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Continuous Extraction Process of Chitin from Discarded Shells of Philippine Blue Swimming Crab (*Portunus pelagicus*)

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Abstract*— Crab shells are often found discarded from local seafood processing plants that end up as waste hauled to landfills. However, high purity chitin has been extracted by chemical methods from this material which is useful in various applications like sutures and scaffolding in medical applications. The viable sources of chitin were selected among the shells of blue swimming crab (Portunus pelagicus), mud crab (Scylla serrata) and tiger prawn (Penaeus monodon). Tiger prawn was shown to have the highest yield of chitin at around 24% while the two varieties of crab yielded only about 10%. However, considering the accessibility and availability of high volume of blue swimming crab shells from crab processing plants, this was chosen as the raw material for subsequent investigations. Moreover, the process that will be established can still be suitable for both sources. Extraction process includes demineralization and deproteinization stages and factors affecting them have been studied. These include acid concentration and shell size for the demineralization stage and alkaline concentration and shell size for the deproteinization stage. Retained inorganic component after demineralization and protein content removed from deproteinization were determined using compositional analysis via Energy Dispersive X-ray Fluorescence technique and Lowry assay, respectively, to understand the effects of these factors on their respective processes. It was found that high acid concentration (greater than 1 N) at room temperature could sufficiently remove the inorganic components of the carapace while high alkaline concentration (greater than 1 N) applied to fine-sized demineralized shells produces high purity chitin similar to commercially-available technical grade chitin.

A refinement of the chitin extraction process was subsequently performed through the development of the bench-scale extraction process. It was found out that some of the stages in the laboratory-scale extraction can be eliminated performing an uninterrupted two-stage chemical extraction process and still be able to produce similar quality of chitin with doubled product recovery.

Keywords*— Chitin Extraction, Chitin, Biodegradable Polymers

1. INTRODUCTION

Several years of uncontrolled production, improper use and irresponsible disposal of nondegradable plastics worldwide lead to currently pronounced deteriorating environmental situation such as the increasing amounts of carbon dioxide in the atmosphere, decreasing availability of landfills and the depletion of fossil-based resources as raw materials. Foreign government administrations and private institutions addressed this problem by pioneering research and promoting the use of biodegradable polymers as alternative to these conventional materials. Locally, several government agencies followed suit by immediately restricting the use of non-biodegradable plastics, however, lacking provision for suitable substitutes, thus, stimulating local research on biodegradable polymers.

Fortunately, the Philippines is endowed with numerous natural resources that may be tapped to develop such materials. Research has revealed that locally-available shells of crustaceans contain chitin, which is one of the most abundant renewable biodegradable polymers. The main biological function of chitin is for support and forms a structural part of the exoskeleton of these organisms. It is known to have

promising properties, hence, it is utilized for a wide range of applications. The crustaceans endemic in the country includes crab, squid, prawn, shrimp and lobster [1].

In 2015, the Philippines had a recorded volume of production of crustaceans of 93,019.48 MT [1]. Almost half of this large production volume is composed of crabs, specifically, blue swimming crabs, 29% and mud crabs, 18%, and the bulk of which are tiger prawns [1]. With these production volume data, an estimated 34,000 MT of crab shell is generated from the crab meat processing industry of the country [1, 2]. On the other hand, the same amount of prawn waste was produced from the domestic consumption [1, 3].

Chitin extraction process is basically composed of demineralization and deproteinization stages. Chemical and biological methods may be employed for the two major stages of this extraction process. In the chemical method, acid and alkaline reagents are employed for the removal of inorganic material and hydrolysis of protein, respectively. On the other hand, the biological method makes use of enzymatic extracts or isolated enzymes and biological fermentation. The product of this method, however, has significantly low quality compared with the chemical method [4] apart from the difficulty posed by handling of microorganisms and disposal of biomass produced in the process.

