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Research Article

The Optimized Ultrasonic Extraction of the Polysaccharide from *Pleurotus eryngii* by Response Surface Methodology and Assessing Its Antioxidant Activity

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Abstract: We aimed to investigate the effects of ultrasonic treatment on the yield of polysaccharide in the extraction and antioxidant activity of polysaccharide. By employing an ultrasonic technique, polysaccharide was extracted from *Pleurotus eryngii* (PPE). The optimal conditions for ultrasonic extraction of PPE were revealed utilizing Response Surface Methodology (RSM) which was adopted to assess the effects of four variables on the extraction of PPE. These variables included temperature, time, ultrasonic power and solvent-solid ratio and were independent to each other. The plots of response surface showed that these variables presented complicated and significant effects on the extraction of polysaccharide. To obtain the highest recovery of polysaccharide, ultrasonic extraction was performed at 95°C for 82 min with a solvent-solid ratio of 20 mL/g and an ultrasonic power of 213 W. The estimated recovery was 14.5%. The determination of extractions in the experiments under optimal conditions showed insignificant differences with the estimated recovery. Following these experiments, the antioxidant activity *in vitro* was assessed by detecting scavenging ability against DPPH, superoxide anions and hydroxyl radicals. A 45.6% removal rate for DPPH was observed at 2.5 mg/ml of PPE.

Keywords: Antioxidant activities, polysaccharide, response surface methodology, ultrasonic treatment

INTRODUCTION

Pleurotus eryngii, is a comestible mushroom originally grown in Mediterranean zones of Europe, North Africa and the Middle East, which also is found in many regions of Asia. *P. eryngii* is a species which is most commonly seen in the genus of oyster mushroom, namely *Pleurotus*, in which there is also the oyster mushroom (*Pleurotus ostreatus*). *P. eryngii* has a white stem which is thick and meaty with a small medium brown cap. *P. eryngii* possibly contains chemical components which can naturally stimulate the immune system. Polysaccharide, as one of the major components of *P. eryngii*, has been proven to have many biological properties as an antioxidant and an antitumor agent.

Extraction of polysaccharide from plant materials is time consuming using hot water. Recently, a new method has been reported for preparing polysaccharide from many plants using an ultrasonic technique (Soria and Villamiel, 2010; Ying *et al*., 2005). Treatment with ultrasound can intensify mass transfer between the solvent and plant and increase extraction of the polysaccharide by destructing cell walls. Whilst ultrasonic treatment also shows adverse effects on polysaccharide. Zhou and Ma (2006) reported that it can lead to changes in the structure of polysaccharide. The structure of polysaccharide is associated with bioactivity including radical scavenging activity (Zhang *et al*., 2004). The mechanism of ultrasonic extraction on polysaccharide, in terms of impact of the physical and chemical properties of the product, needs to be comprehensively understood. Some researches demonstrated that the antioxidant activity of polysaccharides was related to their degree of polymerization and their structure (Chen and Yan, 2005). Currently, no study has been conducted on the influence of ultrasonic extraction on the antioxidant activities of polysaccharide extracted from *P. eryngii*.

Response surface methodology is an effective method for discovering the interaction of various parameters and how they impact the extraction of products (Li *et al*., 2011). At present, although extraction using ultrasonic technique has been extensively utilized in the extraction process from various materials, little research has been performed to determine the optimum extraction parameters for *P. eryngii* in the ultrasonic extraction process.

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This study aimed to investigate the effects of ultrasonic treatment on the yield of polysaccharide in the extraction and antioxidant activity of polysaccharide.

MATERIALS AND METHODS

Materials and chemicals: *P. eryngii* were obtained from a local market of Qinhuangdao, China. DPPH was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). All other reagents and chemicals were of analytical grade and purchased in the local area.

