Utilization of soybean curd residue for polysaccharides by *Wolfiporia extensa (Peck) Ginns* and the antioxidant activities in vitro

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Abstract: To reduce nutrient cost and improve the production of polysaccharides by *Wolfiporia extensa (Peck) Ginns* (*F.A. Wolf*), soybean curd residue (SCR), a food waste, was chosen as the nutrient source in this study. The objective of this research is to determine optimum culture conditions for solid state fermentative production of polysaccharides and evaluate the antioxidant activities. Response surface methodology (RSM) was employed to optimize the fermentation conditions of *F.A. Wolf* for the enhancement of polysaccharides. The optimal conditions were obtained by response surface methodology as follows: fermentation temperature 23.7 ˚C, fermentation time 7.5 days, and inoculum size 15.5 mL. Under optimized conditions, the polysaccharides yield reached 88.93±1.87 mg/g, which was in close agreement with values predicted by the mathematical models. Furthermore, the polysaccharides exhibited positive antioxidant activities. This research provides references for the large-scale production of polysaccharides by *F.A. Wolf* and points to a new direction for SCR utilization.

Keyword： *Wolfiporia extensa*, Polysaccharides, Soybean curd residue, Response surface methodology, Antioxidant activity

1. Introduction

Soybean is one of the most important legumes in the world, particularly in Asian countries like Japan. In 2010, the annual output of soybean exceeded 261 million tons. A FAO report indicates Japan imported soybean amounting to 3.5 million tons in 2009 [1]. Soybean curd residue (SCR) is the main surplus material from soybean products and it is often regarded as waste. About 1.1 kg of fresh SCR is produced from every kilogram of soybeans processed into soymilk or tofu [2]. In Japan, about 800,000 tons of SCR are disposed of annually as by-products of tofu production and the disposal costs around 16 billion yen per annum [3]. SCR is a loose material consisting of a good source of nutrients, including protein, oil, dietary fibre, minerals, along with un-specified monosaccharides and oligosaccharides [4-6]. It is a suitable supporter and carrier because of its porosity, nutrition and cheapness. There have been several reports on the reuse of SCR for fermentation products, such as β-fructofuranosidase, *ganoderma lucidum*, *bacillus subtilis* B_2 and polysaccharides [7-10]. Current polysaccharides production from medicinal fungi is mainly from submerged culture and the fruit body. Submerged fermentation, not only has the problem of more energy-consumption during extraction, but also water-consumption and low yield [11-12]. Extracting polysaccharides from a fruit body takes more than 3 months, which is high cost and time-consuming. Compared with polysaccharides obtained from fruit bodies and mycelia, polysaccharides fermented by SCR have the advantages of waste minimization, time efficiency and high production levels at low cost.

Wolfiporia extensa (Peck) Ginns (*F.A. Wolf*) is a popular fungus of the family *Polyporaceae* that grows on the roots of old, dead pine trees. It has been used in traditional Chinese medicine for many centuries [13]. Polysaccharides isolated from the mycelia of *F.A. Wolf* have recently attracted considerable attention due to their various physiological properties, such as antioxidant, antitumor, antiinflammatory, hypoglycemic, hypocholesterolemic and immunostimulating activities [14-17].

To date, few reports are available in the literature regarding the optimization of fermentation conditions for polysaccharides production by *F.A. Wolf* using SCR as the main nutrient medium. According to previous experiments, the accumulation of polysaccharides by *F.A. Wolf* using SCR is strongly influenced by fermentation conditions, including fermentation temperature, fermentation time and inoculum size. Therefore, further study on the optimal fermentation conditions for polysaccharides is worthwhile. The aim of this work is to apply statistical methods to optimize the fermentation conditions for polysaccharides production by *F.A. Wolf* using SCR and evaluate the antioxidant activities of polysaccharides.

2. Materials and methods

2.1. Chemicals and reagents

Ascorbic acid, hydrogen peroxide, chloride ferric, potassium bromide, sodium salicylate, ethanol, potassium persulphate, ethylene diamine tetraacetic acid (EDTA), glucose, potato extract, agar, monopotassium phosphate and magnesium sulfate heptahydrate were purchased from Wako Pure Chemical (Osaka, Japan). 2,

2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), was purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

2.2. Pre-treatment of SCR

Fresh SCR (moisture content 80 %) was obtained from Inamoto Co., Ltd. (Tsukuba, Japan). Residual water was removed by drying at 60 °C. SCR with a particle size of 600 μm was used. In this study all SCR was obtained from the same batch in the manufacturing process.

2.3. *Strain and culture media*

The strain of *Wolfiporia extensa (Peck) Ginns* ACCC 50876 used in this study was obtained from the Agricultural Culture Collection of China. The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured every three months. The slants were incubated at 25 °C for 10 days. The PDA consisted of the following components: glucose 2.0 %, potato extract 0.4 %, agar 2.0 %, KH_2PO_4 0.3 %, and $MgSO_4 \cdot 7H_2O$ 0.15 %.

