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Extraction optimization of antioxidant polysaccharides from *Auricularia auricula* fruiting bodies

Yu ZOU[1]*, Aili JIANG[1], Mixia TIAN[1]

Abstract

The extraction conditions (liquid-solid ratio, temperature and time) of antioxidant polysaccharides from *Auricularia auricula* fruiting bodies (AAFB) were optimized using response surface methodology (RSM). The Box-Behnken experimental results showed the optimum extraction conditions as follows: a liquid-solid ratio of 38.77 mL/g, a temperature of 93.98 °C and a time of 3.41 h. Under these conditions, the maximal polysaccharide yield was 10.46 g/100 g. In addition, AAFB polysaccharides exhibited stronger antioxidant activities by evaluating of Fe^{2+}-chelating ability and hydroxyl radical scavenging activity with IC_{50} values of 0.43 and 0.38 mg/mL, respectively. These results indicated that AAFB polysaccharides might be potentially used as a natural antioxidant.

Keywords: *Auricularia auricula*; polysaccharide; extraction; antioxidant activity.

Practical Application: *A. auricula* polysaccharides can be potentially used as a natural antioxidant

1 Introduction

Active oxygen and free radicals are increasingly being recognized as being responsible for the pathogenesis of certain human diseases, including cancer, aging and chronic arterial disease (Moskovitz et al., 2002). In order to reduce the oxidative damage of active oxygen and free radicals, some synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) are widely used in food industry. Nowadays, natural antioxidants have become increasingly popular among consumers because synthetic antioxidants are often perceived as undesirable or harmful (Tu et al, 2009). Therefore, there is an increasing interest in studying natural antioxidants that can be used in food processing to improve body's antioxidant defenses and reduce the oxidative stress to human body.

The edible mushroom not only is a good source of nutrients, including protein, vitamin and dietary fiber, but also contains a variety of bioactive substances, such as polysaccharides, cordycepin and lectin (Xie et al., 2010). Previous publications indicate that some mushroom polysaccharides, isolated from *Lentinus edodes*, *Agaricus nevoi*, *Coprinus comatus* and *Daedalea quercina*, are found to have strong antioxidant activity (Asatiani et al., 2008; Chen et al., 2012). In addition, most of mushroom polysaccharides are relatively nontoxic and do not cause significant side effects (Wasser & Weis, 1999; Zhang et al., 2007). Thus, mushroom polysaccharides have great development potential as a natural antioxidant.

Auricularia auricula (*A. auricula*) is a macro-fungus distributed in the Northeast Provinces of China. It has been used as food and drug in China for a long time. Fruiting bodies of *A. auricula* are rich in polysaccharides and are increasingly popular as a health food in China (Zou et al., 2013). Polysaccharide is considered to be one of the most important functional components in these health foods. However, most of this macro-fungus product is only used as cuisine materials, and many of its functional components are not fully developed and employed.

Response surface methodology (RSM) is an affective statistical technique for optimizing complex processes. It is wide used in optimizing the process variables. The extraction conditions of mushroom polysaccharides from *Cordyceps militaris* and *Pleurotus eryngii* have been optimized using RSM with Box-Behnken design (Chen et al., 2014; Zhang et al., 2014). It is considered that liquid-solid ratio, temperature and time are main parameters that can influence polysaccharide yield (Zhang et al., 2015).

The objective of this study was to develop an economical and efficient extraction of antioxidant polysaccharides from *A. auricula* fruit bodies (AAFB), and to investigate the effect of extraction parameters on polysaccharide yield. RSM was employed to optimize extraction conditions (liquid-solid ratio, temperature and time) in order to obtain the maximal polysaccharide yield. Meanwhile, antioxidant activity of AAFB polysaccharides was studied.

