



Utilization of Shellfish Industrial Waste for Isolation, Purification, and Characterizations of Chitin From Crustacean's Sources in Pakistan

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Accepted: 31 December 2020 / Published online: 21 January 2021

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Abstract

Chitin is the most versatile and promising biomaterial after cellulose which can primarily be isolated from crustacean exoskeleton. It has great economic values because of their excellent chemical and biological properties, and their biomedical and industrial applications. This research work aims to report the yield and quality of chitin which has been isolated from crustacean's waste of shellfish industry of Pakistan using chemical method alternating sodium hydroxide, hydrochloric acid, and hydrogen peroxide. The physicochemical properties of chitin extracted from shrimps were compared with commercial standard chitin (C9213) procured from Sigma-Aldrich. The yield obtained of isolated chitin was 23.78% with ash and moisture contents of 0.05% and 6.3% respectively. A low percentage of moisture and ash contents in isolated chitin indicate the efficiency of the extraction protocol employed. This shrimp's chitin compares with standard chitin using FTIR spectroscopy, proton solid-state NMR (¹HSSNMR), X-Ray diffraction (XRD), Scanning electron microscopy (SEM). The thermal behavior of chitin (both standard and samples) was checked with thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC). Both standard and extracted samples of chitins exhibited similar physicochemical and structural properties. The biological behavior of samples was also checked with different biological activities, which showed much dependence on the structure and concentration of samples.

Keywords Chitin · Shellfish industry waste · FT-IR & ¹HSSNMR · Thermal properties · Biological activities

Introduction

Aqua wastes are accessible in a large quantity in the environment, which has the potential for valuable products. During the last few decades, almost 7.3 million tons of biomass wastage has been produced every year [1–3]. If this wastage not treated properly exerts a negative impact on the environment because of their high biological and chemical oxygen demand (BOD, COD), fat-oil-grease (FOG), organic matter, pathogen, total suspended solids, and other nutrients etc. [4,

5]. However, these aqua wastes are considered as a potential source of many by-products.

The concept of environment-friendly technologies of by-products from aquatic organisms may have broad potential to produce different commercial products that are used in various disciplines [6]. Due to less biodegradability of wastage, almost 70% of fish and shellfish processing plants (viscera, heads, cut-offs, bone, skin, etc.) convert the wastage into valuable materials which are used in different fields such as medicine, water treatment, pharmaceutical, agriculture, veterinary, fertilizer, and textile [7]. Recently fishery waste such as shrimps, krill's, crabs, etc. used for the production of chitin, dietary supplements, and other biological compounds. These species contain approximately 25% protein, 25–30% chitin, and 40–50% calcium carbonate [8].

Chitin a homopolymer (1,4)-linked-N-Acetyl-D-glucosamine, is the major polysaccharide (after cellulose) found in crustaceans, insects, and fungi [9]. Globally 10¹²–10¹⁴ tons of chitin is produced every year [10, 11]. Three different polymorphous (α , β , γ), of chitin, are available in nature. Among all these forms α -chitin is a major naturally available

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form; it can be extracted from the exoskeleton of crustaceans (crabs, shrimps) and cell wall of fungi [12, 13]. Chitin in the exoskeleton of crustaceans is closely linked with minerals, protein, pigments, and lipids that have to be eliminated. Several techniques for extraction of chitin have been reported previously [14, 15] in which biological and chemical techniques are mostly used.

The biotechnological processing of crustacean (shrimps, crabs) wastes have many advantages like they are a less polluted, cheap, and relatively simple method, but this method has a huge drawback in which the efficiency of deproteinizations and demineralization is low as compared to chemical extraction [16]. The biological method is a time-consuming process in which microorganisms are grown under specific conditions (pH, temperature, pressure, etc.). Lactic acid bacterial fermentation may not be good for physicochemical properties of extracted chitin as compared to that chitin which is isolated from chemical processing [17]. Biotechnological processing is performed under laboratory conditions and thus may not be applicable at a large scale [16, 18]. Generally, chemical extraction seems to be the most efficient way for isolating the purest chitin because in biological processing impurities easily affect the quality of chitin [19].

At the commercial level, chitin is isolated from crustacean's shells through chemical methods [4, 20]. The chemical process for extraction of chitin from crustacean shells comprises three major steps: (i) removal of the protein contents in alkaline medium (deproteinization) in which shells are treated with a dilute solution of KOH or NaOH; (ii) removal of inorganic contents like salts and minerals (demineralization) by using dilute HCl solution [21]; (iii) decolorization is the last main step in which residue treated with acetone or H₂O₂ [22]. This process can be considered as a contribution to the biotechnological valorization [23].

Due to excellent properties of chitin such as biodegradability, nontoxicity, biocompatibility, capacity to form a foam, adsorption, and chelate metal ions make it an attractive biopolymer which is used in different fields such as medicine, pharmaceutical, agriculture, textile, food industry wastewater treatment, and cosmetics [24–27].

