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Comparison of Chitosan Characterization from Mussel Shell Waste Using Varying Concentration of Solvents

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Abstract:
Massive amounts of mussel shell waste are generated and wasted from the aquaculture processing sectors, resulting in environmental pollution. This material contains chitosan as a valuable compound characterized as a non-toxic structural component with several food processing applications or medicinal applications. In this research, mussel shells were processed using different solvents concentrations in several stages: demineralization, deproteination, decolourization, and deacetylation. Our result showed that the C2 samples gained a high degree of deacetylation (31.8±0.21%) with low moisture and ash content and medium weight of yield. Further research is recommended to purify chitosan using various instrumentation and assess its bioactivity.

Keywords: Chitosan, Degree of Deacetylation (DDA), FTIR, Mussel Shell, Shell Waste

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Introduction

Mussel shell waste is accumulated in massive quantities throughout the aquaculture processing industries and is discarded chiefly, resulting in severe environmental pollution. This waste considered a renewable resource or valuable compound, such as chitosan (Rashid *et al*, 2018). Shell waste is a significant source of chitosan, which accounts for about 40% of the dry weight of mussel shells (Hu *et al*, 2020).

Chitosan is a biopolymer composed of β-(1-4) D-glucosamine units synthesized chemically or enzymatically from the chitin. It is discovered as a structural component of bivalvia and other arthropods, which are biocompatible and non-toxic. As an industrial application, chitosan has various benefits, including food processing, feed supplement, agriculture, antibacterial goods, water treatment, and biomedical applications (Hart *et al*, 2020; Muthu *et al*, 2021).

Different solvents, incubation temperature and concentration, could influence the purity of the chitosan. Therefore, it could be feasible to remove more minerals and proteins from mussel shell waste by using a combination of acid and base, allowing more recovery of chitin to obtain a higher concentration of chitosan (Prameela *et al*, 2017).

The purpose of this research is to demonstrate a methodology for processing shell waste in order to extract chitosan and remove its contaminant. To determine the feasibility of our study, we examined chitosan powder using various solvents with varying incubation times and temperatures to get a higher level of chitosan purity.

Material And Methods

Chitosan Extraction

In general, synthesizing chitosan from mussel shell waste of *Perna viridis* is divided into four stages, namely preparation, demineralization, deproteination, and deacetylation; based on the method of Abdulkarim *et al* (2013) and modified method of Vairamani *et al* (2013).

Shells were cleaned in running water and then dried under the sunlight for 3-4 hours. The shells then were put in an oven at 40°C for 48h. Furthermore, the shells were crushed till smoothed and filtered through a sieve screen using mesh size 1 mm.

The demineralization stage was initiated by adding shell powder to HCl solution (w/v; 1:5) with the concentration of C1, C2, and C3 were 0.68 N, 0.5 N, and 1 N, respectively.

This mixture was heated at 30°C (C1) and 40°C (C2-C3) for 24 hours with a constant stirring at 1500 rpm. The powder was then filtered and washed with distilled water to eliminate any residual HCl.

The deproteination procedure is carried out by mixing demineralized samples with NaOH solutions at a concentration of 0.62 N at 30°C and 1 N at 80°C (C2-C3) with the ratio of sample mass (gr) and solution (ml) was 1:5. Continuous stirring at 1500 rpm was performed for 24 hours. After separating the particles, the solid phase of the deproteination step was filtered and rinsed with distilled water to get the chitin fraction.

The resulting chitin then was subsequently deacetylated by adding 25 M (C1) and 1 M (C2-C3) of NaOH solutions, respectively. For 20h, C1 was heated to 40°C, and C2-C3 were heated for 6h at 110°C with the mass sample (gr) and solution (ml) comparison was 1:5. This mixture was continually stirred at 1500 rpm and cooled at room temperature. Furthermore, this chitosan was rinsed with distilled water and dried for 24h at 40°C to yield chitosan powder in C1.

In chitosan samples of C2 and C3, 10% of CH_3COOH glacial was added and stirred at 1500 rpm for 12 hours at room temperature. The solid phase then was washed twice with distilled water in 24h. The product was centrifuged at 1500 rpm for ten minutes and dried to obtain chitosan powder.

The Weight of Yield

The weight of yield is the ratio between the amount (quantity) of chitosan generated from green mussel shells powder. The yield is expressed in percentages (%). The yield weight was determined by calculating the percentage of chitosan extract (gr)/green mussel shells weight (gr) x 100%.

