

# Extraction and Characterization of Chitosan from the Cuttlebone of Spineless Cuttlefish, *Sepiella inermis*

Rahul Varma <sup>1</sup>, Arnab Pratihar <sup>1</sup>, Nigariga Pasumpon <sup>1,\*</sup>, Sugumar Vasudevan <sup>1,\*</sup>

<sup>1</sup> Department of Oceanography and Coastal Area Studies, Alagappa University, Science Campus, Karaikudi-630003, Tamil Nadu, India

\* Correspondence: [crustacealab@gmail.com](mailto:crustacealab@gmail.com) (S.V.);

Scopus Author ID 14062419600

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**Abstract:** The present study deals with extracting and characterizing chitosan from the cuttlebone of *Sepiella inermis*. The samples were collected from the local fishermen as they are economically important species, and the availability of these samples was not limited. The extracted chitosan was characterized using instrumentations such as Fourier Transform Infrared Spectroscopy (FTIR), X-ray Powder Diffractometry (XRD), Thermogravimetric Analysis (TGA), and Scanning Electron Microscopy (SEM). The FTIR studies revealed the presence of amide I and aliphatic CH stretching, which were recorded at 1382 and 2931  $\text{cm}^{-1}$ , respectively. At the same time, the XRD showed its crystalline nature at the  $2\theta$  value of  $22^\circ$ , which is very common in the case of chitosan. The studies on the thermal stability of the chitosan showed that the chitosan extracted from *S. inermis* has thermal stability at the temperature of 350 to  $450^\circ\text{C}$ . The SEM analysis was carried out to study the chitosan's morphological structure, revealing a smooth texture for the samples.

**Keywords:** cuttlefish; chitosan; characterization; polymer.

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## 1. Introduction

Materials that interact with the biological system are referred to as biomaterials. Natural biomaterials are constantly being discovered to understand their properties better so that they might be used to create more energy-efficient and lightweight materials [1]. Humans have used Marine biomaterials since ancient times, but only recently have they reached an industrial level, thanks to the rapid development of diverse processing technologies [2]. Natural polymers derived from marine resources have gotten a lot of interest in recent years because they are more abundant and physiologically active than conventional polymer sources. Indeed, polysaccharides such as agar, chitin/chitosan, alginate, and glycosaminoglycans are abundant in marine resources such as crustaceans, seaweeds, algae, mollusks, and marine arthropods, with unique traits and properties [3-5].

Polymers are produced by a variety of marine creatures. Biopolymers are used in various sectors and have exceptional and uncommon functional, structural, and biological properties. Apart from the three categories of polysaccharides, proteins, and nucleic acids, polysaccharides—most notably alginate, chitosan, and chitin—are employed for various applications due to their unique properties such as biocompatibility, biodegradability, non-toxicity, and metal ion chelation [6].

Natural polysaccharides are lengthy carbohydrate molecules with repeating monomer units connected by glycoside linkages, having a molecular weight of 100 kDa or more. They feature a pattern that ranges from straight to very branching. They may provide living creatures with the highest levels of energy storage and structural protection [7]. Natural polymers are promising alternatives to synthetic polymers in the creation of materials. As a result, biopolymer science and technology have progressed, allowing researchers to better grasp their basic and applied biological, physicochemical, morphological, and mechanical characteristics. CAZymes, or carbohydrate-active enzymes, are present in microscopic life in the ocean and are connected to the number of marine polysaccharides [8].

Chitin is made up of (1,4)-linked N-acetyl-glucosamine units and is the 'world's second most common polymer after cellulose. Corals, mollusks, worms, sponges, annelids, and arthropods are among the marine phyla that contain them [9]. Chitin is characterized by its long lengths (several nanometers) and microfibrillar shapes (2-5 nanometers) fixed in a protein matrix [6]. Chitin has a highly structured crystalline structure resistant to physical and chemical stimuli. Chitin cannot be dissolved in most common solvents, and as a result of this insolubility, it has been used in a wide range of applications [6]. The most common sources of (1,4)-N acetylglucosamine are alpha, beta, and gamma chitin. Beta chitin, for example, is derived from squid pens and has weak intra-sheet hydrogen bonding. Chitin is made up of a series of parallel and antiparallel chains found in fungi and yeast. Alpha chitin is derived from crustacean exoskeletons and has an antiparallel lineup with strong intra- and inter-sheet hydrogen bonds [10].

Chitosan is a nano-structured, non-toxic polymer made from 1,4-D-glucosamine produced by the deacetylation of chitin. The degree of deacetylation in the deacetylation process is determined by the ratio between the two units, and when the chitosan deacetylation degree reaches around 50%, it becomes soluble in an aqueous acidic environment. Chitosan becomes cationic when it degrades in an acidic environment because the amino groups in the chain protonate, and the polymer becomes cationic, allowing it to interact with a wide spectrum of molecules [11-13]. Enzyme inhibition, immunostimulant, antimicrobial, anticoagulant, anticancer, anticholesterolemic, and wound healing are only a few of the biological properties of chitosan. Due to its inherent properties, chitosan has sparked a lot of scientific curiosity, and there are a lot of studies about it in the literature. Chitosan is considered a versatile biopolymer since it may be used to make gels, films, and nanoparticles [14].

