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RESEARCH ARTICLE

EXTRACTION AND DEACETYLATION PROCESS OF CHITOSAN FROM TACHYPLEUS GIGAS HORSESHOE CRAB OF BALOK AND MUAR, PENINSULAR MALAYSIA

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ABSTRACT

Background: Chitin and chitosan from the exoskeleton of horseshoe crab was reported to have a comparable quality to commercial chitosan. It would become a good alternative for the existing chitosan in the market. Nonetheless, inefficient isolation processes could lead to a waste to this invaluable raw material and incur additional cost. Chitosan isolated from the raw chitin could be affected by the frequency of deacetylation process. At the same time, horseshoe crabs, as known as a living fossil, must be exploited sustainably. ***Objective:*** Thus, it is important to determine if a single deacetylation process can satisfy the quality and amount of extracted chitosan from the exoskeleton. ***Methods:*** In this experiment, exoskeletons of horseshoe crab were obtained from two different area (Balok in the South China Sea coast and Muar in the Straits of Malacca) and processed through several steps of grinding, deproteinization and demineralization before went through a single and double process of deacetylation using 50% NaOH. FT-IR spectra were analysed to ensure the completion of deacetylation process. Statistical analysis was done using IBM SPSS Statistics Version 22. The data were presented as mean ± standard deviation (SD). The p value was obtained from the independent-samples T-Test analysis with $p < 0.05$ was considered significant. ***Results:*** Percentage of chitin and chitosan isolated does not have a significant difference ($p>0.05$) between Balok and Muar. Nonetheless, the degree of deacetylation is significantly different ($p<0.05$) between the study sites despite the insignificant difference between single and double deacetylation processes. ***Conclusion:*** The results suggest that a double process of deacetylation would be efficient to produce chitosan from chitin extracted from the horseshoe crab exoskeleton. The yield was significantly higher when using samples from Balok than Muar which could be related to some other factors that should be further investigated.

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INTRODUCTION

Arthropods are well known as the most dominant group in animal kingdom (Tolaimate, Desbrieres, Rhazi & Alagui, 2003). Horseshoe crab is an arthropod under the subphylum of Chelicerata which also known as the living fossil due to their unchanged feature since existed more than 300 billion years ago. The blue blood from horseshoe crab plays an important role in the biomedical industry. For laboratory and industrial use, chitin usually prepared using crustacean's exoskeleton. Their exoskeleton consists of 50-60 nm chitin fibres (Chen, Lin, Lin, Seki, Stokes, Peyras, Olevsky, Meyers & McKittrick, 2008) that found in layers of parallel arrangement. It functions in supporting the body and protection from harsh environment (Wardiatno, Riyanto, Iskandar, Kleinertz, Funch& Kurniawan, 2021). Table 1 shows the comparison of chitin percentage found in crustacean's exoskeleton, horseshoe crab species and others.

Earlier report from Brine & Austin, (1981) showed that chitin percentage of the Atlantic horseshoe crab *Limulus Polyphemus* of 26.4%.Kassim, Murni, Razak, Omar & Adam (2018) suggested that chitin percentage of *Tachypleus gigas* is at 12.7%. Chitin is not soluble in most solvent because of its compact structure (Sagheer et al., 2009). So, chemical modifications been performed to produce its most common derivative, namely chitosan. Chitosan is the *N*-deacetylated derivative of chitin, produced after deacetylation process. The monomer of chitosan is D-glucosamine and *N*-acetyl-D-glucosamine that linked by β-(1,4)-glycosidic bond (Hamed, Ozogul& Regenstein, 2016). Rinaudo (2006) classified chitosan as when chitin reaches 50% in their degree of deacetylation that eventually give its soluble character. Chitosan can dissolve in dilute acid solutions, that less than pH 6 (No & Meyers, 1995; Duarte, Ferreira, Marvao& Rocha, 2002). Usually, acetic, and formic acids been used to dissolve chitosan. At the same time, chitosan is insoluble in water, alkaline, and organic solvent (No & Meyers, 1995). This property leads to varieties of applications such as in cosmetics, water engineering, food processing, wound dressing, and drug delivery system (Rinaudo,

