Screening factors affecting chitosan extraction from mud crab (Scylla sp.) shell using microwave irradiation for the Response Surface Approach

N Arpi*, Fahrizal, Y M Lubis, Asmawati, M T Fayyadh, Y Atmajaya

Agricultural Product Technology Department, Faculty of Agriculture, Universitas Syiah Kuala, Jalan Tgk. Hasan Krueng Kalee 3, Darussalam-Banda Aceh 23111, Indonesia

*Email: normalina.arpi@unsyiah.ac.id

Abstract. Extracting chitosan from crab shell using conventional method requires processing in strong acid and alkali conditions under high temperature with long reaction time. Microwave-based extraction proposes shorter processing time, and hence energy and cost efficient. This study intended to screen factors affecting the extraction of chitosan from mud crab shell using microwave irradiation. The factors would be used in Response Surface Methodology (RSM) approach to obtain the optimum conditions for chitosan extraction. Microwave irradiation was employed in all of the three steps of chitosan extraction, the demineralization, deproteination, and deacetylation processes. A fractional factorial design was used to screen eight independent factors to determine the most significant ones to be optimized to determine the maximum value of four responses, which were yield, mineral removal, degree of deacetylation and moisture content. The results show that all of the main independent variables were significant in affecting minimal one of the 4 responses (P < 0.05). Mud crab chitosan had the degree of deacetylation of 87.72 – 95.13 %. Results of the screening analysis concluded, the main independent factors that are going to be applied in the optimization study are NaOH concentration, microwave oven power, and reaction time in deacetylation process.

1. Introduction

Indonesia's crab production in 2018 reached 21.6 tons. Crab production and exports continue to increase [1] and the waste produced has built up. The Indonesian Ministry of Maritime Affairs and Fisheries estimates that 56.2 tons of crustacean waste has not been utilized [2]. FAO reported that in 2017 crustaceans shell waste produced was 8.4 million tonnes [3] in which 40-50% of these could have application in the food industries. Crustaceans include prawn waste, and crab shell contain about 15-30% chitin, 30-50% protein, and 15-50% minerals, mainly calcium carbonate on dry basis [4]-[5]. The percentage vary with species and seasons. Currently, the processing of crab waste as fertilizer and animal feed only utilizes a small portion of the available waste. Crab waste containing chitin has the potential for extracting to produce chitosan.

Extracting chitin and chitosan from crab shell needs conditions of strong acid and alkali with high temperature. The production from crab shells goes through the stages of demineralization and deproteination to produce chitin which is followed by deacetylation to yield chitosan. Factors that
affection the characteristics and physico-chemical properties of chitosan during extraction include extraction temperature, alkali and acid concentration and extraction time. Chitosan extraction using conventional heating usually takes a long time employing high temperatures and high alkali concentration which increase costs and generate environmental problems. Study of [6] reported that heating with microwave irradiation can be used as an alternative to conventional heating. Microwaves are able to reach the extraction temperature in a short time and produce uniform molecular motion. Microwave irradiation is the most effective method in terms of time, energy, and cost.

Setyawati et al. [7] reported that the extraction of chitosan from crab shells using the microwave irradiation at 800 Watt for 5 minutes resulted in the highest degree of deacetylation of 86% and yield of 75%. These results are not much different from conventional methods but are obtained in a shorter time. In addition, the molecular weight obtained from the microwave irradiation method at a power of 400, 560 and 800 Watt is lower than that of the conventional method. This result also supported by research by [8]. The molecular weight will be lower with the use of higher microwave power [9]-[7]. Degree of deacetylation (DD) was influenced by the concentration of NaOH in the deacetylation reaction. Increasing the concentration of NaOH will increase the value of the degree of deacetylation [10].

This study intended to screen factors affected the extraction of chitosan from mud crab shell by using microwave irradiation. The factors would be used in Response Surface Methodology (RSM) approach to obtain the optimum conditions for chitosan extraction. In this research, all of the chitosan extraction steps, demineralization, deproteinisation, and deacetylation will be carried out using microwave method.