In Pakistan, the seafood processing industry rapidly increases with the growing culture of seafood. During food processing, disposal of wastage becomes a crucial problem. From a technological point of view, it would be felt necessary to develop a low cost and less hazardous (towards environment and biodiversity) chemical procedure which would be suitable for the extraction of high-quality chitin from crustacean (shrimps) wastage. So, the purpose of this study is to utilize this wastage, for the production of valuable products such as chitin bio-polymeric compound and their derivatives. Therefore, the present project was planned for the extraction and purification of chitin from shrimps wastage and its evaluation of physicochemical and biological

properties following the modified novel chemical method at room temperature. Dissolution of chitin was made with chain scissoring followed by using combination of solvents and hence biological activities of chitin have been reported without converting into chitosan. There are no data concerning the comparison, as the comparative analysis of isolated chitin with that of Sigma-Aldrich is relatively rare. None of the report is available comparing the properties of prepared chitin with Sigma-Aldrich grade chitin. The aim of this research was to utilize the shellfish industrial waste hence isolation, purification, and characterizations of chitin from crustacean's sources in Pakistan was done and results were compared with that of standard chitin procured from Sigma-Aldrich. The structural characterization of extracted chitin and procured chitin (C9213-from Sigma-Aldrich was done using the FT-IR spectroscopy and Solid-state ¹HNMR; morphological analysis with X-Ray diffraction (XRD) and scanning electron microscopy (SEM) and thermal properties using TGA & DSC analysis.

Experimental

Sample Preparation

A huge amount of shrimp, crustacean's wastage are available in Pakistan. Crustacean's waste was collected from the shellfish industry (Karachi, Pakistan). Collected exoskeletons were separated and washed with distilled water, packed in Ziploc bags and stored in a controlled temperature cooling cabinet. After 24 h the samples were dried in controlled temperature until the constant weight was obtained. Approximately 1 kg of the sample (crustacean's waste) was weighed by using an automated weighing balance and converted into powder. The fully grounded skeleton powder was kept in an airtight jar for extraction.

Extraction of Chitin

For extraction of chitin, the chemical procedure described by Puvvada et al. [28] was followed with slight modifications. This established method of chitin extraction has three steps, i.e., deproteination, demineralization, and, decoloration.

Deproteinization

This extraction method was performed at a laboratory scale by using a 1000 mL beaker. A 100 g powdered sample was soaked into 2.0 M solution of sodium hydroxide in the ratio 1:10 (w/v) then left for 50 h at 37 °C with constant stirring during this procedure the pH of the sample was measured and reported (pH = 11.0 to 13.0). After that remaining sample was filtered out and washed with distilled water until

neutral pH (pH 6.5 to 7.5) was achieved. Before the demineralization process, the water was removed in an oven at 50–60 °C temperature.

Demineralization

The deproteinated sample was treated with 1.0 M HCl solution at room temperature in the ratio of 1:10 (w/v) for 28 h with constant stirring and the pH of the solution was maintained at 1.0 to 3.0. Almost, after 24 h, the emission of CO₂ gas was observed which reveals the presence of minerals in the sample [21]. Furthermore, the more emission of CO₂ gas indicated that high quantities of minerals are present in the sample on the other hand, no emission of CO₂ gas confirmed that the demineralization process was completed. Following the above procedure, the reaction sample was washed with distilled water until the neutral pH was obtained (pH 6.5 to 8.0). In the next step, the bleaching was followed by drying of the sample in an oven at 40–50 °C until constant weight obtained.

De-Coloration

The product obtained after the deproteination and demineralization process can be called as chitin. In the decoloration step, the dried sample was treated at room temperature with 10% H₂O₂ in the ratio of 1:10 (w/v) with constant stirring until the colorless sample was obtained [29]. After the removal of all the pigments, the sample was again washed with distilled water until the neutral pH was obtained. The final product was dried in the oven at 40–50 °C and stored in an airtight jar for further use, analysis and characterization. All the extraction protocol are summarized in Fig. 1

Chitin Yield

The yield (%) of freshly extracted chitin was calculated with the following formulas which describe the extracted chitin weight of shrimps relative to the dry weight of total waste [30].

$$\text{Chitin extraction yield (\%)} = \frac{\text{Dried extracted chitin weight (g)}}{\text{Total crustacean's waste (g)}} \times 100$$

Proximate Analysis of Freshly Extracted Chitin

Proximate analysis was conducted to determine the ash and moisture contents of chitin. The analyses were carried out using AOAC standards [31].

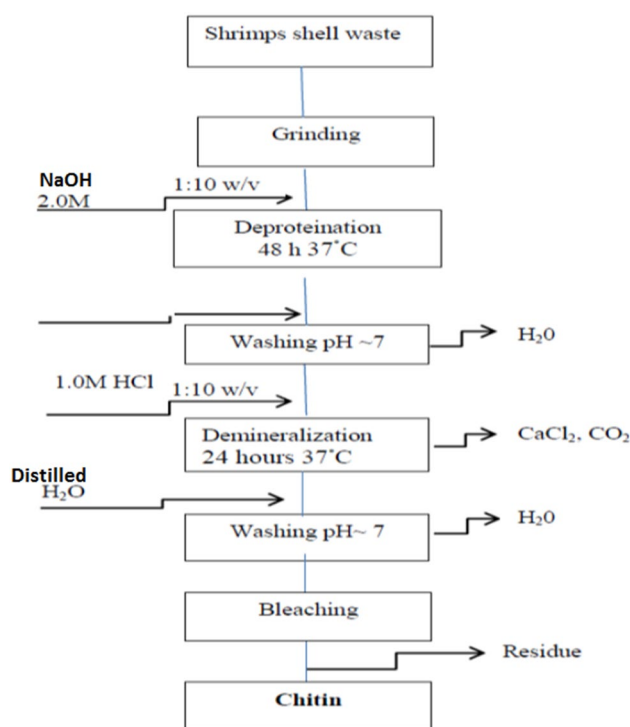


Fig. 1 Flow-sheet for isolation of chitin from crustacean's waste

Characterization of Freshly Extracted Chitin

The following spectroscopic and other evaluation procedures were followed to characterize the freshly extracted chitin.

