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Extraction, purification, and characterization of the polysaccharides from *Opuntia milpa alta*

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Abstract

Polysaccharides production from *Opuntia milpa alta* was carried out. The effects of the extraction parameters of extraction time, extraction temperature and ratio of water to raw material were optimized by using statistical techniques such as a three factor, three level Box–Behnken factorial design (BBD) and response surface methodology (RSM), with a view to maximize the extraction yield under optimal extraction parameters combination. From this study, it could be concluded that the maximum yield of the polysaccharides (0.694%) can be obtained extraction temperature of 86.1 °C, extraction time of 3.61 h, ratio of water to material of 3.72:1. Three isolated fractions of the polysaccharides were characterized by employing High-Performance Liquid Chromatography (HPLC).

Keywords: *Opuntia milpa alta*; Polysaccharides; Extraction; Optimization; Response surface analysis; Purification

1. Introduction

Polysaccharides from plant, epiphyte and animal extracts are an interesting source of additives for several industries, in particular food and drug industry (Forabosco et al., 2006; F-Tischer et al., 2006; Li, Fang, & Zhang, 2007; Nunes, Rocha, Saraiva, & Coimbra, 2006; Ray, 2006; Wang, Luo, & Ena, 2007). Many of these polysaccharides, like those from the Cactaceae family, have been empirically used to modify the rheological properties of some products (Pimienta-Barrios, 1991). In traditional medicine, extracts of polysaccharide-containing plants are widely employed for the treatment of skin and epithelium wounds and of mucous membrane irritation (Bedi & Shenefelt, 2002). Therefore, discovery and evaluation of new polysaccharides from the various *Opuntia* as new safe compounds for functional foods and medicine has become a hot research spot.

Opuntia milpa alta, a member of the cactaceae family, is a tropical or subtropical plant originally grown in South America and cultivated in dry regions as an important nutrient and food source (Habibi, Mahrouz, & Vignon, 2005a). This genus is endemic to America and up to now 377 species have been recognized, 104 have been wild in Mexico, and 60 of which are endemic to this country (Corrales-García, Peña-Valdivia, Razo-Martínez, & Sánchez-Hernández, 2004; Habibi, Mahrouz, & Vignon, 2005b). It often takes the form of a small shrub or creeping plant and it can easily be recognized by its green and thick long pencas, in the shape of a sports racket. In some countries the young stems (nopalitos) is also used for human consumption (Ruiz Pérez-Cacho, Galán-Soldevilla, Corrales García, & Hernández Montes, 2006). The juice obtained from the cladodes contains a large amounts of phenolic components, in particular some flavonoids, (flavan-3-ols, also known as catechins) and proanthocyanidins, such as hydroxycinnamic acids and anthocyanins; antioxidant

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constituents, including Vitamin C (ascorbic acid), Vitamin E (α-tocopherol), carotenoids, glutathione, flavonoids and phenolic acids, as well as other unidentified compounds, many of which possess a variety of biological activities correlated to antioxidant activity and cell matrix interactions (Gaby, 1999; Panico et al., 2005; Shin, Hwang, Kang, & Lee, 2006).

The extract of *Opuntia* cladodes is used in folk medicine for their anti-ulcer and wound healing activities (Corrales Garcia, Franco Moreno, & Rodriguez Campos, 2006; Matsuhiro, Lillo, Sáenz, Urzúa, & Zárate, 2006) and the production of mucilages, often referred as pectin polysaccharides, is characteristic of members of the Cactaceae family (Rivera et al., 1994). Studies found that the extract from *Opuntia* plants was a mixture of a neutral glucan, glycoproteins and an acidic polysaccharide composed of L-arabinose, D-galactose, L-rhamnnose, D-xylose and D-galacturonic acid (Medina-Torres, Brito-De La Fuente, Gómez-Aldapa, Aragon-Piña, & Toro-Vazquez, 2006; Trachtenberg & Mayer, 1981). Studies also found that the mucilage was a neutral polysaccharide that contained arabinose, rhamnose, galactose and xylose (Amin, Awad, & El-Sayed, 1970). Trachtenberg and Mayer (1980, 1981) reported that the mucilage was a polysaccharide, which contained 10% uronic acids, of TCM to promote blood circulation, strengthen the immune system, for the treatment of heart failure congestion, neuralgia, rheumatism and gout, etc., as well as for invigoration and retarding aging.