2. Materials and methods
The main material used in this study was mud crab (Scylla sp.) shells from Ulee Lheeu, Banda Aceh. The chemical used were NaOH, and HCl obtained from Merck (Merck KGaA, 64271Darmstad, Germany).

2.1. Study design
In the screening process of chitosan extraction, a $2^{8-5}$ fractional factorial design was used. Eight independent variables with two different levels were chosen (Tables 1 and 2) based on other results studies [11]-[12]-[13]. The dependent variables measured as a response in the screening process were chitosan yield, degree of deacetylation, percent of ash removal, and chitosan water content.

| Independent variable                        | Symbol | Level -1 | Level +1 |
|---------------------------------------------|--------|----------|----------|
| HCl concentration in demineralization (M)   | A      | 0.5      | 1.5      |
| Demineralization time (minute) at 600 Watt  | B      | 1        | 4        |
| NaOH concentration in deproteinization (%)  | C      | 5        | 10       |
| Deproteinization time (minute) at 600 Watt  | D      | 1        | 4        |
| NaOH : chitin ratio (v/w)                   | E      | 10       | 30       |
| NaOH concentration in deacetylation (%)     | F      | 35       | 55       |
| Microwave oven power (Watt)                 | G      | 400      | 800      |
| Deacetylation time (sec)                    | H      | 55       | 145      |

* represents higher and – represents lower levels
2.2. Chitosan extraction
The crab shells were washed then dried in an oven at 60°C for 6 hours [9]. The dried crab shells were then ground and sifted through 60 mesh. Chitosan extraction of crab shell powder was achieved in three steps, i.e., demineralization, deproteinization, and deacetylation. In this study, all of the chitosan extraction steps were studied using microwave technology. The microwave irradiation heating was used in all steps to replace the conventional heating.

2.3. Demineralization
Crab shell powder were mixed with HCl solution in a ratio of 1:10 (w/v). The varying HCl concentration solutions and heating times of the mixture in a microwave oven at 600 Watt were based on the levels in Table 1. The mixture was then filtered through filter paper, and the solid was washed with distilled water until the pH was neutral. The solid was then dried in an oven at 60°C for 6 hours.

2.4. Deproteinization
The demineralized solids were added with NaOH solution in ratio of 1:10 (w/v) with the varying concentration solutions and heating times in the microwave oven at 600 Watt according to Table 1. The solutions were then filtered, the resulting solids were washed with distilled water until neutral pH was reached, then the solids were dried in an oven at 60°C for 6 hours. The solid products attained were designated as chitin.

2.5. Deacetylation
Chitin powder obtained were treated with the NaOH solution with varying NaOH concentration, ratio, microwave irradiation heating power and time listed in Table 1. The residual solids obtained were washed with distilled water until neutral pH was reached, then dried for 6 hours in an oven at 60°C.

Table 2. Fractional factorial screening design in randomized order.

| Standard order | Run order | Independent variable |
|----------------|-----------|----------------------|
|                |           | A (M)    | B (min) | C (%)  | D (min) | E (v/w) | F (%)  | G (Watt) | H (sec) |
| 15             | 1         | 0.5      | 4       | 10     | 4       | 10      | 55     | 400      | 55     |
| 12             | 2         | 1.5      | 4       | 5      | 4       | 10      | 35     | 400      | 145    |
| 5              | 3         | 0.5      | 1       | 10     | 1       | 30      | 55     | 800      | 55     |
| 1              | 4         | 0.5      | 1       | 5      | 1       | 10      | 35     | 400      | 55     |
| 3              | 5         | 0.5      | 4       | 5      | 1       | 30      | 55     | 400      | 145    |
| 7              | 6         | 0.5      | 4       | 10     | 1       | 10      | 35     | 800      | 145    |
| 11             | 7         | 0.5      | 4       | 5      | 4       | 30      | 35     | 800      | 55     |
| 4              | 8         | 1.5      | 4       | 5      | 1       | 10      | 55     | 800      | 55     |
| 9              | 9         | 0.5      | 1       | 5      | 4       | 10      | 55     | 800      | 145    |
| 8              | 10        | 1.5      | 4       | 10     | 1       | 30      | 35     | 400      | 55     |
| 14             | 11        | 1.5      | 1       | 10     | 4       | 10      | 35     | 800      | 55     |
| 10             | 12        | 1.5      | 1       | 5      | 4       | 30      | 55     | 400      | 55     |
| 6              | 13        | 1.5      | 1       | 10     | 1       | 10      | 55     | 400      | 145    |
| 16             | 14        | 1.5      | 4       | 10     | 4       | 30      | 55     | 800      | 145    |
| 13             | 15        | 0.5      | 1       | 10     | 4       | 30      | 35     | 400      | 145    |
| 2              | 16        | 1.5      | 1       | 5      | 1       | 30      | 35     | 800      | 145    |

