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Original article

# Extraction of polysaccharide from lotus leaf and its anticancer effect

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ABSTRACT

The selenium-enriched lotus leaf sclerotium mycelium fermented in a 20 L bio-tank was used as raw material to optimize the extraction process of selenium polysaccharide in mycelium and analyze the structure of water-soluble selenium polysaccharide through anticancer effect. The optimal extraction conditions of selenium polysaccharide were obtained by experimental and Box-Behnken design method: extraction time was 50 min, extraction temperature was 83 °C, ratio of material to liquid was 1:130 (g:mL), extraction times were 2 times. It is 44.2%, which is 6.2% higher than the predicted value. At this time, the selenium content in the polysaccharide reaches 3.9 μg/g. The purified selenium polysaccharide is analyzed for anticancer effect, which has proliferation inhibition effect on various human cancer cells and inhibits tumor in vivo growing.


## 1. Introduction

*Lyophyllum decastes* (Fr.) Singer is a kind of fungus with high nutritional value and medicinal value (Chen, 2018; Fei, 2018; Das, 2018), and the lotus leaf has a certain selenium-enriching ability. In the process of selenium-enriched fermentation, the addition of selenium can effectively increase the dry weight of mycelium. Selenium is an essential element for life activities (Chen, 2018), and the development of efficient selenium-rich foods is of great significance (Bremer, 2018). The full information and biochemical characteristics of selenium polysaccharides are not fully understood, and direct evidence and experiments on the molecular biology research, structure-activity relationship and mechanism of action of selenium polysaccharides are insufficient (Bremer, 2018).

In this study, the selenium-enriched lotus leaf of Pleurotus ostreatus was fermented in a 20 L bio-tank. Four single-factor tests were conducted to investigate the extraction time, temperature, ratio of material to liquid and the number of extractions. The extraction process was initially optimized, and the extraction process was initially optimized. Then, the response surface test of the Box-Behnken center combination was used to analyze the variance of the polysaccharide extraction rate by Design Expert 8 software. The optimal extraction conditions of the selenium polysaccharide were obtained, and the purified selenium was obtained. The polysaccharides were compared and analyzed for their anticancer effects, and their composition was determined to provide a theoretical basis for the development and utilization of selenium polysaccharides in the mycelium of Pleurotus ostreatus.

## 2. Materials and methods

### 2.1. Materials and reagents

Lotus leaf pleated umbrella, provided by the Applied Fungi Engineering Laboratory of Gansu Province; selenium powder (chemically pure CP), Sinopharm Chemical Reagent Co., Ltd.; anthrone (analytical grade), Shanghai Zhongtai Chemical Reagent Co., Ltd.; 3, 3′ -Diaminobenzidine (chemically pure), Sinopharm Chemical Reagent Co., Ltd.; 95% ethanol, chloroform, n-butanol, glucose, concentrated $H_2SO_4$, concentrated HCl, concentrated $HNO_3$, toluene, NaOH, EDTA-$Na_2$ reagents are of analytical grade The water is distilled water.

### 2.2. Instruments and equipment

V-1200 visible light spectrophotometer (Shanghai Meida Instrument Co., Ltd.); TDL-50 large-capacity low-speed centrifuge (Jintan Yineng Experimental Instrument Field); LGJ-18 freeze dryer



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(Beijing Songyuanhua Technology Development) Co., Ltd.; RE-2000A rotary evaporator (Shanghai Yarong Biochemical Instrument Factory); B260 constant temperature water bath (Shanghai Yarong Biochemical Instrument Factory); DHG-9423A electric heating constant temperature blast drying oven (Shanghai Shenxian Thermostatic Equipment Factory); KQ-250B ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.).

### 2.3. Experimental methods

#### 2.3.1. Fermentation of 20 L bio-tank to prepare lotus leaf pleated mycelium

Preparation of selenium-enriched lotus leaf pleated mycelium: weigh 100 g of soybean, soak it for 12 h, boil for 5 min, cool to 40 °C, add 5% protease, use a 300-mesh sieve after 8 h in constant temperature water bath, and drain the filtrate; 600 g of cornmeal was added to hot water of 65 °C at 1:10 (g:mL), then 5% amylase was added, and the water was heated to the hydrolyzate until the iodine solution was colorless. It was filtered through a 300 mesh sieve and the filtrate was used.