In the present work, we attempted to systematically investigate the extraction, separation, purification and structural analysis of the polysaccharide from *O. milpa alta* and its three isolated fraction were examined using HPLC chromatography.

2. Materials and methods

2.1. Materials

Opuntia milpa alta was obtained from Shanhai LinHui Biotechnology Ltd., in Shanghai, China. All other chemicals and solvents used were of analytical grade.

2.2. Extraction procedure

The fresh cactus (500 g) was agitated into homogenate with a blender and the ground samples were put in boiling water and decocted by a traditional method for Chinese medicinal herbs. After edulcoration with petroleum ether, they were extracted with hot distilled water for 4 h at 90 °C. After centrifuging to remove debris fragments (3000 r/min, 20 min), the solution was concentrated in a rotary evaporator. Protein was removed with the Sevag method (Vilkas & Radjabi-Nassab, 1986). Then, the solution was precipitated with four volumes of 95% ethanol for 48 h at 4 °C. The precipitate was collected by centrifugation (3000 r/min, 20 min) and respectively washed twice with acetone and acetone, and then vacuum-dried at 40 °C, giving a brown powder with a molecular weight of. All experiments were performed at least in duplicate.

Extraction yield of polysaccharides was measured by employing sulphuric acid–phenol method (Hilz, Bakx, Schols, & Voragen, 2005):

Extraction rate (%) = (polysaccharides weight/wet raw material weight) $\times$ 100%.

2.3. Analysis of samples

The polysaccharides was characterized by The FTIR spectrum.

The crude polysaccharide was then separated and sequentially purified through DEAE-cellulose and Sephadex G-75. The chemical composition of three isolated fractions (*F*1, *F*2 and *F*3) of the polysaccharides were measured by a Shimadzu Corporation (Kyoto, Japan) high-performance liquid chromatography (HPLC) with a refractive index (RI) detector and BioRad (Hercules, CA, USA) Aminex HPX-87H column (300 × 7.8 mm) at 45 °C. The eluent was 0.005 M H_2SO_4, at a flow rate of 0.6 ml/min and a sample volume of 20 µl.

2.4. Experimental design

On the basis of single-factor experiment for the polysaccharides production, proper ranges of extraction time, extraction temperature, ratio of water to raw material, extraction number were preliminarily determined. A three level, three variable Box–Behnken factorial design (BBD) (SAS, SAS Institute, Cary, NC, USA) (Aslan & Cebeci, 2007) was applied to determine the best combination of extraction variables for the production of cactus polysaccharides. Based on the investigations on single-factor experiment, the variables considered are extraction time, extraction temperature, ratio of water to raw material in the experimental design. The independent factors and the dependent variables used in this design are listed in Table 1. Table 2 lists definition and coded levels that were carried out for developing the model. Each experiment was performed in duplicate and the average of extraction yield of cactus polysaccharides was taken as the response, *Y*.

Regression analysis was performed, based on the experimental data, and was fitted into an empirical second-order polynomial model as shown below in the following equation.