Extraction process: Reflux of the sclerotia of the *P. eryngii* was carried out at 80°C with 80% (v/v) ethanol for 2 hours and repeated 3 times. After being cut and dried at 60°C, the *P. eryngii* was ground using a grinder. *P. eryngii* powder was preserved at 4°C. The dried residues (5 g) of the *P. eryngii* were utilized in each extraction. The extraction was conducted applying an ultrasonic cleaner (GDS-1012T, China) with varied ultrasonic times (60, 80, 100, 120 and 140 min), temperatures (55, 65, 75, 85 and 95°C), solvent-solid ratios (5, 10, 20, 40, 60) and powers (100, 200, 300, 400 and 500 w). A Whatman Nr 1 filter paper (Whatman Ltd., Kent, U.K.) was used to filter the extract and the filtrate concentrated using a rotary evaporator in vacuum at 50°C. Sevag reagent was used to remove the proteins in the extract (Navarini *et al*., 1999). After removing the Sevag reagent, 95% ethanol was placed in the extract and the obtained mixture preserved at 4°C overnight to precipitate the polysaccharide. The precipitate was obtained by centrifuging the mixture at 3000×g for 10 min. The precipitate was then washed with acetone, petroleum ether and lyophilized. The precipitate was dried to calculate the yield.

In order to further explore the virtues of ultrasonic-assistant extraction, extraction of PPE using hot water without ultrasonic treatment was conducted as a control process for polysaccharide preparation. The subsequent process was similar to that described above.

Response surface methodology: OriginPro (Version 8.0, USA) was used to design the experiments, analyze data and establish the model. A design of regressive rotation with four variables was employed to explore the response patterns and then to construct a model. Each of the four variables including ultrasonic time (X1), temperature (X2), power (X3) and solvent-solid ratio (X4), were with five levels, whilst the extraction of PPE was applied as the dependent variable. Table 1 illustrates the levels and symbols. Seven replicates at the centre of the design were employed to ensure the prediction of a pure error sum of squares. The determinations in triplicate were carried out randomly at each of the design points.

Table 1: Independent variables and their levels in the response surface model design

	Levels				
Parameters	γ	1	0	−1	−γ
X1/min	120	110	100	90	80
X2/°C	95	90	85	80	75
X3 /w	400	350	300	250	200
X4/ml/g	60	50	40	30	20

Detection of DPPH radical scavenging activity: According to the method proposed by Thitilertdecha *et al*. (2008), the removing capacity against DPPH radicals was assayed with some changes. After the samples were incubated at 37°C for 30 min, the absorbance was tested at 517 nm. Using distilled water as the control, the removing activity was computed using the following equation: Scavenging activity (%) = (1-absorbance sample/absorbance control) ×100.

Scavenging ability for superoxide anion radicals: By employing the method put forward by Nishikimi *et al*. (1972), the scavenging abilities of each of the samples and using vitamin C as a control, were evaluated for superoxide radicals. The absorbance was measured at 560 nm against a blank. The control was produced without any sample.

Scavenging for hydroxyl radicals: Non-site specific degradation for hydroxyl radical mediated 2-deoxy-D-ribose was determined based on the method reported by Halliwell *et al*. (1987). The degree of oxidation was predicated according to the absorbance at 532 nm of the solution against a blank control which did not contain any sample.

Reducing capacity: The reducing capacity of the samples was detected based on the method of Yen and Chen (1995). Absorbance was determined at 700 nm. The higher the absorbance of the reaction mixture, the stronger the reducing capacity.

Inhibition on lipid peroxidation: The authors utilized the method proposed by Yin *et al*. (2010) to study the lipid peroxidation. The absorbance of the mixture was measured at 532 nm to compute the inhibition ratio using the following formula: inhibiting ratio (%) = $(1–A)/A_0$

where, A_0 and A are the absorbance values of the control and the samples with polysaccharide, respectively.

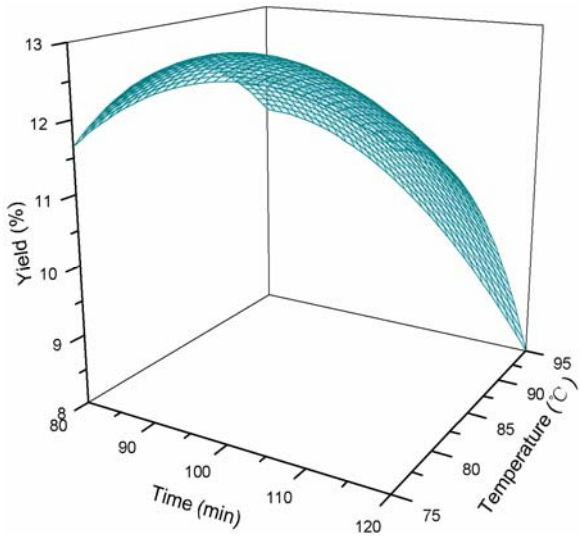
Statistical analysis: Triplicate measurements were conducted and reported as means±standard deviation. The variance was analyzed using ANOVA. To calculate the correlation, statistical calculations were performed utilizing Microsoft Excel 2003 (Microsoft, Seattle,

USA). A significant difference at $p<0.05$ was used for statistical interpretation.