See Table 1 for letter identifications
2.6. Characterization of chitosan

2.6.1. Chitosan yield. The yield was calculated as chitosan dry weight relative to the crab shell weight following the Equation (1).

\[
\text{Chitosan extraction yield (\%)} = \frac{\text{dried chitosan weight (g)}}{\text{dried crab shell powder (g)}} \times 100
\]  

(1)

2.6.2. Mineral removal. The mineral removal was determined by heating a chitosan sample in a furnace at 600°C for 6 hours and weighing the remaining product after cooling in a desiccator.

2.6.3. Degree of deacetylation. The absorption spectra of the FTIR were quantified in KBr pellets in the range 400-4000 cm\(^{-1}\) with 45 scans at resolution of 4 cm\(^{-1}\) using IRprestige-21, Shimadzu, Kyoto-Japan. The sample chitosan was mixed with KBr then the mixture was pressed to form a pellet. The degree of deacetylation was measured by the method of [14] from the intensity of the absorption bands at 1320 cm\(^{-1}\) (amide III band) and 1420 cm\(^{-1}\) as reference band. The DD was determined using the Equation (2) and the DA% was calculated by Equation (3). The study of [14] showed that the band ratio \(A_{1320}/A_{1420}\) results in the narrower experimental error regardless of the technique and the materials state.

\[
\text{DD \%} = 100 - \text{DA \%} \\
\left(\frac{A_{1320}}{A_{1420}}\right) = 0.3822 + 0.03133 \text{ DA}
\]  

(2)

(3)

2.6.4. Moisture content. The moisture content measured by gravimetric method. The moisture mass was calculated by drying the chitosan sample to constant weight and determining the weight of sample before and after drying. Moisture content was determined using the Equation (4).

\[
\text{Moisture content (\%)} = \frac{\text{wet chitosan weight (g)} - \text{dry chitosan weight (g)}}{\text{wet chitosan weight (g)}} \times 100
\]  

(4)

2.6.5. Statistical analysis. For screening purposes, fractional factorial design was used to study the 8 independent variables with 4 dependent variables, as responses. The screening results were analysed using Design Expert 12 for windows. In order to determine independent variables that affect dependent factors at the significant level of 0.05. The screening experiment results support an analysis of all the 8 independent variables according to all 4 dependent variables at once to determine which independent variable(s) affect dependent variables at the significant level of 0.05.

3. Results and discussion

3.1. Yield of chitosan

Yield of chitosan obtained by demineralization, deproteinization, and deacetylation of the mud crab shell using 8 different independent variables are as shown in Table 3, the chitosan yield was 12.5 - 78%. The percent yield varies due to the effectiveness of minerals and proteins removal from the attached crab shells [15]. Demineralization using 1.5 M HCl solution produced low chitosan yield, whereas the yield of 0.5 M HCl solution were high. The high chitosan yield in this study was due to the inefficiency of mineral removal in the demineralization process which was indicated by the low mineral removal when concentration of HCl used was a lower level (0.5 M) compared to the higher level of 1.5 M (Table 4).
Table 3. Fractional factorial screening design with experimental (Exp) and predicted (Pre) results for yield of chitosan from mud crab shell.

