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Original Article

Optimization of chitosan extracted from *Matuta lunaris* shells by using response surface methodology (RSM)

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Abstract

Extraction of chitin and chitosan from natural sources are universally acknowledged as they can be used in many applications. This study aims to determine the optimum extraction condition of the chitosan from moon crab (*Matuta lunaris*) and to determine the yield and chemical properties of the extracted chitosan. Chitosan from moon crab (*Matuta lunaris*) was extracted and optimized by using response surface methodology (RSM) using two variables with five levels which were the deacetylation temperature (60, 70,80, 90 and 100ºC) and deacetylation time (2, 4, 6, 8 and 10 h). Three major steps which were demineralization, deproteination and deacetylation were involved in the extraction process. A full factorial of optimal randomized design was implemented using Design Expert 11 software. Four responses of chitosan extracted were evaluated which were yield, degree of deacetylation, molecular weight and ash content in order to determine the optimum condition of extraction. The deacetylation temperature and time for optimum chitosan extraction condition were suggested at 84.62 ºC and 9.46 h, respectively. The selected conditions (84.62 ºC and 9.46h) gave actual response values28.96 ± 0.93% of chitosan yield, 56.68 ± 1.66% of deacetylation, 567.17± 13.91 kDA of molecular weight and 10.59 ± 0.62% of ash content in chitosan powder. The extracted chitosan will be used as clarifying agent injuice production in the future studies.

Keywords: Matuta lunaris, Moon crab, Extraction, Chitosan, Response surface methodology

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Introduction

Chitin, poly [ß-(1,4)-2-acetamido-2deoxy-D-glucopyranose] is a renewable, natural and biodegradable polymer, acts as a necessary constituent of the exoskeleton of insects, crustaceans and molluscs, and observed also in polysaccharide that is found in the cell wall of fungi (Oduor-Odeto et al., 2005). Chemically almost similar with cellulose, they only differ by the presence or absence of nitrogen which nitrogen is absence in cellulose (Bautista-Baňos et al., 2006). N-deacetylation of chitin synthesized the chitosan by using sodium hydroxide in excess as a reagent and water as a solvent. Generally regarded as

safe (GRAS) compound, chitosan is a nonlethal, biodegradable, and biocompatible compound. Recently, chitosan has received expanded attention in the biomedical, food, and chemical industries for its commercial applications. Chitosan is well recognized as the most researched polysaccharides as it exhibits antifungal properties, inherent antimicrobial and excellent degree of solubility (Benbettaieb et al., 2016). However, chitosan application for the food industry, especially from the moon crab (*Matuta lunaris*) species, was not well documented in Malaysia as well as in the other countries.

This study focuses on the extraction of chitosan from moon crab, *Matuta lunaris* from family of matutidae, a local source of seafood and also aquatic catches. In Malaysia, *Matuta lunaris* is among the most commonly found sandy shore crabs in the typical nursery habitat of Peninsular Malaysia flounder. Locally known as 'ketam bulan' or 'ketam ragi' in certain area, this species is not the main catches of fisherman community in Malaysia despite their common occurrence and the abundance of the species. As a result of rapidly growing crab processing industry, a huge amount of crab shell waste is produced. Normally, only 30% is their flesh while other 70% is the shell, which is then discarded either at landfill or being dump at the coastal area (Samicho and Ramli, 2011). Utilization of crustacean shells for chitosan extraction reduces seafood waste and environmental pollution risks as the decomposition process of shells takes a long time and becomes a pollutant, along with the bad odor and waste product can be controlled. With the concerns of these, the crustacean shells have the potential as a steady supply for chitosan extraction to be further applied in the industry.

The extraction of the compound from the shells is influenced by various process parameters such as temperature and extraction time during the deacetylation. Response surface methodology (RSM) is an efficient experimental procedure for optimizing the whole and complex of extraction. Compared to the line of using "one to one factor" method, response surface methodology (RSM) is a time saving technique and economically better since in response surface methodology (RSM) the several process variables simultaneously interact with each other. The method used in this study is based on the optimal randomized design to predict the optimal conditions of the extraction procedures. To the best of our knowledge, there were no studies focusing on optimizing the parameters for chitosan extraction in moon crab (*Matuta lunaris*) shells. Thus, the aims of this study are to extract chitosan from moon crab (*Matuta lunaris*) shells and to determine the optimum condition for the extraction. The data gained from the study are expected to be used in screening of the clarifying activity potential of chitosan.

Material and Methods

Material

Fresh moon crabs (*Matuta lunaris*) were obtained from Pantai Setiu, Terengganu. Crabs were collected alive at the shore and were kept in an ice box. Then, the sample was transported to Universiti Malaysia Terengganu on the same day for further process. Analytical grade of chemical (sodium hydroxide, calcium chloride, hydrochloric acid) used for this project was purchased from Merck Sdn. Bhd, Malaysia.