Several, foreign, similar chitin extraction studies have been reported that were sourced from shells previously mentioned [4, 5, 6, 7, 8, 9, 10, 11, 12]. The shells were either gathered fresh [6, 9, 12] or collected as discards from local seafood restaurants, shellfish fishing companies and hotels [4, 5, 8, 10] then were scraped of flesh, washed or boiled to remove soluble organics and binding protein, oven-dried and finally ground to obtain flakes of 2-5 mm [4, 9, 10] or powdered with size of about 250 µm to below 80 µm [5, 11, 12]. From the foreign studies on chitin extraction from crustacean shells, process parameters that may affect the removal of both inorganic and organic material may include the acidic and alkaline medium and concentration used, shell particle size, shell-to-acid ratio (g/mL), agitation rate, incubation period and temperature. 0.25 N to 2.0 N hydrochloric acid was most widely used for the demineralization of shells using a shell-to-acid ratio in the range of 1:1 to 1:40 g/mL under continuous agitation at room temperature [4, 5, 6, 8, 9, 10, 11, 12]. As for the incubation period, early studies lasted for two days [9] until it was significantly decreased to 15 minutes [12]. As for the deproteinization stage, aqueous sodium hydroxide solution has been an effective alkaline medium consumed at varying concentrations, shell-to-alkaline ratio and temperature which ranged from 1 N to 15 %, 1:10 to 1:50 (g/mL) and 110 °C down to room temperature, respectively [4, 5, 6, 7, 8, 9, 10, 11, 12]. Improvement of incubation period from 36 hours down to 30 minutes were observed from these experiments, even so, the latter reaction time was repeated several times depending on the clarity of the resulting solution in which they claimed that a clear solution indicated the absence of protein from the treated shells. Washing with distilled water and filtration until neutral was done in both stages of chitin extraction, after demineralization and deproteinization. Drying of the extract at room temperature, higher temperature like 40 °C or even freeze-drying followed [4, 6, 8, 9, 10, 12]. However, some of these studies utilized drastic treatment, if not incomplete removal of inorganic materials and soluble proteins, which leads to depolymerization and deacetylation of the native chitin resulting to low crystallinity index. Also, majority of these studies reported limited characterization to confirm purity of extracted chitin. Moreover, due to the variation in the inorganic material content among the numerous shells sourced for chitin extraction brought about by the different types of species, environmental conditions and season of harvesting further entails the need for a local baseline research to establish a chitin extraction process tailored after utilization of shells available in the country. Furthermore, this research may help pioneer the local manufacturing of this high potential biodegradable polymer, specifically and initially on the scale-up production of chitin. Simultaneously, the study will help utilize the large amount of available shell waste, advance local research and development and help the country realize the potential of being an independent producer of biodegradable polymers.

2. MATERIALS AND METHODS

2.1 Determination of Chitin Viability from Locally-Sourced Shells

Shells of blue swimming crab (BSC), mud crab and tiger prawn were collected to determine the viability of generating chitin from these pre-selected locally-sourced shells. Dried, cooked BSC carapace which originated from a crab meat processing plant in Bacolod, Negros Occidental, Philippines were obtained from local markets. The shells of mud crab and tiger prawn were bought fresh from the same local market. All shells except for BSC were placed in boiling water until the meat was cooked to facilitate easy removal and cleaning of shells. All shells were scraped for any residual tissues and only the shells were retained, cleaned by washing with distilled water followed by air drying.

Viability of chitin extraction from the collected shells was evaluated by determining shell composition using X-ray Diffraction (XRD) analysis using Siemens Kristalloflex 760 X-ray Generator with a copper x-ray tube (Cu Kα wavelength = 1.54056 Å) and a Philips 1080 Vertical Goniometer. The operating voltage was 34 kV and the operating current 20 mA. Thermogravimetric analysis (TGA) was also conducted to validate the composition based on decomposition temperature using Shimadzu TGA-50 instrument at a heating rate of 10 °C/min under nitrogen atmosphere with a flow rate of 50 mL/min. The quantity of each shell component was also estimated from the results obtained from this test.

2.2. Chitin Extraction

The BSC shells as raw materials were subjected to crushing and grinding procedures then washed and dried. A set of Tyler sieves (mesh sizes: 32, 48 and 100) and sieve shaker were used to produce the desired shell size (i.e. 300 μm < x < 500 μm and 150 μm < x < 300 μm).

Chitin extraction by chemical method was conducted by demineralization and deproteinization reactions carried out at different conditions following a 2^2 full factorial design of experiment. Particularly, solvent concentration and shell size were investigated. The demineralization stage was carried out at room temperature for six hours at a 1:15 shell-to-acid solution ratio (g/mL) at 0.8 N and 2.0 N hydrochloric acid solutions and shell sizes as mentioned previously. The resulting solid were filtered and washed several times with distilled water under constant stirring until neutral then oven dried for an hour.

A pretreatment conditioning step preceded the deproteinization step to aid in the extensive washing of the resulting solid to achieve its basic state. After filtration from last wash, samples were spread thinly, about 1 mm thick, into ceramic plates then oven dried until a desired sample moisture content of 19±3 % was achieved.

Removal of protein from demineralized shells was performed at varying concentrations of NaOH solution, 0.5 N and 3.0 N, and shell particle mix of coarse (165 μm < y < 240 μm) and fine (41μm < y < 60 μm) while maintaining a 1:15 solid-to-alkaline solution ratio (g/mL), a three-hour extraction period and constant stirring for all the runs. The retained solid were filtered and washed several times with distilled water under constant stirring until neutral then oven dried.