2 Materials and methods

2.1 Materials

Eighty grams dried fruiting bodies of *A. auricula* (grown in Dongning City and harvested in 2014) were purchased from a local market in Dongning City (Heilongjiang Province, China), pulverized and sifted through a 0.42 mm sieve. The powder

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[1] *College of Life Science, Dalian Nationalities University, Dalian, China*
*Corresponding author: yuzou126@126.com

(moisture content 12-15% on dry basis) stored in dark bags to prevent from moisture and light. BHT was purchased from Sigma Chemicals Co. (St. Louis, USA). All the other chemicals and reagents used in the experiment were of analytical grade.

2.2 Extraction and purification of AAFB polysaccharides

The extraction and purification of AAFB polysaccharides were carried out according to the method of Yan et al. (2014). In short, fruiting bodies powder of *A. auricula* was suspended in distilled water at the established volume and then stirred for extraction at the established temperature and time. The mixture was then centrifuged at 5000 rpm for 20 min. The supernatant was concentrated to 1/5 of the original volume by evaporation (DZF-6020 vacuum oven, Shanghai, China) at 45 °C. Three volumes of absolute ethanol were added into the filtered solution and produced polysaccharide precipitate. The precipitated materials were collected by centrifugation at 5000 rpm for 20 min and then purified using the classic Sevag method (Sevag et al., 1938).

2.3 Experimental design

The Box-Behnken experimental design with three factors and three levels was employed to optimize the extraction conditions in order to obtain the highest polysaccharide yield. Liquid-solid ratio (*A*), temperature (*B*) and time (*C*) were chosen as independent variables in this design. Based on the single-factor experiments (data not shown), *A* (20, 40 and 60 mL/g), *B* (60, 80 and 100 °C) and *C* (1, 3 and 5 h) were determined as critical levels with significant effect on polysaccharide extraction. The complete design consisted of seventeen combinations including five replicates of the center point (Table 1).

The experimental results were analyzed by quadratic stepwise regression to fit the second-order Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{3} B_{ij} X_i X_j \quad (1)$$

where Y stands for polysaccharide yield, X_i, X_j for independent variables, β_0 for the model intercept and B_i, B_{ii}, B_{ij} for regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively. The software Design-Expert 7.0.0 Trial (State-Ease Inc., Minneapolis, USA) was used to obtain the coefficients of the quadratic polynomial model.

2.4 Determination of polysaccharide content

The determination of polysaccharide content was done by phenol-sulfuric acid method (Yan et al., 2014). Briefly, 1 mL of crude polysaccharide solution was mixed with 3 mL concentrated sulphuric acid to initiated the reaction, following 0.6 mL of 5% phenol was added and the mixture was kept at 100 °C for 15 min, after cooling to the room temperature, the absorbance of the reaction mixture was measured at 490 nm using a UV-2802 diode array spectrophotometer (UNIC, Princeton, USA). Polysaccharide content was calculated with d-glucose as standard.

2.5 Assessment of antioxidant activity of AAFB polysaccharide

Assay of Fe^{2+}-chelating activity

The chelating activities of polysaccharides and BHT on Fe^{2+} were determined as reported by measuring the formation of ferrous iron-ferrozine complex (Dinis et al., 1994). Different concentrations of polysaccharides or BHT (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) were mixed with 3.7 mL of deionized water, and then reacted with $FeSO_4$ (2 mM, 0.1 mL). The reaction was allowed to proceed for 30 s. After 0.2 mL of 5 mM ferrozine was added, the solution was mixed, left to stand for 10 min at room temperature, and then the mixture absorbance was determined at 562 nm. The chelating activity of Fe^{2+} was calculated using the formula given below. IC_{50} (inhibitory concentration) was the concentration of the sample required to chelate 50% of Fe^{2+} (Equation 2).

$$\text{Chelating ability (\%)} = (A_0\text{-}A_1)/A_0 \times 100 \quad (2)$$

Table 1. Box-Behnken design and the response for polysaccharide yield extracted from *A. auricula* fruit bodies.