FT-IR Analysis

FT-IR spectra were recorded for freshly extracted chitin on Bruker-IFS 48 Fourier transform infrared spectrophotometer (Ettlingen Germany) linked with ATR (Diamond Crystal). The resolution of the scanner comprised 4 cm⁻¹ with several scans, frequencies ranging from 4000–500 cm⁻¹ at room temperature.

Solid-STATE ¹H-NMR Analysis

Solid-state one-dimensional ¹H-NMR spectrum of freshly prepared chitin was also recorded by using Bruker BioSpin GmbH. The spectrophotometer was worked on different frequencies 400.25 Hz at 294.5 K.

X-Ray Diffraction Analysis

A Crystalline or amorphous pattern of freshly isolated chitin was taken by using Siemens D-5000 X-Ray diffractometer at

25 °C, with K α Cu ($\lambda = 15.4$ nm, 40 kV and 30 mA) source of radiation. The sample scan was recorded in the range of $2\theta = 0$ to 70° at a speed of $2^\circ/\text{min}$.

Thermo-Gravimetric (TG) and Differential Scanning Calorimetry (DSC) Analysis

The TG/DSC analysis of isolated and standard chitin was calculated using SDT Q600 V20.9 Build 20 instrument techniques in which the sample was heated from room temperature to 650°C at the heating rate of $10^\circ\text{C}/\text{min}$ with a continuous flow of dry N_2 gas.

Scanning Electron Microscopy Analysis

Structural features of the chitin sample were also be analyzed by using scanning electron microscopy SEM (model S4800).

Evaluation of Biological Behavior of Freshly Extracted Chitin

The biological aptitude of biological aptitude, including antibacterial, antioxidants, and cytotoxicity of freshly extracted chitin was studied and reported.

Antimicrobial Activity

A well diffusion method was used to evaluate the antibacterial activities of freshly isolated chitin and standard chitin against *Escherichia coli* and *Bacillus subtilis* bacteria [32]. For biological activities 10 mg/ml of chitin solution was prepared by dissolving the chitin in solvents (a mixture of N-methyl-2-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO) with ratio 2:1) with constant stirring at room temperature. For preparation of nutrient agar media almost 30 g agar was dissolved in 1 L distilled water and shaken until a homogenized solution was prepared. For sterilization flask having growth media autoclaved at 120°C for 15 to 20 min and cooled at room temperature. The bacterial strains (100 $\mu\text{L}/100$ mL) poured into growth media and prepared the sterilize Petri plates until the media completely solidify for further use. Afterward, 8 mm wells were prepared into the surface of media with a cork borer, and approximately 150 μL prepared chitin solution was transferred to each well against the streptomycin drug solution, which was used as a standard drug. The plate was incubated at 37°C for 24 h. The zone of inhibition around the sample was measured in millimeters. The bacterial activity was performed in triplicates.

Antioxidant Activity: DPPH Radical Scavenging Activity Assay

The DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical-scavenging activity of freshly extracted chitin as compared to standard chitin was calculated as described by [33]. The 100 μL chitin sample solution which is prepared earlier in a mixture of N-methyl-2-pyrrolidone (NMP) and dimethyl sulfoxide (DMSO) solvents was mixed in DPPH solution with constant shaking. The mixture was incubated in the dark for 30 min at room temperature and DPPH reduction activity was measured at 517 nm against the blank sample in which solvents were used with DPPH solution instead of chitin. Butylated hydroxytoluene (BHT) used as standard. DPPH radical scavenging activity was calculated as follows:

Scavenging activity (%)

$$= \frac{\text{Absorbance of blank sample} - \text{Absorbance of prepared sample}}{\text{Absorbance of blank sample}} \times 100$$

Hemolytic Assay

Hemolytic assay of freshly prepared chitin and standard chitin was studied following the established procedure [34]. A 5 mL fresh human blood was poured into the falcon tube. The plasma layer was separated after 5 min centrifugation and the remaining blood pellet was washed with PBS solution, the process repeats three times. A 20 μL chitin solution and 180 μL of blood cell suspension were mixed in an Eppendorf tube and again centrifuged. A (100 μL) upper layer of sample and blood mixture was taken from Eppendorf and 10 times diluted with 900 μL chilled PBS solution. Then a 100 μL solution from it added into a culture plate. Phosphate buffer solution (PBS) was taken as a negative control and Triton-X was used as a positive control in this process. The absorbance of the chitin sample was recorded at 576 nm using ELISA plate reader. The %age of hemolysis was calculated with the following formula:

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Results and Discussion

The present results demonstrate the structural, biological, functional, and physicochemical properties of freshly extracted chitin concerning their commercial standard chitin (C9213). The collected sample (from the Karachi shellfish industry) was washed dried and processed (as mentioned in the experimental section). The chitin was isolated by

following the three major steps i.e., deproteinization, demineralization, and decoloration (Fig. 1).