The remaining inorganic material (calcium, ppm) was measured by elemental analysis by Energy dispersive x-ray fluorescence (ED-XRF) using the NITON XL3t XRF Analyzer for the demineralization stage while soluble protein removed was measured thru spectrophotometry by Lowry assay using Shimadzu UV-1601 spectrophotometer for the deproteinization stage. The experimental data were

subjected to an analysis of variance for a completely random design at 95% level of confidence using Design-Expert v7.0, Stat-Ease, Inc. 2005.

Samples of highest amount of soluble protein removed was validated by XRD characterization. Morphology of the intermediate and final products of the extraction process was also observed using Chronos USB 2.0 digital microscope.

To make a preliminary assessment of industrial viability, a bench-scale process was conducted by performing the same stages of extraction that included continuous demineralization and deproteinization using a reactor system [13]. The design of the reactor for the bench-scale extraction process considered increasing the feed capacity to 1000 g of BSC shells taking into consideration mass balance and other observed phenomenon from the laboratory scale such as frothing during the demineralization stage. Materials selection was performed to ensure that the assembly will be resistant to degradation when subjected to acidic and alkaline solution. The procedure employed in extracting chitin in the bench-scale is the same as that of the laboratory scale. The filtration set-up was replaced with an industrial dryer to lessen the time for filtration and accommodate larger amount of shells. Additional parametric investigations were also performed to streamline and shorten the duration of the overall extraction process.

3. RESULTS AND DISCUSSIONS

3.1 Viability of Chitin Extraction from Locally-Sourced Shells

Shown in Figure 1 are the selected locally-sourced shells analyzed for viability of chitin extraction. These include the BSC, mud crab and tiger prawn.

Figure 2 shows the XRD profile of the locally-sourced shells. The labeled peaks are characteristic of chitin and calcium carbonate as compared with the reference peaks in the form of calcite for all shells both summarized in Table A.1 and Table A.2 of Appendix A [5, 6, 11, 14, 15, 16, 17, 18, 19]. Only few peaks of chitin were observed in the XRD profiles that may be due to the large amount of calcium carbonate. Among all samples, only shells of the mud crab did not exhibit chitin peaks.

Figure 1. Locally-sourced shells of BSC (a), mud crab (b) and tiger prawn (c) for the determination of chitin viability.

Figure 3 presents the TGA curve of these shells, quantitatively measuring the organic and inorganic contents as summarized in Table 1. All shells exhibited four significant weight losses appearing as shoulders in the curve which correspond to moisture loss, degradation of the organic matrix of chitin and protein, degradation of the inorganic material and the decarboxylation of calcium carbonate and/or recrystallization of any amorphous inorganic material [18].

Table 1. Organic and inorganic contents of the locally-sourced shells.

Shell	Moisture Loss		Degradation of Organic Matrix		Degradation of Inorganic Material	
	Weight Loss (%)	Temp. Range (°C)	Weight Loss (%)	Temp. Range (°C)	Weight Loss (%)	Temp. Range (°C)
BSC	7.19	62-100	22.07	318-372	25.50	621-728
Mud Crab	7.03	48-122	25.51	310-378	22.65	633-727
Tiger Prawn	8.64	54-123	59.25	279-402	5.37	743-763

The moisture loss in the shells ranged from 7.19% to 8.64% at the temperature range of 48-123 °C (Table 1). The next shoulder in the curve (Figure 3) corresponds to the weight loss of chitin-protein network degradation. As can be seen from the XRD profile (Figure 2) of these shells, peaks characteristic of chitin have been identified and also chitin chains form fibrils that are wrapped with proteins [20] which make up the chitin-protein network, hence, the said degradation. Studies on the thermal degradation of chitin observed similar TGA graphs at the temperature range of 326-372 °C [5, 6].