Degree of Deacetylation

The spectra of chitosan samples were obtained using an I.R. Instrument (IR Spirit A22415801432, Shimadzu, Kyoto, Japan). The degree of deacetylation (DDA) in chitosan samples was calculated using baseline (a), which was proposed by Domszy and Roberts (1985) *in* Khan *et al* (2002).

Moisture Content of Chitosan

The moisture content of the samples was measured by drying it in an oven at a temperature of 100-105°C for 3 hours. After cooling in a desiccator, it is weighed (gr).

Ash Content of Chitosan

The ash content was measured by burning the samples in an empty dish of the furnace at a temperature of 600-650°C until the sample was carbon-free, as indicated by the sample being greyish to white. The sample was then cooled overnight in a desiccator and weighed (gr).

Results and Discussion

The extraction of chitosan is accomplished through the deproteination method, which involves eliminating proteins using an alkaline solvent of NaOH. The demineralization process follows the deproteinization method to remove the minerals contained in the HCl solvent. According to Arif (2006), the mineral content of animals is high. The majority of molluscs had $CaCO_3$ and $Ca_3(PO_4)$. The demineralization stage produced CO_2 gas by the creation of air bubbles during the process. The depigmentation process alters the colour of chitin from brown to brownish white. Chitosan then was produced from chitin after a high concentration of NaOH was added into the samples for the chitin deacetylation process. The mixture then was washed with distilled water to remove any solvent after added with CH_3COOH glacial (C2 and C3).

Results show that all extracted chitosan samples were white colour & powdered; All samples had a similar colour to the purified standard of chitosan. The weight of yield from extracted chitosan using various solvents was shown in **Figure 1**. The extracted C1 had the highest quantity with the percentages 39.21±0.36%, while extracted C2 and C3 had 22.1±0.29% and 19.89±0.25%. This figure indicated that the chitosan fraction obtained by chemical method without additional CH_3COOH glacial in C1 sample was presented the highest yield. According to Djaeni *et al* (2003), prolonged operation time could result in a higher yield of chitosan products. It is induced by the extended contact time between chitin and NaOH, which increases the conversion of acetyl groups in chitin to sodium acetic.

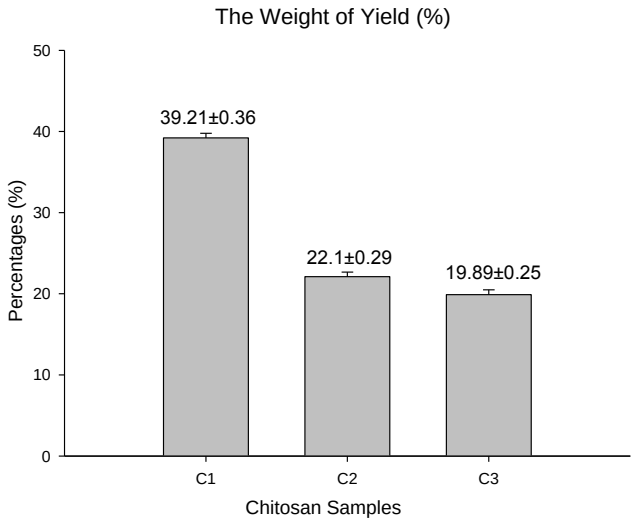

Figure 1. Chitosan yield (%) from green mussels shell using various solvents

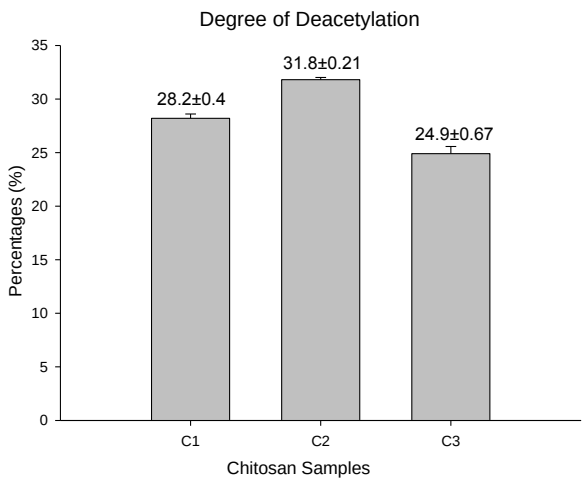

Figure 2. DDA (%) from green mussels shell using various solvents

Regarding chitosan characterization, the deacetylation degree (DDA) is a critical parameter that characterizes their application. The DDA of C1 to C3 in a row were 28.2±0.4%; 31.8±0.21%; and 24.9±0.67%, with the highest percentage was in C2 (Figure 2). Although C1 gave higher chitosan yield by prolonged operation time, the deproteination and deacetylation stage used much lower temperature, indicating lower chitin and its deacetylation results. According to Huang *et al* (2002), the intake temperature of active substances utilized in chitosan preparation ranges between 120 to 170°C. The fluid containing chitosan is subjected to high temperatures for a relatively brief amount of time to increase the chitosan product.

