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Optimization of enzymatic extraction of polysaccharides from some marine algae by response surface methodology

Shengnan Li, Heng Zhang, Dandan Han, and Kyung Ho Row†

Department of Chemical Engineering, Inha University, Incheon 402-751, Korea
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Abstract–A novel enzymatic extraction combined with response surface methodology was developed for polysaccharide extraction from marine algae. Box-Behnken design was employed to optimize concentration of enzyme, ratio of water to raw material and extraction time for high extraction yields. The optimal extraction conditions were as follows: a concentration of enzyme of 778.01 mg/g with ratio of water to raw material of 86.80 and extraction time of 129.93 min for *Gelidium amansii* and a concentration of enzyme of 997.03 mg/g with ratio of water to raw material of 98.76 and extraction time of 117.69 min for *Laminaria japonica Aresch*. Under the optimal conditions, extraction yields were 48.36% and 32.47%, respectively. Experimental data were fitted by multiple regression analysis to a second-order polynomial equation and were statistically analyzed. The predicted model matched the experimental data well with coefficients of determination (R^2) of 0.9864 and 0.9892, respectively.

Key words: Marine Algae, Enzymatic Extraction, Polysaccharides, Response Surface Methodology

INTRODUCTION

Marine algae are potentially abundant sources of diverse bioactive compounds. *Gelidium Amansii* (*GA*: red algae) and *Laminaria japonica Aresch* (*LA*: brown algae) have attracted much interest as new drugs and health foods [1-3]. Many species of marine algae contain significant quantities of polysaccharides that have shown anti-tumor, anti-viral, anti-oxidant, anti-infective effects and anti-mutagenic or hematopoietic activity, especially red and brown algae [4,5]. Polysaccharides have polymeric carbohydrate structures, comprising repeated monosaccharide units joined by glycosidic bonds [6]. Their broad functional and physicochemical properties give these natural biopolymers potential pharmaceutical effect.

However, the cell walls of plants mainly consist of cellulose, which would hamper polysaccharide extraction. Though physical methods such as microwave and ultrasound can disrupt cell walls, they consume much energy with low efficiency. Recently, enzymatic extraction has been widely used in the extraction of bioactive compounds from land plants [7-9]. It is a more attractive alternative, with many advantages such as shorter processing time, less pollution, higher extraction yield, better products at lower cost and less decomposition of target compounds. Cellulase is a complex product which includes β-glucanase, endo-β-glucanase and β-glucosidase and so on. This enzyme can cleave intact glycosaminoglycan from a core peptide by hydrolyzing the xylosyl serine linkage, while it does not cleave cellobiose or *p*-nitrophenyl-β-D-glucoside. Enzymatic hydrolysis can disrupt cell walls, accelerate the migration rates of target compounds from the material to the surroundings and increase the contact area to improve extraction efficiency of polysaccharides. Compared with conventional extraction, less energy is exerted and a higher extraction yield is obtained [10].

†To whom correspondence should be addressed.
E-mail: rowkho@inha.ac.kr

Furthermore, to optimize the extraction conditions, computer modeling has been applied frequently. There are many different kinds of designs in RSM, such as Plackett-Burman design (PBD), Box-Behnken design (BBD), central composite design (CCD), surrogate model, probabilistic design, and so on [11-16]. Box-Behnken design (BBD), which belongs to response surface methodology (RSM), was found to be the easiest and most common design method for optimizing the extraction condition [17,18].

There was no research about the enzymatic extraction of polysaccharide from marine algae using RSM. So in this study, this method was applied to extract polysaccharide from *GA* and *LA*. To simplify the extraction process and obtain the highest extraction yield, the extraction parameters such as extraction time, enzyme concentration and the ratio of water to raw material were optimized by BBD from RSM.

EXPERIMENTAL

1. Materials and Reagents

GA and *LA* were bought from a local market (Incheon, Korea). Cellulase, D-(+)-Glucose (99.5%) and phenol were obtained from Sigma-Aldrich (U.S.A). Ethanol (99.9%) and sulfuric acid were bought from Duksan Pure Chemical Co., Ltd (Korea). All the other solvents used in the experiment were analytical grade.

2. Instrumentation

Deionized water was filtered with a vacuum pump (Division of Millipore, Waters, U.S.A.) and filter (HA-0.45, Division of Millipore, Waters, U.S.A.) before use. Filter paper (No.5A, 110 mm) was from Hyundai Micro Co., Ltd (Korea). Micro high centrifuge (H17R+) was from Hanil Science Industrial Co., Ltd (Korea). UV-VIS-NIR spectroscopy was bought from PerkinElmer (U.S.A.) for analysis of polysaccharides.