Table 1
Definition and coded levels for Box–Behnken design matrix

Factor	Level		
	−1	0	1
Temperature (Z_1)/°C	70	80	90
Time (Z_2)/h	2	3	4
Ratio of water to raw material (Z_3)/mL g^{-1}	2	3	4

Table 2
Box–Behnken design matrix and the responses of the dependent variables of extraction yield

Run	X_1	X_2	X_3	Extraction yield
1	−1	−1	0	0.490
2	−1	1	0	0.567
3	1	−1	0	0.620
4	1	1	0	0.670
5	0	1	−1	0.535
6	0	−1	−1	0.635
7	0	1	1	0.634
8	0	−1	1	0.685
9	−1	0	−1	0.505
10	1	0	−1	0.605
11	−1	0	1	0.561
12	1	0	1	0.693
13	0	0	0	0.651
14	0	0	0	0.655
15	0	0	0	0.660

Experimental polysaccharides productions are averages of duplicates within ±5% error.
Average absolute relative deviation (%) = 5.75.

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

where Y is the response variable, A_0, A_i, A_{ii}, A_{ij} are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables ($i \neq j$).

The responses obtained from each set of experimental design (Table 2) were subjected to multiple nonlinear regression using the software SAS Package,Version 8.01 to obtain the coefficients of the second polynomial model. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance was checked by an F-test. The significances of the regression coefficient were tested by a t-test.

3. Results and discussion

3.1. Result of analysis of samples

The FTIR spectrum of the polysaccharides (Fig. 6) showed a strong band between 1300 and 1000 cm^{-1} attributed to the stretching vibrations of pyranose ring. A characteristic absorption at 897 cm^{-1} was also observed, indicating the β-configuration of the sugar units. There was no absorption to be observed at 890 $cm^{-1}$for the α-configuration in the examination.

The crude polysaccharide was separated and sequentially purified through DEAE-cellulose and Sephadex G-75, each giving three single elution peak (*F*1, *F*2 and *F*3), as detected by the phenol–sulfuric acid assay. The chemical composition of three isolated fractions (*F*1, *F*2 and *F*3) of the polysaccharides were measured by a Shimadzu Corporation (Kyoto, Japan) high-performance liquid chromatography. Results of HPLC showed that molecule weight of *F*1, *F*2 and *F*3 were 4373430, 63366 and 1938 Da, respectively (Figs. 4 and 5). *F*1 is composed of rhamnose, arabinose, xylose, mannose, glucose, galactose with a molar ratio: 1:1.83:0.73:1.44:2.80:24.52. *F*2 is composed of rhamnose, arabinose, xylose, mannose, glucose, galactose with a molar ratio: 11.56:52.83:44.39:0.94:1:44.87. *F*3 is composed of rhamnose, arabinose, xylose, mannose, glucose, galactose with a molar ratio: 18.2:125.0:63.5:1:17.54:46.33.

3.2. Effect of extraction temperature on the yield of the polysaccharides

Here, extraction temperature was respectively set at 70, 75, 80, 85, 90 and 95 °C to examine the influence of extraction temperature on the yield of the synthesized compounds when other extraction conditions were as follows: ratio of water to raw material 3/1, extraction time 3 h, and the extraction number 2. Fig. 1 shows extraction yields of polysaccharides at various temperatures (70–95 °C). The extraction yields ranged from 0.59% to 0.61% with the increasing temperature. The highest yield of polysaccharides was 0.61% obtained at 80 °C. But increasing the temperature from 80 to 90 °C decreased the yield of the polysaccharides because of increasing the hydrolysis of polysaccharides when extraction temperature was higher.

3.3. Effect of extraction time on the yield of the polysaccharides

Here, extraction time was respectively set at 1, 2, 3, 4 and 5 h to examine the influence of extraction time on the yield of the polysaccharides when other extraction conditions were as follows: ratio of water to raw material 3/1, extraction temperature 80 °C, and the extraction number 2. Fig. 2 shows the effect of extraction time on the yield of the polysaccharides. The results indicated that extraction time was proportional to the yield of the polysaccharides when extraction time was between 1 and 4 h. The yield of the polysaccharides was close to the peak value (0.68%) when extraction time was 4 h. After this point, the yield of the polysaccharides started to decrease with increasing the extraction time. Examination of the data in Fig. 2 reveals