Extraction of chitosan

This method was conducted based on method by Mohammed et al. (2013) with slight modifications.

Preparation of the crab shells

Frozen carapace was initially hand washed with hot tap water (60°C) or boiling water (95°C) while stirring to remove free crab flesh residues, lipids and other materials. Washed and dried shells were crushed to small pieces by using laboratory blender (Waring, 8010S HGBTWTS3, USA) and passed through 60-120 µm mesh sieves.

Deproteination

The washed and dried powdered crab shells were treated with 5% sodium hydroxide (NaOH) solution (w/v 1:8) and refluxed at 60 °C for 12h to remove the remaining proteins and other organic materials. Mixture was let to be cooled at room temperature before rinsed until neutral (pH, 7) with distilled water. The deproteinated shells were dried in the oven (Memmert, UF 110, Germany) at 60 °C for 24 h.

Decolourization

Decolourizing was achieved by treating the samples with 2% acetone at room temperature for 24 h to remove pigments. The resulting residues were removed, and shells then washed in running water, rinsed, filtered and dried at 60 °C for 24 h in the oven.

Demineralization

The shells were immersed in 10% hydrochloric acid (HCl) at ambient temperature (28 ± 2 ºC) with a solid to solvent ratio 1:5 (w/v) for 16 h. The residue was washed and soaked in 2% sodium hydroxide (NaOH) until it reaches neutral pH. Then it was rinsed with the distilled water to remove calcium chloride (CaCl). The demineralized shells were dried in oven at 60 °C for 24 h.

Deacetylation

Deacetylation of chitin was conducted by soaking dried chitin in a 50% (w/w) aqueous sodium hydroxide (NaOH) with a ratio of chitin: solution is 1:10 (w/v) at (60, 70, 80, 90, 100°C) temperature for (2, 4, 6, 8, 10) h. In order to obtain chitosan, the chitin was filtered and washed with water until neutral pH (pH, 7). The product, chitosan, was dried at 60 °C for 24 h in the vacuum drying oven (Cole Parmer, 605053-12, Chicago).

Purification of chitosan

Chitosan was treated in 1% acetic acid solution with 1:5 (w/v) ratio and centrifuged (1370 x g for 15 min) by using high speed centrifuge (Gyrozen, 1580R, Korea). Two compounds were completely separated and filtered to remove the insoluble materials. This was followed by gradual addition of 2% sodium hydroxide (NaOH) solution under continuous stirring until precipitate polymer was formed. The chitosan was filtered and washed with water until neutral pH (pH, 7). The final product was dried at 60 °C for 24 h in the oven until it achieved the moisture content of ~8 - 10.

Characterization and optimization of chitosan

Chitosan obtained will be characterized by yield, degree of deacetylation, molecular weight and ash content.

Determination of chitosan yield

The yield of chitosan (%) was calculated as the dry weight of the chitosan relative to the wet weight of shell waste (Nouri et al., 2016).

Chitosan extraction yield (%)
="*Dried extract of chitosan* (g)" /
"*Moon crab shells* (g)" x 100

Determination of chitosan's degree of deacetylation

Degree of deacetylation (DDA) was determined according to the method by Sarbon et al. (2015) with slight modification. Chitosan samples (0.1 g) were completely dissolved in 50 ml of 0.1 M of hydrochloric acid (HCl) at room temperature. Under constant stirring, the solutions were titrated with a 0.1 M sodium hydroxide (NaOH) to pH 3.75. The volume of sodium hydroxide (NaOH) needed was acquired and recorded. Titration was continued to pH 8 and the total volume of sodium hydroxide (NaOH) was recorded. The DDA was calculated using the following equation:

$$\text{Degree of deacetylation} = (161.16 * (V_2 - V_1)N)/W_1$$

where,
161.16 is the mass of chitosan monomer,
V_1 and V_2 are the volumes of NaOH solution used,
N is the strength of NaOH solution (0.1 M),
W_1 is the mass of sample.

Determination of chitosan's molecular weight

Molecular weight (M_v) was determined by obtaining the intrinsic viscosity [η] of chitosan solution, according to the method from Kurniasih and Dewi (2018), with slight modifications. Extracted chitosan was dissolved in a solvent constituted of 0.1 M acetic acid, 0.2 M sodium chloride and water at 26 °C. Analysis was performed using Ostwald type viscometer and value of molecular weight (M_v) was calculated by using the viscosity equation, Mark-Houwink-Sakurada equation:

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

where,
[η] is the instrinsic viscosity,
K and α are 1.81 x 10^{-3} and 0.93, respectively, as they are the empirical viscometric constant value that are specific for a given solvent, polymer and temperature (Cervera et al., 2004).

Determination of chitosan's ash content

Ash content was determined according to AOAC (1990) method. Five grams (5 g) of chitosan powder was heated until no fumes were produced. The sample was then heated at 550 °C overnight. The ash weight was recorded.

