Development and optimization of the demineralization process for the extraction of chitin from Omani *Portunidae segnis*

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Abstract

**Background:** Chitin is an organic polymer and it is rich marine natural polysaccharide after the cellulose. The main natural sources of chitin are exoskeletons of insects, mollusks, the cell walls of certain fungi and crustaceans such as crabs, shrimps and lobsters. The waste of these marine exoskeletons are pollutant for the environment continuously, but this raw material could be used for the production of commercial chitin. The chitin is an important raw material used for water treatment, agricultural, biomedical, biotechnological purposes, food and paper industry and cosmetics. Based on the variety of importance, the present targets of this study is to i. optimize the demineralization process for the removal of calcium and phosphorus from the waste of Portunidae segni (P. segni) by using acid at ambient temperature ii. characterize the isolated demineralized sample as well as the percentage of remaining calcium and phosphorus by using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

**Results:** The coarse powder samples of P. segni were demineralized with seven different concentrations of hydrochloric acid at ambient temperature for 1 hour. All the demineralization samples at different concentrations were analysed by using sensitive ICP-OES. The results based on ICP-OES showed that among the seven different concentrations wused in the demineralization process for the isolation of chitin, the best was 2M of HCl concentration for the production of chitin.

**Conclusion:** The develop optimized demineralization process could be used commercially for the isolation of chitin for the preparation of agriculture, biomedicine, biotechnological purposes.

1. **Introduction**
Since ancient time, the scientists/researchers have been trying to minimize the amount of marine waste material and turn it into commercial sustainable biologically and agricultural products. Chitin is one of the abundant polysaccharide polymers. It is a well-known polysaccharide in nature which is normally transfer to cellulose. As a structural component of animals, chitin is used mainly for exoskeletons of animals like insects and crustaceans such as shrimps, crabs, and lobsters. Previous reports showed that more than 1011 tons of chitin is produced from sea products worldwide. Marine waste is pollutant for the global environment and nowadays it is a burden for the communities.
Marine waste contains several valuable chemicals like chitin, chitosan etc and they are commercially important due to their use in various sectors. However, marine waste could be used as raw material for the production of chitin and chitosan commercially. Therefore, the purpose of this study is to use marine waste for the production/isolation of biologically active products such as chitin and chitosan biopolymeric compounds and their derivatives. Recently, the policy has been developed by the Government of the Sultanate of Oman is to save the environment and nations to protect or use all kinds of marine waste. The main sources of marine waste are crabs, shrimps, squilla and fish waste.

Marine waste contains several biologically active compounds with significant commercial values. Most of the marine waste such as crab, shrimp, squilla and fish waste contains approximately 25–30% chitin, 25% protein, 40–50% calcium carbonate (Marguerite, 2007). Chitin is commercially used in the medical and agriculture sectors. Marine waste contains a significant percentage of chitin. There are three forms of chitin such as α, β, γ-chitin available in the nature and as solid crystalline. Among the three forms, α-chitin is the most commonly found in nature. It can be isolated commercially from the marine waste like shrimp and crab shells (Panariello et al., 2019). It is white colour, hard, inelastic, nitrogenous polysaccharide polymer (Fig. 1). The monosaccharide of chitin is N-acetyl-D-glucosamine (NAG, or GlcNAc) linked with β1α-4 linkages (Mansour et al., 2012; Puvada et al., 2012). It has an orthorhombic crystal and the chains are antiparallel (Panariello et al., 2019). Due to its structural characteristics, the chitin is strong and has other favorable properties. Therefore, it can be used in the industry for making a suitable alternative for plastics. The chitin and its derivative polysaccharides are widely used for medical applications due to their biocompatibility and antimicrobial potency (Brasselet et al., 2019). It is fixed with a protein matrix of a crustacean shell. Recently, commercial chitin is commonly produced by the marine industry from crab and shrimp shells which are cast-off as massive wastes. The physiochemical and toxicological properties of chitin includes solubility, solution, viscosity, polyelectrolyte behavior, polyoxy salt formation, ability to form films, metal chelation, optical, and structural characteristics (Mottari et al., 2018; Achilonu et al., 2018). It has plenty of applications in biomedical, agricultural, biotechnological, wastewater treatment, food, paper and cosmetics industries (Arabia, 2013). However, the major drawback of using chitin in the clinical field is
the insolubility of chitin in most of the organic solvents (Ahyat et al., 2017). Previous reports showed that the extraction of chitin and chitosan with significant amount from *P. pelagicus. P. segnis* is a most common species available in Oman. It occurs in sandy and sandy-muddy areas including mangroves, sea grass and algal beds. The literature showed that there is not enough research available on the phytochemicals and pharmacological study of *P. segnis*. The authors used plenty of methods for the extraction of chitin from marine wastes but these methods were not standardized.