Mix the soybean enzymatic hydrolysate and the corn starch hydrolysate filtrate, add 200 g of glucose, dilute to 20 L fermenter, sterilize at 121 °C for 40 min, rapidly cool to 22 °C, and access 10% lotus leaf pleated umbrella triangle The bottle seed culture solution was passed through sterile air, and the pressure in the tank was 0.02 MPa. On the third day of fermentation, the sterilized $Na_2SeO_3$ solution was added to make the concentration in the fermenter 4 μg/mL, fermented. The fermentation was terminated on the 8th day, and the obtained mycelium was washed 3 times with water and lyophilized in a freeze dryer to obtain a selenium-enriched lotus leaf.

Preparation of non-selenium-rich lotus leaf pleated mycelium: no sterilized $Na_2SeO_3$ solution was added during the fermentation and other processes were the same as the preparation of selenium-rich mycelium.

#### 2.3.2. Extraction method of polysaccharide

Polysaccharide extraction process: selenium-enriched mycelium fermented in 20 L bio-container → dry → dust → ultrasonic extraction → centrifugation → take supernatant → concentration → ethanol precipitation polysaccharide → defattification → Sevage reagent protein removal → constant volume → determination content.

Polysaccharide extraction method: The selenium-enriched lotus leaf of the 20 L bio-tank is placed in a vacuum drying oven for drying, and the powder is ground through a 100 mesh sieve to prepare a standard powder. The sample was accurately weighed 0.5000 g. Ultrasonic extraction after centrifugation at 3000 r/min for 15 min, the supernatant was combined and concentrated under reduced pressure (≤50 mL), and the volume fraction of 3 times volume was 95% ethanol, and it was allowed to stand in a refrigerator at 4 °C for 12 h. After precipitation, the precipitate was washed repeatedly with ethanol, and the protein was removed with Sevage reagent, and finally the supernatant was made up to 50 mL for use.

#### 2.3.3. Determination of polysaccharide content

Place the glucose in an oven at 50–80 °C for 8–10 h, accurately weigh 0.0100 g, add distilled water to 100 mL, prepare 0.1 mg/mL for use. Take 7 15 mL washed and dried with stopper In the test tube, add glucose standard solution 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mL, add distilled water to 2.0 mL, and then accurately add the sulfone sulfone solution (0.1 g of fluorenone, 100 mL of 80% sulfuric acid solution, dissolve and shake) After shaking 6.0 mL, the bath was boiled for 15 min, taken out and placed in an ice bath for 15 min. The absorbance was measured at a wavelength of 625 nm with the corresponding reagent or distilled water as the blank. The glucose content (mg) was plotted on the abscissa and the absorbance was plotted on the ordinate. Draw a standard curve.

Take 15 mL of washed and dried test tube, accurately measure 2.0 mL of the sample solution, then accurately add 6.0 mL of the sulphuric acid ketone solution, shake well, boil water for 15 min, remove and put in an ice bath for 15 min, with the corresponding reagent or The distilled water was blank, the absorbance was measured at a wavelength of 625 nm, and the linear regression equation was substituted to determine the polysaccharide content in the test sample solution, and then the polysaccharide extraction rate of the mycelium sample was calculated.

$$\text{Polysaccharide extraction rate}\% = \frac{Y \times N \times V}{m \times V_S \times 1000} \times 100$$

where: $Y$ is the mass (mg) of the polysaccharide found in the standard curve of the test sample solution; $N$ is the dilution factor; $V$ is the constant volume (50 mL); m is the mass of the test sample (g); $V_S$ is the sample solution Volume (2 mL).

#### 2.3.4. Single factor test method

A. Effect of extraction time on polysaccharide extraction

Accurately weighed 0.5000 g of selenium-enriched mycelium sample, the extraction time was 15, 30, 45, 60, 75 min, the extraction temperature was 60 °C, and the liquid-to-liquid ratio was 1:60, and the ultrasonic extraction was performed once. Centrifuge at 3000r/min for 15 min, concentrate the concentrated solution under reduced pressure (≤50 mL), add 3 times volume of 95% ethanol, and stand for 12 h in 4 °C refrigerator to obtain polysaccharide precipitation. The precipitate is washed repeatedly with ethanol and used Sevage reagent. The protein was removed, and finally the supernatant was adjusted to 50 mL. The content of polysaccharide was determined by anthrone colorimetric method, and the extraction rate of polysaccharide was calculated to study the effect of extraction time on polysaccharide extraction.

