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Extraction optimization and bioactivity of polysaccharides from *Aspergillus fumigatus* AF1

Xianchun Jin [a,*], Yu Ning [b]

[a] School of Sciences, Henan Agricultural University, 450002, China
[b] School of Life Sciences, Henan Agricultural University, 450002, China

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ABSTRACT

Aspergillus fumigatus was used to produce polysaccharide using sodium hydroxide pretreated rice straw as the sole carbon source by submerged fermentation. Response surface methodology (RSM) was used to optimize the extraction of polysaccharide from the mycelia of *A. fumigatus* (PAF). A three-level, three-factor Box–Behnken design (BBD) was applied for experimental design and analyzed the results to obtain the optimal extraction parameters. RSM analysis indicated good correspondence between experimental and predicted values. The optimal conditions for polysaccharide extraction were extraction temperature 50 °C, extraction time 6 h, ratio of dried mycelia mass to water 0.03. PAF showed excellent antitumor activity *in vitro* and exert no damage to the normal cells. PAF also showed prominent antitumor activity *in vivo* against S180 and increased the spleen and thymus index significantly.

1. Introduction

In recent years, a plethora of polysaccharides have been isolated from the microorganisms by extraction (Chen, Wang, Zhang, & Huang, 2012; Gan, Ma, Jiang, Xu, & Zeng, 2011) as many polysaccharides showed a variety of bioactivities such as antitumor, antioxidant and anti-inflammatory effects (Cengiz, Bektas, & Mustafa, 2008; Dentinger, Ammirati, & Both, 2010; He et al., 2012; Mao, Mao, & Meng, 2013; Smiderle et al., 2012). Many polysaccharides have been extracted from various edible fungi such as *Lentinus edodes* (Zhang et al., 2010), *Flammulina velutipes* (Shi, Yang, Guan, Zhang, & Zhang, 2012), *Gomphidius rutilus* (Wang, Zhang, Wang, & Wang, 2013). However, there is few reports about the bioactivity of *Aspergillus fumigatus* (Costachel, Coddeville, Latge', & Fontaine, 2005), as it is usually be thought as the most prevalent airborne fungal pathogen causing fatal invasive infections in immunocompromized patients over the past decade (Latgé, 1999) though Tekaia and Latgé thought that *A. fumigatus* should be a saprophyte (Tekaia & Latgé, 2005).

In recent years, there has been an unprecedented increase in interest in the more efficient utilization of the agro-residues because their application provides an alternative way to reduce the production costs and solve many environmental hazards (Qijun et al., 2008). Rice straw, a by-product of rice production, is discharged as an agricultural waste. 18.4 million tons of rice straw were produced every year in China (Bi, Wang, Wang, Gao, & Wang, 2011), most of which was incinerated, causing much serious air pollution. With the strengthening of environmental legislation, incinerating straw was banned, alternative method of using straw should be found. Using straw to produce enzymes and other valuable products may be such alternative. In the present study, *A. fumigatus* was used to degrade NaOH pretreated rice straw to produce polysaccharide from its mycelia by submerged fermentation as submerged cultures of fungi can give rise to higher productions of mycelial biomass and EPS in a more compact space, within a shorter period of time, and with less chance for contamination (Cho, Oh, Chang, & Yun, 2006). At the same time, response surface methodology was used to optimize the polysaccharide extraction conditions, and the antitumor activity of the polysaccharide both *in vitro* and *in vivo* was also studied.

2. Materials and methods

2.1. Microorganism and culture conditions

A. fumigatus AF1 was isolated from moldy rice straw collected from Chenzhou China. The preservation and sporulation of the strain was done on PDA. To prepare the inoculum, spores from the slant were suspended in 2 mL of 0.9% NaCl (10^7–10^8 spores/mL) and transferred into a 250 mL conical flask containing 50 mL of modified Czapek medium (g/L): sucrose 10, $FeSO_4$ 0.01, KCl 0.5, $MgSO_4{\bullet}7H_2O$ 0.5, K_2HPO_4 1.0, $NaNO_3$ 3.0. Fungal cells were cultured in an orbital shaker (150 rpm) at 40 °C for 20 h before the mycelium pellets was used for inocula. The composition of

* Corresponding author. Tel.: +86 377 63554844; fax: +86 377 63558130.
E-mail addresses: xchjin@163.com, xchjin@gmail.com (X. Jin).