All the extraction methods for chitin are almost similar, only they use different ratio of acid and base, time and the temperature (Cardenas et al., 2004; Pandharipande et al., 2016). Some of them described the extraction of chitin by chemical methods and it was synthesized by chemical method for large scale production (Cardenas et al., 2004). In Oman, marine waste is a major source of environmental pollution in the coastal areas (Marguerite, 2007). Statistical data showed that about 6 to 8 million tons of crab, shrimp and lobster shell waste is produced globally per year. According to the fish production report in Oman, about 90,000 tons of marine waste is produced per year (Qumedia 2016; Oman Observer 2019). These waste shell and other parts are dumped in open places in the coastal areas polluting the environment. Therefore, huge amount of these marine wastes can be used as a raw material for different industries to produce different valuable life-saving, and agricultural drugs. In addition, other components from the marine waste like protein, calcium, potassium, calcium carbonate could also be isolated. Throughout the decades, many methods have been developed for the production of chitin. However, the chemical method is the most effective method found in the literature for the production of industrial chitin. The literature research showed that there has not been such research conducted in Oman. Therefore, the present targets of this study are to i. optimize the demineralization process for the removal of calcium and phosphorus from the waste of *P. segnis* by using acidic medium at ambient temperature ii. characterize the isolated demineralized samples as well as the percentage of remaining calcium and phosphorus minerals by using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES).

2. Materials And Methods
2.1. Chemicals and reagents
Hydrochloric acid used in this experiment were collected from BDH, UK. The other chemicals and reagents used during the process and treatment were analytical grade. The commercial chitin (Purity 98%) was obtained from Sigma-Aldrich Company Limited, Germany.

2.2. Sample collection and process

*P. segnis* crabs include exoskeletons of the crabs waste were collected from the Bahla fish market. The samples were collected from Bahla on September 12, 2018 at 11 am. Soon after collection the samples were carried out to the Chemistry Laboratory and kept in the freezer until the necessary extraction process. Then, the collected samples were washed with tap water and dried in an oven overnight at 45 °C. The dried crab waste samples were ground into coarse powder size 0.30–0.35 mm by using a blender machine. The ground coarse powder was kept in a bottle for the production of chitin.

2.3. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

The sensitive ICP-OES (Optical Emission Spectroscopy, Optima 8000, Perkin Elmer, USA) was used to analyze the demineralized samples. The demineralized samples obtained from the marine crab waste by using chemical treatment with different concentrations of HCl were analyzed by using ICP-OES to detect the concentration of calcium (Ca) and phosphor (P) after digestion of 0.8 g of each demineralized sample by ultra-microwave (Single Reaction Champer Microwave Digestion System, Ultrawave, Mileston, USA).

2.4. Demineralization of crab sample waste

Chemical treatment methods were used for the extraction of chitin from different marine waste samples that were described by several authors (Gadgey et al., 2017; Yadav et al., 2019; Zvezdova, 2010). In the demineralization process, the chemical procedure was used at different concentrations of HCl at ambient temperature incubation for 1 hour.

