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RESEARCH ARTICLE

Extraction and Characterization of Chitosan from the Shell Wastes of Indian Shrimp Using Different Methods of Deacetylation

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Abstract

Objectives: Shrimps are a good source of chitosan, a deacetylated form of chitin, which has several medical and industrial applications. We aimed to convert waste shrimp shells to high-value chitosan product. **Methods:** The waste shells of the Indian shrimp, *Fenneropeanus indicus* were collected from Ukkadam market, Coimbatore, India, and they were subjected to demineralisation, deproteination, and deacetylation under different concentrations of NaOH, at varying temperatures. The derived chitosan was named as LAHT SCS (low alkali high temperature shrimp chitosan), HAHT SCS (high alkali high temperature shrimp chitosan), or HART SCS (high alkali room temperature shrimp chitosan), based on the treatment. **Findings:** The extracted chitosan samples were evaluated for physical characteristics such as moisture, ash content, and degree of deacetylation (DD). In addition, they were subjected to Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis. The HAHT SCS sample was completely soluble in 1% acetic acid and the degree of deacetylation (DD) was higher than that of standard chitosan; the crystallinity was 71%. Therefore, the extraction method employing a higher concentration of alkali (60%) and a higher deacetylation temperature (120°C) for a long duration enhanced the solubility, conferred a higher DD (>70%), a higher crystallinity index (>60%), and less amount of ash content; all these indicate the higher quality of the sample. Among the three samples, HAHT SCS is promising for pharmaceutical and other medical applications. **Novelty:** The extraction of good quality chitosan from the Indian shrimp wastes was standardized. The chitosan has medicinal and industrial applications. In addition, this would reduce the pollution from improperly discarded shrimp wastes.

Keywords: CrI; Chitosan; Chitin; Shrimp; Degree of deacetylation; X-ray diffraction; Fourier transform infrared spectroscopy

1 Introduction

Chitosan, a β 1,4-linked glucosamine polymer, is the deacetylated form of chitin obtained from organisms such as fungi, shrimps, and crab[1]. Chitosan has several applications in biomaterials, pharmaceutical drug delivery systems[2], medical[3], textile[4], agricultural and pesticide applications[5], preservatives[6], wastewater purification[7], and dressing materials for wounds[3]. Chitosan has antibacterial activity; therefore, it is used in making gloves, wound bandages, and sterile textiles[5]. Shrimp is the main product among the exported Indian marine products, contributing to 73% of the value of total exports. Cultured shrimps contribute 90% to the value of total shrimp exports. India exports aquaculture products to almost 76 countries among the total 113 countries that consume Indian marine products[6]. Shrimp processing units generate a massive quantity of shell wastes, which are currently discarded in a manner that creates environmental issues. Shrimp shell wastes from pre-processing units account for 40−50% of the total shrimp weight and are considered as a secondary raw material for various products. Conversion of shell wastes into high value by-products such as chitin and chitosan, and the recovery of minerals and proteins from them is a promising solution for shell waste management; this will reduce the environmental issues associated with these wastes. Shrimp waste, on a dry weight basis, contains up to 65% proteins, 21% ash, and 18% chitin. Therefore, it is particularly critical to convert these by-products into high-value products[1]. The chitin from the exoskeleton is subjected to demineralisation, deproteination, and deacetylation for the synthesis of chitosan. Chitosan is a natural polymer derived from chitin through deacetylation of the glucosamine polymer chain. This polymer is highly linear in structure and is insoluble in water. Chemical extraction using acids and alkali is considered the best method for the industrial production of chitosan because of the shorter reaction times and the ability to completely remove the organic salts and proteins[8]. The biodegradability of the extracted chitosan depends on the proportion of acetylated and non-acetylated glucosamine units. The influence of the treatment times and alkali concentrations on the selected quality characteristics of chitosan extracted from various crustacean species has been evaluated; it is important to understand this influence to elucidate and ensure the quality of the final product[5,9,10].

