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Analytical Characterization of Medium Molecular Weight Chitosan from Pink Shrimp (*Metapenaeus dobsoni*) Shells

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Abstract

Seafood is widely consumed across the globe and serves as a vital source of essential nutrients. However, the processing of seafood generates significant amounts of waste, including shells and scales, which hold potential for the production of valuable byproducts. This study presents an efficient method for synthesizing high-quality chitosan from Pink shrimp (*Metapenaeus dobsoni*) shells. The extraction process involves a novel two-step purification of chitin, followed by an energy-saving freeze-pump-out-thaw (FPT) cycle and optimized deacetylation to obtain chitosan. Physicochemical characterization revealed that the chitosan has an average molecular weight (Mv) of 620 kDa and is soluble in 1% acetic acid. Scanning electron microscopy (SEM) confirmed the conformation of the synthesized chitosan. The degree of deacetylation was determined to be 97.2% using Fourier-transform infrared spectroscopy (FTIR), while the crystallinity index (Icr) was calculated at 69% via powdered X-ray diffraction (XRD). The findings demonstrated that the chitosan extracted from this crustacean source possesses unique physical and chemical properties, making it highly suitable for applications in biomedical fields and food packaging.

Keywords: Chitin, Chitosan, Biopolymer, Shrimp shells waste, Purification, DDA

Introduction

Environmental contamination has become a critical challenge in the modern era, highlighting the need for effective waste management strategies, including waste recycling and reduction. Pollution is driven by both naturally occurring contaminants and human activities, with waste production steadily increasing due to the underutilization of byproducts in various industries, particularly food processing. In the seafood industry, the focus is typically on processing and packaging edible portions, leaving behind significant amounts of waste such as shells, heads, and scales. It is estimated that up to 50% of the biomass from seafood pre-processing, including shrimp, krill, fish, and crab, is discarded as waste [1]. However, this shell waste holds potential for recycling into valuable, eco-friendly products, presenting a

sustainable solution to both waste management and environmental pollution.

Chitosan is a natural polysaccharide obtained from shell waste. Chitosan is extracted from chitin — present in the exoskeleton of insects and other terrestrial arthropods, majorly present in crustaceans, or produced by fungi and some algae [2]. In the published review Pellis et al. [3] reported various raw material sources for chitosan extraction. Around 1 million tons of chitin is produced from shells and the head part of shrimps [4]. As an alternative, this waste could be used for the production of chitosan, a low-cost derivative with great economic value [5].

Shrimp shells are converted into economic wealth through the production of chitosan, which is an economically significant product with a variety of uses. Previous literature has documented the extraction of chitosan from shrimp shells waste through the application of an optimized protocol [6-7]. Moreover, in 2019, the global chitosan market was worth 6.8 billion USD, and it is anticipated to increase at a compound annual growth rate (CAGR) of 24.7% during 2020 to 2027 [8].

Chitosan is a linear polysaccharide which is synthesized through the deacetylation of chitin. The N-Acetyl-D-glucosamine (A-units) and deacetylated D-glucosamine (D-units) units are joined by b-1,4glycosidic links to form the linear random copolymer [9-10]. In each of these repeating units, chitosan molecule possesses three functional groups — primary, secondary, and amine groups which allows chemical reactions to occur under protonation and affect the molecule's physical, mechanical, and biological properties like crystallinity, hydrophilicity, and dissolution ability [11]. Owing to its biodegradable, biocompatible, non-toxic, and anti-microbial characteristics, chitosan can be used in biomedical, agriculture, cosmetic, wastewater and food packaging applications and so forth [12-13].

The objective of this research is to synthesize high-quality chitosan biopolymer at a laboratory scale by developing a modified, simple, and efficient extraction protocol. These techniques will be further optimized for pilot and industrial-scale production. Karachi's geographic location offers a significant advantage, providing access to abundant marine waste, which can support large-scale chitosan production. Currently, this waste is exported at low prices, while finished chitosan products are imported at a high cost. Therefore, establishing an indigenous protocol for chitosan production could significantly enhance the potential for creating a local industry, reducing dependence on imports, and promoting import substitution.