Palk Bay is a small semi-enclosed sea body having a maximum depth of 13 meters [15]. Thondi is part of Palk Bay and is home to a diverse range of fish, crustaceans, cephalopods, seagrass, seaweeds, marine arthropods, and other marine species. It is one of the major fish landing stations found along 'Tamilnadu's Palk Bay coast (Ramanathapuram District). Prawns (*Penaeus semisulcatus*), crabs (*Portunus pelagicus*), cephalopods (*Sepiella spp.*), perches (*Lenthrinus spp.* and *Psammoperca waigiensis*), belone (*Hemirampus spp.*) and rays are among the major kinds of fish found here [16]. *Sepiella inermis* is a demersal benthos-nektonic molluscan mollusk found mostly in Attukanava, Tamil Nadu, and along the Indian coast [17]. *Sepiella inermis* is a cephalopod that lives in shallow water. It has an oval form and is dorso-ventrally compressed, and it can grow from 4.5 cm to 12 cm. From the intertidal to depths of 40 meters, it may be found in Malaysia, Japan, England, 'Singapore's coast, and worldwide. The lack of a feature on the cuttlebone that mimics a spine distinguishes *S. inermis* (spineless cuttlefish) from other cuttlefish genera [18].

The industrial processing of seafood for human utilization, such as squid, shrimp, crab, and krill, as well as fish, produces huge volumes of trash, which accounts for around 50-60% of the weight, with global yearly production estimated to be 1.44 million metric tonnes in dry weight. Low biodegradation rates are a major problem in the seafood processing industry, resulting in massive waste [19,20]. Chemically, the waste is composed of 20-30% chitin, 20-40% protein, 30-60% minerals, and 0-140 percent lipids [21-25].

In India, many cuttlefish (*S. inermis*) internal shells are dumped as garbage. It pollutes the environment and endangers human health, and there is no comprehensive study on how to extract beneficial goods from this “biological waste”. As a result, using waste from industrial operations will decrease environmental pollution while also allowing these waste materials to be used to manufacture usable biomaterials [26]. Therefore, the objectives of this study were: 1) Extraction of chitosan from cuttlebone. 2) Measurement of the degree of deacetylation of chitosan. 3) Utilization of Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD), and Thermogravimetric Analysis (TGA) to characterize the isolated chitosan. 4) Studying the surface topography by Scanning Electron Microscopy (SEM).

## 2. Materials and Methods

### 2.1. Collection of cuttlebones.

The cuttlebone from *Sepiella inermis* was given by local fishermen on the Thondi Coast (9 0 45' N, 79 0 04' E). The bones were collected and dried for seven days before being used. The dried bones were weighed and pulverized for future characterization studies (Fig. 1).



**Figure 1.** Cuttlebone of *Sepiella inermis*.

### 2.2. Extraction of chitin.

Kaya *et al.* (2014) [26] provided instructions for chitin extraction. The extraction method used here comprised four chemical treatment steps, each followed by a distilled water rinse until a neutral pH was reached. In a 40 mL sodium hypochlorite (NaOCl) solution, the samples (10 g of powder sample) were refluxed at 100 °C for 10 minutes (3 percent, v: v). After washing with distilled water, the procedure was repeated. The samples were demineralized by refluxing them for 15 minutes in 20 mL of 1 M HCl at 75°C. To eliminate protein residues, the samples were refluxed for 20 minutes at 100°C in 20 mL of 1 M NaOH (sodium hydroxide) solution. Finally, the extracts were filtered and dried at 60° C until completely dried. The chitin sample's dry mass was determined, and the chitin content was then calculated. The mixture's pH was often neutralized at each stage to obtain a sample of ideal purity.

### 2.3. Extraction of chitosan.

The chitosan extraction was done according to Rasti *et al.* (2017) [27] technique. To extract chitosan, the deproteinized product was diluted in a 45 percent sodium hydroxide solution (15 ml/g) and heated at 110°C for 24 hours. The resulting product was soluble in 2% acetic acid, indicating that it had undergone a significant amount of deacetylation. After that, the resulting product was rinsed with distilled water until the pH was neutral then filtered to remove the solid matter, which was the final product.

### 2.4. Characterization of chitosan.

#### 2.4.1. Fourier transform infrared spectroscopy (FTIR).

A Perkins-Elmer spectrometer (Spectrum RX I, MA, USA) was used to conduct a Fourier Transform Infrared Spectroscopy examination of potassium bromide-supported sample chitosan over the frequency range 4000-400  $\text{cm}^{-1}$  with a resolution of 4  $\text{cm}^{-1}$ . The following equation calculates the degree of acetylation (DA).

$$\text{DA (\%)} = (\text{A}_{1656}/\text{A}_{3468}) * 115 \quad (1)$$

Where  $\text{A}_{1656}$  represents the degree of absorption at 1656  $\text{cm}^{-1}$ , and  $\text{A}_{3468}$  represents the degree of absorption at 3468  $\text{cm}^{-1}$ .

#### 2.4.2. X-ray powder diffractometry (XRD).

X-ray diffraction analysis was used to determine the crystallinity of the isolated components (XRD). The 'X' Pert PRO PAN analytical (Netherlands) equipment was used at 40 kV and 30 mA with  $\text{Cu k} = 1.5406\text{\AA}$ .