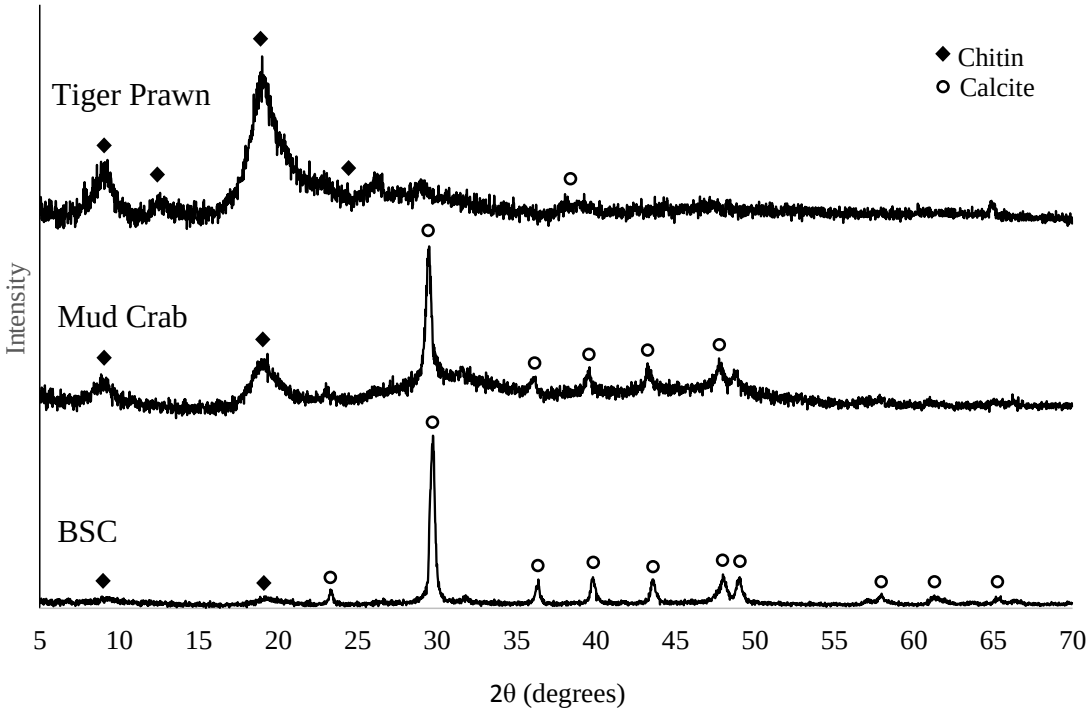

Figure 2. Normalized XRD profile of the selected locally-sourced shell.

The third weight loss in the TGA curve is identified as the degradation of inorganic material, specifically, calcium carbonate. Majority of the peaks shown in the XRD profiles of the locally-sourced shells were of calcium carbonate, in calcite form (Figure 2 and Table A.1). The decomposition of calcium carbonate takes place between 635-865 °C [21] which is similar to the temperature ranges, 621-763 °C, observed in all the shells.

The organic matrix of chitin-protein in the shells of tiger prawn has 59.25% which is more than twice as much as those in the shells of the BSC and mud crab which only have 22.07% and 25.5%, respectively (Table 1).

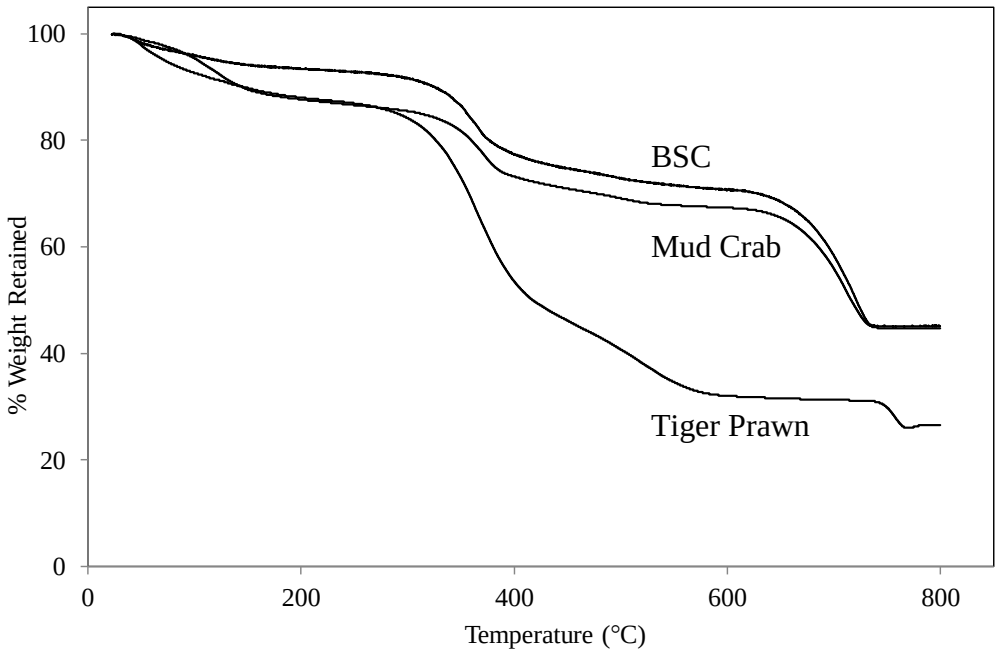

Figure 3. TGA profiles of the locally-sourced shell.

