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ORIGINAL ARTICLE

Chitosan from the carapace of Indian horseshoe crab (*Tachypleus gigas*, müller): Isolation and its characterization

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ABSTRACT
Chitosan is an important amino polysaccharide. This work describes the isolation and characterization of chitosan from dead carapaces of horseshoe crabs which involves a series of steps such as deproteinisation, demineralisation, and deacetylation. After the isolation the average molecular weight of the horseshoe crab chitosan was found to be 497 kDa while degree of deacetylation of chitin was 89.79%. The deacetylation of chitin retrieved was about 34% of chitosan. Moisture, ash, pH, loss on drying and the viscosity of isolated chitosan were 70 and 72% moisture value, 0.25%, >10ppm, 8.5, 9.34%, and 304 cps respectively. A comparison was made between IR spectra of Horseshoe crab chitosan and commercial chitosan to study the structural changes. X-ray diffraction patterns of horseshoe crab chitosan and commercial chitosan were compared with each other to identify the crystalline structure of both chitosan. The morphological structure of both chitosan was obtained by SEM analysis and both the chitosan showed similar morphological structure with flake like appearances on their surface.
***Key words**: Chitosan; Horseshoe; crab; carapace; Characterization*

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INTRODUCTION

Chitin is one of the most abundant amino polysaccharides found in nature next to cellulose; [1]. Chitin is found in the cell wall of fungi, yeast, protists and diatoms [2]. Also, chitin forms the exoskeleton for a broad variety of invertebrates which include sponges, worms, molluscs, bat guano, wings of cockroach and the arthropods [3-5]. It has a wide range of utility including cosmetics, pharmacy, medicine, bioengineering, biological materials science, agriculture; textiles and environmental engineering based upon its nontoxic, eco-friendly, biodegradability and biocompatibility properties. Commercially, chitin is obtained after processing the outer skeleton of crustaceans such as shrimp, crab, prawn and crayfish [6, 7].

Because chitin has a compact structure, it is insoluble in most solvents. In order to make it more useful, there are methods for chemical treatment of chitin and for its appropritate modifications. Among these, the most common derivative is chitosan, derived by partial deacetylation of chitin [8, 9]. When the degree of deacetylation (DDA) reaches higher than 50%, chitosan becomes soluble in acidic aqueous solutions and behaves as a cationic polyelectrolyte.

Potential and already known applications of chitin and its derivatives, particularly chitosan, are estimated to be more than 200 in numbers [10]. These natural and derived polymers of chitin and chitosan have antimicrobial activity, besides being biocompatible and biodegradable [8]. They have a wide range of applications in different fields such as cosmetics, agriculture, food, pharmacy, biomedical, paper industry and also as absorbent materials for wastewater treatment [11].

Among crustaceans, the horseshoe crabs are well known for the chitosan content in their outer hard shells. Presently there are only four species of the horseshoe crabs in the world out of which two species i.e. *Tachypleus gigas* and *Carcinoscorprius rotundicauda* are found in northeast coast of Odisha state, India [12, 13]. Horseshoe crabs have been a model for many biomedical science studies. Medicinal value of horseshoe crabs comes primarily from its blue blood and peri-vitelline fluid (PVF) [14-18]. The horseshoe crab has a hard outer shell or exoskeleton that offers it protection from predators. In the formation of this outer shell millions of tightly interwoven strands of cellulose-like material, the chitin serve as tough and flexible glue which holds together the shell. The chitin in horseshoe crabs is very pure as compared to the chitin derived from other arthropods. Thus in the present work our objective was to isolate the useful polymers of chitin and its derivatives from the carapace of the dead Indian horseshoe crab. Most of these dead animals lie as wastes and get degraded subsequently. With treatment, this purified chitin was converted into a more soluble form i.e. chitosan. The aim of this study was to isolate chitosan, which was characterized by means of FTIR, XRD, TGA, SEM and elemental analyses as also to estimate the extent of their purity. Moreover, the isolated horseshoe crab chitosan was compared with commercially available chitosan to access its qualitative value.

MATERIAL AND METHODS

Sample collection

Extensive surveys have been undertaken since the year of 2013 along coastal areas Balaramgadi, Chandipur and Khandia in the Balasore district of the Odisha state. These surveys were conducted to assess the population density of the horseshoe crab in the locality. It was observed that due to increasing human activities on the spawning beaches; the population of the horseshoe crab has been severely affected, thereby leading to population decrease significantly. Among these, Khandia estuary area was observed to be least affected where in normal occurrence of the horseshoe crabs was seen (Fig. 1). As such this area was identified for regular collection of the carapace.