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Table 1
Coded values and experimental range of extraction variables used in experimental design.

Variables	Symbols	Coded level of variable		
		−1	0	1
Extraction temperature (°C)	X_1	50	70	90
Ratio of dried mycelia to water (g/mL)	X_2	0.03	0.065	0.10
Extraction time (h)	X_3	2	4	6

fermentation medium was 2.0 g of different concentration of NaOH solution pretreated rice straw (2.0 g milled rice straw was mixed with 38.0 mL NaOH solution, autoclaved at 121 °C for 15 min, After cooling to room temperature, the reaction mixture was adjusted to given pH with 10.0 mol/L sulfuric acid and then 50% (w/v) separately autoclaved ammonium sulfate solution was added as the nitrogen source (1% of final concentration)) in 250 mL conical flask. Five milliliters of *A. fumigatus* AF1 was inoculated into each 250 mL flask then cultured at 40 °C for 24 h.

2.2. Extraction of crude polysaccharides from A. fumigatus AF1 mycelia

As the fermentation medium turned to a light yellow liquid in 24 h, *A. fumigatus* mycelia obtained by filtration with a sintered glass crucible (G-3) was washed thoroughly with distilled water. The mycelia were dried at 30 °C. Then, it was grinded and stored in desiccators at room temperature until use. The dried mycelia powders were extracted with distilled water in a 100 mL centrifuge tubes. The centrifuge tube was held in a water bath and exposed to extract polysaccharides for a different time at varying temperatures in different ratios of dried mycelia to water.

2.3. Determination of the yield of polysaccharides from A. fumigatus AF1 mycelia

After extraction for a given time, the extracted slurry was centrifuged at 10,000 × *g* for 10 min, and the supernatant was collected and concentrated under reduced pressure at 40 °C. The solution was precipitated with three volumes of absolute ethanol, and the precipitate was collected by filtration and redissolved in distilled water. After removal of proteins by the Sevag method, the polysaccharide solution was dialyzed extensively against distilled water and lyophilized. Prior to use, the polysaccharide was dissolved in water and diluted into a series of concentrations as indicated. The carbohydrate content of the solution was determined by the phenol–sulfuric acid method. The isolated polysaccharide was referred to as PAF.

2.4. Experimental design

Based on the preliminary results, a three-level–three-factor BBD was used to determine the best combination variables for extraction of polysaccharides from *A. fumigatus* mycelia. Table 1 shows the range of each factor.

Quadratic models considered as response surface model for predicting the optimal points were expressed according to Eq. (1)

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (1)$$

where Y is the dependent variable, β_0 is the model constant, β_i, β_{ii} and β_{ij} are the model coefficients. They represent the linear, quadratic and interaction effects of the variables. Analysis of the experimental design data and calculation of predicted responses was carried out using Design Expert software (version 7.0.0, USA). Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

2.5. Antitumor activity of PAF

2.5.1. In vitro antitumor activity assay

Inhibition activities *in vitro* of crude PAF on such cell lines as human gastric cancer Sarcoma180, human hepatoma cell line (HepG2), human breast adenocarcinoma cell line (MCF-7) and normal human liver cell line (L-02) were evaluated using the MTT method. Briefly, cells in a density of 1.0×10^5 cells/mL in the RPMI-1640 medium supplemented with 10%FBS, 100 U/mL penicillin and 100 μg/mL streptomycin were pipetted into 96-well flat-bottom plate (100 μL/well). After 12 h of incubation at 37 °C in a humidified 5% CO_2 incubator, non-adherent cells were removed by washing three times with RPMI-1640 medium. Then, fresh medium (100 μL/well, control group) or test sample (100 μL/well, crude PAF at different final concentration was added to each well, and the cells were incubated for 24 h. After incubation, MTT solution (10 μL/well, 5 mg/mL) was added to each well, and the plate was incubated for an additional 4 h at 37 °C. Finally, 100 μg/L of 10% SDS in 0.01 M HCl was added to each well and the plate was kept overnight for the dissolution of formazan crystals. The absorbance of each well at 570 nm was measured with an ELISA plate reader (BioTek Instruments Inc., Winooski, VT, USA). The inhibition rate was calculated according to the following formula:

$$\text{Inhibition rate } (\%) = \frac{(A_b - A_s)}{A_b} \times 100$$

where A_s and A_b are the absorbance of treated cells and untreated cells.