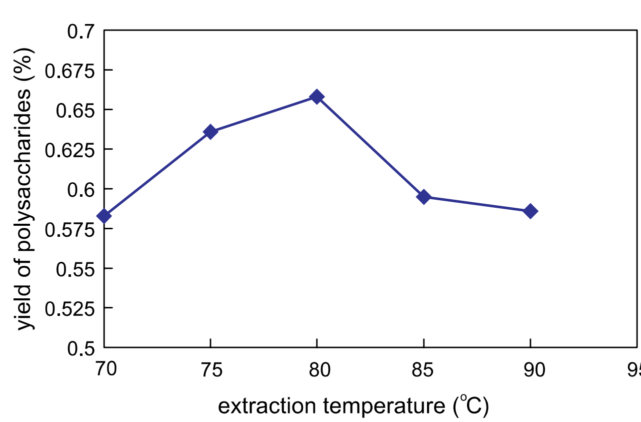


Fig. 1. Effect of extraction temperature on the yield of the polysaccharides.

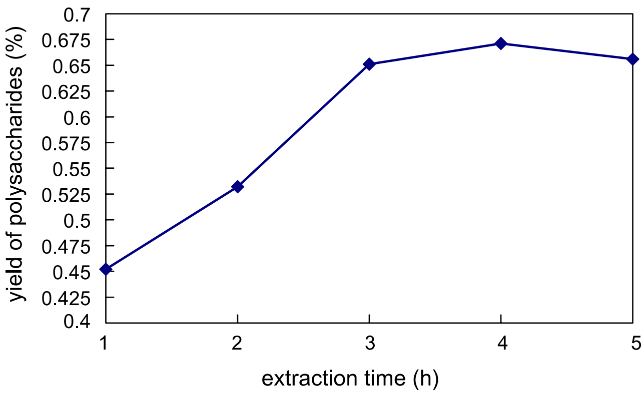


Fig. 2. Effect of extraction time on the yield of the polysaccharides.

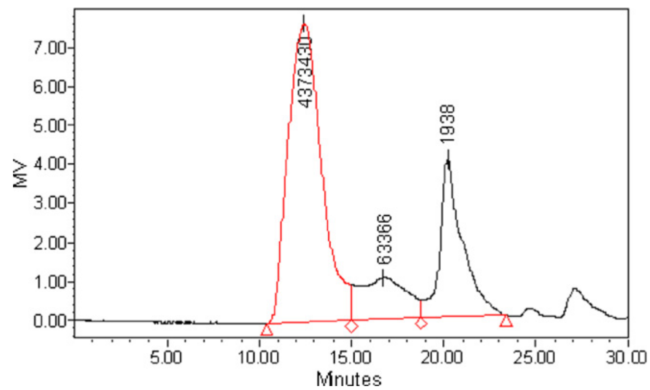


Fig. 4. HPLC of polysaccharides.

that increasing the extraction time may enhance the yield of the polysaccharides. However, excessively lengthening extraction time will also induce the change of polysaccharides molecule structure, as a result of which extraction yield of polysaccharides instead decreased.

3.4. Effect of ratio of water to raw material on the yield of the polysaccharides

In this research, ratio of water to raw material was set at 1, 2, 3, 4 and 5 to investigate the influence of ratio of water to raw material on the yield of the polysaccharides when other reaction conditions were similar to those described in Section 3.2. The results were given in Figs. 3, 5 and 6, we could observe that, as the ratio of water to raw material was in the range of 1–3, the extraction yield rapidly increased with the ration; however, as the ratio continued to increase the yield increased slowly. Therefore from the results we concluded that high extraction yield could be obtained with ratio of water to raw material in the range of 3–4.