Ash (%) = (Weight of ash (g))/
(Weight of sample (g)) x 100

Optimization of chitosan extraction

The best conditions of chitosan extraction were chosen after the characterization steps. Response Surface Methodology (RSM) was applied to determine the optimum conditions in deacetylation step for the chitosan extraction process. In the present study, two parameters which were temperature of deacetylation and time of deacetylation were chosen as the independent variables (5 levels each) and employed at three equidistant levels (-1, -0.5, 0, +0.5, +1).D – optimal design was chosen in this study as it produced the best estimates effects of the factor, with 5 levels each. Using 2-factor-5-level design with 5 replicates at central point, sixteen experimental runs was employed to develop predictive models for different responses. Yield, degree of deacetylation (DDA), molecular weight, and ash content were selected as the responses (dependent variables) of the study. All analysis were conducted and the results of response surface methodology (RSM) experiments were analyzed using the statistical software Design Expert software (Version 11, Stat-Ease, USA). The data obtained was subjected to the Analysis of Variance (ANOVA) to determine the significance of the models. The responses obtained in optimal randomized design were subjected to regression analysis for obtaining models that relate the response to the independent factors. Three dimensional response surface and contour graphs were drawn to illustrate the main and interactive effects of independent variables. The optimum values of each independent variable for maximum response variable were determined using response surface curves and desirability profile. Verification of optimized conditions and predicted values were done in triplicate to confirm the validity of the models.

Results and Discussion

Yield of chitosan

Yield of chitosan in this study was ranged from 24.04 to 34.5%. Parallel to previous studies, the yield obtained were 17 and 41.37% for *Pachygrapsus mamoratus* and *Sesarma plicatum*, respectively (Abdelaziz, 2012).The yield obtained in the extraction of chitosan from mud crabs (*S. olivacea*) was 44.57 ± 3.44 % with the deacetylation conditions of 2 h and 105°C (Sarbon et al., 2015). The yield of chitosan (deacetylated with 65% (w/v) sodium hydroxide, NaOH, at 30 °C for 3 days) extracted from shrimp and crab shells was recorded at 46% (Rajendran et al., 2015). The obtained yield of chitosan from this study is comparatively less than some of previous studies which may be affected by the high mineral content in *Matuta lunaris'* shells (Hossain and Iqbal, 2014).

The second order polynomial equation showed a significant response (i.e. relationship) (p-value < 0.05) between the independent variables of time of deacetylation (h) (X_1), temperature of deacetylation (°C) (X_2) and the dependent variable yield (%) as:

$$\text{Yield (\%)} = 33.50 - 2.86\,X_1 - 1.04\,X_2 + 0.5904 X_1 X_2 - 1.90\,X_1^{\,2} - 4.47\,X_2^{\,2} \quad (1)$$

$R^2 = 0.9631$

From the equation (1), yield was found to have quadratic relationship with independent variables of deacetylation's time and temperature (Figure 1). Time and temperature of deacetylation affected significantly ($p < 0.05$) the yield of chitosan extracted. The R^2 value indicated that 96.31 % of the total variation was explained by the model. The "predicted R^2" of 0.9155 is in reasonable agreement with the "adjusted R^2" of 0.9446 with the difference is less than 0.2. The model F – value of 64.83 implies the model is significant and there is only a 0.01 % chance that an F – value this large could occur due to noise.

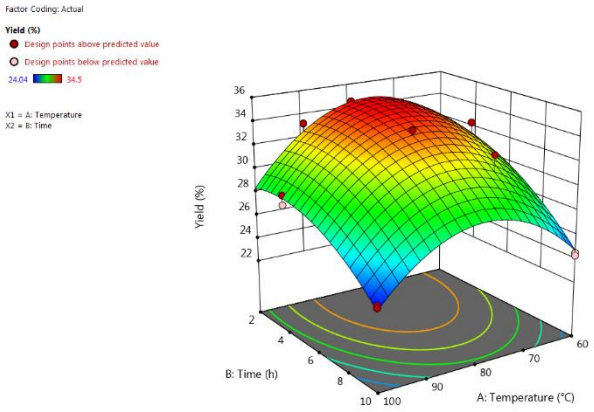

Figure-1: Contour 3D plot of chitosan extracted from *Matuta lunaris* at different time (h) and temperature (°C) on yield (%)

Based on the Table 1, for chitosan extraction, the extraction temperature and time during deacetylation play an important role in determining chitosan yield. The effects of extraction time and temperature are shown in Figure 1.