In this study, the Indian shrimp, *Fenneropenaeus indicus* was chosen because the shells of this crustacean are available in abundance in the local market where it is discarded in large amounts as a waste. We aimed to convert this waste into a valuable product, by standardising the extraction procedure using different concentrations of acid and alkali for the demineralisation, deproteination, and deacetylation protocols to extract good-quality chitosan. We performed various physico-chemical and analytical analyses to confirm the quality of the isolated chitosan. The effect of different methods of deacetylation in enhancing the quality of the extracted chitosan was analysed; the quality of the extracted chitosan was compared with that of standard commercially available chitosan.

2 Materials and Methods

2.1 Collection and Transport of Fresh Shrimp Shells

The waste shells of the Indian shrimp, *Fenneropenaeus indicus* were freshly collected from Ukkadam fish market, Coimbatore, Tamil Nadu, in a clean container. The waste was transported to the laboratory at ambient temperature.

2.2 Extraction of Chitin

The shrimp shells were washed in boiling water to remove the remanent flesh. They were then air dried and oven dried at 80°C for 24 h. The dried samples were finely ground into powder using an electric blender and stored in an airtight container. The shrimp shell powder was divided into 3 aliquots. The demineralisation, deproteination, and the deacetylation for the extraction of chitin were performed with different concentrations of HCl and NaOH.

2.2.1 Low Alkali High Temperature Method

An aliquot (20 g) of the shrimp powder was treated with 1.5 M HCl in the ratio 1:10 (W/V) at 30±2°C for 30 min. The powder was washed with tap water until the pH of the treated sample became neutral. The sample was deproteinized with 2 N NaOH in the ratio 1:10 (W/V) for 2 h at 65°C. The sample was deacetylated with 50 % NaOH in the ratio 1:10 (W/V) for 3 h at 90°C, neutralised with water, treated with 80% alcohol, and dried in an oven at 80°C overnight.

2.2.2 High Alkali High Temperature Method

An aliquot (20 g) of the shrimp powder was treated with 1 N HCl in the ratio 1:15 (W/V) at 30±2°C for 30 min. The sample was washed with tap water until the pH of the treated sample became neutral. The sample was deproteinized with 3.5% NaOH in

the ratio 1:10 (W/V) for 2 h at 75°C. The sample was treated with 60% NaOH in the ratio 1:10 (W/V) at 120°C for 3 h, followed by washing and neutralisation with tap water, and drying at 60°C for 4 h.

2.2.3 High Alkali Room Temperature Method

An aliquot (20 g) of the shrimp powder was treated with 4% HCl in the ratio 1:10 (W/V) at 30±2°C for 12 h. The sample was deproteinized with 4 % NaOH in the ratio 1:10 (W/V) at 30±2°C for 24 h, with constant stirring. The sample was washed with tap water until the pH of the treated sample became neutral. The sample was treated with 65% NaOH for 72 h at 30±2°C and then washed with tap water until the pH reached neutral. The samples were then allowed to dry at room temperature.

2.3 Physical Characterization

2.3.1 Solubility Test

The samples were subjected to solubility test by dissolving 0.1 g of the prepared chitosan in 10 ml of 1% acetic acid, in triplicates, in centrifuge tubes of known weight. The samples were incubated in a shaker incubator at 25° C at 240 ×g for 30 min. The mixture was heated for 10 min in a boiling water bath, cooled to 25° C, and centrifuged at 5000 ×g for 10 min. The supernatant was discarded and the sediment was washed with 25 ml of distilled water. It was then dried in an oven and the mass was measured.

$$Solubility = \frac{initial\ weight\ of\ the\ (tube + chitosan) - final\ weight\ of\ the\ (tube + chitosan)}{Initial\ weight\ of\ the\ (tube + chitosan) - weight\ of\ empty\ tube} x\ 100$$

