06 mg/mL. The scavenging rate increased with the concentration of mycelia polysaccharides (Figure 10).

Conclusion

The mycelia from *M. esculenta* were able to undergo liquid fermentation to produce abundant mycelia polysaccharides using soybean residues. The fermentation media and conditions were optimized using the response surface methodology and a three-factor – three-level orthogonal design. The mycelia polysaccharides from *M. esculenta* had strong antioxidant

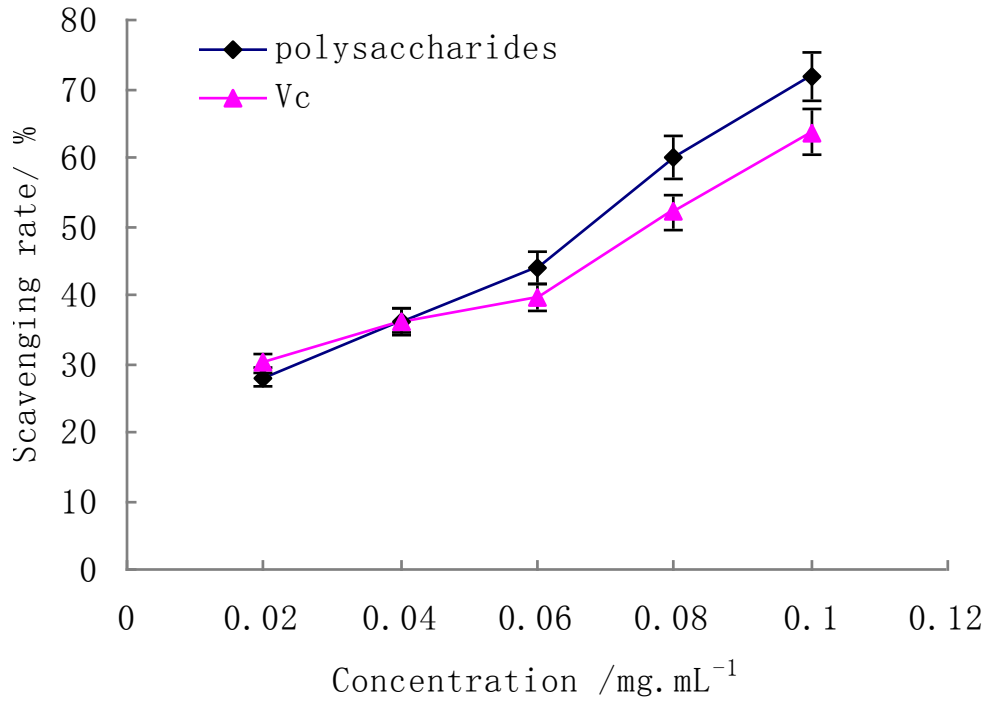

Figure 8. The scavenging effect of *M. esculenta* mycelia polysaccharides and Vc on hydroxyl radicals. Data are presented as means ± SD; n=3.