Table-1: ANOVA table for response of yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	220.05	5	44.01	52.13	< 0.0001	significant
A-Temperature	9.23	1	9.23	10.94	0.0079	
B-Time	64.26	1	64.26	76.12	< 0.0001	
AB	2.05	1	2.05	2.43	0.1502	
A^2	47.27	1	47.27	56.00	< 0.0001	
B^2	7.96	1	7.96	9.43	0.0118	
Residual	8.44	10	0.8442			
Lack of Fit	7.54	5	1.51	8.41	0.0178	significant
Pure Error	0.8976	5	0.1795			
Cor Total	228.50	15				

Both of the variables played prominent role in extraction efficiency for chitosan yield, so by increasing the temperature and time of deacetylation, the yield increases significantly to the certain point. The best peak of chitosan yield obtained was at condition of temperature 80 °C with time 6 h. The decreasing percent of chitosan yield when higher temperature was applied might be affected by excessive removal of acetyl group during the depolymerisation of chitosan polymer (Hossain and Iqbal, 2014).

The high yield obtained in this study justifies the potential of moon crab usage as an economic source for the production of chitosan on an industrial scale due to the availability of non seasonal moon crab and the low cost of the source. However, based on Khan et al. (2002), loss of weight of chitosan during the removal of acetyl group during deacetylation also affected the yield obtained. Other factors that concern the yield of chitosan are the different concentration used of acid and alkaline during the process of demineralization and deproteination (Samar et al., 2012). Extraction process sequences also proved to affect the responses (Lertsutthiwong, 2002). If deproteination precedes others, protective layer of protein is removed and the unprotected chitin is exposed to the hydrochloric acid (HCl), ruling to efficient demineralization but disadvantage to the yield obtain as more hydrolysis will happen and loss of material in chitin fraction. If demineralization is prior to deproteination, adhering protein protects the chitin resulting in less hydrolysis and higher yield. The yield of chitin and chitosan is varying also in different animals of the same group or different groups (Majekodunmi et al., 2017).

Degree of deacetylation

Removal of acetyl groups from the molecular chain of chitin happens at the process of deacetylation, residing behind a compound (chitosan) with a high degree of chemical reactive amino group (-NH2) (Baskar and Kumar, 2009). In any case, the degree of deacetylation (DDA) can be employed to differentiate between chitin and chitosan because it figures the content of free amino groups in the polysaccharides (Sarbon et al., 2015). The degree of deacetylation (DDA) is a parameter that affects chitosan properties such as chemical reactivity, covalent linking ability, solubility, viscosity and biodegrability (Lamarque et al., 2005 ; Lertsutthiwong, 2002).Degree of deacetylation obtained in this studywas ranged from 52.12 to 57.82%. The following linear, first orderpolynomial equation explained a significant response (i.e. relationship) (p-value < 0.05) between the independent variables of time of deacetylation (h) (X_1), temperature of deacetylation (°C) (X_2) and the dependent variable degree of deacetylation (DDA) (%) as:

$$\text{Degree of deacetylation (DDA)} = 55.24 + 1.01X_1 + 1.57X_2 \quad (2)$$

$R^2 = 0.9587$

Degree of deacetylation (DDA) was found to have linear relationship with independent variables of deacetylation's time and temperature (Figure 1). Time and temperature of deacetylation affected significantly ($p < 0.05$) the degree of deacetylation (DDA) of chitosan extracted. The R^2 value indicated that 95.87% of the total variation was explained by the model. The "predicted R^2" of 0.9372 is in reasonable agreement with the "adjusted R^2" of 0.9524 with the difference is less than 0.2. The model F – value of 150.99 implies the model is significant and there is only a 0.01 %

chance that an F – value this large could occur due to noise.

There are various strong alkali reagents used in deacetylating chitin such as sodium hydroxide (NaOH), potassium hydroxide (KOH) and sodium borohydride/ tetrahydridoborate ($NaBH_4$). In this study, sodium hydroxide (NaOH) was chosen as the alkali reagent, due to the more effective treatment compared to others (Younes et al., 2014). Degree of deacetylation (DDA) more than 50% shows the presence of amino group at the C – 2 position instead of acetamido group and high numbers of chitosan monomers are in the state of deacetylation form (Palma-Guerrero et al., 2010). Previous study showed that the degree of deacetylation of mudcrab under 40% sodium hydroxide (NaOH) for 2 hours at 105 °C was 53.40% (Sarbon et al., 2015). The degree of deacetylation (DDA) values are highly reliant on the type of source and different method of extraction, as well as the type of analytical methods employed, sample preparation and various other conditions that may influence the degree of deacetylation analysis. Based on Table 2 and Figure 2, results obtained in this study was in agreement with previous studies done by Tsaih and Chen (2003), the degree of deacetylation (DDA) of the chitosans obtained increased with increasing reaction time (h) and temperature (°C).In agreement with previous one, Chang et al. (2012) studied about the influence of sodium hydroxide (NaOH) concentration, temperature and solution ratio and reported that increasing of temperature and sodium hydroxide (NaOH) solution caused an increment in degree of deacetylation (DDA).

