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Different Processing Sequential Protocols for Extraction, Quantification and Characterization of Chitosan from Cray Fish

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Abstract*: Chitosan is produced from chitin by a chemical process involving demineralization (DM), deproteinization (DP), decolorization (DC), and deacetylation (DA). Commercially, chitosan is produced via the chemical deacetylation of crustacean chitin under treatment with strong alkali. The functions and roles of chitosan in industrial setting is enormous, therefore there is needs to further research on chitosan in order to maximize chitosan production. Little work has been done to evaluate the effects of modifying or excluding any of the production processing steps on chitosan characteristics. The present study was undertaken to examine the effects of process alteration on quantification, extraction and characterization of chitosan produced from cray fish. Cray fish were collected from Ilesha, Osun state and sundry for 24hrs before grinding with blender. 12g of powdered form of Cray fish shells was used for chitin extraction and chitosan production. Chitosan was produced by prepared by changing of the order of the four sequential preparation processes (DCMPA, DMCPA, DMPCA, DMPAC, and DPMCA,). DPMCA denotes sequential steps of deproteinization + demineralization + decolorization + deacetylation. DPMCA was taken as the traditional processing method (control sample). Physicochemical and functional properties such as Nitrogen content, ash content, moisture content, viscosity, solubility, degree of deacetylation, emulsion capacity, bulk density, water binding capacity and fat binding capacity. The results showed that 7.24 and 3.55g of chitin and chitosan were produced with 60.33 and 49.03% by DCMPA methods. Physicochemical and functional properties recorded were: Nitrogen content (1.25%), ash content (0.12%), moisture content (4.46%), viscosity (720cP), solubility (88.55%), degree of deacetylation (23.73%), emulsion capacity (9.33%), bulk density (0.87g/ml), water binding capacity (716.33%) and fat binding capacity (455.35%). Decolorization, demineralization, deproteinization and deacetylation (DCMPA) produced the best chitosan in terms of quantities and properties with respect to physicochemical and functional.*

Keywords: *Chitosan*, chitin, Cray fish shells, Decolourization, demenirelaization, deproteinization and deacetylation

1. Introduction

Majority of fish processing factories produces various types of wastes ranging from bones, shells, skin, head and meat. The wastes produced are sources of pollution in riverine side and coastal environment (Islam *et al*., 2004). Parts of the wastes generated through this source are starting materials for chitin production which is major derivative used in chitosan production process. So, most of wastes produced from fish and fisheries products are useful for chitin production.

Sources of chitins are available in Nigeria and are abundant in the rural and urban areas of Nigeria (Amos, 2000; Amos 2007). These materials litter the banks of rivers side constituting environmental pollution because they are underutilized despite their vast industrial applications. More so, the products of the crustacean are also discarded after processing and these are valuable sources of chitin, which can be further, processed into chitosan.

Astacus leptodactylus (cray fish) is a member of Astaciadea family, crustacean species with high economic value, originating from Turkey and it has a wide area of distribution around the world (Erol *et al*., 2010). Studies showed that nearly 80% of crayfish comprises wastes shells (Hunner, 1994). The composition of shell in the wastes of Cray fish is 30 % protein, 40% calcium carbonate and 30 % chitin (Ghannam *et al*., 2016). It has been reported that Cray fish shell might have physicochemical properties with superior polymer compared with what is available in other crustacean wastes (No et al., 1989).

The chitosan are obtained from Chitin while Chitin is commonly extracted from crustacean shell waste. The process involves three basic steps: demineralization (separation of calcium carbonate and calcium phosphate), deproteinization (Protein separation), decolorization (pigments of removal) and deacetylation (removal of acetyl groups) (Ghannam *et al*., 2016). These three steps are traditional standard sequence for chitin production. Subsequently, conversion of chitin to chitosan (deacetylation) is achieved by treatment with concentrated sodium hydroxide solution (40-50%) at 100°C or higher temperature to remove acetyl group from the chitin (Kumari and Kumar Rath, 2014).

Chitosan is a biodegradable, non-toxic and biocompatible polymer. Over the last several years, chitosan have received increased attention as one of the promising renewable polymeric materials for their extensive applications in the pharmaceutical and biomedical industries for enzyme immobilization and purification, in chemical plants for wastewater treatment, and in food industries for food formulations as binding, gelling, thickening and stabilizing agent (Kumari and Kumar Rath, 2014)..

Despite numerous works reported on chitosan production, there is limited research on extraction and quantification of chitosan from chitin of Cray fish of Nigerian origin while there is no report on alteration of earlier sequence involves in traditional production process of chitosan extracted from Nigerian Cray fish. The current study is focus on examine the effect of production procee modification on extraction; quantification and characterisation of Chitosan produced from chitin obtained from Cray fish.

2. Materials and Methods

2.1 Materials

Shell materials were obtained from the wastes of crayfish (Astacus leptodactylus) obtained from Ilesha, Osun State. The area was within the latitude and longitude of 7° 37' 40.40" N and 4° 44' 29.80" E respectively. The samples collected were put in ice for storage during transportation to the laboratory. Shells were completely separated from the crayfish samples, washed in pure water and dried at 60°C in an oven.

Other materials include Sodium hydroxide (NaOH) and Hydrochloric acid (HCl, 36.5g/mol). All chemical were of analytical grades. Five Cray fish Chitosan labeled (DCMPA, DMCPA, DMPCA, DMPAC, and DPMCA,) were prepared by changing of the order of the four sequential preparation processes. For example, DPMCA denotes sequential steps of deproteinization + demineralization + decolorization + deacetylation. DPMCA was taken as the traditional processing method (control sample).