3.5. Effect of extraction number on the yield of the polysaccharides

In this research, extraction number was in turn set at 1, 2 and 3. Other reaction conditions were similar to those described in Section 3.2. The effect of extraction number on the yield of the polysaccharides was tested. We can found that, as the extraction number was 1, the yield of the polysaccharides was 0.654%, and that as the extraction number was 2, the yield was 0.671%. However, when extraction number exceeding twice, its influence on the extraction yield becomes insignificant. Therefore, taking the extraction yield and processing costs into consideration, twice is sufficient for the extraction of the polysaccharides.

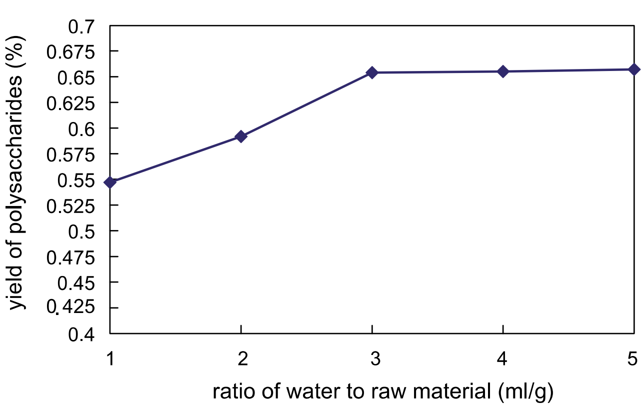
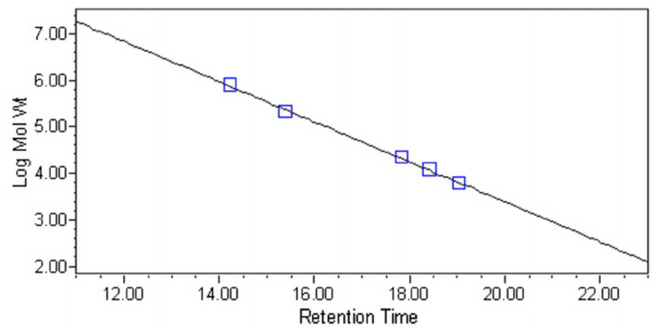


Fig. 3. Effect of ratio of water to raw material on the yield of polysaccharides.

3.6. Box–Behnken design results and response surface analysis

Using the relationships in Table 1, the actual levels of the variables for each of the experiments in the design matrix were calculated and experimental results obtained as given in Table 2. Multiple regression analysis was performed on the experimental data and the coefficients of the model were evaluated for significance with the Student *t*-test. The values of the coefficients are presented in Table 3. The analysis of variance (ANOVA) for the BBD is shown in Table 4. The *F*-ratio in this table is the ratio of the mean square error to the pure error obtained from the replicates at the design centre. The significance of the *F*-value depends on the number of degrees of freedom (DF) in the model, and is shown in the *P*-value column (95% confidence level). Thus, the effects lower than 0.05 in this column are significant. This is emphasized in Table 2, which reveals three significant coefficients affecting the extraction (within the chosen limits), namely extraction time, extraction temperature and ratio of water to raw material. The lack of significance of the cross product terms suggests the absence of interaction between variables in the zone studied. From the experimental results listed in Table 2 and regression analysis, the second-order response functions representing (Y) can be expressed as a function of extraction temperature (X_1), extraction time (X_2) and ratio of water to raw material (X_3). The relationship between responses (Y) and variables were obtained for coded unit for one size fraction as follows:



	R	R^2	Standard Error	Equation
1	0.999098	0.998197	4.383942e-002	Log Mol Wt = 1.20e+001 - 4.32e-001 T^1

Fig. 5. Standard curve of molecular weight of polysaccharides.