3. Extraction of Polysaccharides

To remove the alcohol soluble composition (alkaloid, flavone gly-

coside, cardiac glycoside, saponins, pigment), *GA* (100 g) and *LA* (100 g) were dipped in ethanol (200 mL) at an 80 °C water bath for 2 h [19]. According to the property of cellulase, enzymic hydrolysis was conducted under 37 °C at pH 5.5 for 2 h, and then cellulase was inactivated by heating at 100 °C for 10 min. Each pretreated sample was extracted by water at various extraction times, concentrations of enzyme, and ratios of water to raw material. The aqueous extraction solutions were precipitated after 1 day by addition of 80% ethanol. Insoluble residue was separated by centrifugation (10,000 rpm, 20 min). The precipitate was filtered and dried at 50 °C for 24 h. The dried crude polysaccharides were refluxed three times with acetone and filter to remove lipids. The crude product was dissolved in water and then filtered. The obtained sample was used for the following analysis [6].

4. Quantitative Analysis of Polysaccharides

Polysaccharides were analyzed as described in *Phenol-Sulfuric acid methods* [20]. 400 μL samples and 400 μL 5% phenol solutions mixed with 2,000 μL 95% H_2SO_4. The mixture was strongly vortexed under room temperature for 20 min. D-(+)-Glucose was used as a standard sample for measurement of the total sugar content. The absorbance of samples was measured by UV-VIS-NIR spectroscopy at 490 nm. The polysaccharide yield (%) was then calculated using the following equation:

$$\text{Polysaccharide yield } (\%) = \frac{C \times N \times V}{W \times 1000} \times 100\% \quad (1)$$

Where C is the concentration of polysaccharide calculated from the calibrated regression equation (mg/mL); N is the dilution factor; V is the total volume of extraction solution (mL); and W is the mass of raw material (g).

5. Experimental Design

The extraction conditions of polysaccharides from marine algae were optimized by a 15-run BBD method. Although there are many factors such as temperature, extraction solvent and pH which could affect the extraction efficiency, according to the product description, cellulase (Sigma-Aldrich, USA) should be conducted at less than 37 °C at pH 5.5. In this case, concentration of enzyme, extraction time and the ratio of water to raw material are the most effective factors to extraction yields. Because both cellulose and polysaccharides can be well dissolved in water, water was selected as the extraction solvent. Therefore, the independent processing variables were concentration of enzyme (X_1), ratio of water to raw material (X_2) and extraction time (X_3). A central composite design was used for the optimization of each variable at three levels with 15 runs, including three replicates at the central point. To predict the global optimum conditions, the first step of this work was to select a suitable range of each independent factor, and to make sure that the optimum is located within the boundaries of the experimental limits. The range and levels of the independent variables and code values are listed in Table 1 and were based on the results of preliminary experiments. Yields of polysaccharides from *GA* and from *LA* were used as the responses for each combination of the independent variables given in Table 2. Three experiments at each condition were carried out with their mean listed as the observed response. Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses.

The experimental design was analyzed with Design-Expert software (v.8.0.4, Stat-Ease, Inc, Minneapolis, USA) and fitted to a second-order polynomial regression model containing coefficients of linear, quadratic, and two-factor interaction effects. The model equation of response (Y) to the three independent variables (X_1, X_2, and X_3) is,

Table 1. Independent variables and experimental domain of Box-Behnken design (BBD)

Independent variables		Levels		
		−1	0	1
X_1	Concentration of enzyme (mg/g)	200	600	1000
X_2	Ratio of water to raw material	40	70	100
X_3	Extraction time (min)	40	120	200

Table 2. Box-Behnken design and the response values for yields of polysaccharides

Run	Coded variable levels			Yield of polysaccharides (Y_1, %)		Yield of polysaccharides (Y_2, %)	
	X_1	X_2	X_3	Experimental values	Predicted values	Experimental values	Predicted values
1	0	0	0	38.93	38.93	21.94	21.94
2	−1	−1	0	14.30	13.94	10.17	10.05
3	−1	1	0	27.77	26.44	15.27	14.49
4	1	1	0	41.16	41.50	27.68	27.81
5	0	−1	−1	13.76	11.75	10.01	8.88
6	0	−1	1	19.21	20.25	12.81	13.22
7	−1	0	−1	15.29	17.64	10.46	11.71
8	0	0	0	38.93	38.93	21.94	21.94
9	−1	0	1	30.85	30.16	17.69	17.41
10	1	0	1	47.55	45.20	30.94	29.69
11	0	0	0	38.93	38.93	21.94	21.94
12	0	1	−1	21.29	20.25	13.42	13.00
13	0	1	1	43.26	45.27	28.02	29.14
14	1	−1	0	19.16	20.48	11.32	12.15
15	1	0	−1	23.51	24.20	14.65	14.91

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} A_{ij} X_i X_j \quad (2)$$

where Y is the dependent variable, A_0 is a constant, A_i is the linear coefficient (main effect), A_{ii} is the quadratic coefficient, and A_{ij} is the two-factor interaction coefficient.