2.2 Extraction of chitosan

a.Deproteinization

With a ratio of ground shell to the solution of 1:20 (w/v) and a constant stirring for 2 h at 90 °C to remove the protein, the Crayfish shells was treated with 2.0 % of potassium hydroxide (KOH) solution. The filtrates were washed with tap water for 30 mins until pH neutral (pH, 7) after the samples were filtered under vacuum. This was followed by drying of deproteinized shells in the oven for 24 hrs at 60 °C (Shahidi and Synowiecki, 1991).

b. Demineralization

Demineralization of the deproteinized Crayfish shells was carried out through addition of 2.5 % (w/v) of hydrochloric acid (HCl) in a ratio of ground shell to the solution of 1:20 (w/v) at room temperature (20 °C) for 6 h to remove the mineral content. The samples were washed for 30 mins with tap water until pH neutral (pH, 7) after filtration under vacuum. The demineralized shells were dried in the oven for 24 h at 60 °C (Shahidi and Synowiecki, 1991).

c. Decolouration and dewatering

Decolourizing was carried out by treating the samples with acetone for 10 mins and dried at ambient temperature for 2 h and followed by removal of the resulting residues. Cray fish shell chitin was obtained by washing the decolourized shells in the running tap water, rinsed, filtered and dried for 24h at 60 °C (Shahidi and Synowiecki, 1991).

d. Deacetylation of chitin

This was done according to the method described by Yen *et al*. (2009). The chitin was treated with 40 % (w/w) aqueous sodium hydroxide (NaOH) with a ratio of chitin to the solution of 1:15 (w/v) at 105 °C for 2 h. Then, the filter pump was used to filtered the chitin and the chitosan was obtained by washing the filtered chitin with the deionized water until pH neutral (pH, 7). The chitosan obtained was then dried at 60 °C for 24 h in the oven.

2.3 Characterization of chitosan

a. The yield

This was determined by comparing weight measurements of the Chitosan obtained after treatment and that of the raw material.

A yield was calculated as follows:

Yield of chitin (%) = $\frac{(\text{Extracted chitin (g)}}{\text{Grinded Snail shells (g))}}$ x100 (1)

Yield of chitosan (%) = $\frac{(\text{Extracted chitosan(g)}}{\text{Extracted chitin(g))}}$ x100

b. Moisture, Ash and Nitrogen Contents

This was determined according to the method described by standard method (AOAC, 1990) with minor modification. The samples were dried for 24h at 60°C until the weights are constant. It was then calculated by percentage of weight loss comparing to the initial weight of the samples. Ash and nitrogen contents of chitosan were measured according to a previously described procedure (AOAC, 1990).

c. Determination of degree of deacetylation (DD)

This was determined by method of direct titration described by Kjartansson (2008) with some modification. Chitosan samples (0.1 g) were dissolved in 25 ml of 0.06 M HCl at room temperature for an hour. The solution was diluted to 50 ml before being titrated with a 0.1 N NaOH under constant stirring to pH 3.75. The volume of NaOH at pH 3.75 was acquired and recorded. Titration was continued to pH 8 and the total volume of NaOH (0.1 M) was recorded. The degree of deacetylation was then calculated using the following equation:

DD= $\frac{(161.16*(V2-V1)N}{W1}$

where, 161.16 is the mass of chitosan monomer, V1 and V2 are the volumes of NaOH solution used, *N* is the strength of the NaOH solution (0.1 M) and W1 is the mass of sample after correction for moisture. The degrees of deacetylation (DD) of the samples were be determined in triplicate.

d. Water binding capacity

This was measured as described by method of Ocloo *et al*. (2011). To a centrifuge tube, 0.5 g of chitosan and 10 ml of distilled water ware added. To dissolve the chitosan, the mixture was vortexed for 1 min before left for 30 mins at an ambient temperature and the tube was shaken for 5 s every 10 minutes before being centrifuged for 25mins at 3,200 rpm. After supernatant decantation, the tube was weighed again. The water binding capacity was then calculated as the following equation:

WBC (%) = $\frac{\text{Water bound(g)}}{\text{Sample weight(g))}}$ x 100

e. Fat binding capacity

This was measured using a modified method of Wang and Kinsella (1976). This was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of oil (five types of oil: soybean oil (Pure Wesson® Congra Foods, Irvine, CA. USA), canola (Pure Wesson®), corn (Pure Wesson®), sunflower (Pure Wesson®), and olive (San Marc' Can-America Inc. Tampa, FL. USA)) and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking for 5 s every 10 min and centrifuged (Model # Z383K, HERMLE-National Labnet Company, Woodbridge, NJ. USA) at 3,500 rpm (6,000 x *g*) for 25 min. After the supernatant was decanted, the tube was weighed again. FBC was calculated as follows:

FBC (%) = $\frac{\text{Fat bound(g)}}{\text{Sample weight(g))}}$ x 100

All experiments were triplicated.

f. Solubility

This was determined according to the method described by Fernandez-kim (2004). Chitosan powder (0.1 g in triplicate) was then placed into a centrifuge tube (known weight) then dissolved with 10 ml of 1 % acetic acid for 30 mins using an incubator shaker operating at 240 rpm and 25 °C. The solution was then immersed in a boiling water bath for 10 mins, cooled to room temperature (25 °C) and centrifuged at 10,000 rpm for 10 mins. The supernatant was decanted. The un-dissolved particles was washed in distilled water (25 ml) and then centrifuged at 10,000 rpm. The supernatant was removed and undissolved pellets were dried at 60 °C for 24 h. Finally, the particles were weighed and the percentage of the chitosan solubility was determined. The solubility of chitosan was calculated using the following equation:

Solubility (%)=(Initialweightoftube+chitosan)(Finalweightoftube+chitosan)×100/(Initialweightoftube+chitosan)–(Initialweightoftube)

g. Viscosity

The viscosity of extracted chitosan was determined according to the method by Ocloo *et al*. (2011). The extracted chitosan was diluted in 1 % of acetic acid at 1 % concentration on a dry basis. The viscosity of the extracted chitosan was determined using a Brookfield viscometer (Spindle no. 2 at 50 rpm at 25 °C) with values reported in centipoise units (cP).

g. Emulsifying capacity

This was determined based on method of Yasumatu *et al*, (1972). 1g of each sample, 50 ml of cold distilled water (4 ^{o}C) and 50 ml of sunflower oil were used for emulsion preparation. The gelatine sample was dispersed with a homogenizer/blender. Each blended samples was equally added into 50 ml of centrifuge tubes. One centrifuge tubes was directly centrifuge for 10mins at 4000 × g while after heating in a water bath for 30mins at 80^{o}C and cooling to room temperature (25 ^{o} C), the other tube was centrifuged under the same conditions. The height of emulsified layer, as a percentage of the total height of material in the unheated tubes, was used to determine the emulsifying capacity.