2.4. *Inoculum preparation*

For preparation of the inoculum, the mycelia of *F.A. Wolf* was transferred from a slant into a sterile petri-dish (diameter: 100 mm) containing 20 mL of PDA. It was incubated at 25 °C for 6 days. The 100 mL liquid culture was undertaken in a 300 mL flask containing four units of mycelial agar 5 mm × 5 mm square obtained using a self-designed cutter. Then it was put in a rotary shaker at 120 rpm at 25 °C for 5 days

and activated in the liquid medium. The flask of the liquid culture medium was composed of the following components: 2.0 %, potato extract 0.4 %, KH_2PO_4 0.3 %, and $MgSO_4 \cdot 7H_2O$ 0.15 %. Then the seed in the liquid culture was transferred to the solid culture by pipette. The solid-state culture experiment was performed in a 200 mL flask with 7.0 g SCR and 0.5 g rice bran, (moisture content 75 %) under different culture conditions. All of the media were autoclaved at 121 °C for 15 min.

2.5. *Analytical methods*

2.5.1. Determination of total sugars

Treatment of the fermented SCR was according to a procedure from the literature with a few modifications [10]. The fermented SCR from different cultured conditions were harvested and dried in a convection oven at 60 °C. Then it was ground into powder and passed through a 60 mesh. The crushed powder (500 mg) was mixed with 15 mL distilled water and extracted under boiling water for two hours. The carbohydrate was precipitated by adding fourfold volumes of 99.5 % ethanol and stored at 4.0 °C overnight. Then the total sugars were determined by the phenol-sulfuric acid method [18]. The results were expressed as mg of glucose equivalent per g of fermented SCR.

2.5.2. Determination of reducing sugars

Reducing sugars content was analyzed by dinitrosalicylic (DNS) colorimetric method [19], using D-glucose as the standard. For each 0.4 mL of the sample 0.8 mL of DNS reagent was added. The mixture was heated in boiling water for 2 min and then cooled to room temperature in a water bath. After this 4.8 mL deionized water was added. The

absorbance was measured at 540 nm. The concentration of total reducing sugars was calculated based on a standard curve obtained with D-glucose.

2.5.3. Determination of polysaccharides

The yield of polysaccharides was defined as the amount of total sugars minus the amount of reducing sugars.

2.5.4. Fourier-transform infrared spectrometric analysis

FT-IR spectrum was recorded on a Jasco FTIR 3000 spectrometer (Jasco,Wakayama, Japan). The dried sample was ground with potassium bromide (KBr) powder and pressed into pellets for spectrometric measurement at a frequency range of 4000-400cm^{-1}.

2.6. *Optimization for polysaccharides production*

Based on results of the previous test, fermentation temperature, fermentation time and inoculum size were chosen for the optimization of polysaccharides by Box-Behnken design. The experimental design including name, symbol code, and level of the variables are shown in Tables 1 and 2. The test factors were coded according to the following equation (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (1)$$

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

2.7. Extraction of polysaccharides from fermented SCR

157 Fermented SCR at optimal condition was harvested and dried in a convection oven at
158 60 °C. The obtained product was sieved with a 60 mesh sieve. The sieved powder 1.0 g
159 was added to 30.0 mL distilled water and extracted under boiling water for two hours.
160 After being treated with sevage reagent and dialyzed, the water-soluble polysaccharides
161 were precipitated by adding fourfold volumes of 99.5 % ethanol. The precipitated
162 polysaccharides were collected after being centrifuged at 7500×g for 15 min and
163 lyophilized to powder form, which was applied to detect the antioxidant activities in
164 vitro.

165 *2.8. Assay for antioxidant activities of polysaccharides*

166 *2.8.1. Hydroxyl radical scavenging activity*

167 Hydroxyl radical scavenging activity was measured according to the procedures of
168 Winterbourn and Sutton with a few modifications [20]. The reaction mixture (2.5 mL)
169 contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6.0 mM), 0.15 mL of sodium
170 salicylate (20.0 mM), and 1.0 mL of different concentrations of polysaccharides.
171 Ascorbic acid was used as the positive control. After incubation for 1 h at 37 °C, the
172 absorbance of the formed hydroxylated salicylate complex was measured at 562 nm.
173 The percentage scavenging effect was calculated using equation (2):

174 $$\text{HO}\cdot \text{ scavenged (\%)} = [1 - \frac{A_1 - A_2}{A_0}] \times 100\% \quad (2)$$

175 where A_0 is the absorbance of the solvent control, A_1 is the absorbance of the sample or
176 ascorbic acid and A_2 is the absorbance of the reagent blank without sodium salicylate.