Run	*A*: Liquid-solid ratio (mL/g)	*B*: Temperature (°C)	*C*: Time (h)	Polysaccharide yield (g/100 g)
1	40	80	3	10.07±0.31
2	20	100	3	7.39±0.30
3	20	60	3	5.86±0.19
4	40	100	1	6.41±0.26
5	60	100	3	6.46±0.25
6	40	80	3	10.13±0.51
7	20	80	5	4.96±0.13
8	60	80	5	4.85±0.17
9	20	80	1	4.59±0.18
10	60	60	3	6.21±0.30
11	40	80	3	10.30±0.49
12	60	80	1	5.11±0.17
13	40	80	3	10.29±0.46
14	40	60	1	4.59±0.16
15	40	100	5	9.63±0.38
16	40	80	3	7.74±0.23
17	40	60	5	4.13±0.16

where A_0 was the absorbance of the control (deionized water, instead of sample), and A_1 was the absorbance of the test sample mixed with reaction solution.

Assay of hydroxyl radical scavenging activity

The scavenging activity of AAFB polysaccharides on hydroxyl radical was determined by the method previously reported by Sun & Kennedy (2010). Reaction mixtures in a final volume of 1.0 mL contained deoxyribose (60 mM), phosphate buffer (pH 7.4, 20 mM), ferric trichloride (100 μM), ethylene diamine tetraacetic acid (100 μM), H_2O_2 (1 mM), and different concentrations of polysaccharides or BHT (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL). The reaction solution was incubated for 1 h at 37 °C, and then 1 mL of 1% thiobarbituric acid and 1 mL of 20% (v/v) HCl were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the resulting mixture was measured at 532 nm. The scavenging activity of hydroxyl radical was calculated according to the formula given below. IC_{50} (inhibitory concentration) was the concentration of the sample required to scavenge 50% of hydroxyl radicals (Equation 3).

$$\text{Scavenging activity (\%)} = (A_B\text{-}A_S)/A_B \times 100 \quad (3)$$

where A_B was the absorbance of the control (deionized water, instead of sample), and A_S was the absorbance of the test sample mixed with reaction solution.

2.6 Statistical analysis

The experimental results were expressed as means ± standard deviation (SD) of triplicates. Statistical analysis was performed using Fisher's *F*-test and $p<0.05$ was regarded as significant.

3 Results and discussion

3.1 Analysis of Box-Behnken experiment

The extraction conditions including liquid-solid ratio, temperature and time as independent variables were optimized for the maximum polysaccharide yield. The Box-Behnken design and the corresponding response values are shown in Table 1.

A second-order polynomial model describing the correlation between polysaccharide yield and the three variables in this study was obtained in Equation 4 below:

$$Y = -22.99 + 0.53A + 0.41B + 2.34C - 0.01A^2 - 0.01B^2 - 0.64C^2 - 0.01AB - 0.01AC + 0.02BC \quad (4)$$

The statistical significance of Equation 4 was checked by *F*-test, and the results of analysis of variance (ANOVA) are shown in Table 2. The model *P*-value of 0.0145 obtained by ANOVA indicated that the model was significant ($p<0.05$). Meanwhile, the lack of fit *P*-value of 0.3836 indicated that the lack of fit was not significant ($p>0.05$). For the model fitted, the coefficient of determination (R^2) was 0.9135 implying that the sample variation of 91.35% for the polysaccharide yield was attributed to the independent variables. These results suggested that the developed model could adequately represent the real relationship among the parameters chosen.

3.2 Effects of liquid-solid ratio, temperature and time on polysaccharides extraction

As is shown in Table 2, temperature had significant linear effect ($p<0.05$) on polysaccharides extraction; liquid-solid ratio and time had significant quadratic effect ($p<0.05$) on polysaccharides extraction. However, none of the independent variables (liquid-solid ratio, temperature and time) interacted significantly ($p>0.05$).