2.5.2. In vivo antitumor activity assay

Animal care and handling were according to the Committee for the Purpose of Control and Supervision of Experiments on Animal's guidelines after research project approval by the Institutional Animal Ethics Committee.

BALB/C mice (male, 7–9 weeks old, weighing 20 ± 1.0 g) were purchased from the Animal Center of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

Each of polysaccharides sample as well as control agents was tested on an individual group of animals consisting of 6 BALB/C mice.

Sarcoma180 tumor cells (5×10^6 cells/mL) were transplanted subcutaneously into the right groin of the mice. After 24 h of tumor inoculation, PAF samples were dissolved in 0.9% aqueous NaCl, then injected intraperitoneally to mice in experimental group every other day for 20 days. The equivalent volume of 0.9% aqueous NaCl was injected intraperitoneally in the negative control group. The mice were killed on the next day of the last injection, and the tumors, spleens, thymuses and livers were excised and weighed.

The inhibition ratio ξ and enhancement ratio of body weight f were calculated as follows:

$$x = \left[\frac{(W_c - W_t)}{W_c}\right] \times 100\%$$

$$f = \left[\frac{(W_a - W_b)}{W_b}\right] \times 100\%$$

W_c is the average tumor weight of the negative control group. W_t is the average tumor weight of PAF group; W_b and W_a are the body weight of mice before and after the assay.

The indexes of spleen, liver and thymus were defined as the ratio of corresponding organ's weight (mg) to the body weight (g).

Table 2
The Box–Behnken design matrix and the results for extraction yield of crude polysaccharides.

Run numbers	X_1 extraction temperature (°C)	X_2 ratio of dried mycelia to water (g/mL)	X_3 extraction time (h)	Response (μg/g)
1	50	0.065	6	9986.67
2	70	0.065	4	6218.26
3	90	0.065	2	6658.12
4	90	0.065	6	8390.68
5	90	0.1	4	6753.15
6	70	0.065	4	6225.38
7	70	0.065	4	6238.18
8	50	0.03	4	7610.69
9	50	0.065	2	6894.35
10	70	0.1	6	8455.52
11	70	0.1	2	6462.13
12	70	0.065	4	6279.38
13	70	0.065	4	6241.08
14	50	0.1	4	7054.97
15	70	0.03	6	8815.99
16	90	0.03	4	6080.29
17	70	0.03	2	5984.51

2.6. Statistical analysis

All data are expressed as means ± standard deviation of a minimum three separate experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA).

3. Results and discussion

3.1. Statistical analysis and the model fitting

The most common and efficient design used in response surface modeling is the Box–Behnken design. Compared to the central composite and Doehlert designs, Box–Behnken presents some advantages such as requiring few experimental points for its application (three levels per factor) and high efficiency (Costa Ferreira et al., 2007). Based on preliminary results, a three-factor–three-level response surface methodology of BBD was used to optimize the polysaccharide extraction. The experimental design and results were shown in Table 2. The whole design consisted of 17 experimental points carried out in a random order, which included 12 factorial points and 5 central points. Five replicates (runs 2, 6, 7, 12, 13) at the center of the design were used to allow for estimation of a pure error sum of squares. The results were fitted with a second order polynomial equation. The values of regression coefficients were calculated, and the fitted equation (in terms of actual values) for predicting yield (Y) was given as below:

$$Y = 16221.1809 - 225.1957X_1 - 22224.2990X_2 - 904.2005X_3 + 438.7786X_1X_2 - 8.4985X_1X_3 - 2993.1786X_2X_3 + 1.4840X_1^2 + 33225.1020X_2^2 + 287.0952X_3^2 \quad (2)$$

