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Optimization of production conditions for mushroom polysaccharides with high yield and antitumor activity

Bing-zhao Zhang [a], Pei-sheng Yan [a,b,*], Hao Chen [b], Jian He [b]

[a] School of Municipal and Environmental Engineering, Harbin Institute of Technology, Harbin 150001, China
[b] School of Ocean, Harbin Institute of Technology at Weihai, Weihai 264209, China

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ABSTRACT

Single factor experiment and response surface methodology were conducted to optimize the production conditions for polysaccharide with higher antitumor activity from mushroom fermentation broth. Based on the results of single factor experiments, a central composite design was applied to the following optimization and second-order polynomial regression models were established. As the yield and antitumor activity of the polysaccharides were considered with equal weight, the optimal conditions were that ethanol concentration and pH of the broth was adjusted to 85.00% and 7.70 respectively, then precipitated at 12 °C for 1 h and dried the precipitates at 40 °C. The verification experiments completed under the optimal conditions gave the polysaccharides yield of 9.843 ± 0.217 g/l, and the cancer cell growth inhibitory rate of 83.69 ± 0.167% by the polysaccharides, which were in close agreement with values predicted by the models (RSD < 5%).

1. Introduction

In the 1960s, mushroom polysaccharides were proved to have antitumor activity for the first time (Brander, Clarke, & Stock, 1958). It caused a wide public concern to antitumor activity of polysaccharides derived from medicinal mushroom. Afterwards, scientists isolated polysaccharides with antitumor activity from kinds of edible and medicinal mushroom successively (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Cun et al., 1994; Ma, Mizuno, Ito, 1991; Miyazaki & Nishijima, 1981). It pushed research about polysaccharides from medicinal mushroom to modern scientific frontier. Both submerged culture and fruit body were the main sources to obtain polysaccharides from medicinal mushroom. In traditional conditions, crude polysaccharides were usually produced by adding fourfold of the volume of 95% ethanol (v/v), precipitated at 4 °C overnight, centrifuged at 4000–10,000 rpm and freeze-dried (Oh, Cho, Nam, Choi, & Yun, 2007; Sun et al., 2009; Yang, Gao, Han, & Tan, 2005). Usually the yield of the polysaccharides rises with the final ethanol concentration and does not change after the ethanol concentration reaches a certain value (Sandford, Watson, & Knutson, 1978; Whistler & Lauterbach, 1958). However, effects of the final ethanol concentration to antitumor activity of polysaccharides were investigated rarely. Precipitation at 4 °C for overnight and lyophilization, which were widely applied for preparation of polysaccharides were high energy and time-consuming and not suitable for low-cost production in industrial scale. Therefore, it is necessary to investigate the effects of precipitation temperature, precipitation time and dry temperature on the yield and antitumor activity of polysaccharides. Furthermore, in acidic or alkaline environments, some polysaccharides were difficult to get because of degradation, selecting an appropriate pH range was important to the yield and antitumor activity of polysaccharides.

Response surface methodology (RSM) is an effective method widely applied to optimization (Gan, Abdul Manaf, & Latiff, 2010; Wu, Cui, Tang, & Gu, 2007), especially the central composite design (CCD), being used for investigating effects of the factors and their interaction terms to response values and give a precise description about the relationships between independent and dependent variables (Guo et al., 2009; Sun, Liu, & Kennedy, 2010).

In order to establish a simple and economic procedure for producing mushroom polysaccharide with higher yield and antitumor activity, single factor experiment and response surface methodology were conducted to optimize the production conditions for extra-cellular polysaccharide (EPS) from fermentation broth of *Hypsizigus marmoreus*. The results would provide a theoretical and practical basis for large-scale production of medicinal polysaccharides with higher yield and antitumor activity, and low input.

* Corresponding author at: West Culture Road 2, School of Ocean, Harbin Institute of Technology at Weihai, Weihai 264209, China. Tel.: +86 631 5687230; fax: +86 631 5687230.
E-mail address: psyan6@hotmail.com (P.-s. Yan).

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2. Materials and methods

2.1. Materials

The fungus *H. marmoreus* JB06 was screened and preserved in our Lab. It was initially cultured on PDA medium (fresh potato 20%, glucose 2% and agar 1.5%) in a Petri dish at 25 °C for 10 days. Ten agar plugs, 10 mm in diameter with young mycelia were punched out by a puncher from the marginal culture and inoculated into 250 ml Erlenmeyer flasks containing 100 ml of seed culture medium including 3.5% of glucose, 11% of potato, 1.75% of yeast extract, 1.75% of beef extract, 0.1% of KH_2PO_4 and 0.05% of $MgSO_4$, and then cultivated on a rotary shaker at 150 rpm, 25 °C for 9 days. The fermentation experiments were performed in 500 ml flasks containing 300 ml of fermentation culture medium including soya peptone 2.5%, glucose 2.346%, sweet potatoes power 1.0%, corn power 0.688%, flour 1.0%, soy power 1.0%, $CaCO_3$ 0.088%, KH_2PO_4 0.172%, pH 7.0 and was inoculated with 15% (v/v) of the seed medium culture on above rotary shaker at 25 °C for 8 days. Five batches of the fermentation were carried out at different time when necessary. The broth from the first batch was used for single factor experiments and the second was used for RSM investigation. Finally, the other three batches were used for the verification experiments. Human gastric cancer cells SGC-7901 were purchased from Cell Resource Center; Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences. All chemicals used in the study were of analytical grade.