#### 2.4.3. Thermogravimetric analysis (TGA).

The Mettler Toledo TGA 2 was used to determine the change in mass of the chitosan sample. Temperatures ranging from 0 to 600°C were used to assess the 'material's thermal stability.

#### 2.4.4. Scanning electron microscopy (SEM).

For analysis, the sample was wrapped in carbon tape and spun-coated with gold. The TESCAN Oxford was used to make the Scanning Electron Micrographs, which were collected at various magnifications.

## 3. Results and Discussion

### 3.1. Extraction of chitin and chitosan.

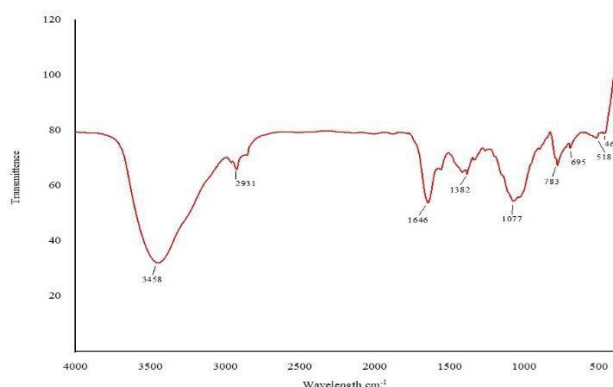
With an average weight of 50 g, chitin and chitosan were isolated from *Sepiella inermis*, and 100 g of pulverized powder, 17.50% and 6.22 percent of chitin and chitosan were recovered, respectively. In this study, chitosan derived from *Sepiella inermis* was rich in minerals and calcium carbonate ( $\text{CaCO}_3$ ). The yield of chitosan is 6.22 percent in this case. Previous research found that the production of chitosan from *Lavocardium attenuatum* and *Mytilus edulis* was 43.8 and 51.8 percent, respectively, exceeding that of *S. inermis* [28,29]. In a study of the cuttlebone of *S. officinalis*, which contained 20% chitin [30]. Tolaimate *et al.*

(2000) [31] utilized 40% sodium hydroxide to deacetylate the fungal chitin, yielding 23.7, 21.8, and 24 percent fungal chitosan, respectively. Prior research found 45.01 percent chitosan production from *Pinna deltoidea* shells [32]. Chitosan production is greater in the current study than in *N. crepidularia* (35.43 percent), *Metapeneus affinis* (19.13 percent), and *Penaeus monodon* (16.75 percent) [33,34].

### 3.2. Characterization of chitosan.

#### 3.2.1. Fourier transform infrared spectroscopy (FTIR).

Fourier transform infrared spectroscopy of chitosan revealed significant bands ranging from 700 to 3000  $\text{cm}^{-1}$ . Chitosan absorbance bands were found in the samples at 464, 518, 695, 783, 1077, 1382, 1646, 2931, and 3458  $\text{cm}^{-1}$ . Secondary stretching of C-O was detected at 1646  $\text{cm}^{-1}$ , whereas amide I and aliphatic CH stretching were recorded at 1382 and 2931  $\text{cm}^{-1}$ , respectively. The presence of an asymmetrical C-O-C with saccharide rings was shown by the peak at 1077  $\text{cm}^{-1}$ . The spectral analysis of *S. inermis* bone chitosan revealed a strong bond at 3458  $\text{cm}^{-1}$ , which corresponds to the angular deformation of OH present in the structure of chitosan hydroxyl asymmetrical stretching vibration and -NH<sub>2</sub> stretching vibration. Peak 2931  $\text{cm}^{-1}$  was ascribed to C=O stretching and amide I. The peak at 1646  $\text{cm}^{-1}$  was assigned to CO-stretching of amide I, confirming chitosan deacetylation. The FTIR spectrum was used to determine the degree of deacetylation of chitosan isolated from *Sepiella inermis*. The isolated chitosan was found to have 59.76 percent deacetylation (Fig. 2).



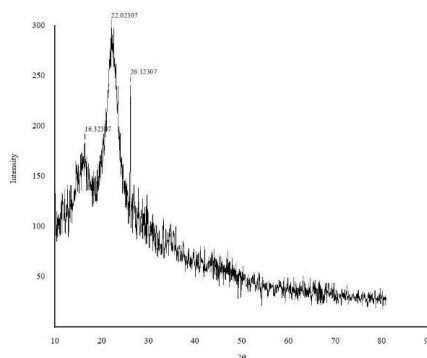
**Figure 2.** FTIR spectra of chitosan extracted from *S. inermis*.

