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Extraction and optimization of chitosan from razor clam (*Ensis arcuatus*) shells by using response surface methodology (RSM)

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Abstract

Chitin can be found in animal source especially arthropods such as crustacean, mollusk and insect, as well as in plant source such as fungi. Chitosan is obtained from chitin after the acetyl group is removed from chitin structure through deacetylation step and has wide application in various fields (food, cosmetics and pharmaceutical). In this study, chitosan was extracted from razor clam shells, where the extraction conditions were optimized. Two- factors of randomized D-optimal design was used to determine the optimum condition for the extraction of chitosan from razor clam (*Ensis arcuatus*) by using response surface methodology (RSM). The chemical extraction was optimized using five levels with two factors which were the deacetylation time (2,4,6,8,10 hrs) and deacetylation temperature (50, 60, 70, 80 and 90ºC). A randomized design suggested by Design Expert software was implemented with four responses evaluated: yield (%); degree of deacetylation (%); molecular weight (kDA); and ash content (%). Time (h) and temperature (ºC) of the deacetylation significantly ($p<0.05$) affected the yield (%), degree of deacetylation (DDA) (%), molecular weight (Mw) (kDA) and ash content (%) of the chitosan extracted. The optimum conditions for the chitosan extraction were at the respective deacetylation time and temperature of 6h and 70ºC with actual values of yield (%), degree of deacetylation (%), molecular weight (kDA) and ash content (%) of 19.903±2.367, 50.113±0.902, 476.727±13.603, 8.517±2.094, respectively. The optimum condition for the chitosan extraction was experimentally verified and valid for further analysis.

1. Introduction

Razor clam is classified in genus *Ensis* or known as 'Siput buluh' among the locals in Malaysia. This species is abundant in the intertidal sandy beaches and mudflats along the western coast of Malaysia (Na, 2008; Rabuyan and Bakar, 2016), mostly in Selangor and Johor as well as at the eastern coast, Kuching and Samarahan and collected by the locals as the economic sources. As razor clam had been widely commercialized in Malaysia, the waste particularly the shells of this species may cause environmental pollution. By utilizing the shells for chitosan extraction, the source of pollution may be reduced significantly. Previous study proved that chitosan extracted from razor clam can act as a natural coagulant and able to remove the turbidity in water in a water treatment process (Adenan, 2009). In this study, chitosan was extracted from razor clam at the optimized condition to be used as an edible film in the food industry.

In nature, chitin is usually extracted from the exoskeleton of crustaceans, squid pens, fungi, and mollusc. Chitosan is a universal natural polysaccharide, mainly consisting copolymers of glucosamine and N-acetylglucosamine, and can be acquired by the partial deacetylation process of chitin which included enzymatic means or alkali deacetylation method. Optimization by response surface methodology has been employed to carry out optimization studies as it can assist in determining the interactive impact of process variables and in plotting a mathematical model that precisely describes the whole process (Mourabet *et al*., 2017). The objective of this study was to statistically optimize the independent process parameters such as deacetylation time (hrs) and deacetylation temperature (ºC) by randomized D-optimal design in response surface methodology and to evaluate the chemical properties of chitosan extracted from razor clam shells.

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2. Materials and methods

2.1 Materials

Fresh razor clam, *Ensis arcuatus* was obtained from Pulau Ketam, Selangor, Malaysia. Razor clam was collected fresh from the seashore and was kept at 4ºC prior to use. Analytical grade Hydrochloric acid (HCl) (20%) and sodium hydroxide (NaOH) (2%, 10%, and 50%) were used in chemical extraction. The chemical used for this project was acquired from Merck Sdn. Bhd, Malaysia.

2.2 Extraction of chitosan

2.2.1 Preparation of the razor clam shells

The razor clam flesh was separated from the shells. The shell was washed before dried using the oven (Memmert UF 110, Germany) at 70°C for 4 hrs or until the moisture content (%) reached between 8-10%. Chitosan was extracted according to Zainal *et al*. (2014) with slight modifications. Dried shell was crushed by using a blender (Waring 8010S-HGBTWTS3, USA) and then passed through 60-120 µm mesh sieves to obtain finer and consistent particles powder.

2.2.2 Demineralization

The powder was soaked and agitated in 20% HCl for 16.5 hrs at the ratio of 1:5 of shell powder to the acid solution at room temperature. This step was repeated twice (Majekodunmi, 2016). The powder was then treated with 2% NaOH until neutral pH was obtained.

2.2.3 Deproteination

The powder obtained from the demineralisation process was then immersed in 10% NaOH solution at the ratio of 1:5 of sample to the solution at 70°C in water bath shaker (Memmert SV 1422, Germany) for 2 hrs. The mixture was cooled at room temperature (~26°C) and rinsed using distilled water until neutral pH was obtained.