| Standard Order | Run Order | A (M) | B (min) | C (%) | D (min) | E (% v/w) | F (%) | G (Watt) | H (sec) | Yield (%) |
|---------------|----------|-------|---------|-------|---------|-----------|-------|----------|---------|-----------|
| 15            | 1        | 0.5   | 4       | 10    | 10      | 55        | 400   | 55       |         | 48        |
| 12            | 2        | 1.5   | 4       | 5     | 4       | 10        | 35    | 400      | 145     | 55        |
| 5             | 3        | 0.5   | 1       | 10    | 1       | 30        | 55    | 800      | 55      | 53        |
| 1             | 4        | 0.5   | 1       | 5     | 1       | 10        | 35    | 400      | 55      | 53        |
| 3             | 5        | 0.5   | 4       | 5     | 1       | 30        | 55    | 400      | 145     | 60        |
| 7             | 6        | 0.5   | 4       | 10    | 1       | 10        | 35    | 800      | 145     | 50.6      |
| 11            | 7        | 0.5   | 4       | 5     | 4       | 30        | 35    | 800      | 55      | 48        |
| 4             | 8        | 1.5   | 4       | 5     | 1       | 10        | 55    | 800      | 55      | 12.5      |
| 9             | 9        | 0.5   | 1       | 5     | 4       | 10        | 55    | 800      | 145     | 54        |
| 8             | 10       | 1.5   | 4       | 10    | 1       | 30        | 35    | 400      | 55      | 15        |
| 14            | 11       | 1.5   | 1       | 10    | 4       | 10        | 35    | 800      | 55      | 16.4      |
| 10            | 12       | 1.5   | 1       | 5     | 4       | 30        | 55    | 400      | 55      | 16        |
| 6             | 13       | 1.5   | 1       | 10    | 1       | 10        | 55    | 400      | 55      | 22.5      |
| 16            | 14       | 1.5   | 4       | 10    | 4       | 30        | 55    | 800      | 145     | 16.05     |
| 13            | 15       | 0.5   | 1       | 10    | 4       | 30        | 35    | 400      | 145     | 43        |
| 2             | 16       | 1.5   | 1       | 5     | 1       | 30        | 35    | 800      | 145     | 19        |

Effects of independent variables on yield: ** = Significant at the level of P < 0.05. ns = Not significant.

It could be noticed from Table 3 that the chitosan yield significantly affected by all independent variables except for factors C, E, and H. Figure 1 shows that factors A, B, and D had negative effects on yield. On the other hand, factors F and G positively affected yield.

Figure 1. Normal plot of the independent variables effect on chitosan yield obtained from the screening analysis.
The negative effect of factor A (HCl concentration) could be observed in Table 3. Higher concentration of 1.5 M HCl solution and longer the demineralization time of 4 minute resulted in lower yield of chitosan, ranging 12.5-22.5%, while HCl concentration of 0.5 M at 1 minute caused higher yield of 43-78%. These high chitosan yields might due to the high mineral content of the chitosan caused by HCl ineffective mineral removal. The low concentration of HCl solution caused low percentage of mineral removal which in turn produced high yield of chitosan. Study by [12] show that the mineral removal percentage increases with the increasing HCl concentration from 1-2.5 M. The same trend was observed for the effects of time in the deproteinisation process (factor D). In the decatetlation process, increasing the concentration of NaOH and microwave oven power will increase the yield of chitosan. This result in line with the report by [11]-[16] showing that chitosan yield increased with the increase in the NaOH concentration, microwave oven power, and irradiation time. Similar tendency also found by [16] that the yield of chitosan raised significantly as NaOH concentration increased, with the highest yield obtained from 50% NaOH solution.

3.2. Removal of mineral
Table 4 shows that the experimental results of mineral removal in the extracted chitosan were 16.76 – 95.20%. It could be noted that factor A, the independent variable of HCl concentration in the demineralization process, significantly affected the mineral removal. Furthermore, Figure 2 shows that factor A, the HCl concentration, positively affect the chitosan mineral removal.

