EXTRACTION AND CHARACTERIZATION OF CHITIN FROM MARINE BYCATCH CRUSTACEANS EMPLOYING FERMENTATION METHOD

Kamalesan Jaganathan¹, Sirajudeen Mohammed Raffi² and Peyil Soundarapandian¹*

¹Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai- 608 502, Tamil Nadu, India.
²Kerala University of Fisheries and Ocean Studies, Panangad, Kochi- 682 506, Kerala, India.

ABSTRACT
Chitin is an important biopolymer next to cellulose, extracted in the present study. The exoskeleton of marine bycatch brachyuran crabs, namely Calappa lophos, Dromia dehaani, Dorippe facchino and also from stomatopod Squilla spp. were used to extract chitin through fermentation methods by employing two bacterial strains such as Pseudomonas aeruginosa, Serratia marcescens. The yield of chitin was 44.24%, 37.45%, 11.56% and 27.24% in C. lophos, D. dehaani, D. facchino and Squilla spp. respectively. FT-IR spectra of the produced chitin exhibit peaks which is more or less coherent to that of standard chitin which is further analysed by Scanning Electron Microscope. The quality of produced chitin was assessed through moisture, protein, ash and lipid content analysis ensured that chitin obtained from trash crustaceans are on par with that of standard chitin.

KEYWORDS: chitin, fermentation, bycatch, Calappa lophos, Pseudomonas aeruginosa, FT-IR.

INTRODUCTION
Demersal trawling is the most destructive forms of fishing that reduces the structural heterogeneity of benthic habitats of a range of fish and invertebrates.¹,² Along with the target fishes, this type of fishing will indiscriminately catch quite a large number and biomass of non-target species (bycatch) which is estimated to be between 6.8 and 20 million tons per
annum globally.\cite{3,4} Production of byproducts from the non-edible crabs and stomatopods will amplify its cost-effectiveness. Marine trash fish are considered as a potential economic resource and is no more considered as waste as the production of valuable substances and compounds from bycatch resources gained momentum in recent past.\cite{5} Trash crustacean exoskeleton shells are rich chitin content.\cite{6} Based on this evidence the present study focused on the production of chitin from trash crustaceans.

Chitin is mainly used as a raw material to produce chitin-derived products, such as chitosan, chitin/chitosan derivatives, oligosaccharides and glucosamine. An increasing number of useful products derived from chitin have tremendous industrial and pharmaceutical applications.\cite{7} An estimated 75% of chitin produced is used to manufacture products of nutraceutical importance. Currently, the major driving force in the market is the increasing sales of glucosamine as a dietary supplement.\cite{8} Commercially the chitin occupies high economic value. Practical grade chitin rated at a price of 220 USD per kilogram in the international market.

Production of chitin employing chemical method is widely used worldwide. The common procedure for isolating chitin from crustacean shells involves demineralisation, deproteinisation and decoloration. Demineralisation is generally performed by acids and deproteinisation by alkaline treatments. Moreover, these chemical treatment methods bring about quite a large question of waste water and its disposal pave way to several environmental problems. Above all, the cost of the chemicals involved in these process is another drawback\cite{9}, makes it less lucrative.

An interesting alternative method involves in the production of chitin is the biological method, through the process of fermentation by employing microbes such as bacteria and fungi. Application of bacterial proteolytic enzymes for protein removal from chitin rich fractions gains momentum in recent past as it ensures effective conversion of crustacean shell waste into useful chitin fractions. In the present study two bacterial strains were used to produce chitin. \textit{P. aeruginosa}, is used for the production of organic acids by fermenting the D-Glucose. The \textit{S. marcescens} is used to produce protease enzyme for the deproteinization step.
MATERIALS AND METHODS

Collection and preparation of raw material
The non-edible crabs viz C. lophos, D. dehaani and D. facchino and also stomatopod Squilla spp. were procured from Mudasalodai fish landing centre (Lat. 11°29′N; Long. 79°46′E), Parangipettai, Cuddalore district, Tamilnadu. The specimens were thorough and repeatedly washed in seawater to remove all the dirt and sand. They were then taken to the laboratory and the viscera and tissues were removed. The exoskeletons of the crustaceans were thoroughly washed with running tap water with ample care so as to remove sand adhered to it, and placed in hot air oven at 60°C for 24 hours.