2.4 Statistical analysis

Data were determined in triplicate. The mean of the data was determined with standard deviation

3. Result and Discussion

3.1 Different Processing Sequential modifications on Quantity and yield of Chitin and Chitosan produced from Cray fish waste shells

Figure 1a showed the quantity of Chitin produced from Cray fish shells using sequential modification process. The different Chitins, labeled DPMCA, DMCPA, DMPCA and DCMPA, were prepared by altering the order of the four sequential preparation processes. For example, DPMCA denotes sequential steps of deproteinization + demineralization + decolorization + deacetylation. DPMCA represents the traditional processing method and was selected as the control sample.

The quantities of chitin produced depended on the Chitin extraction method, as DCMPA gave the highest chitin (7.24g from 12 g of grinded Cray fish shells) and DMPAC gave the lowest (5.22g from 12 g of powdered Snail Cray fish shells) chitin (Figure 1a). The highest yield of chitin (60.33%) was also observed with DCMPA as extraction sequence while the least chitin yield (43.5%) was recorded with DMPAC as production process method (Figure 1b).

The maximum quantity of chitosan (3.55g from 7.24g of chitin) recorded in this study was observed by DCMPA as sequential production process but DMPAC as method gave the minimum quantity of chitosan (1.65g from 31.61 g of chitin) Figure 2a.The best and least yields of chitosan (49.03% and 31.61%) were obtained with DCMPA and DMPAC as procedural production step (Figure 2b).

The above result was due to the fact that when decoloration and demineralization steps were performed before demineralization and deproteinization, Chitin and chitosan production will be slightly increased.

This result is in accordance with work of Gaikwad *et al*. (2015) who reported 53%,49%, 52%, 41% and 42% of chitosan from crab (*Scylla serrata)* by different chemical processing sequence (DCMPA, DMCPA, DMPCA, DMPAC and DPMCA).

3.2 Characterization of Chitosan produced by different processing Modification Process

a.Ash content

Ash measurement is an indicator of the effectiveness of the demineralization (DM) step for removal of calcium carbonate. The ash content in chitosan is an important parameter. The ash content obtained from chitosan in this study had excellent low ash content (0.12 to 0.86%) figure 3. This is due to fact that DM step was effective in removing minerals content of Cray fish shells. The above study is similar to the work of Tajik *et al*. (2008) that reported low ash content of between 0.19 to 0.51% in chitosan sequential production process.

ii. Moisture

The above study showed a slightly variation and a significant difference in the % of moisture content (2.35-5.63%) between the chitosan produced from Cray fish shells by varied production process Figure 3. The low moisture content recorded in this study is of advantages because moisture adsorptions affect water holding capacity of chitosan in relation to its processing and several applications (Chandumpai *et al*., 2004). In similar view, Gaikwad *et al*. (2015) reported that there was significant difference in the % moisture (2.37 and 5.4 %) between the five Chitosan prepared from crab chitosan.

iii. Nitrogen content

The percentage of nitrogen content of Cray fish chitosan reported in this study varied from 1.25 to 1.96% (figure 5). The above study is related to the work Gaikwad *et al*. (2015) that showed that the nitrogen content of the chitosan products was in the 0.9% - 1.91% range. Related view were reported by Tajik *et al*. (2008) and No and Meyers (1995) with nitrogen content 7.06 to 7.97% and 7.32-7.51% respectively.

iv. Solubility

This above study showed an excellent solubility of chitosan ranging from 88.55 to 94.74 % for Cray fish shells with significance variation; the least solubility was recorded with DCMPA (88.55%) as production sequence while chitosan produced by DMPCA (94.74%) methods had the highest solubility (Figure 6). The higher solubility as recorded in this study indicates complete removal of protein (Brine and Austin, 1981). This study is related to the work of Gaikwad *et al*. (2015), they demonstrated an excellent solubility ranging from 81.78 to 88.78 % with significant difference, while the DCMPA showed lower solubility (81.78%) in crab chitosan samples.

v. Degree of Deacetylation

The DD is an important parameter affecting solubility, chemical reactivity, and biodegradability. This study (Figure 7) revealed that, DD was < 70% for all Chitosan obtained through different sequential modification. The results from the present work showed that the Cray fish shells chitosan had degree of deacetylation ranging from 21.46 to 42.26% (Figure 7). A lower degree of acetylation reduces the amount of positively charged groups available for flocculating a negatively charged material e.g., bacteria (Shepherd *et al*., 1997).

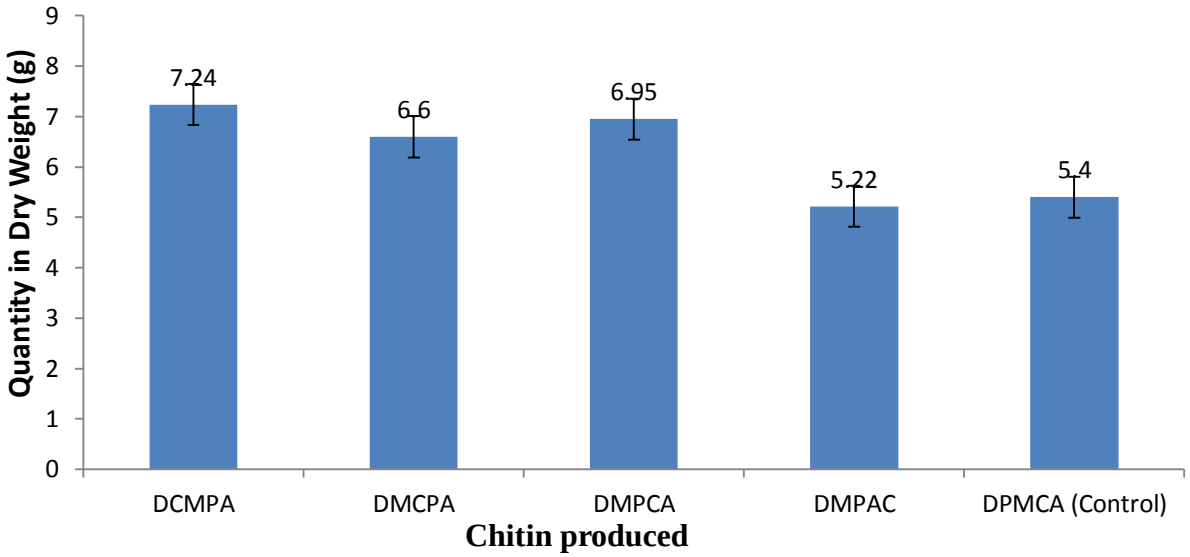

Figure 1a: Quantity of Chitin produced from Cray fish shells using different sequential modification process