2.5. Demineralization process

The crab coarse powder samples each (5 gm) were treated with seven various concentrations such as 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, and 2.25 M of hydrochloric acid. The powder samples (5 gm) were put in a beaker and various concentration of HCl were added separately. The samples and acid ratio was 1:15. The samples were kept at ambient temperature for 1 hour incubation with constant stirring.
to remove the phosphate and carbonate contents from the crab shell powder (Gadgey et al., 2017). There were three samples for each concentration. After demineralization, the products were pale pink color and the average masses of each concentration process were measured. The demineralized product of each sample was digested in ultra-wave digestion system at 230 °C at 120 atmospheric pressure for 15 minutes than the samples were analyzed by using ICP-OES. The concentration was measured as parts per million (ppm) from 0.8 g after digestion and dilution in 50 ml of distilled water for each treatment (Fig. 2).

3. Results
The huge amount of P. segnis crabs include exoskeletons of the crab waste were available in Oman. The sample was collected from the fish market in Bahla. The collected crab waste samples were washed with water and dried in an oven at 45°C. The dried samples were ground into coarse powder.

3.1. Demineralization process
The carb samples were demineralized by using the chemical methods which was previously described by several authors (Gadgey, & Dey, S., 2017; Yadav et al., 2019; Zvezdova, 2010). The crab shell coarse powder was demineralized by various concentrations of HCl at ambient temperature for 1 hour incubation. The results are presented in Table 1. The highest percentage of demineralized samples was obtained using 2M HCl and the lowest was when 0.75M HCl was used.

| Concentration of HCl (M) | 1       | 2       | 3       | Average Demineralized sample yield (gm) | Percentage of yield (%) | Percentage of demineralization (%) | Color     |
|-------------------------|---------|---------|---------|----------------------------------------|-------------------------|-----------------------------------|-----------|
| 0.75                    | 1.648   | 1.614   | 1.571   | 1.611                                  | 32.22                   | 67.78                             | Slightly pink |
| 1                       | 0.965   | 0.908   | 0.938   | 0.937                                  | 18.74                   | 81.26                             | Slightly pink |
| 1.25                    | 0.983   | 0.970   | 0.976   | 0.976                                  | 19.53                   | 80.47                             | Slightly pink |
| 1.50                    | 0.935   | 0.925   | 0.951   | 0.937                                  | 18.74                   | 81.26                             | Slightly pink |
| 1.75                    | 0.899   | 0.914   | 0.929   | 0.914                                  | 18.28                   | 81.72                             | Slightly pink |
| 2.00                    | 0.921   | 0.912   | 0.894   | 0.909                                  | 18.18                   | 81.82                             | Slightly pink |
| 2.25                    | 0.984   | 0.953   | 0.963   | 0.967                                  | 19.33                   | 80.67                             | Slightly pink |
| 2.50                    | 1.019   | 1.000   | 1.011   | 1.010                                  | 20.20                   | 79.80                             | Slightly pink |

3.2. Determination of calcium and phosphate in the demineralized samples
The calcium (Ca) and phosphorus (P) after the demineralization of crab samples by applied the seven concentration were determined by using the sensitive ICP-OES. The average concentration was calculated using the established equation described by several authors (Gadgey, & Dey, S., 2017; Yadav et al., 2019; Zvezdova, 2010). All the obtained results for calcium and phosphate after
demineralization process are presented in Table 2. Table 2 shows that demineralization with 2M HCl gave smaller concentration of calcium and phosphate compared to other concentrations applied in this experiments.