2.2. Routine procedures for preparation of crude polysaccharides

The fermentation cultures were separated by centrifugation at 4500 rpm for 20 min, and the aqueous broth with pH 5.3 was obtained. Fourfold of the volume of 95% ethanol (v/v) was added to the broth and the mixture was maintained overnight at 4 °C to precipitate the polysaccharide. The precipitates were collected by centrifugation and rewashed twice with anhydrous ethanol. After lyophilizing the weight was measured and the yield of crude polysaccharides was calculated as the weight of precipitates from 1.0 L fermentation broth.

2.3. Antitumor activity of the polysaccharides

The crude polysaccharides were dissolved in phosphate buffer solution (PBS) and carbohydrate content in the supernatant was determined by phenol sulfuric method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The growth inhibitory rates of the crude polysaccharides to human gastric cancer cells were evaluated in vitro by MTT assay (Chen et al., 2006). Absorbance at 570 nm was detected by a spectrophotometer (Thermo Multiskan MK3; Thermo.). 5-Fluorouracil (5-Fu) was treated as positive control (pc) and PBS was taken as negative control (nc). The growth inhibitory rate (IR%) was calculated according to the equation below:

$$\text{IR}\ (\%) = \frac{A_{\text{nc}} - A_{\text{exp}}}{A_{\text{nc}}} \times 100 \quad (1)$$

where A_{nc} is the absorbance of negative control group and A_{exp} is the absorbance of experimental group.

2.4. Optimization by single factor experiments

Five single factors were optimized respectively, including the final ethanol concentration of the broth (50%, 60%, 70%, 80% or 90%), the final pH value of the broth (1.3, 3.3, 5.3, 7.3, 9.3 or 11.3), precipitation temperature (4, 12, 20, 28 or 36 °C), precipitation time (1, 2, 4, 12 or 24 h) and precipitates-dried temperature (lyophilizing at −40 °C, dry at 40, 60, 80 or 100 °C). The procedures for polysaccharide production were the same as in Section 2.2, but substituted by a tested single factor wherever necessary. Influences of these factors to yield and antitumor activity of the polysaccharides were analyzed by SPSS 16.0.

Table 1
Factorial levels and results of RSM.

No.	Coded factors		Experimental factors		Yield (g/l)		Growth inhibitory rates (%)	
	$x_1 \cdots x_2$		$X_1 \cdots X_2$		y_1 [a] $\cdots Y_1$ [m]		y_2 [b] $\cdots Y_2$ [n]	
1	0.00	0.00	80.00	7.30	8.299	8.51	81.53	84.97
2	1.00	−1.00	85.00	5.80	9.490	9.17	70.68	67.46
3	1.414	0.00	87.07	7.30	9.597	10.01	80.36	82.51
4	0.00	−1.414	80.00	5.18	7.529	7.62	58.48	62.00
5	0.00	0.00	80.00	7.30	8.974	8.51	86.73	84.97
6	1.00	1.00	85.00	8.80	10.329	10.01	78.05	77.10
7	0.00	1.414	80.00	9.42	8.320	8.42	61.09	61.41
8	−1.00	−1.00	75.00	5.80	5.414	5.55	79.22	76.33
9	0.00	0.00	80.00	7.30	8.359	8.51	87.40	84.97
10	−1.00	1.00	75.00	8.80	5.695	5.83	66.49	65.86
11	0.00	0.00	80.00	7.30	8.666	8.51	84.92	84.97
12	0.00	0.00	80.00	7.30	8.254	8.51	84.25	84.97
13	−1.414	0.00	72.93	7.30	4.727	4.50	79.12	80.82

[a] Means measured yield.
[b] Means measured growth inhibitory rates.
[m] Means predicted yield.
[n] Means predicted growth inhibitory rates.

2.5. Optimization by response surface methodology

The final ethanol concentration (X_1) and pH of the broth (X_2) were further optimized by RSM as they showed significant influences on yield and antitumor activity of prepared polysaccharide. CCD was employed in the regard; the range and center point values of the two independent variables were based on the results from single factor experiments. CCD in the experimental design consists of eight factorial points and five replicates of the central point. Yield and growth inhibitory rate of the crude polysaccharides were taken as the response values for the combination of final ethanol concentration and pH of the broth given in Table 1. Experimental runs were randomized to estimate a pure error sum of squares in the observed responses. The precipitation temperature, precipitation time, and precipitates-dried temperature were set at 12 °C for 1 h and 40 °C respectively in the procedures of polysaccharide production.

