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Optimization of fermentation conditions for crude polysaccharides by *Morchella esculenta* using soybean curd residue

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Abstract: In this study, orthogonal experimental design and response surface methodology were employed to optimize the fermentation conditions for crude polysaccharides (MPS) production from the strain *Morchella esculenta* (*M.esculenta*) by soybean curd residue (SCR). The MPS yield varied depending on the nutrition contents added in SCR and fermentation time, fermentation temperature and inoculum size by *M.esculenta* during solid-state fermentation. The optimal fermentation conditions achieved for MPS production 95.82±1.37 mg/g were glucose 4%, $(NH_4)_2SO_4$ 1.5%, water 75% and $MgSO_4 \cdot 7H_2O$ 0.2%, fermentation temperature 22.6 $^{\circ}$C, fermentation time 21 days and inoculum size 2.67%, respectively. Furthermore, purified polysaccharides (PMPS) exhibited a positive antioxidant activity. The results provide a reference for large-scale production of polysaccharides by *M. esculenta* using SCR in the medical and food industries.

Keyword: Antioxidant activity, *Morchella esculenta*, Polysaccharides, Response surface methodology, Soybean curd residue

1. Introduction

Soybean curd residue (SCR), the byproduct of soybean curd and soymilk processing, is a porous and cheap available resource in Asian countries. Currently, SCR is used as stock feed, fertilizer or dumped in landfill (Wong et al., 2001). Particularly in Japan, about 800,000 tons of SCR are disposed of annually as by-products of tofu production. The expense for SCR disposal costs around 16, 000 million yen per annum (Muroyama et al., 2006). However, SCR is a relatively inexpensive material that is widely recognized for its high nutritional and excellent functional properties (Wang and Cavins, 1989; Rovaris et al., 2012).

Mushrooms have become attractive as functional foods, and a source of physiologically beneficial medicine recently (Mau et al., 2004). Polysaccharides from fruiting bodies, cultured mycelium or culture media have potential antitumor, immunomodulation and antioxidant properties (Ooi and Liu, 2000; Wasser, 2002; Masuda et al., 2009). For centuries, *M. esculenta* has been consumed and appreciated for its nutritional value as well as medicinal properties (Wahid et al., 1988). The crud polysaccharides isolated from *M. esculenta* mycelia have been proven to possess potential antioxidant properties (Elmastas et al., 2006). Currently, *M. esculenta* is highly valued in China, partially due to its biological activity, rareness and cultivation difficulty via traditional methods.

So far, there are no literature reports on the polysaccharides of *M. esculenta*, which use SCR as the main nutrient media. In this study, SCR was used as substrate in order to reduce the cost of polysaccharides production as well as the pollution brought

about by it. The objective of this study was to maximize MPS production, by optimizing the culture media, fermentation time, fermentation temperature and inoculum size. Then, the antioxidant activities in vitro of PMPS against 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, ferrous metal ions and the 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS radical cation) were investigated.

2. Materials and methods

2.1. Pre-treatment of SCR

Fresh SCR (80% moisture content) was obtained from Inamoto Co., Ltd., Tsukuba, Japan. The fresh SCR was dried at 60 °C, powdered and sieved through a NO. 60 mesh. In this study all SCR was obtained from the same batch in the manufacturing process.

2.2. Strain and culture media

The strain of *M. esculenta (ACCC 50764)* was obtained from the Agricultural Culture Collection of China, Beijing, China. The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were incubated at 25 °C for 10 days and then stored at 4 °C, and sub-cultured every 3 months. The culture was prepared with distilled water containing (g/L): glucose 20.0, potato extract 4.0, agar 20.0, KH_2PO_4 3.0, and $MgSO_4 \cdot 7H_2O$ 1.5.

2.3. Inoculum preparation

For preparation of the inoculum, the mycelia of *M. esculenta* were transferred from a slant into a sterile Petri dish (diameter: 100 mm) containing 20 mL of PDA. It was

incubated at 25 °C until mycelium permeated the culture dish. The 100 mL liquid culture was undertaken in a 300 mL flask containing ten units of mycelial agar 5 mm×5 mm square obtained using a self-designed cutter. The submerged cultivation was the same as PDA in the absence of the agar. Then it was put in a rotary shaker at 120 rpm and 25 °C for 7 days and used as a seed for solid-state fermentation. After SCR added different nutrition it was autoclaved at 121 °C for 15 min, the solid-state culture experiment was performed in a 200 mL flask. Three replications for all investigated factors were used.