2006). Several steps were suggested to extract chitosan from chitin compound. Four essential steps outlined by No, Meyers, & Lee (1989) including demineralization, deproteinization, decolouration and deacetylation to isolate chitosan from crawfish shell. Abdou, Nagy, &Elsabee (2008) proposed three crucial steps which are demineralization, deproteinization and deacetylation with no decolouration been performed for their local source of chitin. Younes &Rinaudo, (2015) portrayed deproteinization step as a difficult step where the chemical bonds between chitin and proteins need to be disrupted. Abdou et al., (2008) repeated the deproteinization step several times depending on the clarity of the solution. To convert chitin into chitosan, acetyl group of chitins need to be removed. The process is called deacetylation. As the process been performed, depolymerization reaction occurs too, hence molecular weight of chitosan reduced (Younes &Rinaudo, 2015). To get a higher degree of deacetylation (DD), this process can be done twice as been performed by Ahing&Wid (2016) for the shrimp waste samples. Degree of deacetylation is essential as this will affect the properties of chitosan produced in every aspect including the physical chemical and biological properties (Hussain, Iman & Maji, 2013). The report on the isolation or extraction of chitosan from horseshoe crab is still very limited but the exploitation of horseshoe crab as exotic food become increasing in Malaysia. The abundance of their exoskeleton as waste would increase as well. This study aims to explore if extraction of chitosan from chitin of horseshoe crab would be affected by the repetition of deacetylation process which could incur additional cost and unnecessary usage of exoskeleton supply. The geographic factor on the supply or source of the exoskeleton will also be investigated.

MATERIALS AND METHODS

Collection of horseshoe crab exoskeleton was carried out in Balok River estuary (facing the South China Sea) in Kuantan, Pahang and in the Kesang River estuary (facing the Straits of Malacca) in Muar, Johor. A total of 74 and 64 samples were collected from Balok and Muar respectively. Only the carapace part of the exoskeleton (covered the head and body part of the animal) was used in this study while the legs and telson were discarded. Isolation of chitin and chitosan from the horseshoe crab carapace involves several steps including deproteinization, demineralization and deacetylation as summarised in Figure 1. After being washed thoroughly, it was dried in the oven for 24 hours at 60º. The carapace is then being pulverised using commercial blender and then passed through 250µm sieve mesh to get a uniform 250µm in size (No & Meyers, 1995). The powder is then washed, filtered, and dried again in the oven at 60°C for another 24 hours. Deproteinization is important to separate protein from chitin, the main building block of horseshoe crabs' carapace. In deproteinization step, the carapace powder was treated using 1M NaOH in ratio 1:10 (w/v) with constant stirring for 6 hours at 60°C. The residue been washed to neutrality using tap water, rinsed with deionized water, and filtered. Demineralization was performed to separate calcium carbonate and calcium phosphate from chitin. Demineralization was performed by adding 1M HCl in ratio 1:15 (w/v) to the powder with constant stirring for 4 hours at 50°C. Washing, filtering and drying processes were performed as before. At this step, chitin was produced. Weight of chitin was measured and recorded. For deacetylation step, 50% NaOH in ratio 1:10 (w/v) was added to chitin, then the mixture was autoclaved at pressure 15 psi for 30 minutes. The resulting chitosan will undergo the same washing, filtering, and drying process. Weight was recorded. The deacetylation step has been done twice. 50% NaOH in ratio 1:10 (w/v) was added to the sample for second time, before been autoclaved at pressure 15 psi for 30 minutes again. Calculation of chitin and chitosan percentage and preparation of samples for FT-IR was carried out following Kassim et al. (2018). The absorbance of FT-IR used is between 4000 cm^{-1} to 400 cm^{-1} (Zvezdova&Stoeva, 2010) and resolution at 4 cm^{-1}. As the sample been analysed by FT-IR, a spectrum will be produced. The absorption band used will be between 4000 cm^{-1} to 400 cm^{-1}.