Extraction of Chitin

Chitin is a natural biopolymer found in fungi, crustaceans, and insects. The chemical analysis showed that shrimp's waste contains about 23.7% of chitin. Therefore, shrimps considered a major source of chitin production. Chitin is bounded with different compounds like protein and minerals, (calcium carbonate, calcium phosphate, etc.) so, the extraction of chitin is a very crucial process [35–37]. During the chemical reaction, the protein-chitin covalent bond complex destroyed with alkali treatment at ambient temperature [38, 39]. Elemental analysis and clarity of the sample showed that protein contents decrease by increasing the time duration for 52 h. In the isolation of chitin from shrimps waste, 40 to 50% of minerals should be removed in the second stage. For removing inorganic contents deproteinized shrimps exoskeleton, treated with dilute 1.0 M solution of hydrochloric acid (HCl) at room temperature for 28 h. In the demineralization process, calcium carbonate is removed at the initial stage and other inorganic contents are removed after that. The CO₂ gas bubbles are produced when acid penetrates the surface of shrimp's shells. The maximum emission of CO₂ gas exhibits that a high quantity of minerals is present in the sample, so minimal or no emission of CO₂ gas confirmed that the demineralization process is completed. In our study, extracted chitin has a pale yellow color which was further treated with 10% H₂O₂ for decolorization purpose. After the completion of the due procedure, the color of isolated chitin is white which indicates the minimum amount of protein and inorganic contents in the sample [40].

Physicochemical Properties of Chitin

The isolated chitin possessing fewer amounts of ash (minerals) and moisture contents are enough in comparison to commercial international standards [41]. The proximate composition of chitin varies with species (raw material), weather conditions, a methodology that follows for the extraction process, and many other factors [42]. Quality of chitin depends on the presence of ash and moisture contents, lower the level of these contents indicates the better quality of chitin.

The yield of chitin calculated from the biomass manifested that the yield of the chitin depends on the concentration of HCl (less amount of HCl minimum removal of minerals), extraction time, size of particles, and temperature, etc. [43, 44]. In this study, chitin is isolated by treating the sample with alkali and acid possessing a yield of approximately 23.78% in the total weight of dried shrimps shell waste presented in Table 1. Srinivasan et al. [45] investigated

Table 1 Physicochemical parameters of freshly isolated chitin from crustacean (Shrimps) source

Proximate composition	%Age of contents
Yield	23.78
Ash	0.05
Moisture	6.3

the 30% yield of chitin in dried *P. monodon* shells. Since the literature survey revealed that the yield from crustacean shells (α -chitin) is 7–40% depending on the parent source [46, 47]. Ash and moisture contents are also tabulated in Table 1. Ash and moisture contents are analyzed by AOAC 1990 (Association of official analytical chemist) method [31]. The moisture contents varied in different species, depending on humidity, the intensity of sunlight, and season of harvesting [48, 49]. Ash contents indicated the appropriateness of the demineralization process for removing the mineral (inorganic) contents. Ash contents are found about 0.05% and this is less than that detected by Isa et al. [50]. Studies revealed that the moisture content is 6.3% slightly lower as compared to the literature [28, 51] which was 9.6% and 9.34% respectively.

Characterization of Freshly Isolated Chitin and Commercially Available Standard Chitin (C9213)

FT-IR Analysis

Chemical analysis of chitin showed that shrimps are a good source for the chitin extraction. FTIR spectrum of the freshly isolated chitin identified the different functional groups in the range of 4000 to 400 cm⁻¹. Considering the study of FTIR, strong peaks observed at 3438 cm⁻¹ which indicated the vibrating and stretching of the hydroxyl (–OH) group, which is more visible in the chitin spectrum. Bands that appeared at 3256 to 3100 cm⁻¹ show the strong stretching of N–H group [40]. The absorptions band at 2876 cm⁻¹ detected the stretching vibration of aliphatic compounds (–CH₃ and –CH₂). There are three bands in spectra which confirm the presence of α -chitin. Besides, the splitting of amide I band in isolated chitin spectrum give two bands at 1651 cm⁻¹ and 1618 cm⁻¹ which demonstrate the presence of intramolecular hydrogen bonds of CO · · · HN and CO · · · HOCH₂, and bands at 1552 cm⁻¹ and 1375 cm⁻¹ correspond to the bands of amide-II (N–H stretching) and amide-III (C–N bending) respectively [52]. In addition, a peak appears at 1375 cm⁻¹ exhibited the acetamide linkage, and an intense band at 1000 to 1259 cm⁻¹ was attributed the stretching vibration for (C–O–C) of the 2-(acetylamino)-2-deoxy-D-glucose ring. The absorption peaks appearing at 1067 cm⁻¹ and 1007 cm⁻¹ indicated the presence of C=O and C=C bending vibration, while a peak appeared

at 895 cm^{-1} indicate the ring stretching for β -1,4 glycosidic bonds. The FTIR spectra of standard commercial shrimp chitin (C9213) bought from Sigma-Aldrich is also shown in Fig. 2. The FTIR peaks of isolated chitin in this study were very similar to those of standard commercial chitin. All the reported results regarding the extraction of chitin and its characterization with FTIR spectroscopy are in accord with previous studies [43, 52]. It can be concluded and confirmed from the diamond internal reflection element (IRE) that the isolated chitin from Crustacean's waste having an α -chitin structure.