Fourier Transform Infrared (FT-IR) spectroscopy revealed that the peaks at 1653  $\text{cm}^{-1}$  suggested the possibility of C-O secondary stretching in chitosan [35]. Stretching was detected at 2931  $\text{cm}^{-1}$  in the previous work, indicating CH bending in chitosan. Similar peaks in the cuttlefish (*Sepia officinalis*), shrimp (*Penaeus kerathurus*), and crab (*Carcinus mediterraneus*) were also reported [15]. In the current investigation, the FTIR spectra of chitosan isolated from *S. inermis* revealed a characteristic- C-O-C glycosidic linkage of 1066  $\text{cm}^{-1}$  and an OH-strong bond. At 3449  $\text{cm}^{-1}$ , the presence of NH<sub>2</sub> stretching vibration is equivalent to that of *Brachytrupes portentosus* was observed [36]. Characteristic peaks for chitosan at 3400  $\text{cm}^{-1}$  have been found to be related to stretching vibration and intermolecular hydrogen bonding of NH<sub>2</sub> and OH groups [37]. Fourier transform infrared spectroscopy analysis revealed a characteristic -CO stretching at 2931  $\text{cm}^{-1}$  [38]. The current 'study's findings were comparable to those found in *Sardina pilchardus* scales [39].

The DDA of chitosan recovered from the bone of *S. inermis* was determined using FT-IR measurements and found to be 54.91 percent. While *S. kobienis* has a greater degree of deacetylation (85.55 percent) [25], *Sepia prashadi* has a similar degree of deacetylation (55.95 percent) [40]. Research using sardine scales, *Sardina pilchardus*, found that the DDA of chitosan was 86.81 percent [41]. Tsung *et al.* (2009) [42] discovered that increasing deacetylation at 110°C for 2 hours dramatically boosted DDA (78 percent). However, when an 8-hour deacetylation period was applied, it climbed by up to 87 percent. A similar result was obtained when chitin was deacetylated at 130°C, with the maximum DDA (90 percent) obtained when deacetylation was carried out for 8 hours at 130°C. The results revealed that increasing the deacetylation temperature and duration enhanced the degree of deacetylation. The DDA was also affected by the alkaline concentration and solid-solvent ratio.

### 3.2.2. X-ray powder diffractometry (XRD).

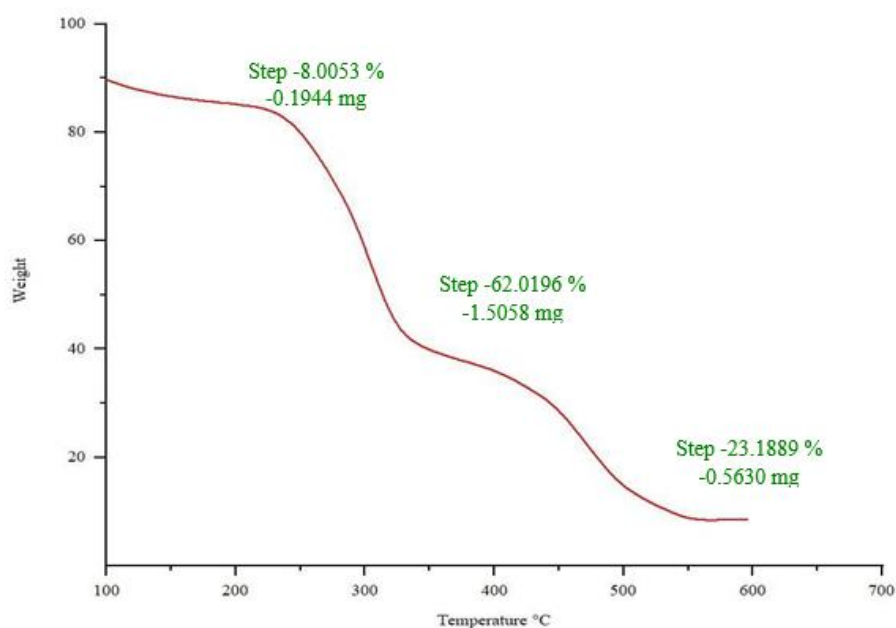
The material is blasted with X-rays during an XRD analysis, and the diffraction pattern created is recorded. In the case of chitosan, three significant diffraction peaks were found at 16.3°, 22.0°, and 26.1° crystal planes. Significant levels of chitosan were found in the range of  $2\theta$  to roughly 20° -27° (1002.2count/s). The sharper chitosan peaks of 22.0° in the current experiment demonstrate the hefty crystalline structure (Fig. 3). The largest peaks of chitosan discovered by X-ray powder diffractometry (XRD) were observed in the range of  $2\theta$  to around 22.0°, and respectively from commercial chitosan. Previous research in *S. kobiensis* has revealed two large chitosan peaks at  $2\theta= 10.2^\circ$  and  $20.4^\circ$ , which correspond to the XRD peaks in the current study [26]. Ibitoye *et al.* (2018) [36] published an XRD investigation of *Brachytrupes protentosus* (house cricket) chitosan that revealed three distinct chitosan peaks at 9.6°, 19.6°, and 22.3° with three clear, crystalline reflections. In the wide-angle X-ray diffraction patterns, shrimp chitosan displayed two large distinctive peaks at  $2\theta= 9.9^\circ - 10.7^\circ$  and  $19.8^\circ - 20.7^\circ$ , comparable to the current study [43].



**Figure 3.** XRD spectra of chitosan extracted from *S. inermis*.

### 3.2.3. Thermogravimetric analysis (TGA).

Figure 4 depicts the thermal activity of chitosan isolated from *S. inermis*. The thermogravimetric analysis of *S. inermis* reveals three phases of mass loss. At 250°C, the primary mass loss stage is seen, with a mass loss of approximately 8.0053 percent. At 340°C, the second stage of weight deficit was identified, with a percentage of mass loss of 62.0196 percent. At 450°C, the third segment of mass loss was detected with a mass loss percentage of 23.1889 percent. The initial step of mass loss is caused by the evaporation of liquid content, whereas the second and final stages are caused by polysaccharide breakdown.