2.4. Determination of crude polysaccharides

The fermented SCR from different cultured conditions was harvested and dried in a convection oven at 60 °C. MPS was assayed using phenol-sulfuric acid method (Shi et al., 2012). The result was expressed as mg of glucose equivalent per g of fermented SCR.

2.5. Experimental design

The content changing of culture media including glucose, $(NH_4)_2SO_4$, water and $MgSO_4 \cdot 7H_2O$ were optimized to improve MPS yield using orthogonal design. Every factor is matched with three levels and the orthogonal experiments design is shown in Table 1. During the culture media optimization process, fermentation temperature 25 °C, fermentation time 18 days and inoculum size 4% were used. Meanwhile the Box-Behnken design was applied to investigate the influence of fermentation temperature, fermentation time and inoculum size on the yield of MPS. Levels and codes of variables in the Box-Behnken design are shown in Table 2.

2.6. Extraction and purification of polysaccharides

In order to compare the antioxidant activity of polysaccharides before and after fermentation, the purified polysaccharides from unfermented SCR (PUPS) and PMPS were extracted according to Meng et al (2010) with some modifications. Briefly, unfermented and fermented SCR were ground in a sample mill to pass through NO. 60 mesh after oven drying for 4 days at 60 °C. The powdered material was refluxed in 80% ethanol for 6 h to remove some colored materials, monosaccharides, oligosaccharides, and small molecule materials. Then the cooled extract was discarded and the residue was washed with 95% ethanol, anhydrous ethyl alcohol, acetone and diethyl ether respectively. The residue was dried at room temperature for 24 h prior to extraction. Subsequently, the extraction was carried out using boiling water for 2 h. After that, the syrup was centrifuged at 7500×g for 15 min and the residue was re-extracted under the same conditions. The combined supernatant fluids were concentrated to minimum volume using a rotary evaporator at 60 °C under low pressure. The protein in the concentrated solution was removed by Sevag reagent (chloroform and n-butanol in 4:1 ratio) (Staub, 1965). The extract was dialyzed by the deionized water for 72 h. To obtain the purified polysaccharides, the extract was precipitated with 4 volumes of anhydrous ethanol at 4 °C for overnight and the precipitation was centrifuged at 7500×g for 15 min. The precipitate was dissolved in distilled water, collected, frozen and freeze-dried, then the PMPS and PUPS was obtained to study the antioxidant activities.

2.7. Assay for antioxidant activities

130 *2.7.1. Radical scavenging activity on DPPH*

131 Radical scavenging activities on DPPH were evaluated using the method described
132 by Blois (2002) with a slight modification. Aliquots (0.5 mL) of various
133 concentrations (0.156-10.00 mg/mL) of PMPS and PUPS were mixed with 2 mL (25
134 μg/mL) of a MeOH solution of DPPH. Then the mixture was shaken vigorously and
135 allowed to stand in the dark for 30 min. The absorbance was measured at 517 nm
136 against a blank. Decrease in the DPPH solution absorbance indicated an increase of
137 the DPPH radical-scavenging activity. Ascorbic acid was used as the positive control.
138 The radical scavenging activity on DPPH was calculated according to the following
139 equation:

140 $$\text{Scavenging activity (\%)} = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100 \quad (1)$$

141 where A_1 was the absorbance with the presence of DPPH and sample; A_0, with the
142 presence of DPPH but without sample; and A_2, with the presence of sample but
143 without DPPH.