Table 2: The concentration of calcium and phosphate after the demineralization of carboxy samples by applied the seven concentrations

| Conc. HCl | 1  | 2  | 3  | Conc of Ca |
|-----------|----|----|----|------------|
| 0.75      | 84.24 | 0  | 90.01 | 0  | 78.47 | 0  | 84.24 |
| 1         | 42.36 | 95.54 | 39.16 | 43.28 | 41.61 | 68.76 | 41.04333 |
| 1.25      | 32.85 | 81.61 | 38.51 | 53.48 | 34.03 | 20.24 | 35.13 |
| 1.5       | 32.83 | 24.19 | 32.81 | 11.27 | 33.68 | 8.77 | 33.10667 |
| 1.75      | 26.86 | 5.998 | 25.51 | 20.55 | 23.86 | 11.24 | 25.41 |
| 2         | 23.89 | 6.458 | 24.98 | 17.53 | 25.16 | 10.87 | 24.67667 |
| 2.25      | 24.13 | 20.23 | 24.82 | 22.69 | 24.48 | 21.46 | 24.47667 |
| 2.5       | 25.92 | 17.76 | 25.33 | 35.75 | 29.12 | 6.771 | 26.79 |

4. Discussion

One of the major natural polymers is chitin that is detected first in mushrooms by Braconnot in 1811. According to the list of natural polymers, chitin is the second abundant polymer after cellulose. The annual production of chitin is approximately 1010 to 1011 tons (Gooday, 1994). Based on the structure, chitin is a derivative of cellulose only different at carbon-2 in the chemical structure. In chitin structure, carbon-2 contains a hydroxyl group while cellulose has an acetamido group (Rinaudo M, 2006). Chitin is widely abundant in invertebrates, plants and fungi. It is the main chemical compound in the exoskeletons of arthropods and insects. Nowadays more than 75% of the total weight of marine wastes like shrimp, crabs, prawns, lobster, and krill are used commercially for the production of chitin (Kuddus & Ahmad, 2013).

Since the Roman time, chitin is extracted traditionally from various marine wastes by chemical methods which include three simple process viz. (i) deproteinisation, (ii) demineralization and (iii) bleaching. The marine samples were demineralized by reactions in acidic medium such as HCl, HNO₃,
H$_2$SO$_4$, CH$_3$COOH and HCOOH at various temperatures within the range of 90-100 °C (Percot et al., 2003). The molecular formula of chitin is poly ($\beta$-(1$\rightarrow$4)-N-acetyl-D-glucosamine). The chitin as a polymer is isolated mainly from living organisms (Yadav et al., 2019). It is the second most widespread polimer aster cellulose. It is odorless and it is crystalline solid (Fig. 1). However, the main natural sources of chitin are marine sources especially from crab and shrimp shell wastes. The chitin and its derivatives have several medicinal, agricultural and industrial applications. In the medical sector, it is used widely as a wound dressing, monitoring bleedings, antitumor, etc (Yadav et al., 2019).

The previous several studies have been conducted by several authors on isolation or extraction of chitin from the available marine sources showed that most of the scientists are used the same methods (Mottari et al., 2018; Cardenas et al., 2004; Pandharipande et al., 2016; Gadgey et al., 2017; Zvezdova, 2010). However, in our present experiment, we used the same acidic method with slight modification. We have tried to optimize the demineralization process by using various concentrations of HCl at constant temperature. The main aim for demineralization process is to remove the minerals, predominantly calcium carbonate, calcium and phosphorus. Several acids such as HCl, HNO$_3$, H$_2$SO$_4$, CH$_3$COOH and HCOOH were used to demineralize the marine waste samples. Among the methods, most of the authors used diluted hydrochloric acid for demineralization, to remove various minerals from the crab sample. After the demineralization process, the mineral content in the samples are varied. These variation mainly depends on process time, temperature, particle size, acid concentration and samples/solvent ratio. Finally, the acid requires two molecules of HCl to convert calcium carbonate into calcium chloride for complete the demineralization process. The equal amount or even greater of acid needed to demineralize equal amount of minerals (Auerswald & Gäde, 2008; Raabe et al., 2007).