Figure 1 shows the effect of liquid-solid ratio and temperature on polysaccharides extraction from *A. auricula* fruit bodies at a constant time of 3 h. At a fixed liquid-solid ratio, the polysaccharide yield increased rapidly when temperature reached a certain value (approximately 95 °C), and then leveled off. At a fixed temperature, the polysaccharide yield first increased and then decreased when the liquid-solid ratio was raised, but the variety of polysaccharide yield was slight when the temperature exceeded 85 °C. This indicated that extraction temperature was the principal effect on the polysaccharide yield. At a higher temperature, the solubility of polysaccharides in *A. auricula*

Table 2. Analysis of variance (ANOVA) of the response surface regression model.

Source	Sum of squares	Degree of freedom	Mean squares	*F*-value	*P*-value
Model	73.61	9	8.18	5.90	0.0145
A	0.01	1	0.01	0.01	0.9607
B	10.35	1	10.35	7.46	0.0293
C	1.03	1	1.03	0.74	0.4174
A^2	21.68	1	21.68	15.63	0.0055
B^2	3.85	1	3.85	2.78	0.1394
C^2	27.58	1	27.58	19.89	0.0029
AB	0.41	1	0.41	0.30	0.6037
AC	0.09	1	0.09	0.07	0.7968
BC	3.39	1	3.39	2.44	0.1622
Residual	9.71	7	1.39		
Lack of Fit	4.84	3	1.61	1.32	0.3836
Pure Error	4.87	4	1.22		
Corrected Total	83.32	16		$R^2 = 0.9135$	

fruit bodies could be enhanced and the viscosity of the solvent decreased. Therefore, the whole extraction of polysaccharides was accelerated. However, increasing extraction temperature might result in more solvent volatilization, more energy cost and more impurities extraction (Lianfu & Zelong, 2008). Therefore, the optimum extraction temperature should be about 95 °C in the present study.

Figure 2 shows the effect of temperature and time on polysaccharide extraction from *A. auricula* fruit bodies at a constant liquid-solid ratio of 40 mL/g. When time was set, the polysaccharide yield increased rapidly when temperature was raised, which also implied that polysaccharide yield was significantly influenced by temperature. The polysaccharide yield increased when extraction time was extended from 1 to 3.5 h but slowly decreased when time continued to be extended. This phenomenon could be explained in terms of polysaccharides degradation. Biomacromolecule, such as polysaccharides, polyphenols and colorants, might degrade due to long time treatment under high temperature condition (Zou et al., 2010). Therefore, extraction time should not exceed 3.5 h in the present work.

Figure 3 shows the effect of liquid-solid ratio and time on polysaccharide extraction from *A. auricula* fruit bodies at a constant temperature of 80 °C. The increase of both liquid-solid ratio and time accelerated extraction of polysaccharides. However, above the optimal liquid-solid ratio (about 40 mL/g) and time (about 3.5 h), the increase in liquid-solid ratio and time would not further increase the polysaccharide yield. This result was similar to those previously reported by Rodrigues et al. (2008). These findings make the whole process of polysaccharides extraction economically more feasible and efficient in the potential application in food industry.

3.3 Optimization of extraction conditions and verification of model

According to the RSM test results, the optimal extraction conditions to obtain the highest polysaccharide yield were determined as follows: a liquid-solid ratio of 38.77 mL/g, a temperature of 93.98 °C min and a time of 3.41 h. The verification of the model was performed by the method previously reported by Derringer & Suich (1980). Under the optimal extraction conditions, the polysaccharide yield was 10.46±0.34 g/100 g, and this value was not significantly different (p>0.05) from the predicted value of 10.41 mg/100 g. These data proved that the model designed in this study was valid.