144 *2.7.2. Hydroxyl free radical scavenging activity*

145 Hydroxyl free radical scavenging activity was measured according to a literature
146 procedure with a few modifications (Nicholas et al., 1989). Hydroxyl free radicals
147 were generated from $FeSO_4$ and H_2O_2, and detected by their ability to hydroxylate
148 salicylate. The reaction mixture (2.5 mL) contained 0.5 mL $FeSO_4$ (1.5 mM), 0.35 mL
149 of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1 mL of different
150 concentrations of PMPS. Ascorbic acid was used as the positive control. After
151 incubation for 1 h at 37 °C, the absorbance of the hydroxylated salicylate complex

was measured at 562 nm. The percentage scavenging effect was calculated as:

$$\text{Scavenging activity}(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100 \quad (2)$$

where A_0 was the absorbance of the solvent control, A_1 was the absorbance of the sample or ascorbic acid, whereas A_2 was the absorbance of the reagent blank without sodium salicylate.

2.7.3. Ferrous metal ions chelating activity

Ferrous metal ions chelating activity of PMPS was measured according to a literature procedure with a few modifications (Yuan et al., 2008). A sample of ethylenediaminetetraacetic acid (EDTA) solution (1 mL) were mixed with 50 μL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM), shaken well, settled for 10 min at room temperature, and the absorbance of the mixture was determine d at 562 nm. EDTA was included as the positive control. The ion chelating activity was calculated as:

$$\text{Chelating activity}(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\% \quad (3)$$

where A_0 was the absorbance of the control (without sample), A_1 was the absorbance in the presence of the sample and A_2 was the absorbance without ferrozine.

2.7.4. ABTS radical scavenging activity

ABTS assay was based on the method of Re et al. (1999). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of

173 0.70 ± 0.02 at 734 nm.

174 Then 0.15 mL of various concentration of the sample (0.156-10.00 mg/mL) was

175 mixed with 2.85 mL of $ABTS^{\bullet+}$ solution. Finally, the absorbance was measured at

176 734 nm after incubation at room temperature for 10 min. The scavenging activity of

177 ABTS free radical was calculated using the following equation:

178 $$\text{Scavenging activity (\%)} = (1 - \frac{A_1}{A_0}) \times 100 \quad (4)$$

179 where A_0 is the absorbance of control without sample and A_1 is the test sample

180 without $ABTS^{\bullet+}$.

181 *2.8. Statistical analysis*

182 All experiments were carried out in triplicate. Data were processed and analyzed

183 using Design Expert Software (version 8.0.6, Stat-Ease. Inc., Minneapolis, USA) and

184 Data Processing System (version 7.05 Fujitsu Ltd). P-values below 0.01 were

185 regarded as statistically significant.

186 **3. Results and discussion**

187 *3.1. Culture media optimization*

188 Based on the results of single-factor experiment, glucose, $(NH_4)_2SO_4$, water and

189 $MgSO_4 \cdot 7H_2O$ added in SCR were selected and applied to optimize the culture media

190 composition using orthogonal experimental design. The design of the

191 four-factor-three-level orthogonal experiment and the results are described in Table 1.

192 As shown in Table 1, all these substrates showed significant influence on MPS

193 content ($P < 0.01$), and the four factors affecting MPS content in descending order are:

194 water, glucose, $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$. The optimal culture media added in

SCR was obtained as follows (%): glucose 4.0, $(NH_4)_2SO_4$ 1.5, water 75.0 and $MgSO_4 \cdot 7H_2O$ 0.2. Using the optimal culture media, the verifying experiment indicated that the yield of MPS was 87.36±1.73 mg/g under the following conditions, fermentation temperature 25 °C, fermentation time 18 days and inoculum size 4%.

3.2. Optimization of the culture conditions

Response surface methodology was used to establish the relationship between the variables with the obtained responses. According to single factor analysis, MPS yield varied depending on the fermentation conditions, including fermentation temperature, inoculum size and fermentation time. The MPS yield was taken as the response value, a Box-Behnken design with factors of the fermentation temperature (X_1), fermentation time (X_2) and inoculum size (X_3) at three levels were considered. The experimental design including name, symbol code, and actual level of the variables are shown in Tables 2 and 3. The test factors were coded according to the following equation (5):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (5)$$

where x_i is the coded value of the *i*th independent variable, X_i is the uncode value of the *i*th independent variable, X_0 is the uncoded value of the *i*th independent variable at the centre point and ΔX_i is the step change value.