2.6. Statistical analysis

The second-order polynomial regression model was expressed by the following quadratic equation:

$$Y_k = A_{k0} + A_{ki}X_i + A_{kii}X_i^2 + AkijX_iX_j \quad (2)$$

in which Y_k is the dependent variable, A_{k0} is the center point of the system, A_{ki}, A_{kii}, and A_{kij} are the coefficients estimated by the model for the linear, quadratic and interactive terms. X_i, X_i^2 and X_iX_j are the linear, quadratic, and interaction terms of the independent variables, respectively. The model was expressed as surface and contour plots to explain the relationship of each independent variable and the response (Triveni, Shamala, & Rastogi, 2001). Analysis of the experimental data and calculation of predicted data were carried out using Design-Expert 7.0 software. Finally, three verification experiments were conducted to verify the validity of the statistical experimental strategies.

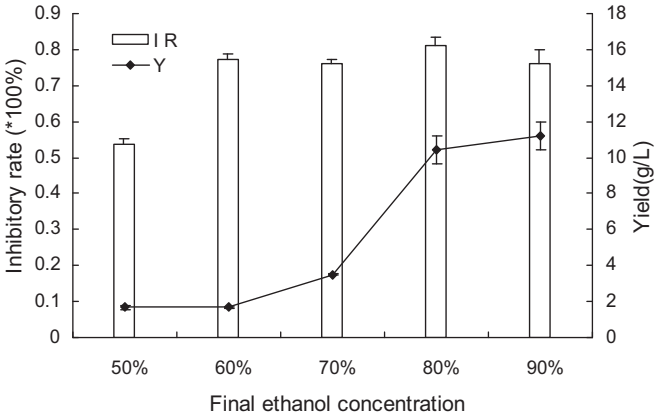

Fig. 1. Effects of ethanol concentration on yield and antitumor activity of crude polysaccharides (Y, yield; IR, inhibitory rate).

3. Results

3.1. Optimization by single factor experiments

3.1.1. Effects of final ethanol concentration on yield and activity of the polysaccharides

The effects of different ethanol concentration on yield and activity of polysaccharides are shown in Fig. 1. With increasing of final ethanol concentration, the yield of crude polysaccharides increased. The yield of polysaccharides obtained in 80% ethanol concentration was significantly higher than that obtained with ethanol concentration of 50–70%, but not significantly different from that obtained with ethanol concentration of 90% ($p = 0.129$). The highest growth inhibitory rate obtained at ethanol concentration of 80% was of 81.59% which was significantly higher than those obtained from other ethanol concentrations ($p < 0.05$). Therefore, the final ethanol concentration held near 80% was good for producing more polysaccharides with higher antitumor activity.

3.1.2. Effects of pH on yield and activity of the polysaccharides

The effects of pH of broth on yield and activity of the polysaccharides are shown in Fig. 2. The cancer cell growth inhibitory rate was the highest for polysaccharide obtained from broth with pH 7.3, and significantly different from those obtained from broth with other pH values except of pH 5.3 ($p = 0.072 > 0.05$). It indicated that antitumor activity of the polysaccharide would decrease as the conditions for producing polysaccharide change to acidic or alkaline. The yield of the polysaccharides obtained from broth with pH 7.3 was not significantly different from that obtained from broth with pH 9.3, but significantly different from those obtained from the other pH values. Therefore, neutral environment was good for production of polysaccharides with higher antitumor activity.

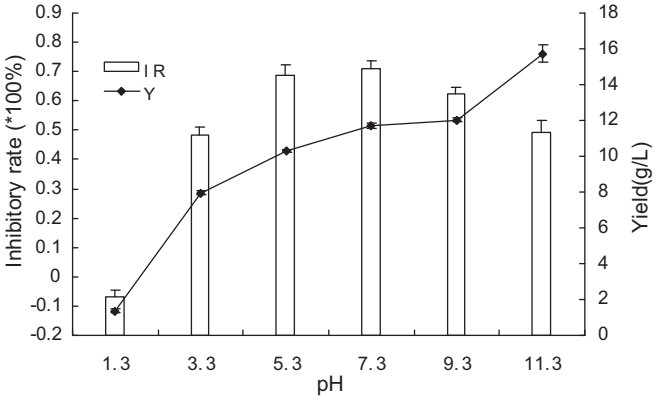

Fig. 2. Effects of pH on yield and antitumor activity of crude polysaccharides (Y, yield; IR, inhibitory rate).