The chitin is extracted from the marine sources by hydrochloric acid treatment to dissolve the calcium carbonate. The calcium carbonate was decomposed and form calcium chloride along with carbon dioxide gas is involved in demineralization as shown:
CaCO₃(s) + 2HCl(aq) → CaCl₂(aq) + CO₂(g) + H₂O

In the present experiment, the crab coarse powder samples were treated with seven different concentrations of HCl for one hour and then the demineralized sample were analyzed ICP-OES (Fig. 2). In our target through this experiment is to optimize the concentration of HCl in the demineralization process of the crab samples for the production of chitin commercially. Therefore, to achieve this target, after the demineralization of crab samples, the percentage of calcium and phosphorus present in the demineralized samples is measured to reveal the quality of chitin. Therefore, the measurement of the percentage of calcium and phosphorus present in the demineralize samples was conducted by ICP-OES. Table 2 shows that among the applied seven different concentrations of HCl, the best results for demineralization were obtained by the use of 2M HCl. However, the percentage of yield of the demineralized samples was different which could be due to the strength of acid, time of incubation and particles size etc (Table 1). It showed that the concentration of calcium (Ca) and phosphorus (P) in the crab waste samples was different when different concentration of acid was applied for the demineralization process. Table 2, clearly shows that the concentration of Ca decreased when the concentration of HCl was increased from 1 to 2 M. However, if the concentration was increased to 2.25 M and above then the concentration of Ca also increased. Similarly, P concentration also decreased as the concentration of HCl was increased from 0.75 to 2 M. Again similar trend observed when the concentration of HCl was increased to 2.25M and above. Therefore, in conclusion, the optimized concentration of HCl is 2.0 M for the demineralization process of the crab samples Several studies conducted by several authors globally, however, the optimized acid concentrations for demineralization process are not similar (Suryawanshi, et al., 2019). Their optimized concentration of HCl for demineralization process is different from each other or even from our results. Even though, they used the same method for the demineralization process of marine samples. In addition, the same process done in other country for the quantification of minerals in the demineralized crab samples by using ICP-OES analysis (Yadav et al., 2019), but the concentration of Ca and P are not same as our results. Therefore, in our present experiment, the optimized concentration is 2M of HCl for the extraction of chitin from Omani *P. segnis*. The variation of results for demineralized samples, Ca and
P, it could be due process time, temperature, particle size, acid concentration and samples/solvent ratio.

5. Conclusion
The current target is to extract high percentage of chitin with low concentration of Ca and P from the Omani _P. segnis_ marine wastes by using different chemical methods which was collected from Bahla. Based on the observation of demineralization method, it is concluded that the products obtained from 2M concentration of HCl at room temperature with duration of 1 hour is the best optimized method for the extraction of chitin from Omani _P. segnis_. In addition, the percentage of yield chitin, and the ICP-OES analysis of the calcium and phosphore content, also concluded that the present optimized demineralization process could be used successfully for the extraction of best quality of chitin from Omani _P. segnis_ waste. Therefore, the optimized demineralization process could be used for the extraction of chitin from crab shells which will be used as raw material for industrial, medical and pharmaceutical industries. However, our future study will be carried out on the selection of best samples/solvent ratio and temperature during the demineralization process it will be also useful to demineralize the minerals from the crab samples for the use in medicine and other sectors.

Declarations

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

All experimental data used and analyzed in this present study are available from the corresponding author.

**Competing interests**

The authors declare that they do not have any competing financial interests or institutional and personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Noura Hamed Khalifa Al Shaqsi: Data curation, Investigation. Horiya Ali Said Al Hoqani: Data curation, Investigation. Mohammed A. Hossain: Review & Editing. Mohammed Abdullah Al Sibani: Conceptualization, Project administration, and Supervision.