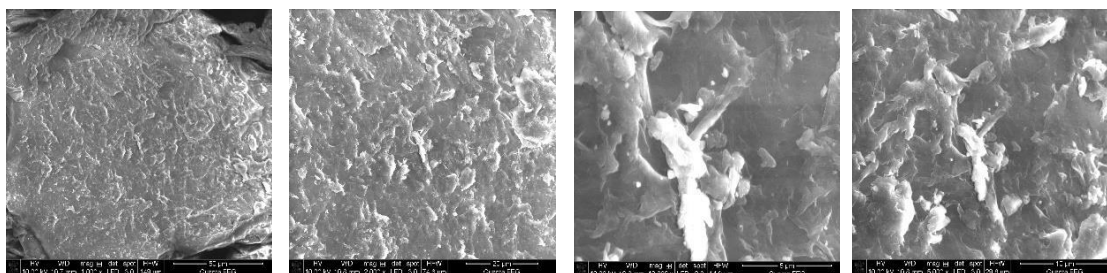


**Figure 4.** TGA of Chitosan extracted from *S.inermis*.

The thermogravimetric analysis (TGA) of *S. inermis* chitosan revealed a significant stage of mass loss at 250°C, consistent with comparable research of crab chitosan with varying degrees of DDA, such as 83.3–93.3 percent, which revealed an endothermic melting peak in the region of 152.3 – 159.2°C [44]. The TGA curve of pure chitosan revealed two phases of weight loss with temperatures ranging from 47 to 450°C, with the first stage occurring in the range of 47-100°C due to the loss of water molecules with a weight loss of roughly 9% [45]. The initial degradation of pure chitosan began at 247°C and was completed at around 450°C with a weight loss of around 34% [46]. In the current investigation, the first step occurred at 250°C and resulted in mass loss owing to water molecule loss. The second phase with mass loss at 340°C owing to polysaccharide and bond breakage. At 450°C, the chitosan was discovered to have completely dissolved. The data show that chitosan has lost its heat stability. The thermal degradation trend in the current study resembles that of prior studies [47,48].

### 3.2.4. Scanning electron microscopy (SEM).

To better comprehend the geometry of the chitosan sample, it was examined using a scanning electron microscope (SEM). Figure 5 shows electron micrographs of distinct sections of chitosan isolated from *S. inermis* at various magnifications.



**Figure 5.** SEM micrographs of chitosan extracted from *S. inermis*.

Chitosan has a smooth, porosity-free texture, as revealed by SEM, which is consistent with prior findings [49,50]. A non-porous, smooth, membranous phase with dome-shaped orifices, microfibrils, and crystallites may be seen in SEM images of pure chitosan and

chitosan-chromone derivatives [45]. Smooth, rough, flaky, porous, and fibrous chitosan surface morphology was isolated from house cricket by Ibitoye *et al.* (2018) [36]. Chitosan isolated from the saltwater clam *Macra veneriformis* was shown to have a smooth texture in previous work [7].

#### 4. Conclusions

For the isolation of chitosan from the wastes of marine food processing industries, a viable and cost-effective approach has been established. This research aimed to convert seafood waste material into a biomaterial. Technological advancements are driving demand for alternative materials in numerous fields of biotechnology and industry, promoting the increased usage of biopolymers and chitosan as the most abundant and renewable polysaccharides, gaining more attention from researchers. The process of demineralization, deproteinization, and deacetylation determines the maximal production of chitosan. The chitosan DDA in this study was 54.91 percent, which had a wide range of industrial uses. Chitosan has gained popularity as a means of accelerating tissue engineering research. The pharmacological characteristics of chitosan generated from marine waste products will be the focus of our future research.

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#### Conflicts of Interest

The authors declare no conflict of interest.