RESULTS AND DISCUSSION

Effects of ultrasonic power, time, temperature and solvent-solid ratio on the extraction of polysaccharide: Figure 1 demonstrates the effects of time, temperature, ultrasonic power and solvent-solid ratio on the extraction of PPE, together with their interactions. Surface plots in 3D were drawn to display the major and interactive effects of the independent variables on the dependent one. Each factor showed a complex relationship with the extraction process. The effects of ultrasonic temperature (X2) and ultrasonic time (X1) on PPE yield are illustrated in Fig. 1a. A higher yield of polysaccharide was obtained when the ultrasonic treatment was performed for a longer time at a lower ultrasonic temperature. In Fig. 1a, the PPE recovery increased to a certain value with an increase in the ultrasonic time which then decreased with a fixed ultrasonic power and within the partial scope of the ultrasonic temperature used in the experiment.

The effects of ultrasonic time (X1) and ultrasonic power (X3) on PPE yield are shown in Fig. 1b. As the ultrasonic power increased, the recovery of polysaccharide grew when a low ultrasonic power was applied to the extraction. In contrast, the yield declined if the extraction was performed at a high ultrasonic power. The extension of ultrasonic time caused a higher recovery in the extraction whilst the PPE recovery reduced with the application of a high ultrasonic power and an increase of ultrasonic time. This could potentially be explained due to the ultrasonic wave



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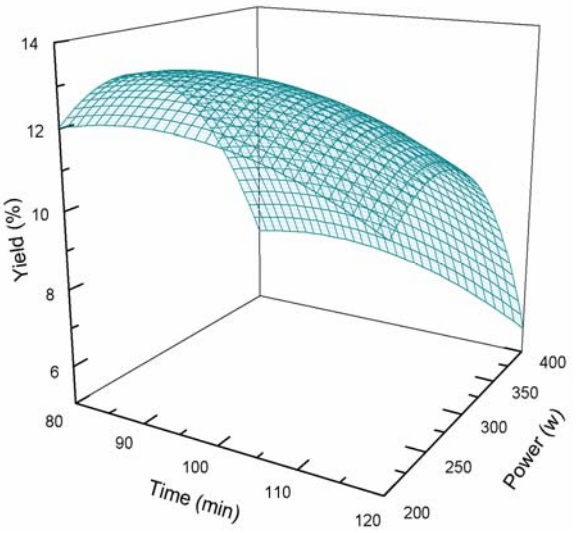

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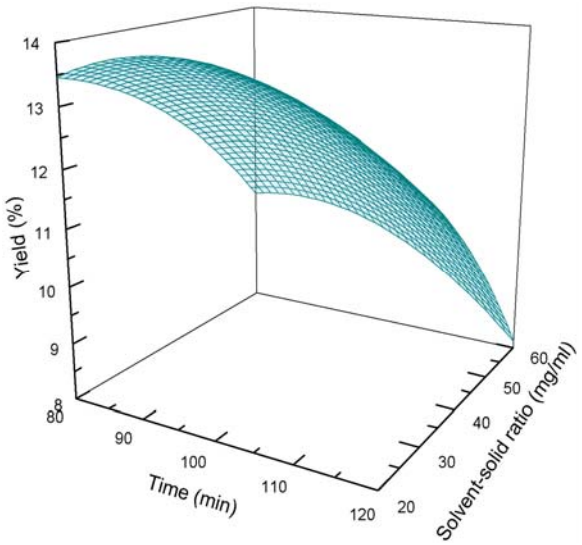

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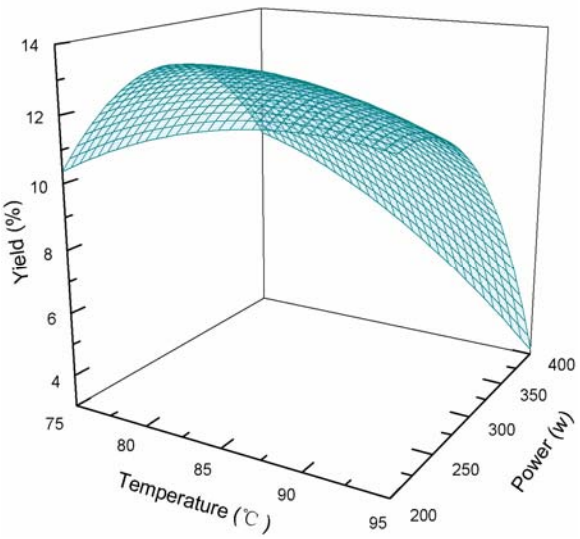