2.2.4 Deacetylation

The powder was soaked in 50% NaOH solution at the ratio of 1:5 of sample to the solution and then was treated with NaOH at (50, 60, 70, 80 and 90°C) for (2,4, 68, and 10 hrs) in water bath shaker, then filtered in purpose to obtain the solid matter which is chitosan powder.

2.2.5 Purification of chitosan

Chitosan powder was treated with 0.01M HCl solution at the ratio of 1:5 of sample to the solution. The mixture was centrifuged at 1370 x *g* for 15 mins. The collected, supernatant, was dried in the vacuum oven (Protech Model-ADP 21, Japan) at 60°C for 4 hrs or until the moisture content reached between 8-10%.

2.3 Characterization of extracted chitosan powder

2.3.1 Percentage yield

Yield (%) of chitosan was calculated as the total weight of chitosan powder to the total razor clam shells used.

$$\text{Yield (\%)} = \frac{\text{Chitosan powder (g)}}{\text{Razor clam shells (g)}} \times 100$$

2.3.2 Degree of deacetylation

Degree of deacetylation was determined by direct titration according to Sarbon *et al*. (2015). The calculation of degree of deacetylation was made by using the following equation:

$$\text{Degree of deacetylation (\%)} = \frac{161.16 * (V2 - V1)N}{W1}$$

Where V1 = final volume of NaOH solution; V2 = initial volume of NaOH solution; N = strength of the NaOH solution (0.1 M); and W1 = mass of sample after correction for moisture. Mass of chitosan monomer is 161.16.

2.3.3 Molecular weight

Molecular weight (kDA) was determined according to Kurniasih and Dewi (2018). Molecular weight (kDA) was calculated using Mark Houwink-Sakurada equation:

$$[\eta] = K (M_v)^{\alpha}$$

Where [η] = intrinsic viscosity; M_v = Molecular weight; and K and α are 1.81 x 10^{-3} and 0.93 respectively.

2.3.4 Ash content

Ash content was determined according to AOAC (1990) method.

2.4 Optimization of extracted chitosan

Optimization of chitosan extraction was done using Design Expert Software (Version 11.1.2.0), Stat Ease, USA. Response surface methodology (RSM) using D-optimal design of 2-factors-5-levels was employed to develop predictive models for different responses. The experimental design produced 16 runs (conditions) in which was carried out to obtain the optimum condition of the factors. In this study, factors of time (X_1) and temperature of deacetylation (X_2) were chosen as the independent variables, with five levels at (2,4,6,8,10 hrs) and (50, 60, 70, 80 and 90ºC). Yield (%), degree of deacetylation (DDA) (%), molecular weight (kDA), and ash content (%) were selected as the response (dependent variable) of the study. Optimal design was set to five levels, equivalents to levels -1, -0.5, 0, 0.5, and 1 in order to obtain a lot of information about the main effects in a

relatively few number of runs besides obtaining more precise of optimum point. All the statistical testing of the model was performed by ANOVA analysis with F-test. The F-values for each model was confirmed significantly with p-value ≤ 0.05.

3. Results and discussion

3.1 Yield

The second-order polynomial equation showed a relationship between the independent variables time (h) (X_1), and temperature of deacetylation (ºC) (X_2) and the dependent variable yield (%) as:

$$Y = 19.36 + 0.8453X_1 - 1.72X_2 - 2.21X_1X_2 + 1.64X^2_1 - 5.85X^2_2 \quad (1)$$

From the equation (1), the yield (%) was found to have a quadratic relationship with the two variables. The R^2 value (0.9406) (Table 1), being a measure of the goodness of fit of the model. 3D contour plot clearly showed that the yield (%) of chitosan decreased at 90ºC as the color gradient changed from red to blue. The yield of chitosan obtained for all the 16 runs ranged from 10.87 to 22.61%. The yield obtained from this study was in ranged with Shanmugam *et al*. (2012) where chitosan extracted from *Donax scortum* shells yielded 18.8% respectively. In this study, yield (%) of chitosan increased with the increasing time (h) and temperature (° C) of deacetylation but decreased when the time and temperature reached 8 hrs and 90ºC respectively. The same trend was showed by Patria (2013). The decreased of yield (%) at 90ºC and 8 hrs for razor clam shells, probably due to excessive removal of polymers' acetyl group during deacetylation, depolymerization of the chitosan polymer, causing loss of sample mass/weight and chitosan particles loss during washing (Hossain and Iqbal, 2014).