According to multiple regression analysis of the experimental data, the independent variables and the dependent variables were related by the following second-order polynomial equation (6):

$$Y=-1647.98+26.49X_1+6.97X_2+1026.36X_3+0.14X_1X_2+5.76X_2X_3-0.66X_1^2-0.59X_2^2$$

$-215.55X_3^2$ (6)

where Y is the predicted response, that is the polysaccharides yield (mg/g) and X_1, X_2 and X_3 are the uncoded values of the test variables, fermentation temperature (°C), time (days) and inoculum size (%), respectively. The statistical significance of Eq. (6) was checked by F test, and the analysis of variance for response surface quadratic model is summarized in Table 4. The adjusted determination coefficients (R^2Adj) were measured for testing the goodness-of-fit of the regression equations. The value of (R^2Adj) for this equation was 0.9726 as shown in Table 4, which indicated a high degree of correlation between the experimental and predicted values.

The 3D response surface plots are employed to determine the interaction of the fermentation conditions and the optimum levels that have the most significant effect on MPS production. The response surfaces plots based on the model are depicted in Fig. 1, which shows the interactions between two variables by keeping the other variable at zero level for MPS production. It is clear from Fig.1 (a) that yield of MPS increased and later decreased with the increase in time and temperature. When inoculum size was fixed at 2.60% level, fermentation time and fermentation temperature displayed a quadratic effect on MPS yield. Fig. 1 (b) demonstrates the effects of temperature and inoculum size on MPS production. It was observed that the MPS production varied significantly with the variation of temperature. It is evident that MPS production significantly increased with increasing temperature up to about 22 °C but decreased sharply beyond this, reaching its maximum yield at 22 °C - 23 °C. However, the effect of inoculum size on the production of MPS is insensitive within

239 the tested range. MPS yield increased gradually when inoculums size increased. As
240 can be seen from Fig. 1(c), the MPS yield was significantly affected by fermentation
241 time. It increased when time increased up to 21 days and decreased sharply beyond
242 this. This observation can be attributed to the autolysis of mycelia as time increases.
243 However, the effect of inoculum size was also insensitive compared with time. The
244 optimum ranges of fermentation time and inoculum size for the maximum yield of
245 MPS lies between 20 days - 22 days, and 2.60% - 2.70%, respectively.

246 By solving the inverse matrix using Design Expert software, the optimum values of
247 the test variables in uncoded units were obtained, i.e. fermentation temperature
248 22.6 $^{\circ}$C, fermentation time 21 days, and inoculum size 2.67%, respectively. The
249 predicted optimal MPS production corresponding to these values was 96.10 mg/g. In
250 order to validate the suitability of the model equations for predicting optimum
251 response values, a group of verification experiments were carried under the optimum
252 condition predicted respectively for highest yield. These triplicate experiments
253 produced MPS yield of 95.82±1.37 mg/g, confirming the good fit between the
254 predicted and experimental values and also the validity of the model. As a result, the
255 models developed were considered to be accurate and reliable for predicting the
256 production of MPS using SCR as main nutrient medium.

257 *3.3. Evaluation of antioxidant activity*

258 The material with antioxidant activity may fight inflammation, neutralize the free
259 radicals that damage cells and can prematurely age, which plays an important role in
260 body's health (Tehranifar et al., 2011). After purification, the extraction rate of PMPS

and PUPS were 9.03% and 2.44%, respectively. To compare the antioxidant activity of the PUPS and PMPS, as the main index of antioxidant activities in vivo, several methods have been used for the determination of the antioxidant activities such as ABTS assay, DPPH test, hydroxyl radical scavenging activities and ferrous metal ions chelating activity method.

DPPH is a free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares, et al., 1997). The effect of polysaccharides on DPPH free radical scavenging activity was believed to be due to their hydrogen donating ability (Chen et al., 2008). The results of DPPH free radical scavenging activity of the PUPS and PMPS are shown in Fig. 2 (a) and compared with ascorbic acid (Vc) as control standard. As can be seen from Fig. 2 (a), the DPPH radical scavenging activity increased from 11.96% to 93.94%, when the concentration of the PMPS increased from 0.15 to 10 mg/mL. While the PUPS concentration increased from 0.156 mg/mL to 10 mg/mL, the DPPH radical scavenging ration increased from 12.69% to 27.51%. Compared with PUPS, the results indicated that PMPS had significant DPPH radical scavenging activity.

