Optimization and characterization of chitosan extracted from *Mucor rouxii*

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Abstract. Chitosan is a non-toxic, biodegradable and biocompatible biopolymer comprising of (1,4)-linked amino-deoxy-β-D-glucan. The unique characteristics of biodegradability and non-toxicity led to versatile application of this biopolymers in various fields. The aim of this study was to identify factors which could optimize the yields and physicochemical characteristics of chitosan extracted from *Mucor rouxii* using different incubation period and temperature. Three combinations of incubation temperatures (98°C, 110°C and 121°C) and three incubation periods (15 minutes, 20 minutes, 25 minutes) were used in the alkaline treatment and the extracted chitosan were analysed for the dry weight, molecular weight and degree of deacetylation. Results shown that incubation temperature had a significant effect on the yield of chitosan (p<0.05). Kruskal-Wallis test shown that, different incubation temperatures and periods had no significant effects on the molecular weight and degree of deacetylation (p>0.05).

1. Introduction

Chitosan is the deacetylated derivative of chitin, which is the second most abundant natural polysaccharide after cellulose. Due to its unique characteristics such as biocompatibility, non-toxicity and biodegradability, chitosan has been widely used for different biological and biomedical application [1,2]. Antitumor, antimicrobial and antioxidants activities that chitosan possessed allow it to be used used for different application. This includes the use of chitosan as wound-healing materials, pharmaceutical excipient or drug carrier, and in dietary supplement [3,4].

The Zygomycetes fungi is the most potential source for chitosan production. They are fast-grower and primary utilizers of carbon sources such as sugar and starch. *M. rouxii* is one of the common fungal species investigated for chitosan production. The wider application of chitosan is also due to their physicochemical characteristics which are solubility, molecular weight and degree of deacetylation. The interaction between chitosan and its derivatives with bacteria cell wall molecules had resulted in the low molecular weight which had an indirect impact in greater antimicrobial activity [6,7], while the high degree of deacetylation (>85%) have strong positive charge in aqueous solution with pH below 6 [8]. The maximum yield of chitosan from fungal mycelium depend very much on the optimization of extraction and characterization of chitosan from *Mucor rouxii*. Therefore, in this study, we aimed to identify factors that will optimize the extraction and characterization of chitosan from *Mucor rouxii*.
The optimization in extraction was determined by comparing the chitosan production from three different temperature during alkaline treatment and different incubation period. Meanwhile, the characterization of chitosan was performed by comparing the molecular weight and the degree of deacetylation of chitosan between different incubation period and temperature.

2. Materials and methods

2.1. Preparation of spore and fungi culture

2.1.1. Organism. *Mucor rouxii* was obtained from the Microbiology Department, Hospital Universiti Sains Malaysia (HUSM). Fungi was cultured on Potato Dextrose Agar (PDA) and allowed to grow for five days at temperature 30°C in dark place for spore formation. The plates were covered with parafilm to avoid contamination. Combinations for three incubation period and three incubation temperature were used in this study; 15, 20 and 25 minutes and 98°C, 110°C and 121°C respectively.

2.1.2. Chemicals and reagents. The following chemical and reagents have been used in this study: Yeast Peptone Glucose (YPG) medium, (3 g yeast, 10g glucose, 10 mg FeSO4.7H2O, 10g peptone, 3 mg ZnCl2, 4 mg CoCl2.6H2O, 4 mg MnSO4.H2O & 20 µl HCl), distilled water, 95% ethanol (HmbG, Malaysia), 1M Sodium hydroxide, 0.5% acetic acid, 2M Sodium hydroxide, 1% acetic acid, 0.01 M acetic acid and N-acetylglucosamine powder (Sigma).

2.1.3. Preparation of growth medium. A 500 ml distilled water was filled with 195 ml of YPG growth medium. Sterilization was done on autoclave at 110°C for 10 minutes.

2.1.4. Preparation of spore formation. Spore was fermented by submerging the surface of the culture agar on each plate with 5 ml of sterilized distilled water. The fermented spores were collected before being inoculated onto YPG medium.

