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PORPHYRA HAITANENSIS CHANG *ET* ZHENG: OPTIMIZATION OF POLYSACCHARIDE EXTRACTION BY RSM AND ITS ANTI-OXIDANT ACTIVITY

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Key words: Porphyra haitanensis, Polysaccharide, Extraction, Microwave, Response surface methodology, Antioxidant

Abstract

Response surface methodology (RSM) was used to investigate the optimum conditions for extracting polysaccharides from *Porphyra haitanensis* Chang *et* Zheng. Results indicated that a ratio of water to raw material of 60 ml/g, pH 8.0 and a microwave-assisted processing time of 5 min at 90°C. Under the conditions the yield was 16.38 ± 0.12% (n = 5), the value within close range of predicted yield value. Moreover, the polysaccharide, at a concentration of 8 mg/ml, showed significant antioxidant activity *in vitro*, where scavenging activity of hydroxyl radicals (•OH), superoxide anions ($\cdot O_2^-$) and DPPH free radicals were 33.81, 51.78 and 31.67%, respectively.

Introduction

In China, there are dozens of *Porphyra* species of which *P. yezoensis* Ueda and *P. haitanensis* Chang *et* Zheng are the main commercially farmed species (Guo *et al.* 2014). *P. haitanensis* is endemic to temperate regions, and abundant at Zhejiang, Fujian and the East China Sea coast along Guangzhou (Na *et al*. 2012). Polysaccharides in addition to traditional industrial uses, also have physiological effects. They act as free-radical scavengers and antioxidants, and can help lower blood glucose and cholesterol. They also have anti-clotting, anti-thrombosis, anti-tumour (Shen *et al*. 2013), and anti-inflammatory properties, and can strengthen cardiac functioning (Hiqashi-Okaja *et al.* 1999), prevent ulcers, and enhance cellular and immunological functions (Figueras *et al*. 2011, Guo and Liu 2012, Jia *et al*. 2014).

There are many existing reports on traditional methods of extracting polysaccharides from seaweed (Wu *et al*. 2004, Yoshizawa *et al*. 1995). However, long extraction times and low yields limit the application of these methods. A recently developed microwave-assisted extraction technique has been increasingly used in the extraction of plant polysaccharides (Wang *et al*. 2012, Samavati and Manoochehrizade 2013). In the present study, microwave-assisted extraction of polysaccharides has been optimized through a combination of univariate (single-factor) experiments and response surface methods (RSM).

Materials and Methods

Porphyra haitanensis Chang *et* Zheng used in this experiment was obtained from Dongtou county in Zhejiang province, China. All chemicals used in the experiment were of analytical grade.

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In this study we used a UV/V-16/18 UV-Visible spectrophotometer, Midea microwave, electric air blowing drying oven, and R206 rotary evaporator.

The seaweed was first dried in an oven for 4 hrs at 60°C. Then it was ground in a blender and passed through a 40 mesh sieve, and then soaked in water. The sample was placed in the microwave at 25°C preset time adjusted pH. The seaweed sample was then placed into a hot water bath, coarsely filtered, centrifuged and then treated with TCA to remove the proteins. Finally, the sample was concentrated using a rotator evaporator, ethanol-precipitated, centrifuged and then freeze dried. In all experiments three replicates were used.

The concentration of polysaccharides was measured by the phenol-sulphuric acid colorimetric method (at 485 nm) using glucose as the standard (Wu *et al*. 2004). The measured absorbance value was plotted against the glucose concentration and the glucose standard curve (Fig. 1). The absorbances were linearly related to the glucose concentration as: $y = 0.0016x - 0.0209$ ($R^2 = 0.9992$), where y is the absorbance value and x is the glucose concentration (µg/ml) (Fig. 1).