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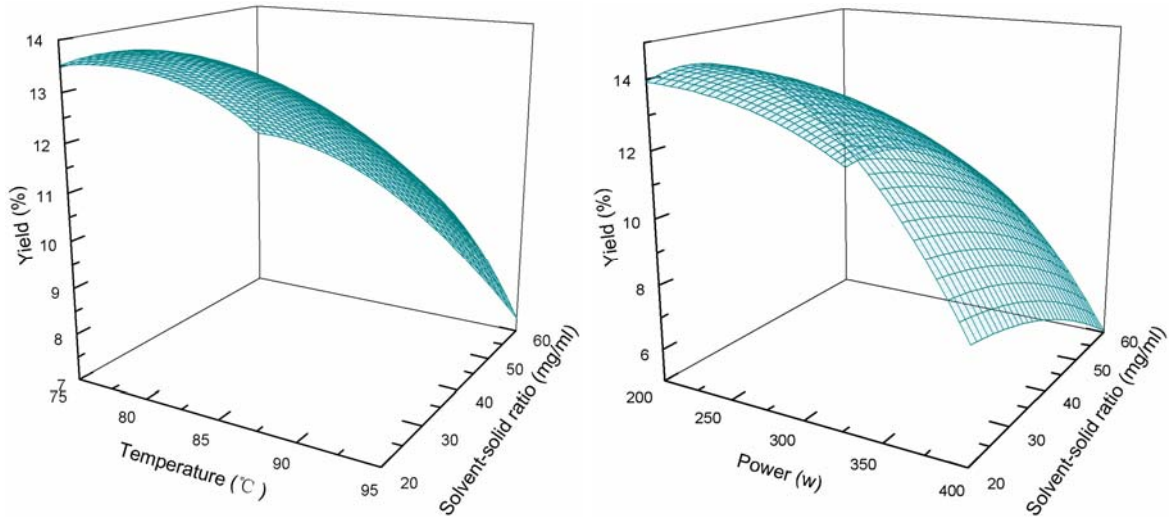


(e) (f)

Fig. 1: Response surface plots showing the effect of ultrasonic power, time, temperature and solvent-solid ratio on the recovery of PPE and their interactions. Two of the variables were kept at 0 level whilst the remaining two variables were changed within experimental range

induced degradation of polysaccharide. Both the liquid density and viscosity declined and exhibited fast mass transfer at high ultrasonic temperature (Hemwimol *et al*., 2006). Meanwhile, due to the high ultrasonic temperature, the surface contact area and the number of bubbles increased. Thus, the efficiency of extraction can be strengthened at a high ultrasonic temperature.

The temperature of ultrasonic treatment exhibited a positive effect on the extraction of PPE with an ultrasonic power of 200 w (Fig. 1d), whilst a different effect of the ultrasonic temperature was discovered with 250 w of ultrasonic power. If the ultrasonic power was relatively high, the temperature of ultrasonic treatment exerted an adverse influence on the PPE yield. This had been caused by the excessively high temperature and the degradation effect of the ultrasonic waves.

Figure 1c, 1e and 1f display the effects of ultrasonic time, power, temperature and solvent-solid ratio on the extraction rate of PPE. The ratio of solvent-solid shows a linear effect on the extraction rate of PPE, whereas the others factors displayed a quadratic effect. Figure 1f shows the effects exerted on PPE yield by ultrasonic power (X3) and solvent-solid ratio (X4). As the ultrasonic power reduced, the recovery of polysaccharide grew to a value and the decreased with a high solvent-solid ratio, whilst with a subsequent low ratio of solvent-solid it rose constantly.

In summary, when all the four factors increased within a certain extent, the recovery of polysaccharide could be improved. However, the yield was likely to be reduced with larger ultrasonic power and solvent-solid ratio, accompanied with longer ultrasonic time or higher temperature of ultrasonic treatment.