2.1.5. Preparation of fungi submerged culture. A total of 5 ml of fermented spores containing 1 x 107 spores/ml were transferred aseptically into 500 ml flask that contain 195 ml of YPG medium. Spores were incubated for growth at 30°C for 60 hours with constant agitation at 150 rotation per minute (rpm).

2.2. Extraction of mycelium and biomass

2.2.1. Extraction of mycelium. Culture media was incubated for 72 hours and extracted for every 12 hours. The pH of media was measured every 12 hours. Extracted fungi was washed with distilled water and dried using freeze-dried machine. Weight of mycelium for each extraction was measured to plot the graph of dry weight of mycelium vs incubation time.

2.2.2. Extraction of biomass for extraction of chitosan. Biomass was separated from growth medium by using Whatman paper no 1 and vacuum filter funnel. Then, distilled water was used to wash the biomass until clear filtrate was formed. Mycelium was then dried in hot oven at 80°C overnight. Next, the mycelium was dried using freeze-drying machine. The dried mycelium was grinded to form powder.

2.3. Extraction of chitosan from fungi

2.3.1. Alkaline treatment. An amount of 40 ml of 1 M NaOH was added to each gram of dried biomass powder mixture, then autoclaved at 98°C, 110°C and 121°C for 15, 20 and 25 minutes. Mixture was filtered to get the alkaline insoluble materials (AIM). Distilled water and 95% alcohol were used alternately to wash the AIM obtained with the processes repeated for three times. AIM that have been washed were freeze-dried.
2.3.2. **Acid treatment.** Chitosan were extracted from AIM by adding 100 ml 0.5% acetic acid for each gram of AIM powder. The mixture was then incubated at 95°C with constant agitation at 80-100 psm for demineralisation step. After 14 hours, mixture was filtered, and chitosan were suspended by increasing the pH of supernatant to 8.5-9.0 with 2 M NaOH. The suspended medium was centrifuged at 10,000 psm for 10 minutes to obtain the chitosan pellet. Chitosan pellet was then washed with distilled water and freeze-dried. Dry pellet was stored in the freezer.

2.4. **Characterization of chitosan**

2.4.1. **Determination of average molecular weight of chitosan according to the time solvent flow and solubility of chitosan.** Chitosan (75mg) were solubilised in 30 ml of 1% acetic acid to make 0.25% solubilised chitosan. Then, 10 ml of solvent was placed into the Ubbelohde tube. Next, 1% acetic acid was allowed to flow through the Ubbelohde tube and the flow time from point A to point B was determined by using a stopwatch. Flow time of the solvent was recorded as T₀. An amount of 2.5 ml of solubilised chitosan were placed into the Ubbelohde tube to make the volume of solubilised mixture into 15 ml. The flow time from point A to point B was recorded as T₁. An amount of 2.5 ml of the 0.75% chitosan mixture was placed into the Ubbelohde tube to make the volume into 15 ml mixture. The flow of the mixture is recorded as T₂. The steps were repeated with different concentrations of acetic acid, 0.5% and 0.25% to get T₃ and T₄, respectively. \( \eta_{\text{rel}} / C \) were extrapolated to zero concentration to obtain intrinsic viscosity. C is the chitosan concentration. The molecular weight of chitosan is calculated by using Mark-Houwink equation:

\[
[\eta] = KM^a
\]  
where K and a are coefficients related to the Ubbelohde tube, meanwhile M is the molecular weight of sample. \([\eta]\) is the intrinsic viscosity and K and a used are based on the previous study, \( K = 3.04 \times 10^{-5} \) and \( a = 1.26 \) [9] [10].

2.4.2. **Determination of Degree of Deacetylation (DD) (N-acetylglucosamine calibration curve)**

N-acetylglucosamine powder was reconstituted in 0.01M acetic acid to produce solubilised N-acetylglucosamine at different concentration (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/L. Optical density (OD) reading was taken by using spectrophotometer at two wavelengths (i.e 190 nm to 240 nm). The value of absorbance was used to plot a N-acetylglucosamine calibration curve, the absorbance vs N-acetylglucosamine concentration. Then, an equation derived from the calibration curve was used to calculate the degree of deacetylation.