Tiger prawn had the highest organic matrix content and lowest fraction of inorganic material which would support the selection of these shells as raw materials for the determination of the chitin extraction design. However, despite possibility of higher chitin yield and high volume of production, the collection of sufficient amount of these shells for this study would become the major obstacle during the chitin extraction process experiments, leaving the selection to a choice between BSC and mud crab. The contents of the organic and inorganic components of the shells of these two crabs are almost similar. The difference would now lie on the volume of production of the BSC shell amounting to 26,290.19 MT while only more than half of this volume was recorded for the mud crab which is 17,095.29 MT [1]. As a result, the shells of the BSC having much higher volume of production and export would be more suitable and more viable to be utilized as raw materials for the systematic study and design of the extraction process of chitin. The extraction process that will be developed can still be suitable for the chitin extraction of the other two shell-sources.

3.2 Chitin Extraction

3.2.1 Demineralization Stage

Complete removal of calcium carbonate from BSC shell particles was observed after demineralization reaction using high concentration of dilute HCl acid based from the results of XRF characterization (Table 2). Furthermore, statistical analysis by analysis of variance showed that regardless of shell particle size the quantity of inorganic component removed shows no trend (Table 3). Hence, from these apparent observations solvent concentration is identified as a significant factor in the demineralization stage of BSC-derived chitin extraction.

Table 2. Amount of inorganic material (calcium in ppm) retained in demineralized BSC shell particles.

Process Parameters		Response Variable
HCl Concentration, N	Average Shell Particle Size (x), μm	Ca, ppm
2.0	300 μm < x < 500 μm	<LOD
2.0	150 μm < x < 300 μm	<LOD
0.8	300 μm < x < 500 μm	158,761.78
0.8	150 μm < x < 300 μm	173,825.68
Limit of detection (LOD) ≤ 330 ppm		

Table 3. Analysis of variance of the demineralization stage design of experiment.

Source	Sum of Squares	Degrees of Freedom	Mean Square	F Value	p-value Prob > F
Model	1.4098E+11	7	2.0140E+10	244.2880	< 0.0001
A-HCl Concentration	1.4079E+11	1	1.4079E+11	1,707.6602	< 0.0001
B-Shell Size	6.4149E+07	1	6.4149E+07	0.7781	0.3908
C-Agitation Rate	3.1787E+07	1	3.1787E+07	0.3856	0.5434
AB	6.4149E+07	1	6.4149E+07	0.7781	0.3908
AC	3.1787E+07	1	3.1787E+07	0.3856	0.5434
BC	1.1834E+06	1	1.1834E+06	0.0144	0.9061
ABC	1.1834E+06	1	1.1834E+06	0.0144	0.9061
Pure Error	1.3191E+09	16	8.2444E+07		
Corrected Total	1.4230E+11	23			

3.2.2 Deproteinization Stage

The filtrate after deproteinization stage was collected to measure the amount of soluble protein that was removed as summarized in Table 4. Results of statistical analysis suggest a significant difference in the amount of protein removed as a function of shell size while the effect of solvent concentration has been evaluated irrelevant for this stage in the extraction process (Table 5). However, interestingly, the expected effect of each of the levels of the shell size exhibited different trends such that lower amounts of protein were removed from fine shell particles that should have a higher contact area with the solvent.

Optical images of the fine and coarse demineralized BSC shell particles is presented in Figure 4. For the coarse shell particles (Figure 4a), samples may be described to have plate-like shapes detached from each other. On the other hand, the images for the fine shell particles (Figure 4b) presented large agglomerated components. This most likely formed during drying step of the pretreatment stage of demineralized samples before deproteinization. At this condition, the size of the individual particles in the agglomerate becomes insignificant because the NaOH solution apparently is not able to permeate through the formed agglomerate [22].

Milling, thus, became necessary to reduce the agglomerates into finer sizes if not to break down the agglomerate to individual particles (Figure 4c).

Table 4. Amount of soluble protein removed in BSC shells after deproteinization stage.

Process Parameters		
NaOH Concentration, N	Shell Particle Mix (y), μm	Amount of soluble protein removed, mg
0.5	coarse	206.185
3.0	coarse	226.906
0.5	fine	158.637
3.0	fine	139.255

Note: Since resulting particle size is not controllable after demineralization stage, arbitrary selection of coarse (165 μm < y < 240 μm) and fine (41 μm < y < 60 μm) particle sizes were made for deproteinization.