3.4 Fe^{2+}-chelating activity of AAFB polysaccharides

The previous research reported some transition metals, such as Fe^{2+}, Cu^{+} and Co^{2+}, could trigger process of free radical reaction to magnify the cellular damage (Gungor & Sengul, 2008). Among these metal ions, Fe^{2+} was known as the most powerful pro-oxidant due to its high reactivity, which accelerated lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton reaction (Sun & Kennedy, 2010). In this study, ferrozine could react with Fe^{2+} to form red complexes of ferrozine-Fe^{2+}. When there was other chelating agent, the ferrozine-Fe^{2+} formation was disrupted which

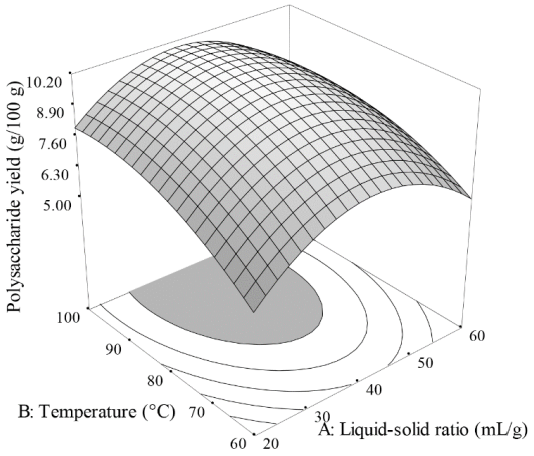

Figure 1. Response surface plot showing the effect of liquid-solid ratio and temperature on polysaccharides extraction from *A. auricula* fruit bodies. The time was constant at 3 h.

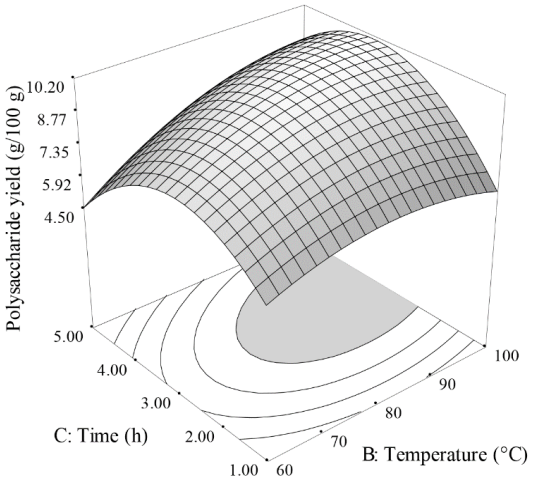

Figure 2. Response surface plot showing the effect of temperature and time on polysaccharides extraction from *A. auricula* fruit bodies. The liquid-solid ratio was constant at 40 mL/g.

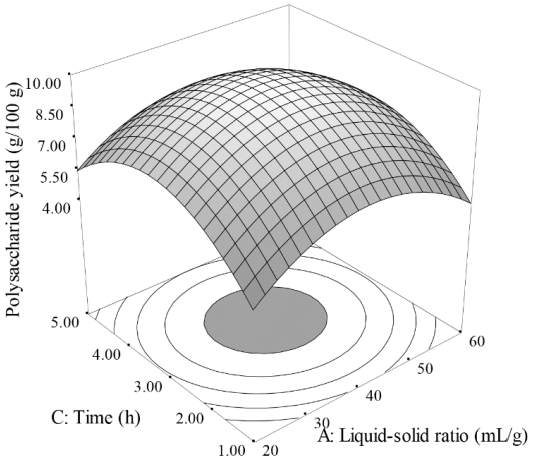

Figure 3. Response surface plot showing the effect of liquid-solid ratio and time on polysaccharides extraction from *A. auricula* fruit bodies. The temperature was constant at 80 °C.

resulted in decrease of red complexes. Fe^{2+}-chelating activity of antioxidant could be estimated by measuring absorbance of reaction solution at 562 nm.

The chelating activities of AAFB polysaccharides and BHT on Fe^{2+} are shown in Figure 4. AAFB polysaccharides possessed higher (p<0.05) Fe^{2+}-chelating activity than BHT in a concentration-dependent manner. IC_{50} value (0.43 mg/mL) of AAFB polysaccharides with Fe^{2+}-chelating activity was significant lower (p<0.05) than BHT (0.79 mg/mL). At an identical concentration, chelating activity of AAFB polysaccharides on Fe^{2+} was stronger compared to that of polysaccharides from four mushroom *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus* fruit bodies (Kozarski et al., 2011).