Fermentation method
The demineralization\cite{10} and deproteinization\cite{11} steps were carried out for the production of chitin. The bacterial strains P. aeruginosa and S. marcescens were isolated from sediment samples procured from Vellar estuary, Parangipettai (11°29′N; 79°46′E). Thirty percentage of powdered exoskeleton was mixed with 10.0% inoculum of P. aeruginosa in a 500 ml flask containing 200 ml of 10.0% D-glucose. The fermentation was carried out at 30°C in an orbital shaker at 180 RPM for 7 days. After 7 days the shell powder was washed with distilled water and dried at 60°C for 4 hours; subsequently 2-3 drops of 1% H$_2$SO$_4$ was added with 1 mg of fermented crab shell powder to confirm the demineralization. The foam appearance indicates the presence of minerals. The demineralization step was extended until complete removal of minerals. All the experiments were done in triplicate.

The S. marcescens inoculum was prepared for demineralization process. The washed powdered exoskeleton was mixed with 10.0% of inoculum in a flask (500 ml) containing 200ml of 10.0% D-glucose. The fermentation was carried out at 30°C an orbital shaker at 180 RPM for 7 days. After that the sample was washed with distilled water and dried at 60°C for 4 hours, subsequently 2-3 drops of I$_2$/KI-solution (0.2 g I$_2$ in 100 ml of 5% KI solution) was added with a pinch of fermented exoskeleton powder to confirm the chitin production. The appearance of brownish-yellow colour indicates the removal of protein and non appearance of colour indicates protein presence. The deproteinization step was extended until complete protein removal. All the experiments were done in triplicate.
FTIR spectral analysis
IR characterization of chitin was performed with PERKIN ELMER SPECTRUM RX1 type FT-IR instrument in the Central Instrumentation Laboratory, Annamalai University. The standard grade chitin (Marine Chemicals, Cochin, India) was compared with that of the chitin obtained from all the species. The chitin sample was dried in hot air oven at 60°C for 6 hours for dehydration and was subjected for FTIR analysis.

Scanning Electron Microscope Analysis
The physical structure and nature of chitin was obtained with the help of Scanning Electron Microscope (SEM) (Model: JEOL.JSM 5160 with INCAEDS, version 1.1, Japan) at the Central Instrumentation Laboratory, Annamalai University. Powdered chitin was well dried in hot air oven at 60°C for 6 hours. Prior to analysis the chitin samples were sprinkled onto Carbon tapes which are adhesive and supported on metallic disks and coated with Au. Images of the sample surfaces were recorded at different areas and magnifications. Chitin produced from C. lophos alone were subjected to SEM analyse; since it fetched higher yield.

Proximate analysis
The extracted chitin from various species was subjected to moisture and ash content analysis.[12] Protein[13] and Lipid[14] estimations were carried out by following the standard methodologies.

RESULTS AND DISCUSSION
Production of Chitin
The results of chitin extracted from the exoskeleton of trash crustaceans by fermentation method are presented in table 1. The average yield of chitin from 30 g of raw exoskeleton of C. lophos, D. dehaani, D. facchino and Squilla spp. were 13.27 g, 11.233 g, 3.47 g and 8.17 g respectively. The percentage yield of chitin also followed similar trend as average yield of chitin. Among the species used in the present study, chitin obtained from C. lophos, showed higher yields of 44.24% and minimum yield fetched from D. facchino as 11.57%.
Table 1: Table showing the average yield of chitin produced from marine trash crustaceans.