2.4.3. **Determination Degree of Deacetylation for chitosan mixture (Muzzarelli & Rochetti, 1985).**

UV spectrophotometer was used to determine degree of deacetylation. This was done by adding 0.04 g of chitosan with 10 ml of 0.1M acetic acid mixture. Final volume of 1000ml were achieved by adding up distilled water. Absorbance value of the sample was measured at wavelength 190 nm to 240 nm. Degree of deacetylation was determined by percentage of glucosamine in the sample. The absorbance of chitosan was taken at 200 nm. DD was calculated using the equation:

\[
\text{DD} = 100\% - (C_1/C)
\]  
Where \( C_1 \) is the acetyl concentration and the C is concentration of sample. Concentration of samples were calculated by using this formula:

\[
C = (\text{mass/volume}) \times (1/\text{MW})
\]  
Where molecular weight was obtained from the Mark-Houwink equation.

2.5. **Statistical analysis**

Kruskal-Wallis test was applied to the data since the sample size was small (n=9). The test was performed to determine if there were any differences in chitosan dry weight, molecular weight of chitosan and the degree of deacetylation with different incubation period and incubation temperature. The test was performed using IBM SPSS Statistics 24.
3. Result and Discussion
In this study, incubation temperature of 121°C and incubation period of 25 minutes was recorded to produce the highest yield of chitosan (Table 1). Table (2) shows that there was no significant difference in chitosan dry weight across incubation period (p-value>0.05). Table 3 shows that there was a significant difference in dry weight of chitosan between incubation temperature (p-value<0.05).

Table 1. The dry weight of chitosan using alkali treatment at different incubation period and temperature (n=9)

| Incubation period (min) | 98°C | 110°C | 121°C |
|------------------------|------|-------|-------|
| 15                     | 91.48| 133.85| 139.69|
| 20                     | 94.50| 135.37| 148.25|
| 25                     | 102.18| 140.25| 158.0 |

Table 2. Comparison of dry weight of chitosan between different incubation time (n=9)

| Variable       | Incubation time (min) | n  | Median (IQR) | F- stats (df) | p-value |
|----------------|-----------------------|----|--------------|----------------|---------|
| Dry weight (mg)| 15                    | 3  | 133.85 (48.21)| 1.422 (2)      | 0.491   |
|                | 20                    | 3  | 135.37 (53.75)|                |         |
|                | 25                    | 3  | 140.25 (54.86)|                |         |

*Kruskal-Wallis test

Table 3. Comparison of dry weight of chitosan between different incubation temperature (n=9)

| Variable       | Incubation temperature (°C) | n  | Median (IQR) | F stats (df) | p-value |
|----------------|----------------------------|----|--------------|---------------|---------|
| Dry weight (mg)| 98                        | 3  | 94.50 (10.7) | 6.489 (2)     | 0.039b  |
|                | 110                       | 3  | 135.37 (6.4) |               |         |
|                | 121                       | 3  | 148.25 (8.31)|               |         |

*Kruskal-Wallis test bPost hoc analysis with Bonferroni corrections shows significant difference between 98° C and 121° C (p=0.034) and between 110° C and 121° C (p=0.035)

Table 4 shows that the temperature of 110° C and 20 minutes incubation period recorded the highest molecular weight of chitosan. The comparison of chitosan molecular weight shows no significant difference between incubation time and temperatures when p-value was greater than 0.05 (Table 5) and (Table 6).

Table 4. Molecular weight of chitosan obtained from alkali extraction at different incubation time and incubation temperature.

| Incubation period (min) | 98°C | 110°C | 121°C |
|------------------------|------|-------|-------|
| 15                     | 8.26 | 8.84  | 8.99  |
| 20                     | 9.15 | 9.29  | 8.48  |
| 25                     | 9.08 | 9.12  | 8.86  |
Table 5. Comparison of molecular weight of chitosan between different incubation time (n=9)

| Variable     | Incubation time (min) | n | Median (IQR) | F- stats (df) | p-value |
|--------------|-----------------------|---|--------------|---------------|---------|
| Molecular weight | 15                    | 3 | 8.84 (0.89)  | 2.489 (2)     | 0.288   |
|              | 20                    | 3 | 9.15 (0.81)  |               |         |
|              | 25                    | 3 | 9.08 (0.26)  |               |         |