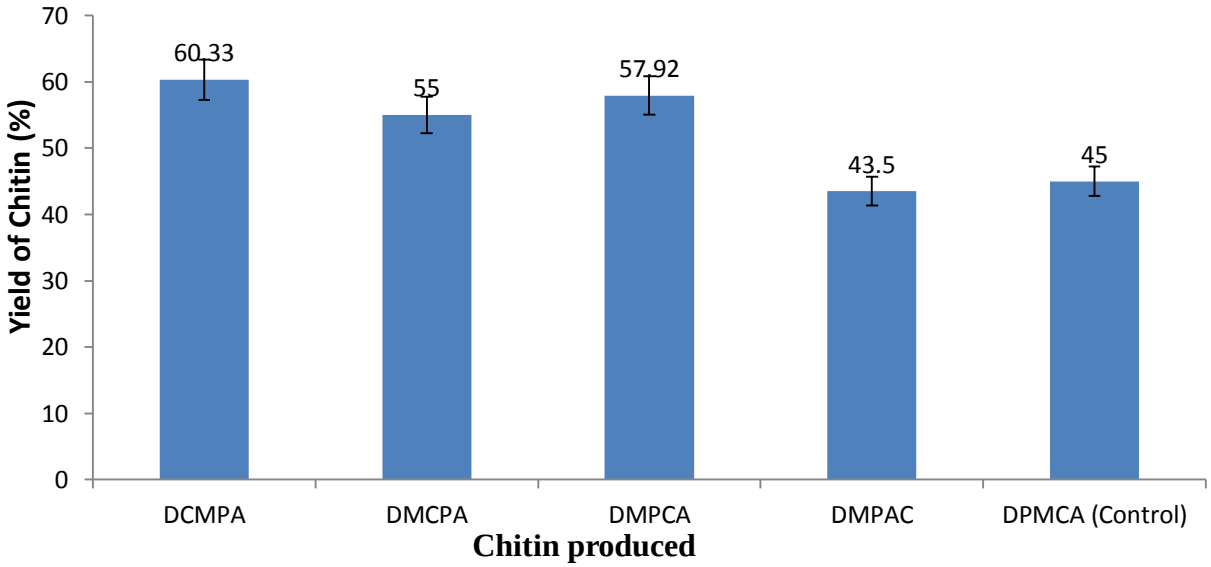

Figure 1b: Yields of Chitin produced from Cray fish shells using different sequential modification process

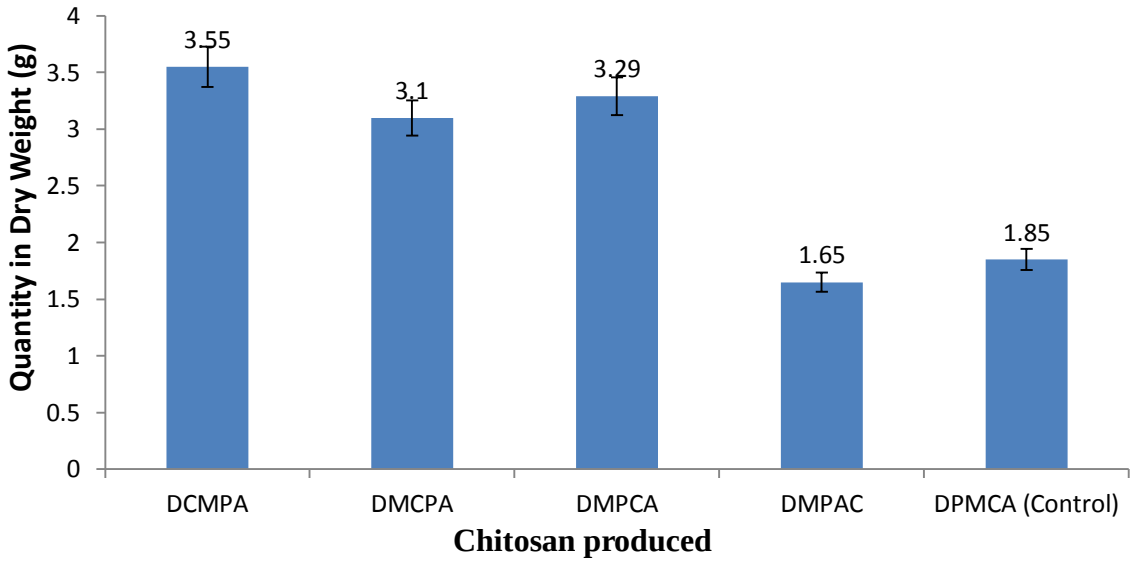

Figure 2a: Quantity of Chitosan produced from Cray fish shells using different sequential modification process