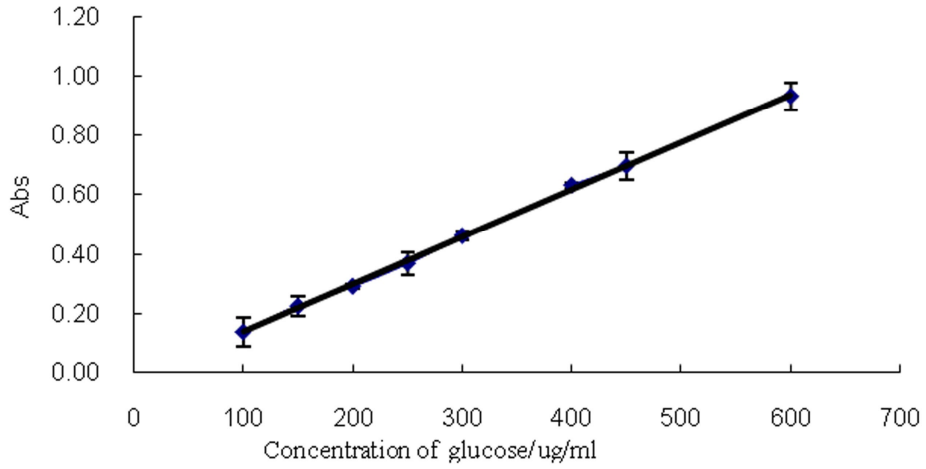

Fig. 1. Glucose standard curve.

The yield of extracted polysaccharides was calculated as:

Yield (%) = Glucose concentration in the polysaccharides × dilution factor × polysaccharide extract volume × 100/the amount of dried seaweed material.

Based on the results from the single-factor experiments, a Box-Benhnken design (BBD) was used to analyze the response surface of each factor described below (Table 1). The polysaccharide yield was set as the dependent variable Y, and the program Design Expert 8.06 was used to create a linear regression model to determine optimum extraction conditions.

The degree of scavenging activity of polysaccharides on hydroxyl radicals (•OH) was determined by the salicylic acid method, based on a modified Smirnoff procedure (Li and Cao 2014). The measured absorbance reflects the concentration of •OH. 3 ml of the reaction mixture was used containing 9 mmol/l of $FeSO_4$, 9 mmol/l of salicylic acid, and 1 ml of the other sample solutions at various concentrations. Later 0.2 ml of 8.8 mmol/l of H_2O_2 was added to initiate the

Table 1. Experimental design for the response surface analysis.

Factor	Code	Level		
		–1	0	1
Ratio of water to material (ml/g)	A	40:1	50:1	60:1
pH	B	7.0	8.0	9.0
Microwave time (min)	C	2	4	6
Extraction temperature (°C)	D	80	90	100

reaction. Distilled water was used as the blank control and vitamin C was used as the positive control. After 30 minutes in the constant temperature water bath at 37°C, the absorbance was measured at a wavelength of 510 nm (A_x), the control absorbance (A_0), and the absorbance of the sample with distilled water replaced by H_2O_2 (A_{x0}). Scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0-(A_x-A_{x0})}{A_0} \times 100\%$$

The scavenging activity on superoxide anion radicals (**•O_2-**) was determined using the pyrogallol auto-oxidation assay (Li and Cao 2014). ΔA_0 represents the pyrogallol auto-oxidation rate and ΔA is the pyrogallol auto-oxidation rate after sample addition at absorbance increments per minute. Each group was measured three times, averaged, and expressed as:

$$\text{Scavenging activity (\%)} = \frac{\Delta A_0-\Delta A}{\Delta A_0} \times 100$$

The scavenging activity on 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was determined using the same method employed by Leong and Shui (2002). A_x represents the absorbance after the addition of the sample and DPPH, A_{x0} is the initial sample absorbance, and A_0 is the absorbance of DPPH with distilled water. The scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0-(A_x-A_{x0})}{A_0} \times 100$$

Results and Discussion

Based on the values determined by the single-factor experiments, the extraction parameters for the independent variables (ratio of water to material [A], pH [B], microwave time [C], and extraction temperature [D]) were set up using the Box-Behnken factorial design at three levels (-1, 0, 1) (Table 2). Polysaccharide extraction yield was set as the response variable Y.

