# 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

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 $^{\circ}$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 $^{\circ}$C for 10 days and

80 then stored at 4 $^{\circ}$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, $KH_2PO_4$

82 3.0, and $MgSO_4 \cdot 7H_2O$ 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 $^{\circ}$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 $^{\circ}$C for 7 days and used as a seed for solid-state fermentation. After

91　SCR added different nutrition it was autoclaved at 121 $^{\circ}$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 $^{\circ}$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, $(NH_4)_2SO_4$, water and

101　$MgSO_4·7H_2O$ 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 $^{\circ}$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 $^{\circ}$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 $^{\circ}$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 $^{\circ}$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*

*2.7.1. Radical scavenging activity on DPPH*

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

$$\text{Scavenging activity (\%)} = (1 - \frac{A_1 - A_2}{A_0}) \times 100 \tag{1}$$

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

*2.7.2. Hydroxyl free radical scavenging activity*

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

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

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

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 μL 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 determine d at 562

163 nm. EDTA was included as the positive control. The ion chelating activity was

164 calculated as:

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

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 (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 ABTS$^{\cdot+}$ solution was diluted with ethanol to an absorbance of

173     $0.70 \pm 0.02$ 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 $ABTS^{\cdot+}$ 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            $$\text{Scavenging activity (\%)} = (1 - \frac{A_1}{A_0}) \times 100 \qquad (4)$$

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

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

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

195　　SCR was obtained as follows (%): glucose 4.0, $(NH_4)_2SO_4$ 1.5, water 75.0 and

196　　$MgSO_4 \cdot 7H_2O$ 0.2. Using the optimal culture media, the verifying experiment

197　　indicated that the yield of MPS was $87.36 \pm 1.73$ mg/g under the following conditions,

198　　fermentation temperature 25 $^\circ$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 ($X_1$),

205　　fermentation time ($X_2$) and inoculum size ($X_3$) 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 $$x_i = \frac{X_i - X_0}{\Delta X_i} \tag{5}$$

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

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 (°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 ($R^2$Adj) were

223     measured for testing the goodness-of-fit of the regression equations. The value of

224     ($R^2$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 $^{\circ}$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

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.

**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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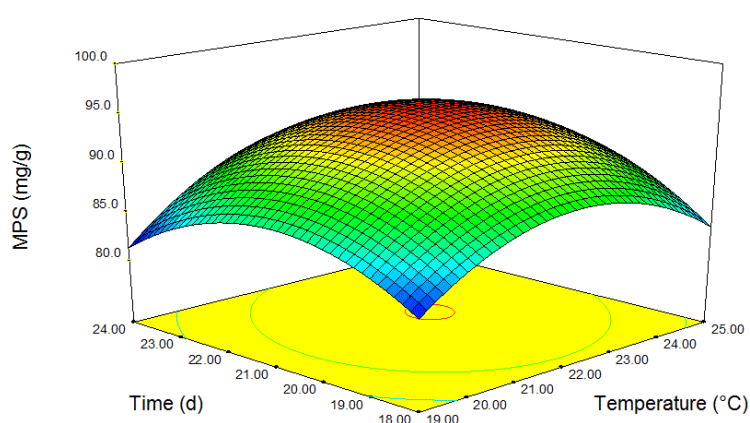
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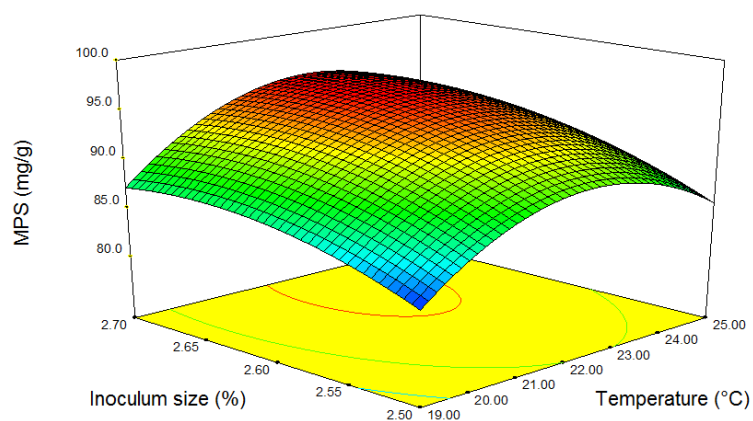
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381    Yuan, J.F., Zhang, Z.Q., Fan, Z.C., Yang, J.X., 2008. Antioxidant effects and cytotoxicity of three