The variables were coded according to the equation:

$$x = \frac{(X_i - X_0)}{\Delta X} \quad (3)$$

where X_i is the (dimensionless) coded value of the variable X_i, X_0 is the value of X_i at the center point, and ΔX is the step change of the independent variable. Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

RESULTS AND DISCUSSIONS

1. Fitting the Models

Polysaccharide extraction in the BBD was optimized over 15 runs with three levels. The values of the independent variables (X_1, X_2 and X_3) and the measured and predicted polysaccharide yields (Y_1: from *GA*, Y_2: from *LA*) are listed in Table 2. The experimental data were used to calculate the coefficients of the second-order polynomial equation and close agreement between actual and predicted values. *GA* had a wide range of yields from 13.76% to 47.55%, with the maximum yield found at X_1=1,000 mg, X_2=70 and X_3=200 min. Yields of polysaccharides from *LA* ranged from 10.01% to 30.94%, with the maximum yield found under the same conditions. The conditions of the optimal process were analyzed so as to obtain high extraction yields.

By applying multiple regression analysis to the experimental data, the fitted quadratic models for the response (extraction yields) and the tested variables (in coded units) are given by the following predictive equations:

$$Y_1 = 38.93 + 5.40X_1 + 8.38X_2 + 8.38X_3 - 4.21X_1^2 - 9.13X_2^2 - 5.42X_3^2 + 2.13X_1X_2 + 2.12X_1X_3 + 4.13X_2X_3 \quad (4)$$

$$Y_2 = 21.94 + 3.87X_1 + 5.01X_2 + 5.12X_3 - 1.73X_1^2 - 4.10X_2^2 - 1.78X_3^2 + 2.82X_1X_2 + 2.27X_1X_3 + 2.95X_2X_3 \quad (5)$$

where Y_1 and Y_2 are the polysaccharide yields and X_1, X_2, and X_3 are the linear terms of concentration of enzyme, the ratio of water to raw material, and the extraction time, respectively.

Table 3. Analysis of variance of the experimental results of the Box-Behnken design (BBD)

Source	Sun of squares	DF	Mean square	*F*-value	*P*-value
Yield (Y_1)					
Model	1888.55	9	209.84	40.36	0.0004
X_1	232.96	1	232.96	44.80	0.0011
X_2	561.96	1	561.96	108.08	0.0001
X_3	561.46	1	561.46	107.99	0.0001
X_1X_2	18.19	1	18.19	3.50	0.1203
X_1X_3	17.98	1	17.98	3.46	0.1221
X_2X_3	68.23	1	68.23	13.12	0.0152
X_1^2	65.33	1	65.33	12.56	0.0165
X_2^2	307.53	1	307.53	59.15	0.0006
X_3^2	108.62	1	108.62	20.89	0.0060
Residual	26.00	5	5.20		
Lack of fit	26.00	3	8.67		
Pure error	0.00	2	0.00		
Cor total	1914.55	14			
Yield (Y_2)					
Model	693.65	9	77.07	50.89	0.0002
X_1	120.13	1	120.13	79.32	0.0003
X_2	200.80	1	200.80	132.60	<0.0001
X_3	209.31	1	209.31	138.21	<0.0001
X_1X_2	31.70	1	31.70	20.93	0.0060
X_1X_3	20.52	1	20.52	13.55	0.0143
X_2X_3	34.81	1	34.81	22.99	0.0049
X_1^2	11.05	1	11.05	7.30	0.0427
X_2^2	62.07	1	62.07	40.99	0.0014
X_3^2	11.63	1	11.63	7.68	0.0393
Residual	7.57	5	1.51		
Lack of fit	7.57	3	2.52		
Pure error	0.00	2	0.00		
Cor total	701.22	14			

Table 3 summarizes the results of fitting the models to the data by the F-test and *p*-value. The results of analysis of variance (ANOVA) indicate that the contribution of the quadratic model was significant for responses of extraction yields. For any of the terms in the model, the corresponding variables would be more significant if the absolute *F*-value becomes greater and the *p*-value becomes smaller (Gan and Latiff, 2011).