The statistical significance of the regression model was checked by F-test, and the analysis of variance (ANOVA) for the response surface quadratic model was shown in Table 3. ANOVA of regression model demonstrates that the model is highly significant, as is evident from the Fisher's F-test with a very low probability value [(p model > F) <0.0001]. As shown in Table 3, the value of R^2 and adjusted R^2 are 0.9999 and 0.9998, which shows a very high correlation between the observed values and the predicted ones (Fig. 1). The results clearly revealed that there is a satisfactory correlation between experimental values and predict ones. The p values are used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller the p value, the more significant is the corresponding coefficient. It can be seen from Table 3 that all the linear coefficients, all the quadratic term coefficients and cross product coefficients were significant, with very small p values ($p < 0.0024$). "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 291.666 was obtained indicating an adequate signal. Hence, these models can be used to navigate the design space.

Table 3
ANOVA for selected Box–Behnken design for PAF extraction.

Source	Sum of squares	DF	Mean square	F value	p-value prob > F	
Model	21,779,313	9	2,419,924	7560.251	<0.0001	Significant
X_1	1,678,515	1	1,678,515	5243.965	<0.0001	
X_2	6861.476	1	6861.476	21.43641	0.0024	
X_3	11,639,709	1	11,639,709	36,364.42	<0.0001	
X_1X_2	377,352.2	1	377,352.2	1178.912	<0.0001	
X_1X_3	462,236.8	1	462,236.8	1444.106	<0.0001	
X_2X_3	175,598.7	1	175,598.7	548.6001	<0.0001	
X_1^2	1,483,716	1	1,483,716	4635.381	<0.0001	
X_2^2	6974.952	1	6974.952	21.79093	0.0023	
X_3^2	5,552,751	1	5,552,751	17,347.73	<0.0001	
Residual	2240.596	7	320.0851			
Lack of fit	2.5E−05	3	8.33E−06	1.49E−08	1.0000	Not significant
Pure error	2240.596	4	560.1489			
Cor. total	21,781,553	16				
R^2	0.9999					
Adj. R^2	0.9998					

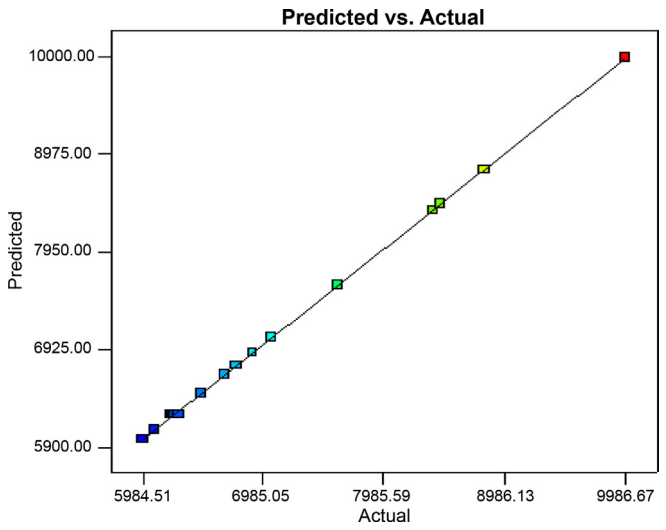

Fig. 1. Predicted values *versus* experimental values of PAF.

3.2. Graphical interpretation of the response surface model

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable to reach a maximum polysaccharide yield. The response surfaces plots are presented in Fig. 2a–c. Each figure demonstrates the effect of two factors while the third one was fixed at zero level. As shown in Fig. 2a and b, at the lowest level of temperature, the PAF yield was found to decrease rapidly at the beginning but with a slower rate toward the end. As the extraction time increased, the PAF yield increased obviously, especially at the lowest extraction temperature as shown in Fig. 2b and c. It was reported that an extended extraction time favors the production of PAF (Hou & Chen, 2008). This might be due to the time requirement of the exposure of the PAF to the release medium where the liquid penetrated into the dried powdered mycelia, dissolved the PAF and subsequently diffused out from the mycelia. As can be seen in Fig. 2a and c, dried mycelia mass to water slightly influenced the PAF yield. Considering all the responses, it is evident that extraction temperature, extraction time and the quadratic effects among them had a significant effect on the PAF yield while the effect of dried mycelia mass to water was more limited. The results agree well with Table 3.