XRD profile of the sample obtained from the run using the set of deproteinization process parameters with highest amount of soluble protein removed is presented in Figure 5. This resulting sample with peaks characteristic only to chitin can now be identified as high purity, BSC-derived chitin. These are peaks at *d-spacing* (*Å*) equal to 9.75, 7.12, 4.63, 3.43, 3.15 and 2.59. Obtained XRD peaks are similar to the results of chitin extracted by Sagheer et al. [5], Abdou et al. [6], Lavall et al. [14], Stawski et al. [16], and Cardenas et al. [23]. Also shown in Figure 5 is the XRD profile of commercially-available chitin where peaks of crystalline protein still appear, thereby, making it of less quality compared to the one produced in this study.

Table 5. Analysis of variance of the deproteinization stage design of experiment.

Source	**Sum of Squares**	**Degrees of Freedom**	**Mean Square**	**F Value**	**p-value Prob > F**
Model	29,676.6030	7	4,239.5147	20.2823	0.0002
A-NaOH Conc.	23.9366	1	23.9366	0.1145	0.7438
B-Shell Size Fraction	28,388.0377	1	28,388.0377	135.8115	< 0.0001
C-Temperature	396.7068	1	396.7068	1.8979	0.2056
AB	81.7668	1	81.7668	0.3912	0.5491
AC	47.8518	1	47.8518	0.2289	0.6451
BC	216.7520	1	216.7520	1.0370	0.3383
ABC	521.5514	1	521.5514	2.4952	0.1529
Pure Error	1,672.2026	8	209.0253		
Corrected Total	31,348.8056	15			

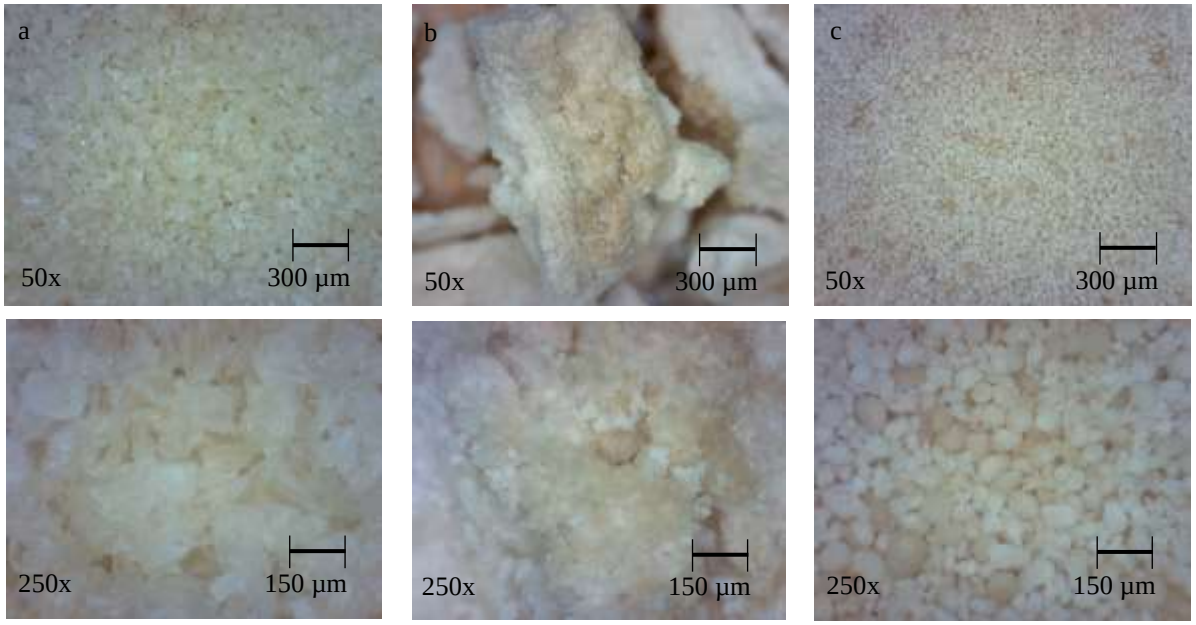

Figure 4. Digital images of the demineralized shells with different particle size at 50x (top row) and 250x (bottom row): coarse mix (a), agglomerated fine mix (b) and milled agglomerated fine mix (c).