1-1. Extraction Yield of GA

It can be observed that the variables with the largest effect ($p<0.05$) on extraction yield were linear terms of ratio of water to raw material (X_2) and extraction time (X_3) followed by inear term of concentration of enzyme (X_1), interaction terms of ratio of water to raw material and extraction time (X_2X_3) and all the quadratic terms ($X_2^2<X_3^2<X_1^2$) (Table 3 (Yield Y_1)). However, the interaction terms of concentration of enzyme and ratio of water to raw material (X_1X_2) and concentration of enzyme and extraction time (X_1X_2) were found insignificant to this contribution. The model F-value of 40.36 implies the model was significant, and there was only a 0.04% chance that a "Model *F*-value" this large could occur due to noise. Table 4 shows that the coefficient of determination (R^2) was 0.9864, and the R^2_{Pred} of 0.7827 is in reasonable agreement with the R^2_{Adj} of 0.9620. The signal to noise ratio, measured by the "Adeq Precision", greater than 4 is desirable. The ratio of 18.003 indicated an adequate signal and the model can be used to navigate the design space. These values would give a relative good fit to the mathematic model in Eq. (4).

1-2. Extraction Yield of LA

Linear term of ratio of water to raw material (X_2) and extraction time (X_3) gave the largest effect ($p<0.0001$) on extraction yield followed by concentration of enzyme (X_1) in Table 3 (Yield Y_2). In addition, all the interaction terms ($X_2X_3<X_1X_2<X_1X_3$) and all the quadratic terms ($X_2^2<X_3^2<X_1^2$) showed significant effect ($p<0.05$). The model *F*-value of 50.89 suggested that the model is significant, and there was only a 0.02% chance that a "Model *F*-value" could occur because of noise. According to Table 4, the coefficient of determination (R^2) in this response was 0.9892, the R^2_{Pred} was 0.8272 and the R^2_{Adj} was 0.9698, which suggested an excellent fit to the mathematical model in Eq. (5). At the same time, the coefficient of variation (C.V.-6.88%) had a low value, indicating a high precision and reliability of the experimental values. Furthermore, the Adeq Precision of 20.701 implies an adequate signal and the model can be used to navigate the design space. The predicted models seemed to reasonably describe the observed values. Thus, the responses were sufficiently explained by the models.

2. Analysis of Response Surface

The relationship between independent and dependent variables was illustrated by the three-dimensional (3D) representation of the response surfaces and the two-dimensional contours (2D) generated by the model (Figs. 1-6). The 3D response surfaces and 2D contour plots, which were the graphical representations of regression equation, provide a method to visualize the relationship between

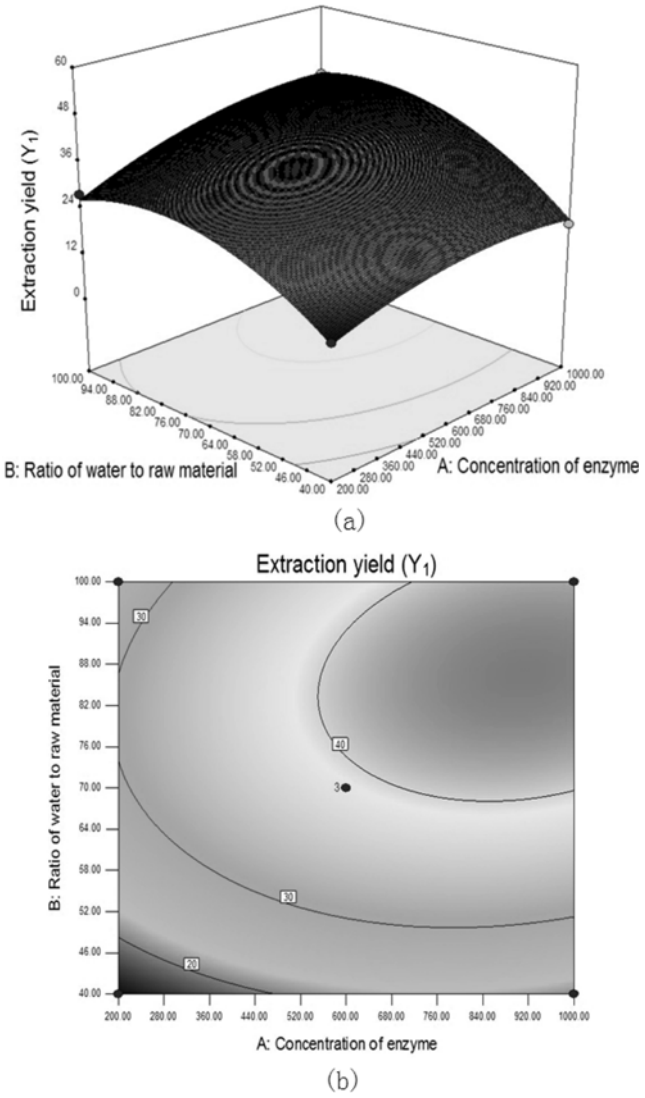

Fig. 1. Response surface plots (a) and contour plots (b) showing the effect of concentration of enzyme (X_1) and ratio of water to material (X_2) on the yield of *GA* polysaccharides.

responses and experimental levels of each variable as well as the type of interactions between two test variables. According to circular or elliptical contour plots, the mutual interactions between the variables can be estimated. The interactions between the corresponding variables were negligible due to the circular contour plot, while an elliptical contour plot indicates that the interactions between the corresponding variables are significant.