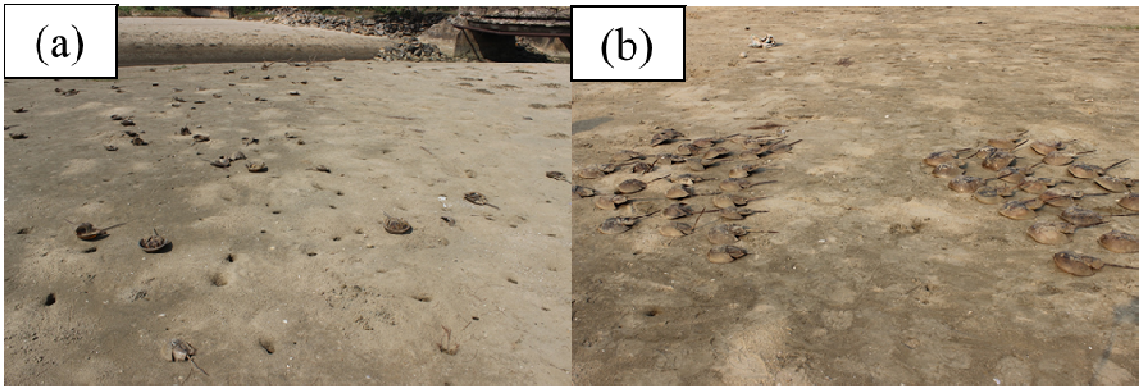

Fig- 1 (a) Khandia Estuary (b) Dead Carapace sample collected for Experiment

Raw materials

Horseshoe crab carapaces were obtained from Khandia estuary (Near Chandipur, Balasore, Odisha, India) along the north east coast of Odisha state. The GPS coordinates of the estuary are 21°19'1.65"N and86°53'32.99"E [19]. Pictures of estuary and horseshoe crab carapaces have been shown in Fig. 1. Almost all of the collected horseshoe crab carapaces belonged to the species *Tachypleus gigas.* Chemicals used in this study were NaOH, HCL and acetic acid etc. those were obtained from HiMedia, Mumbai, India.

Preparation of chitosan from horseshoe crab

Cleaning process

The horseshoe crabs were washed several times and carapace were cleaned free of tissues and other impurities. The carapace was then crushed into small pieces and soaked in deionized water at room temperature for two days.

De- Mineralization

De-mineralization process consisted of removing minerals, mainly calcium carbonate. Generally, demineralization was carried out by acid treatment using HNO_3, H_2SO_4, HCl, CH_3COOH and HCOOH. Between these acids, the preferred acid was dilute hydrochloric acid [20]. In the present study demineralization was carried out using HCL. Carapace was kept submerged in 2N HCL for 24 hours. Later

it was washed with deionised water until a neutral pH was observed. This was followed by drying at room temperature and pulverisation. Thus, dried chitin was obtained.

De- Proteinization

The deproteinization is a complex and difficult process. The horseshoe crab carapace is in tightly bound with proteins. However from the possible literature survey, the most common method for deproteinization was found to be the alkali method by using sodium oxide [21]. The demineralized raw chitin obtained previously were washed twice with water to remove the excess acid which is present in the chitin, as it might affect and lowers the activity of sodium hydroxide. It was then treated with 4% NaOH solution for 1 hour to break down the albumin into water-soluble amino acids.

De- Acetylation

Deacetylation was carried out to convert chitin to chitosan. It is the process of removing acetyl groups from chitin. It is achieved by treating the chitin with alkalis such as NaOH or KOH at 40-50% concentration. Usually, NaOH is used for chitin deacetylation process [22]. The obtained de-proteinized chitin was treated with 50% NaOH at 100°C for 2 hours on a hot plate. After deacetylation samples were cooled to room temperature and washed continuously with 50% NaOH. The solid materials were filtered and dried at 110°C for 6 hours. The dried chitosan was tested for solubility by using 1% acetic acid with the composition of 1:100 (v/v). The procured chitosan was characterized and tested for its chemical and physical properties using advanced instruments including FTIR, XRD, TGA and SEM.

Yield of chitosan

The yield of chitosan was determined by comparing the weight of raw chitosan material with the isolated chitosan, obtained after the treatment. The yield was determined according to the methods described earlier [23]. The following equation has been used to determine the yield of chitosan.

$$\text{The yield of isolated chitosan (\%)} = \frac{\text{Dried extratced horseshoe crab chitosan weight (g)}}{\text{Raw horseshoe crab chitosan (g)}} X\ 100$$

Characterization of Horseshoe crab chitosan

Purification of chitosan

The level of purity of the chitosan influences its various properties when used for biomedical applications. The samples were purified before taking it for characterization. Briefly, the chitosan powder was first solubilized by adding 2M of the acetic acid solution and adjusting the pH to 4-5.5. The chitosan solution thus obtained was kept overnight under the stirring condition to achieve complete solubilisation of chitosan with water [24].

Fig- 2Horseshoe crab Chitosan for characterization

Characterization of the chitosan samples

Determination of ash content

The ash content was determined using laboratory muffle furnace (Fourtech, Mumbai, India) as per AOAC method. In brief, 1 g of each sample was taken in pre-weighed crucible with lid and placed in muffle furnace. Temperature was maintained at 575 ± 10 °C for 6 h. After cooling, the crucibles were removed from the furnace and were placed in the desiccators. The above process of heating and cooling was repeated until a constant weight was obtained. The ash with crucible and lid was weighed when sample turns to gray. The percent ash was calculated as follows:

$$\text{Ash (\%)} = \frac{\text{Weight of ash (g)}}{\text{Sample weight (g)}} X\ 100$$

Loss of mass on drying

Loss on drying of the prepared chitosan was determined by the gravimetric method. The water mass lost was determined by drying the sample till it achieves a constant weight and measuring the weight of sample before and after drying. The water mass (or weight) was the difference between the weights of the wet and oven dry samples [25].