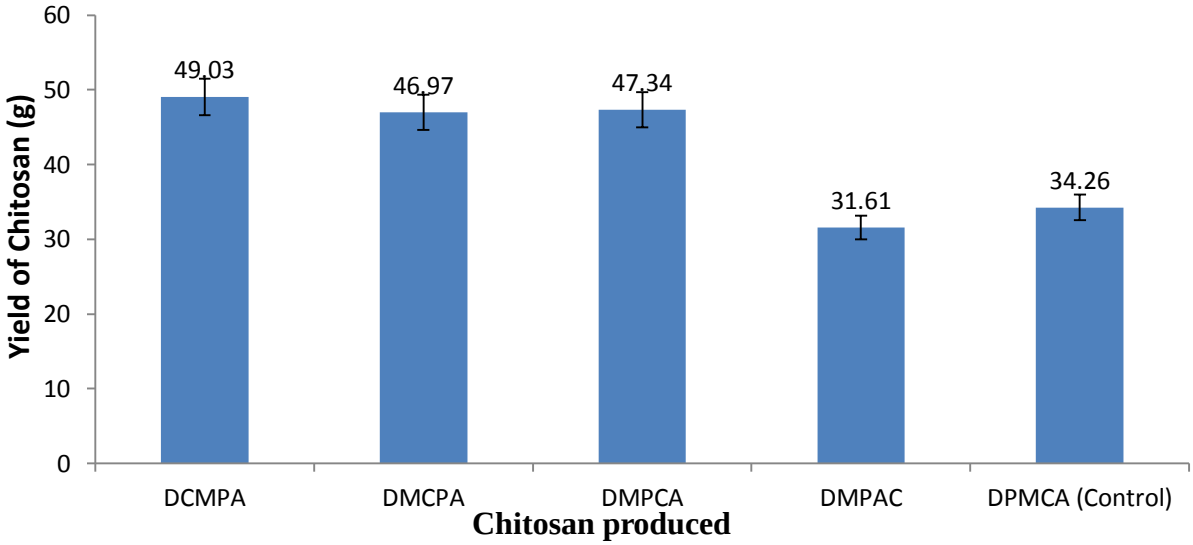

Figure 2b: Yields of Chitosan produced from Cray fish shells using different sequential modification process

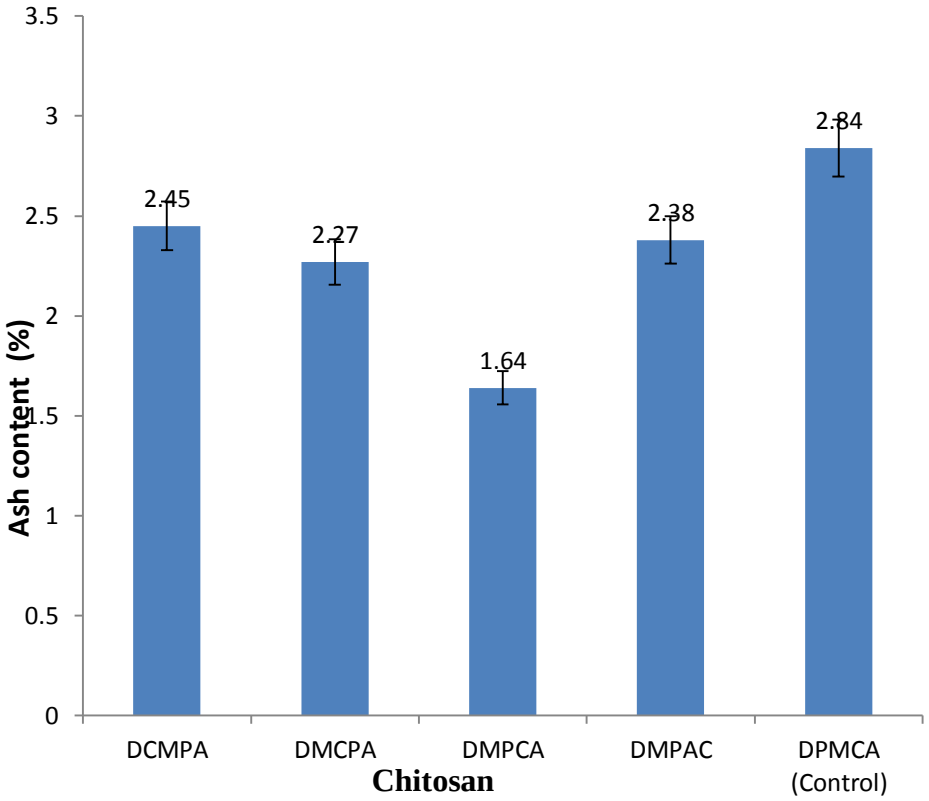

Figure 3: Percentage composition of Ash content in Chitosan produced from Cray fish shells with different sequential modification process

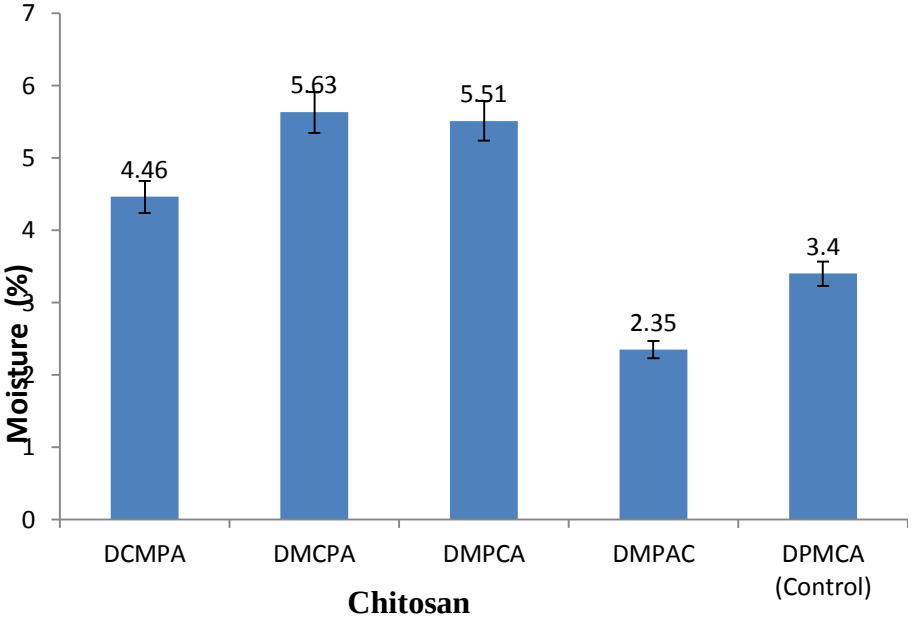

Figure 4: Percentage composition of Moisture content in Chitosan produced from Cray fish shells with different sequential modification process