2-1. Response Surface Analysis of GA

The interaction relationships of concentration of enzyme with the ratio of water to raw material and extraction time on yield of polysaccharides are shown in Figs. 1-3, which indicates the three

Table 4. Analysis of variance for the fitted quadratic polynomial model of extraction of polysaccharides

Item	Std. Dev.	Mean	C.V. %	Press	R^2	R^2_{Adj}	R^2_{Pred}	Adeq precision
Y_1	2.28	28.93	7.88	415.95	0.9864	0.9620	0.7827	18.003
Y_2	1.23	17.88	6.88	121.15	0.9892	0.9698	0.8272	20.701

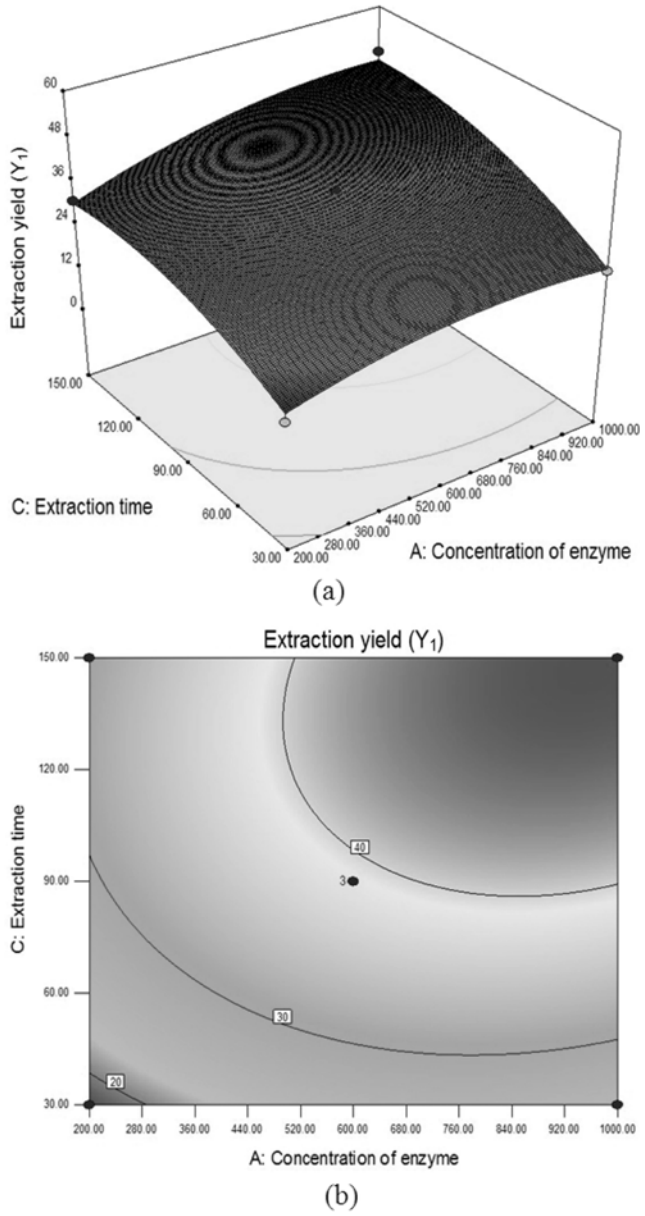

Fig. 2. Response surface plots (a) and contour plots (b) showing the effect of concentration of enzyme (X_1) and extraction time (X_3) on the yield of *GA* polysaccharides.