**Table 4.** Fractional factorial screening design with experimental (Exp) and predicted (Pre) results for mineral removal of chitosan from mud crab shell.

| Standard Order | Run Order | A (M) | B (min) | C (%) | D (min) | E (v/w) | F (%) | G (Watt) | H (sec) | Mineral removal (%) |
|----------------|-----------|-------|---------|-------|---------|---------|-------|---------|---------|---------------------|
| 15             | 1         | 0.5   | 4       | 10    | 4       | 10      | 55    | 400     | 55      | 39.24               |
| 12             | 2         | 1.5   | 4       | 5      | 4       | 10      | 35    | 400     | 145     | 95.20               |
| 5              | 3         | 0.5   | 1       | 10     | 1       | 30      | 55    | 800     | 55      | 16.76               |
| 1              | 4         | 0.5   | 1       | 5      | 1       | 10      | 35    | 400     | 55      | 51.71               |
| 3              | 5         | 0.5   | 4       | 5      | 1       | 30      | 55    | 400     | 145     | 35.53               |
| 7              | 6         | 0.5   | 4       | 10     | 1       | 10      | 35    | 800     | 145     | 38.98               |
| 11             | 7         | 0.5   | 4       | 5      | 4       | 30      | 35    | 800     | 55      | 53.75               |
| 4              | 8         | 1.5   | 4       | 5      | 1       | 10      | 55    | 800     | 55      | 94.68               |
| 9              | 9         | 0.5   | 1       | 5      | 4       | 10      | 55    | 800     | 145     | 20.27               |
| 8              | 10        | 1.5   | 4       | 10     | 1       | 30      | 35    | 400     | 55      | 92.08               |
| 14             | 11        | 1.5   | 1       | 10     | 4       | 10      | 35    | 800     | 55      | 93.75               |
| 10             | 12        | 1.5   | 1       | 5      | 4       | 30      | 55    | 400     | 55      | 93.84               |
| 6              | 13        | 1.5   | 1       | 10     | 1       | 10      | 55    | 400     | 145     | 86.94               |
| 16             | 14        | 1.5   | 4       | 10     | 4       | 30      | 55    | 800     | 145     | 90.63               |
| 13             | 15        | 0.5   | 1       | 10     | 4       | 30      | 35    | 400     | 145     | 44.06               |
| 2              | 16        | 1.5   | 1       | 5      | 1       | 30      | 35    | 800     | 145     | 90.26               |

**Effects of independent variables on mineral removal**

| A | B | C | D | E | F | G | H | Mineral removal (%) |
|---|---|---|---|---|---|---|---|---------------------|
| **| **| **| **| **| **| **| **| **                      |

A= HCl concentration in demineralization, B= Time in demineralization, C= NaOH concentration in deproteinization, D= Time in deproteinization, E= NaOH : chitin ratio in decatetylation, F= NaOH concentration in decatetylation, G= Microwave oven power, H= Time in decatetylation. **Significant at the level of P < 0.05.

High HCl concentration solution of 1.5 M in demineralization process resulted in high mineral removal of 86.94 – 95.20% which however, insufficient in total removing of all minerals. Study by [12] found that the removal of mineral bounding to shrimp shells required reaction using 2.5 M HCl in
demineralization steps. The lower HCl concentration of 0.5 M can only remove minerals as much as 16.76 - 53.75%. Review of [17] suggested that higher acid concentration have a thorough reaction and assure all minerals, primarily calcium carbonate, totally removed. However, it was stated that drastic acid treatments may cause polymer degradation as acetylation degree and the molecular weight (Mw) was lowered. The usual acid used in conventional demineralization process is hydrochloric acid (HCl), although demineralization is usually also accomplished using sulfuric acid (H2SO4), nitric acid (HNO3), hydrochloric acid (HCl), or acetic acid (CH3COOH).

Concentration of NaOH in deacetylation process, factor F, also affected mineral removal (Table 4) and has negative effect (Figure 2). Therefore, low NaOH concentration of 35% produced higher mineral removal than the 55% one. Unlike the use of high HCl concentrations in demineralization process which causes polymer degradation, the use of high NaOH concentrations up to 75% in deacetylation process causes only minimal degradation [17].

3.3. Degree of deacetylation
The experimental results for degree of deacetylation of mud crab chitosan were 87.72 – 95.13 % (Table 5). All of the independent variables A, C, D, E, F, G, and H, except demineralization time (factor B), significantly affected chitosan degree of deacetylation. Figure 3 shows that all of the affecting factors had negative effects on degree of deacetylation, beside deproteinisation time (factor D).