$$\text{\% Loss on drying} = \frac{(\text{Wet weight} - \text{Dry weight})}{\text{Dry weight}} \text{X } 100$$

Determination of moisture content

The moisture content of the isolated chitosan samples was determined by gravimetric method. The moisture content in terms of water mass was the difference between the weights of the wet and dried chitosan were expressed in percentage [26].

$$\text{\% Moisture content} = \frac{\text{Wet weight (g)} - \text{Dry weight (g)}}{\text{Wet weight (g)}} \text{ x } 100$$

Viscosity

Viscosity of the chitosan was determined with a Brookfield viscometer (Model DV-IIþ Brookfield) as described by [27] with few modifications. Chitosan solution was prepared using 1% acetic acid at 1% concentration on a dry weight basis. Measurement was made in triplicates using spindle number 2 at 50 rpm at 28º C for each solution and the values were reported in centipoises (cPs) units.

Solubility

The solubility of the isolated chitosan samples were determined by dissolving 0.1g of deacetylated chitosan in 100ml of 1% acetic acid solution with continuous mixing for 30 minutes by magnetic stirrer at 240 rpm. The chitosan solution was then filtered using vacuum pump and dried. The procedure was repeated for 3 times and the dried particles were weighed [28]. The solubility percentage of chitosan was calculated by the following equation.

$$\text{Insoluble (g)} = \text{Final weight of filter paper} - \text{Initial weight of filter paper (g)}$$

$$\text{Insoluble \%} = \frac{\text{Insoluble (g)}}{\text{Sample weight (g)}} \text{ x } 100$$

$$\text{Solubility (\%)} = 100 - \text{\% Insoluble}$$

Degree of deacetylation of chitosan by using FTIR

The degree of deacetylation is an important property of chitosan and it plays an important role in determining the utility of chitosan. The degree of deacetylation of chitosan was determined byusing FTIR (Fourier Transform Infrared) Spectroscopy instrument Model: NEXUS- 870. The FTIRwas operated in the frequency range of 400-4500 cm^{-1}. The degree of deacetylation (DD) of the chitosan was calculated using the baseline by [29]. The computation equation for the baseline is given below:

$$DD = 100 - [(A_{1655} / A_{3450}) \text{ X } 100 / 1.33]$$

Where A_{1655} and A_{3450}were the absorbance at 1655 cm^{-1} of the amide-I band as a measure of the N-acetyl group content and 3450 cm^{-1} of the hydroxyl band was as an internal standard to correct the film thickness. The factor '1.33' denoted the value of the ratio of A_{1655} / A_{3450} for fully N-acetylated chitosan [27].

Fourier Transform Infrared (FT-IR) Spectroscopy

FTIR spectroscopy has been one of the most effective technique for a few decades when it comes to characterization of polymer materials like chitin and chitosan owing to its ease of access, simplicity, and low cost. In FTIR, infrared rays at different wavelength are allowed to pass through the sample, wherein certain wavelengths are absorbed by the constituent molecules of the sample and the remaining wavelengths are transmitted out through the sample [30].

The infrared spectrum indicates the absorption peak of the sample. The peaks are produced because of the vibrational frequencies created between the bonds of atoms arranged in the material. The spectrum of each material is unique owing to their different atomic arrangements and do not repeat the same spectrum. Therefore, FT-IR spectroscopy provides very sharp qualitative results for every kind of materials. The peak's size is directly proportional to the density of the particular material. [30].

The structural changes and the percentage of deacetylation in bothHorseshoe crab chitosan (HCH) and Commercial Shrimp chitosan (SCH) were measured by FTIR spectroscopy. The powdered form of chitosan preparations (HCH&SCH) was mixed up with KBr. FTIR analysis was performed using FT-IR Spectrometer Model: NEXUS- 870. The FTIR instrument was operated in the frequency range of 400-4500 cm^{-1} and the spectra were recorded at the resolution of 4 cm^{-1}.

Before carrying out the FTIR spectroscopy, moisture content present in both chitosan samples were to exclude the interruption of water molecules with the chitosan. The powdered form of HCH and SCH were dried in a hot air oven for 3 days before the proceeding to FTIR spectroscopy. The moisture-free samples were then analysed by FTIR spectroscopy [24].

X-ray Diffraction (XRD) analysis

X-ray diffraction or X-ray crystallography is most widely used, versatile analytical technique to determine the crystalline structure using phase identification and dimension of a unit cell [30]. Generally, two types of structural data can be obtained by XRD: Geometric structure and electronic structure. In geometric structure, the location of atoms in a molecule can be identified at atomic resolution, whereas in an electronic structure characterization is focused on core and valence electrons. To determine the geometric and electronic structure of a material various spectroscopic techniques are employed in XRD.

The spectroscopic techniques utilized in XRD are X-ray photoelectron spectroscopy (XPS), X-ray absorption spectroscopy (XAS), X-ray emission spectroscopy (XES), and X-ray Auger spectroscopy. The type of X-ray spectroscopy to be used depends upon the structure or the oxidation state of the materials. For every crystalline material the diffraction pattern is unique and so XRD is versatile in identifying the crystallinity of different substances [31].