2.3.2 Determination of Sulphated Ash and Inorganic Content in the Sample

The inorganic content in the sample was determined as described in the *Brazilian pharmacopeia*; 1 g of the sample was taken in a crucible that was previously heated and weighed. The sample was moistened with 1 ml of concentrated sulphuric acid and heated gently to char. The sample was cooled and 1 ml of sulphuric acid was added to the crucible and the crucible was heated to 600°C and maintained at this temperature until the residue was completely incinerated. The crucible with the sample was then cooled in a desiccator and weighed. The heating and cooling were repeated until a constant weight was obtained. The sulphated ash content was calculated as follows:

Sulphated ash (%) =W3-W1/W2-W1×100

Where W1 is weight of empty crucible, W2 is weight of crucible with substance, and W3 is weight of crucible with substance after drying.

2.3.3 Water Binding Capacity (WBC)

An aliquot (0.5 g) of the prepared chitosan was added to 10 ml of distilled water in a centrifuge tube and mixed thoroughly. The sample was incubated at ambient temperature for 30 min with intermittent shaking (5 s) every 10 min and then centrifuged at 3500 ×g for 25 min. The supernatant was discarded, and the tube was weighed. WBC was calculated as follows:

$$WBC\ (\%) = \frac{(Water\ bound)\ g}{(Initial\ sample)\ g} x\ 100$$

2.3.3 Fat Binding Capacity (FBC)

An aliquot (0.5 g) of the prepared chitosan was mixed with 10 ml of soya bean oil in a centrifuge tube and thoroughly mixed. The samples were incubated at ambient temperature for 30 min with intermittent shaking (5 s) every 10 min and then centrifuged for 25 min at 3500 ×g. The supernatant was discarded, and the tube was weighed. FBC was calculated as follows:

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

2.3.5 Determination of the Degree of Deacetylation using FTIR Spectra

Infrared spectra of the purified samples were obtained using a Shimadzu spectrometer at room temperature, using the KBr pellet scanning method. Pellets were scanned at 25°C±2°C in the spectral range of 400−4000 cm^{-1}. FTIR was used to confirm the formation of chitosan; 4 g of the sample was mixed with 200 mg of KBr and this mixture was made into pellets. The degree of deacetylation (DD) of chitosan was calculated by applying the Domzy and Robertz and Burnerotto equation [11,12].

$$DA\ (\%) = \frac{A_{1655}}{A_{3455}} x\ 100/1.3$$

where, A_{1655} is and A_{3455} are the absorbance at wave number of 1655 cm^{-1} and 3450 cm^{-1}.

2.3.6 X-Ray Diffraction

The structural characterization of the chitosan was performed using an X-ray diffraction meter (XRD 6000 Shimadzu). The intensity of the diffracted X-rays was measured as a function of the diffraction angle 2Θ and the specimen's orientation. This

diffraction pattern was used to identify the specimen's crystalline phases and to measure its structural properties.

3 Results and Discussion

3.1 Physical Characterization of the Shrimp Shell Chitosan

The chitosan obtained using the three methods were named as LAHT SCS (low alkali high temperature shrimp chitosan), HAHT SCS (high alkali high temperature shrimp chitosan), and HART SCS (high alkali room temperature shrimp chitosan).

Fig 1. Shrimp shell

The yield of the extracted chitosan was in the range of 35 to 37 %; this was higher than that obtained by Santos et al[13], where microwave extraction with 30 min of irradiation was used. In addition, the yield was higher than that by Öğretmen et al., who obtained a yield of 18.82%[14] using 50% NaOH at 121°C.

The extraction of chitosan under strong alkali (60%) and high temperature (120°C for 3 h) enhanced deacetylation; the hydrolysis of the acetamido groups of the acetylglucosamine in chitin resulted in the formation of chitosan. When the deacetylation time and the alkali concentration increased with temperature, there was a decrease in the resulting chitosan yield. Similar results were observed for chitosan extracted from squid pen[7] and Pacific white shrimp shells[15].