The yield of polysaccharide recovered with the application of the ultrasonic technique was higher than that (8.7%) using hot water. Considering the enhancement of the extraction using ultrasonic technique, it resulted from favorable solvent penetration, the strengthening of mass transfer and cell destruction. Therefore, due to its high efficiency, ultrasonic treatment is evidently an optimal approach for extracting PPE.

Model fitting: Generally, optimizing a response surface which is fitted is likely to generate misleading or poor results only if the model presents a good fit, which inspects the adequacy of the model. The optimization conditions for extracting PPE were acquired from the design of the test, as displayed in Table 2. The experiments were run 31 times with the levels represented as $-\gamma$, -1, 0, $+1$, γ for each variable in the statistical analysis. The PPE yield served as the dependent variable. Table 2 shows the yields of PPE. According to the results, different contents of PPE were obtained in varied conditions.

The mathematical model which represented the extraction of PPE as a function of the independent variables within the region investigated was represented as the equation below:

$$Y = -179.6+0.81X_1+2.61X_2+0.31X_3+0.32X_4-0.0048X_1X_2-0.000015X_1X_3-0.00086X_1X_4-0.00203X_2X_3-0.003225X_2X_4+0.000165X_3X_4-0.00204X^2_1-0.008874X^2_2-0.0002787X^2_3-0.00131X^2_4$$

where, Y is the recovery of PPE and X_1, X_2, X_3 and X_4 are the coded variables of ultrasonic time, temperature,

Table 2: Levels of the main factors in the quadratic regression rotation design

Run number	X1/time (min)	X2/temperature (°C)	X3/ power (w)	X4/Solvent-solid ratio(mL/g)	Y/polysaccharides yield (%)
1	1	1	1	1	6.200
2	1	1	1	-1	8.500
3	1	1	-1	1	9.500
4	1	1	-1	-1	12.10
5	1	-1	1	1	9.200
6	1	-1	1	-1	10.80
7	1	-1	-1	1	10.40
8	1	-1	-1	-1	12.40
9	-1	1	1	1	7.800
10	-1	1	1	-1	9.800
11	-1	1	-1	1	11.10
12	-1	1	-1	-1	13.30
13	-1	-1	1	1	9.850
14	-1	-1	1	-1	11.10
12	-1	1	-1	-1	13.30
13	-1	-1	1	1	9.850
14	-1	-1	1	-1	11.10
15	-1	-1	-1	1	11.03
16	-1	-1	-1	-1	12.70
18	-2	0	0	0	11.70
19	0	2	0	0	9.500
20	0	-2	0	0	11.90
21	0	0	2	0	6.400
22	0	0	-2	0	11.20
23	0	0	0	2	9.100
24	0	0	0	-2	13.03
25	0	0	0	0	11.80
26	0	0	0	0	11.30
27	0	0	0	0	12.01
28	0	0	0	0	11.70
29	0	0	0	0	11.73
30	0	0	0	0	11.50
31	0	0	0	0	11.20

Table 3: Variance analysis

Source	SS	DF	MS	F value	Significance F	Multiple R
Gression	56.17	14	4.01	5.546	0.000817	0.997
Remainder	11.57	16	0.72			
Sum	67.74	30				

power and the ratio of solvent-solid. The results of variance analysis using the model implied a significant relationship between the PPE and the variables at a p-value less than 0.1 with a correlation coefficient of 0.997 (Table 3). The coefficient of determination, which is expressed as R^2 refers to the ratio of the explained variation to the total variation and is applied to measure the extent of the fit. The smaller the R^2, the poorer the correlation of the independent variables in the model. The model shows preferable fit to the measured data as R^2 tends to be unity. By analyzing the variance, the value of R^2 in the model for the extraction of PPE was calculated as 0.9945. This indicated that the actual behavior of the system could be determined favorably using the regression model.

From the calculations and predictions, to obtain the maximum yield of PPE, the extraction needs to be conducted at 95°C with a solvent-solid ratio of 20 mL/g and an ultrasonic power of 213 W for 82 min. These parameters are the optimal conditions for extracting PPE. By extracting PPE under such conditions, 14.1% of PPE was extracted, which showed an insignificant difference with the estimated value of 14.5% based on a 95% confidence interval.