The molecular weight is an important parameter which reflexes the quality of chitosan. Lower molecular weight is more favorable as it possesses better anti – bacterial, antioxidant and coagulating potential (Patria, 2013). There is no standardized value for molecular weight of chitosan, however it is known that low molecular weight chitosan is less than 50 kDA, medium molecular weight chitosan is 50 to 100 kDA, while the high molecular weight chitosan is more than 150 kDA (Goy et al., 2009). Molecular weight of chitosan in this study was ranged from 540 kDA to 710 kDA. In agreement with Ahamed et al. (2015), the molecular weight of chitosan extracted from Indian crab shell was found to be 600 ± 10 kDA.

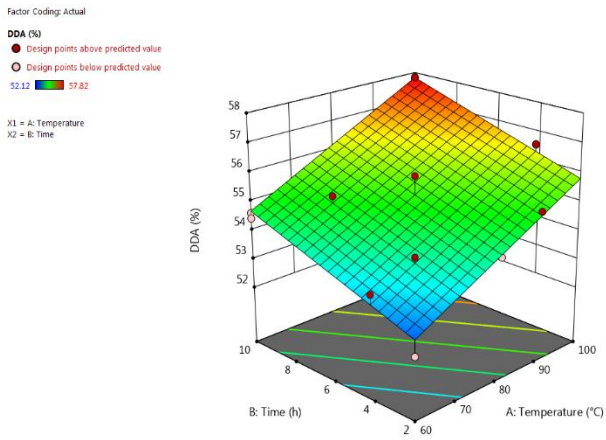

Figure-2: Contour 3D plot of chitosan extracted from *Matuta lunaris* at different time (h) and temperature (°C) on degree of deacetylation (%).

Molecular weight

Hwang et al. (2002) studied the optimization of extraction condition for deacetylation degree and molecular weight of chitosan. The depolymerized chitosan was in range from 100 to 1100 kDA by sodium hydroxide (NaOH) alkaline treatment.

Table-2: ANOVA table for response of DDA

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	31.50	2	15.75	150.99	<0.0001	significant
A-Temperature	22.07	1	22.07	211.53	<0.0001	
B-Time	8.62	1	8.62	82.64	<0.0001	
Residual	1.36	13	0.1043			
Lack of Fit	0.7447	8	0.0931	0.7612	0.6523	not significant
Pure Error	0.6114	5	0.1223			
Cor Total	32.86	15				

The first order polynomial equation showed a significant response (i.e. relationship) (p-value < 0.05) between the independent variables of time of deacetylation (h) (X_1), temperature of deacetylation (°C) (X_2) and the dependent variable, molecular weight (kDA), as:

Molecular weight = $622.13 - 63.49X_1 - 17.29X_2$ (3)
$R^2 = 0.8799$

From the equation (3), molecular weight obtained was found to have linear relationship with independent variables of deacetylation's time and temperature (Figure 1). Time and temperature of deacetylation affected significantly ($p < 0.05$) the yield of chitosan extracted. The R^2 value indicated that 96.31 % of the total variation was explained by the model. The "predicted R^2" of 0.9155 is in reasonable agreement with the "adjusted R^2" of 0.9446 with the difference is less than 0.2. The model F – value of 64.83 implies the model is significant and there is only a 0.01 % chance that an F – value this large could occur due to noise.
Based on Table 3, for chitosan extraction, the extraction temperature and time during deacetylation significantly affected ($p<0.05$) the molecular weight. The effects of extraction time and temperature are shown in Figure 3. Increment in temperature and time during the deacetylation process caused decrement in molecular weight of chitosan. This is in line with previous study done by Weska et al. (2007), as they reported increase in chitosan's molecular weight happened when the temperature was decreased. Tsaih and Chen (2003) applied the conditions of deacetylation at 99 °C or 140 °C for 1 to 9 h with 50% sodium hydroxide (NaOH) solution. They proved the point that molecular weight of chitosan decreased as the reaction time and temperature increased. In present study, the lowest value of molecular weight was found in the combination of higher time (+1) and higher temperature (+1). In this condition, the amino groups predominated to substitute the acetyl groups in the polymeric chains, besides the depolymerisation, resulting to the low molecular weight value (Weska et al., 2007). According to Prashanth et al. (2002), without the polysaccharides chain's degradation, presence of alkalis prevents the removal of acetyl groups of chitins. Depolymerization will be the result from the reagent's high temperature and reaction times required to obtain a complete deacetylation.