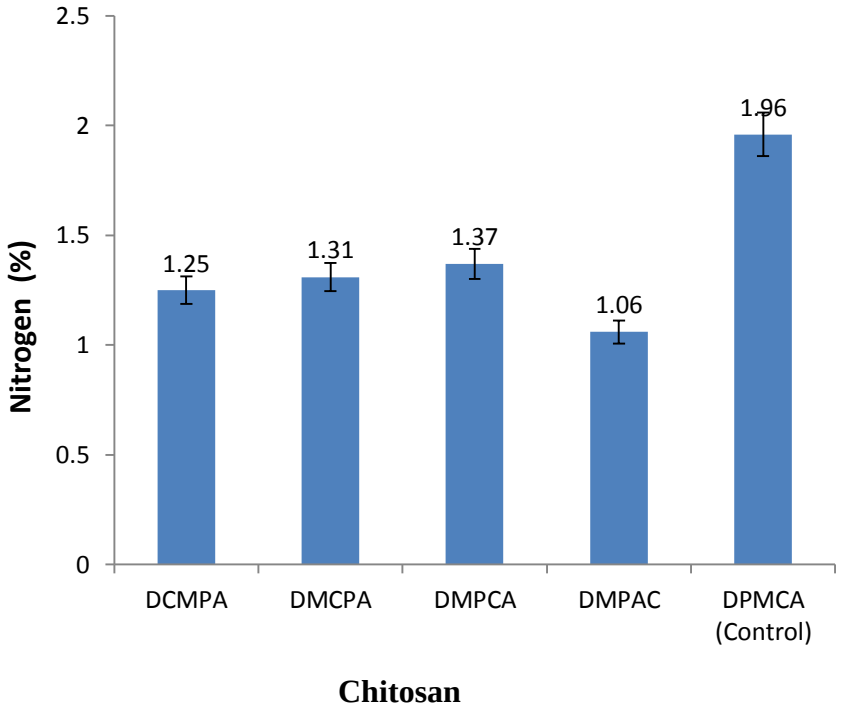

Figure 5: Percentage composition of Nitrogen content in Chitosan produced from Cray fish shells with different sequential modification process

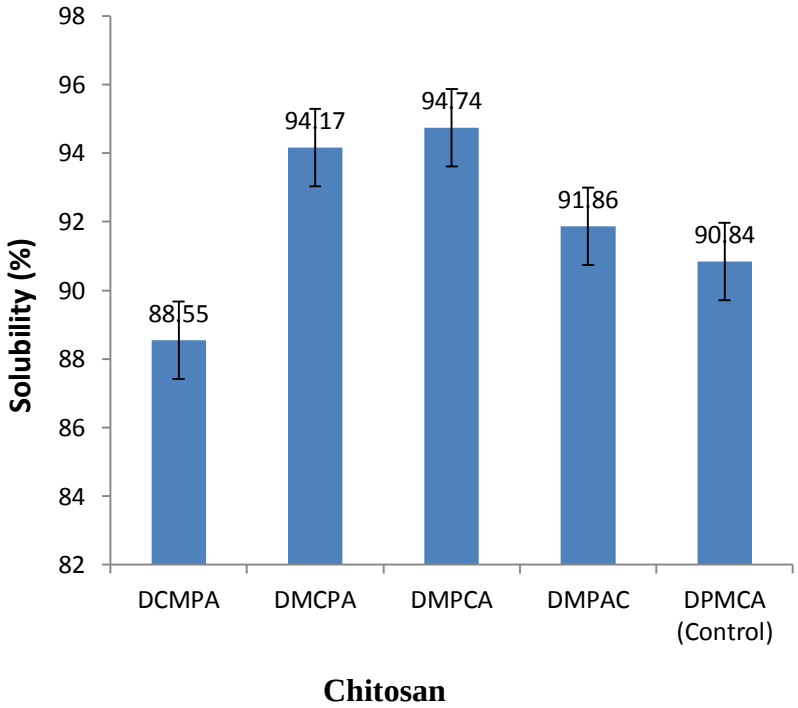

Figure 6: Solubility of Chitosan produced from Cray fish shells with different sequential modification process

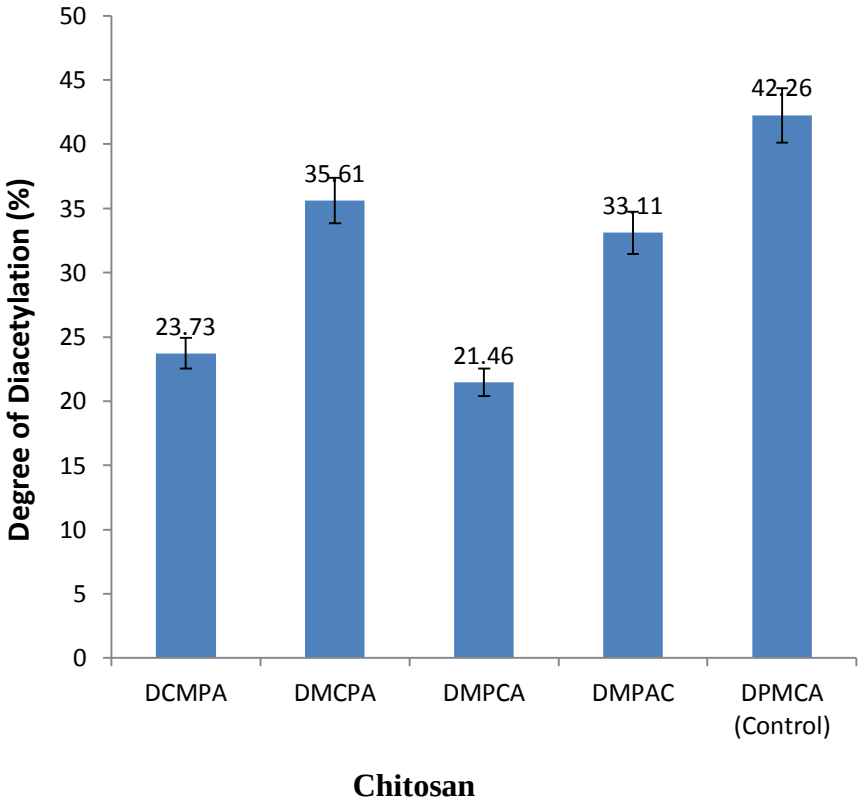

Figure 7: Degree of Deacetylation of Chitosan produced from Cray fish shells with different sequential modification process

vi. Emulsion Capacity

This study showed that Cray fish shells chitosan samples had an emulsion capacity ranging from 4.46 to 10.22 % (Table 8). The lowest emulsion capacity was observed with DMPAC (4.46%) as production process while highest was recorded by chitosan produced through DMPCA (10.22%) figure 4.8. Del Blanco *et al.,* (1999) stated that the degree of deacetylation is a determining factor in the emulsifying properties of chitosan, and chitosan with intermediate DD is a less effective emulsifier while chitosan with higher DD tends to produce poor emulsification. The optimum %DD of chitosan for sunflower oil emulsification is 81 and 89.