177 *2.8.2. Ferrous metal ions chelating activity*

Ferrous metal ions chelating activities of the polysaccharides were measured according to a procedure from the literature with a few modifications [21]. The sample or EDTA solution (1.0 mL) was mixed with 50 μL of ferrous chloride (2.0 mM) and 0.2 mL of ferrozine (5.0 mM), shaken well, and allowed to settle for 10 min at room temperature. The absorbance of the mixture was determined at 562 nm with EDTA as the positive control. The ion-chelating activity was calculated using equation (3):

$$\text{Chelating rate (\%)} = [1 - \frac{A_1 - A_2}{A_0}] \times 100\% \quad (3)$$

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

2.8.3. ABTS radical scavenging assay

The radical scavenging activities of the polysaccharides against radical cations ($ABTS^+$) were measured using the methods of Pellegrini et al. with some modifications [22]. ABTS was dissolved in distilled water at a final concentration of 7.0 mM. $ABTS^+$ was produced by reacting 7.0 mmol/L of $ABTS^+$ solution with 2.45 mmol/L of potassium persulphate, and the mixture was kept in the dark at room temperature for 16 h. At the moment of use, the $ABTS^+$ solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm.

Samples (0.15 mL) of various concentrations (0.16-10.00 mg/mL) were mixed with 2.85 mL of $ABTS^+$ solution and mixed vigorously. Finally, the absorbance was measured at 734 nm after incubation at room temperature for 10 minutes. The

198 scavenging activity of the ABTS free radicals was calculated using the following
199 equation (4):

200 $$\text{ABTS scavenging activity (\%)} = [1 - \frac{A_1}{A_0}] \times 100\% \quad (4)$$

201 where A_0 is the absorbance of control without sample and A_1 is the test sample without
202 $ABTS^+$.

203 *2.9. Statistical analysis*

204 All experiments were carried out in triplicate. Data were processed and analyzed
205 using Design Expert Software (version 8.0.6, Stat-Ease. Inc., Minneapolis, USA).
206 P-values below 0.05 were regarded as statistically significant.

207 **3. Results and discussion**

208 3.1 *Optimization of the yield of polysaccharides*

209 For prediction of the optimal point, a second-order polynomial function was fitted to
210 correlate the relationship between independent variables and response. A Box-Behnken
211 design with 3 levels for all the 3 factors: fermentation temperature (X_1), fermentation
212 time (X_2) and inoculum size (X_3) were used for this purpose. A total of 17 experiments
213 with associated combinations were undertaken. The range of the variables is given in
214 Table 1. The experimental design and the results obtained from experiments are shown
215 in Table 2. The results of these experiments were fitted with a second order polynomial
216 equation. Judging from the regression coefficients and considering the significant terms,
217 the fitted equation (in terms of coded values) for predicting polysaccharides production
218 (Y) is given below:

$$Y=86.62-5.86x_1+6.02x_2+1.61x_3-3.78x_1x_2+2.38x_1x_3-10.44x_1^2-21.15x_2^2-6.08x_3^2 \quad (5)$$

where Y is the predicted response that is the yield of polysaccharides, and x_1, is fermentation temperature; x_2, the fermentation time and x_3, the inoculum size. Table 3 shows the verification of the model based on the results of an F-test and analysis of variance.

The value of probability (P) was less than 0.0001, which indicates that the selected factors and their ranges have significant influence on the yield of polysaccharides. From Tables 2 and 3, the coefficient of determination (R^2=0.99) shows a good fit and the accuracy of the model between the predicted and actual responses [23-25]. Regression analysis of the data shows that the value of the adjusted determination coefficient (R^2_{Adj} = 0.99) was also high enough to indicate the significance of the model. The accuracy of the model is also verified by the data in Table 2, in which the obtained polysaccharides yields is almost equal to the predicted values. The *P*-values are used not only as a tool to check the significance of each coefficient but also an indication of the interaction strength between each independent variable. The smaller the *P*-values, the larger are the significance of the corresponding coefficient. It can be seen from Table 3 that all regression coefficients are significant except for the interactions of two cross-product coefficients between fermentation time and inoculum size. When their absolute values were compared, the individual effects of fermentation time and fermentation temperature were stronger than inoculum size. Considering the quadratic coefficient, the square of fermentation time has the strongest effect on polysaccharides production.