The extracted chitosan was in the form of creamy white flakes. It was soluble in 1 % acetic acid (Table 1). The HAHT SCS was easily soluble in 1 % acetic acid, compared to HART SCS and LAHT SCS. Lower solubility suggests incomplete removal of proteins[16]. The moisture content in the extracted chitosan was in the range of 4.3% to 4. 7%. The moisture content is in accordance with the hygroscopic nature of chitosan. This could vary with the moisture absorption during storage. The ash content was in the range of 1.19% to 1.21%. The WBC of the isolated samples was in the range of 450 to 650. The ash value of the HAHT SCS samples was lesser than that of the HART SCS and LAHT SCS samples. Low ash content (1.20%) is indicative of effective demineralization[17].

D-degree of deacetylation, WBC-water binding capacity, FBC-fat binding capacity

WBC of HAHT SCS was 654.34. The WBC of chitosan was influenced by the dissimilarities in crystallinity, availability of salt forming groups, and the residual protein in the chitosan samples[18]. Fat binding capacity (FBC) was in the range of 384 −400. The FBC value depends on the extracted chitosan as well as its sources[14].

Table 1. Physical characteristics of the samples

Parameters	LAHT SCS	HAHT SCS	HART SCS
Yield (%)	35	36	37
Moisture (%)	4.3	4.4	4.7
Ash	1.21	1.19	1.22
pH	7.4	7.3	7.2
DD	67.91	79.57	61.25
Solubility in 1 % acetic acid	91	100	93
WBC	461.024	654.34	452.72
FBC	387	400	384

3.2 FTIR Spectrum

HAHT SCS showed peaks at 3450 cm^{-1}, 2877 cm^{-1}, 1425 cm^{-1}, 1087 cm^{-1}, and 898 cm^{-1}, indicating the presence of amino stretching, CH stretching, amide C=O stretching, CO stretching, and ring stretching, respectively (Figure 2). The LAHT sample exhibited peaks at 3441 cm^{-1}, 2924 cm^{-1}, 1435 cm^{-1}, 1072 cm^{-1}, and 879 cm^{-1} representing amino stretching, CH stretching, amide C=O stretching, CO stretching, and ring stretching, respectively (Figure 2). The sample HART SCS showed peaks at 3441 cm^{-1}, 2924 cm^{-1}, 1427 cm^{-1}, and 879 cm^{-1}, indicating the presence of amino stretching, CH stretching, amide C=O stretching, CO stretching, and ring stretching, respectively (Figure 2). The HAHT SCS sample peaks were in close relation with that of the standard chitosan, compared to that of the other two samples (Figure 3 andTable 2).

Table 2. Assignments of the relevant bands of the FTIR spectra of the extracted samples

LAHT SCS	HAHT SCS	HART SCS	Wave number (CM^{-1}) std chitosan from Sigma	Possible assignment of absorption bond
3441	3450	3441	3429.57	NH_2 stretching
2924	2877	2924	2878.47	CH stretching
1435	1425	1427	1417.48	Aliphatic CH stretching
1072	1087	Not identified	1083.04	Amide C=O stretching
879	898	879	867.97	Ring stretching