In this study, the crystallinity of the Horseshoe crab chitosan (HCH) and Commercial Shrimp chitosan (SCH), were evaluated by wide-angle X-ray diffraction (WAXD) analysis using XRD (Rigaku Corp; Japan). The powdered form of HCH and SCH are mounted on the goniometer and rotated gradually while bombarding with the X-rays. The X-ray diffractometer was operated with CuKα radiation (40 kV, 30 mA) at wavelength λ= 1.54nm. The diffraction patterns were scanned in continuous mode at a rate of 1°/min from 5°- 45° over the 2θ range.

The crystallinity index of the isolated chitosan was obtained from the ratio of the area of the crystalline contribution (A_{crist}) to the total area of the diffractogram (A_{total}). The crystallinity index was obtained by subtracting the amorphous contribution from A_{total} [32].

$$\% \text{ Cristallinity index (Crl)} = A_{crist} / A_{total} \times 100$$

Thermal analysis

Thermal analysis is the most widely used study to reveal the material's thermal properties and to optimize its properties. The techniques employed for thermal analysis are thermogravimetric analysis (TGA), derivative thermogravimetric (DTG) analysis, differential thermal analysis (DTA) and differential scanning calorimetry (DSC).

TGA is used to identify the thermal stability, material purity and to determine the humidity of a material. In TGA the amount of weight, loss of a substance is monitored isothermally or under a controlled temperature with the time basis in the air, He, N_2, and other gas or in vacuum condition.

DTA is a technique in which analyte and reference materials are heated at identical thermal cycles, while any sudden change in temperature of an analyte material is recorded. It is used to study the exothermic and endothermic transition of samples with corresponding to the reference.

DSC is also similar to DTA but here the change in the amount of heat required to increase the temperature of a sample and reference material with respect to the temperature. The most commonly used reference material in these techniques is alumina and it is inert to have the acceptable temperature as with processing temperature [33]. In this study, the techniques used to acquire the thermal behaviour of HCH and SCH are TGA, DTA, and DTG.

Thermogravimetric analysis (TGA)/ Derivative Thermogravimetric (DTG) analysis

TGA and DTG were performed using Perkin Elmer Pyris Diamond DSC with a heating rate of 10°C/minute and a dynamic synthetic atmospheric air with the flow of 100ml/minute was set as a standard condition. The heating rate was started and ended from 50°C to 650°C. At the initial time, the weight of the HCH and SCH were weighed around 5.4mg and 5.2mg. TGA was implemented to measure the change in mass of the sample when it is subjected to an isothermal temperature in a controlled way. DTG was employed to examine the derivative of TGA. It also used to obtain the accurate temperature at which the weight loss takes place.

Differential thermal analysis (DTA)

DTA was evaluated using Perkin Elmer Pyris Diamond DSC under a flow of 100ml/min of dynamic atmospheric air with the heating rate of 10°C/minute from 50°C to 650°C constantly. It was performed to evaluate the amount of heat flux required for a glass transition and crystallization of HCH&SCH either exothermically or endothermically.

Scanning Electron Microscopy (SEM)

The scanning electron microscopy gives the information about the morphology and composition of the produced material. SEM visualizes the selected point locations in solid surface and the powder particles by emitting high energy electrons on the surface of the material and also with the help of some functional device it determines the dispersed chemical composition of the material. When the electron beam hit the solid surface with high kinetic energy, it produces secondary and backscattered electrons. These electrons will produce 2D SEM images and find the structure and chemical composition of the material [34].

The chitosan from horseshoe crab (HCH) and commercial chitosan (SCH) was observed under scanning electron microscopy. The images were captured using POLARON-SC7620, Carbon Accessory, Model-CA7625, manufactured by Carl ZEISS SMT, Germany. The samples were kept on carbon tape and it was coated with gold using a sputter coater in the vacuumed atmosphere before the analysis. SEM was operated at the accelerated voltage of 20kV. The topography of the chitosan samples was analyzed from the image.

RESULTS

Yield of Chitosan

The molecular weight of the chitosan obtained from the horseshoe crab carapace was found to be 497 kDa. The chitosan yield was dependent on the acid and alkali concentrations that were used during the extraction process. The chitin was removed by deacetylation process using 50% NaOH. It was noticed that the chitin particle size decreased, while the yield of chitosan was increased when the chitin was treated with 50% NaOH. Finally, the deacetylation of chitin resulted in the higher yield of chitosan. The final yield of chitosan after purification was found to be 75% of the total isolated chitosan from the horseshoe crab carapace.

Characterization

Degree of deacetylation from FTIR spectroscopy

The degree of deacetylation is dependent on the number of glucosamide bonds in the biopolymer chain with respects to the total number of units, which represents the formation chitosan from chitin. The degree of deacetylation was calculated from FTIR data for both the chitosan samples. The bands at 1221 cm^{-1} and 1505 cm^{-1} were chosen as measuring and reference bands respectively. DDA was found to be 83.90% chitosan which is obtained from Horseshoe crab (HCH) and commercial chitosan (SCH) the percentage of DDA was found to be 80%.

Ash content

The prepared chitosan had an ash content of 0.350±0.044%. The ash content in chitosan is an important parameter that affects its solubility, viscosity and also other important characteristics. Low ash content was achieved in the chitosan derived from horseshoe crabs, since theydo not have high calcium carbonate in their shells.