Solid-State ^1H NMR Analysis

Solid-state NMR (SSNMR) spectroscopy is a kind of nuclear magnetic resonance (NMR) spectroscopy characterized by the presence of anisotropic (directionally dependent) interactions. In solution NMR, spectrum consists of a series of very sharp transitions, due to averaging of anisotropic NMR interactions by rapid random tumbling. By contrast, solid-state NMR spectrum is very broad, as the full effects of anisotropic or orientation-dependent interactions are observed in the spectrum. Despite the elaborate attempts by physical chemists to understand the molecular origins of solid-state polymer properties, all quantitative theories have been limited to describing general and ideal behavior. With the advent of methods such as neutron scattering and solid state nuclear magnetic resonance (NMR) made scientists able to directly probe the molecular characteristics of polymers in the solid state. The NMR spectroscopy is a powerful tool used to characterize the chemical composition of chitin. As the chitin is not soluble in most common organic solvents, hence structure of chitin and its purity was evaluated using solid-state ^1H NMR spectroscopy. The ^1H solid state NMR spectra of standard chitin (procured from Sigma-Aldrich) and the extracted and purified chitin are shown in Fig. 3a and

b. In both these spectra, the chemical shift value of protons attached to sp^3 hybridized carbon lies in the range -0.5 to 15 ppm. The values of NH group and $=\text{C}-\text{H}$ group attached protons lies in the range of 15 to 65 ppm. As can be seen both the chitins had practically identical spectra. Each spectrum consisted of five well-defined and two very small signals. Three signals (two well defined and one very small) corresponding to protons of 2-(acetylamino)-2-deoxy-D-glucose ring were observed between 20 and 90 ppm, indicating high structural homogeneity. It means that the chemical structure of chitin has caused minimal damage during isolation and that the chitin is then suitable for high yield *N*-acetyl glucosamine production [53]. The signal corresponding to

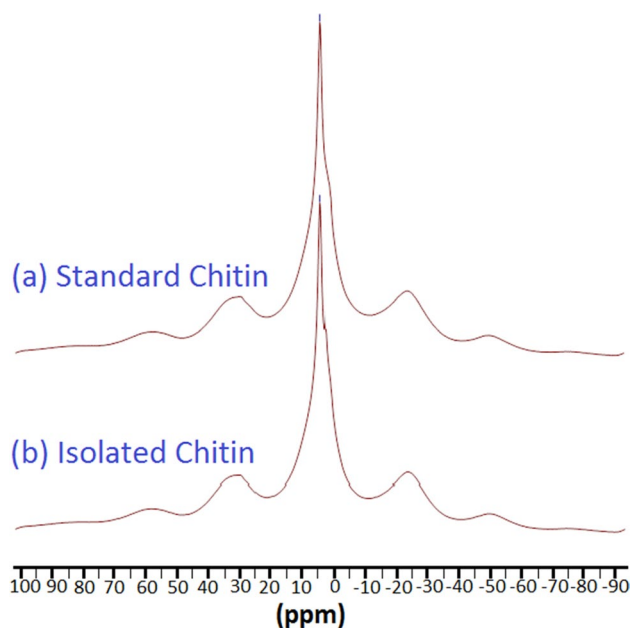
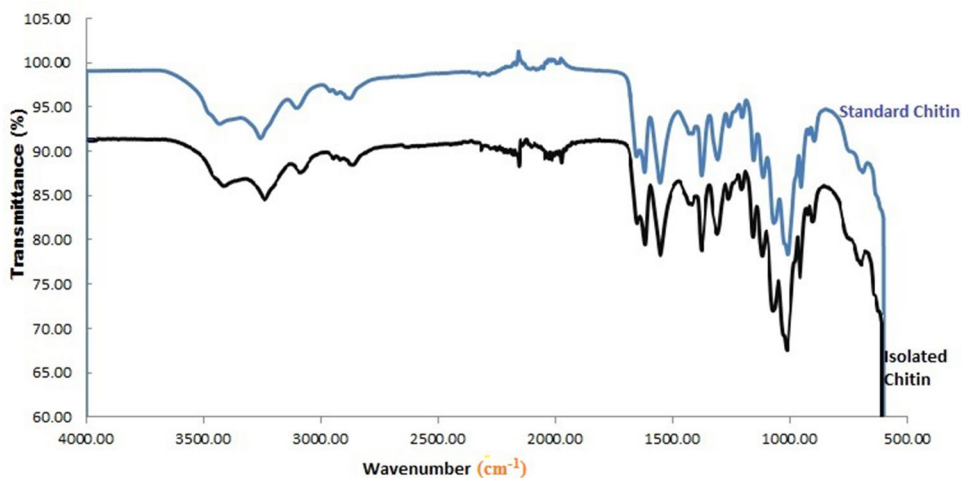


Fig. 3 Solid state ^1H NMR spectra (a) standard chitin (b) isolated chitin

Fig. 2 FTIR spectra of isolated & standard chitin



the methyl substituent is located at – 25 ppm. The aim of substituting enzymatic method by the chemical one for chitin extraction process is mainly providing pure chitin retaining a structure as close as possible to the native form (less deacetylated and less depolymerized).