High HCl and NaOH concentrations in deminerlalization process and in deproteinisation process, respectively, and high level of NaOH:chitin ratio, NaOH concentration, microwave oven power, and time in deacetylation process resulted in chitosan with low degree of deacetylation. Whereas high level of deproteinization time produced high degree of deacetylation chitosan.
Table 5. Fractional factorial screening design with experimental (Exp) and predicted (Pre) results for chitosan Degree of Deacetylation (DD).

| Standard Order | Run Order | A (M) | B (min) | C (%) | D (min) | E (v/w) | F (%) | G (Watt) | H (sec) | DD (%) |
|----------------|-----------|-------|---------|-------|---------|---------|-------|----------|---------|--------|
| 15             | 1         | 0.5   | 4       | 10    | 4       | 10      | 55    | 400      | 55      | 94.98  |
| 12             | 2         | 1.5   | 4       | 5     | 4       | 10      | 35    | 400      | 145     | 92.99  |
| 5              | 3         | 0.5   | 1       | 10    | 1       | 30      | 55    | 800      | 55      | 90.00  |
| 1              | 4         | 0.5   | 1       | 5     | 1       | 10      | 35    | 400      | 55      | 95.13  |
| 3              | 5         | 0.5   | 4       | 5     | 1       | 30      | 55    | 400      | 145     | 91.44  |
| 7              | 6         | 0.5   | 4       | 10    | 1       | 10      | 35    | 800      | 145     | 91.99  |
| 11             | 7         | 0.5   | 4       | 5     | 4       | 30      | 35    | 800      | 55      | 93.78  |
| 4              | 8         | 1.5   | 4       | 5     | 1       | 10      | 55    | 800      | 55      | 88.56  |
| 9              | 9         | 0.5   | 1       | 5     | 4       | 10      | 55    | 800      | 145     | 92.55  |
| 8              | 10        | 1.5   | 4       | 10    | 1       | 30      | 35    | 400      | 55      | 90.14  |
| 14             | 11        | 1.5   | 1       | 10    | 4       | 10      | 35    | 800      | 55      | 89.76  |
| 10             | 12        | 1.5   | 1       | 5     | 4       | 30      | 55    | 400      | 55      | 91.59  |
| 6              | 13        | 1.5   | 1       | 10    | 1       | 10      | 55    | 400      | 145     | 87.72  |
| 16             | 14        | 1.5   | 4       | 10    | 4       | 30      | 55    | 800      | 145     | 88.50  |
| 13             | 15        | 0.5   | 1       | 10    | 4       | 30      | 35    | 400      | 145     | 92.98  |
| 2              | 16        | 1.5   | 1       | 5     | 1       | 30      | 35    | 800      | 145     | 89.28  |

Effects of independent variables on degree of deacetylation

A = HCl concentration in demineralization, B = Time in demineralization, C = NaOH concentration in deproteinization, D = Time in deproteinization, E = NaOH : chitin ratio in deacetylation, F = NaOH concentration in deacetylation, G = Microwave oven power, H = Time in deacetylation. ** Significant at the level of P < 0.05.

Figure 3. Normal plot of the independent variables effect on chitosan degree of deacetylation obtained from the screening analysis.
It has been reported that the degree of deacetylation depend on alkali concentration, particle size, previous treatment, and chitin density [17]. In addition [18] summarized that degree of deacetylation and molecular weight of chitosan are generally affected by temperature, concentration of NaOH, duration of reaction and alkaline process recurrence. Contrary to the results of this study, [16] reported that the degree of deacetylation of chitosan raised by increasing NaOH concentration in deacetylation process. They reported that 50% NaOH produced the highest degree of deacetylation of 95.19%. Although high NaOH concentration of 60% resulted in the highest deacetylated chitosan with maximum solubility, 50% NaOH treatment could also produced high-quality chitosan [17].

3.4. Moisture content

The chitosan moisture content found to be 0.81 – 19.95%. The independent factors of NaOH concentration, microwave oven power, and time in the deacetylation process significantly affected chitosan moisture content. All of the significant factors had positive effects on the moisture content (Table 6 and Figure 4). The low concentration of 35% NaOH in deacetylation process resulted in low chitosan moisture contents of 0.81 – 1.77%, while high concentration of 55% NaOH produced chitosan moisture contents of 1.66 – 19.95%. The highest moisture content of 19.95% induced by the highest microwave oven power and deacetylation time of 800 Watt and 145 second, respectively.