Loss of mass on drying

Loss of mass on drying the prepared chitosan was determined by the gravimetric method. The water mass loss was determined by drying the sample to constant weight and measuring the sample after and before drying. The obtained loss of dry weight was found to be 10%.

pH

The pH measurements of the chitosan solutions were carried out using a microprocessor pH meter. The obtained pH was 8.5 for both the chitosan's.

Solubility

The solubility of the chitosan is important for its quality, where the higher solubility leads togood quality chitosan. Chemical method was followed to determine the solubility of horseshoe chitosan and commercial chitosan. The solubility of horseshoe crab chitosan was found to be 93%, whereas for commercial chitosan was 87% which is lower than the isolated chitosan. It is due to the incomplete removal of proteins from the chitosan in the deproteinization steps.

Viscosity

The viscosity is the important parameter in the determination of molecular weight of the chitosan. Higher molecular weight of chitosan often leads to highly viscous solutions, which may not be desirable for industrial handling. Chitosan viscosity decreases with an increased time of demineralization. Viscosity of chitosan in acetic acid tends to increase with decreasing pH but decreases with decreasing pH in HCl, thus giving rise to the definition of 'Intrinsic Viscosity'. The obtained viscosity of Horseshoe chitosan and commercial chitosan was found to be 304 cPs.

Moisture content

Results of the moisture content of the isolated chitosan and commercial chitosan value was found to be 70% and 72.1% respectively.

Fourier Transform Infrared (FT-IR) Spectroscopy

FTIR was used to determine the structure of the polymer. It was recorded for both samples of chitosan, commercial and horseshoe crab carapace. The different absorption bands ranging from 4000 cm^{-1} to 500 cm^{-1} was recorded in the FTIR spectra of chitosan.

Fig.3 shows the FTIR spectra of horseshoe crab chitosan (HCH) and commercial chitosan (SCH). The Fourier spectrum of HCH was compared with SCH and analyzed for its similarity of the functional groups which is present in the two forms of chitosan.

The characteristic peak at 3435.81 cm^{-1} corresponds to the hydroxyl (-OH) stretching bands of both the HCH and SCH respectively. The peak at 2995 cm^{-1}& 2988.94 cm^{-1} represents the presence of C-H group. The band at 1878 cm^{-1} has the stretching of C=O band in pyranose ring of isolated chitosan, while for commercial chitosan the stretching of C=Oband was at 1864.56 cm^{-1}. The absorption bands of isolated chitosan were similar to commercial chitosan. In addition, the stretching of primary NH group band at 1512.65 cm^{-1} (HCH) and 1505.47 cm^{-1} (SCH) was observed and this indicates to the presence of primary amine group. The bands at 1221 cm^{-1} and 1214.20 cm^{-1} were observed, which could be inferred as a complex of NHCO with the secondary amines (Tab. 1).

From the FTIR spectrum results, chitosan derived from of horseshoe crab chitosan and commercially available chitosan were compared. The results showed that the both chitosan's were considerably similar in structures and functional groups. The Comparison of the results shows the similar structure with the slight variation in the band shifts.

X-ray Diffraction (XRD) analysis

The XRD analysis was carried out to identify the degree of crystallinity of the isolated chitosan and the commercial chitosan. TheXRD patterns obtained from the chitosans are shown in Fig.4. It provides accurate information about the crystalline content, which plays a great role in physical and biological properties of the polymer materials. The strong reflections peaks were observed at2θ= 9.7° and 2θ= 19.81° for horseshoe chitosan and at 2θ= 8° and 2θ= 20° for commercial chitosan respectively, which indicates that both the chitosans are semi crystalline in nature (Tab. 2). This could be due to the incorporation of water molecule in the lattice space of the crystals in chitosan. The crystallinity found in the chitosan is owing to the linkage of large number of hydroxyl and amine groups in its structure. These groups forms strong intermolecular hydrogen bonds which lead to strong regularity in chitosan structure and results in the crystalline appearance. From the obtained results, it was observed that there is a slight change in the peak values between HCH and SCH. Those slight changes may be due to the chitosan from different sources.

Crystallinity index

The crystallinity index was calculated from the XRD data. The Crl of the isolated chitosan was found to be 86%, while for commercial chitosan was found between 70% - 85%. The crystallinity index was obtained by subtracting the amorphous contribution from $A_{total.}$

Thermal analysis

Thermal gravimetric (TG), Derivative thermogravimetric (DTG) and Differential thermal analysis (DTA) were obtained for the samples of commercial chitosan and Horseshoe crab chitosan are shown in Fig.5.It was carried out under the temperature from 50° C to 650° C with the heating rate of 10°C/min. Under the dry atmospheric air, polymers have three main steps in weight loss. In the first step the polymer undergoes degradation process and in the second step the polymer undergoes decomposition and later the residues remain. The initial weight loss occurs at 100°C which is due to the evaporation of water molecules from the sample. DTA involves three events, the first even are endothermic that is dehydration and next two events are related to exothermic decomposition in according to TG.