<table>
<thead>
<tr>
<th>Source</th>
<th>Raw material (g)</th>
<th>Yield of chitin in three replicates</th>
<th>Average yield of the chitin</th>
<th>Percentage Yield of Chitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. lophos</td>
<td>30</td>
<td>13 13.6 13.2</td>
<td>13.27</td>
<td>44.24</td>
</tr>
<tr>
<td>D. dehaani</td>
<td>30</td>
<td>11 11.1 11.6</td>
<td>11.233</td>
<td>37.45</td>
</tr>
<tr>
<td>D. facchino</td>
<td>30</td>
<td>3.4 3.5 3.5</td>
<td>3.47</td>
<td>11.56</td>
</tr>
<tr>
<td>Squilla spp.</td>
<td>30</td>
<td>7 9 8.5</td>
<td>8.17</td>
<td>27.24</td>
</tr>
</tbody>
</table>

The strains of *P. aeruginosa* have been applied for demineralization process. This strain exhibits high protease activity (~50 U/ml) in the culture medium and produced organic acids, mainly of lactic, succinic and citric acids (roughly in the ratio of 1:1:1, w/w/w, about 0.3 mg/ml each). It is unraveled that the strains also secreted substantial amounts of chitinase (0.5 U/ml) but little chitosanase.[15,16]

The demineralization rate was gradually increased for the samples during culture. During 7th day, the demineralization rate was low for the exoskeleton of *C. lophos*. To rectify this, secondary incubation with fresh strains of *P. aeruginosa* was conducted for another 7 days at same parameters to achieve complete demineralization. The pH (pH7) was gradually decreased from the second day of incubation and finally reduced to pH3. Double fermentation with *P. aeruginosa* alone achieved higher level of demineralization. It is interesting to note that, during fermentation, *P. aeruginosa* produce sufficient amount of organic acids to solubilize CaCO₃; and it produce less quantum of protease, which indicates that the partial deproteinization occurs in the first step itself.

The parameters such as initial glucose amount, inoculation level and culture time have a significant effect on organic acid production.[16, 26] Since, crab shell waste is a poor source of fermentable carbon, so an additional energy source like glucose, lactose, malt, or whey powder must be added to the medium for better growth of bacterial strains. [18] The concentration of glucose as carbon source were standardised for the tune of 10.0%. Supplementation of 10.0% of glucose resulted in better efficiency for demineralization and deproteinization.[10] In the present study, nearly 91% of demineralization rate attained on the 8th day which was more or less similar with previously reported that the highest demineralisation value of 90% in lactic acid fermentation (with 7.2% of TTA) of crayfish waste.[19]
In order to improve the extraction efficiency of chitin from crab shell waste, deproteinisation was conducted using protease producing bacterium *S. marcescens* secretes mainly proteases but not sufficient amount of organic acids.\(^{[11]}\) In this step, the pH reached to 5 at the 4\(^{th}\) day. Deproteinisation reached its peak at 14\(^{th}\) day of incubation for *C. lophos*; for *Squilla* spp. it achieved on 10\(^{th}\) day, whereas for *D. facchino* it was achieved on the 7\(^{th}\) day. Earlier works stated that 23\% of chitin extracted from crab shells using *P. aeruginosa*\(^{[10]}\) and 33\% of chitin from red crab shells by fermentation method using *Lactobacillus paracasei* KCTC-3074 and *S. marcescens* FS-3.\(^{[11]}\) In the present study, the yield obtained for *C. lophos*, *D. dehaani*, *D. facchino*, and *Squilla* spp. were to the tune of 44.24\%, 37.45\%, 11.56 and 27.24\% respectively by employing fermentation method. From the results it is suggested that fermentation method produced higher yield in all the species.

**FTIR Analysis**

The results of FT-IR *C. lophos*, *D. dehaani*, *D. facchino* and *Squilla* spp. were represented in Fig. (1-5). FT-IR investigation proved the existence of the helical arrangement of chitin. The amide-A band of standard chitin was recorded at 3449 cm\(^{-1}\) showed that there were OH groups involved in free hydroxyl bonds. The amide-B band of chitin was found at 2960 cm\(^{-1}\) which is related to asymmetric and symmetric stretching H-C-H, whereas amide-I, amide-II and amide-III bands were observed at 1425 cm\(^{-1}\), 1418 cm\(^{-1}\) and 1261 cm\(^{-1}\) respectively.

