

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

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20 **Abstract:** In this study, orthogonal experimental design and response surface
21 methodology were employed to optimize the fermentation conditions for crude
22 polysaccharides (MPS) production from the strain *Morchella esculenta* (*M.esculenta*)
23 by soybean curd residue (SCR). The MPS yield varied depending on the nutrition
24 contents added in SCR and fermentation time, fermentation temperature and inoculum
25 size by *M.esculenta* during solid-state fermentation. The optimal fermentation
26 conditions achieved for MPS production 95.82 ± 1.37 mg/g were glucose 4%,
27 $(\text{NH}_4)_2\text{SO}_4$ 1.5%, water 75% and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.2%, fermentation temperature
28 22.6°C , fermentation time 21 days and inoculum size 2.67%, respectively.
29 Furthermore, purified polysaccharides (PMPS) exhibited a positive antioxidant
30 activity. The results provide a reference for large-scale production of polysaccharides
31 by *M. esculenta* using SCR in the medical and food industries.

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

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42 **1. Introduction**

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

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

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

64 about by it. The objective of this study was to maximize MPS production, by
65 optimizing the culture media, fermentation time, fermentation temperature and
66 inoculum size. Then, the antioxidant activities in vitro of PMPS against 1,
67 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical, ferrous metal ions and the 2,
68 2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS radical cation) were
69 investigated.

70 **2. Materials and methods**

71 *2.1. Pre-treatment of SCR*

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

76 *2.2. Strain and culture media*

77 The strain of *M. esculenta* (ACCC 50764) was obtained from the Agricultural
78 Culture Collection of China, Beijing, China. The stock culture was maintained on
79 potato dextrose agar (PDA) slants. The slants were incubated at 25 °C for 10 days and
80 then stored at 4 °C, and sub-cultured every 3 months. The culture was prepared with
81 distilled water containing (g/L): glucose 20.0, potato extract 4.0, agar 20.0, KH₂PO₄
82 3.0, and MgSO₄·7H₂O 1.5.

83 *2.3. Inoculum preparation*

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

86 incubated at 25 °C until mycelium permeated the culture dish. The 100 mL liquid
87 culture was undertaken in a 300 mL flask containing ten units of mycelial agar 5
88 mm×5 mm square obtained using a self-designed cutter. The submerged cultivation
89 was the same as PDA in the absence of the agar. Then it was put in a rotary shaker at
90 120 rpm and 25 °C for 7 days and used as a seed for solid-state fermentation. After
91 SCR added different nutrition it was autoclaved at 121 °C for 15 min, the solid-state
92 culture experiment was performed in a 200 mL flask. Three replications for all
93 investigated factors were used.

94 *2.4. Determination of crude polysaccharides*

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

99 *2.5. Experimental design*

100 The content changing of culture media including glucose, (NH₄)₂SO₄, water and
101 MgSO₄·7H₂O were optimized to improve MPS yield using orthogonal design. Every
102 factor is matched with three levels and the orthogonal experiments design is shown in
103 Table 1. During the culture media optimization process, fermentation temperature
104 25 °C, fermentation time 18 days and inoculum size 4% were used. Meanwhile the
105 Box-Behnken design was applied to investigate the influence of fermentation
106 temperature, fermentation time and inoculum size on the yield of MPS. Levels and
107 codes of variables in the Box-Behnken design are shown in Table 2.