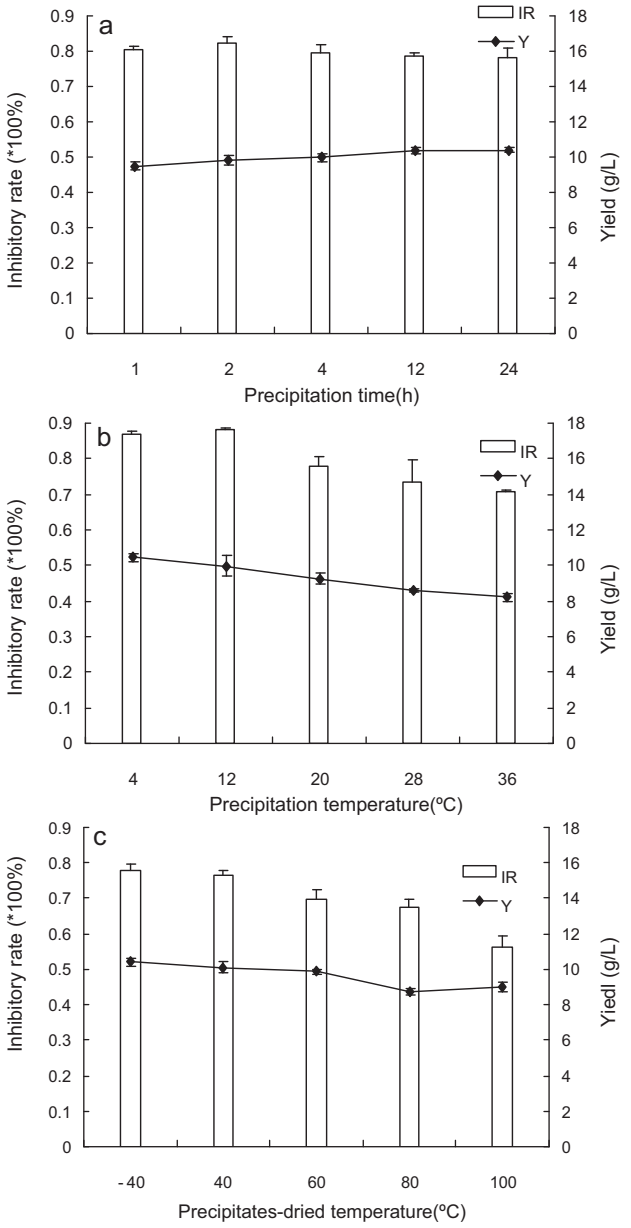

Fig. 3. Effects of precipitation time, precipitation temperature and precipitates-dried on yield and antitumor activity of crude polysaccharides (Y, yield; IR, inhibitory rate).

3.1.3. Effects of precipitation time on yield and activity of the polysaccharides

The results of precipitation time affecting the yield and antitumor activity of the polysaccharides are shown in Fig. 3a. The yield and activity of the polysaccharides did not vary significantly with increase of precipitation time ($p > 0.05$). It indicated that precipitation time was the minor factor affecting yield and antitumor activity of the polysaccharides. For improving production efficiency, precipitation time could be held for 1 h.

3.1.4. Effects of precipitation temperature on yield and activity of the polysaccharides

The effects of precipitation temperature on yield and activity of the polysaccharides are shown in Fig. 3b. With precipitation temperature increase, the yield of polysaccharides decreased slowly. The highest polysaccharide yield was obtained from precipitation temperature at 4 °C, but there was no significant difference between precipitating at 4 °C and 12 °C. The polysaccharide with highest growth inhibitory rate was precipitated at 12 °C, but again no significant difference between precipitating at 4 °C and 12 °C ($p = 0.106$) and significantly different from those precipitated at other temperatures. These results indicated that low temperature was good for preparation of polysaccharides, but ice cold was not necessary, 12 °C was the appropriate temperature for obtaining more polysaccharides with higher antitumor activity and low energy input.

3.1.5. Effects of precipitates-dried temperature on yield and activity of the polysaccharides

The effects of precipitates-dried temperature on yield and activity of the polysaccharides are shown in Fig. 3c. The yield of the crude polysaccharides decreased a little bit with increase of dry temperature. The cancer cell growth inhibitory rate of polysaccharide also decreased with increase of the dry temperature. But there was no significant difference between the activity of polysaccharides dried at −40 and 40 °C ($p = 0.109$). These results indicated that drying at low temperature was favored to keep higher antitumor activity of the polysaccharides, but there was no significant difference between the effects of drying at 40 °C and lyophilizing. Therefore, drying at 40 °C was chosen for polysaccharide production because of its lower energy consumption and low cost for equipments.

3.2. Optimization by response surface methodology

3.2.1. Optimization by RSM

RSM is an economic and reasonable design that integrates test analysis and mathematical modeling, and widely applied in the optimization research work to investigate the effects of various variables (Liu, Weng, & Zhang, 2003). When the yield and growth inhibitory effect on anticancer cell in vitro of the polysaccharides were taken as the response values, a central composite design with two factors of the final ethanol concentration (X_1) and pH of the broth (X_2) at five levels were considered. The process variables, predicted and measured results are shown in Table 1. The yield and cancer cell growth inhibitory rates of the polysaccharides ranged from 4.50 to 10.01 g/l and 61.09% to 87.40%, respectively.