X-ray Diffraction Analysis (XRD)

The crystalline pattern of α -chitin has been reported Minke et al. [54] that the unit cell is orthorhombic, with dimension $a = 0.474$ nm (4.74 Å), $b = 1.886$ nm (18.86 Å), and $c = 1.032$ nm (10.32 Å) (fiber axis). The space group is $P2_12_12_1$ and this unit cell contains two chains of disaccharides sections that have antiparallel arrangement due to the presence of strong hydrogen bonding. The crystallinity and amorphous patterns of freshly isolated chitin from shrimp’s comparing with standard chitin (C9213) are presented in Fig. 4. It can be observed that the XRD pattern of fresh chitin showed maximum reflection peaks at 19.8°, 20.89°, and 23.7°, and minor diffraction peaks at 26.1°, 34.0°, and 38.9°. From the results, standard commercial chitin also shows the same diffraction peaks in the same position comparing with freshly isolated chitin. These peaks are mostly confirmed the structural pattern of rigid crystalline α -chitin. Huang et al. [55] reported similar results of crustacean chitin at the range of $2\theta = 9.08$ to 27.98° , which shows the α -chitin crystallinity and amorphous patterns.

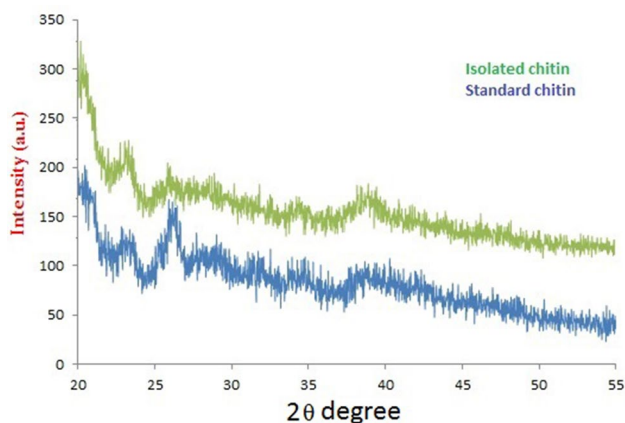


Fig. 4 XRD analysis of isolated & standard chitin

Thermal Analysis of Chitin

TGA: The TGA technique was used to evaluate thermal stability of the isolated and standard chitin (Table 2). It can be observed that the decomposition temperature for 10% weight loss was in the range 298–302 °C and for 40% weight loss was in the range 378–382 °C. The results revealed that overall isolated chitin is slightly more stable as compared to standard chitin (C9213). The maximum decomposition temperature was in the range 630–632 °C corresponded to the formation of char. It was quite clear that isolated chitin sample was thermally more stable than standard chitin (C9213). We can successfully state that the early stage degradation occurred mainly in the side chains of chitin where dehydration of the material underwent to depolymerization, which resulted in individual monomers, and then, their further reaction produced carbon dioxide. The results revealed that the thermal degradation of chitin takes place within 398 to 406 °C (Table 2). The TG curves for both the samples show one-step degradation and the differences between both the sample (isolated and standard chitin) are relatively small.

Thermogravimetric profiles of fresh shrimp’s chitin comparing with commercial chitin evaluate the structure of α -chitin concerning to degree of degradation and thermal stability (Fig. 5). The results of TGA analysis indicate the two degradation steps. In first step weight loss occurs below 100 °C due to the endothermic desorption of water

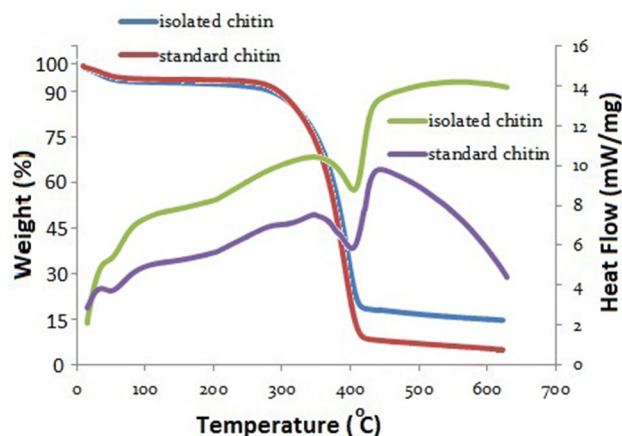


Fig. 5 Thermogravimetric and DSC analysis of isolated & standard chitin

Table 2 Thermal stability data of the isolated and standard chitin

Sample	T_{10} (°C)	T_{25} (°C)	T_{40} (°C)	T_{55} (°C)	T_{70} (°C)	T_{max} (°C)	T_g (°C)
1 Isolated chitin	298	351	382	392	406	632	–
2 Standard chitin	302	345	378	389	399	630	–

T_{10} , T_{25} , T_{40} , T_{55} , T_{70} , and T_{85} are the temperatures of 10, 25, 40, 55 and 70% weight losses, respectively, obtained from TGA; and T_{max} is the maximum decomposition temperature obtained from TGA curves

molecules. The second degradation (begins at 280 °C) corresponds to the endothermic degradation of isolated polymeric structure which is primarily associated with the deterioration of acetylated chitin and saccharide ring dehydration [54, 56–58]. The TGA curves of isolated and standard chitin revealed that the final degradation above the 400 °C indicates the complete volatilization of the chitin sample. Earlier literature showed the TGA results for isolated chitin samples were quite similar to the results reported in the current study [40, 58, 59].

