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Deracination and characterization of chitin and chitosan from waste crab shell

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Abstract

Chitin has been extracted from Four different methods. The acquired chitin was transformed into the more beneficial soluble chitosan by way of marinating into solutions of NaOH of a number of concentrations and for prolonged periods of time, then the alkali chitin was heated in an autoclave which dramatically reduced the time of deacetylation. Chitosan's special tiers of deacetylation can be bought from the deacetylation of chitin in sturdy sodium hydroxide solution at one-of-a-kind durations of time after extraction from crab shells, which waste crab shells in Bangladesh and this is a preliminary find out about to consider a number stages of deacetylated chitin for various functions. The obtained chitosan has been characterized by way of spectral analysis, X-ray diffraction, and thermogravimetric analysis.

keywords: Chitin/Chitosan extraction; Food waste; Degree of Deacetylation; Characterization

1. Introduction

The name Chitin (C8H13O5N) n came from "chiton", a Greek word that means a coat of mail. Chitin and chitosan are aminoglucopyrans composed of N-acetylglucosamine (GlcNAc) and glucosamine (GlcN) residues. (V.K. Mourya) Chitin is a copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked with β-(1-4) glycosidic bonds. In this polymeric chain, N-acetyl-D-glucosamine units are predominant. The structure of Chitin is similar to cellulose but with 2-acetamido-2-deoxy-β-D-glucose monomer units.

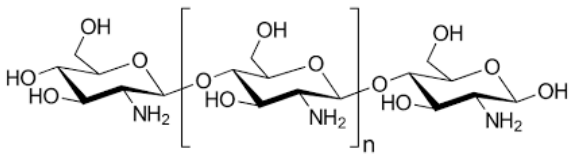


Figure: Chemical structure of chitosan.

Chitin is a white, rigid, inelastic, nitrogen-containing polysaccharide and it is obtained from

the cell walls of several fungi, the exoskeleton of crustaceans, the internal structure of invertebrates and the periphery of insects. (Hui Niu, 2003) Chitin is the second most important natural polymer in the world. The main sources exploited are two marine crustaceans, crab and crabs. (MargueriteRinaudo, 2006) It is the most abundant of the renewable polysaccharides in the marine environment and one of the most abundant on Earth after cellulose. (Crini, 2019) Of late, the most bountiful natural biopolymer chitin and chitosan have become cynosure of all party because of an unusual combination of biological activities plus mechanical and physical properties. (V.K.Mourya, 2008) Chitin has restricted usage due to its acetyl groups, thus, chitin is transformed into chitosan through deacetylation. During the deacetylation, the acetyl group present in chitin is converted into hydroxyl (-OH) and amino (-NH2) groups in the chitosan. The modification of the reactive functional groups present in chitosan opens the possibility of broad application in many fields. (Tran Thi Bich Quyen, 2021) Chitin and chitosan have excellent properties such as biodegradability, biocompatibility, and nontoxicity. (Suneeta Kumari R. K., 2020)

Figure: Sources of Chitosan.

Chitin is usually isolated from the exoskeletons of arthropods' chitin-based tissue (30%-40% protein, 30%-50% calcium carbonate, and 20%-30% chitin), such as crustaceans, mollusks, insects, and certain fungi. It is a biological nano composite material strictly hierarchically organized which reveals various structural levels. At the molecular level is the polysaccharide chitin itself. (Entsar S.Abdou, 2008) The next structural level is the arrangement of c. 18-25 of such molecules in the form of narrow and long crystalline units, which are wrapped by proteins, forming nan fibrils of about 2-5 nm diameter and about 300 nm length. The next step in the scale consists of the clustering of some of these nan fibrils into long chitin-protein fibers of about 50-300 nm diameter. Chitin is mainly occurring in three different polymeric α-, β-, and γ-forms. The chains are arranged in stacks or sheets in α-chitin and adjacent sheets along the c-axis have the same direction in a parallel arrangement. The α-chitin occurs in the exoskeletons of crustaceans (e.g., crabs, lobsters, and prawns). In the case of β-chitin, the adjacent sheets along the c-axis present in opposite directions in an antiparallel arrangement and it can be found in squid pen, certain diatoms, and vestimentiferans (a class of deep-sea animal). However, every third sheet is in the opposite direction to the preceding sheets in γ-chitin. It mainly exists in fungi and yeast. (Suneeta Kumari R. K., 2020) Chitosan, which is a natural and linear polysaccharide made from chitin by a chemical process involving deproteinization, demineralization, decolouration and deacetylation, has received considerable attention because of its properties. (Alaa Jabbar Al-Manhel, 2018) Due to its fungicidal effects and elicitation of defense mechanisms in plant tissue, chitosan has become a useful and highly appreciated as a natural biodegradable high molecular polymer compound which is a nontoxic and bioactive agent. (Min-Woo KIM, 2016)