B. Effect of extraction temperature on polysaccharide extraction

The sample (standard powder) was accurately weighed to 0.5000 g, and the extraction temperature was 50, 60, 70, 80, 90 °C, the extraction time was 45 min, and the liquid-to-liquid ratio was 1:60, and the ultrasonic extraction was performed once. 3 Determination of polysaccharide content, and calculate the polysaccharide extraction rate, in order to study the effect of extraction temperature on polysaccharide extraction.

C. Effect of different ratios of liquid to liquid on polysaccharide extraction

Weigh accurately sample (standard powder) 0.5000 g, add distilled water to each flask when the ratio of material to liquid is 1:40, 1:80, 1:120, 1:160, 1:200, the extraction time is 45 min Ultrasonic extraction was carried out once at a constant temperature of 80 °C. After extraction, the content of polysaccharide was determined in the same manner as in 1.3.3, and the polysaccharide extraction rate was calculated to study the effect of the ratio of the liquid to the polysaccharide extraction.

D. Effect of extraction times on polysaccharide extraction

Weigh accurately sample (standard powder) 0.5000 g, the extraction times are 1, 2, 3, 4 times, the extraction time is 45 min, the ratio of material to liquid is 1:120, 80 °C under constant temperature conditions, after extraction, the same 1.3 0.3

Determine the content of polysaccharides and calculate the polysaccharide extraction rate to study the effect of extraction times on polysaccharide extraction.

*2.3.5. Response surface test method*

Based on the four single factor experiments, the influencing factors were select and the factors were determined. The Box-Behnken center combination principle was used to design the response surface test table. The best extraction process conditions for polysaccharide extraction were determined by using the polysaccharide extraction rate as a reference index. The four factors affecting the extraction of polysaccharides were selected: extraction time, extraction temperature, ratio of material to liquid and number of extractions, respectively, represented by A, B, C and D. The design of test factors was shown in Table 1.

*2.3.6. Detection of anticancer effects of selenium polysaccharides*

Take 1 mg of dried and purified selenium-enriched mycelium and non-selenium-rich mycelium polysaccharide samples, and 100–200 mg dry KBr powder is gently ground in a mortar, operated under infrared light, and pressed by tablet press. In the form of flakes, the anticancer effect was measured at 4000–400 $cm^{-1}$.

## 3. Results and analysis

*3.1. Glucose standard curve*

Taking the absorbance value (OD value) as the ordinate and the polysaccharide mass (mg) as the abscissa, the regression equation $Y = 4.69X - 0.0081$ and the correlation coefficient $R^2 = 0.9992$ were obtained, which meet the accuracy requirements, as shown in Fig. 1.

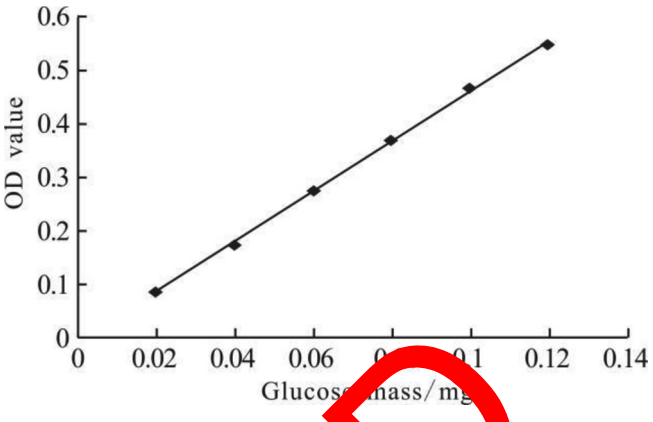


**Fig. 1.** Glucose standard curve.

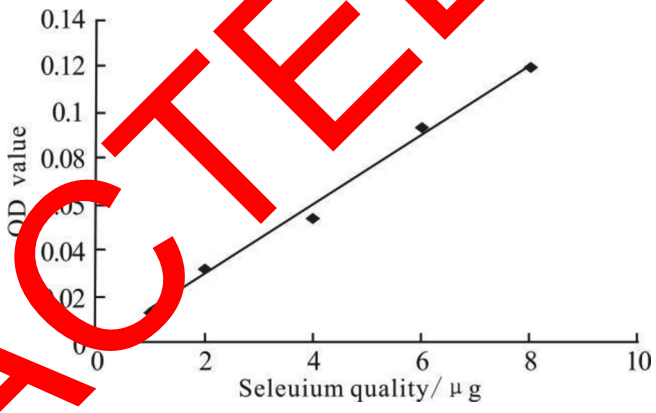


**Fig. 2.** Selenium standard curve.

*3.2. Selenium standard curve and selenium content*

The regression equation of the selenium standard curve: the absorbance value (OD value) is taken as the ordinate, and the selenium content (μg) is plotted on the abscissa, and the standard curve is drawn (Fig. 2).