Hydroxyl radical removal is important for the protection of living systems. It can damage virtually all types of macromolecules in our body such as carbohydrates, nucleic acids, lipids and amino acids, which makes it a very dangerous compound to an organism (Gulcin, 2006; Ke et al., 2009). Therefore, it is important to discover chemicals with good scavenging capacity for these reactive oxygen species. The hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant

activity (Babu et al., 2001). Fig. 2 (b) depicts the scavenging activity of a hydroxyl radical. The scavenging ratio of PMPS and PUPS correlated well with increasing concentrations, increasing from 16.57% to 100%, 6.58% to 42.13% when the concentration increased from 0.156 mg/mL to 10 mg/mL. The scavenging activity of PMPS was lower than Vc, but still higher than that of PUPS. The results indicated that PMPS exhibits strong antioxidant effects than PUPS on hydroxyl radical activity.

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Katalinic et al., 2006; Huang et al., 2008). In our experiment, the scavenging ability of the PMPS and PUPS on ABTS free radical is shown in Fig. 2 (c). The PMPS and PUPS were found to have the ability to scavenge hydroxyl radicals at concentrations between 0.156 mg/mL and 10 mg/mL compared to the same concentration of Vc. PMPS had a higher scavenging effect for hydroxyl radicals than PUPS. Their scavenging powers correlated well with increasing concentrations, but were significantly lower than ascorbic acid when the concentration was below 5.0 mg/mL.

Chelation of metal ions has an antioxidant effect because the transition metals iron and copper promote oxidative damage at different levels (Saiga et al., 2003). As shown in Fig. 2 (d), the metal chelating activity of PMPS and PUPS increased with increasing concentrations used in the test. Compared with EDTA, the chelating activity of the samples on ferrous ion was weaker when the concentration was below 5.0 mg/mL. The result showed that PUPS had negligible Fe^{2+} chelating activity, and the maximal chelating activities of PMPS and PUPS were 95.94% and 27.51% at 10

mg/mL, respectively.

4. Conclusions

In this study, the effects of culture media and fermentation conditions on the yield of MPS were investigated for the first time. The optimal fermentation conditions achieved for MPS production 95.82±1.37 mg/g were glucose 4.0%, $(NH_4)_2SO_4$ 1.5%, water 75.0% and $MgSO_4 \cdot 7H_2O$ 0.2%, fermentation temperature 22.6 $^{\circ}$C, fermentation time 21.0 days, and inoculum size 2.67%. The obtained PMPS demonstrated greater positive antioxidant activities than PUPS. The results will provide references for the large-scale production of polysaccharides by *M.esculenta* and point to a new direction for the utilization of SCR. This affords a theoretical foundation for low-cost production of polysaccharides on an industrial scale. Further purification and characterization of the polysaccharides are necessary to investigate the function and structure of polysaccharides from the fermentation media of medicinal mushroom. In addition, it is necessary to establish the relationship between the function and structure of the polysaccharides and expand their application.

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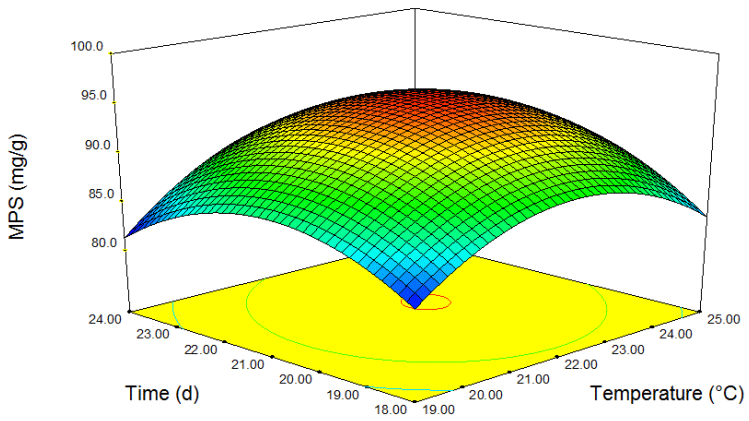
383 a