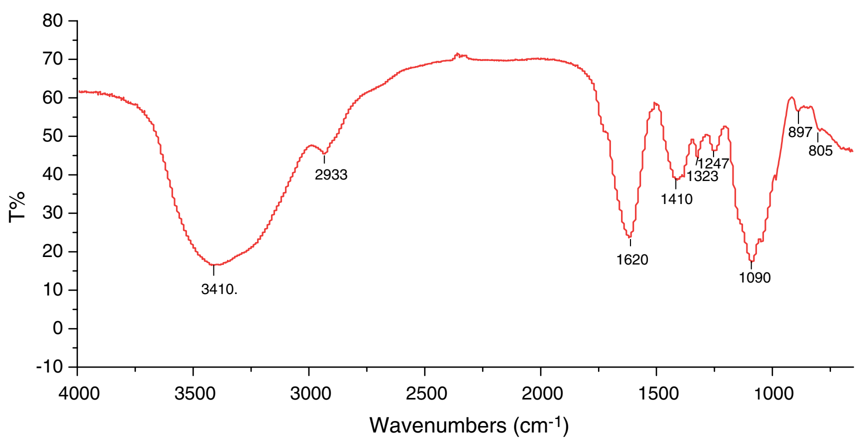


Fig. 6. IR of polysaccharides.

Table 3
Estimated coefficients of the fitted second-order polynomial model for the polysaccharides production

Term	Coefficients estimated	*t*-value	*P*-value	Significance
A_1	0.058375	35.08205	<0.0001	**
A_2	0.034875	20.95909	<0.0001	**
A_3	0.03675	22.08592	<0.0001	**
A_{11}	−0.049625	20.2611	<0.0001	**
A_{12}	−0.00625	2.65597	0.045102	*
A_{13}	0.008	3.399641	0.019259	*
A_{22}	−0.018125	7.40014	0.000709	**
A_{23}	−0.0125	5.31194	0.003161	**
A_{33}	−0.014375	5.86908	0.002037	**

** Significance level 5%; *significance level 1%.

The yield of polysaccharides extracted by boiling water $(Y) = 0.655 + 0.058375X_1 + 0.034875X_2 + 0.03675X_3 - 0.00625X_1X_2 - 0.0125X_2X_3 + 0.008X_1X_3 - 0.049625X_1^2 - 0.18125X_2^2 - 0.014375X_3^2$ where $X_1 = (Z_1 - 80)/10$ (Z_1 denotes extraction temperature), $X_2 = (Z_2 - 3)/1$ (Z_2 denotes extraction time) and $X_3 = (Z_3 - 3)/1$ (Z_3 denotes ratio of water to raw material). The response surface for this polynomial is represented in Figs. 7–9 where a maximum at the positive extremes is clearly shown.

Extraction temperature is the major factor affecting the yield of the polysaccharides. As time increases, the yield increases almost linearly. Extraction time and ratio of water to raw material affect also the yield in the same way as the extraction temperature. Although The determinant coefficients (R^2) of the regression equation is equal to 0.9981, the value of lack-of-fit for regression equation is very small ($F = 0.81 < F_{0.05}(3,2) = 19.16$), indicating that the equation has a good fitness, and that both response value (Y) and factors had no simple linear effect on the model. The model *F*-value analysis of the regression equation indicate that there was a strong and significant influence of regression linear coefficients (A_1 A_2 and A_3), the quadratic factors and intercross term on the response value (Y).

To determine optimal levels of the variables for the polysaccharides production, three dimension surface plots were constructed. Fig. 7a and b shows the effect of extraction time and extraction temperature on polysaccharide production. While the yield of the polysaccharides reached a maximum near the central condition of extraction time at a fixed extraction temperature, increase in extraction temperature at a fixed extraction time led to an increase in the yield of the polysaccharides. The general form of three dimensional relationship demonstrated that the yield of the polysaccha-

Table 4
Analysis of variance of the second-order polysaccharides production model

	Sum of squares	Degree of freedom	Mean square	F value	Significance
Linear	0.047796	3	0.01593	637.3	**
Quadratic	0.010193	3	0.003398	135.9	**
Cross product	0.0010373	3	0.0003458	13.83	**
Lack of fit	0.0000608	3	0.0000203	0.81	
Pure error	0.00005	2	0.00005		
Total error	0.059136	14			
Coefficient of determination (R^2)	0.9981				

Note: $F_{0.05}(3,2) = 19.16$, $F_{0.01}(3,2) = 99.17$, $F_{0.01}(9,5) = 10.16$.
**Significance level 1%.