The experiments were performed according to the levels set in Table 2. The multiple regression test data was fitted using a quadratic equation with maximum yield Y as the objective function. In the experimental equation, A is the ratio of water to material, B is the pH, C is the microwave time, and D is the extraction temperature:

$Y = 15.81 + 1.08A + 1.04B + 1.01C + 1.99D – 0.47AB – 0.19AC + 0.34AD – 0.076BC – 0.12BD – 0.26CD – 0.76A^2 – 0.76B^2 – 0.63C^2 – 1.84D^2$.

The size of each coefficient directly reflects the influence of the factor on the yield, while the sign of the coefficient shows whether the factor has a positive or negative impact on the yield.

Table 2. Experimental design and results of response surface methodology.

No.	Ratio of water to material ml/g (A)	pH (B)	Microwave time min (C)	Extraction temeprature °C (D)	Yield% (Y)
1	(-1) 40:1	(-1) 7.0	(0) 4	(0) 90	11.8092
2	(+1) 60:1	(-1) 7.0	(0) 4	(0) 90	14.8869
3	(-1) 40:1	(+1) 9.0	(0) 4	(0) 90	14.7462
4	(+1) 60:1	(+1) 9.0	(0) 4	(0) 90	15.9255
5	(0) 50:1	(0) 8.0	(-1) 2	(-1) 80	10.1261
6	(0) 50:1	(0) 8.0	(+1) 6	(-1) 80	12.6753
7	(0) 50:1	(0) 8.0	(-1) 2	(+1) 100	14.6148
8	(0) 50:1	(0) 8.0	(+1) 6	(+1) 100	16.1377
9	(-1) 40:1	(0) 8.0	(0) 4	(-1) 80	10.4195
10	(+1) 60:1	(0) 8.0	(0) 4	(-1) 80	11.8872
11	(-1) 40:1	(0) 8.0	(0) 4	(+1) 100	13.6902
12	(+1) 60:1	(0) 8.0	(0) 4	(+1) 100	16.5309
13	(0) 50:1	(-1) 7.0	(-1) 2	(0) 90	12.1908
14	(0) 50:1	(+1) 9.0	(-1) 2	(0) 90	14.4469
15	(0) 50:1	(-1) 7.0	(+1) 6	(0) 90	14.3702
16	(0) 50:1	(+1) 9.0	(+1) 6	(0) 90	16.3223
17	(-1) 40:1	(0) 8.0	(-1) 2	(0) 90	12.1418
18	(+1) 60:1	(0) 8.0	(-1) 2	(0) 90	14.7419
19	(-1) 40:1	(0) 8.0	(+1) 6	(0) 90	14.5192
20	(+1) 60:1	(0) 8.0	(+1) 6	(0) 90	16.3407
21	(0) 50:1	(-1) 7.0	(0) 4	(-1) 80	10.0389
22	(0) 50:1	(+1) 9.0	(0) 4	(-1) 80	12.415
23	(0) 50:1	(-1) 7.0	(0) 4	(+1) 100	14.2706
24	(0) 50:1	(+1) 9.0	(0) 4	(+1) 100	16.1786
25	(0) 50:1	(0) 8.0	(0) 4	(0) 90	15.654
26	(0) 50:1	(0) 8.0	(0) 4	(0) 90	15.8254
27	(0) 50:1	(0) 8.0	(0) 4	(0) 90	15.7978
28	(0) 50:1	(0) 8.0	(0) 4	(0) 90	15.9405
29	(0) 50:1	(0) 8.0	(0) 4	(0) 90	15.8161

The regression analysis and variance of coefficients related to the polysaccharide extraction yield values are given in Table 3.

The single factors A, B, C, D and the interaction factors AB and AD have extremely significant regression coefficients, demonstrating that ratio of water to material, pH, microwave time, extraction temperature, and the interaction between ratio of water to material and pH, and the interaction of ratio of water to material and extraction temperature all have very large influences on polysaccharide yield.