TG&DTG

The TG curve of HCH and SCH is shown in Fig.5. It was found that the thermal degradation started at 50° C up to 120° C which is due to the loss of water molecules from the sample and the second degradation occurred at 260° C to 300° C which is due to the depolymerisation of polysaccharides of the chitosan. The third degradation occurred from 350° C to 500° C which is due to the residual molecules from the chitosan. Residues are the minerals impurities from the raw material. The mass loss occurred at two phases, in the first phase it was 5% between the temperatures of50° C to 120° C and in the second phase it was15% between the temperature 260° C to 350° C (DTG_{max}: 290°C). The final weight loss was 75% between the temperatures 350° C to 550° C. While for the SCH chitosan, all degradation steps occurred at the similar temperature of HCH with slight change in DTG_{max} temperature at 300° C. Both the chitosan

material had similar thermal temperatures, structure and composition which were confirmed by thermal analysis, XRD and FTIR.

From the obtained temperature, it was observed that both HCH and SCH have same degradation temperature profiles.

DTA

The DTA curves of horseshoe crab chitosan and commercial chitosan are given in Fig.5.The decomposition of chitosan is an exothermic reaction with the peak at 315°C and 305°C of both HCH and SCH, respectively and occurs between the temperature ranges 260°C to 330°C. It can be due to the decomposition of the residues such as amino and N- acyl groups from chitosans. Both the chitosan materials have similar decomposition temperature, though it has same structure and compositions.

Scanning Electron Microscopy (SEM)

The morphological structure was examined by using scanning electron microscopy. The morphology has smooth surface and crystalline structure. It shows the chitosan structures were shaped like nanofibers. The SEM images of the surface morphologies of the chitosan extracted from the horseshoe crab and commercial chitosan are easily differentiable from one another (Fig.6 a, b). The surface of the chitosan was irregular with flake like appearance. SEM micrographs exhibit no significant difference between commercial and horseshoe crab chitosan and for both samples (Fig.6 a, b) the surface appears as fibres.

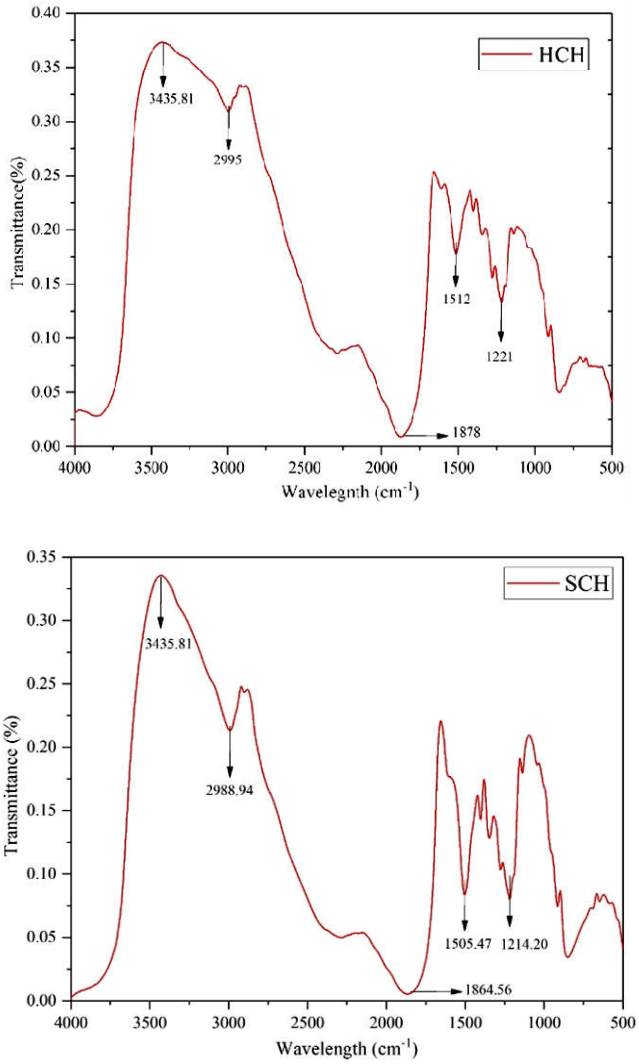

Fig- 3 FTIR Spectra of Horseshoe crab chitosan (HCH) and Commercial chitosan (SCH)

Table.1- FTIR bands of Horseshoe crab chitosan (HCH) and Commercial chitosan (SCH)

Wavenumber (cm^{-1}) frequency		Vibration modes
HCH	SCH	
3435.81 cm^{-1}	3450 cm^{-1}	(-OH) stretching bands
2995 cm^{-1}	2988.94 cm-1	C-H group in pyranose ring
1878 cm-1	1864.56 cm-1	Stretching bands of C=O
1512.65 cm-1	1505 cm^{-1}	Stretching bands of primary NH groups
1221 cm-1	1214.20 cm-1	Complex vibration of NHCO groups