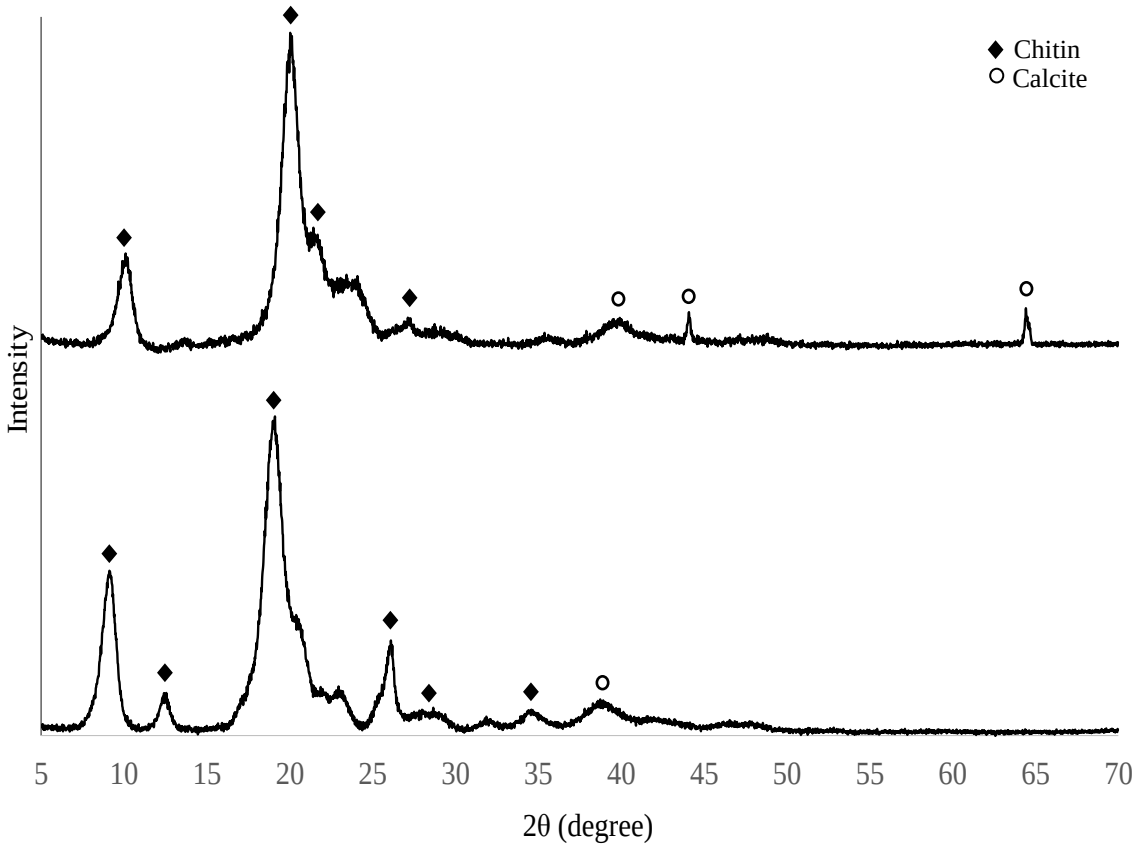

Figure 5. XRD profiles of high-purity BSC shell-derived chitin and commercially-available chitin.

3.2.3 Bench Scale Extraction of Chitin

For a 1000 g shell sample while maintaining a 1:15 solid-to-liquid ratio (g/mL) plus the volume of frothing generated during demineralization reaction, a reactor volume equivalent to 30-L reactor was designed and fabricated (Figure 6). Other features of the reactor include suspended stirrer with motor replacing the lab scale magnetic stirrer and baffles on the side of the mixing tank to sustain suspension of particles. Results of material selection for the reactor mixing tank showed that medium density polyethylene is the best material given that it is known to have high resistance to degradation even exposure to highly acidic and alkaline solution [13].

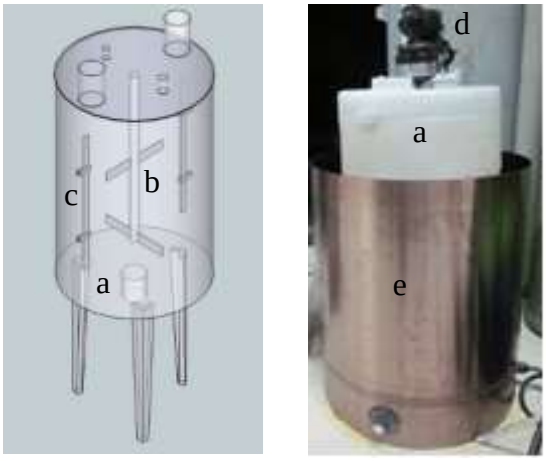

Figure 6. Isometric drawing and fabricated mixing tank for bench scale chitin extraction showing the reactor vessel (a), stirrer (b), baffles (c), motor (d) and water bath (e).