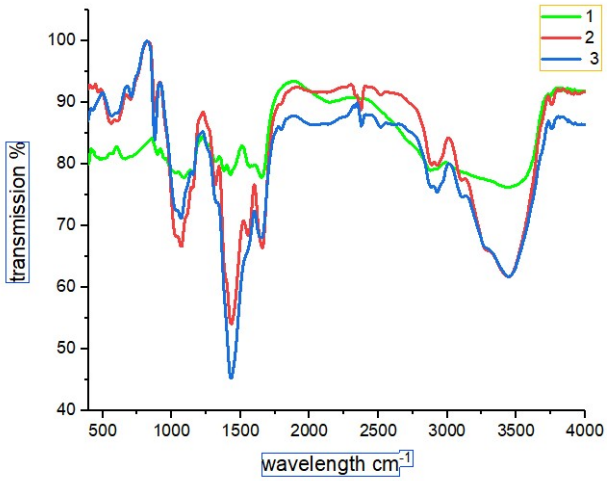

Fig 2. FTIR peaks of three samples.1-HAHT SCS, 2. LAHT SCS, 3. HART SCS

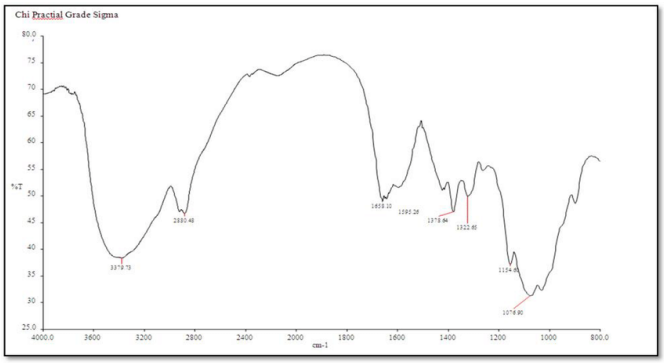

Fig 3. Chitosan Standard

The peak above 3000 cm^{-1} to 3450 cm^{-1} revealed the symmetric stretching vibration of hydrogen-bonded OH and amines (NH_2), ranging from 3070 to 3450 cm^{-1} [19]. The HAHT SCS sample showed stretching at 3450 cm^{-1}. The higher frequency shift revealed the presence of a higher order structure in the chitosan sample [20]. The peaks at 2924.09 cm^{-1}, 2923.09 cm^{-1}, and 2877.79 cm^{-1} indicated the N-H stretching in the samples, HAHT SCS, LAHT SCS, and HART SCS, respectively. The bands around 2920 cm^{-1} represent C-H groups stretching. [14] The absence of (-C-O) containing carbonyl and carboxyl groups can be confirmed through the absence of bands in the 1900−1660 cm^{-1} range. The bands at 1658.78 cm^{-1}, 1651 cm^{-1}, and 1649.14 cm^{-1} in the samples indicated the bending vibration of (NH of R-NH_2) primary amine groups, the bending vibrations of the amide groups of NH_2, hydroxyl, and the N-H bond. This was in accordance with the results in an earlier report [14]. It confirms the deacetylation of chitin. The peaks at 1435.04 cm^{-1}, 1427.32 cm^{-1}, and 1425.40 cm^{-1} indicated the presence of amide band II and N-H bending in the samples. The peaks at 1087.85 cm^{-1},1072 cm^{-1}, and 1064 cm^{-1} in the FTIR spectrum indicated the C-O in the secondary stretching OH-group. The peaks at 1072 and 1028 cm^{-1} could be assigned to the absorbance of C-N in CH_3CONH (amide III band) and symmetrical deformation of CH_2 and CH_3 [19].

3.3 Degree of Deacetylation (DD) as Ascertained using FTIR

The DD values of the samples were 79.57%, 61.25%, and 67.91%, for HAHT SCS HART SCS and LAHT SCS respectively. The DD % of HAHT SCS was higher than that for the other two samples, LAHT SCS and HART SCS. The degree of deacetylation (DD) is influenced by NaOH concentration. The bound acetyl groups in chitin are difficult to remove. Therefore, high concentration of NaOH and a high temperature are required. In this case, the high NaOH concentration increases the deacetylation to 79 %. A high deacetylation grade of 70% was reported at high NaOH concentrations [21].