3D response surfaces plots were employed to determine the interaction of the fermentation conditions and the optimum levels that have the most significant effect on polysaccharides production. The response surfaces plots based on the model are depicted in Fig. 1, which show the interactions between two variables by maintaining the other variable at zero level for polysaccharides production. The main goal of response surface is to hunt efficiently the optimum values of the variables so that the response is maximized. It is clear from Fig. 1 that the minimum response of polysaccharides production (45.4 mg/g) occurred when time was at its lowest level. Polysaccharides production increased considerably as time increased, indicating that time in polysaccharides production has a significant effect on the responses. As the time increased, the responses were maximal near the median temperature. Fig. 2 demonstrates the effects of temperature and inoculum size on polysaccharides production. It could be observed that the polysaccharides production varied significantly with the variation in temperature. It is evident that the polysaccharides yield significantly increased with increasing temperature up to about 24 $^{\circ}$C but decreased sharply beyond this, reaching its maximum yield at 22.6-24.6 $^{\circ}$C. However, inoculum size didn't show a similar significant effect on the production of polysaccharides within the tested range of the fermentation temperature. Polysaccharides yields increased gradually when inoculum size increased from 10 to 15 mL, but negligibly after 15 mL. As can be seen from Fig. 3, the polysaccharides yield was significantly affected by fermentation time. Production increased when time increased up to 7 days but decreased

sharply beyond this. This observation can be attributed to the autolysis of mycelia as time increases. However, the effect of inoculum size on the production of polysaccharides is insensitive within the tested range. The optimum ranges of fermentation time and inoculum size for the maximum yield of polysaccharides lie between 7.1-7.5 days, and 14.1-16.3 mL, respectively.

3.2. Validation of the models

By solving the inverse matrix (from Eq. (5)) using Design Expert software, the optimum values of the test variables in uncoded units were obtained, i.e. fermentation temperature 23.7 $^{\circ}$C, fermentation time 7.5 days, and inoculum size 15.5 mL, respectively. The predicted optimal polysaccharides production corresponding to these values was 87.98 mg/g. To confirm the accuracy of the model for predicting maximal polysaccharides production, additional experiments in triplicate using these optimized fermentation conditions were carried out. These triplicate experiments produced polysaccharides yield of 88.93±1.87 mg/g, confirming a good fit between the predicted and experimental values and also the validity of the model. The polysaccharides yield obtained after optimization increased to 3.54 folds. It is higher than 13.8 mg/g obtained from the fruit body of *F.A. Wolf* [17]. As a result, the models developed were considered to be accurate and reliable for predicting the production of polysaccharides by *F.A. Wolf ACCC 50876* using SCR as the nutrient medium.

3.3. A Fourier transform infrared spectroscopy (FT-IR) of polysaccharides

The polysaccharides were characterized by FT-IR spectroscopy as shown in Fig. 4.

282 The strong bands at 3000-3500 cm^{-1} are characteristic of glycosidic structures and are
283 related to OH stretching. The sample exhibited a specific absorption peak at 1720 cm^{-1},
284 suggesting the presence of uronic acid [26]. It exhibited the characteristic absorption of
285 polysaccharides at 1650 and 1400 cm^{-1} [27]. The C–O–C stretching has characteristic
286 absorption at 1124 cm^{-1}. The band at 890 cm^{-1} is characteristic for the β-glycosidic
287 linkage [28]. It has been reported that the β-glycosidic linkage is the essential structural
288 feature for immunostimulatory and antitumoral effects [29-31]. The absorption peak at
289 800 cm^{-1} for the sample was the characteristic absorption of mannose [32].

290 *3.4. Antioxidant activities in vitro of polysaccharides*

291 Antioxidant activities have been attributed to various reactions and mechanisms, such
292 as radical scavenging, reductive capacity, prevention of chain initiation, binding of
293 transition metal ion catalysts, etc. [33, 34]. Polysaccharides isolated from *Pleurotus*
294 *ostreatus* and I*nonotus obliquus* exhibited significant antioxidant effects on scavenging
295 DPPH and hydroxyl radical [35, 36]. To investigate the antioxidant activities of the
296 polysaccharides, antioxidant properties based on ABTS radical-scavenging activity,
297 ferrous metal ions chelating activity and hydroxyl radical scavenging activity assays
298 were carried out. The antioxidant results of polysaccharides are described in Figs. 5-7.

299 It is important to remove hydroxyl radicals for antioxidant defense because hydroxyl
300 radicals are one of the reactive oxygen species generated in the body. As shown in Fig. 5,
301 the polysaccharides exhibited a concentration-dependent $HO^{\cdot}$ scavenging activity.
302 Polysaccharides showed higher hydroxyl scavenging activity than that of the ascorbic

303 acid under the low the concentrations. The polysaccharides exhibited 93.88 % of the
304 hydroxyl radical scavenging activity at a concentration of 2.5 mg/mL, approximately
305 equal to that of 2.5 mg/mL Vc (100 %). Moreover, the hydroxyl radical scavenging
306 activity of polysaccharides was superior to those found in other fermented soybean
307 products [37].

308 Metal chelating capacity is an index to evaluate antioxidant behavior since it reduces
309 the concentration of transition metals that may act as catalysts to generate the first few
310 radicals and initiate the radical-mediated oxidative chain reactions in biological or food
311 systems. The ferrous metal ions chelating activity of polysaccharides is shown in Fig. 6.
312 Compared with EDTA, the chelating ability of the samples on ferrous ion was weaker.
313 The Fe^{2+}-chelating activity showed no siginificant increase with the increase in
314 polysaccharides concentration and just reached 21.65 % at 10 mg/mL. The result was
315 consistent with soybean products fermented using *Bacillus subtilis* [38].