Regression equation $Y = 0.015X - 0.0014$, correlation coefficient $R^2 = 0.9934$, has a good correlation.

According to the standard curve of selenium, it is concluded that the selenium content in the mycelium of the lotus leaf is 114.2 μg/g, which indicates that the selenium is rich in selenium. Because the selenium content determination method is complicated, the cost is complicated. After a long period of time, after comprehensive consideration, the change of polysaccharide extraction rate is used to illustrate the change of selenium polysaccharide extraction in the mycelium of Pleurotus ostreatus.

*3.3. Effect of extraction time on polysaccharide extraction*

With the extension of time, the extraction rate of selenium polysaccharide increased gradually. The extraction rate of polysaccharide reached 10.97% at 45 min. Then the extraction rate of polysaccharide decreased with the extension of extraction time (see Fig. 3). The extraction process is closely related to time. When the ultrasonic time is short, the product is not dissolved enough. When the time is too long, the macromolecular polysaccharide degrades under the strong shearing action of ultrasonic waves, which leads to the loss of polysaccharide. From saving energy and reducing production cycle Consider that the extraction time is around 45 min.

*3.4. Response surface test results and analysis*

*3.4.1. Model establishment and significance test and analysis*

According to Box-Behnken's central combination experimental design principle, comprehensive single factor influence test results, using 4 factors and 3 levels of response surface analysis method for experimental design, analysis factors and design are shown in Table 1, the data in the table is statistically designed by Design-Expert 8 The analysis software was used for multiple regression analysis. The design results were analyzed by Design Expert 8 software. After regression fitting the factors, the quadratic regression

**Table 1**
Box-Behnken design test factors and levels.

| Factor | Code code value | Level | | |
|---|---|---|---|---|
| | | −1 | 0 | 1 |
| Extraction time | A | 30 | 45 | 60 |
| Extraction temperature | B | 70 | 80 | 90 |
| Material to liquid ratio | C | 1:80 | 1:120 | 1:160 |
| Number of extractions | D | 1 | 2 | 3 |

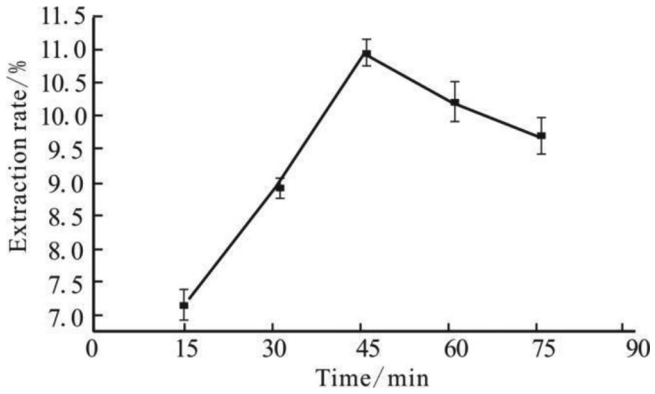


**Fig. 3.** Effect of extraction time on polysaccharide extraction rate.

equation between the four factors of polysaccharide extraction rate was obtained:

$$Y = -334.64364 + 1.42204A + 5.95762B + 1.25132C + 12.91923D + 8.48614 \times 10^{-3}AB - 1.94563 \times 10^{-4}AC + 0.033333AD + 1.50053 \times 10^{-3}BC + 0.06233BD - 0.021828CD - 0.02648A^2 - 0.040372B^2 - 5.15448 \times 10^{-3}C^2 - 3.55101D^2$$

The results of analysis of variance and significance test for the regression equation are shown in Table 1. Where $Y$ is the polysaccharide extraction rate, and $A$, $B$, $C$ and $D$ are the encoded values of the above four variables. In the regression term, $p < 0.0001$, indicating that the selected model is highly significant; the missing term $p = 0.9744 > 0.05$, that is, the difference of the missing items is not significant, indicating that the quadratic regression model significantly fit the test; the regression model $R^2 = 0.9456$ indicates that The model can account for 94.56% of the response change, and only 5.44% of the variation cannot be explained by the model. The model is therefore extremely significant and reliable and can predict polysaccharide extraction.