vii. Viscosity

Among the four samples and the control used in this study, DCMPA showed the highest viscosity (720cP) while the sample (DMPAC) had the lowest viscosity (184 cP) which suggests a decrease of MW Figure 9. This was similar to the findings of No and Meyers (1995), who demonstrated that the viscosity of CSs varied considerably, from 60 to 5110 cP, depending on the species and preparation methods used. In above study, significant differences were found between the viscosity of DCMPA samples (720 cP) and others sequential process methods Figure 9. The viscosity obtained with the DCMPA method was 2 and 3-folds higher than with the DMCPA (365 cP) and DPMCA (247 cP) methods, respectively. Compared to other crustaceans (Tharanathan and Kittur, 2003) the viscosity of Chitosan obtained from Cray fish shells was higher. The higher viscosity of chitosan enhance its applicability as a thickening and suspending agent for medical, cosmetic and food applications while Lower viscosity functions in opposite direction to aforementioned applications.

viii. Bulk Density

In the present study, bulk density of Cray fish shells chitosan samples were in the range of 0.64 – 1.06 g/ml (Figure 10). The highest bulk density of Cray fish shells chitosan was recorded at DMPAC method (1.06g/ml) (Figure 10). Cho and No, (1999)

noted that lower bulk density may indicate that the chitosan is more porous and may have been subjected to a lower alkali concentration treatment for deproteinization

ix.Water Binding Capacity (WBC)

Water binding capacity observed in this study for Cray fish shells ranged from 625.33 to 716.33% (Figure 11). A similar result has been reported by Cho *et al.* (1998) but No *et al.* (2003) reported lower results of 355 - 611%. The highest WBC was observed for DCMPA (716.33%), followed by DMPCA, DMCPA, DPMCA and DMPAC (716.33, 707.67, 673, 643.67 and 625.33%, respectively) (Figure 11). As shown in Figure 11, reversing the sequence of steps had marked effects on WBC. An increase in WBC was observed when demineralization was conducted prior to deproteinization followed by deacetylation, whilst this was not detected when deproteinization was performed prior to demineralization, followed by deacetylation. A similar result has been reported by Rout (2001) in crawfish. He also reported that the process of decoloration causes a decrease in WBC of chitosan than those of unbleached crawfish chitosan.

x. Fat Binding Capacity (WBC)

The fat binding capacity of five Cray fish shells chitosan was measured using olive oil. As shown in Figure 12, FBC of Cray fish shells chitosan ranged from 410.01 to 484.19%. The range of FBC found in our study (410.01 – 484.19%) was slightly similar to that reported by Cho *et al.* (1998) and 217 - 403% observed by Li *et al*., (1992).

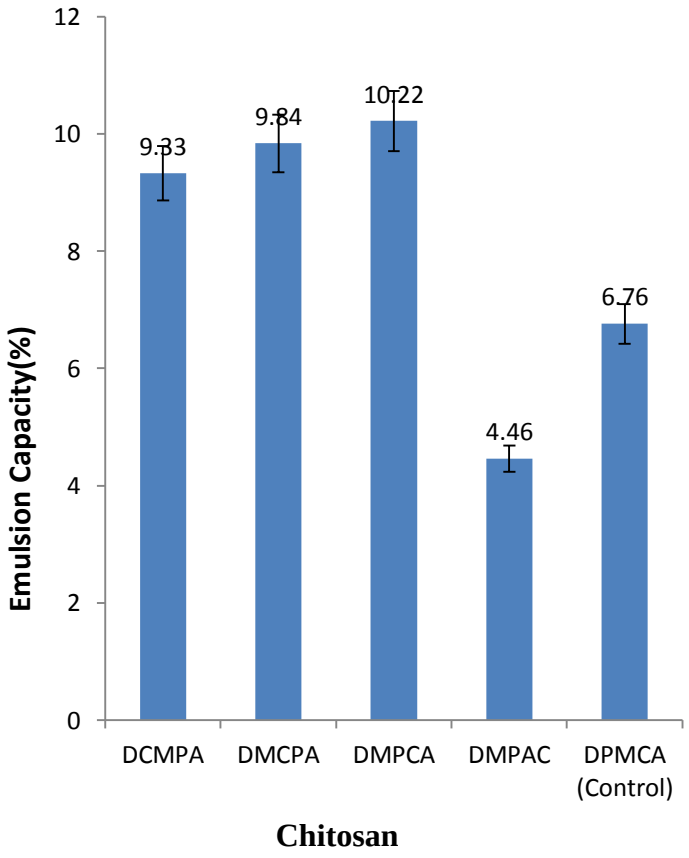

Figure 8: Emulsion Capacity of Chitosan produced from Cray fish shells with different sequential modification process

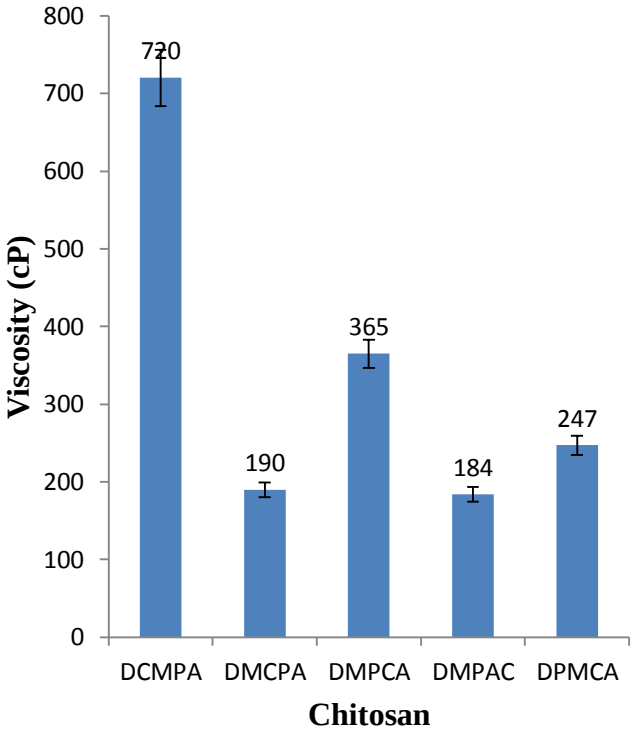

Figure 9: Viscosity of Chitosan produced from Cray fish shells with different sequential modification process