3.3. Optimization response and verification of predictive model

The second-order polynomial model obtained (Eq. (2)) in this study was utilized for response optimization by using Design Expert software (version 7.0.0, USA). The maximum PAF mass of 10,514.7 μg/g was predicted at the following optimum

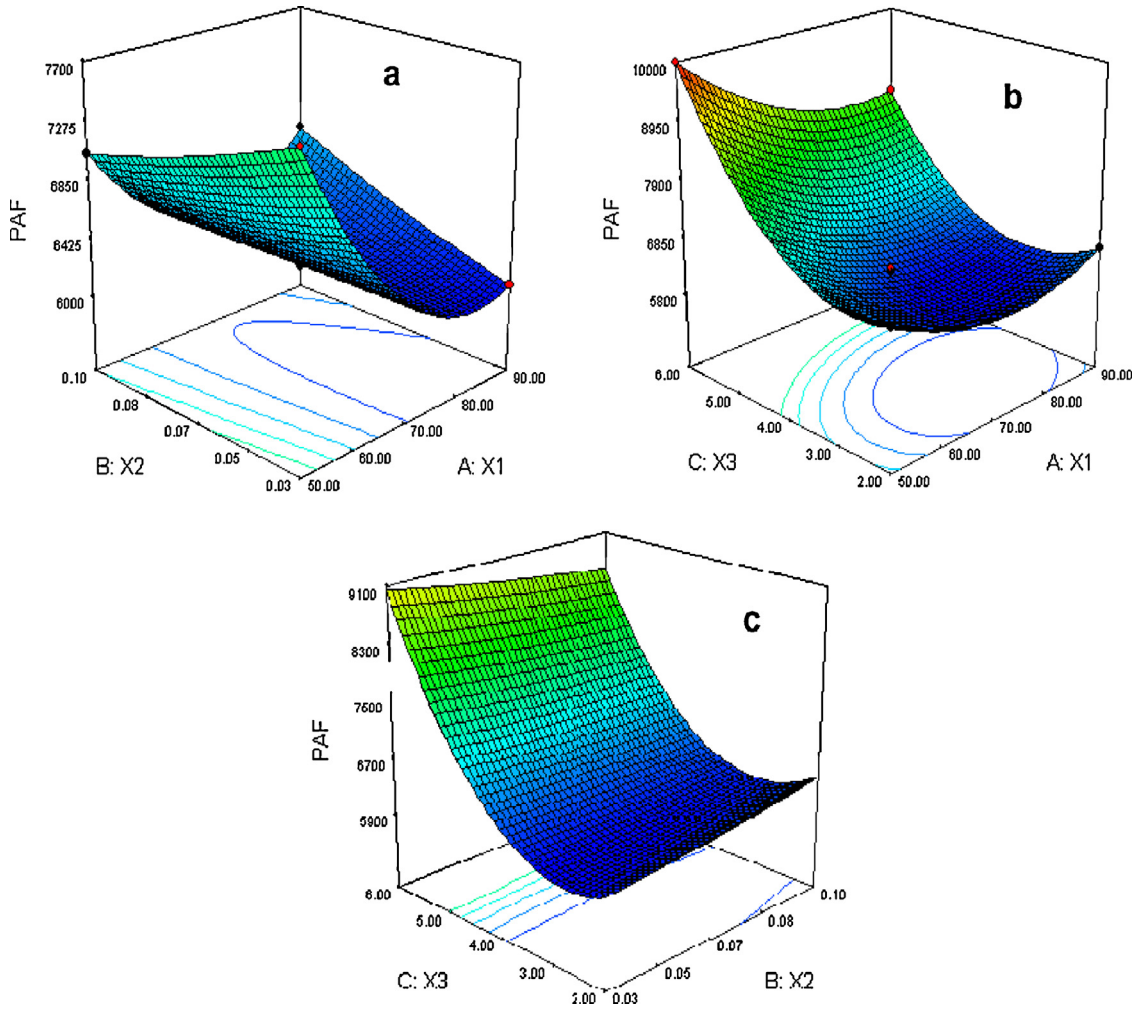

Fig. 2. Response surface plots showing the interaction between variables in the PAF: (a) interaction between extraction temperature and ratio of dried mycelia to water; (b) interaction between extraction time and ratio of dried mycelia to water; and (c) interaction between extraction temperature and extraction time.