Materials and Methods

Raw Sample and Materials

Pink shrimp (*Metapenaeus dobsoni*) shells were procured from Karachi, Pakistan's seafood processing market.

Sodium hydroxide (NaOH) (Merck, 99%), Hydrochloric acid (HCl), and Acetic acid (CH_3COOH) were obtained from Sigma Aldrich. Instruments used were laboratory test sieve (Fine: mesh no. 170), shaking water bath (Vision: VS- 120SW1), hot plate and magnetic stirrer (Bibby: HB502), dry air oven (Memmert: UM 400), analytical balance (Mettler, Monobioc B204-S), weighing balance (Mettler, Monobioc 2002-S), centrifuge (Beckman Coulter: Allegra X-22), Scanning Electron Microscope (Jeol: JSM-6380), Fourier Transform Infrared Spectrophotometer (Thermo Scientific: Nicolet Summit LITE FTIR), and X-ray Diffractometer (D8 Advance XRD, Bruker).

Extraction of Chitosan

Raw sample processing

The collected shell waste was given a thorough wash utilizing tap water (Fig. 1) and then dried at 90 ℃ for 6 h in a dry air oven. Further, shells were ground to approximately 100 µm size and stored in polyethylene bags at ambient temperature.

Figure 1. **Washed shrimp shells**

Pretreatment

Pretreatment of shells was carried out by immersing ground shells in 2% NaOH solution at ambient temperature for 30 min, after which the shells were washed with running water several times using strainer of mesh size 170 (retaining ability of 88 µm particle diameter). At the end, the shells were dried at 60 °C.

Demineralization

Demineralization was performed by adding 1 L of 11% HCl solution in 100 g of dried pretreated shells [10] at room temperature under agitation for 4 h at 80 rpm. Afterwards, the demineralized shells were sifted through a sieve, completely washed until pH was neutral and then dried in air dry oven at 50 °C.

Deproteinization

Deproteinization was accomplished by introducing a 1:10 (g/mL) solid/liquid mixture of 1 M NaOH to demineralized [10], dried shells at 65 ℃ for 4 h in an automated shaking water bath at 80 rpm. After repeated washing of the product to the neutral pH, it was dried in a 50 °C oven.

Purification of chitin

The chitin sample was purified by soaking it in a 2% NaOH solution for 30 min at ambient temperature and then washing it with tap water until the pH was neutralized. After following washing, in 1% HCl solution the sample was soaked for 30 min at ambient temperature. Again, washed utilizing tap water until neutral pH and dried at 50 ℃.

Production of chitosan

Chitin was deacetylated by treating the sample with solution of 12.5 M NaOH in 1:15 (g/mL) ratio [10]. After cooling, the mixture was held frozen at –4 ℃ for 24 h. After removing the sample from the freezer, it was kept at 90 °C, 80 rpm, shaking water bath for 4 h (Fig. 2). Chitosan, the end product, was rinsed with tap water after being filtered through a sieve to remove excess NaOH until pH attained neutrality. Afterwards, for moisture removal, the sample was parched in an air-dry oven at 65 °C.

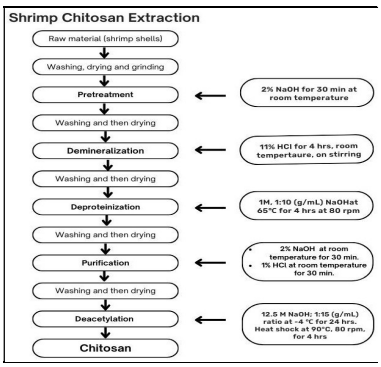

Figure 2. **Chitosan production flow scheme from shrimp shells**

Characterization of Extracted Chitosan Solubility

The chitosan solubility was tested according to a reported method [14]. Briefly, chitosan sample was dissolved in acetic acid (1%). The mixture was stirred at 240 rpm for 30 min at 25 °C before being heated in a water bath for 10 min at 100 °C and cooled at ambient temperature. Afterwards, for 10 min the sample was centrifuged at 10,000 rpm. Finally, the collected powder was weighed and the percentage solubility was determined after the pellet was dried at 60 ℃ for 24 h.