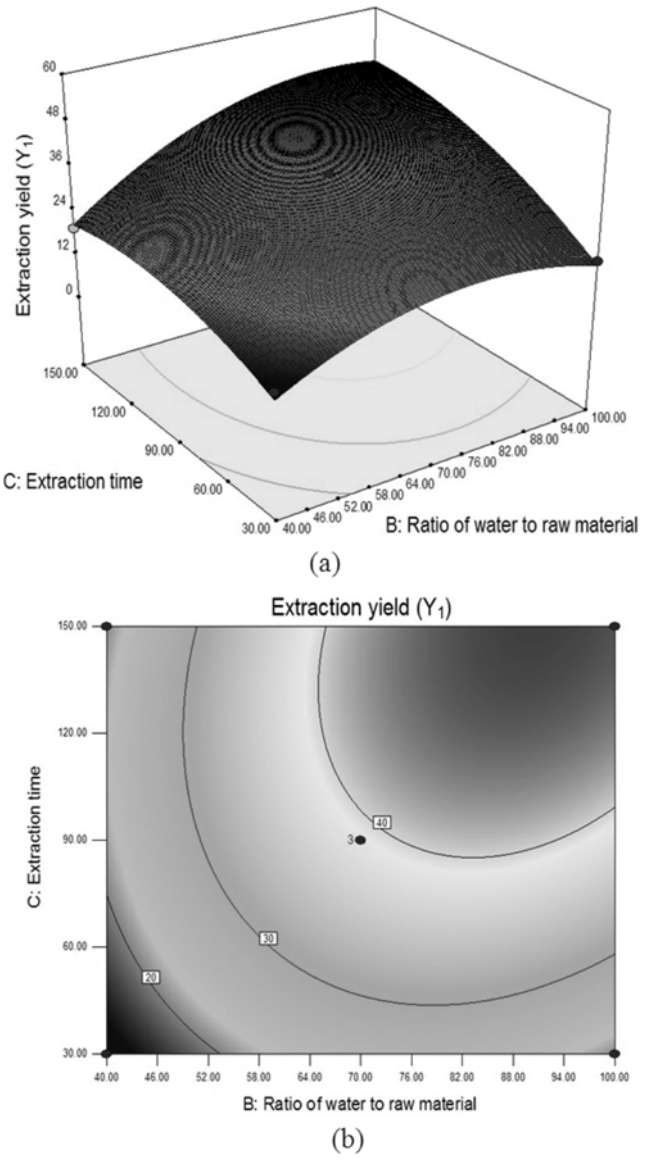

Fig. 3. Response surface plots (a) and contour plots (b) showing the effect of ratio of water to material (X_2) and extraction time (X_3) on the yield of *GA* polysaccharides.

variables all had significant effect on the extraction yield of polysaccharides. Each contour curve represents an infinite number of combinations of two test variables, while the other factor was fixed at the zero level. In Fig. 1(a) and (b), the ratio of water to raw material demonstrated a quadratic increase of this response (from 14.30% to 41.16%) when concentration of enzyme increased smoothly and the extraction time was fixed at 90 min. As shown in Fig. 2(a) and (b), both concentration of enzyme and extraction time displayed a positive impact on the response when the ratio of water to material was fixed at 70. The extraction yield rapidly improved with increasing extraction time and reached 30.85%, and then the yield changed slightly during the concentration of enzyme in the range of 200 mg/g to 1,000 mg/g. Likewise, the response increased from 13.76% to 43.26% with the ratio of water to raw material and extraction time increasing (Fig. 3(a) and (b)).

2-2. Response Surface Analysis of LA

The significant effects of the three variables are given in Figs. 4-6. Fig. 4(a) and (b) showed that when the extraction time fixed at 90 min, the extraction yield increased rapidly ranging from 10.17% to 27.68, and the range of concentration of enzyme was 200 to 1,000 mg/g, ratio of water to material was 40 to 100, correspondingly. According to Fig. 5(a) and (b), both f concentration of enzyme and extraction time gave a positive impact on the response, which ranged from 10.46% to 30.94% when the ratio of water to raw material was fixed at 70. In Fig. 6(a) and (b), the extraction yield increased when the ratio of water to raw material ranged from 40 to 100, and there was a quadratic effect on the response with the extraction time increasing from 30 to 150 min. All these results could be explained that enzymatic extraction with an advisable time and ratio of water to material favors the extraction of polysaccharides through disrup-