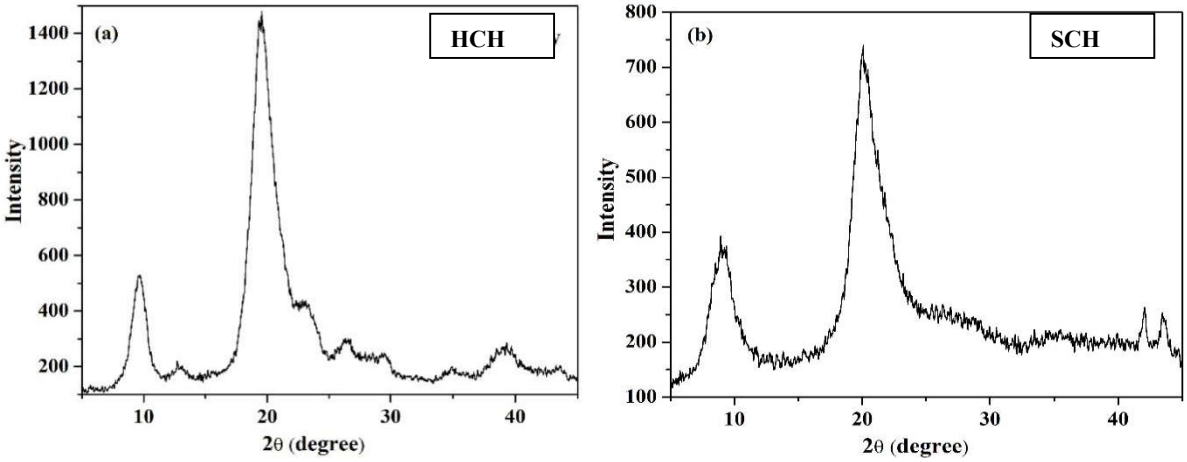

Fig- 4XRD patterns of a) Horseshoe crab chitosan (HCH) and b) commercial chitosan (SCH)

Table.2- Data obtained from XRD analysis of HCH and SCH

Chitosan	Peaks	
	HCH	SCH
	9.7° and 19.81°	8° and 20°

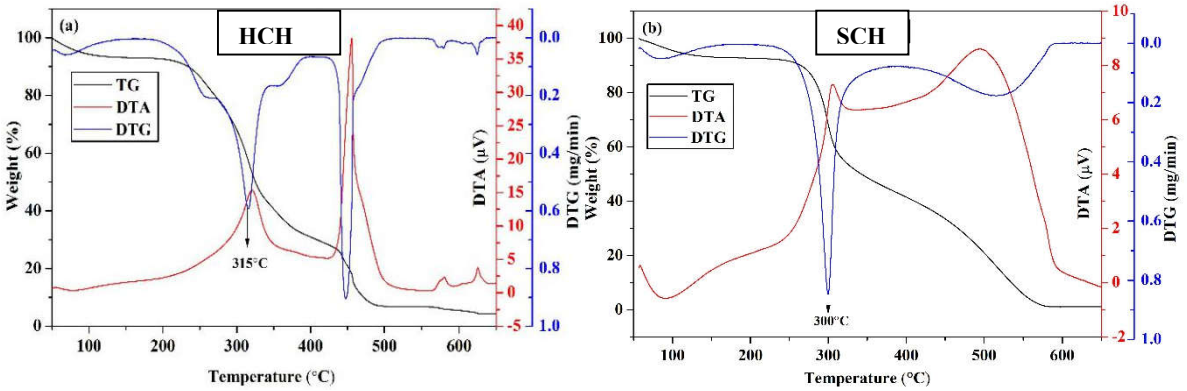

Fig- 5 TG, DTG, DTA curves of Horseshoe crab chitosan (HCH) and Commercial chitosan (SCH)

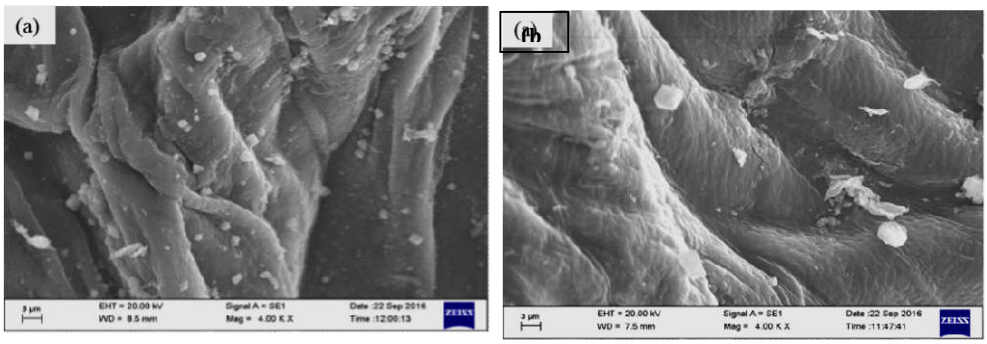

Fig- 6 SEM micrographs of Horseshoe chitosan (HCH) and commercial chitosan at 4.00KX magnification.

Table. 3- Characteristics of chitosan obtained from the Horseshoe crab.

Characteristics of isolated chitosan	Obtained values
Chitosan yield (%)	75%
Average molecular weight	497 kDa
Degree of deacetylation (%)	75 %
Viscosity	304 cps
Ash (%)	0,350±0,044%
Loss on drying (%)	10%
pH	8.5

DISCUSSION

Chitosan is a versatile natural amino polysaccharide, which happens to be the second most abundant natural polymer. Many biochemists have found chitosan to be biocompatible, biodegradable and nontoxic, thus making possible its wide applicability in food industries and pharmaceutics as a potential formulation excipient. Chitosan was prepared by processing dead horseshoe waste (shells) whichincludespartial deacetylation of chitin. For the chitosan isolated from the horseshoe crab shells, yield was found to be about 75% and its physicochemical properties were also characterized. In a previous study [35] the chitosan was isolated from shrimp waste (shells) and the obtained molecular weight of the chitosan was found to be 35.49%, which was less than our present finding where the value was 77.15%.