Ash Content

The ash content was calculated as described in the AOAC (Association of Official Analytical Chemists) official method [15]. The chitosan sample was desiccated at 90 °C for 5 h until constant weight was achieved. Afterwards, 1 g of chitosan was placed in a porcelain crucible and burned in a muffle furnace at 500 ℃ for 2 h. Following equation used for determining the ash percentage:

$$\text{Ash}\,\% = \frac{\text{P - p}}{\text{M}} \times 100 \quad (1)$$

Where M = mass of the sample, P = weight of the crucible containing the calcined material (g), and p = weight of the crucible when it is empty (g). The results represent the average values of three independently performed determinations.

Moisture Content

The gravimetric method was used to calculate the content of moisture [16]. The chitosan sample (1 g) was placed in the oven at 110 °C and dried for 2 h. Moisture values were calculated according to the following equation:

$$\text{Moisture content}\,\% = \frac{(\text{Initial weight - Dry weight})}{\text{Initial weight}} \times 100 \quad (2)$$

Molecular Weight

Molecular weight is the utmost parameter to determine the functional properties of chitosan [17]. Average molecular weight (Mv) was calculated by determining the intrinsic viscosity [η], and comparing the flow times of two liquids of equal volumes from a capillary viscometer. Determinations were performed in triplicate solution of chitosan in 0.25 M acetic acid/0.25 M sodium acetate at 25 °C [18] and Mv was calculated using the Mark–Houwink equation:

$$[\eta] = \kappa \text{M}_{\nu}^{\alpha} \quad (3)$$

Where M_{ν} = polymer viscosity average molecular weight, α=0.83 and k=1.4×10^{-4}.

Scanning Electron Microscopy (SEM)

The extracted sample conformation was analyzed by SEM under a high voltage of 15 kV, working distance of 4.4 mm and display mode secondary electrons, and having high vacuum under ambient temperature. Layers of gold particles were added to the sample by the Mini Sputter Coater. The magnification is 150–20,000 X, vacuum is high, and distance of 4–5 mm was maintained between the sample and the objective.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was performed for the functional groups observation of chitosan [19]. FTIR Spectrometer equipped with ATR diamond crystal for spectroscopy. Transmittance was determined as a function of wave number, between 4000 and 400 cm^{-1}.

Degree of Deacetylation

The degree of deacetylation (DDA) was calculated by FTIR analysis. The DDA was calculated by comparing the bands intensity of at 1320 cm^{-1} and 1420 cm^{-1}, according to the equation (4) proposed by Brugnerotto et al. [20]. Further, equation (5) was used to estimate DDA.

$$A_{1320}/A_{1420} = 0.3822+0.03133\ DA \quad (4)$$

$$DAA = 100 - DA \quad (5)$$

Diffraction Analysis

The crystallinity of the extracted chitosan was determined by powder X-ray diffraction analysis using a Bruker smart apex II diffractometer functional with CuKα, λ = 1.54060 Å, in the 2θ angle range from 5-60°. The diffractometer was operated with a step length of 0.036° in continuous mode at 40-kV and 25 mA at room temperature (25 °C). The crystallinity index (*Icr*) was determined by the following equation (6):

$$Icr = \left[\frac{I_{100} - I_{am}}{I_{am}}\right] \times 100 \quad (6)$$

Where I_{110} = maximum intensity at 2θ = ~ 20° and I_{am} = diffraction intensity in the amorphous area at 16° [21].