According to multiple regression analysis of the experimental data, the independent variables and the dependent variables were related by the following second-order polynomial equations:

$$\text{Eq. 1: } Y_1 = -179.532 + 4.267X_1 + 0.292X_2 + 0.019X_1X_2 - 0.025X_1^2 - 0.109X_2^2 \quad (3)$$

$$\text{Eq. 2: } Y_2 = -230.195 + 5.792X_1 + 21.702X_2 + 0.67X_1X_2 - 0.066X_1^2 - 5.168X_2^2 \quad (4)$$

The relationships between the dependent and independent variables were represented by Eq. 1 and 2. The adjusted determination coefficients (R^2_{Adj}) were measured for testing the goodness-of-fit of the regression equations. The values of (R^2_{Adj}) for the two equations were 0.9754 and 0.9446 as shown in Tables 2 and 3, and it indicated a high degree of correlation between the experimental and predicted values. The p-values of the lack of fit for the two equations were 0.2964 and 0.1982, both of which were higher than 0.05, indicated a high degree of precision and reliability of the experimental values. For the model of yield, the linear and quadratic effects of final ethanol concentration were significant (p-value < 0.05), and the linear effects of pH were marginally significant (0.05 < p-value < 0.10). With increase of ethanol concentration in range of the experiments, the yield increased accordingly; but the coefficients of quadratic term of ethanol concentration were negative, it means that the yield would reach a maximum value with increase of ethanol concentration. For the model of growth inhibitory rates, the coefficients of the quadratic term of pH and interaction term of ethanol concentration and pH were significant (p-value < 0.05). In model 2, the linear effect of pH was not significant, and coefficients of the quadratic term were negative and significant, it means that there was an appropriate pH range to keep an ideal growth inhibitory activity of the polysaccharides.

Three-dimensional response surface plots were constructed by Design-Expert 7.0 software to estimate the effects of the factors and their interactions on the yield and antitumor activity of the polysaccharides. As presented in Figs. 4 and 5, with increase of the two factors, the yield of the polysaccharides increased even faster on the effects of the ethanol concentration. The optimum production condition for the highest yield of the polysaccharides was predicted as the final ethanol concentration was of 85% and pH was of 8.58. As a result, the yield of the polysaccharides was predicted as 10.01 g/l. The elliptical contours in Fig. 5 showed that the growth inhibitory rate rose up with pH ranged from 5.18 to 7.3, and declined with pH ranged from 7.3 to 9.42. The optimal conditions predicted for the highest antitumor activity of the polysaccharides were final ethanol concentration of 81.23% and pH of 7.37. As a result, the highest growth inhibitory rate was predicted as 85.04%. Finally, when the yield and antitumor activity of the polysaccharide were considered with equal weight, the model predicted that the optimal production conditions were final ethanol concentration of 85.00% and pH of 7.70. Under the optimal conditions, the yield and

Table 2
Analysis of variance for the yield of polysaccharides.

Source	Sum of squares	df	Mean square	F value	p-Value Prob > F
Model	34.04	5	6.81	55.50	<0.0001
x_1	30.41	1	30.41	247.93	<0.0001[a]
x_2	0.63	1	0.63	5.11	0.0583
x_1x_2	0.078	1	0.078	0.63	0.4518
X_1^2	2.74	1	2.74	22.30	0.0022[a]
X_2^2	0.42	1	0.42	3.42	0.1069
Residual	0.86	7	0.12		
Lack of fit	0.49	3	0.16	1.74	0.2964
Pure error	0.37	4	0.093		
Cor total	34.90	12			
R^2_{Adj}			0.9754		

[a] Means significance (p-value of Prob > F less than 0.05).

Table 3
Analysis of variance for antitumor activity of polysaccharides.

Source	Sum of squares	df	Mean square	F value	p-Value Prob > F
Model	1045.17	5	209.03	23.85	0.0003[a]
x_1	2.84	1	2.84	0.32	0.5867
x_2	0.35	1	0.35	0.040	0.8480
x_1x_2	101.08	1	101.08	11.53	0.0115[a]
X_1^2	18.96	1	18.96	2.16	0.1848
X_2^2	940.77	1	940.77	107.34	<0.0001[a]
Residual	61.35	7	8.76		
Lack of fit	40.03	3	13.34	2.50	0.1982
Pure error	21.32	4	5.33		
Cor total	1106.52	12			
R^2_{Adj}			0.9446		

[a] Means significance (p-value of Prob > F less than 0.05).

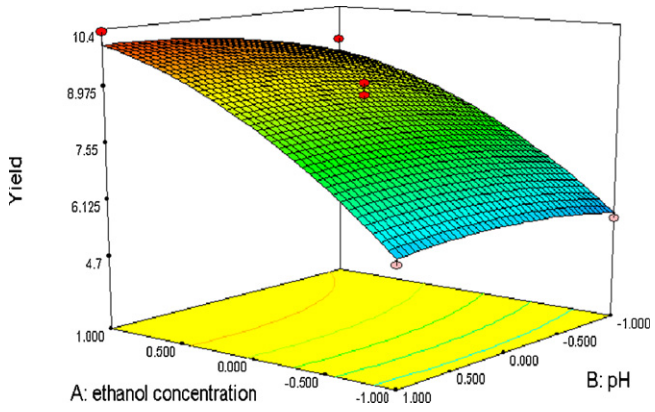

Fig. 4. Response surface contour of the model for yield of crude polysaccharides.

growth inhibitory rate of the polysaccharides were predicted as 9.93 g/l and 84.37%.