108 *2.6. Extraction and purification of polysaccharides*

109 In order to compare the antioxidant activity of polysaccharides before and after
110 fermentation, the purified polysaccharides from unfermented SCR (PUPS) and PMPS
111 were extracted according to Meng et al (2010) with some modifications. Briefly,
112 unfermented and fermented SCR were ground in a sample mill to pass through NO.
113 60 mesh after oven drying for 4 days at 60 °C. The powdered material was refluxed in
114 80% ethanol for 6 h to remove some colored materials, monosaccharides,
115 oligosaccharides, and small molecule materials. Then the cooled extract was
116 discarded and the residue was washed with 95% ethanol, anhydrous ethyl alcohol,
117 acetone and diethyl ether respectively. The residue was dried at room temperature for
118 24 h prior to extraction. Subsequently, the extraction was carried out using boiling
119 water for 2 h. After that, the syrup was centrifuged at 7500×g for 15 min and the
120 residue was re-extracted under the same conditions. The combined supernatant fluids
121 were concentrated to minimum volume using a rotary evaporator at 60 °C under low
122 pressure. The protein in the concentrated solution was removed by Sevag reagent
123 (chloroform and n-butanol in 4:1 ratio) (Staub, 1965). The extract was dialyzed by the
124 deionized water for 72 h. To obtain the purified polysaccharides, the extract was
125 precipitated with 4 volumes of anhydrous ethanol at 4 °C for overnight and the
126 precipitation was centrifuged at 7500×g for 15 min. The precipitate was dissolved in
127 distilled water, collected, frozen and freeze-dried, then the PMPS and PUPS was
128 obtained to study the antioxidant activities.

129 *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:

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

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

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

153
$$\text{Scavenging activity (\%)} = \left(1 - \frac{A_1 - A_2}{A_0} \right) \times 100 \quad (2)$$

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

157 2.7.3. Ferrous metal ions chelating activity

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

165
$$\text{Chelating activity (\%)} = \left(1 - \frac{A_1 - A_2}{A_0} \right) \times 100\% \quad (3)$$

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

168 2.7.4. ABTS radical scavenging activity

169 ABTS assay was based on the method of Re et al. (1999). ABTS radical cation
170 ($\text{ABTS}^{\cdot+}$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium
171 persulphate and allowing the mixture to stand in the dark at room temperature for
172 12-16 h before use. The $\text{ABTS}^{\cdot+}$ 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^{•+} 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:

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

179 where A_0 is the absorbance of control without sample and A_1 is the test sample
180 without ABTS^{•+}.

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, (NH₄)₂SO₄, water and
189 MgSO₄·7H₂O 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, (NH₄)₂SO₄ and MgSO₄·7H₂O. The optimal culture media added in

195 SCR was obtained as follows (%): glucose 4.0, (NH₄)₂SO₄ 1.5, water 75.0 and
196 MgSO₄·7H₂O 0.2. Using the optimal culture media, the verifying experiment
197 indicated that the yield of MPS was 87.36±1.73 mg/g under the following conditions,
198 fermentation temperature 25 °C, fermentation time 18 days and inoculum size 4%.

199 3.2. Optimization of the culture conditions

200 Response surface methodology was used to establish the relationship between the
201 variables with the obtained responses. According to single factor analysis, MPS yield
202 varied depending on the fermentation conditions, including fermentation temperature,
203 inoculum size and fermentation time. The MPS yield was taken as the response value,
204 a Box-Behnken design with factors of the fermentation temperature (X₁),
205 fermentation time (X₂) and inoculum size (X₃) at three levels were considered. The
206 experimental design including name, symbol code, and actual level of the variables
207 are shown in Tables 2 and 3. The test factors were coded according to the following
208 equation (5):

$$209 \quad x_i = \frac{X_i - X_0}{\Delta X_i} \quad (5)$$

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

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

$$216 \quad Y = -1647.98 + 26.49X_1 + 6.97X_2 + 1026.36X_3 + 0.14X_1X_2 + 5.76X_2X_3 - 0.66X_1^2 - 0.59X_2^2$$

217 $-215.55X_3^2$ (6)

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

226 The 3D response surface plots are employed to determine the interaction of the
227 fermentation conditions and the optimum levels that have the most significant effect
228 on MPS production. The response surfaces plots based on the model are depicted in
229 Fig. 1, which shows the interactions between two variables by keeping the other
230 variable at zero level for MPS production. It is clear from Fig.1 (a) that yield of MPS
231 increased and later decreased with the increase in time and temperature. When
232 inoculum size was fixed at 2.60% level, fermentation time and fermentation
233 temperature displayed a quadratic effect on MPS yield. Fig. 1 (b) demonstrates the
234 effects of temperature and inoculum size on MPS production. It was observed that the
235 MPS production varied significantly with the variation of temperature. It is evident
236 that MPS production significantly increased with increasing temperature up to about
237 22°C but decreased sharply beyond this, reaching its maximum yield at 22°C - 23°C .
238 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 °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