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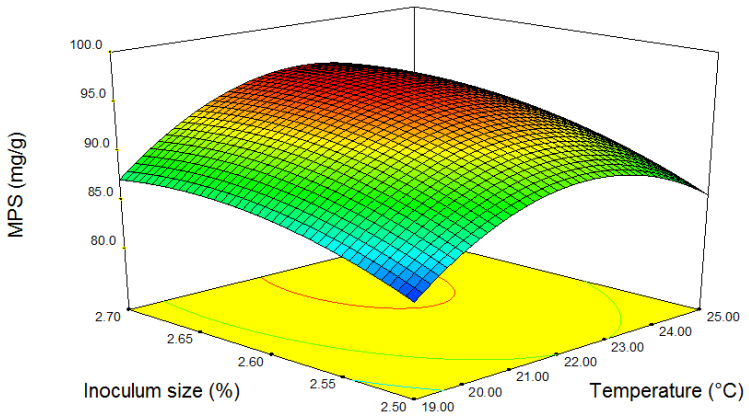

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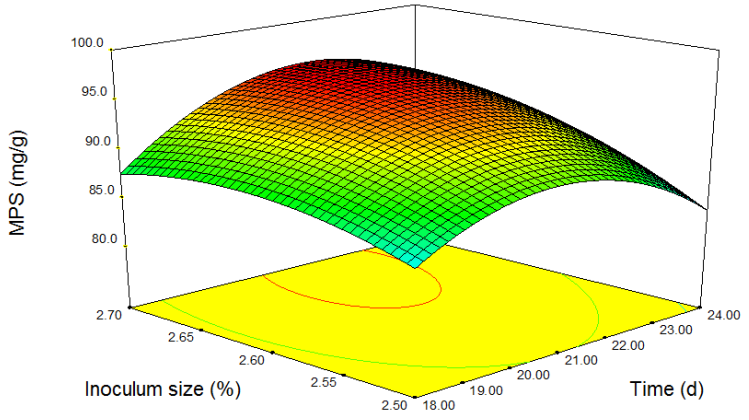

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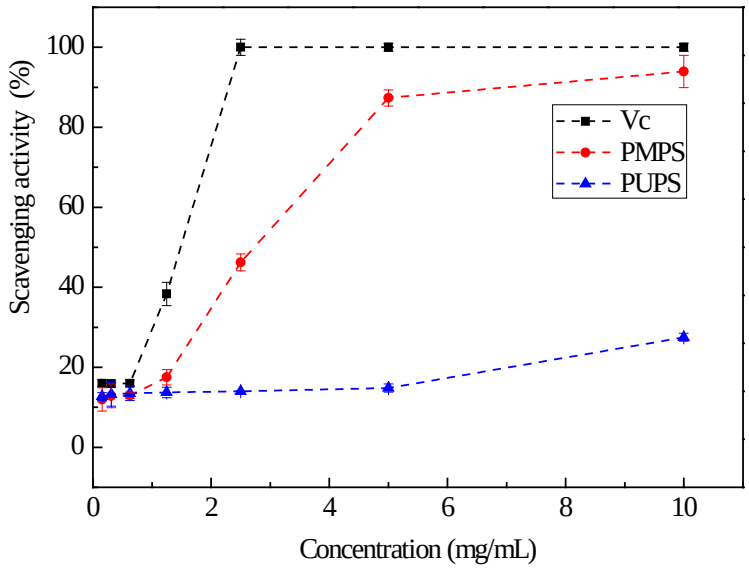
391 Fig. 1. Response surface plot for the MPS yield in terms of the effects of (a) time and temperature, (b)

392 temperature and inoculum size, and (c) inoculum size and time.