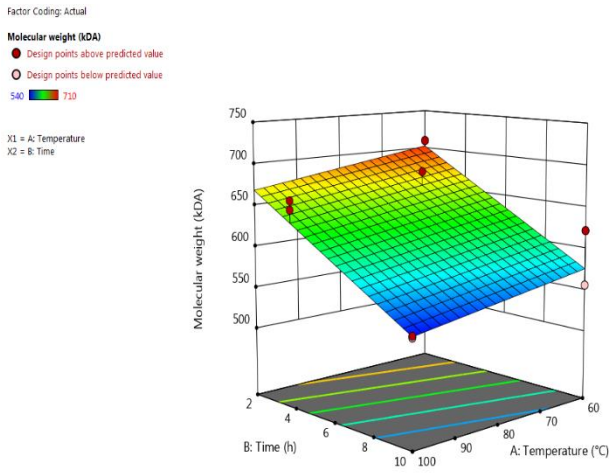

Figure-3: Contour 3D plot of chitosan extracted from *Matuta lunaris* at different time (h) and temperature (°C) on molecular weight (kDA)

Ash content

The ash content in chitosan is an important parameter as it can affect its solubility and viscosity, as well as other important characteristics. Low ash content values indicate the efficiency of demineralization step followed in the preparation of the chitosan sample by removing the minerals. Ash content obtained in this study ranged from 10.55 to 11.95%.

Table-3: ANOVA table for molecular weight

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	37504.89	2	18752.45	47.62	< 0.0001	significant
A-Temperature	2688.89	1	2688.89	6.83	0.0215	
B-Time	34236.86	1	34236.86	86.95	< 0.0001	
Residual	5118.86	13	393.76			
Lack of Fit	2852.36	8	356.54	0.7866	0.6375	not significant
Pure Error	2266.50	5	453.30			
Cor Total	42623.75	15				

Previous studies recorded that ash content of chitosan from shrimp waste deacetylated by 70% NaOH for 72 h at room temperature was 32.27% (Ghannam et al., 2016). Chitosan extracted from mud crab (*Scylla olivicea*) shells, was deacetylated with 40% of sodium hydroxide (NaOH) at 105 °C for 2 h, showed ash content of 5.97% (Sarbon et al., 2015). Experimental data obtained for ash content of chitosan were fitted into linear relationship with time of deacetylation (h) (X_1) and temperature (°C) (X_2), were described by the following second order polynomial equation;

$$\text{Ash} = 11.23 - 0.6216X_1 - 0.0255X_2 \quad (4)$$

$R^2 = 0.9801$

From the equation (4), ash content obtained was found to have linear relationship with independent variables of deacetylation's time and temperature (Figure 4). Time of deacetylation affected significantly ($p < 0.05$) the ash content of chitosan extracted. The R^2 value indicated that 98.01% of the total variation was explained by the model. The "predicted R^2" of 0.9710 is in reasonable agreement with the "adjusted R^2" of 0.9770 with the difference is less than 0.2. The model F – value of 319.61 implies the model is significant and there is only a 0.01% chance that an F – value this large could occur due to noise.

Based on Table 4, p-value less than 0.05 indicated that independent variable (time of deacetylation) interact with the ash content in the chitosan extraction. Pertubation plot obtained showed that variable of temperature gave only a slight variation of ash content compared to the time of deacetylation, which proved the residual amount of calcium carbonate ($CaCO_3$) is further removed in the process of deacetylation. Most of the minerals in crab shells were removed in the demineralization step. An increase in treatment time during demineralization process or an increase in the concentration of hydrochloric acid (HCl) during demineralization is among the possibilities for reduction in the ash content (Pillai et al., 2009). However, in this study, the demineralization time was set to 16 h because prolonged demineralization time, even in 24 h, will result in very slight drop of ash content but can cause polymer degradation resulting in declining yield. Ash content of chitosan also affected significantly by the type of species and seasonal variations (Pachapur et al., 2016). The mineral content in the crab shells is higher because of strong bond between chitin and mineral, indicating the hard structure of the crab shells. Different concentration of hydrochloric acid (HCl) used during the demineralization process also affect the ash content (Sarbon et al., 2015).

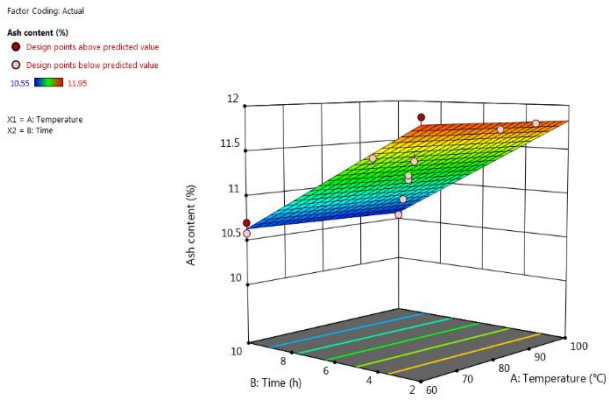

Figure-4: Contour 3D plot of chitosan extracted from *Matuta lunaris* at different time (h) and temperature (°C) on ash content (%)

Optimization

For the chitosan extraction from crab shells,by comparing slope of every responses, it was seen that temperature of deacetylation is more dominant factor compared to time of deacetylation in terms of the influence it had on yield, degree of deacetylation and moisture.