#### References

1. Green, D.W.; Lee, J.-M.; Jung, H.-S. Marine Structural Biomaterials in Medical Biomimicry. *Tissue Engineering Part B: Reviews* **2015**, *21*, 438-450, <https://doi.org/10.1089/ten.TEB.2015.0055>.
2. Khrunyk, Y.; Lach, S.; Petrenko, I.; Ehrlich, H. Progress in Modern Marine Biomaterials Research. *Marine Drugs* **2020**, *18*, <https://doi.org/10.3390/md18120589>.
3. Krishnan, S.; Chakraborty, K.; Dhara, S. Biomedical potential of  $\beta$ -chitosan from cuttlebone of cephalopods. *Carbohydrate Polymers* **2021**, *273*, <https://doi.org/10.1016/j.carbpol.2021.118591>.
4. Abhinaya, M.; Parthiban, R.; Kumar, P.S.; Vo, D.-V.N. A review on cleaner strategies for extraction of chitosan and its application in toxic pollutant removal. *Environmental Research* **2021**, *196*, <https://doi.org/10.1016/j.envres.2021.110996>.
5. De Simone, N.; Pace, B.; Grieco, F.; Chimienti, M.; Tyibilika, V.; Santoro, V.; Capozzi, V.; Colelli, G.; Spano, G.; Russo, P. Botrytis cinerea and Table Grapes: A Review of the Main Physical, Chemical, and Bio-Based Control Treatments in Post-Harvest. *Foods* **2020**, *9*, <https://doi.org/10.3390/foods9091138>.
6. Joseph, B.; Mavelil Sam, R.; Balakrishnan, P.; J. Maria, H.; Gopi, S.; Volova, T.; C. M. Fernandes, S.; Thomas, S. Extraction of Nanochitin from Marine Resources and Fabrication of Polymer Nanocomposites: Recent Advances. *Polymers* **2020**, *12*, <https://doi.org/10.3390/polym12081664>.
7. Pakizeh, M.; Moradi, A.; Ghassemi, T. Chemical extraction and modification of chitin and chitosan from shrimp shells. *European Polymer Journal* **2021**, *159*, <https://doi.org/10.1016/j.eurpolymj.2021.110709>.
8. Veetil, U.T.; Olza, S.; Brugerolle de Fraissinette, N.; Bascans, E.; Castejón, N.; Adrien, A.; Fernández-Marín, R.; Nardin, C.; Fernandes, S.C.M. Contributions of Women in Recent Research on Biopolymer Science. *Polymers* **2022**, *14*, <https://doi.org/10.3390/polym14071420>.

9. El Knidri, H.; Belaabed, R.; Addaou, A.; Laajeb, A.; Lahsini, A. Extraction, chemical modification and characterization of chitin and chitosan. *International Journal of Biological Macromolecules* **2018**, *120*, 1181-1189, <https://doi.org/10.1016/j.ijbiomac.2018.08.139>.
10. Cuong, H.N.; Minh, N.C.; Van Hoa, N.; Trung, T.S. Preparation and characterization of high purity  $\beta$ -chitin from squid pens (*Loligo chenis*). *International Journal of Biological Macromolecules* **2016**, *93*, 442-447, <https://doi.org/10.1016/j.ijbiomac.2016.08.085>.
11. Román-Hidalgo, C.; Aranda-Merino, N.; López-Pérez, G.; Sánchez-Coronilla, A.; Villar-Navarro, M.; Martín-Valero, M.J. Chitosan biofilms: Insights for the selective electromembrane extraction of fluoroquinolones from biological samples. *Analytica Chimica Acta* **2021**, *1179*, <https://doi.org/10.1016/j.aca.2021.338832>.
12. Lizardi-Mendoza, J.; Argüelles Monal, W.M.; Goycoolea Valencia, F.M. Chapter 1 - Chemical Characteristics and Functional Properties of Chitosan. In: *Chitosan in the Preservation of Agricultural Commodities*. Bautista-Baños, S.; Romanazzi, G.; Jiménez-Aparicio, A. Eds.; Academic Press: San Diego, **2016**; pp. 3-31, <https://doi.org/10.1016/B978-0-12-802735-6.00001-X>.
13. Bedian, L.; Angel, M.; Rodriguez, V.; Gustavo, H.V.; Saldivar, R.P.; Iqbal, H.M.N. Bio-based Materials with Novel Characteristics for Tissue Engineering Applications. *Int. J. Biol. Macromol.* **2017**, *98*, 837- 846, <https://doi.org/10.1016/j.ijbiomac.2017.02.048>.
14. Hajji, S.; Younes, I.; Rinaudo, M.; Jellouli, K.; Nasri, M. Characterization and In Vitro Evaluation of Cytotoxicity, Antimicrobial and Antioxidant Activities of Chitosans Extracted from Three Different Marine Sources. *Applied Biochemistry and Biotechnology* **2015**, *177*, 18-35, <https://doi.org/10.1007/s12010-015-1724-x>.