316 ABTS assay is often used in evaluating the total antioxidant power of single
317 compound and complex mixtures of various plants [39]. In our experiment, the
318 scavenging ability of the polysaccharides on ABTS free radicals is shown in Fig. 7 and
319 compared with ascorbic acid as control standards. Their scavenging powers correlated
320 well with increasing concentrations, increasing from 22.63 % to 100 %, when the
321 concentration of the polysaccharides increased from 0.16 to 10.00 mg/mL. The results
322 indicate that the obtained polysaccharides had strong potential ABTS radical scavenging
323 activity.

4. Conclusions

In conclusion, optimization of the fermentation process is an efficient technology of SCR for polysaccharides. An optimized fermentation condition was obtained at: fermentation temperature 23.7 °C, fermentation time 7.5 days, and inoculum size 15.5 mL. Under optimized conditions, the polysaccharides yield reached 88.93±1.87 mg/g. Further experiments showed that the experimental values agreed with the predicted values well. The obtained polysaccharides demonstrated positive antioxidant activities. The results obtained will provide a reference for the large-scale production of polysaccharides by *F.A. Wolf* and point to a new direction for the utilization of SCR.

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Figures Captions:

Fig. 1. Response surface plot for the yield of polysacchriades by *P.cocos* in terms of the effects of time and temperature.

Fig. 2. Response surface plot for the yield of polysacchriades by *P.cocos* in terms of temperature and inoculum size.

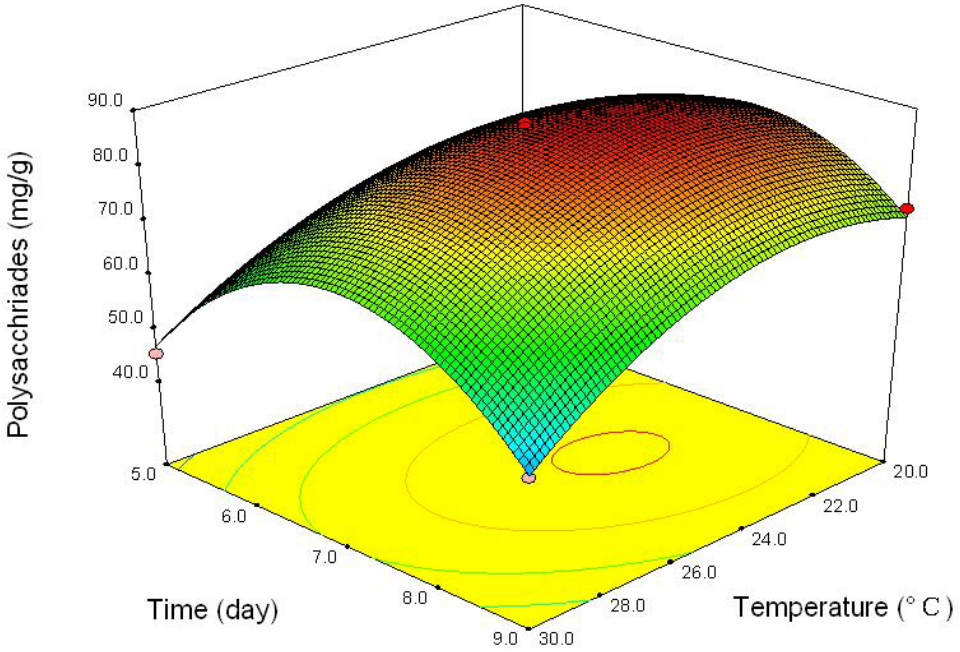
Fig. 3. Response surface plot for the yield of polysacchriades by *P.cocos* in terms of inoculum size and time.

Fig. 4.FT-IR spectrum of the polysaccharides.

Fig. 5. Hydroxyl radical scavenging activitiy of polysaccharides.

Fig. 6. Ferrous metal ions chelating activity of polysacchriades.