2. Material and Methodology (Crab Shell)

The aim of this research is to extract chitosan from crab shell and from crab shell. (Djaeni, Optimization Of Chitosan Preparation From Crab Shell Waste, 2017) Crab shell waste from seafood restaurant is potential to be used as chitosan source. This material containing 20-30% of chitin which could be converted into chitosan through deacetylation process. While, chitin could be isolated from crab shell by deproteination and demineralization. Chitosan is fine chemical used to adsorb fat from body, heavy metal adsorbent, and medicine. (Islem Younes, 2015)

2.1 Extraction of chitosan from crab (C. sapidus): Chitin separation from the shell requires the elimination of two major constituents, protein and minerals.

- Protein is removed by the deproteinization process.
- Minerals are removed by the demineralization process.
- Decolonization process is carried out as an additional process to remove pigments.

Then Chitin is converted into Chitosan by the deacetylation process. (Djaeni, Optimization Of Chitosan Preparation From Crab Shell Waste, 2017)

Figure: Crabs

2.1.1 Deproteinization:

Cuticles of various crustaceans like crabs, crabs, lobster, and fish scales are the major sources of chitin. Crustaceans carry an exoskeleton composed of proteins, chitin, and calcium carbonate which bind together to form an external shell. (San-LangWang, 1998)

Hence, Chitin's separation from the shell requires the elimination of two major constituents, protein and minerals.

Protein is removed by a deproteinization process. Deproteinization means the removing of protein. [Chitin is usually isolated from the exoskeletons of arthropods' chitin-based tissue (30%-40% protein, 30%-50% calcium carbonate, and 20%-30% chitin), such as crustaceans, mollusks, insects, and certain fungi.] Protein and chitosan bind together and a small part of the protein is available in the polymer complex. These proteins are removed from the shell by deproteinization process which is carried out by using an alkali treatment. (Gopi, Thomas, & Pius) The deproteinization step is difficult due to disruption of chemical bonds between chitin and proteins. This is performed heterogeneously using chemicals which also depolymerize the biopolymer. (Entsar S. Abdou, 2008)

Deproteinization of mud crab shells is carried out by using an alkali treatment with common reagents such as NaOH, the protein is removed from demineralized shells using NaOH with constant stirring for 2 hr at around 90^0C. The samples were then filtered under vacuum and the filtrates were washed with tap water for 30mins until pH neutral (pH, 7). The deproteinized shells were dried in the oven at 60 °C for 24 h. (Marwa Hamdi, 2019)

Reagents	Concentration	Temperature	Number of Baths	Duration
NaOH	98%	90^0C	1	2 hr

2.1.2 Demineralization:

Demineralization is the process by which the minerals of shells are removed. Demineralization of shells is based on acidic treatment to remove minerals like calcium carbonate and calcium phosphate. (Carla-Cezarina Pădureț u, 2019)

$$2HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2$$

This reaction uses hydrochloric acid to decompose calcium carbonate into calcium chloride with the release of water and carbon dioxide. (Kyung-TaekOh, 2007) Similarly, minerals also react with the acid and produce soluble salts. The salts are removed by filtration. Chitin is recovered by washing with distilled water and drying. (W. J. Jung, 2005)