3.2.2. Verification experiments

In order to validate the suitability of the model equations for predicting optimum response values, a group of verification experiments were carried out using broth from three batches of fermentation under the optimum conditions predicted respectively for highest yield, highest antitumor activity and both for yield and antitumor activity. Under the optimal production conditions for highest yield of polysaccharide, the measured yield was 9.923 ± 0.236 g/l, which was very close to the predicted yield of 10.01 g/l. Under the optimal production conditions for highest antitumor activity, the measured cancer cell growth inhibitory rate was 87.13 ± 0.194%, which was very close to the predicted inhibitory rate of 85.04%. Under the optimal production conditions both for highest yield and antitumor activity of polysaccharide, the measured yield and cancer cell growth inhibitory rate were 9.843 ± 0.217 g/l and 83.69 ± 0.167% respectively, which were also very close to the predicted yield of 9.93 g/l and inhibitory rate of 84.37%. The significant difference at 5% level was not found between the predicted and measured values. The results indicated that the optimized conditions were suitable for high yield production of polysaccharide with higher antitumor activity, and the established model equations could accurately predict the yield and antitumor activity of produced polysaccharide.

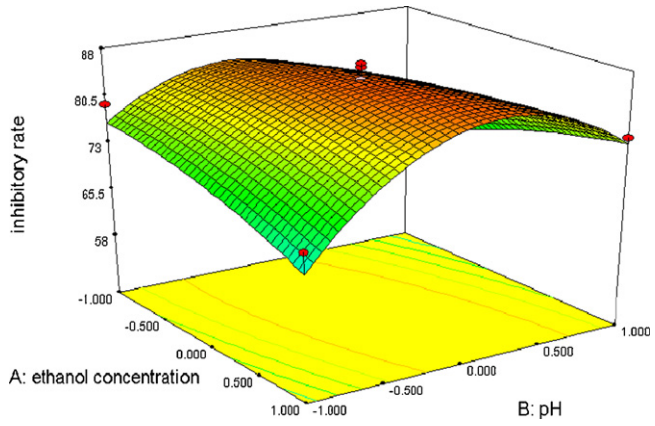

Fig. 5. Response surface contour of the model for antitumor activity of crude polysaccharides.

4. Discussion

Single factor experiments showed that final ethanol concentration and pH of the broth were the main factors influencing yield of the polysaccharides. In most case, it is generally to add 4–5 volumes of ethanol and keep the final ethanol concentration between 76% and 83.3% for precipitation of polysaccharides (Li, Yuan, & Rashid, 2009; Schepetkin et al., 2008). That was in agreement with the result of our single factor experiments about the suitable ethanol concentration. Furthermore, as ethanol concentration and pH of broth were optimized by RSM, the second-order polynomial regression models were established. The regression models indicated that the highest yield of polysaccharides could be got as the ethanol concentration was controlled at 85% and pH as 8.58. When the yield and antitumor activity of the polysaccharides were considered with equal weight, the optimum ethanol concentration was also predicted to 85% and pH as 7.70. Furthermore, the contour in Fig. 4 showed that the yield of polysaccharides rose up with ethanol concentration increase ranging from 80% to 85%. These results indicated that keeping the ethanol concentration as 85% was more suitable to get the highest yield of the polysaccharides.

Investigation about pH of broth indicated that neutral environment was favorable to keep higher antitumor activity of the polysaccharides. Furthermore, the yield of polysaccharides rose up with increase of pH value of the broth. Polysaccharides were easy to degrade in acidic environment, the glycosidic bonds disconnect and the polysaccharide molecules break into lots of micromolecule or oligosaccharides. This is the main reason that low pH is not in favorable to higher yield and antitumor activity of the polysaccharides. In general, distilled water or alkali solution was used for extraction of medicinal fungal polysaccharides (Kim, Park, Nam, Lee, & Lee, 2003; Kim, Choi, Lee, & Park, 2004; Leung, Fung, & Choy, 1997). However, our results suggested that the antitumor activities of the polysaccharides prepared from alkaline environment were significantly lower than that prepared in neutral environment. This might attribute to that addition of sodium hydroxide increasing the hydrophobic interactions not only between polysaccharide molecules but also between polysaccharides and impurities. Therefore, lots of the impurities co-precipitated with polysaccharides during preparation procedure. Perhaps, the hydrophobic interaction between polysaccharides and impurities affects antitumor activity of the polysaccharides.

During conventional preparation procedure, polysaccharide is usually precipitated overnight (Chen, Xie, Nie, Li, & Wang, 2008; Yang, Gao, Han, & Xu, et al., 2005) and rarely precipitated for 4 h (Pang et al., 2007). Long precipitation time would prolong the production cycle and is not suitable for greater production efficiency. Our results indicate that precipitation time from 1 to 24 h has little influence on the yield and antitumor activity of the polysaccharides. Precipitation for 1 h is enough to obtain the highest yield and antitumor activity of the polysaccharides.

In most of procedures, polysaccharide is usually precipitated at 4 °C, and dried through lyophilizing (Qin, Huang, & Xu, 2002; Xie, Schepetkin, & Quinn, 2007). The single factor experiments suggested that there was no significant difference for yield and antitumor activity of the polysaccharides precipitated at 4 °C and 12 °C. Therefore, precipitation at ice-cold environment is not irreplaceable. Similarly, there was no significant difference for yield and antitumor activity of the polysaccharides dried at 40 °C and lyophilized, all of which favor to obtain more polysaccharides with higher antitumor activity. For these reasons, precipitating at 4 °C overnight and lyophilizing could be replaced by precipitating at 12 °C for 1 h and dry at 40 °C which were less time and energy consuming.