*Kruskal-Wallis test

Table 6. Comparison of molecular weight of chitosan between incubation temperature (n=9)

| Variable     | Incubation temperature (°C) | n | Median (IQR) | F- stats (df) | p-value |
|--------------|-----------------------------|---|--------------|---------------|---------|
| Molecular weight | 98                        | 3 | 9.08 (0.89)  | 1.422 (2)     | 0.491   |
|              | 110                        | 3 | 9.12 (0.45)  |               |         |
|              | 121                        | 3 | 8.86 (0.51)  |               |         |

*Kruskal-Wallis test

Table 7 shows the degree of deacetylation for different incubation time and temperature. Temperature of 98°C and 15 minutes incubation period were observed as the optimum incubation temperature and period whereby the degree of deacetylation was 91.60%. There were no significant differences in degree of deacetylation between incubation time and temperature (p >0.05) (Table 8 and Table 9).

Table 7. Degree of deacetylation for fungal chitosan extracted with different incubation time and incubation temperature.

| Incubation period (min) | 98°C | 110°C | 121°C |
|-------------------------|------|-------|-------|
| 15                      | 91.60| 89.26 | 88.36 |
| 20                      | 90.32| 88.46 | 87.91 |
| 25                      | 90.00| 87.31 | 87.05 |

Table 8. Comparison of the degree of the deacetylation between different incubation time (n=9)

| Variable                  | Incubation time (min) | n | Median (IQR) | F- stats (df) | p-value |
|---------------------------|-----------------------|---|--------------|---------------|---------|
| Degree of deacetylation   | 15                    | 3 | 89.26 (3.24) | 1.867 (2)     | 0.393   |
|                           | 20                    | 3 | 88.46 (2.41) |               |         |
|                           | 25                    | 3 | 87.31 (2.95) |               |         |

*Kruskal-Wallis test

Table 9. Comparison of the degree of the deacetylation between different incubation temperature (n=9)

| Variable                  | Incubation temperature (°C) | n | Median (IQR) | F- stats (df) | p-value |
|---------------------------|-----------------------------|---|--------------|---------------|---------|
| Degree of deacetylation   | 15                          | 3 | 90.32 (1.60) | 5.956 (2)     | 0.051   |
|                           | 20                          | 3 | 88.46 (1.95) |               |         |
|                           | 25                          | 3 | 87.91 (1.31) |               |         |

*Kruskal-Wallis test
In this study, temperature of 121°C with incubation period of 25 minutes recorded the highest yield of chitosan (158 mg). No significant difference was observed in the amount of chitosan across incubation period (p>0.05). Conversely, a significant difference in the amount of chitosan was observed across incubation temperature (p<0.05). The non-significant result of dry chitosan when extracted with different incubation period may due to the short time interval, thus it did not affect the chitosan production. Meanwhile, the significant differences in the amount of dry chitosan with different temperature was observed because of the depolymerization process of biopolymer chitosan [11]. A range of 8.26 x 10^{-3} g/mol to 9.29 x 10^{-3} g/mol for the chitosan molecular weight was recorded in this study. A study shown that chitosan with medium molecular weight possess an – anti cholesterol property compared to chitosan with higher molecular weight [12]. The result of this study shown that there was no significant difference on molecular weight between alkali incubation time and incubation period (p>0.05). However, previous study shows that the different incubation temperature gave a significant effect on the molecular weight of chitosan [13]. The degree of deacetylation obtained from this study ranged from 91.60% to 87.05%. Generally, chitosan with higher degree of deacetylation has higher purity. This could be explained by lower ash and protein content [14]. Kruskal-Wallis test shown that the incubation period and incubation temperature did not affect the degree of deacetylation of chitosan, indicating that the deacetylation process was consistent in different temperature and incubation period (Table 9). A similar finding was reported when no significant difference was observed in the degree of deacetylation with different temperature even though the degree of deacetylation of chitosan increased with increasing temperature [14].

4. Conclusion
In conclusion, the highest yield of chitosan was obtained at incubation temperature of 121°C and incubation period of 25 minutes (158 mg). The alkali incubation temperature was the only factor that influence the production of dry weight chitosan. Further study concerning different methods to optimize the chitosan extraction should be conducted to ensure the better quality of chitosan characterization.

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