Demineralization treatments are often empirical and vary with the mineralization degree of each shell, extraction time, temperature, particle size, acid concentration and solute/solvent ratio. (Vijayalakshmi Shankar, 2017) The latter depends on the acid concentration, since it needs two molecules of HCl to convert one molecule of calcium carbonate into calcium chloride. In order to have a complete reaction, acid intake should be equal to the stoichiometric amount of minerals, or even greater. Since, it is difficult to remove all minerals (due to the heterogeneity of the solid), larger volume or more concentrated acid solution is used. Demineralization can be followed by acidimetric titration: the evolution of pH towards

neutrality corresponds to acid consumption but the persistence of acidity in the medium indicates the end of the reaction. (GADGEY, 2017)

The deproteinized mud crab shells were demineralized with most common reagents are HCl at room temperature (20 °C) for 6 h to remove the mineral content. The samples were then filtered under vacuum and washed for 30 mins with tap water until pH neutral (pH, 7). The demineralized shells were dried in the oven at 60 °C for 24 h. (Horiya AliSaid Al Hoqani, 2020)

2.1.3 Decolorization:

Decolorization is an additional process carried out to remove pigments from the chitin.

Decolorizing was achieved by treating the samples with acetone for 10 mins and dried for 2 h at ambient temperature and the resulting residues were then removed. The decolorized shells were then washed in running tap water, rinsed, filtered and dried at 60 °C for 24 h in the oven to obtain crab chitin. (Cansu Metin Y. A., 2019)

2.1.4 Deacetylation of chitin:

Deacetylation is the process of removing acetate group from chitin. Chitin is a copolymer of N-acetyl-D-glucosamine. The deacetylated form of chitin refers to chitosan. Chitin has limited applications because of its acetyl groups, but through the deacetylation process chitin is converted into chitosan. During the deacetylation process, the acetyl group present in chitin is converted into hydroxyl (-OH) and amino ($-NH_2$) groups in the chitosan. (Tarun Kumar Varun, 2016)

The dry chitin is a raw material to produce chitosan through deacetylation process. The chitin powder is mixed with high concentration of NaOH to remove acetyl groups bounded in amine groups of chitins. Here, the acetyl reacted with NaOH forming sodium acetic. The sodium acetate will dilute in solution, while deacetylated chitin namely chitosan could be obtained as solid product. The mixture was separated by vacuum filter to obtain chitosan as solid phase (cake). The cake was washed by pure water and then dried in electric oven for 2-4 at 105°C. (He, 2011)

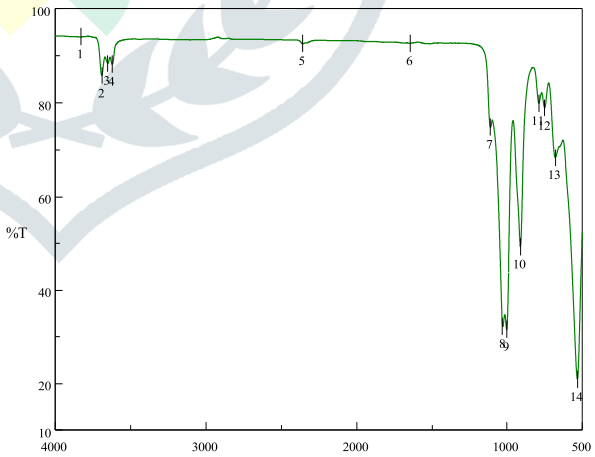
Reagents	Concentration	Temperature	Number of Baths	Duration
HCl		20 °C	1	6 h

From a chemical point of view, either acids or alkalis can be used to deacetylate chitin. However, glycosidic bonds are very

susceptible to acid; therefore, alkali deacetylation is used more frequently.