DSC: Carbohydrates especially polysaccharides have a strong bonding with a water molecule and may have structurally disordered in the solid-state which can be hydrated easily. Hydration properties of these macromolecules depend on their structure. Figure 5 presents the differential scanning calorimetry (DSC) curve of the isolated and standard chitin. It can be observed that the glass transition temperature of both the isolated and standard chitin is almost same i.e., 75.1 °C. The broad endothermic peaks are observed at various temperatures indicating the crystallization of the material as well as evaporation of water in the films and decomposition of side chain. Due to intra-sheet and intersheet hydrogen bonding, the samples show a rigid crystalline structure. On comparing the DSC curves of both isolated and standard chitin, it was found that the endothermic peaks, the glass transition temperatures and maximum

decomposition temperatures are shifted to slightly higher values. It confirms that isolated chitin present a higher thermal stability pattern than the standard chitin with the formation of different crystalline forms. Similar degradation pattern has also been reported in the literature [54, 60]

SEM Analysis

Scanning electron microscopy used to elucidate the surface structure (layers, folds, homogeneity, roughness, porosity, and shapes or size of partials) of the materials. Figure 6 presents the SEM images of standard and isolated chitin, which was taken at various magnifications such as 650× and 350×. All the captured images exhibited a rough thick surface of non-homogeneous shrimp's chitin with irregular shapes and sizes of particles under the electron microscope. Similar morphological analysis of α -chitin in a beetle (*Holotrichia parallela*) and crustacean's have been reported in previous literature [35, 46].

Biological Activities of Freshly Extracted Chitin

Antimicrobial Activity of Chitin

The natural antimicrobial characteristics of standard and isolated chitin against microorganisms have been considered as one of the most important properties linked

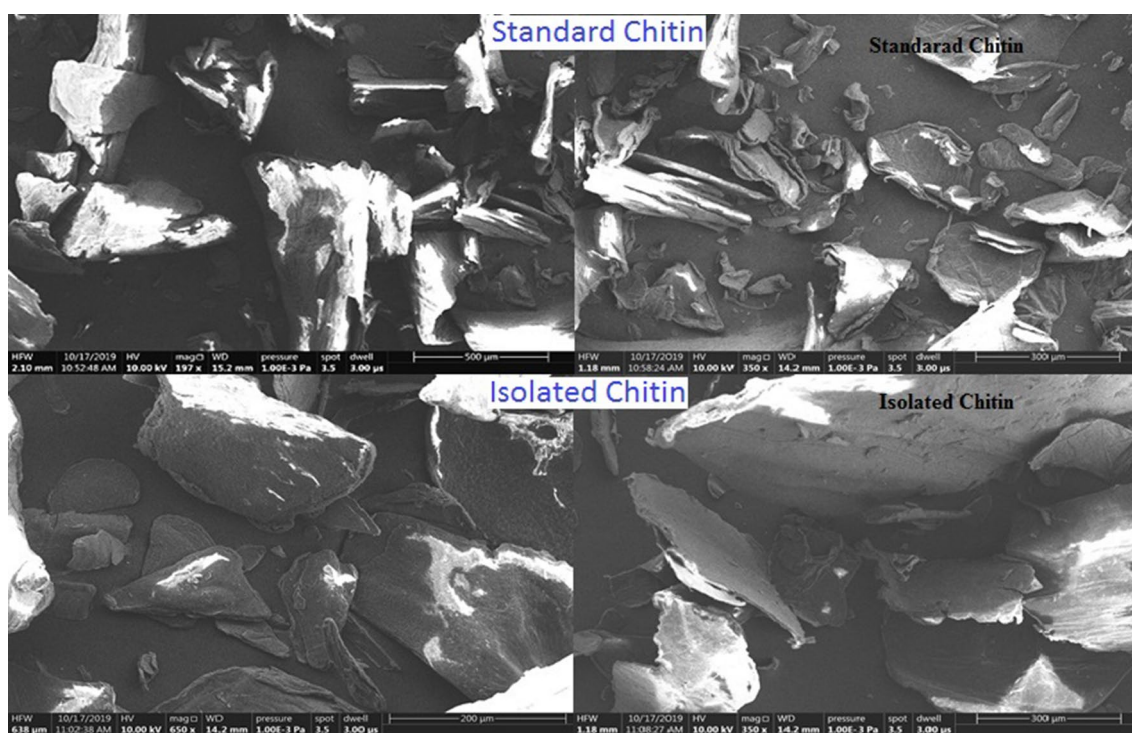


Fig. 6 SEM analysis of isolated & Standard chitin

directly to their biological applications. It is worth to mention here that the –NHCO– linked into acetamide group attached at C-2 position of chitin is responsible for biocompatibility and antimicrobial. When the chin is converted into chitosan, the amino functionality located at C-2 position plays the vital role in promising antimicrobial activity of the material. In this study, the antibacterial activities of both chitins were evaluated against gram-positive (*B.subtilis*) and gram-negative (*E. coli*) bacteria using a well diffusion method (Fig. 7, Table 3). The chitin samples inhibited the growth of bacteria and show an effective inhibitory effect against both gram-negative and gram-positive bacteria. Generally, the samples have more effective inhibition on *B.subtilis* than *E. coli*, because the structure and composition of their cell wall are different from each other. *B. subtilis* bacteria have a porous peptide polyglycogen cell wall which allows the foreign molecules to enter into the cell easily. But the cell wall of gram-negative (*E.Coli*) bacterium is made up of a thin layer of polyglycogen and another outside layer which constituted with phospholipids, lipoprotein, and lipopolysaccharides, this bilayer structure creates a potential barrier against the foreign materials [61]. When inhibition zone of chitin samples compared to the inhibitory effect of standard drug streptomycin it clearly shows the less inhibition growth activity as compared to the positive control.