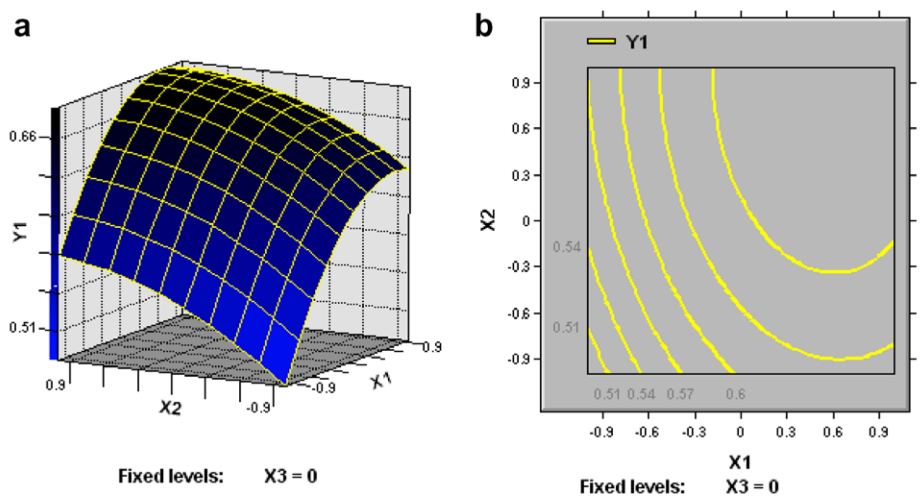


Fig. 7. Response surface (3D) and contour plots showing the effect of extraction temperature (X_1) and extraction time (X_2) added on the response Y.

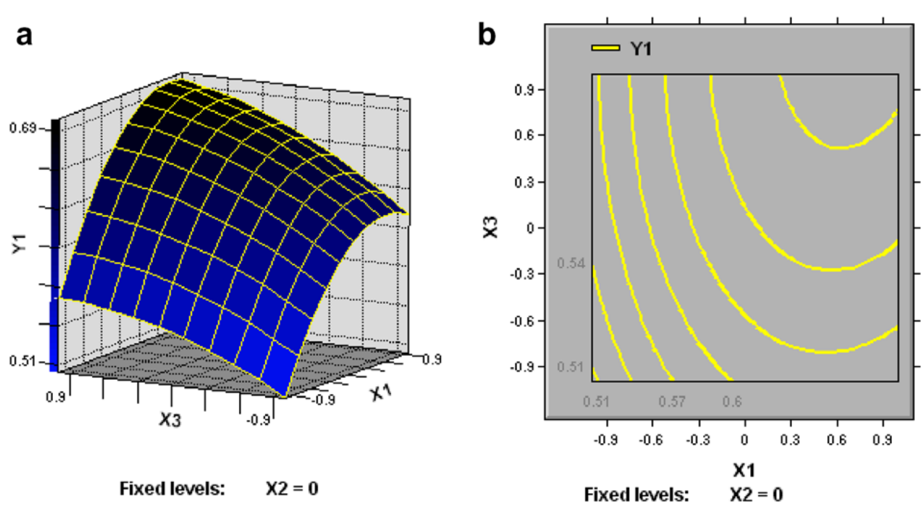


Fig. 8. Response surface (3D) and contour plots showing the effect of extraction temperature (X_1) and ratio of water to raw material (X_3) added on the response Y.