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

266 DPPH is a free radical that accepts an electron or hydrogen radical to become a
267 stable diamagnetic molecule (Soares, et al., 1997). The effect of polysaccharides on
268 DPPH free radical scavenging activity was believed to be due to their hydrogen
269 donating ability (Chen et al., 2008). The results of DPPH free radical scavenging
270 activity of the PUPS and PMPS are shown in Fig. 2 (a) and compared with ascorbic
271 acid (Vc) as control standard. As can be seen from Fig. 2 (a), the DPPH radical
272 scavenging activity increased from 11.96% to 93.94%, when the concentration of the
273 PMPS increased from 0.15 to 10 mg/mL. While the PUPS concentration increased
274 from 0.156 mg/mL to 10 mg/mL, the DPPH radical scavenging ration increased from
275 12.69% to 27.51%. Compared with PUPS, the results indicated that PMPS had
276 significant DPPH radical scavenging activity.

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

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

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

298 Chelation of metal ions has an antioxidant effect because the transition metals iron
299 and copper promote oxidative damage at different levels (Saiga et al., 2003). As
300 shown in Fig. 2 (d), the metal chelating activity of PMPS and PUPS increased with
301 increasing concentrations used in the test. Compared with EDTA, the chelating
302 activity of the samples on ferrous ion was weaker when the concentration was below
303 5.0 mg/mL. The result showed that PUPS had negligible Fe^{2+} chelating activity, and
304 the maximal chelating activities of PMPS and PUPS were 95.94% and 27.51% at 10

305 mg/mL, respectively.

306 **4. Conclusions**

307 In this study, the effects of culture media and fermentation conditions on the yield
308 of MPS were investigated for the first time. The optimal fermentation conditions
309 achieved for MPS production 95.82 ± 1.37 mg/g were glucose 4.0%, $(\text{NH}_4)_2\text{SO}_4$ 1.5%,
310 water 75.0% and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2%, fermentation temperature 22.6°C , fermentation
311 time 21.0 days, and inoculum size 2.67%. The obtained PMPS demonstrated greater
312 positive antioxidant activities than PUPS. The results will provide references for the
313 large-scale production of polysaccharides by *M.esculenta* and point to a new direction
314 for the utilization of SCR. This affords a theoretical foundation for low-cost
315 production of polysaccharides on an industrial scale. Further purification and
316 characterization of the polysaccharides are necessary to investigate the function and
317 structure of polysaccharides from the fermentation media of medicinal mushroom. In
318 addition, it is necessary to establish the relationship between the function and
319 structure of the polysaccharides and expand their application.

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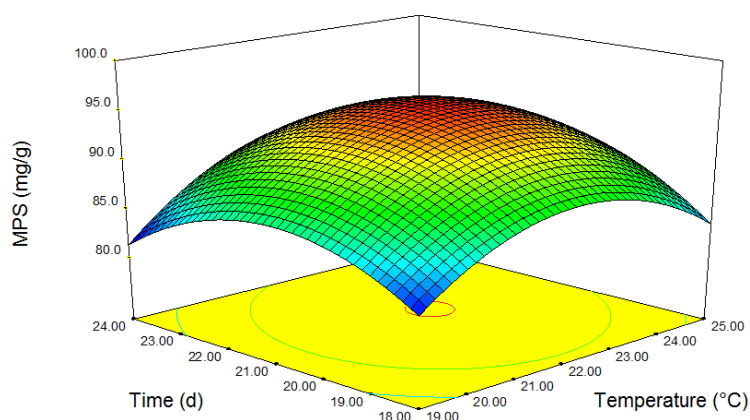
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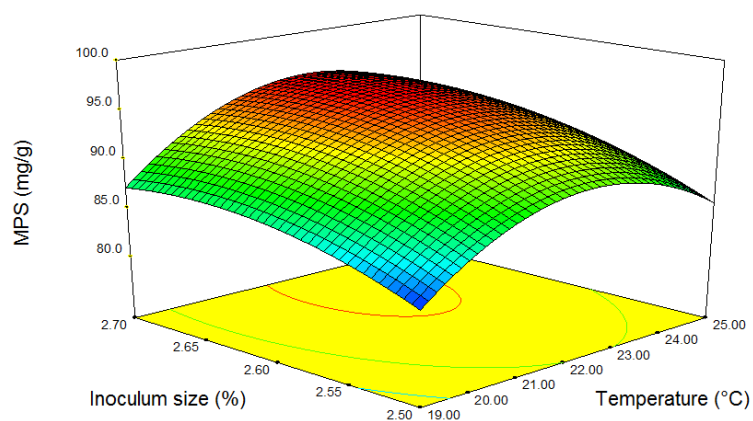