The following formula will be used to determine the degree of deacetylation.

$$DD = 100 - [\frac{A^{1655}}{A^{3450}} x \frac{100}{1.33}]$$

A_{1655} refers to the absorbance at 1655 cm^{-1} that shows the amide-I band in cm^{-1}. A_{3450} however, refers to the hydroxyl band that act as an internal standard. Factor 1.33 is the value for ratio of A_{1655}/A_{3450} that indicates fully *N*-acetylation chitosan. If the chitin undergoes 100% deacetylation, the value of this ratio is going to be 0 (Domszya & Roberts, 1985; Benhabiles et al., 2012). FT-IR absorption bands that belongs to both processes of chitosan were compared with standard bands that proposed by Zvezdova&Stoeva (2010). Statistical analysis was done using IBM SPSS Statistics Version 22. The data were presented as mean ± standard deviation (SD). The p value was obtained from the independent-samples T-Test analysis with $p < 0.05$ was considered significant.

RESULTS

Percentage of Chitin and Chitosan: Chitin and chitosan percentage was higher in samples from Muar than Balok. Nonetheless, the Independent-sample T-test showed that there was no significant different ($p>0.05$) in the scores for Muar and Balok (Table 2). These results suggested that different area where horseshoe crab lives do not affect the percentage of chitosan isolated from their carapace.

Table 1. Different percentage of chitin and chitosan produced from various animal species

Source	Chitin (%)	Reference
Horseshoe crab (*Limulus polyphemus*)	26.4	(Brine & Austin, 1981)
Horseshoe crab (*Tachypleus gigas*)	12.7	(Kassim et al., 2018)
Crab (Carcinusmediterraneus) Shrimp (*Penaeus kerathurus*) Cuttlefish (*Sepia officinali*)	27.4 37.2 5.8	(Hajji, Younes, Ghorbel-Bellaaj, Hajji, Rinaudo, Nasri&Jellouli, 2014)
Crab (*Chionoecetes opilio*) Krill (*Euphausia superb*) Shrimp *(Crangoncrangon)* Crawfish (*Procambarusclarkii)*	26.6 24 17.8 13.2	(Synowiecki& Al-Khateeb, 2003)

Table 2. t-test results comparing two different sources of horseshoe crab exoskeleton (Balok and Muar) on percentage of chitin and chitosan. Different capital letters between area indicate the significant differences ($p<0.05$)

Sources	Chitin (%)	Chitosan (%)
Muar	24.73 ± 4.49^{A}	20.33 ± 4.90^{A}
Balok	19.61 ± 3.53^{A}	15.12 ± 2.76^{A}

Degree of Deacetylation: Chitosan obtained from horseshoe crab from Balok and Muar were analysed using FT-IR in order to determine the functional groups to indicate its degree of deacetylation. Identical bands to standard chitosan will give a confirmation of the chitosan structure (Figure 2). Based on the value for ratio of A_{1655}/A_{3450} that indicate the fully deacetylation of chitosan, n independent-sample t-test was conducted to compare the degree of deacetylation of chitosan from two different areas, Muar and Balok. Figure 3 shows the insignificant different of degree of deacetylation between the single and twice of deacetylation process for samples from both areas. Nonetheless, obviously the values of degree of deacetylation were significantly different between areas. The values for Balok were significantly higher than Muar. These results suggest that area from where horseshoe crab comes from does influence the percentage of degree of deacetylation of chitosan. In another note, the result showed that by repeating the deacetylation process, DD could achieve the values of more than 50% that indicate the pure chitosan have been extracted.