rides depends more on extraction temperature than on extraction time. The effect of extraction temperature and ratio of water to raw material shown in Fig. 8a and b for the production of the polysaccharides, demonstrated that the yield of the polysaccharides increased with the increase of extraction temperature at a fixed ratio of water to raw material (Fig. 8a and b) or with the increase of ratio of water to raw material at a fixed extraction temperature. Fig. 9a and b shows the response surface plot at various extraction time and ratio of water to raw material. A linear increase in the yield of the polysaccharides with increase in extraction time and ratio of water to raw material was

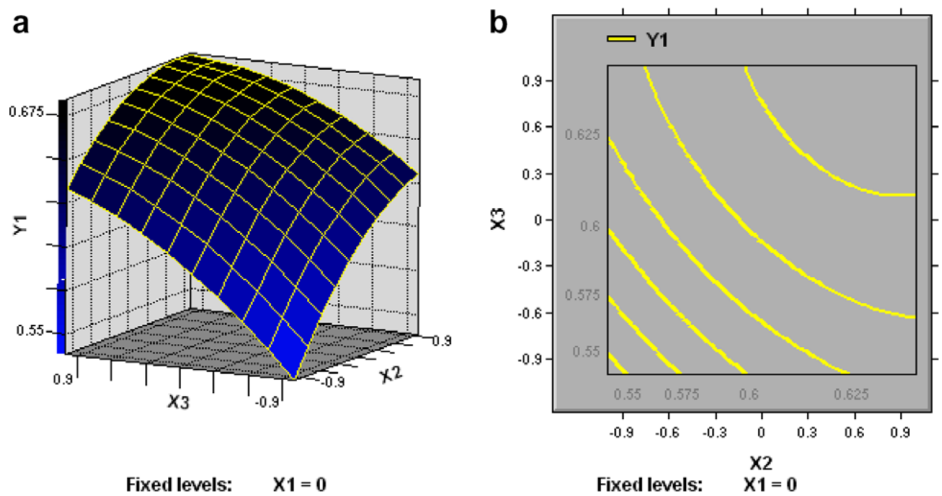


Fig. 9. Response surface (3D) and contour plots showing the effect of extraction time (X_2) and ratio of water to raw material (X_3) added on the response Y.

observed. Particularly, increase in extraction time at a fixed ratio of water to raw material led to a marked increase in the yield of the polysaccharides. The behaviour of response surface graphs indicated that increasing extraction time up to 4 h had a positive effect on the yield of the polysaccharides. However, there seemed to be less effect of increased ration of water to raw material on the extraction yield.

To further validate optimal values, first partial derivative of regression equation was taken and made to be zero. Calculating the equation gave the following results: $X_1 = 0.61$, $X_2 = 0.61$, $X_3 = 0.73$. The optimal values of the variables affecting the yield of the polysaccharides given by the software are the following: extraction temperature: 86.1 °C, extraction time: 3.61 h and ratio of water to raw material: 3.72. Under these conditions, the model gave predicted values of Y (the extraction yield) being 0.698%. As expected, and according to the response surface, the efficiency of the extraction in terms of yield in polysaccharides increases by increasing all the three factors. Among the three parameters, extraction temperature is the most significant factor to affect the extraction yield.

To test validity of response surface analysis (RSA) method, the polysaccharides was extracted under the optimal condition and the extraction yield was 0.694%. The value was close to the theoretical predicted values, indicating that the experimental design matrix may better reflect the extraction parameters of the polysaccharides.

4. Conclusion

This present study indicates that the polysaccharides from *O. milpa alta* are able to be produced by employing boiling water extraction. Results of HPLC indicated that molecular weight of three fractions of the polysaccharides were 4373430, 63366 and 1938 Da, respectively. The optimal conditions obtained by BBD and RSA for production of the polysaccharides from *O. milpa alta* include the following parameters: extraction temperature 86.1 °C, extraction time 3.61 h, and ratio of water to raw material 3.72/1. Under these conditions, the experimental yield was 0.698%, which is well matched with the predictive yield.

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