Based on the less time and energy consumption production conditions, ethanol concentration and pH value of the broth which

were found to have significant influence on the yield of polysaccharides were optimized by RSM. According to RSM analysis, the second-order polynomial regression models were established to predict the optimal polysaccharide production conditions. As the final ethanol concentration was predicted as 85.00% and pH as 8.58, it was suitable to get the highest yield of the polysaccharides with 10.01 g/l. When the final ethanol concentration was predicted as 81.23% and pH as 7.37, it was more suitable to obtain the crude polysaccharides with highest antitumor inhibitory rate (85.04%). Consideration of the yield and antitumor activity with equal weight, the optimal final ethanol concentration and pH of the broth were predicted as 85.00% and 7.70. The verification experimental values conducted under each optimal condition as above were all in close agreement with values predicted by the models. According to the models, the yield of the polysaccharides increased rapidly with increase of ethanol concentration and the coefficients of quadratic term of ethanol concentration was negative; those means that the yield will reach a maximum value with increase of the ethanol concentration. It was in close agreement with the results that the yield of the polysaccharides had a tendency to keep constant when the ethanol concentration increased from 80% to 90% in the single factor experiments. The second-order polynomial regression model finally predicted that the optimum ethanol concentration was 85% and it was not only good for highest yield but also favorable to the higher antitumor activity of the polysaccharides. In contrast, pH of the broth showed larger effects to antitumor activity of the polysaccharides. In model 2, the coefficient of its linear term was not significant, but that of the quadratic term was negative and significant. It means that there is an appropriate pH range to keep an ideal antitumor activity. This result was in agreement with the single factor experiments. The polynomial regression model finally predicted that the optimal pH was 7.70 when yield and antitumor activity of the polysaccharides were considered with equal weight.

5. Conclusion

In this study, the effects of precipitation time, precipitation temperature and dry temperature on the yield and antitumor activity of the polysaccharides were investigated for the first time. Single factor experiments indicated that the conventional operation such as precipitation at 4 °C overnight and lyophilizing could be instead by precipitating at 12 °C for 1 h and dry at 40 °C which were less time and energy consumption. Furthermore, based on the less time and energy consumption conditions, RSM was employed to optimize the production conditions of polysaccharide with higher yield and antitumor activity. Our novel production procedure is that ethanol concentration and pH of the broth are adjusted to 85.00% and 7.70 respectively, then precipitated at 12 °C for 1 h and dried the precipitates at 40 °C. Under these optimal production conditions, the measured yield and cancer cell growth inhibitory rate of the polysaccharides were 9.843 ± 0.217 g/l and 83.69 ± 0.167% respectively, which were more higher than the yield of 7.637 ± 0.049 g/l and inhibitory rate of 78.32 ± 0.657% of the polysaccharides produced under the traditional production conditions described in Section 1. Compared with traditional production procedure, although more ethanol needed, the novel production procedure is not only more efficient to obtain polysaccharides with higher yield and antitumor activity, but also less time and energy consuming. It affords a theoretical foundation for low-cost production of polysaccharides in industrial scale. Further purification and characterization of the polysaccharides are on the march to investigate the function and structure of polysaccharide from fermentation broth of medicinal mushroom. And the relationships between the function and structure of the polysaccharides are also necessary to establish and to expand their application.

Acknowledgements

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References

Brander, W. T., Clarke, D. A., & Stock, C. C. (1958). Stimulation of host defence against experimental cancer. I. Zymosan and Sarcoma 180 in mice. *Cancer Research*, *18*, 347–351.

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y., & Fukuoka, F. (1970). Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes*. *Cancer Research*, *30*, 2776–2782.

Cun, Z., Mizuno, T., Ito, H., Shimura, K., Sumiya, T., & Kawade, M. (1994). Antitumor activity and immunological property of polysaccharides from the mycelium of liquid-cultured *Grifola frondosa*. *Journal of the Japanese Society for Food Science and Technology*, *41*, 724–733.

Chen, Y. L., Lin, S. Z., Chang, J. Y., Cheng, Y. L., Tsai, N. M., Chen, S. P., et al. (2006). In vitro and in vivo studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells. *Biochemical Pharmacology*, *72*, 308–319.

Chen, Y., Xie, M. Y., Nie, S. P., Li, C., & Wang, Y. X. (2008). Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. *Food Chemistry*, *107*, 231–241.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Gan, C. Y., Abdul Manaf, N. Hj., & Latiff, A. A. (2010). Optimization of alcohol insoluble polysaccharides (AIPS) extraction from the *Parkia speciosa* pod using response surface methodology (RSM). *Carbohydrate Polymers*, *79*, 825–831.

Guo, W. Q., Ren, N. Q., Wang, X. J., Xiang, W. S., Ding, J., You, Y., & Liu, B. F. (2009). Optimization of culture conditions for hydrogen production by *Ethanoligenens harbinense* B49 using response surface methodology. *Bioresource Technology*, *100*, 1192–1196.