3.4 XRD Analysis

The X-ray diffraction pattern of the chitosan samples exhibited a strong reflection in the 2Θ values at 9° and 20°, in HAHT SCS sample. The LAHT SCS and HART SCS patterns exhibited crystalline peaks with slightly fluctuated diffraction angles other than 9° and 20° at 2Θ values. The crystallinity values of the extracted samples were in the range of 60 to 71%. The HAHT SCS sample exhibited a higher crystallinity value of 71.45%, while that of HART SCS and LAHT SCS were 60.32 % and 65.61 %, respectively.

These variations could be attributed to the incomplete removal of proteins or calcium from the shrimp shells. The crystallinity percentages were: HAHT SCS, 71%; HART SCS, 60 %; and LAHT SCS, 65%. The varying concentrations of HCL and NaOH and the temperature used for the deacetylation process could have an impact on the quality of the extracted chitosan. The concentration of the acid and alkali conferred little variation in the crystallinity peak. The role of the base is to remove the calcite from shrimp shells; however, it mildly affects the crystallization because the protein structure is disrupted [8]. The CrI index of the samples was increased in HAHT and LAHT SCSs, where a higher concentration of alkali was used. The CrI value of the extracted and refined chitosan from shrimp wastes was 74.36, while that of raw chitosan from shrimp and crab shell wastes was 64.0% and 60.0%, respectively [20]. The treatment conditions adopted during the conversion of chitin to chitosan, polymorphism, and the nature of the species from which the chitin is extracted are the factors that influence the crystallinity of chitosan [14].

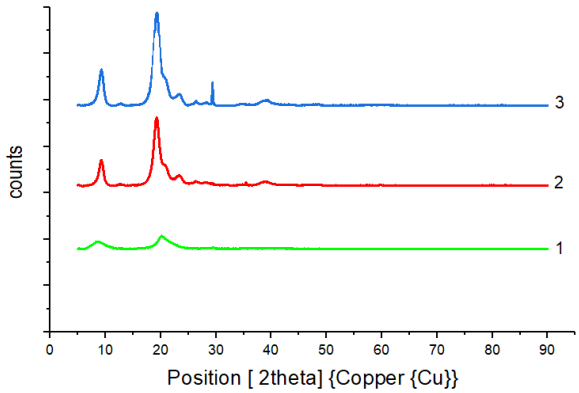

Fig 4. X ray diffraction pattern for three samples.1-HAHT SCS, 2. LAHT SCS, 3. HART SCS

Significance: The waste from Indian shrimp was transformed into the valuable biopolymer chitosan, and the extraction process from Indian shrimp was standardized to produce chitosan with a good grade of DD 80%. This grade of chitosan is utilized in pharmaceutical products and as a delivery system for medications used to treat tumors.

Limitations: Here, sodium hydroxide and hydrochloric acid are used as the approach. In terms of environmental protection, this is a massive buildup of chemicals even though it is effective at extracting chitosan. Use of sustainable extraction techniques is required.

Future directions In the future, chitin-rich shrimp waste can be converted to chitosan using techniques that don't harm the environment by using microbial enzymes or microbial consortia.

4 Conclusion

Chitosan was extracted from the shell wastes of Indian shrimp employing low alkali high temperature, high alkali high temperature, and high alkali room temperature, with different concentrations of HCl and NaOH, deacetylation at different alkali ratios, and at different temperature parameters. The use of high alkali and high temperature enhanced the production of chitosan with a DD of 80%. The chitosan was completely soluble in 1% acetic acid. The solubility and degree of deacetylation indicated the good quality of the chitosan obtained. The crystallinity index of the sample was 74 %. In the HART SCS sample, the wave peak that represents the amide stretching was not identified. This iterates that a high alkali concentration along with high temperature with extended hours of treatment is necessary for the extraction of good-grade chitosan. The HAHT SCS sample is of the best grade among the three samples, and is promising for pharmaceutical applications. The quality of the chitosan extracted using high alkali and temperature is good; however, the use of large quantities of alkali could be overcome by using a suitable microbial enzyme in future studies.

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