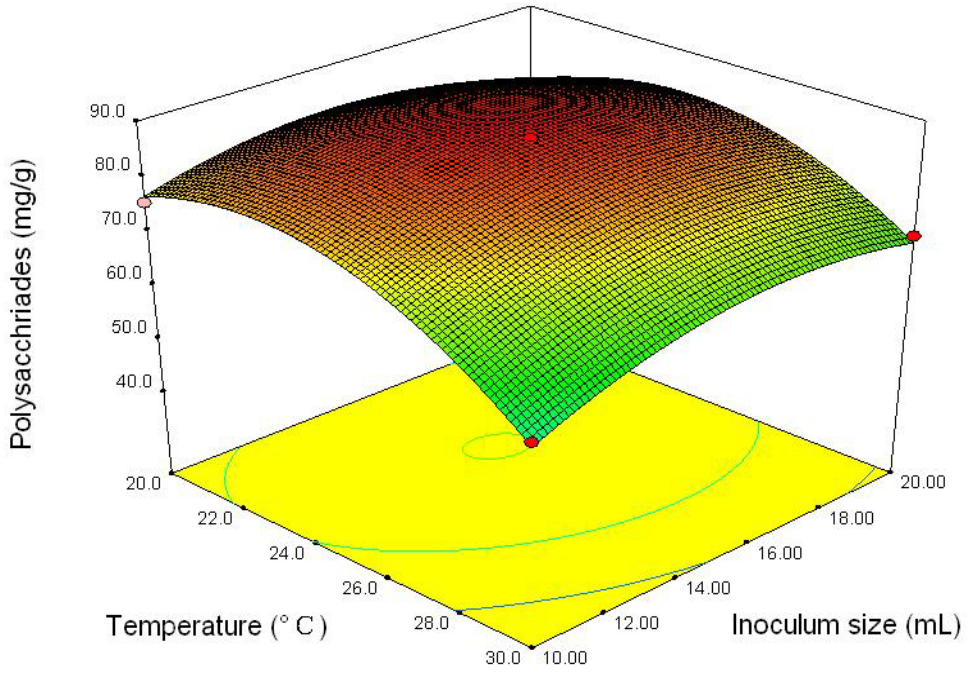
Fig. 7. Inhibition effects of polysaccharides on the stable ABTS free radicals.



449

450 **Fig. 1.** Response surface plot for the yield of polysacchriades by *P.cocos* in terms of the effects of

451 time and temperature.



452

453 **Fig. 2.** Response surface plot for the yield of polysacchriades by *P.cocos* in terms of temperature and

454 inoculum size.

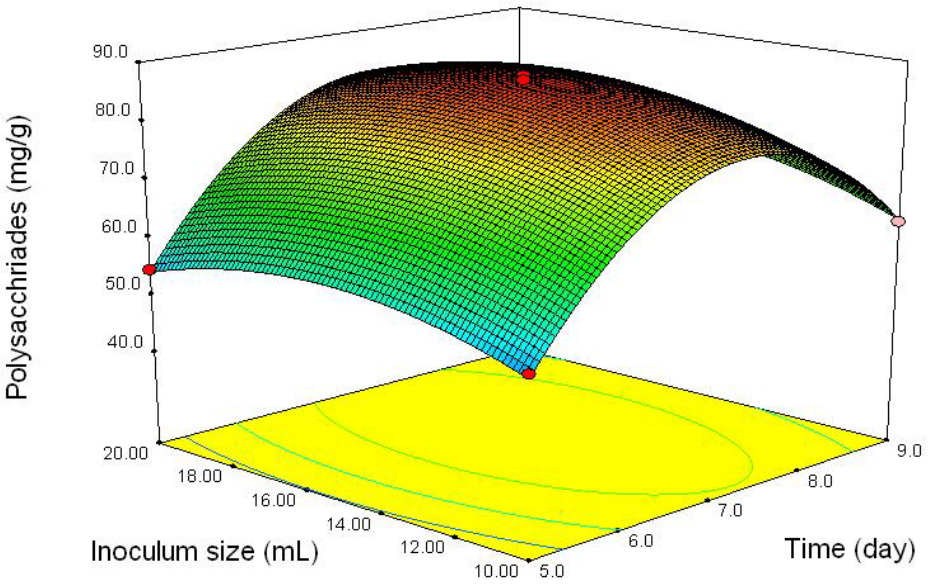


Fig. 3. Response surface plot for the yield of polysacchriades by *P.cocos* in terms of inoculum size and time.