Kim, G. Y., Choi, G. S., Lee, S. H., & Park, Y. M. (2004). Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-α from peritoneal macrophages. *Journal of Ethnopharmacology*, *95*, 69–76.

Kim, G. Y., Park, H. S., Nam, B. H., Lee, S. J., & Lee, J. D. (2003). Purification and characterization of acidic proteoheteroglycan from the fruiting body of *Phellinus linteus* (Berk & M. A. Curtis) Teng. *Bioresource Technology*, *89*, 81–87.

Leung, M. Y., Fung, K. P., & Choy, Y. M. (1997). The isolation and characterization of an immunomodulatory and anti-tumor polysaccharide preparation from *Flammulina velutipes*. *Immunopharmacology*, *35*, 255–263.

Li, F., Yuan, Q. P., & Rashid, F. (2009). Isolation, purification and immunobiological activity of a new water-soluble bee pollen polysaccharide from *Crataegus pinnatifida Bge*. *Carbohydrate Polymers*, *78*, 80–88.

Liu, J. Z., Weng, L. P., & Zhang, Q. L. (2003). Optimization of glucose oxidase production by *Aspergillus niger* in a benchtop bioreactor using response surface methodology. *World Journal of Microbiology Biotechnology*, *19*, 317–323.

Ma, Y., Mizuno, T., & Ito, H. (1991). Antitumor activity of some polysaccharides isolated from a Chinese mushroom, huangmo, the fruiting body of *Hohenbuehelia serotina*. *Agricultural and Biological Chemistry*, *55*, 2701–2710.

Miyazaki, T., & Nishijima, M. (1981). Studies on fungal polysaccharides. XXVII. Structural examination of a water-soluble, antitumor polysaccharide of *Ganoderma Lucidum*. *Chemical & Phamaceutical Bulletin*, *29*, 3611–3625.

Oh, J. Y., Cho, E. J., Nam, S. H., Choi, J. W., & Yun, J. W. (2007). Production of polysaccharide-peptide complexes by submerged mycelial culture of an entomopathogenic fungus. *Process Biochemistry*, *42*, 352–362.

Pang, X. B., Yao, W. B., Yang, X. B., Xie, C., Liu, D., Zhang, J., & Gao, X. D. (2007). Purification, characterization and biological activity on hepatocytes of a polysaccharide from *Flammulina velutipes* mycelium. *Carbohydrate Polymers*, *70*, 291–297.

Qin, C. G., Huang, K. X., & Xu, H. B. (2002). Isolation and characterization of a novel polysaccharide from the mucus of the loach, *Misgurnus anguillicaudatus*. *Carbohydrate Polymers*, *49*, 367–371.

Sandford, P. A., Watson, P. R., & Knutson, C. A. (1978). Separation of xanthan gums of differing pyruvate content by fractional precipitation with alcohol. *Carbohydrate Research*, *63*, 253–256.

Schepetkin, I. A., Xie, G., Kirpotina, L. N., Klein, R. A., Jutila, M. A., & Quinn, M. T. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from *Opuntia polyacantha*. *International Immunopharmacology*, *8*, 1455–1466.

Sun, H. H., Mao, W. J., Chen, Y., Guo, S. D., Li, H. Y., Qi, X. H., Chen, Y. L., & Xu, J. (2009). Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium* sp. F23-2. *Carbohydrate Polymers*, *78*, 117–124.

Sun, Y. X., Liu, J. C., & Kennedy, J. F. (2010). Application of response surface methodology for optimization of polysaccharides production parameters from the roots of *Codonopsis pilosula* by a central composite design. *Carbohydrate Polymers*, *80*, 949–953.

Triveni, R., Shamala, T. R., & Rastogi, N. K. (2001). Optimised production and ultilization of exopolysaccharide from *Agrobacterium radiobacter*. *Process Biochemistry*, *36*, 787–795.

Whistler, R. L., & Lauterbach, G. E. (1958). Isolation of two further polysaccharides from Corn Cobs. *Archives of Biochemistry and Biophysics*, *77*, 62–67.
Wu, Y., Cui, S. W., Tang, J., & Gu, X. H. (2007). Optimization of extraction process of crude polysaccharides from boat-fruited *sterculia* seeds by response surface methodology. *Food Chemistry*, *105*, 1599–1605.
Xie, G., Schepetkin, I. A., & Quinn, M. T. (2007). Immunomodulatory activity of acidic polysaccharides isolated from *Tanacetum vulgare* L. *International Immunopharmacology*, *7*, 1639–1650.
Yang, X. B., Gao, X. D., Han, F., & Tan, R. X. (2005). Sulfation of a polysaccharide produced by a marine filamentous fungus *Phoma herbarum* YS4108 alters its antioxidant properties in vitro. *Biochimica et Biophysica Acta*, *1725*, 120–127.
Yang, X. B., Gao, X. D., Han, F., Xu, B. S., Song, Y. C., & Tan, R. X. (2005). Purification, characterization and enzymatic degradation of YCP, a polysaccharide from marine filamentous fungus *Phoma herbarum* YS4108. *Biochimie*, *87*, 747–754.