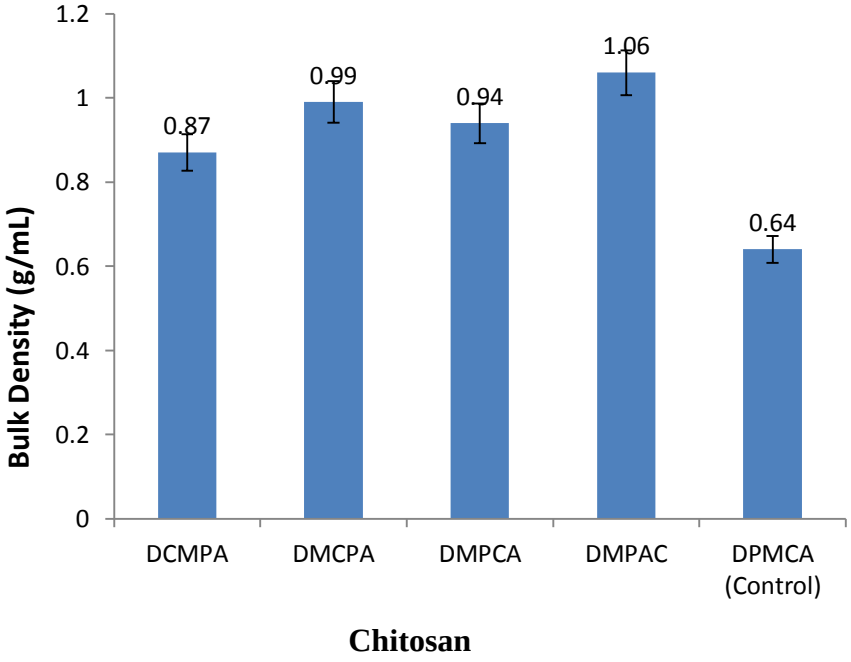

Figure 10: Bulk Density of Chitosan produced from Cray fish shells with different sequential modification process

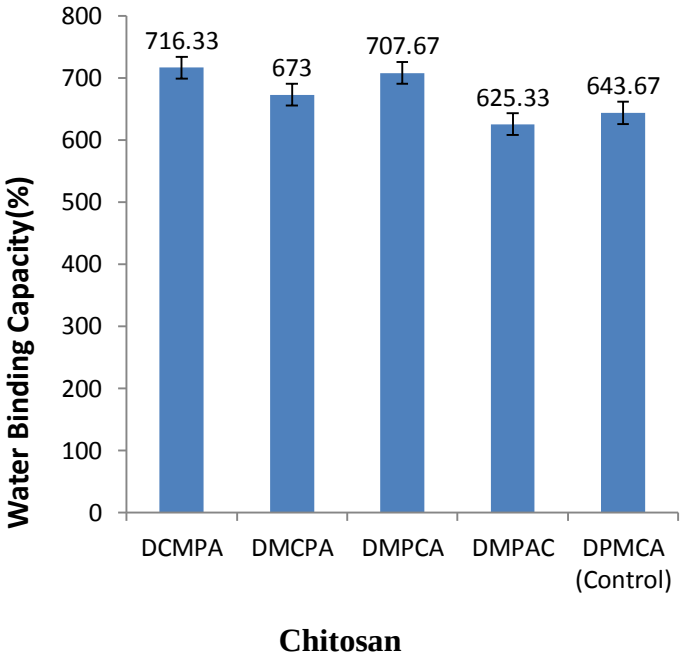

Figure 11: Water Binding Capacity of Chitosan produced from Cray fish shells with different sequential modification process

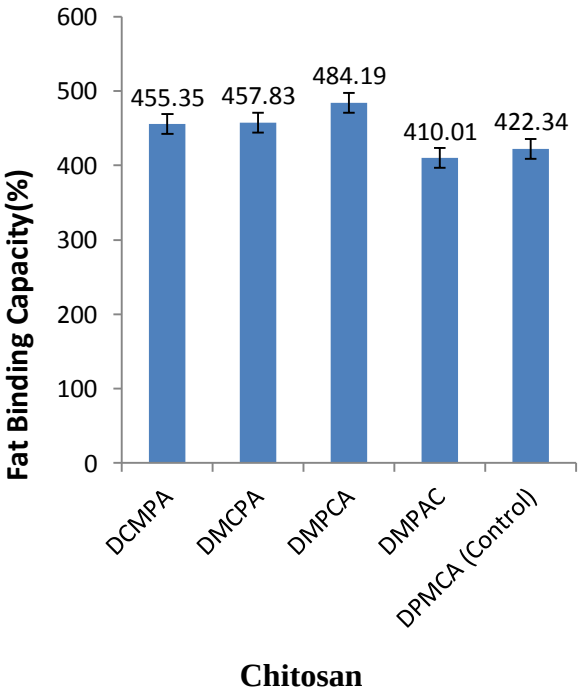

Figure 12: Fat Binding Capacity of Chitosan produced from Cray fish shells with different sequential modification process

4. Conclusion

The study is based on enormous and several applications of chitosan in industrial set up, despite arrays of chitosan of uses links to chitosan, the quantity and quality of chitosan produced is still major concerns. This study jettisoned the traditional protocols for

chitosan production in preference to modification of steps involved in earlier production process. The choice of Cray fish is based on high percentage of waste shells reported in several literatures for Cray fish. This is of advantage in a bid to enhance the quantity of Chitosan produced. The results of this study revealed that alteration in chitosan traditional production process from crayfish positively affected the extraction; quantification and characterization of chitosan to an advantage as this modification process concurrently enhance and improve the quantity and quality of chitosan produced against the backdrop of the then traditional existing methods

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