The streamlining of the laboratory scale process of extracting chitin aimed to obtain a higher percent yield, enhance mixing reactions and eliminate unnecessary stages [13]. It was found out that drying in between the demineralization and deproteinization stages can be done at temperatures around 60 °C instead of 100 °C in an effort to prevent formation of agglomerated particles which actually improved particle suspension during the deproteinization stage. The moisture content of the dried sample was measured to be at 9% which became the basis for the determination of the liquid-to-solid ratio necessary for deproteinization. Also, the neutralization of demineralized shells prior to deproteinization, which is similarly performed by related studies as previously mentioned, was removed as it is possible that the demineralized BSC shells being neutralized are also undergoing the process of deproteinization. The solution after demineralization was simply filtered out of the reactor then addition of the basic solution for the deproteinization followed. The conduct of process refinement observed an improvement of the percent yield from 4.80% from the laboratory scale methodology to 9.62%.

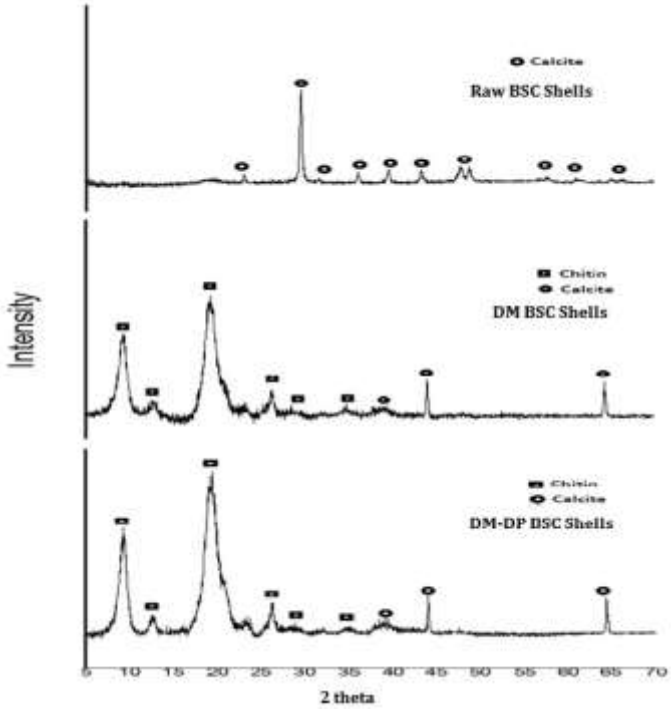

Figure 7. X-ray diffraction profiles of BSC shells in its raw form, after undergoing the demineralization stage (DM BSC Shells) and demineralization and deproteinization stages (DM-DP BSC Shells).

In spite of this significant improvement of the percent yield in the bench-scale extraction of chitin, XRD profiles presented in Figure 7 still proved the comparable high purity of the isolated material to that produced from the laboratory scale. Furthermore, results of the XRD analysis was also used to determine the degree of acetylation (DA) of the extracted chitin using Eq. 1 [24]. The DA is indicative of the purity of chitin wherein high values would correspond to a high purity material. The extracted chitin has a computed DA of 68.16% for the laboratory scale, 78.09% for the bench scale and the commercially-available chitin with only 30.99%. It has been reported that the expected DA for chitin samples is at least 55% [24].

$$I_{CR} = \frac{I_{020} - I_{am}}{I_{020}} \; x \; 100 \text{ and } DA = 100 - \left(\frac{103.97 - I_{CR}}{0.7529}\right) \qquad (1)$$

Where:

I_{CR} - crystalline index
I_{020} - intensity of the peak at (020) at 2θ < 13°
I_{am} - intensity of peak at 2θ ≅ 28°

4. CONCLUSION

Among the possible local sources of chitin namely BSC (*Portunus pelagicus*), mud crab (*Scylla serrata*) and tiger prawn (*Penaeus monodon*), tiger prawns consisted significant amount of chitin at 24%. However, the BSC shell was selected as raw materials for the subsequent chitin extraction process considering its accessibility and abundance.

A high acid concentration turned out to be the critical factor influencing the efficient removal of inorganic materials, specifically, calcium carbonate in calcite form, in BSC carapace producing a highly, if not completely, demineralized shell. It was also found out that for the deproteinization stage, the shell size is the significant factor where the mix of coarse shell size showed higher removal of protein content. Fine shell mix was found to form an impervious agglomerate, thereby, preventing sufficient contact with the alkaline solvent.

The demineralization and deproteinization stages were then streamlined through the development of the bench-scale extraction process which was able to refine unnecessary stages, namely, the drying stage and neutralization; and enhance mixing reaction by experimental set-up design modifications. Hence, obtaining higher product recovery from 4.80% to 9.62% while maintaining similar quality of chitin extracted from the laboratory scale.

Thus, this study was able to establish process conditions for the demineralization and deproteinization stages of bench-scale chitin extraction process from the shells of BSC which generated a high-purity chitin suitable for the manufacture of chitin having qualities better than the commercially-available technical grade chitin.

5. ACKNOWLEDGEMENTS

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