Table-4: ANOVA table for response of ash content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.30	2	1.65	319.61	< 0.0001	significant
A-Temperature	0.0059	1	0.0059	1.13	0.3062	
B-Time	3.28	1	3.28	636.02	< 0.0001	
Residual	0.0671	13	0.0052			
Lack of Fit	0.0425	8	0.0053	1.08	0.4881	not significant
Pure Error	0.0246	5	0.0049			
Cor Total	3.37	15				

For dependent variable of ash content, it plays a minimal influence. In the present study, the values of determination coefficient (R^2) for all models are in range of 87.99 - 98.01%, indicate that only 1.99–12.01% value of variation are not explained by the model due to the other factors which are not included in the model.Lack-of-Fit is the variation due to the model inadequacy; either proposed statistical model fits well or not. The lack of fit for this present study was not significant for all models, thus indicate that the models are good and adequately explain the variation in the responses.

The suggested optimum condition of deacetylation for chitosan extraction was temperature of 84.62 °C and time of 9.46 h. The yields, degree of deacetylation, molecular weight and ash content predicted from the optimum conditions of extraction were 29.24%, 56.48%, 563.26 kDA and 10.69%, respectively. In order to validate responses, an additional experiment was conducted with three replicates for each response. The results obtained were 28.96 ± 0.93% for yield, 56.68 ± 1.66% for degree of deacetylation, 567.17 ± 13.91 kDA of molecular weight and 10.59 ± 0.62% for ash content. T-test was done and the obtained p-value for all responses were higher than 0.05, indicated that there was no significant difference between predicted and actual values. Therefore, the suggested optimum condition by response surface methodology is suitable for chitosan extraction in moon crab (*Matuta lunaris*) shells.

Conclusion

In conclusion, the response surface methodology (RSM) applied suggested that the optimum condition of deacetylation for chitosan extraction was temperature of 84.62 and time of 9.46 h. At this deacetylation time and temperature, the yield, degree of deacetylation, molecular weight, and ash content actual values were 28.96 ± 0.93%, 56.68 ± 1.66%, 567.17± 13.91 kDA, 10.59 ± 0.62%, respectively. After validation test (t-test) was conducted, it was found that the result is not significant (p-value > 0.05) and in similar agreement with the point prediction value generated by response surface methodology (RSM). This study proved that moon crab (*Matuta lunaris)* can be used as potential source for chitosan extraction with optimized deacetylation conditions for future studies.

Contribution of Authors

Abdullah QH: Data collection and manuscript writing, Data interpretation
Zamri AI: Statistical analysis, Manuscript final reading and final approval, Data interpretation
Chilek TZT: Conceived idea and manuscript final approvaL
Ahmad F: Statistical analysis
Razak SBA: Literature search,
Farhaten WN: Manuscript writing, Designed research methodology

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References

Abdelaziz MEE, 2012. Production and characterization of chitosan. PhD Thesis, Department of Food Science, Faculty of Agriculture, Cairo University, Egypt.

Ahamed MN, Sankar S, Kashif PM, Basha SH and Sastry TP, 2015. Evaluation of biomaterial containing regenerated cellulose and chitosan incorporated with silver nanoparticles. Int. J. Biol. Macromol. 72: 680-686.

AOAC, 1990. Official methods of analysis of the Association of Official Analytical Chemistry, 15th edition. The Association of Official Analytical Chemistry, Washington, DC, USA.

Baskar D and Kumar TS, 2009. Effect of deacetylation time on the preparation, properties and swelling behavior of chitosan films. Carbohydr. Polym. 78(4): 767-772.

Bautista-Baños S, Hernandez-Lauzardo AN, Velazquez-Del Valle MG, Hernández-López M, Barka EA, Bosquez-Molina E and Wilson CL, 2006. Chitosan as a potential natural compound to control pre and postharvest diseases of horticultural commodities. Crop Prot. 25(2): 108-118.

Benbettaieb N, Chambin O, Assifaoui A, Al-Assaf S, Karbowiak T and Debeaufort F, 2016. Release of coumarin incorporated into chitosan-gelatin irradiated films. Food Hydrocoll. 56: 266-276.

Cervera MF, Heinämäki J, Räsänen M, Maunu SL, Karjalainen M, Acosta ON and Yliruusi, 2004. Solid-state characterization of chitosans derived

from lobster chitin. Carbohydr. Polym. 58(4): 401-408.

Chang JJ, Lee YH, Wu MH, Yang MC and Chien CT, 2012. Electrospun anti-adhesion barrier made of chitosan alginate for reducing peritoneal adhesions. Carbohydr. Polym. 88(4): 1304-1312.

Ghannam HE, Talab AS, Dolganova NV, Hussein AM and Abdelmaguid NM, 2016. Characterization of Chitosan Extracted from Different Crustacean Shell Wastes. J. Appl. Sci. 16(10): 454-461.

Goy RC, Britto DD and Assis OB, 2009. A review of the antimicrobial activity of chitosan. Polimeros. 19(3): 241-247.

Hossain MS and Iqbal A, 2014. Production and characterization of chitosan from shrimp waste. J. Bangladesh Agric. Univ. 12(1): 153-160.