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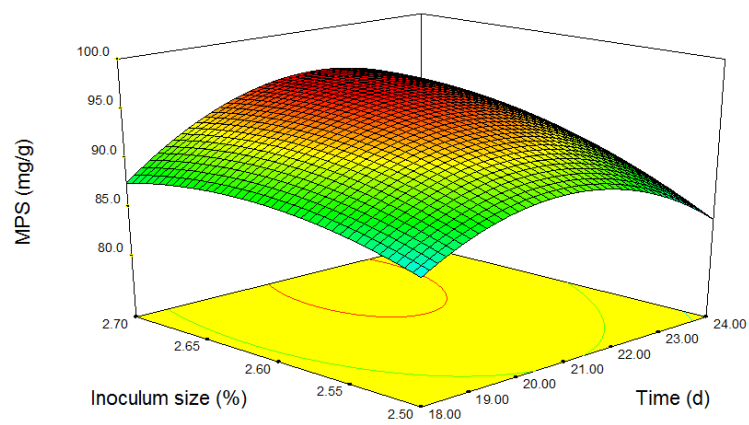
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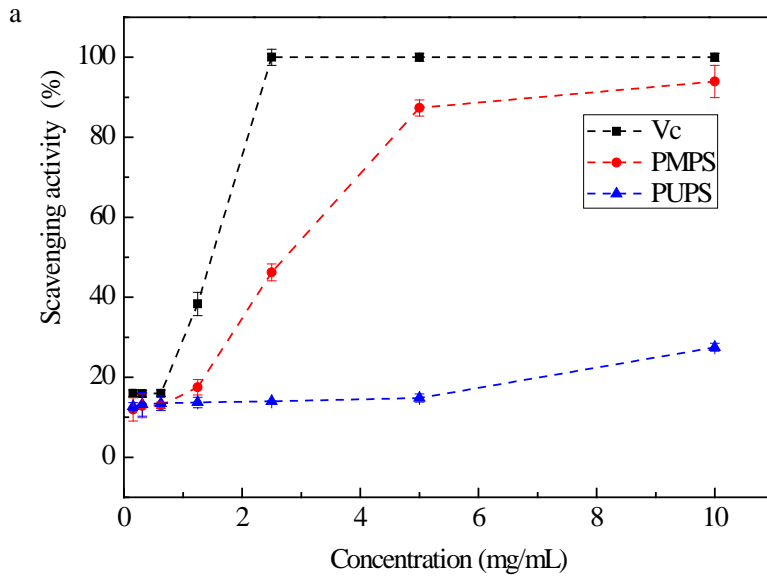
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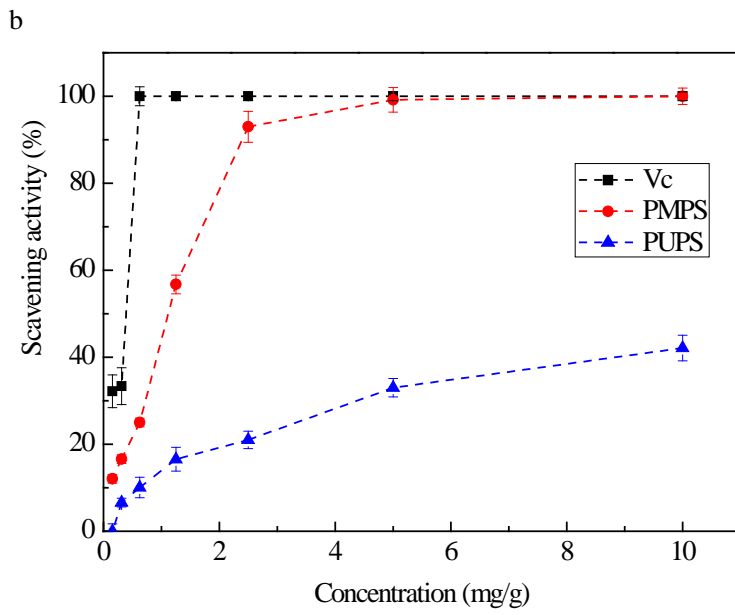
391 Fig. 1. Response surface plot for the MPS yield in terms of the effects of (a) time and temperature, (b)