3. Characterization of chitosan from Crab Shell

3.1 Fourier transform infrared spectroscopy (FTIR) Fourier transform infrared spectroscopy (FTIR) Samples were characterized with infrared spectroscopy by using KBr pellets in the scanning range of 400–4,000 cm-1 (FTIR-8400S, Shimadzu). KBr pellets were prepared (1 mg chitosan with 100 mg of KBr) and stabilized under controlled relative humidity before acquiring the spectrum. (Suneeta Kumari S. H., 2017)



Wavenumber [cm-1]

3.2 Thermogravimetric Analysis (TGA) Thermogravimetric analysis of the synthesized chitosan was performed on a TGA instrument (Perkin Elmer TGA 4000, Perkin Elmer, Waltham, MA). Samples were heated from 30 to 600 °C at a rate of 10 °C min−1 under a nitrogen flow rate of 20 mL min−1. (Cansu Metin Y. A., 2019)

3.3 X-ray powder diffraction (XRD)

The starting materials, prepared chitin and chitosan samples were characterized by Xray diffraction (XRD) technique using an X-ray diffractometer (XRD-7000, Shimadzu,) with CuKa radiation (k = 40 kV, 30 mA). The present X-ray diffractometer feature a high precision vertical θ-θ goniometer that can accept up to 400 mm (w) x 5500 mm (d) x 400 mm (h) maximum. The measurements taken were in the scanning range of 5–80 at a scanning speed of 50 s-1 . The degree of crystallinity was be estimated according to the reports of Focher et al., [13]. Therein, the computed formula of crystallinity is based on the parameter of the crystalline index. The crystalline index is determined as follows:

$Crystalline\ index$ (%) = $I_{110} - I_{am} / I_{110} \times 100$. Where I_{110} is the maximum intensity at 20°. Iam is the intensity of amorphous diffraction at 16°.

3.4 Scanning electron microscope (SEM)

Starting materials, chitin and chitosan were examined by scanning electron microscopy (SEM) having a magnification range of 5,000x and accelerating voltage 20 kV. The S3700 N (Hitachi High Technologies) has a huge sample chamber and can accommodate samples as large as 300 mm in diameter and 110 mm high.

3.5 Elemental analysis

The elemental analysis of prepared chitosan was estimated by using the Thermo Finnigan elemental Analyzer. The percentage of carbon and nitrogen was calculated. The chromatographic elemental analyzer uses flash combustion in an oxygen atmosphere for the determination of carbon, hydrogen, nitrogen and sulfur. The

4. Materials & Method (Crab Shell)

4.1 Materials

Raw crabs stated as large size were purchased from Bangladeshi (Market, New Market, Dhaka), Sodium hydroxide (NaOH) (Market, New Market, Dhaka), Hydrochloric acid (HCl) (Market, New Market, Dhaka), and acetic acid (Market, New Market, Dhaka). They were then diluted to the concentration required for the methodology with distilled water. All chemicals were used without further purification. (Nadia G. Kandile, 2018)

Figure: Crab Shell

4.2 Measurements

The infrared spectra were measured on Perkin-Elmer-1430 infrared spectrophoto-meter using the potassium bromide Wafer technique. X-ray diffractograms of polymers were obtained with a Phillips X-ray radiation

unit (Generator PW-1390) and Ni-filtered Cu. Thermogravimetric analysis (TGA) was carried out in a nitrogen atmosphere using a Shimadzu TGA-50H. The morphology of the different hydrogels was investigated using JXA 850 prop micro analyzer scanning electron microscope (SEM). The solubility of the polymers was examined using 0.02 g of polymer in 5 ml solvents at room temperature 25˚C. (Nadia G. Kandile, 2018)

4.3 Methods (Extraction of Chitosan)

The extraction of chitosan can be carried out by different four methods under different conditions after removing the loose tissue from the crab shells then washed, dried and grind to obtain dry powder. The major procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong

alkaline solution at different period of time. (Nadia G. Kandile, 2018)

Extraction of Chitosan from Crab Shell

4.3.1 Method 1

Deproteinization process

The deproteinization was occurred by heating of 3 gm of crab shells powder after adding 2 N NaOH with ratio of 12ml:1g (w/v) at 70˚C for 4 h. The product was neutralized by washing under running tap water. The solid was collected and washed with distilled water. The solid product was dried in vacuum and weighed with analytical balance. (Nadia G. Kandile, 2018)

Demineralization process

The dry solid was treated with 10% HCl (3.25 N) with ratio of 14ml:1g (w/v) at room temperature and kept for 4 h. The solid product was collected and washed with distilled water. The solid was then dried. (Nadia G. Kandile, 2018)