Results and Discussion

Extraction of Chitosan

By following a modified protocol, the yield obtained was 21.62% chitosan from shrimp shells waste (Fig. 3). The results were comparatively better than that of Abdulkarim et al. [22], in which 15% of chitosan yield was achieved after deacetylation with 50% NaOH. Shrimp shells for this study were collected from the coast of Arabian Sea, similar study depict yield of chitosan from different shrimp species was ranged between 18% - 21% [23].

Figure 3. **Extracted chitosan from shrimp shells**

Solubility

Solubility holds an utmost importance in determining the quality of chitosan. The high solubility in aqueous acetic acid is directly related to high deacetylation degree due to the removal of acetyl group and leaving only amine group [8, 24]. Moreover, to widen the application of chitosan solubility is a crucial parameter to be considered for modification. In this study, the average solubility of chitosan, listed in Table 1, is in concordance with the outcome obtained by Renuka et al. [25]. In other study, the solubility ranges from 17.43 to 95.29%, following chemical method of extraction from shrimp shell waste [26]. Higher solubility increases the application versatility and quality of chitosan.

Ash Content and Moisture Content

In this investigation, chitosan's ash content was 0.26% (±0.058), Table 1 in correspondence to the value reported by Firdous and Chakraborty [27]. Moreover, for high-quality chitosan, the maximum allowable ash value should be less than 1%, which shows the effectiveness of demineralization step. Moisture content of chitosan holds a

paramount importance in determining the shelf life and hydrogen bonding capability of the polymer, which should be 6% (w/w) [23]. While in this study the average value of moisture content was 4.56% (±0.83) shows the stability of the extracted chitosan. Although, the permitted chitosan moisture level is below 10% [16] to avoid damage to the polymer. The chemical composition of shrimp shells however varies from geographical location and harvesting season.

Molecular Weight

The molecular weight is a crucial aspect to take into account for the functional activities of polymers. The molecular weight of biopolymer is reported to augment the antibacterial and antifungal properties [28]. According to its Mv, chitosan can be divided in: high molecular weight >700 kDa, medium molecular weight 150-700 kDa, and low molecular weight <150 kDa chitosan [29]. Commercial chitosan from crustacean's source exhibit Mv between 50 to 2000 kDa [30]. In the present study the viscosity–average Mv (Table 1) shows that chitosan extracted from shrimp shells waste has medium molecular weight and can be utilized in various ways such as in bioplastic fabrication, cosmetics, aqua culture, drug delivery and tissue engineering.

Table 1. **Characteristics of extracted chitosan.**

Parameters	Value ± SD
Solubility (%)	98.1 ± 0.1
Ash value (%)	0.26 ± 0.058
Moisture content (%)	4.56 ± 0.83
Average molecular weight (kDa)	620
Degree of deacetylation (%)	97.2

Scanning Electron Microscopy

The morphology of shrimp shells extracted chitosan was analyzed by SEM. The results showed that chitin exhibit a heterogeneous morphology and rough structure with distinct round white spots (Fig. 4c). After treatment El-araby et al. [31] reported the top smooth surface and fibrous structure of chitosan biopolymer extracted from shrimp shells as visualized in the present work at 2000x magnification (Fig. 4f). According to an investigation of El Knidri et al. [17], the high degree of deacetylation of chitosan, which exposes more sheaths and removes some bonding agents, is responsible for the layered smooth surface of the biopolymer.