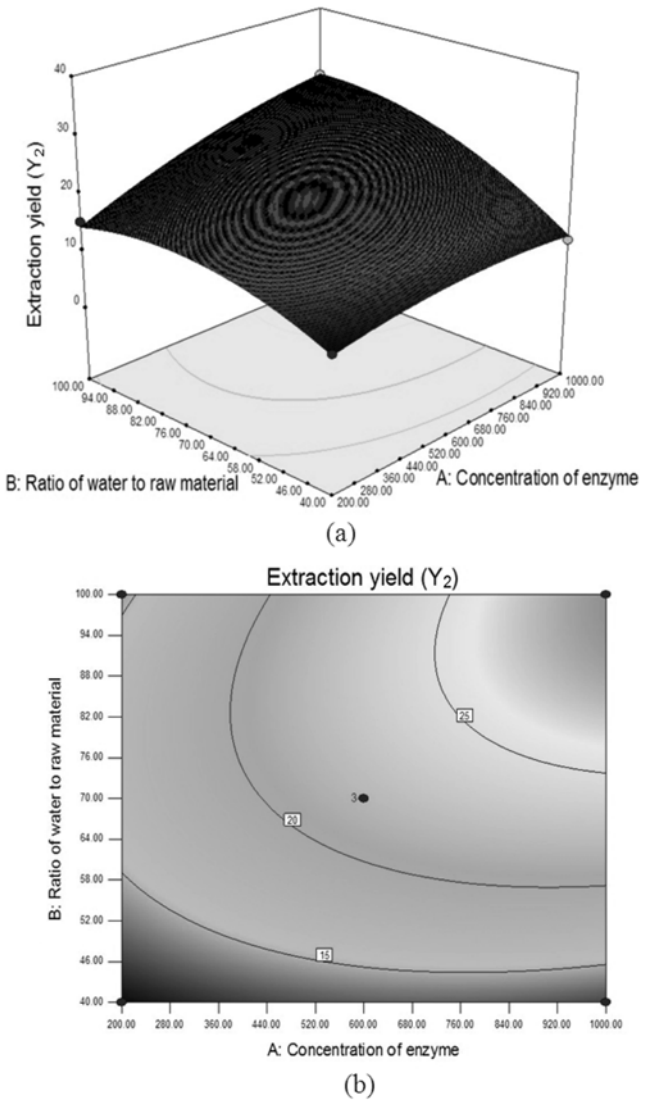

Fig. 4. Response surface plots (a) and contour plots (b) showing the effect of concentration of enzyme (X_1) and ratio of water to material (X_2) on the yield of *LA* polysaccharides.

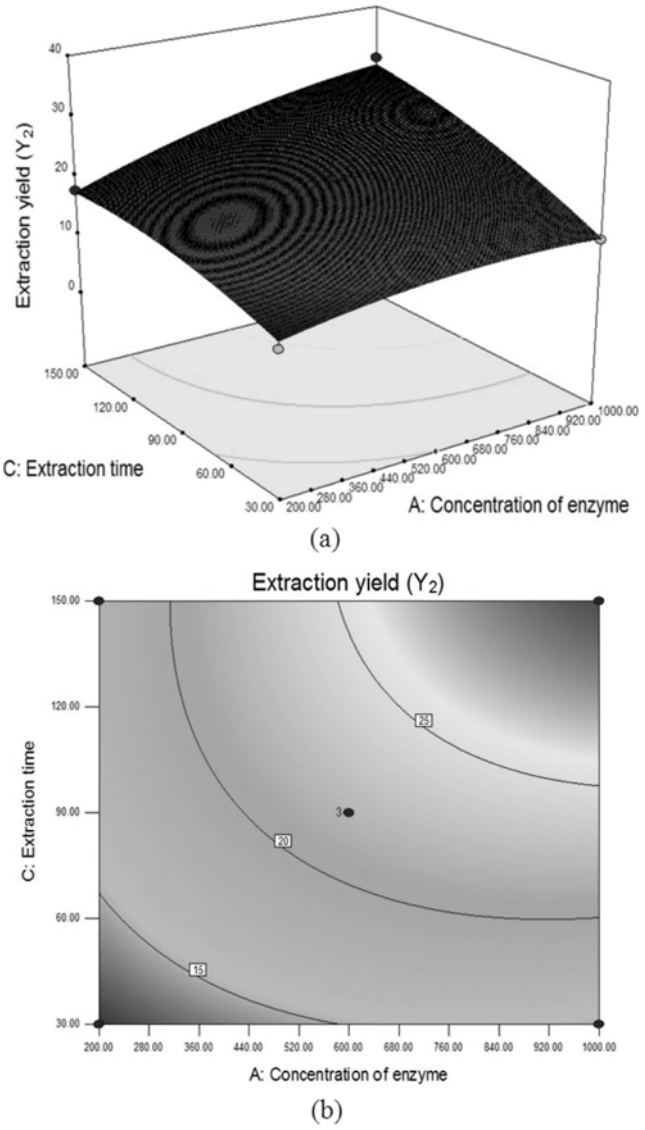

Fig. 5. Response surface plots (a) and contour plots (b) showing the effect of concentration of enzyme (X_1) and extraction time (X_3) on the yield of *LA* polysaccharides.

tion the cell wall as well as the mass transfer rates to prompt the migration of target compounds from the material to the surroundings and to bring about a higher extraction efficiency of polysaccharides (Qiao et al., 2009).