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References

1. Achilonu M, Alabaraoye E, Hester R (2018) Biopolymer (Chitin) from various marine seashell wastes: isolation and characterization. J Poly Environ 26: 2207–2218.
2. Ahyat NM, Mohamad F, Ahmad A, Azmi AA (2017) Chitin and chitosan extraction from portunus pelagicus. Malays. J Anal Sci 21: 770–777.
3. Arbia W, Arbia L, Adour L, Amrane A (2013) Chitin extraction from crustacean shells using biological methods–a review. Food Tech Biotech 51(1): 12–25.
4. Auerswald L, Gäd, G (2008) Simultaneous extraction of chitin and astaxanthin from waste of lobsters Jasus lalandii, and use of astaxanthin as an aquacultural feed additive. Afr J Mar Sci 30:35–44.
5. Brasselet C., Pierre G., Dubessay P, Dols-Lafargue M, Coulon J, Maupeu J, Vallet-Courbin A, de Baynast H, Doco T, Michaud P, Delattre C (2019) Modification of Chitosan for the Generation of Functional Derivatives. Appl Scie 9(7):.1321–1327.
6. Cárdenas G, Cabrera G, Taboada E, Miranda SP (2004) Chitin characterization by SEM, FTIR, XRD, and 13C cross polarization/mass angle spinning NMR. J Appl Poly Scie 93(4):1876–1885.
7. Gadgey KK, Dey S (2017) Development of chitin and chitosan from Narmada riverside
crab shells. Inter J Mech. Eng Tech 8(7):298–307.
8. Gooday GW (1994) Physiology of microbial degradation of chitin and chitosan. In Biochemistry of microbial degradation (pp. 279-312), Springer Netherlands.
9. Kuddus M, Ahmad IZ (2013) Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J Genet Eng Biotechnol 11:39–46.
10. Marguerite R (2007) Chitin and Chitosan Properties and Application. PP,425.
11. Moattari M, Moattari F, Kaka G, Kouchesfehani HM, Sadraie H, Naghd M (2018) Application of chitosan in textile's. Annals Mat Sci. & Eng 3(1): 1032–1034.
12. Oman Observer (2019) Oman’s food consumption to reach 3.9 m tonnes in 2021, Available at: /(Accessed: Sunday, September 15, 2019).
13. Pandharipande SL, Bhagat PH (2016) Synthesis of chitin from crab shells and its utilization in preparation of nanostructured film. Inter J Scie Eng Tech Res 5: 1378-1383.
14. Panariello L, Coltelli MB, Buchignani M, Lazzeri A (2019) Chitosan and nanostructured chitin for biobased anti-microbial treatments onto cellulose based materials. Eur Poly J 113: 328–339.
15. Percot A, Viton C, Domard A (2003) Optimization of chitin extraction from shrimp shells. Biomacromol 4:12-18.
16. Puvvada YS, Vankayalapati S, Sukhavasi S (2012) Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry. Inter Curr Pharma J 1(9): 258–263.
17. Raabe D, Al-Sawalmih A, Yi SB, Fabritius H (2007) Preferred crystallographic texture of α-chitin as a microscopic and macroscopic design principle of the exoskeleton of the lobster Homarus ameri-canus. Acta Biomaterialia 3:882–895.
18. Rinaudo M (2006) Chitin and chitosan: properties and applications. Prog Polym Sci
31:603–632.

19. Salman S, Mansour S, Al-Mosharfi M, Al-Humaidi A, Yahya N, Al-Mahrami I (2012) Crabs of Sultanate of Oman, 1st edn., Muscat: Ministry of Agriculture and Fisheries Wealth Marine Science Fisheries Centre.

20. Squimedia (2016) CAMS Study Indicates Novel Use for Shrimp Waste, Available at: (Accessed: 11/28/2016).

21. Suryawanshi N, Jujjavarapu SE, Ayothisran S (2019) Marine shell industrial wastes—an abundant source of chitin and its derivatives: constituents, pretreatment, fermentation, and pleiotropic applications—a revisit. Inter J Environ Scie & Technol 1-22.

22. Yadav M, Goswami P, Paritosh K, Kumar M, Pareek N, Vivekanand V (2019) Seafood waste: A source for preparation of commercially employable chitin/chitosan materials. Biore & Bioproc 6(1): 8–12.

23. Zvezdova D (2010). Synthesis and characterization of chitosan from marine sources in black Sea. Annual Proceedings," Angel Kanchev" University of Ruse. 49(9.1): 65–69.

Figures
Figure 1

Chemical structure of Chitin

Figure 2

Process of carb samples for the measurement of chitin

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