382        purified polysaccharides from Ligusticum chuanxiong Hort. Carbohydr. Polym. 74, 822-827.
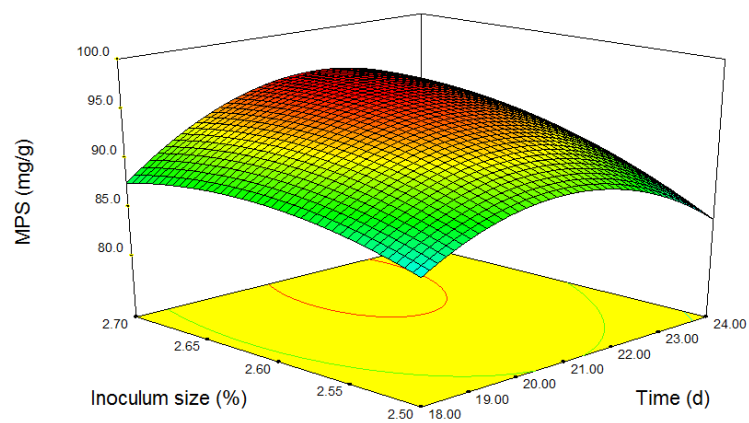
383        a



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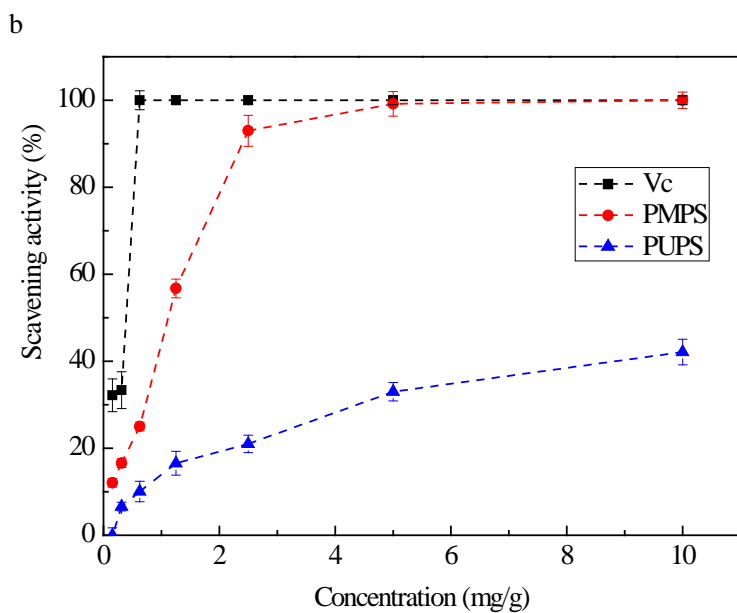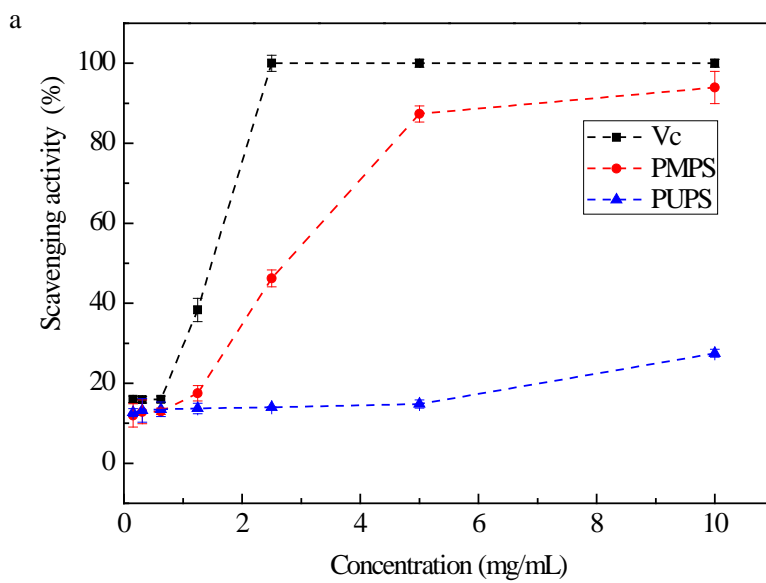389        c
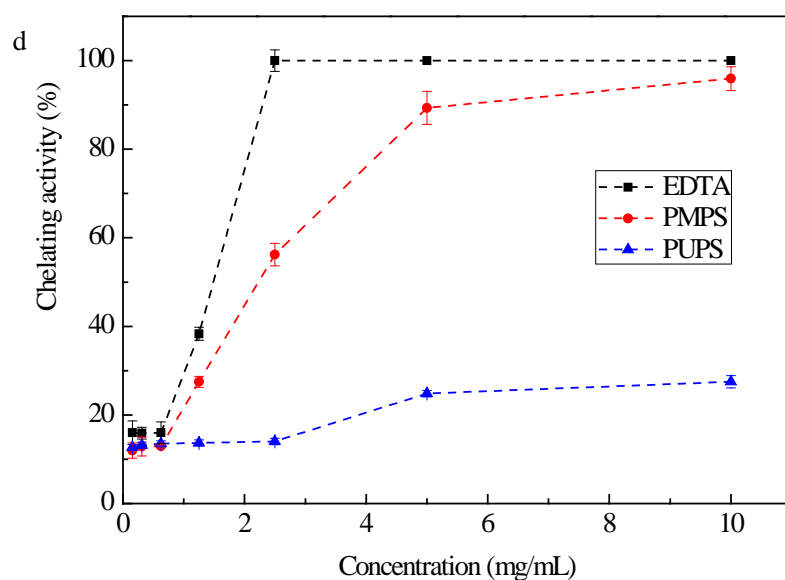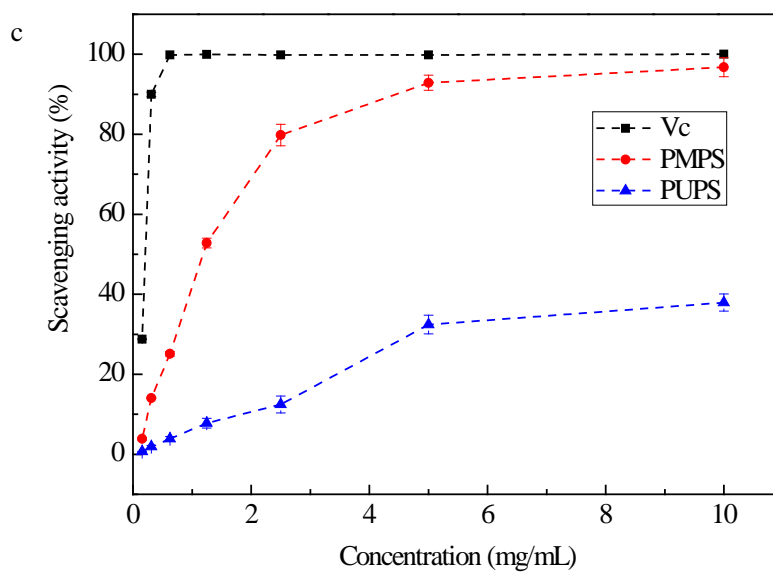


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Fig. 1. Response surface plot for the MPS yield in terms of the effects of (a) time and temperature, (b)

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

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

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

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 ($^{\circ}$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 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 |

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 |
| $X_1X_2$ | 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 | $R^2$Adj= 0.9726 | | | | |

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