The F-value was calculated as 825.12, demonstrating that the result is significant. The lack of fit F-value is 0.91, which shows that the result is not significant relative to the pure error. This indicates that the model equation is adequate for predicting polysaccharide extraction yield under any combination of variables' values.

Using the data from Table 2, the optimum conditions were determined based on the response surface analysis within the experimental boundaries. The effects of the ratio of water to material, pH, microwave time, and extraction temperature on yield based on the response surface and contour plots (Fig. 2).

Table 3. Variance analysis of the regression equation.

Source	Sum	Degrees of freedom	Mean square	F value	Pr>F
Model	112.06	14	8.05	825.12	<0.0001
A	14.06	1	14.06	1440.92	<0.0001
B	12.95	1	12.95	1328.04	<0.0001
C	12.21	1	12.21	1251.45	<0.0001
D	47.44	1	47.44	4863.99	<0.0001
AB	0.90	1	0.90	92.36	<0.0001
AC	0.15	1	0.15	15.54	0.0015
AD	0.47	1	0.47	48.32	<0.0001
BC	0.023	1	0.023	2.37	0.1461
BD	0.055	1	0.055	5.62	0.0327
CD	0.26	1	0.26	27.00	0.0001
A2	3.73	1	3.73	381.99	<0.0001
B2	3.77	1	3.77	386.80	<0.0001
C2	2.61	1	2.61	267.59	<0.0001
D2	21.95	1	21.95	2250.67	<0.0001
Residual	0.14	14			
Lack of fit	0.095	10		0.91	0.5922
Error	0.042	4			
Total deviation	112.80	28			

Pr < 0.001 indicates a distinctly significant difference; Pr < 0.01 indicates a high significant difference;

Pr < 0.05 indicates a significant difference.

Comparing the data within this figure, yield increases in direct relation to both ratio of water to material and extraction temperature, and shows a significant interaction between these two factors (Fig. 2C). Increasing the ratio of water to material increased the liquid volume, which improved the efficiency of the dissolution of the polysaccharides into the extracting medium. According to the kinetic energy theory, increasing the extraction temperature allows for a greater dispersion of the polysaccharides. As a result, combining an increase in both the ratio of water to material and the extraction temperature contributed to greater yields. The effect of pH is slightly more significant than that of the other factors, as evidenced by the greater curvature of the response plot (Fig. 2A, D, E). In contrast, microwave time had the least significant effect on the production of polysaccharides.