Table 6. Fractional factorial screening design with experimental (Exp) and predicted (Pre) results for chitosan moisture content (MC).

| Standard Order | Run Order | A (M) | B (min) | C (%) | D (min) | E (v/w) | F (%) | G (Watt) | H (sec) | MC (%) |
|----------------|-----------|-------|---------|-------|---------|---------|-------|----------|---------|--------|
| 15             | 1         | 0.5   | 4       | 10    | 4       | 10      | 55    | 400      | 55      | 1.66 -0.33 |
| 12             | 2         | 1.5   | 4       | 5     | 4       | 10      | 35    | 400      | 145     | 1.98 0.98 |
| 5              | 3         | 0.5   | 1       | 10    | 1       | 30      | 55    | 800      | 55      | 5.34 6.61 |
| 1              | 4         | 0.5   | 1       | 5     | 1       | 10      | 35    | 400      | 55      | 0.97 -0.49 |
| 3              | 5         | 0.5   | 4       | 5     | 1       | 30      | 55    | 400      | 145     | 3.49 6.75 |
| 7              | 6         | 0.5   | 4       | 10    | 1       | 10      | 35    | 800      | 145     | 1.06 1.31 |
| 11             | 7         | 0.5   | 4       | 5     | 4       | 30      | 35    | 800      | 55      | 1.07 -0.46 |
| 4              | 8         | 1.5   | 4       | 5     | 1       | 10      | 55    | 800      | 55      | 4.68 6.31 |
| 9              | 9         | 0.5   | 1       | 5     | 4       | 10      | 55    | 800      | 145     | 13.97 13.69 |
| 8              | 10        | 1.5   | 4       | 10    | 1       | 30      | 35    | 400      | 55      | 1.77 3.15 |
| 14             | 11        | 1.5   | 1       | 10    | 4       | 10      | 35    | 800      | 55      | 2.77 3.77 |
| 10             | 12        | 1.5   | 1       | 5     | 4       | 30      | 55    | 400      | 55      | 0.86 -0.05 |
| 6              | 13        | 1.5   | 1       | 10    | 1       | 10      | 55    | 400      | 145     | 11.34 10.98 |
| 16             | 14        | 1.5   | 4       | 10    | 4       | 30      | 55    | 800      | 145     | 19.95 17.33 |
| 13             | 15        | 0.5   | 1       | 10    | 4       | 30      | 35    | 400      | 145     | 0.81 1.28 |
| 2              | 16        | 1.5   | 1       | 5     | 1       | 30      | 35    | 800      | 145     | 1.32 1.60 |

Effects of independent variables on moisture content: ns ns ns - - ** ** ** **

A= HCl concentration in demineralization, B= Time in demineralization, C= NaOH concentration in deproteinization, D= Time in deproteinization, E= NaOH : chitin ratio in deacetylation, F= NaOH concentration in deacetylation, G= Microwave oven power, H= Time in deacetylation. **Significant at the level of P < 0.05. ns= Not significant.

On the contrary to the results of the present study, most of chitosan moisture content reported were very low [19]- [20], and can be as low as 0.0004% [21]. However, [22] published that moisture content of >10% may be found in commercial chitosan products. In addition, [23] found that mud crab chitosan
had moisture content of 9.48±0.59%, which was significantly lower than moisture content of commercial chitosan, which was 14.15±0.75%.

Chitosan is very hygroscopic in nature which during storage affected by moisture absorption that could lead to lump and activate fungal growth. A lower moisture content of chitosan tends to have better storage stability and enhances the quality [24]. Chitosan for used in application has standard moisture content ranged from 5.0%–15.0% depend on the humidity and the form of chitosan, powder or flake [23].