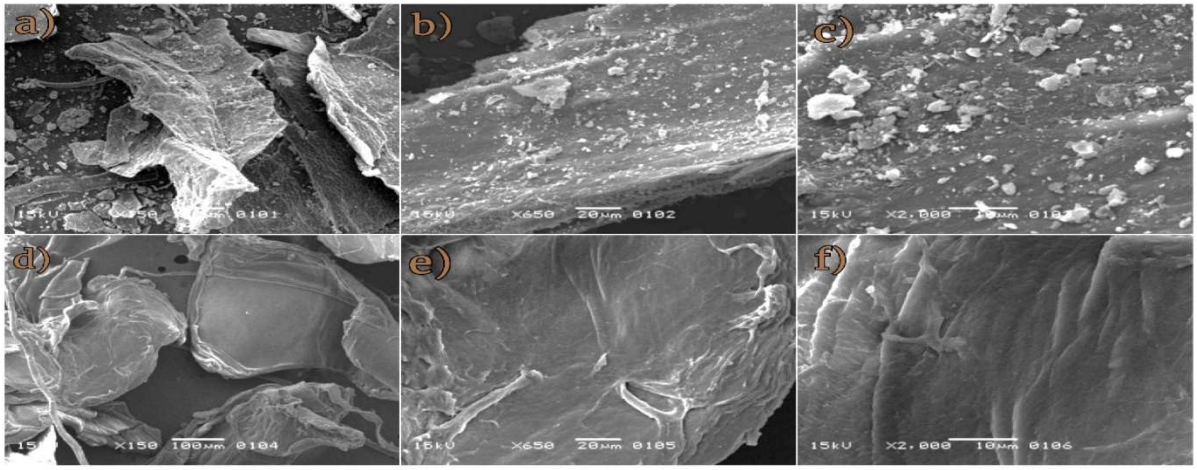

Figure 4. **SEM images of shells and the final material obtained after processing. Shells: (a) 150× (b) 650× (c) 2000×; Chitosan: (d) 150× (e) 650× (f) 2000×**

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of extracted chitosan samples obtained and standard chitosan are shown in Fig. 5.

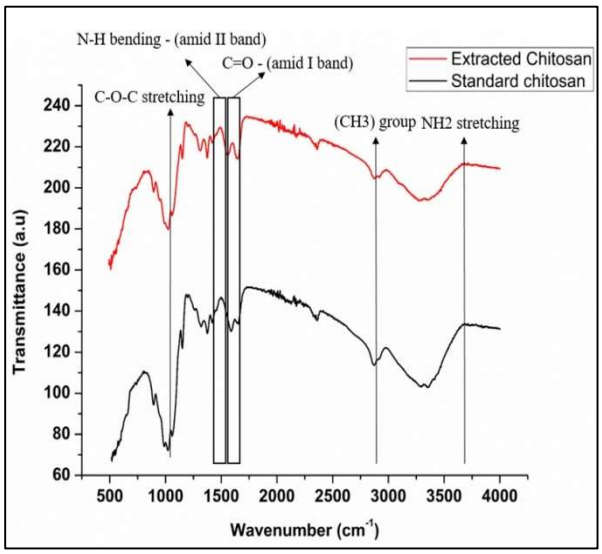

Figure 5. **FTIR spectra of standard and extracted chitosan**

Table 2. **FTIR main bands wavelength of standard and shrimp shell extracted chitosan.**

Vibration modes	Standard chitosan (cm^{-1})	Extracted chitosan (cm^{-1})
NH_2 stretching	3353	3354
(CH_3) - $NHCOCH_3$ group	1374	1373
C=O - (amid I band)	1652	1646
N-H bending - (amid II band)	1587	1559
C-N stretching - (amid III band)	1318	1312
CH_2 - CH_2OH group	2871 1418	2873 1418
C-O-C stretching - glycosidic linkage	1150	1150

The peak at 3354 cm^{-1} indicating hydroxyl group (OH stretching) vibration, -NH_2 of amine and hydrogen bonding, as compared to the peak at 3353 cm^{-1} that of standard chitosan. The standard chitosan absorption band at 1652 cm^{-1} (amide I), 1587 cm^{-1} (amide II), and 1318 cm^{-1} (amide III) shows slight comparability at peak of extracted chitosan at 1312 cm^{-1}, however, shows variation at 1646 cm^{-1}, and 1559 cm^{-1}, previously reported in [19, 32-33] respectively, as the deacetylation process occur in extracted chitosan. The characteristic band at 1646 cm^{-1} express the amid bond (C=O) stretching in the structure of extracted chitosan from chemical method. The $–CH_2$ group in CH_2OH peaks were observed at 2873 cm^{-1} and 1418 cm^{-1} in extracted chitosan coincided with standard at 2871 cm^{-1} and 1418 cm^{-1}.