15. Varma, R.; Vasudevan, S. Extraction, Characterization, and Antimicrobial Activity of Chitosan from Horse Mussel *Modiolus modiolus*. *ACS Omega* **2020**, *5*, 20224-20230, <https://doi.org/10.1021/acsomega.0c01903>.
16. Central Marine Fisheries Research Institute Cochin, India. Marine Fisheries Information Service. Indian Council of Agriculture Research, **1998**, No. 153.
17. Visweswaran, B. Is *Sepiella inermis* 'Spineless'? *J. Pharm. Biol. Sci.* **2017**, *12*, 51-60.
18. Reid, A.L.; Chung, C.L. A new cuttlefish, *Sepia filibrachian* from the South China Sea with a redescription of *Sepia mestus* Gray, 1849 (Cephalopoda: Sepiidae) from eastern Australia. *Zootaxa* **2005**, *911*, 1-22.
19. Ramasamy, P.; Sekar, S.; Paramasivam, S.; Suri, P.; Chinnaiyan, U.; Singh, R.; Tanguturi Raghavaiah, B.P.; Seshadri, V.D. Sulfation of chitosan from *Sepia kobsiensis* as potential anticoagulant and antibacterial molecule. *Natural Product Research* **2022**, *36*, 3216-3222, <https://doi.org/10.1080/14786419.2021.1956492>.
20. Nunes, B.D.; Rosendo, R.A.; Filho, A.A.; Fook, M.V.; de Sousa, W.J.; Barbosa, R.C.; Pina, H.D.; da Silva Neto, J.E.; Amoah, S.K.; Fontana, C.E.; Bueno, C.E.; De Martin, A.S. Chitosan-Based Biomaterial, Calcium Hydroxide and Chlorhexidine for Potential Use as Intracanal Medication. *Materials* **2021**, *14*, 1-13, <https://doi.org/10.3390/ma14030488>.
21. Hobel, C.F.V. Access to biodiversity and new genes from thermophiles by special enrichment methods. Ph. D thesis, University of Iceland, **2004**.
22. Kurita, K. Chitin and chitosan: functional biopolymers from marine crustaceans. *Mar. Biotechnol.* **2006**, *8*, 203-26, <https://doi.org/10.1007/s10126-005-0097-5>.
23. Crini, G.; Badot, P.M.; Guibal, E. Chitine et Chitosane. Du Biopolyme`re a` l`Application. Paris : Presses Universitaires de Franche-Comté, **2009**.
24. Molina-Ramírez, C.; Mazo, P.; Zuluaga, R.; Gañán, P.; Álvarez-Caballero, J. Characterization of Chitosan Extracted from Fish Scales of the Colombian Endemic Species *Prochilodus magdalenae* as a Novel Source for Antibacterial Starch-Based Films. *Polymers* **2021**, *13*, <https://doi.org/10.3390/polym13132079>.
25. Ramasamy, P.; Subhapradha, N.; Shanmugam, V.; Shanmugam, A. Extraction, characterization and antioxidant property of chitosan from cuttlebone *Sepia kobsiensis* (Hoyle 1885). *International Journal of Biological Macromolecules* **2014**, *64*, 202-212, <https://doi.org/10.1016/j.ijbiomac.2013.12.008>.
26. Kaya, M.; Baran, T.; Mentés, A.; Asaroglu, M.; Sezen, G.; Tozak, K.O. Extraction and Characterization of  $\alpha$ -Chitin and Chitosan from Six Different Aquatic Invertebrates. *Food Biophysics* **2014**, *9*, 145-157, <https://doi.org/10.1007/s11483-013-9327-y>.
27. Rasti, H.; Parivar, K.; Baharara, J.; Iranshahi, M.; Namvar, F. Chitin from the Mollusc Chiton: Extraction, Characterization and Chitosan Preparation. **2017**, *16*, 366-379, <https://doi.org/10.22037/ijpr.2017.1963>.
28. Majekodunmi, S.; Olorunsola, E.; Ofiwe, C.; Udobre, A.; Akpan, E. Material properties of chitosan from shells of *Egeria radiata*: Drug delivery considerations. *Journal of Coastal Life Medicine* **2017**, *5*, 321-324, <https://doi.org/10.5923/j.ajps.20170701.03>.
29. Hanafy, N.A.N.; Leporatti, S.; El-Kemary, M.A. Extraction of chlorophyll and carotenoids loaded into chitosan as potential targeted therapy and bio imaging agents for breast carcinoma. *International Journal of Biological Macromolecules* **2021**, *182*, 1150-1160, <https://doi.org/10.1016/j.ijbiomac.2021.03.189>.
30. Arrouze, F.; Desbrieres, J.; Lidrissi, H.S.; Tolaimate, A. Investigation of  $\beta$ -Chitin Extracted from Cuttlefish: Comparison with Squid  $\beta$ -Chitin. *Polym. Bull.* **2021**, *78*, 7219-7239, <https://doi.org/10.1007/s00289-020-03466-z>.