a



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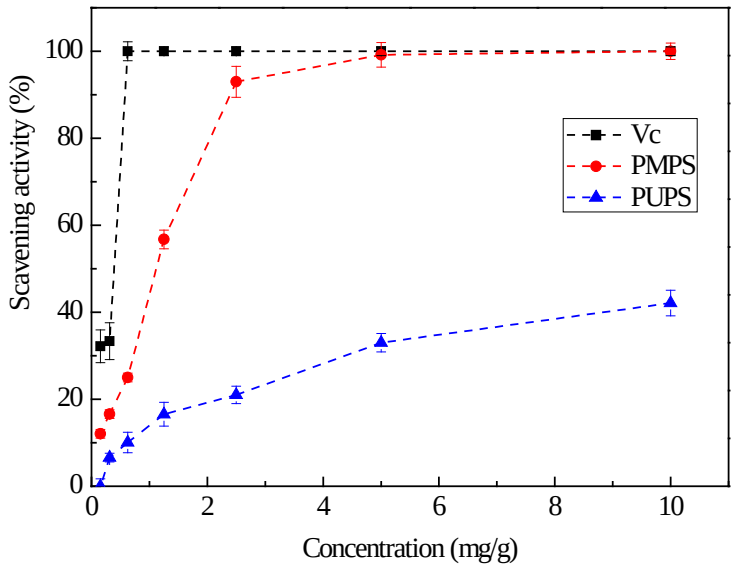

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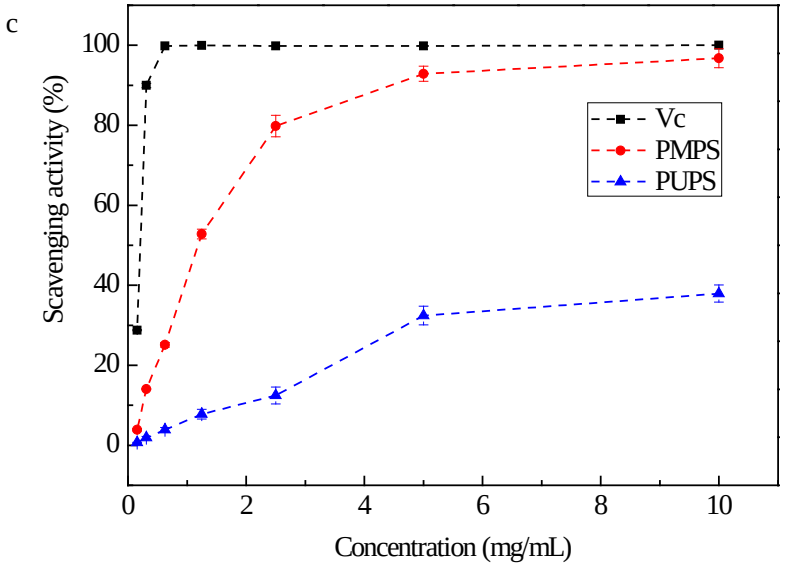

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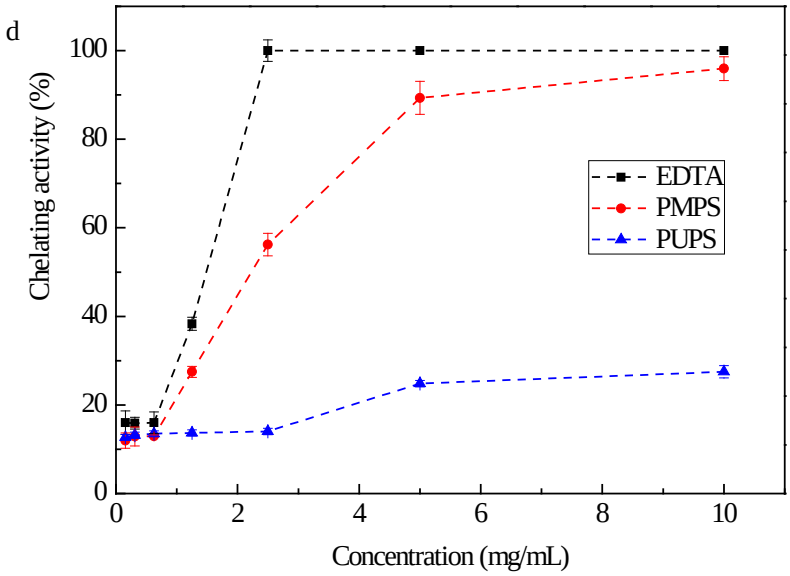

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402 **Fig. 2.** Antioxidant activities of PMPS and PUPS. (a) Scavenging activity of the PMPS and PUPS

403 on DPPH radical. (b) Scavenging activity of PMPS and PUPS on hydroxyl radical. (c) Scavenging

404 activity of the PMPS and PUPS on ABTS radical. (d) Chelating activity of the PMPS and PUPS.