Similarly, asymmetric expansion of oxygen bridge in glycosidic linkage indicated at peak 1150 cm^{-1} in standard, exactly overlapped by extracted at 1150 cm^{-1} [34]. Thus, the existence of the full band stretching of extracted chitosan from shrimp shell as compared with that of standard chitosan confirmed the extracted sample is chitosan (Table 2).

Degree of Deacetylation (DDA)

The degree of deacetylation is a crucial factor in evaluating chemical, physical, and biological characteristics of chitosan biopolymer. The acetyl groups removed from chitin and the number of free -NH_2 in chitosan is represented by deacetylation degree. The term chitosan as a biopolymer is only recognized when it exhibits a DDA greater than 70% [35]. In this study, equation (4) and (5) were used to estimate degree of deacetylation of extracted biopolymer from IR spectra (Table 1). Similarly, using 50% NaOH for deacetylation of shrimp shells waste DDA value of 89.79% was reported by Puvvada et al. [16]. However, the reported deacetylation value ranged between 97-61% of chitosan extracted from different species of shrimp shells [37].

X-ray Diffraction

As shown in Fig. 6 from X-ray diffraction data the crystallinity index calculated was 69%, for shrimp-extracted chitosan. In chitosan molecules, as the crystallinity increases the molecular chains become more homogenized which characterized water sorption ability of the polymer. However, medium molecular-weight chitosan exhibits a wide degree of crystallinity [38].

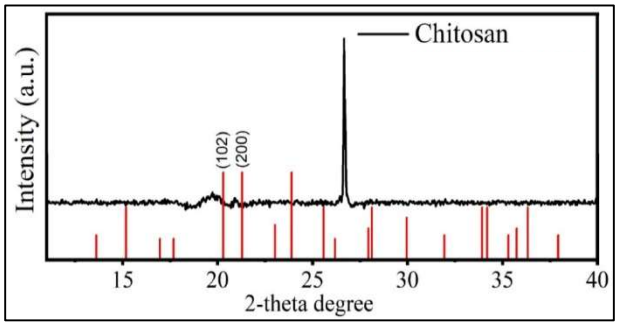

Figure 6. **X-ray diffraction patterns of shrimp shell extracted chitosan**

The XRD pattern in Fig. 6 is compared with the JCPDS no. 039-1894 and presented that brags angle at 20.3° is very much similar to the XRD pattern. This indicates that the quality of the extracted powder is very good.

The extracted sample shows characteristic crystalline planes at 2θ = 9.5° and 2θ = 19.8°. The inter-plane distances of 0.92 nm and 0.44 nm, correspondingly, displaying a semi-crystalline structure of chitosan. Likewise, sharp plane was observed at 2θ = 26.6°, as reported around 20.2° and 22.4° for shrimp-extracted chitosan [39]. This basis of having variable peaks may be due to the sources of chitin.

Conclusion

It was demonstrated that the method developed in the study for extracting chitosan biopolymer from Pink shrimp (*Metapenaeus dobsoni*) shell waste is efficient for obtaining biopolymer with quality attributes. Extracted samples of chitosan showed impressive physical and chemical properties such as, moisture content below 10%, ash value of 0.26% and high solubility in 1% acetic acid. Similarly, the extracted chitosan exhibit medium molecular weight (MMW), *Icr* value of 69% and high DD manifest its application in pharmaceutical, food and agriculture industry. The primary limitation of this study lies in the variability of chitosan properties due to differences in shrimp species and geographical origin. Future research should focus on investigating the potential of medium molecular weight chitosan for biomedical applications, particularly in drug delivery and tissue engineering. Therefore, utilization and extraction of chitosan from sea waste pave a path for clean and green environment to be lived in.

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Conflict of Interest

The authors declare no conflict of interest.

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