Hwang KT, Jung ST, Lee GD, Chinnan MS, Park YS and Park HJ, 2002. Controlling molecular weight and degree of deacetylation of chitosan by response surface methodology. J. Agric. Food Chem. 50(7): 1876-1882.

Khan TA, Peh KK and Ch'ng HS, 2002. Reporting degree of deacetylation values of chitosan: the influence of analytical methods. J. Pharm. Pharmaceut. Sci. 5(3): 205-212.

Kurniasih M and Dewi RS, 2018. Toxicity tests, antioxidant activity, and antimicrobial activity of chitosan. In IOP Conference Series: Mater. Sci. Eng. 349(1): 012037.

Lamarque G, Cretenet M, Viton C and Domard A, 2005. New route of deacetylation of α-and β-chitins by means of freeze− pump out− thaw cycles. Biomacromol. 6(3): 1380-1388.

Lertsutthiwong P, How NC, Chandrkrachang S and Stevens WF, 2002. Effect of Chemical Treatment on the Characteristics of Shrimp Chitosan. J. Metal. Mater. Miner. 12(1): 11-18.

Majekodunmi SO, Olorunsola EO, Ofiwe UC, Udobre AS and Akpan EE, 2017. Material properties of chitosan from shells of *Egeria radiata*. Drug delivery considerations. J. Coastal Life Med. 5(7):321-324.

Mohammed MH, Williams PA and Tverezovskaya O, 2013. Extraction of chitin from prawn shells and conversion to low molecular mass chitosan. Food Hydrocoll. 31(2): 166-171.

Nouri M, Khodaiyan F, Razavi SH and Mousavi M, 2016. Improvement of chitosan production from Persian Gulf shrimp waste by response surface methodology. Food Hydrocoll. 59: 50-58.

Oduor-Odeto PM, Struszezyk MH and Peter MG, 2005. Characterisation of chitosan from blowfly larvae and some crustacean species from kenyan marin waters prepared under different conditions. West Indian Ocean J. Mar. Sci. 4(1): 99-108.

Pachapur VL, Guemiza K, Rouissi T, Sarma SJ and Brar SK, 2016. Novel biological and chemical methods of chitin extraction from crustacean waste using saline water. J. Chem. Technol. Biotechnol. 91(8): 2331-2339.

Palma-Guerrero J, Lopez-Jimenez JA, Pérez - Berná AJ, Huang IC, Jansson HB, Salinas J and Lopez-Llorca LV, 2010. Membrane fluidity determines sensitivity of filamentous fungi to chitosan. Mol. Microbiol. 75(4): 1021-1032.

Patria A, 2013. Production and characterization of Chitosan from shrimp shells waste. Aquac. Aquar. Conserv. Legis. 6(4): 339-344.

Pillai CKS, Paul W and Sharma CP, 2009. Chitin and chitosan polymers: Chemistry and fiber formation. Prog. Polym. Sci. 34(7): 641-678.

Prashanth KH, Kittur FS and Tharanathan RN, 2002. Solid state structure of chitosan prepared under different N-deacetylating conditions. Carbohydr. Polym. 50(1): 27-33.

Rajendran R, Abirami M, Prabhavathi P, Premasudha P, Kanimozhi B and Manikandan A, 2015. Biological treatment of drinking water by chitosan-based nanocomposites. Afr. J. Biotechnol. 14(11): 930-936.

Sarbon NM, Sandanamsamy S, Kamaruzaman SFS and Ahmad F, 2015. Chitosan extracted from mud crab (Scylla olivicea) shells: physicochemical and antioxidant properties. J. Food Sci. Technol. 52(7): 4266-4275.

Samar MM, El-Kalyoubi MH, Khalaf MM and Abd El-Razik MM, 2012. Physicochemical, functional, antioxidant and antibacterial properties of chitosan extracted from shrimp wastes by microwave technique. Ann. Agric. Sci. 58(1): 33–41.

Samicho Z and Ramli A, 2011. Extraction of chitosan & its film-forming properties: A review. In Business, Engineering and Industrial Applications (ISBEIA). pp. 576-580.

Tsaih ML and Chen RH, 2003. The effect of reaction time and temperature during heterogenous alkali deacetylation on degree of deacetylation and molecular weight of resulting chitosan. J. Appl. Polym. Sci. 88(13): 2917-2923.

Weska RF, Moura JMD, Batista LDM, Rizzi J and Pinto LADA, 2007. Optimization of deacetylation

in the production of chitosan from shrimp wastes: Use of response surface methodology. J Food Eng. 80(3): 749-753.

Younes I, Hajji S, Frachet V, Rinaudo M, Jellouli K and Nasri M, 2014. Chitin extraction from shrimp shell using enzymatic treatment. Antitumor, antioxidant and antimicrobial activities of chitosan. Int. J. Biol. Macromol. 69: 489-498.