Szymańska and Winnicka (2015) also noted that using high temperature, the enormous surface area of the chitosan exposed to a heat stream could lead to a more significant concentration of hydrolysis products on the surface particles and faster agglomeration. However, this condition was vulnerable to thermal degradation and change of the physicochemical properties of the polymers.

Another crucial factor in chitosan extraction was maceration time. Although longer operation time (≤24 h) could enhance chitosan yield, nevertheless, more than 3 hours, the optimization process could not improve the chitosan yield; even it could reduce the product (Romero *et al*, 2018). This result is because the acetyl group content of chitin decreases. As a result, large concentrations of NaOH would introduce the chitin component, causing its chitosan product to be degraded.

The concentration of NaOH regulates DDA of chitosan. The acetyl groups bonded in chitin were extremely tough to be removed. To improve the quality of the chitosan, a high concentration of NaOH and a high temperature are required (Moosa *et al*, 2016). Although using different concentrations and temperatures, DDA of C1 to C3 were not significantly different. According to Djaeni (2003), it needs an optimal concentration and temperature of NaOH during the deacetylation procedure. Too high a concentration of NaOH, the chitosan product could decrease. This phenomenon is caused by using an excessively high dose of NaOH to remove acetyl groups from chitin. As a result, the residual OH- will attack the chitin ring, causing the chitin complex to degrade.

However, when the concentration of NaOH is reduced by less than 20%, the interaction between NaOH and acetyl groups cannot continue because the OH- concentration is insufficient. These findings were consistent with Hossain & Iqbal (2014) results. Using different concentrations of NaOH (40-80%) would optimize the deacetylation process, with the deacetylation grade reached 73.4% at NaOH concentration was 60%.

The addition of CH_3COOH glacial in C2 and C3 aims to increase the deacetylation process of chitin to chitosan. The protonation of NH_2^+ of chitosan in acidic media allowing the chitosan polymer to work as a thickener or suspending agent. It is also noted that acetic acid would increase its solubility (Thomas *et al*, 2015). When combined with acetic acid, chitosan improves gelation and drug entrapment in the sol-gel method (Essel *et al*, 2018). This method resulted in higher solubility of C2-C3 than C1 due to additional CH_3COOH glacial at the end of the process.

No	Wave number (cm^{-1})	Possible assignment of functional groups	Nature of peaks
1	3435.74	H-bonded NH_2 & OH Stretching	Broad
2	2983.63-2855.28	Aliphatic CH-stretching	Medium
3	1787.04	Amide C=O stretching	Sharp
4	1457.59	NH Bending	Broad
5	1082.49	C-O-C stretching	Sharp
6	861.43-711.68	C-N stretching	Sharp
7	509.16	C-O Bending	Medium