**Fig.1:** FT-IR spectral analysis of standard grade chitin.
Fig. 2: FT-IR spectral analysis of chitin produced from *C. lophos*.

Fig. 3: FT-IR spectral analysis of chitin produced from *D. dehaani*.

Fig. 4: FT-IR spectral analysis of chitin produced from *D. facchino*.
In the case of *C. lophos*, *D. dehaani*, *D. facchino* and *Squilla* spp. chitin, amide-I were showed at 1638 cm⁻¹, 1638 cm⁻¹ and 1639 cm⁻¹ respectively in fermentation method against those of standard chitin which is 1653 cm⁻¹. Amide-II was represented at 1425 cm⁻¹ - 1425 cm⁻¹, 1413 cm⁻¹ - 1426 cm⁻¹, 1379 cm⁻¹ - 1328 cm⁻¹ and 1411 cm⁻¹ for *C. lophos*, *D. dehaani*, *D. facchino* and *Squilla* spp. respectively against 1418 cm⁻¹ for standard chitin representing CH₂ bend and CH₃ deformation. The amide-III band position of standard chitin was in the range of 1262 cm⁻¹, 1261 cm⁻¹ – 1262 cm⁻¹, 1262 cm⁻¹ – 1262 cm⁻¹ and 1258 cm⁻¹ – 1262 cm⁻¹ respectively.

The stretching bands of the OH groups involved in hydrogen bonds O-3-H…O-5 occurs at 3440 cm⁻¹.²⁰, ²¹ The C=O stretching region of the amide-moiety between 1600 and 1500 cm⁻¹ for chitin, the Amide-I band is split at 1656 and 1621 cm⁻¹ for β-chitin.²¹ From the results of the FT-IR spectral analysis it is evident that, the chitin produced from the *C. lophos*, *D. dehaani*, *D. facchino* and *Squilla* spp. showed helical arrangement and their functional properties more or less matching with that of standard grade chitin.

**SEM Analysis**

The SEM images of the chitin produced from *C. lophos* with different magnifications and different surface areas of chitin are shown in Fig.6. It was observed that chitin biopolymer produced in the present study exhibits porous and fibril structures. The structure of chitin as crystalline and dense those were extracted from the exoskeleton of shrimps, *Penaeus semisulcatus*, (de Haan), *Metapenaeus affinis* (Milne-Edwards); from brachyuran crabs
Portunus pelagicus (Linne), from lobster Thenus orientalis, (Lund) and from cephalopod Sepia spp.[22]

Fig.6: SEM images of chitin produced from C. lophos by fermentation method (a. 500X and b. 750X).

Proximate Composition and Quality Analysis of Chitin

The extracted chitin contains very little amount of moisture, lipid, protein, and ash content and is satisfactory in line with commercial international standards (Fig.7). The proximate composition of chitin used to vary in its chitin and non–chitinous fractions which might be due to the variation in parent source (raw material), difference and modifications in the methodologies, hygiene, less sand content, etc. The quality of chitin is assessed based on its proximate composition, moisture content, ash content in such a fashion that lower the levels of protein, lipid, moisture and ash contents were low indicates a better quality of chitin. Non – chitinous materials negatively affects the quality and property of chitin by interacting with it.[23] The proportion of chitin and the non–chitinous fractions varies with species.[17]

Fig.7. Moisture content, Protein content, Ash content and Lipid content of chitin produced from C. lophos, D. dehaani, D. facchino and Squilla spp.
CONCLUSION
The chitin extracted, contains very little amount of moisture content, lipid, protein, and ash content and is satisfactory in line with commercial international standards. Moreover, this method involves little application of chemicals, thus ensures an economically feasible and an ecofriendly method. In general, it is evident that the chitin produced from the trash crustaceans showed better yield and the quality assessment proved that the chitin produced from trash crustaceans are in line with that of the standard grade chitin available at international markets.

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REFERENCES
1. Frid CLJ, Hall SJ. Inferring changes in North Sea benthos from fish stomach analysis. Marine Ecological Progress Series, 1999; 184: 183–188.