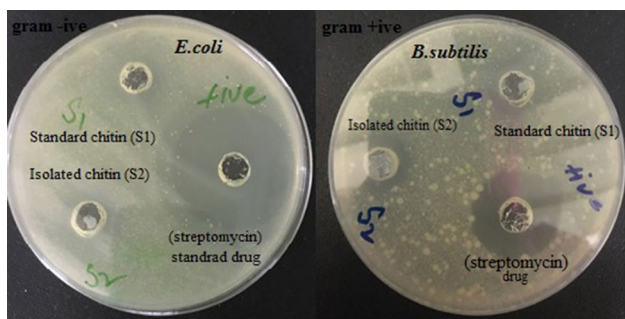


Fig. 7 Antimicrobial activity of isolated & standard chitin

Table 3 Antibacterial activity of freshly isolated and standard chitin against *Bacillus subtilis* and *Escherichia coli*

Sample	Zone of inhibition with 8 mm diameter		Zone of Inhibition in (mm)	
	<i>Escherichia coli</i> (mm)	<i>Bacillus subtilis</i> (mm)	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>
Standard chitin (S1)	17	19	9 ± 1.3	11 ± 1.5
Isolated chitin (S2)	17	19	9 ± 1.1	11 ± 1.2
Standard drug (streptomycin)	36	31	28 ± 1.19	23 ± 1.17

Antioxidant Activity of Isolated Chitin: DPPH Free Radical-Scavenging Assay of Freshly Isolated Chitin

DPPH free radical-scavenging assay of freshly isolated chitin, standard chitin, and BHT at different concentrations (0, 0.5, 1.0, 1.5, 2.0 mg/mL) is presented in Fig. 8. DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radical encounters a proton donating substrate such as an antioxidant, the radical is scavenged and the absorbance is reduced [23, 42]. Generally, the standard chitin showed significantly less scavenging activity than isolated chitin from shrimps as the purple color of the solution mixture faded. The free radicals of DPPH solution react with the amino group of chitin and convert them into stable molecules, and terminates the chain reaction. DPPH also reduced when the hydroxyl group of solvent (methanol) reacts with DPPH and purple color faded with concentrations. Chitin and BHT scavenging activities are concentration-dependent, therefore the present results suggested that at higher concentrations both samples and standard (BHT) exhibit high antioxidant activity against DPPH.

Hemolytic Assay

When physical contact comes in between biopolymer and blood cells, they interact with the RBCs which can lead to RBC dysfunctioning, so biocompatibility is one of the

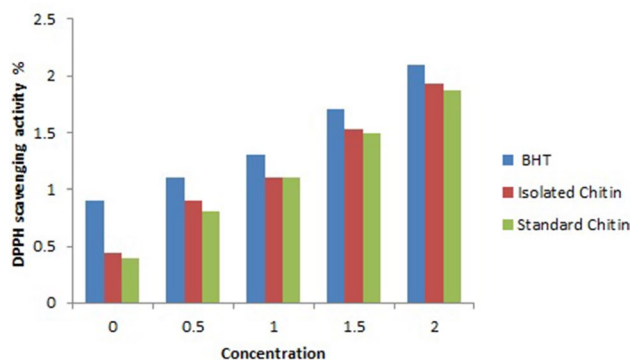


Fig. 8 DPPH free radical scavenging activity of isolated & standard chitin

Table 4 Hemolytic assay of freshly isolated and standard chitin (The scale was as follows: nontoxic, 1–10; slightly toxic, 11–25; moderately toxic, 26–50; and highly toxic, 50–100)

Sample	Hemolytic activity %	Result
Standard chitin (S1)	5.9 ± 1.39	Non-toxic
Isolated chitin (S2)	5.2 ± 1.15	Non-toxic
Negative control (PBS ^a)	0.09 ± 0.01	Non-toxic
Positive control Triton-X-100 ^b	100 ± 1.23	Highly toxic

Hemolysis values are mean ± SD of three samples investigated independently in triplicate

^aNegative control

^bPositive control

major tests to be performed. Interactions of chitin samples and negatively charged red blood cells were elucidated using biocompatibility of blood [62]. The quantitative analysis of hemoglobin indicates the membrane-damaging property of the samples. The results of this research are shown in Table 4. The hemolytic activity of the freshly extracted and standard chitin was evaluated against the red blood cells (RBC) and results were compared with PBS (negative control) as well as with Triton-X-100 (positive control) [34]. Chitin samples and PBS exhibited 5.2%, 5.9%, and 0.09 cytotoxicity values respectively, which were within the range of non-toxicity, and no sample displayed any toxic behavior towards the living cells.

Conclusion

The chemically isolated chitin possessing little amounts of ash (minerals) and moisture contents with better yield is enough in comparison to commercial international standards. The extracted chitin from shrimps wastage compare with standard chitin by using FTIR, ¹HSSNMR, XRD, SEM, and thermal analysis and the results revealed that the isolated chitin from the exoskeleton of shrimps in alpha form and showed good quality assessment. Due to bacteriostatic nature, both chitins have excellent antimicrobial activities against gram-positive (*Bacillus subtilis*) bacteria. Additionally, these biopolymers have good antioxidant and biocompatible properties. The antioxidant value on DPPH solution of isolated chitin was increased with the increase of concentration. In general, it is evident that the chitin isolated from wastage of crustaceans have excellent properties (biodegradability, nontoxicity, biocompatibility), and has the potential to be used in different applications such as medicine, pharmaceutical, agriculture, textile, food industry wastewater treatment, and cosmetics.

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