*3.4.2. Determination and verification test of the optimal extraction process*

With the help of Design Expert 8 statistical analysis software, the optimal extraction conditions were obtained: extraction time 50.45 min, extraction temperature 83.3 °C, ratio of material to liquid 1:127.47 (g:mL), extraction times 2.39 times, theoretical polysaccharide extraction rate It is 44.60%. For the convenience of practical operation, the optimal extraction process conditions are as follows: extraction time 50 min, extraction temperature 83 °C, ratio of material to liquid 1:130 (g:mL), extraction times 2 times. Under this condition, the load is The selenium polysaccharide in the mycelium of the pleats was extracted, and the polysaccharide extraction rate was 44.62% through three parallel experiments, which was very different from the predicted theoretical value. The correctness of the regression model was verified again. The content of selenium was determined by spectrophotometry with '-diaminobenzidine. The selenium content was calculated from the standard curve of selenium to be 31.9 μg/g. This indicates that the equation is consistent with the actual situation. The experimental results fully verify the correctness of the model and indicate the lotus leaf. The optimization process of the extraction process of selenium polysaccharide in the pleam mycelium is effective.

*3.5. Anticancer structure analysis of selenium polysaccharide in the mycelium of Pleurotus ostreatus*

Selenium as a characteristic part of selenium polysaccharide may exist in selenium polysaccharides in the form of Se-H and $R_1$-Se-O-$R_2$ (Tiemei, 2008), and the selenium-free lotus leaf is not selenium-rich After purifying the water-soluble polysaccharide of Pleurotus ostreatus, the anticancer effect was analyzed in 4000–400 $cm^{-1}$. Selenium-enriched did not change the main anticancer structure of water-soluble polysaccharide, which showed that the two had the same polysaccharide. Characteristic peak: A broad peak at 3600–3200 $cm^{-1}$ is O—H stretching vibration, and a group of 3000–2800 $cm^{-1}$ is C—H stretching vibration of sugar $CH_3$, $CH_2$, CH, etc. 1650–1550 $cm^{-1}$ There is a strong absorption peak at 1 position, which is the stretching vibration of C=O and the asymmetric stretching vibration of C—O. The polysaccharide may contain —COOH, which is an acidic polysaccharide. Since the absorption peak of 800–900 $cm^{-1}$ is not obvious, Therefore, it is impossible to judge the type of glycosidic bond. The absorption peak of 1400–1200 $cm^{-1}$ is the angular vibration of C—H. 890 $cm^{-1}$ is the characteristic absorption peak of typical glucopyranose and β-type glycosidic linkage, indicating the selenium-enriched Flammulina There is a β-type glucan configuration in the polysaccharide of Mycobacterium sinensis. After selenium enrichment, the absorption peak of the pyran ring changes, indicating that selenium participates in the process of selenium enrichment. p The synthesis of polysaccharides causes changes in the anticancer structure of polysaccharides.

## 4. Discussion

In this experiment, the effects of extraction temperature, extraction time, ratio of material to liquid and extraction times on polysaccharide extraction were studied by four single factor experiments in a 20 L biological culture tank. Four-factor and three-level response surface experiments were designed by Box-Behnken center combination principle. Data was processed by Design Expert 8 software, and multiple regression analysis was carried out. Four regression models of single factor and polysaccharide extraction rate were obtained. The effect of factor rendezvous on the extraction rate of polysaccharides in the mycelium of Pleurotus ostreatus, and finally the optimal extraction conditions for the model optimal value: extraction time 50.45 min, extraction temperature 83.3 °C, ratio of material to liquid 1:127.47 (g:mL), the number of extractions was 2.39 times, and the polysaccharide extraction rate was predicted to be 44.60%. For the feasibility of the test operation, the extraction time was adjusted to 50 min, the extraction temperature was 83 °C, the ratio of material to liquid was 1:130 (g:mL), and the number of extractions was Two times, the polysaccharide extraction rate was 44.62%, and the selenium content in the polysaccharide reached 31.9 μg/g. In this paper, the mathematical model between each factor and response value was established by response surface analysis, which can be seen visually. Factor The interaction, targeted adjustment, can reduce the number of trials, improve efficiency, and has a wide range of application value in actual production. Through the analysis of the anticancer structure of two forms of selenium polysaccharide before and after selenium enrichment, it is known that Human cancer cells have a proliferation inhibitory effect and inhibit tumor growth in animals.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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