3.5 Hydroxyl radical scavenging activity of AAFB polysaccharides

Among all reactive oxygen radicals, hydroxyl radical was known as the most powerful radical. It could induce severe damage to adjacent biomolecules in the body, which result in cell damage that caused ageing, cancer and several other diseases (Yang et al., 2014). The removal of hydroxyl radical was probably one of the most effective ways to defense oxidative damage of human body. Therefore, hydroxyl radical scavenging activity was considered to be one of the most important antioxidant mechanisms.

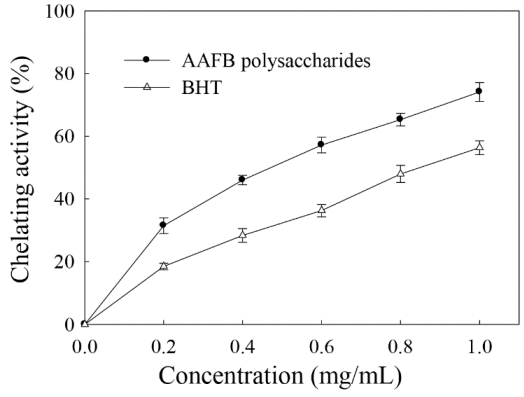

Figure 4. Chelating activity of AAFB polysaccharides on Fe^{2+}. Values are means ± SD of three independent determinations.

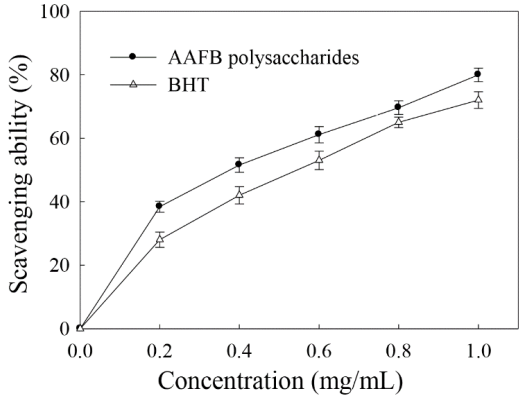

Figure 5. Scavenging activity of AAFB polysaccharides on hydroxyl radical. Values are means ± SD of three independent determinations.

The scavenging abilities of AAFB polysaccharides and BHT on hydroxyl radical are shown in Figure 5. With the increase of concentration, the scavenging abilities of AAFB polysaccharides and BHT on hydroxyl radical also increased. At the concentration range of 0.2-1.0 mg/mL, AAFB polysaccharides showed significantly stronger (p<0.05) scavenging activities than BHT. Meanwhile, IC_{50} value of AAFB polysaccharides was 0.38 mg/mL, which was significant lower (p<0.05) than BHT (0.56 mg/mL). At an identical concentration, scavenging activity of AAFB polysaccharides on hydroxyl radical was slightly stronger than that of polysaccharides from *Gloeostereum incarnatum* fruit bodies (Zhang et al., 2015). These results suggested that AAFB polysaccharides were better natural antioxidant than BHT in scavenging hydroxyl radical.

4 Conclusions

The extraction conditions of AAFB antioxidant polysaccharides were optimized by a three variable, three level Box-Behnken experiment design. Correlation analysis of the quadratic polynomial regression model indicated that the model could be employed to optimize conditions for polysaccharides extraction. The combination of liquid-solid ratio (38.77 mL/g), temperature (93.98 °C) and time (3.41 h) was determined to obtain the highest polysaccharide yield (10.46 g/100 g). The antioxidant activities of AAFB polysaccharides were evaluated by Fe^{2+}-chelating ability and hydroxyl radical scavenging assay. It exhibited stronger antioxidant activity compared to BHT. Results from this study indicated that AAFB polysaccharides could be potentially used as a natural antioxidant.

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