3. Optimization of Extraction Parameters and Verification of the Model

Based on the above study, an optimization study was performed to evaluate the optimal operating conditions for the extraction with the highest yield of *GA* and *LA*. First, we input the data (factors and levels) into software (Table 1); the software designs experiment runs (random order). After finishing all the given experiments, 15 experiment values were obtained and the predicted values were calculated by equations. Input experimental values and predicted values into the software (Table 2), the software will predict the optimal value. As shown in Table 5, the optimal conditions for GA were concentration of enzyme (X_1=778.01 mg/g), ratio of water to raw material (X_2=86.80) and extraction time (X_3=129.93 min). The optimal conditions for *LA* were concentration of enzyme (X_1=997.03 mg/g), ratio of water to raw material (X_2=98.76) and extraction time (X_3=117.69 min). Under these conditions, the extraction yields of *GA* and *LA* were predicted by RSM models to be 48.36% and 32.47%, respectively.

4. Verification of the Model

The suitability of the model equation for predicting the optimum response values was verified using the optimal condition. The experimental value was 48.36% and 32.47% (Table 4), and all the experiments were repeated three times. Only small deviations were found between the actual values and predicted values. Thus, the model can be used to optimize the process of polysaccharides extraction from marine algae.

CONCLUSION

RSM was used to determine the optimum process parameters

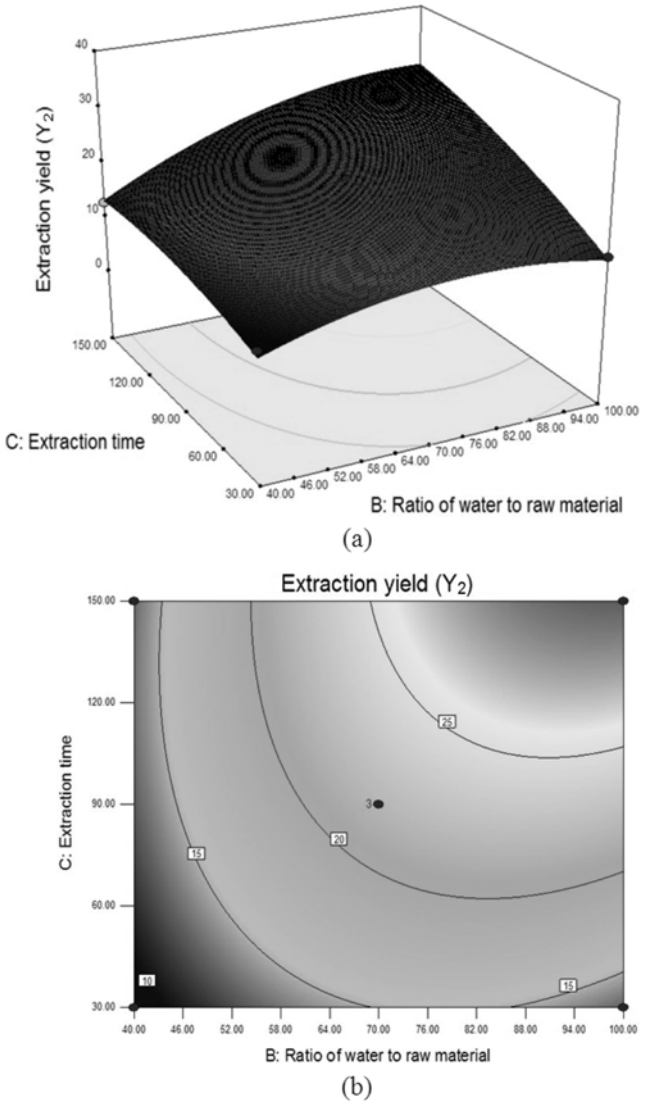

Fig. 6. Response surface plots (a) and contour plots (b) showing the effect of ratio of water to material (X_2) and extraction time (X_3) on the yield of *LA* polysaccharides.

that gave a high extraction yield of polysaccharides for *GA* and *LA*. ANOVA showed that the effects of all variables were significant and quadratic models were obtained for predicting the responses. The optimal conditions were the following: concentration of enzyme of 778.01 mg/g with ratio of water to raw material of 86.80 and extraction time of 129.93 min for *GA* and concentration of enzyme of 997.03 mg/g with ratio of water to raw material of 98.76 and extraction time of 117.69 min for *LA*. Under these conditions, the experimental yields were 48.20% and 32.12%, with the predicted model matching well with the coefficients of determination (R^2) of 0.9864 and 0.9892, respectively. Thus, the model can be used to optimize the process of polysaccharides extraction from marine algae.

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Table 5. Optimum conditions and the predicted and actual value of response at the optimum conditions

Responses	Concentration of enzyme (mg/g)	Ratio of water to raw material	Extraction time (min)	Yield of polysaccharides (predicted, %)	Yield of polysaccharides (actual, %)
Y_1	778.01	86.80	129.93	48.20	48.36
Y_2	997.03	98.76	117.69	32.12	32.47