Table 4
In vitro inhibition rate of PAF on the carcinoma cells.

Samples dosage (μg/mL)	Inhibition rate (%) S180	HepG2	MCF-7	L-02
0	ND	ND	ND	ND
6.25	58.56	45.81	52.69	ND
12.5	79.85	62.54	76.57	ND
25	100	92.35	96.85	ND
50	100	100	100	ND
100	100	100	100	ND

conditions: extraction temperature 50 °C, ratio of dried mycelia to water 0.03 and extraction time 6 h. Another three experiment sets were carried out to confirm the prediction. An average PAF mass of 10,506.3 μg/g was obtained, an excellent fit with the predicted value of 10,514.7 μg/g. This result therefore demonstrates the validity of the response model.

3.4. In vitro antitumor activity assay

The antitumor activity of PAF on human gastric cancer Sarcoma180, human hepatoma cell line (HepG2), human breast adenocarcinoma cell line (MCF-7) and human normal liver cell line (L-02) were tested in this experiment. As shown in Table 4, the PAF was effective dose-dependently in all the assayed carcinoma cells but showed no damage to the normal human liver cell line (L-02). This study indicated that *A. fumigatus* polysaccharide probably had no inhibitory effect on noncancerous cells.

3.5. In vivo antitumor activity assay

The results of antitumor activities of PAF against S180 tumor-bearing BALB/C mice *in vivo* are summarized in Table 5. After 20 days treatment, the growth of transplantable S180 in mice was significantly inhibited by PAF compared with the negative control ($p < 0.05$), with the inhibitory rate being 55.93%, 67.79% and 78.81% at the PAF concentration of 10, 20 and 40 mg/kg, respectively. The spleen and thymus indexes were obviously enhanced by PAF treatment than those of the negative control group ($p < 0.05$), which was quite in accordance with the antitumor activity. Little change in liver weights in the experimental groups and those of negative control ones indicating that PAF did not cause liver damage, furthermore, the enhancement ratios of body weight indicated that the polysaccharide might not have cytotoxicity, which kills normal cells as well as cancer cells, which would greatly promote the development of antitumor polysaccharides from *A. fumigatus*. At present, the proposed mechanisms which polysaccharides exert antitumor effect include cancer-preventing, immuno-enhancing and direct tumor inhibition (Fan, Ding, Ai, & Deng, 2012). These results suggested that activating immune responses in the host might be one of the mechanisms of antitumor activity of PAF, as many antitumor polysaccharides found in the world (Ding, Hou, & Hou, 2012).

Table 5
Antitumor effects of PAF on S180 tumor-bearing BALB/C mice *in vivo*.

Sample	Negative control	PAF		
Dose (mg/kg days)		10 × 20	20 × 20	40 × 20
Enhanced ratio of body weight (%)	26.8	42.5	46.8	45.9
Tumor weight (g)	1.18 ± 0.33	0.52 ± 0.09	0.38 ± 0.05	0.25 ± 0.03
Tumor inhibitory rate (%)		55.93	67.79	78.81
Spleen index (mg/g)	5.78 ± 2.11	8.62 ± 2.41	8.85 ± 3.21	8.64 ± 2.65
Liver index (mg/g)	60.37 ± 3.68	60.48 ± 3.24	61.09 ± 3.56	60.54 ± 3.87
Thymus index (mg/g)	1.44 ± 0.72	4.95 ± 0.32	8.25 ± 0.42	7.98 ± 0.46

4. Conclusion

The production of polysaccharide from *A. fumigatus* using NaOH pretreated rice straw as sole carbon source was investigated in the submerged cultures and the optimized extraction conditions for polysaccharides were obtained by response surface methodology. Optimum conditions for polysaccharide extraction were: extraction temperature 50 °C, ratio of dried mycelia to water 0.03 and extraction time 6 h. Under the optimized conditions, the experimental PAF mass well agreed with the predicted one. The polysaccharide exhibited good antitumor activity both *in vitro* and *in vivo* with no expressive toxicity. This activity seems relative to immuno-enhancing and direct tumor inhibition.

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