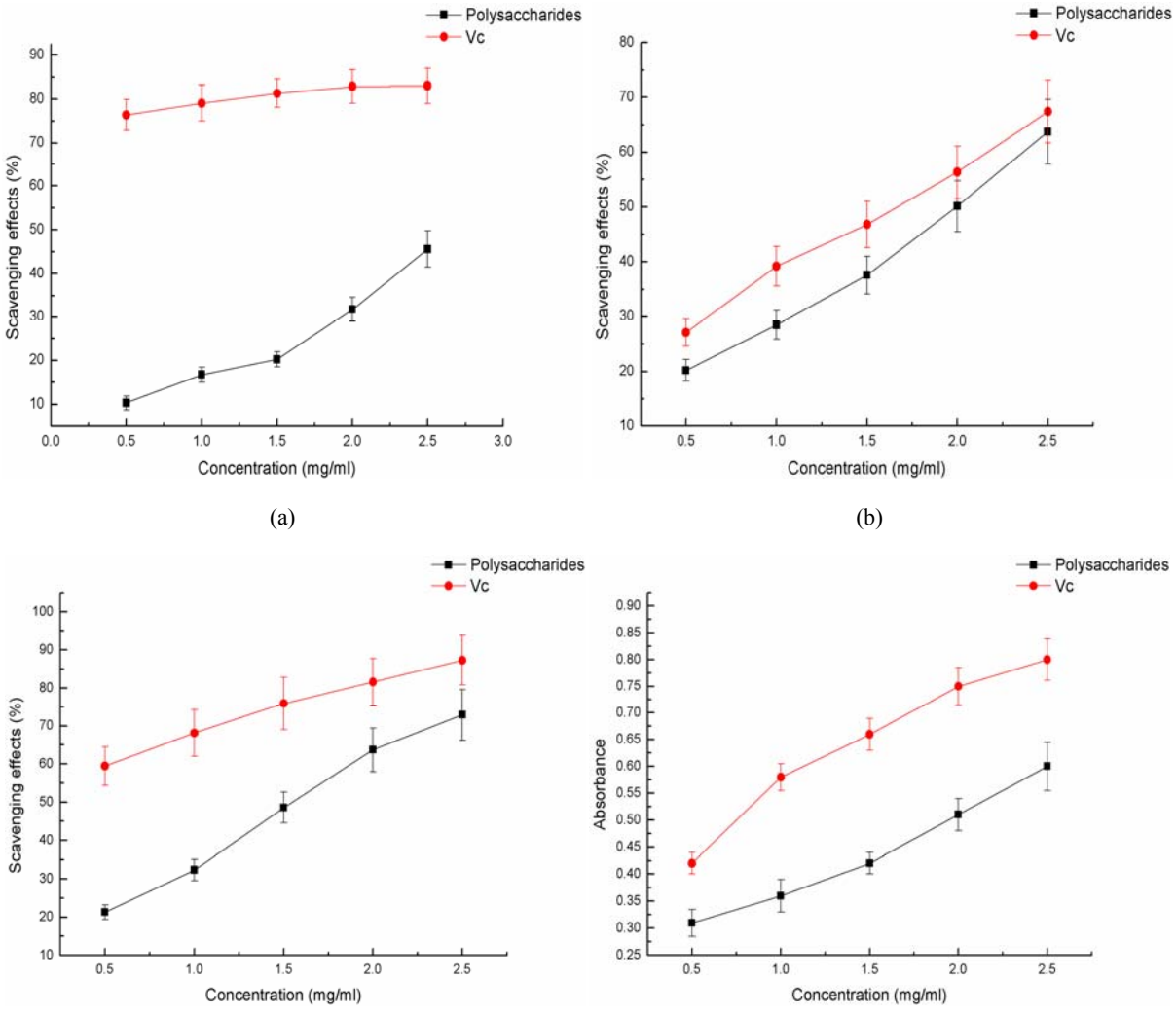
According to the statistical analysis, it can be concluded that, followed by the remarkable interaction effects between two variables (X2 and X3; X2 and X4), the four independent variables and the quadratic of X1, X2, X3 and X4 significantly affected the recovery of PPE ($p<0.01$). It revealed that optimum conditions can be found for extracting PPE.