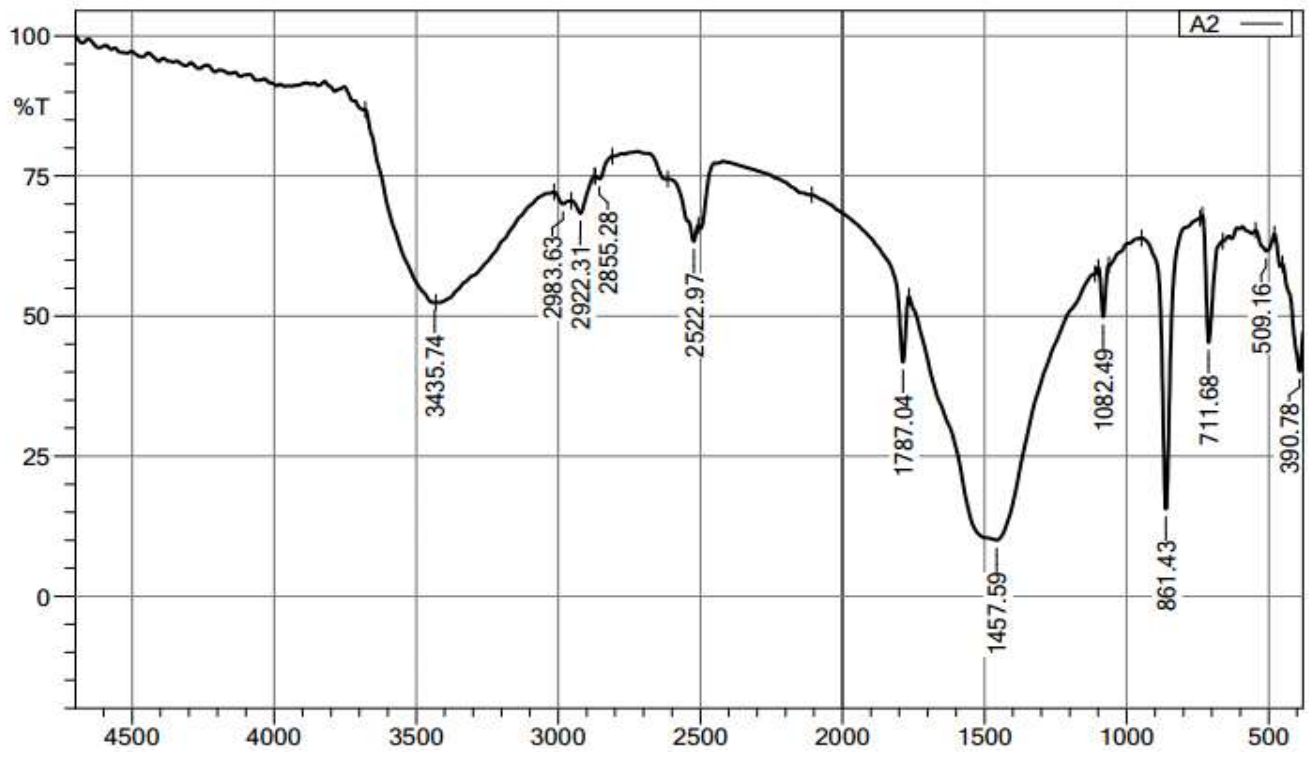

Figure 3. FTIR Spectra (cm^{-1}) of green mussels shell C3

The FTIR spectrum of chitosan on all samples has a chitosan functional group in a similar wavelength, indicated that the chitosan fraction was successfully extracted from shell mussels of *P. viridis* (data were not shown). In C3 chitosan, broad peaks were seen in the FTIR spectra of chitosan at 3435.74 cm^{-1} were H-bonded NH_2 & O-H stretching functional groups. Medium peaks were seen in 2983.63, 2922.31, and 2855.28 cm^{-1} were aliphatic CH-stretching. A sharp peak of amide C=O stretching was seen at 1787.04 cm^{-1} . A broad peak of NH bending was seen at 1457.59 cm^{-1}. Another sharp peak of C-O-C stretching was seen at 1082.49 cm^{-1}. At 861.43, and 711.68 cm^{-1} were identified as C-N stretching, shown as sharp peaks. Lastly, C-O bending & ring stretching was shown at 509.16 cm^{-1} with a medium peak. Using another mussel from horse mussel *Modiolus modiolus*

FTIR spectra of chitosan by Varma and Vasudevan (2020) revealed vibrational mode of O-H stretching at 3594 cm^{-1}, CH_2 stretching at 2865 cm^{-1}, amide C-O stretching at 1604, 1598, and 1592 cm^{-1}, C-O-C stretching at 1174 cm^{-1}, NH bending at 711 cm^{-1}, and C-O bending at 564 cm^{-1}. Similarly, FTIR spectra of shrimp shells waste gave characteristic bands of NH_2 and O-H stretching at 3425.58 cm^{-1}, N-H stretching at 3271.27, symmetric (CH_3) & asymmetric CH_2 stretching at 2924.09 cm^{-1}, C=O in the $NHCOCH_3$ group (amide I band) at 1654.92 cm^{-1}, Amide II band at 1558.48, CH_2 bending & CH_3 deformation at 1423.47 cm^{-1}, C-O stretching at 1033.85, and ring stretching at 894.97 cm^{-1} (Moosa *et al*, 2016).

The moisture content of chitosan in all samples was shown in **Figure 4**, in the range of 5.58±0.61 to 7.87±0.47%. This water content is still appropriate to use as the standard chitosan has less than 8% moisture content. This result indicated that various concentrations of solvents did not affect the water content of chitosan

significantly. Lower percentages of moisture content would keep chitosan dry and minimize the microorganisms in the samples. This result also indicated that the drying time and temperature were optimized to minimize the moisture content of chitosan.