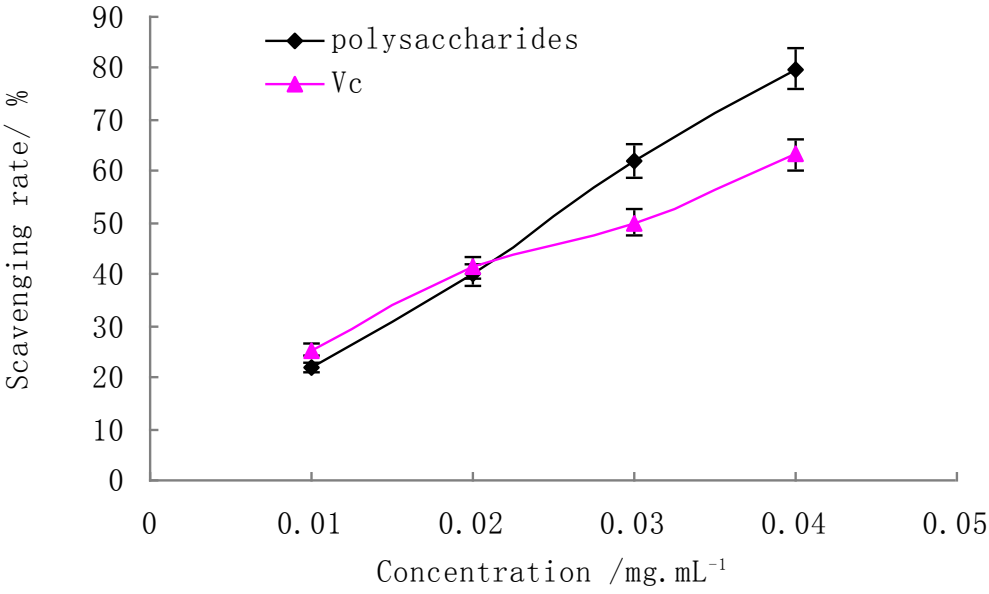

Figure 9. The scavenging effect of *M. esculenta* mycelia polysaccharides and Vc on superoxide anion radicals. For each point, values are presented as the mean ± SD from three replicates.

activities *in vitro*, which provide an explanation for their beneficial therapeutic effects. Our results provide a reference for large-scale isolation of polysaccharides by *M. esculenta* using industrial fermentation.

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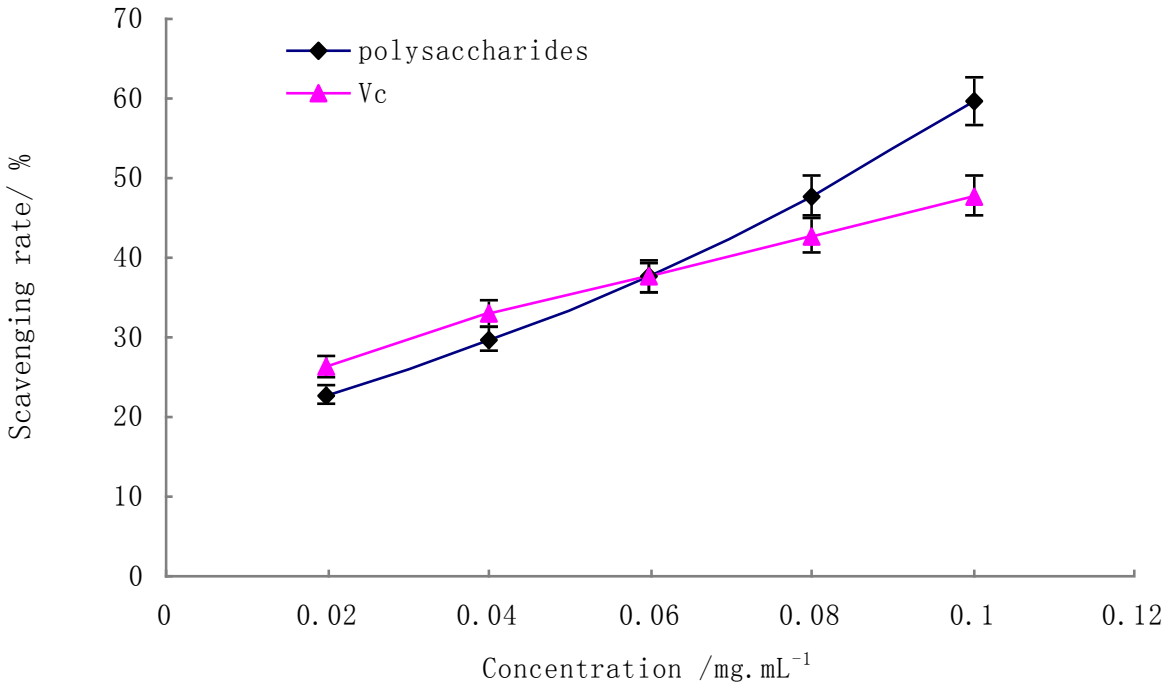

Figure 10. The scavenging effect of *Morchella esculenta* mycelia polysaccharides and Vc on DPPH radicals. The means and standard deviations (±SD) of the three independent experiments are presented.

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