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409 **Table 1** Results of orthogonal experiments for media optimization.

NO.	Glucose (%)	$(NH_4)_2SO_4$ (%)	Water (%)	$MgSO_4 \cdot 7H_2O$ (%)	MPS content (mg/g)
1	2	0.05	65	0.1	64.94±2.05
2	2	0.10	70	0.2	76.48±1.28
3	2	0.15	75	0.3	83.64±0.71
4	4	0.05	70	0.3	78.30±1.74
5	4	0.10	75	0.1	88.99±0.69
6	4	0.15	65	0.2	81.84±1.59
7	6	0.05	75	0.2	84.89±1.08
8	6	0.10	65	0.3	76.42±0.87
9	6	0.15	70	0.1	83.00±0.56
K_1	225.06	228.13	223.20	236.93	
K_2	249.13	241.88	237.78	243.21	
K_3	244.31	248.48	257.52	238.36	
R	24.07	20.35	34.33	6.28	
P	0.0001^{**}	0.0001^{**}	0.0001^{**}	0.0032^{**}	

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Table 2 Levels and codes of variables in the Box-Behnken design.

Variables	Symbol		Coded and uncode levels		
	Uncoded	Coded	-1	0	1
Fermentation temperature (°C)	X_1	x_1	19	22	25
Fermentation time (d)	X_2	x_2	18	21	24
Inoculum size (%)	X_3	x_3	2.5	2.6	2.7

Table 3 Experimental and predicted values of polysaccharides based on Box-Behnken design.

Runs	X_1: Fermentation temperature (°C)	X_2:Fermetation time (d)	X_3: Inoculum size (%)	Polysaccharides content (mg/g)	
				Experimental	Predict
1	22	18	2.5	86.55±0.79	85.76
2	19	24	2.6	82.19±1.50	81.31
3	22	21	2.6	94.23±0.66	94.91
4	22	24	2.5	83.28±1.04	83.81
5	25	18	2.6	82.66±1.99	83.54
6	25	24	2.6	88.03±0.68	87.59
7	22	24	2.7	91.79±0.68	92.58
8	22	18	2.7	88.14±0.79	87.61
9	22	21	2.6	95.23±0.57	94.91
10	22	21	2.6	94.99±1.65	94.91
11	19	21	2.5	82.43±1.76	82.34
12	25	21	2.5	85.72±0.29	86.07
13	22	21	2.6	94.57±2.13	94.91
14	19	21	2.7	87.12±0.29	87.65
15	19	18	2.6	81.90±2.41	82.35
16	22	21	2.6	95.54±1.42	94.91
17	25	21	2.7	92.17±2.00	91.38

The experimental results were means ± standard deviation (SD) of triple determinations.

435 **Table 4** The result of analysis of variance (ANOVA) for the selected model.

Source	Sum of squares	Degree of freedom	Mean square	F- value	Probability＞F
Model	420.11	8	52.51	71.99	< 0.0001
X_1	27.87	1	27.87	38.20	0.0003
X_2	4.57	1	4.57	6.26	0.0368
X_3	56.38	1	56.38	77.29	< 0.0001
X_1X_2	6.46	1	6.46	8.86	0.0177
X_2X_3	11.96	1	11.96	16.39	0.0037
X_1^2	146.46	1	146.46	200.78	< 0.0001
X_2^2	119.04	1	119.04	163.19	< 0.0001
X_3^2	19.56	1	19.56	26.82	0.0008
Residual	5.84	8	0.73		
Lack of fit	4.76	4	1.19	4.41	0.0899
Pure error	1.08	4	0.27		
Corrected total	425.94	16			
R=0.9863	R^2Adj= 0.9726				

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