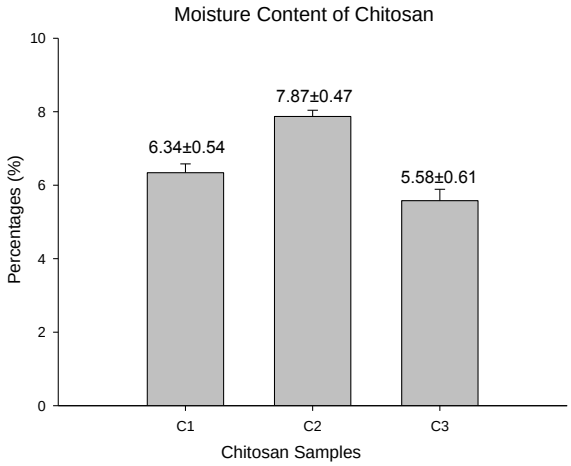

Figure 4. Moisture content (%) from green mussels shell using various solvents

The ash content of all chitosan samples was shown in **Figure 5**. The ash content was ranged from 50.39±0.5% (C2) to 66.79±0.38% (C1). Kurniawan *et al* (2019) studied the effect of HCl concentration on the demineralization process of chitosan shell crab. Different concentrations of HCl (1-1.5M) resulted in different demineralization effectiveness. The smaller the HCl solvents, the greater chitosan purity produced.

The high ash content in this research, especially on C1 and C3 was attributed to an inefficient demineralization concentration and stirring throughout the extraction and washing process. Furthermore, mixing HCl solution with shells powder stage needs to be added gradually to minimize the bubbles (CO_2 gas) that minimize the demineralization process. A poor washing procedure will result in released minerals that may be reattached to the surface of the chitin molecule. Minerals that have been liberated from the substance were bonded to the solvent and discarded and dissolved with the water. This result also could impact the ash content of the chitosan. The constant stirring would perfectly distribute the solvent could bind the minerals perfectly (Avadi *et al*, 2004).

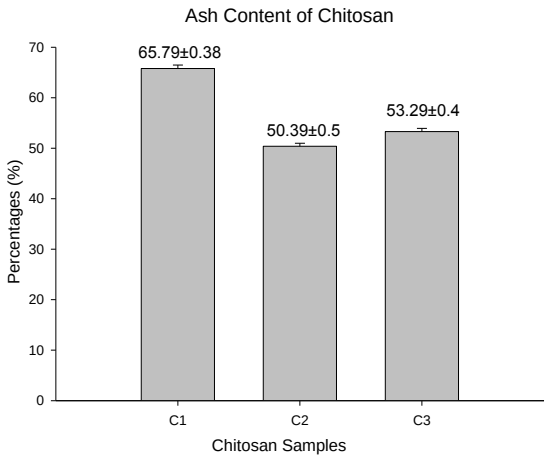

Figure 5. Ash content (%) from green mussels shell using various solvents

Higher ash content was linear with a lower chitosan deacetylation degree. In our result, the C1 sample had a high percentage of ash content and chitosan yield, contrary to the degree of deacetylation percentage. Otherwise, in another sample of C2, although the ash content was still high due to extraction procedure and solvent concentration, its deacetylation degree and the weight of yield were still higher than other samples.

Thus, according to our research, C2 has the optimum condition to gain better chitosan quality from shell mussels waste. Several factors must be addressed throughout the chitosan process, including a lower concentration of HCl, middle (40-60%) concentration of NaOH, high temperature with shorter maceration time, additional of CH_3COOH glacial to increase deacetylation of chitin, constant stirring during the whole extraction process, and high technique of washing procedure to gained purified chitosan.

Conclusion

This study was demonstrated the various chemical characterization of chitosan from mussel shell waste using different solvents concentrations. The optimal condition to extract chitosan is using HCl 0.5 N at 40°C in the demineralization stage, NaOH 1 N at 80°C in the deproteination stage, NaOH 1 M 110°C for 6 h in deacetylation stage, and additional CH_3COOH glacial 10% at room temperature. The resulting chitosan gained high deacetylation (31.8±0.21%), low moisture and ash content, and medium weight of yield.

The FTIR spectra of chitosan were also identified as several functional groups, such as:

H-bonded NH_2 & OH Stretching, Aliphatic CH-stretching, Amide C=O stretching, NH Bending, C-O-C stretching, C-N stretching, C-O bending, and ring stretching. Basic formulation of the DDA, the weight of yield, moisture and ash content, of the mussel shell waste can efficiently generate chitosan to be high yield. Further research is needed to increase chitosan purity using the different instruments (such as sonication) or implement this chitosan as an additional supplement to evaluate its bioactivity.

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