3.2 Degree of deacetylation

Based on equation 2, it was found that DDA to have 2FI relationship with the two variables (time and temperature of deacetylation). The following second order polynomial equation showed a relationship between the independent variables time (h) (X_1), and temperature (°C) of deacetylation (X_2) and the dependent variable DDA as :

$$Y = 48.22 + 5.30X_1 - 5.99X_2 - 3.311X_1X_2 \quad (2)$$

High F-value and p-value less than 0.05 indicated that the model was significant. R^2 value (0.9301) (Table 1) indicated that 93.01% of the total variation was explained by the model. Contour plot ascertained showed that the DDA% of chitosan increased when both factors (time and temperature) increased. The DDA of chitosan extracted from razor clam shells from all the 16 runs ranged between 32.34 to 55.76%. The value obtained in this study was in range with the previous study by Majekodunmi *et al*. (2017) where the DDA of chitosan two samples of bivalve shells, *Laevicardium attenuatum* and *Mytilus edulis* were 37.3 % and 69.6% respectively. The DDA of chitosan varies from 50% to 99% (Kalut, 2008).

3.3 Molecular weight (kDA)

Equation 3 showed that molecular weight have a linear relationship with time (h) (X_1) and temperature (ºC) (X_2) of deacetylation as described by the following equation:

$$Y = 482.38 - 79.04X_1 - 110.87X_2 \quad (3)$$

R^2 (Table 1), indicated that 86.16% of the total variation was explained by the model. Molecular weight decreased as the time (h) and temperature (ºC) increased. The molecular weight of chitosan extracted from razor clam obtained from all the 16 runs ranged from 326.571 to 716.285 kDA. The molecular weight values obtained in this study was similar to the previous studies by Devikrishna and Remya (2015) and Shanmugam *et al*. (2012). The present study showed that the molecular weight of chitosan from razor clam decreased as time and temperature of deacetylation increased. The trend was similar to previous study by Patria (2013). Longer duration of alkali treatment and high temperature may result in the higher formation of amine groups, hence the molecular chains of chitosan depolymerized that eventually cause decreases in molecular weight (Patria, 2013).

3.4 Ash content (%)

Equation 4 showed that ash content (%) to have a linear relationship with time (h) (X_1) and temperature (ºC)(X_2) of deacetylation as described by the following

Table 1. Model for responses of extracted chitosan with different conditions

Response	Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	R^2	F-value	p-value
Yield	Quadratic	< 0.0001	0.2532	0.9109	0.8720	0.9406	31.69	<0.0001
DDA	2FI	0.0077	0.2391	0.9126	0.8679	0.9301	53.19	<0.0001
Mw	Linear	< 0.0001	0.2963	0.8403	0.7939	0.8616	40.46	<0.0001
Ash	Linear	< 0.0001	0.4681	0.8632	0.8258	0.8814	48.32	<0.0001

Where DDA = degree of deacetylation (%), Mw = Molecular weight (kDA).

equation:

$$\text{Ash (\%)} = 8.08 - 1.37X_1 - 0.094X_2 \quad (4)$$

Contour plot showed that the temperature (ºC) did not give significant effect to the ash content (%), while as the time (h) increased, the ash content (%) decreased. This is because mineral is heat resistant until 800ºC. The residual amount of $CaCO_3$ in the shells left from demineralization step was further removed in the process of deacetylation (Lertsutthiwong *et al*., 2002). Ash content of chitosan obtained from this study ranged between 6.94 - 9.89% which was in ranged in between with the previous studies by Ibitoye *et al*. (2018) and Sarbon *et al*. (2015) which showed that the ash content of extracted chitin and chitosan from commercial shrimps' shells was 11.77% and chitosan extracted from mud crab (*Scylla olivicea*) shells at 5.97%. The concentration of HCl used during demineralization step affect the ash contents (Sarbon *et al*., 2015).

3.5 Optimization

The optimum condition for the chitosan extraction are deacetylation time and temperature at 6 h and 70ºC respectively with predicted values of yield (%), DDA (%), molecular weight (kDA) and ash content (%) were 19.36%, 48.22%, 482.38 kDA, and 8.08% respectively. After validation by t-test, the actual values of yield (%), degree of deacetylation (%), molecular weight (kDA) and ash content (%) at the optimum point were 19.903±2.367, 50.113±0.902, 476.727±13.603, 8.517±2.094 respectively.

5. Conclusion

The optimum conditions for the extraction of chitosan from razor clam shells were able to be determined. The physicochemical properties of chitosan extracted such as degree of deacetylation (DDA), moisture content and ash content were analyzed as the responding variables to determine the optimum condition for the extractions. The optimum condition for the chitosan extraction is deacetylation time and temperature at 6 h and 70ºC respectively with predicted values of yield (%), DDA (%), molecular weight (kDA) and ash content (%) were 19.36%, 48.22%, 482.38 kDA, and 8.08% respectively.

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