31. Tolaimate, A.; Desbrières, J.; Rhazi, M.; Alagui, A.; Vincendon, M.; Vottero, P. On the influence of deacetylation process on the physicochemical characteristics of chitosan from squid chitin. *Polymer* **2000**, *41*, 2463-2469, [https://doi.org/10.1016/S0032-3861\(99\)00400-0](https://doi.org/10.1016/S0032-3861(99)00400-0).
32. Rahul, V.; Sugumar, V.; Stella, C.; Anandhan, N. Synthesis and Physicochemical Characteristics of Chitosan Extracted from *Pinna deltoidea*. *Lett. Appl. NanoBioSci.* **2021**, *11*, 4061–4070, <https://doi.org/10.33263/ianbs114.40614070>.
33. Palpandi, C.; Shanmugam, V.; Shanmugam, A. Extraction of chitin and chitosan from shell and operculum of mangrove gastropod *Nerita (Dostia) crepidularia Lamarck*. *Int. J. Med. Med. Sci.* **2009**, *1*, 198–205.
34. Sagheer, F.A.A.; Al-Sughayer, M.A.; Muslim, S.; Elsabee, M.Z. Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf. *Carbohydr. Polym.* **2009**, *77*, 410–419, <https://doi.org/10.1016/j.carbpol.2009.01.032>.
35. De Queiroz Antonino, R.S.; Lia Fook, B.R.; De Oliveira Lima, V.A.; De Farias Rached, R.Í.; Lima, E.P.; Da Silva Lima, R.J.; Peniche Covas, C.A.; Lia Fook, M.V. Preparation and Characterization of Chitosan Obtained from Shells of Shrimp (*Litopenaeus vannamei* Boone). *Marine Drugs* **2017**, *15*, <https://doi.org/10.3390/md15050141>.
36. Ibitoye, E.B.; Lokman, I.H.; Hezmee, M.N.M.; Goh, Y.M.; Zuki, A.B.Z.; Jimoh, A.A. Extraction and physicochemical characterization of chitin and chitosan isolated from house cricket. *Biomedical Materials* **2018**, *13*, <https://doi.org/10.1088/1748-605X/aa9dde>.
37. Dahmane, E.M.; Taourirte, M.; Eladlani, N.; Rhazi, M. Extraction and Characterization of Chitin and Chitosan from *Parapenaeus longirostris* from Moroccan Local Sources. *International Journal of Polymer Analysis and Characterization* **2014**, *19*, 342-351, <https://doi.org/10.1080/1023666X.2014.902577>.
38. Rasweefali, M.K.; Sabu, S.; Sunooj, K.V.; Sasidharan, A.; Xavier, K.A.M. Consequences of chemical deacetylation on physicochemical, structural and functional characteristics of chitosan extracted from deep-sea mud shrimp. *Carbohydrate Polymer Technologies and Applications* **2021**, *2*, <https://doi.org/10.1016/j.carpta.2020.100032>.
39. Aboudamia, F.Z.; Kharroubi, M.; Mounsef, N.; Fadna, A.; Hanoune, S.; Bouchdoug, M.; Jaouad, A. Potential of discarded sardine scales (*Sardina pilchardus*) as chitosan sources. *J. Air Waste Manag. Assoc.* **2020**, *70*, 1186-1197, <https://doi.org/10.1080/10962247.2020.1813840>.
40. Jothi, N.; Kunthavai R.N. Identification and Isolation of Chitin and Chitosan from Cuttle Bone of *Sepia prashadi*. *Glob. J. Biotechnol. Biochem.* **2013**, *8*, 33-39.
41. Sathiyabama, M.; Akila, G. Water Soluble Chitosan Extraction from Mycelium of *Alternaria solani* and its Field Evaluation on Tomato Plants. *Carbohydr. Polym. Technol. App.* **2021**, *2*, <https://doi.org/10.1016/j.carpta.2021.100101>.
42. Ming-Tsung, Y.; Joan-Hwa, Y.; Jeng-Leun, M. Physicochemical characterization of chitin and chitosan from crab shells. *Carbohydr. Polym.* **2009**, *75*, 15–21, <https://doi.org/10.1016/j.carbpol.2008.06.006>.
43. Santosh, K.; Joonseok, K. Physicochemical, Optical and Biological Activity of Chitosan-Chromone Derivative for Biomedical Applications. *Int. J. Mol. Sci.* **2012**, *13*, 6102-6116, <https://doi.org/10.3390/ijms13056102>.
44. Kumar, S.; Dutta, P.K.; Koh, J. A physicochemical and biological study of novel chitosan- chloroquinoline derivative for biomedical applications. *Int. J. Biol. Macromol.* **2011**, *49*, 356–361, <https://doi.org/10.1016/j.ijbiomac.2011.05.017>.
45. Xin, Z.; Qingyin, D.; Xi, H.; Zhiyong, Q. Preparation and characterizations of antibacterial–antioxidant film from soy protein isolate incorporated with mangosteen peel extract. *De Gruyter* **2021**, *21*, 575-589, <https://doi.org/10.1515/epoly-2021-0058>.
46. Burgos-Díaz, C.; Opazo-Navarrete, M.; Palacios, J.L.; Barahona, T.; Mosi-Roa, Y.; Anguita-Barrales, F.; Bustamante, M. Synthesis of New Chitosan from an Endemic Chilean Crayfish Exoskeleton (*Parastacus Pugnax*): Physicochemical and Biological Properties. *Polymers* **2021**, *13*, <https://doi.org/10.3390/polym13142304>.
47. Riaz, A.; Lagnika, C.; Abdin, M.; Hashim, M.M.; Ahmed, W. Preparation and Characterization of Chitosan/Gelatin-Based Active Food Packaging Films Containing Apple Peel Nanoparticles. *Journal of Polymers and the Environment* **2020**, *28*, 411-420, <https://doi.org/10.1007/s10924-019-01619-4>.
48. Ling, C.W.; Liu, Q.D.; Rui, L.; Hao, W. Characterizations and microsphere formulation of polysaccharide from the marine clam (*Macra veneriformis*). *Carbohydr. Polym.* **2013**, *92*, 106–113, <https://doi.org/10.1016/j.carbpol.2012.08.084>.
49. Hossain, M.S.; Iqbal, A. Production and characterization of chitosan from shrimp waste. *J. Bangladesh Agric. Univ.* **2014**, *12*, 153–160, <http://dx.doi.org/10.3329/jbau.v12i1.21405>.
50. Rashki, S.; Asgarpour, K.; Tarrahimofrad, H.; Hashemipour, M.; Ebrahimi, M.S.; Fathizadeh, H.; Khorshidi, A.; Khan, H.; Marzhoseyni, Z.; Salavati-Niasari, M.; Mirzaei, H. Chitosan-based nanoparticles against bacterial infections. *Carbohydrate Polymers* **2021**, *251*, <https://doi.org/10.1016/j.carbpol.2020.117108>.