3.5. Screening and significant variables
The main and interaction effects of chitosan extraction independent factors on the responses, dependent factors, are shown in Table 7. The main and/or the interaction effects of the independent variables having P values of < 0.05 were significant. The dependent factor of yield significantly (P < 0.05) affected by B, D, F, G main factors, and by almost all interaction factors, except for AE and AH. In contrast with mineral removal, which significantly affected only by A, F, and AF factors. However, all of the main independent factors, except for B factor, were significant factors affecting chitosan degree of deacetylation (P < 0.05). The interaction effect of AE was also significant for the degree of deacetylation. In addition, the main and the interaction effects of F, G, H, AB and AC were significant for moisture content (P < 0.05).

The results of the screening analysis summarized that all of the main and interaction independent variables, except for AH, were significant factors affecting minimal one of the 4 responses (P < 0.05). In order to limit independent factors in the optimization study, only the main independent factors that affect two responses or more at once will be included. The factors are HCl concentration in the demineralization process (A), reaction time in the deproteinization steps (D), also factors F, G, and H which were NaOH concentration, microwave oven power, and reaction time in the deacetylation process, respectively. The demineralization, and deproteinization processes are separate steps prior to the deacetylation process. Therefore, the best level of HCl concentration in the demineralization process

![Figure 4. Normal plot of the independent variables effect on chitosan moisture content obtained from the screening analysis.](image)
and reaction time in the deproteinization steps will be determined before the optimization study. The results of the screening analysis concluded that the main independent factors that are going to be used in the optimization study are NaOH concentration (F), microwave oven power (G), and reaction time (H) in the deacetylation process.

Table 7. The P values for the main and interaction effects of independent variables on the dependent variables obtained from the screening analysis.

| Variable | Yield   | Mineral Removal | Degree of Deacetylation | Moisture Content |
|----------|---------|-----------------|-------------------------|-----------------|
| A        | 0.0015** | < 0.0001**      | < 0.0001**              | 0.1337          |
| B        | 0.0015** | 0.0008**        | 0.0353**                | 0.1325          |
| C        | 0.5221  | 0.0112**        | 0.0232**                | 0.0102**        |
| D        | 0.0044** | 0.0009**        | 0.0254**                | 0.0065**        |
| E        | 0.0380** | 0.0292**        | 0.0052**                | 0.0065**        |
| F        | 0.0564*  | 0.0194**        | 0.0032**                | -               |
| G        | 0.0014** | 0.0226**        | 0.0032**                | -               |
| H        | 0.0044** | 0.0009**        | 0.0232**                | 0.0102**        |
| AB       | 0.0564*  | 0.0194**        | 0.0032**                | -               |
| AC       | 0.0300** | 0.0292**        | 0.0052**                | 0.0065**        |
| AD       | 0.0380** | 0.0292**        | 0.0052**                | 0.0065**        |
| AE       | 0.0014** | 0.0226**        | 0.0032**                | -               |
| AF       | 0.0044** | 0.0009**        | 0.0232**                | 0.0102**        |
| AG       | 0.0564*  | 0.0194**        | 0.0032**                | -               |
| AH       | 0.0300** | 0.0292**        | 0.0052**                | 0.0065**        |

A= HCl concentration in demineralization, B= Time in demineralization, C= NaOH concentration in deproteinization, D= Time in deproteinization, E= NaOH : chitin ratio in deacetylation, F= NaOH concentration in deacetylation process, G= Microwave oven power, H= Time in deacetylation. ** Significant at the level of P < 0.05.

4. Conclusions
All the main and interaction independent variables, except for interaction effect of HCl concentration in demineralization process and reaction time in the deacetylation steps (AH) were significant in affecting minimal one of the 4 responses (P < 0.05). The response of chitosan yield and degree of deacetylation affected by almost all the main independent variables. The yields of mud crab chitosan were high, 12.5-78 % due to inefficient mineral removal, which ranged 16.76 – 95.20%. The mineral removal affected by HCl concentration in the demineralization steps and NaOH concentration in deacetylation process. HCl concentration solution of 1.5 M was insufficient in total removing of all minerals, higher HCl concentration is required. Based on the screening analysis, independent factors of NaOH concentration (F), microwave oven power (G), and reaction time (H) in the deacetylation process are going to be utilized in the optimization study are.

Acknowledgement
We wish to thank Universitas Syiah Kuala for research funding through the Calon Profesor scheme.

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