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



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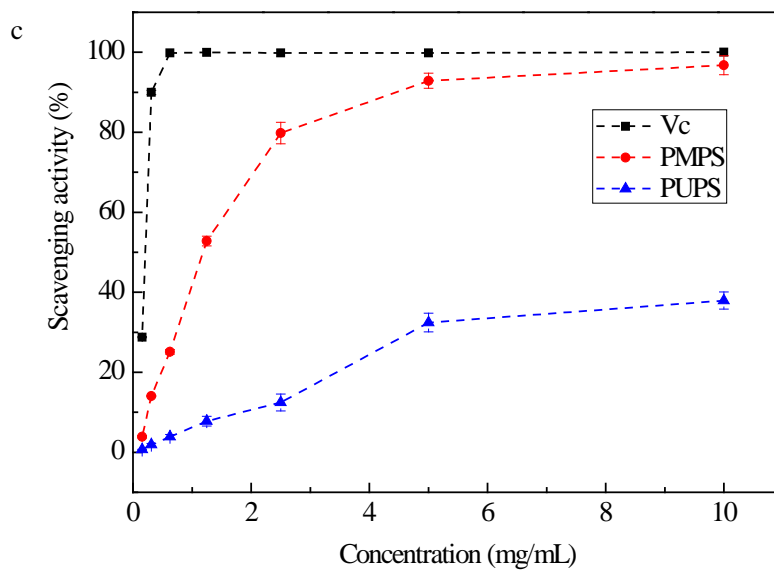
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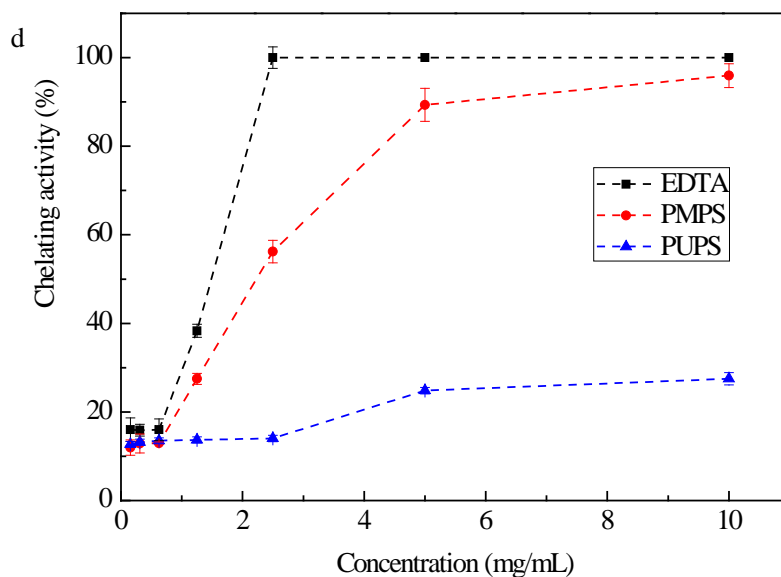
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402 **Fig. 2.** Antioxidant activities of PMPS and PUPS. (a) Scavenging activity of the PMPS and PUPS

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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Table 1 Results of orthogonal experiments for media optimization.

NO.	Glucose (%)	(NH ₄) ₂ SO ₄ (%)	Water (%)	MgSO ₄ ·7H ₂ O (%)	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

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

Runs	X ₁ : Fermentation	X ₂ : Fermentation	X ₃ : Inoculum	Polysaccharides content (mg/g)	
	temperature (°C)	time (d)	size (%)	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

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

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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 ₁ X ₂	6.46	1	6.46	8.86	0.0177
X ₂ X 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 ² Adj= 0.9726					

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