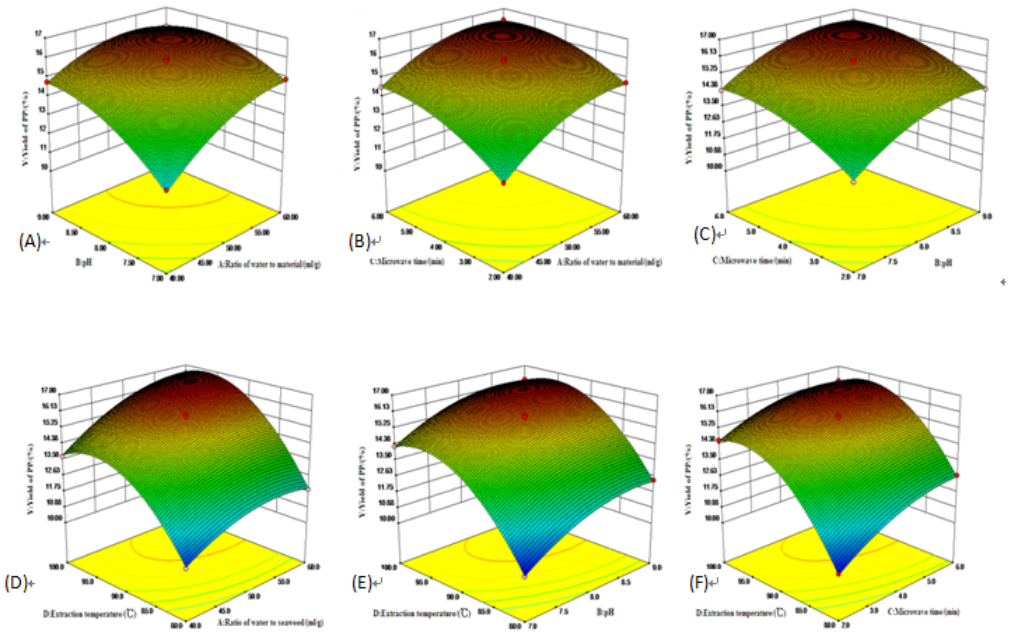

Fig. 2. Response surface plots of extraction parameters on the yield of polysaccharide: (A) ratio of water to material and pH, (B) ratio of water to material and microwave time, (C) ratio of water to material and extraction temperature, (D) pH and microwave time, (E) pH and extraction temperature and (F) microwave time and extraction temperature.

The software's numerical function was used for optimization, starting from the lowest experimental boundaries and selecting a maximum value for the response variable. Based on the data, it was determined that the optimum conditions for polysaccharide extraction are: a ratio of water to material of 56.36 ml/g, pH 8.41, a microwave time of 5.12 minutes, and an extraction temperature of 95.48°C. Under these conditions, a maximum yield of 17.11% was attained. For practical purposes, the optimum conditions were adjusted as follows: a ratio of water to material of 60 ml/g, pH 8.0, microwave time of 5 minutes, and an extraction temperature of 90°C. Under these adjusted conditions, after five replications, the optimized extraction yield was 16.38 ± 0.12%, differing from the predicted yield by 4.27%. Since the experimental and predicted yields are within 5%, we determined the model suitable for use.

Using Vc as a comparison, the polysaccharide scavenging activity for the hydroxyl radical (•OH) in a concentration range of 2.0 to 10.0 mg/ml (Fig. 3). This result shows that the polysaccharide has a scavenging effect on •OH at all concentrations. Furthermore, a proportional relationship was observed between polysaccharide concentration and scavenging activity. A maximum scavenging activity of 34.69% was achieved with a polysaccharide concentration of approximately 10.0 mg/ml, although the scavenging activity was still significantly lower compared to that of Vc.

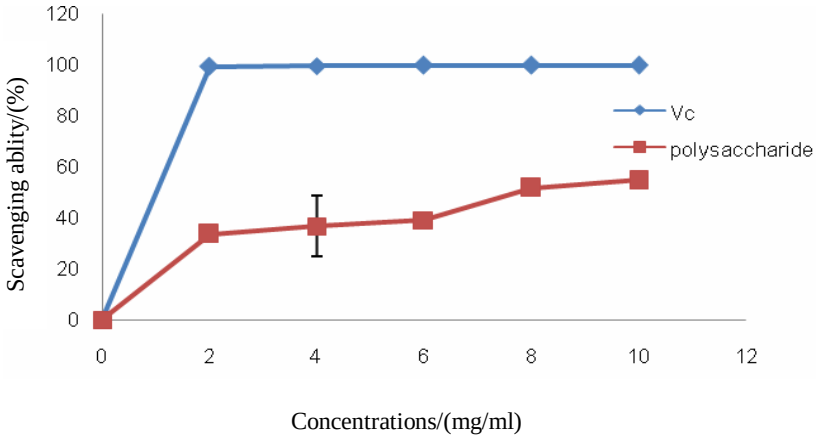

Fig. 3. Scavenging effect of polysaccharide on hydroxyl radicals (•OH).

Scavenging activity of the polysaccharide (2.0 to 10.0 mg/ml) on superoxide anions (O_2-) in Fig. 4. As with •OH, the data shows that the polysaccharide has a scavenging effect on O_2- at all concentrations. A maximum scavenging activity of 54.73% was achieved with a polysaccharide concentration of 10.0 mg/ml, although the scavenging activity was again lower than the activity of Vc.