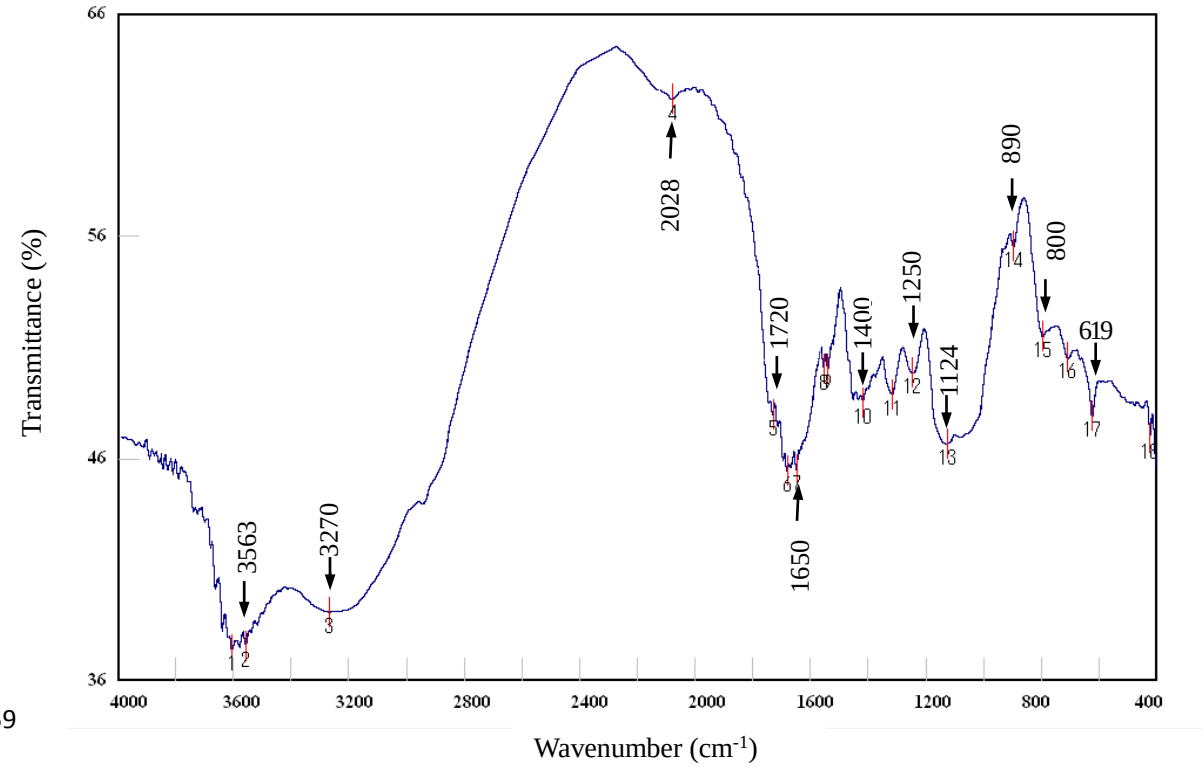


Fig. 4. FT-IR spectrum of the polysaccharides.

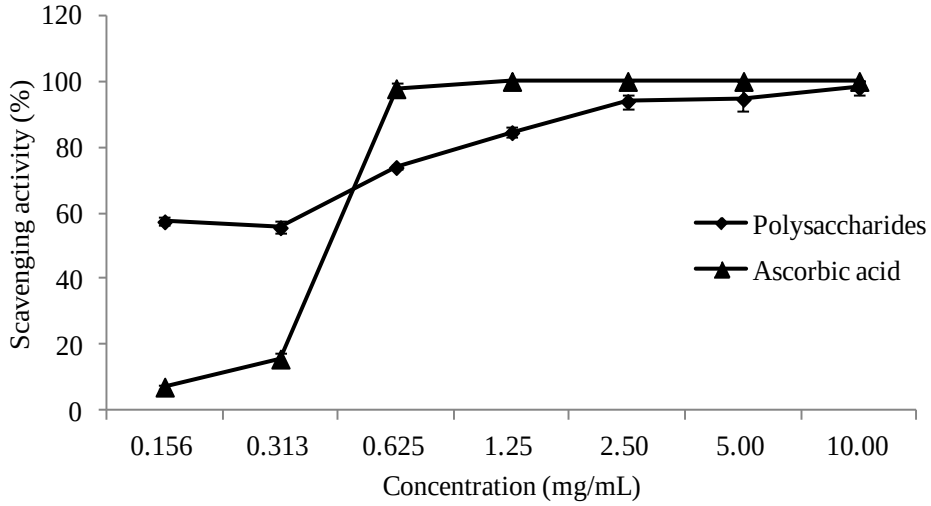


Fig. 5. Hydroxyl radical scavenging activity of polysaccharides.

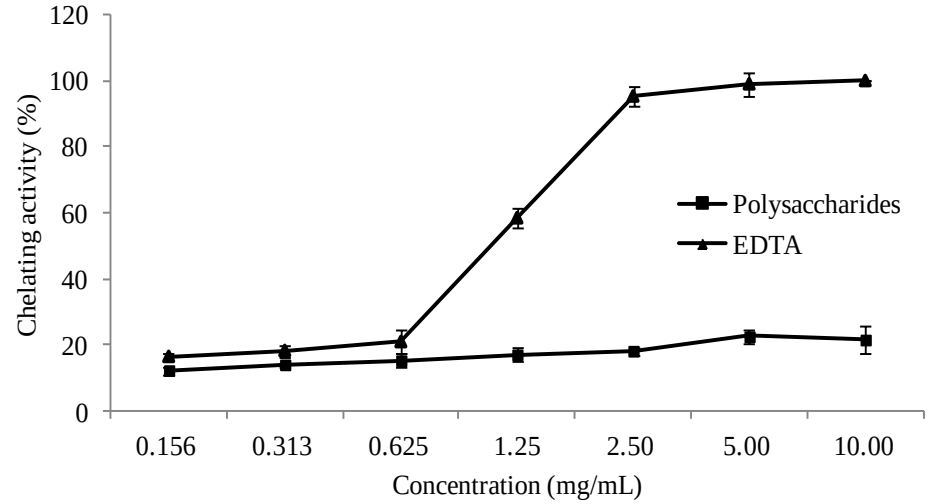


Fig. 6. Ferrous metal ions chelating activity of polysacchriades.