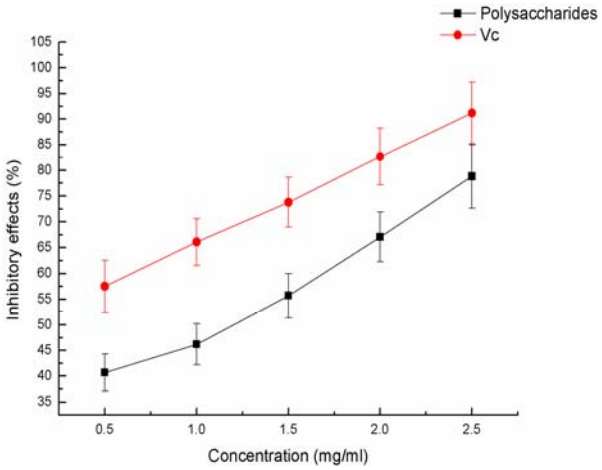
Scavenging activity of polysaccharide for DPPH radical: In this section, the scavenging ability of the extracted polysaccharide against free radical DPPH was tested *in vitro*. The measurement of the ability to remove DPPH describes the ability of a compound to provide the free radicals and ROS with an electron or a hydrogen. Detecting the scavenging capacity for DPPH radicals is a technique extensively applied for assessing

the antioxidant activity of polysaccharide. DPPH, is organic nitrogen radical, it has an ultraviolet-visible absorption at 517 nm and a color which fades with reduction. Figure 2a illustrates the results. In the test, vitamin C was adopted as a positive control. In Fig. 2a, the scavenging activity of the polysaccharide for DPPH radical was dose-dependent. However, the scavenging capacities for radicals of extracts were notably inferior to that of vitamin C which was used as a reference antioxidant. It has been shown that polysaccharide can decrease DPPH radicals which are stable and the antioxidant activity of polysaccharide is closely associated with its chemical structure. Owing to the hydroxyl group of the monosaccharide unit, it can reduce the DPPH radicals by supplying protons. The development of a methoxyl group decreases the property of the monosaccharide unit in providing hydrogen (Yang *et al*., 2010). The reason why PPE serves as an antioxidant may possibly be due to the power of electron donation to the free radicals, thereby ending the reaction of the radical chain.

Scavenging capacity for superoxide anion radicals: Despite showing weak oxidizing ability in the majority of organisms, superoxide is able to degrade continuously and generate other active ROS, induce peroxidation of lipids and thereby trigger pathological incidents. Thus, scavenging superoxide radicals is of significant importance.

- Figure 2b demonstrates the scavenging of vitamin C and the polysaccharide for superoxide anion radicals. The scavenging capacity of polysaccharide for superoxide anions grew with an increase in the concentration of polysaccharide. The EC50 value of polysaccharide, the concentration of samples which are requested to remove 50% of the free radicals, was shown to be





(e)

Fig. 2: Physical and chemical properties of polysaccharides; a: Scavenging effect on DPPH free radicals of PPE; b: Scavenging effect on superoxide radicals of PPE; c: Scavenging effect on hydroxyl radicals of PPE; d: Reducing power of PPE; e: Inhibitory effect of PPE on lipid peroxidation compared to that of ascorbic acid. Results were means ± SD of three parallel measurements

around 2 mg/mL. Qi *et al*. (2006) proposed that the EC50 value of sulfated polysaccharide which is extracted from *Ulva pertusas* varies in the range of 0.06 to 1.60 mg/mL. These values approximates to that of the samples analyzed in the current study.

According to the values of EC50, vitamin C presented a higher scavenging capacity for superoxide anions than polysaccharide. The scavenging activity for superoxide radicals of vitamin C was more potent than that of polysaccharide. However, polysaccharide extracted from *P. eryngii* applying the ultrasonic technique is a possible scavenger of superoxide radicals.

The antioxidant activity of the polysaccharide is potentially associated with molecular size, monosaccharide component and structure. The monosaccharide is a strong reducing agent in the polysaccharide due to its ability to supply hydrogen. When a radical combines the hydrogen, a stable radical can be generated to end the radical reaction. The effect of the polysaccharide removing superoxide anions may be related to the dissociation energy of O-H bond as a larger amount of electrons which are attached to the polysaccharide with attracting groups indicates a weaker energy of the O-H bond. According to existing research, the effect of polysaccharide removing superoxide anions is potentially related to the existence of certain categories of electrophilic groups such as keto- or aldehydes in the former, which accelerate the removal of hydrogen from O-H bonds and therefore stabilize superoxide anion (Lin *et al*., 2009). The antioxidant activity of PPE is not attributed to a single factor but results from a combination of multiple factors.