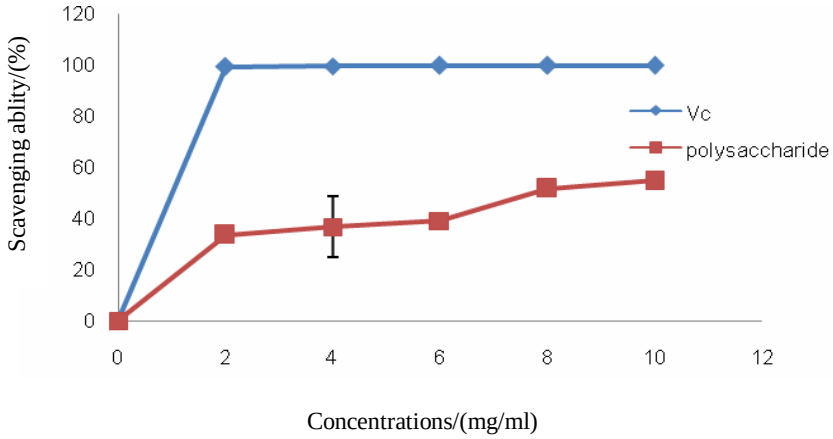

Fig. 4. Scavenging effect of polysaccharide on superoxide anions (•O_2-).

The scavenging activity of the polysaccharide increases as concentration increases (Fig. 5). The maximum scavenging activity was achieved at a concentration of 6 mg/ml. However, the scavenging activity was still considerably lower than Vc.

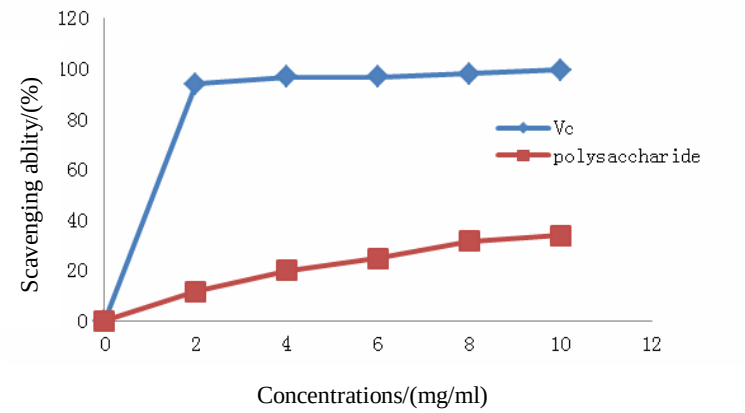

Fig. 5. Scavenging effect of polysaccharide on DPPH free radicals.

This study determined the optimal extraction parameters of multiple variables, including the ratio of water to material, pH, microwave time, and extraction temperature using single factor experiments. Moreover, we utilized RSM and linear regression to produce a statistically significant model, which could be further utilized to predict the effects of these factors on polysaccharide yield.

Our results confirmed that all four of the parameters had a significant effect on the extraction yield. The optimum conditions for extraction were found to be : a ratio of water to material of 56.36 ml/g, a pH of 8.41, a microwave time of 5.12 minutes, and an extraction temperature of 95.48°C. Under these conditions, the ideal extraction yield was 17.11%. Subsequently, for practical purposes, the optimum conditions were set as 60 ml/g, pH 8.0, 5 minutes, and 90°C, respectively. Using these conditions in five replication experiments, the extraction yield was 16.38%, a value with a difference of 4.27% from the ideal extraction yield. Therefore, this model is suitable for use in predicting extraction yields within the boundaries of these parameters.

The polysaccharide's antioxidant activity, including its scavenging activities on hydroxyls (•OH), superoxide anions (•O_2-), and DPPH free radicals, was also studied. Results revealed that the scavenging activity was dependent on polysaccharide concentration; antioxidant activities increased as polysaccharide concentration increased. At a concentration of 8 mg/mL, the scavenging activities of the polysaccharide on •OH, O_2- and DPPH were 33.81, 51.78, and 31.67% respectively, demonstrating the antioxidant activity capability of the polysaccharide.

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