In another earlier study [37], chitosan was developed from crab shell and was investigated for its physical and chemical properties. The optimal conditions for deproteinization of crab shell waste were found to be 3.5% NaOH at 65°C and demineralization with 1N HCL at ambient temperature for 30 min with a solid solvent ratio of 1:15 (w/v). Chitin deacetylation was carried out at 15 psi/121°C using 50% sodium hydroxide solution for 15 min and resulted in the range from 23.63% to 52.17%. In our present research work the deacetylation was carried out by using 50% NaOH which resulted in higher yield of chitosan.

The results obtained reveal that carbon to nitrogen ratio of the chitosan extracted was 5.9 with the degree of deacetylationat60.69% and 60.66%, calculated from the elemental analysis and the FTIR spectra of chitosan respectively. Also the values of DDA was obtained from the FTIR spectra and it was found to be 83.90% for HCH and 80% for SCH which was quite higher than [37]. It was due to the reduction of molecular weight caused the higher degree of deacetylation.

Chitosan is known to be produced from shrimp waste by chemical method involving demineralization, deproteinization and deacetylation, where in the results showed that 3% HCI and 4% NaOH were suitable concentration for demineralization and deproteinization, respectively at ambient temperature (28±2°C). In our study, chitosan with a high degree of deacetylation (81.24%) and high solubility (97.65%) was obtained by deacetylation with 60% NaOH for 24 hours at 60°C. Purified chitosan was characterized for intrinsic viscosity (13.2dl/g), molecular weight (1.05×106 Dalton), FBC (427.98%), WBC (537.29%) as well as yield (15.4%)[38]. Thus our in research findings, the higher percentage of deacetylation showed higher solubility for isolated chitosan, which was found to be 93% for horseshoe crab derived chitosan and 87% for commercial chitosan. This slight difference may be attributed to the presence of protein molecules and other impurities in the sample. Since it is based on a chemical method including reaction with amino group, the presence of 50 protein contaminants remaining in the sample during the analysis process could adversely interfere with the results. In the commercial chitosan, the highest residues left

over are about 8% -N. This indicates that the commercial chitosan had some protein impurities left over with it.

As for the viscosity, it was found to be 304 cPs, which is suitable for designing drug delivery system [35]. In our present research findings, the extracted chitosan shows 20% better viscocity which can be used for food preservation in food industries. When compared to the previous study the isolated chitosan has fewer amounts of proteins which may be preferable for food preservation.

The XRD patterns were investigated for isolated chitosan and standard chitosan. From the XRD results it was found to have similar crystalline peaks as reported in the earlier study [40]. The raw material chitosan was evaluated for XRD patterns and the results exhibited the crystalline peaks at 10.75° and 20.14°. For deacetylated chitosan the crystalline peaks were found to be at 10.97° and 20.14° which has similar values. The degree of crystallinity is a function of DDA value as reported [41]. In our present findings it was found to be having values such as2θ= 9.7° and 2θ= 19.81° for raw chitosan and for deacetylated chitosan it has 2θ= 10° and of2θ= 20°, which is preferable. The crystallinity was due to the presence of large number of hydrogen and amine groups which leads to forming of strong intermolecular structures in the chitosan as reported by [42].

A thermal property of the chitosan was investigated by using TGA [43]. All the samples exhibited the melting endothermic peak in the range of 118° C to 135° C. Chitosan has different endothermic ranges that indicates to different water holding capacities and strength of water polymer interaction.In our finding chitosan from horseshoe crab and commercial chitosan showed different structures when compared to previous studies. The surface of commercial chitosan appears as fibres whereas horseshoe crab chitosan appears layer of flakes like structures.

The chitosan were extracted from different crustaceans shell wastes and investigated for it solubility, ash and moisture were also determined and compared with the commercial chitosan. The chitosan from different shells had varied moisture content due to the hydroscopic nature of chitosan whereas commercial chitosan has 10% less moisture [44]. In our present study the ash content for isolated chitosan was 0.350±0.044% which is less than the commercial chitosan thus it indicates the isolated chitosan had less calcium.

The chitosan extracted from crab carapace had crystallinity index of 87% while the chitosan derived from shrimps varied between 66 and 85.8% [45]. This proves that the deacetylation process was efficient, due to removal of a portion of the acetyl groups from the chitin structure and also owing to the greater presence of primary amine groups in the chitosan [46].

CONCLUSION

Highly pure chitosan was isolated from horseshoe crab carapace by the chemical treatment method. The isolated biopolymers were characterized and confirmed by different analytical tools like FTIR, XRD, TGA, SEM and compared with the commercial available chitosan. As per our observation and finding, the horseshoe crab carapaces can be considered to be good and dependable alternative source of chitin and chitosan to crab, shrimp, crayfish, krill and can be used in various application as per the need.

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