Scavenging capacity for hydroxyl radicals: Hydroxyl radicals are the most harmful ROS. They are able to readily penetrate cell membranes and react with the majority of biomolecules such as carbohydrate, protein, DNA and lipids in cells, resulting in damage to tissues. The scavenging of hydroxyl radicals is of significant importance for protecting living systems. By applying vitamin C as a positive control, Figure 2c illustrates the scavenging activities of PPE for hydroxyl radicals. It was observed that PPE presented capacity to remove activity against hydroxyl radicals in a manner relying on the concentration as the removing effect improved with the concentration of PPE. The EC50 values of polysaccharide and vitamin C were respectively higher than 1.5 mg/mL and lower than 0.5 mg/mL. Their performance was shown to decreasing in order as vitamin C> polysaccharide according to the EC50. The mechanism of antioxidants for scavenging hydroxyl radicals was potentially because of hydroxyl groups of the monosaccharide in the polysaccharide donating hydrogen for combining with hydroxyl radicals to realize the removing effect. According to Smith *et al*. (1992), molecules which show inhibition of function on the degradation of deoxyribose are those which could chelate iron ions to make them inactive or poorly active in a Fenton reaction. It is commonly know that hydroxyl radicals can be produced in a Fenton reaction. The underlying chelating capacity for metals of the crude extracts obtained in the experiments could also inhibit the oxidation of deoxyribose.

Chen *et al*. (2011) proposed that the antioxidant effect is probably due to the polysaccharide providing hydrogen that binds with radicals to produce more stable radicals. In this way, the reaction of the radical

chain is terminated. There is another assumption that the hydrogen supplied by the polysaccharide binds with the radical ions including Cu^{2+}, which therefore leads to an antioxidant effect (Qi *et al*., 2005). These results imply that PPE can be utilized as a donor of electrons or hydrogen for the removal of hydroxyl radicals.

Reducing capacity: As the reduction of Fe^{3+} can indicate the activity of donating electrons, it is assumed as a significant mechanism for antioxidant activity. To measure the reducing capacity, the transformation of Fe^{3+} to Fe^{2+} using vitamin C and polysaccharide was studied. In Fig. 2d, vitamin C displayed a higher reducing capacity than polysaccharide.

Inhibition of lipid peroxidation: It can be seen from Fig. 2e that PPE displayed an intense inhibitory function on lipid peroxidation and that this effect varied with concentration. The PPE showed an inhibition rate that changed from 40.7 to 78.8 % as the concentration ranged from 0.5 to 2.5 mg/mL, which was less than that of ascorbic acid. A 40.7% inhibition rate was achieved for PPE with a concentration of 0.5 mg/mL, which exceeded those which have been reported for other polysaccharides (Yin *et al*., 2010). The data obtained in this study reveal that PPE exhibits an important inhibitory effect on lipid peroxidation. PPE can be applied in the food industry as an underlying natural antioxidant with high effectiveness and marginal side effects.

CONCLUSION

According to the response surface plots, ultrasonic time, temperature, power and the ratio solvent-solid showed complex and crucial effects on the extraction of polysaccharide. These factors significantly influence the recovery of polysaccharide. To obtain the maximum yield of polysaccharide, the ultrasonic treatment needs to be conducted under optimal ultrasonic conditions at 95°C with 213 w and 20 mg/mL of solvent-solid ratio for 82 min. By applying ultrasonic extraction, a higher yield of polysaccharide was acquired than that obtained for extraction using hot water. Ultrasonic technique was therefore effective in extracting polysaccharide with bioactive activities from *P. eryngii*. By conducting experiments under the optimal ultrasonic conditions mentioned above, a yield of 14.1±0.95% was obtained, without a significant difference ($p>0.05$) with the estimated yield. This explained the favorable estimation ability of the response surface methodology which determined the non-linear characteristics between the extraction of polysaccharide and the ultrasonic conditions. In this study the specific correlation between the structure of polysaccharide and its antioxidant activity was not clarified. Therefore, there is a need for more comprehensive research to accurately determine the mechanism which changes the bioactivity which is the subject of ongoing investigation in our laboratory. It has been verified that the PPE has preferable potential in enhancing the antioxidant capacity based on the detections including the scavenging activity against DPPH which implying that the extraction of PPE exhibits positive effects on anti-oxidation.

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