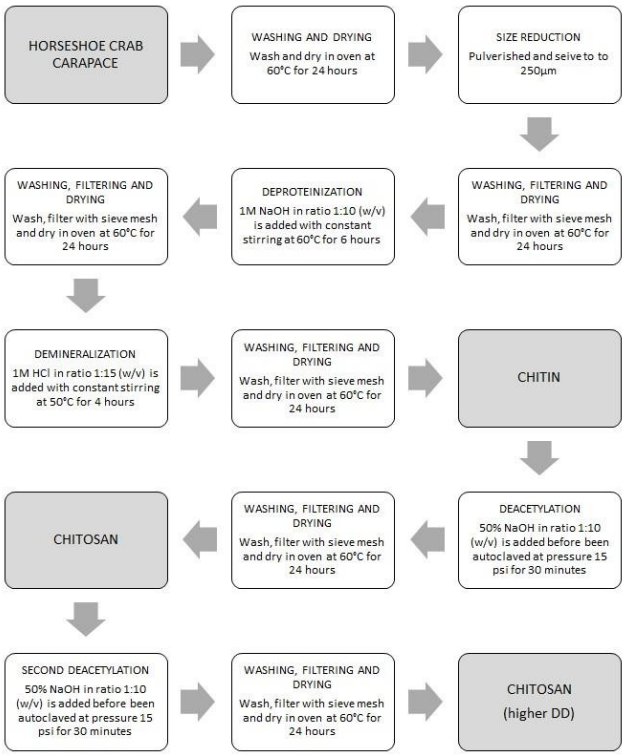

Figure 1. The diagram shows the overall process to produce chitin and chitosan

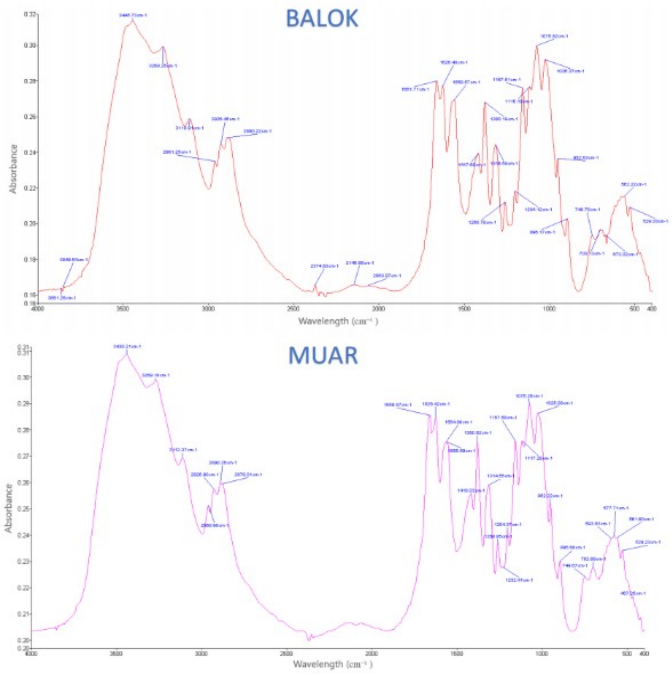

Figure 2. FT-IR Spectra of horseshoe crab chitosan from Muar and Balok

DISCUSSION

Yield of chitin ranges from 19.61% to 24.73%, is a promising result to be compared with percentage of chitin in *Limulus polyphemus* which is at 26.4% (Brine & Austin, 1981). This is based on the foundation study that stated crustacean shell waste mainly consists of protein (30-40%), calcium carbonate (and calcium phosphate) (30-50%), and chitin (20-30%) (No & Meyers, 1995). Brine & Austin (1981) suggested that these proportions vary with species and horseshoe crab was indicated as experiencing minimal denaturation compared to blue crab, stone crab and red crab.

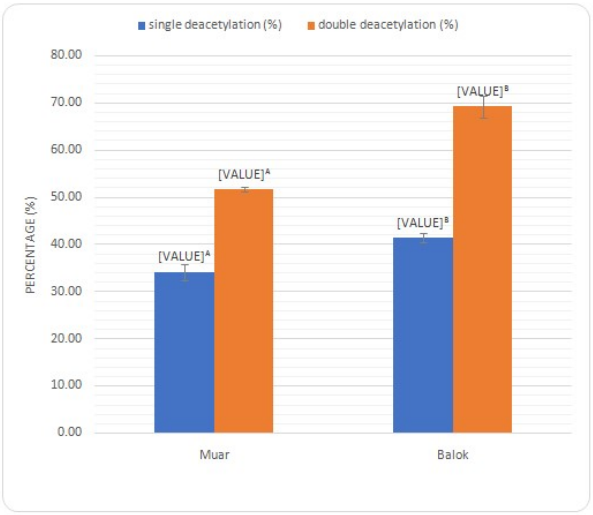

Figure 3. Degree of deacetylation (%) of chitosan when single and double deacetylation process has been done on Muar and Balok horseshoe crabs. Different capital letters between area indicate the significant differences ($p<0.05$)

The components in the carapace including chitin, proteins and other minerals also depends on their stage of reproductive cycle, nutrition and it is suggested that older specimen has lower chitin percentage with more calcified exoskeleton (Rkhaila et al., 2021). The processes including deproteinization and demineralization also contribute to the decrease in chitin yield because deproteinization let the matrix unprotected due to the removal of protein layer beneath them while demineralization extensively remove inorganic material in chitin fraction (Lertsutthiwong, How, Chandkracang & Stevens, 2014; Ibitoye, Lokman, Hezmee, Goh, Zuki & Jimoh, 2018). Percentage of chitosan for Balok and Muar, however, are 20.33% and 15.12% respectively.