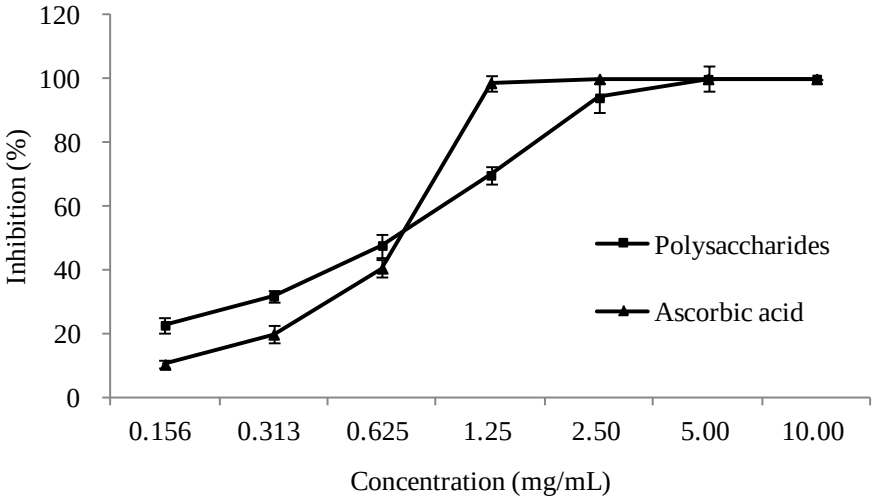


Fig. 7. Inhibition effects of polysaccharides on the stable ABTS free radicals.

Table Captions:

Table 1 Levels and codes of variables in the Box-Behnken design.

Table 2 Experimental and predicted values of polysaccharides based on Box-Behnken design.

Table 3 The result of analysis of variance (ANOVA) for the selected model.

Table 1 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	20	25	30
Fermentation time (d)	X_2	x_2	5	7	9
Inoculum size (mL)	X_3	x_3	10	15	20

487 **Table 2** Experimental and predicted values of polysaccharides based on Box-Behnken design.

Runs	X_1: Fermentation temperature	X_2:Fermetation time	X_3: Inoculum size	Polysaccharides content(mg/g)	
				Experimental	Predict
1	-1 (20)	0 (7)	1 (20)	74.41±0.37	75.60
2	0 (25)	1 (9)	-1 (10)	62.63±1.20	63.00
3	-1 (20)	1 (9)	0 (15)	72.22±0.67	70.70
4	0 (25)	0 (7)	0 (15)	86.25±1.32	86.60
5	0 (25)	-1 (5)	1 (20)	54.54±0.81	54.20
6	-1(20)	-1 (5)	0 (15)	51.49±1.07	51.60
7	0 (25)	0 (7)	0 (15)	86.81±1.23	86.60
8	1 (30)	0 (7)	-1 (10)	61.01±0.54	60.20
9	1 (30)	0 (7)	1 (20)	69.40±0.74	68.20
10	0 (25)	1 (9)	1 (20)	67.06±1.32	67.80
11	-1(20)	0 (7)	-1 (10)	75.56±0.69	77.10
12	0 (25)	0 (7)	0 (15)	85.53±1.58	86.60
13	0 (25)	-1 (5)	-1 (10)	53.33±1.25	52.60
14	1 (30)	-1 (5)	0 (15)	45.40±0.45	46.90
15	1 (30)	1 (9)	0 (15)	51.01±0.93	51.40
16	0 (25)	0 (7)	0 (15)	87.65±0.88	86.60
17	0 (25)	0 (7)	0 (15)	86.86±0.20	86.60

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

489 **Table 3** 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	3374.48	9	374.94	204.49	< 0.0001
X_1	274.52	1	274.52	149.72	< 0.0001
X_2	289.89	1	289.89	158.11	< 0.0001
X_3	20.74	1	20.74	11.31	0.012
X_1X_2	57.2	1	57.2	31.2	0.0008
X_1X_3	22.75	1	22.75	12.41	0.0097
X_2X_3	2.6	1	2.6	1.42	0.2721
X_1^2	459.08	1	459.08	250.38	< 0.0001
X_2^2	1882.88	1	1882.88	1026.91	< 0.0001
X_3^2	155.79	1	155.79	84.97	< 0.0001
Residual	12.83	7	1.83		
Lack of fit	10.34	3	3.45	5.33	0.0611
Pure error	2.5	4	0.62		
Corrected Total	3387.32	16			
R^2= 0.99	R^2_{Adj} = 0.99				

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