This is considered high when compared with 12.7% (Kassim et al., 2018) but much lower when compared to 75% of chitosan after purification (Pati, Shahimi, Edinur, Nelson, Acharya & Dash, 2020) in the other study. Lower yield of chitosan has been suggested may due to the depolymerization that occurs on the polymer where excessive removal of acetyl groups takes place (Hossain & Iqbal, 2014). Method used to conduct the experiment can also affect chitin and chitosan percentage (Hossain & Iqbal, 2014; Kassim et al., 2018). Loss of chitin and chitosan particles can occur during washing and drying process. During deacetylation process, removal of acetyl group in excess will decrease sample weight. Source of chitin should be taken into consideration as well (Kucukgulmez, Celik, Yanar, Sen, Polat&Kadak, 2011; Kassim et al., 2018). Different species will have different percentage of chitin hence different percentage of chitosan will be observed. Degree of deacetylation is very important. Chitosan nanoparticles with higher degree of deacetylation showed increased in uptake capacity by cells and having higher affinity to bind to them (Huang, Khor & Lim, 2004). Main factor that influences the degree of deacetylation is NaOH concentration due to the difficulty in removing the acetyl group. It was suggested that higher concentration of NaOH, as high as 50% (Pati et al., 2018) to 60% (Hossain & Iqbal, 2014) will results in higher percentage on degree of deacetylation because it can be obtained with the reduction of chitosan molecular weight. Sagheer et al. (2009) performed heating in deacetylation process by using microwave compared to conventional method. The results boost the degree of deacetylation up to 90%. Based on the results of the present study, second deacetylation process did not show significant different from the single deacetylation process in increasing the degree of deacetylation itself. Nonetheless, the percentage obviously higher after the second process which achieved more than 50% of DD. This is in agreement with Rinaudo (2006). Previous studies showed that when deacetylation process been performed twice, the degree of deacetylation will be higher. The difference in the results could be due to the type of animal used, for example Ahing & Wid (2016) used crawfish shell and not horseshoe crab carapace.

The different result between samples taken from different area could also support the fact that species and geographic location would contribute to the variability in the DD which also related to the percentage of the chitin and chitosan. Razali & Kassim (2018) reported on the significant difference in the morphometric data that indicate differences in age of horseshoe crab from different area around the Peninsular Malaysia.

CONCLUSION

Extraction and deacetylation process of chitosan from *Tachypleus gigas* horseshoe crab collected in Balok and Muar, Peninsular Malaysia was made possible following the procedures of the established methods recommended by previous reports. The difference on the percentage of chitin and chitosan obtained was not significant when comparing between Balok and Muar. Nonetheless, the degree of deacetylation significantly varied between area which could be associated with certain morphometric data. The degree of deacetylation increased when the process was repeated twice and obtained the DD of more than 50% which satisfied the character of chitosan. Further study on the effect of temperature and concentration of NaOH would give more insight into the process optimization in extracting chitosan from animal samples.

Conflict of Interest Statement: There is no conflict of interest involved in this project.

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KEY POINTS

- Chitin and chitosan from the carapace of a horseshoe crab, *Tachypleus gigas* could be extracted following the standard procedure as for other source animal such as crustacean and mollusks.
- Exoskeleton collected from Balok and Muar showed insignificant difference in chitin and chitosan content but significantly differed in the degree of deacetylation.
- Repeating the deacetylation process would increase the degree of deacetylation, thus increase the chitosan production.
- Glossary of Abbreviations
- Arthropod Arthropods are invertebrate animals having an exoskeleton, a segmented body, and paired jointed appendages
- Carapace The upper surface or dorsal part of exoskeleton
- Crustacean A group of arthropods having jointed appendages, and three body sections
- ExoskeletonRigid or articulated envelope that support and protect soft tissues of the animal
- FT-IRFourier-transform infrared spectroscopy
- NaOHSodium hydroxide
- SpectraBands of colours

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