Deacetylation process

Then the demineralized product was treated with 35% NaOH (8.75 N) with ratio of 14ml:1g (w/v) at room temperature for 75 h. with stirring. The deacetylated solid was filtered then collected and washed with distilled water. The deacetylated product was dried in a vacuum to give 1.51 gm and then labeled as Cs1. (Nadia G. Kandile, 2018)

4.3.2 Method 2

Demineralization process

The demineralization was carried out by weight 3 gm of crab shells powder by using 4% HCl (1.3 N) with ratio of 14ml:1g (w/v) at room temperature for 24 h. The product was washed to neutrality under running tap water. The solid was collected and washed with distilled water, then dried in a vacuum. (José Carlos Vilar Junior, 2016)

Deproteinization process

Deproteinization was carried out using 5% NaOH (1.25 N) with ratio of 12ml:1g (w/v) at 90˚C for 24 h. The deproteinized product was collected and washed with distilled water.

Deacetylation

The product was deacetylated with 70% NaOH (17.5 N) with the ratio of 14ml:1g (w/v) at room temperature for 75 h. with stirring. The solid was collected and washed with distilled water. The deacetylated product was then dried in a vacuum, producing 2.04 gm and labeled as Cs2. (Nadia G. Kandile, 2018)

4.3.3 Method 3

Deproteinization process

The deproteinization process was carried out by the weight of 3 gm of crab shells powder by using 5% NaOH (1.25 N) with a weight to volume ratio of 1g:8ml (w/v). The solution with crab shells was refluxed at 70˚C for 3 h. The product was collected and washed until a clear solution. It was then dried in a vacuum. The product was decolorized with pure acetone for 24 h. The product was collected and washed to neutrality, then dried.

Demineralization process

The decolorized product was demineralized by using 1% HCl (0.32 N) with a weight to volume ratio of 1g:10ml for 24 h. at room temperature. The product was collected and washed to give light brown powder.

Deacetylation

The N-deacetylation of the demineralized product was carried out by using 55% NaOH (12.5 N) with a weight to volume ratio of 1g:5ml at 100˚C for 12 h. The product was washed with distilled water and dried to produce 1.69 gm and then labeled as Cs3.

4.3.4 Method 4

Demineralization process

Weight 3 gm of crab shells powder, then the powder was treated by 1 N NaOH (4%) with a weight to volume ratio of 1 g: 10 ml for 24 h. at room temperature. It was washed and dried in a vacuum.

The solid from the alkaline treatment was then demineralized by using 1 N HCl (3%) with weight to volume ratio of 1g:10ml for 24 h. at room temperature. It was washed and dried in vacuum. (M.D. Teli, 2012)

Deproteinization process

The demineralized product was deproteinized by using 1 M NaOH (4%) with weight to volume ratio of 1g:10ml for 24 h. at room temperature. It was washed and dried in vacuum. The product from deproteinization was decolorized using pure acetone with for 24 h. at room temperature. It was washed and dried in vacuum.

Deacetylation

From decolorization, the product was then deacetylated by using 50% NaOH with weight to volume ratio of 1g:10ml for 24 h. at room temperature. The product was washed and dried in vacuum to produce 1.14 gm and then labeled as Cs4.

5. Result and Discussion

The major procedure for extraction of chitosan from crab shells powder, which is waste crab shells in Egypt, is a preliminary study to evaluate various levels of deacetylated chitin for different applications as pharmaceutical processes and medical and agricultural drugs, and the extraction is based on the alkaline deacetylation of chitin with a strongly alkaline solution via deproteinization, demineralization and deacetylation of crab shells powder at different conditions to give the following chitosan samples: Cs1, Cs2, Cs3, and Cs4 respectively.

5.1 Characterization of the Prepared Chitosan

The chitosan samples: Cs1, Cs2, Cs3, and Cs4 were characterized by (FT-IR) to identify the functional groups in chitosan. X-ray diffractometry (XRD) is to analyze the crystallinity of the product; thermogravimetric analysis (TGA) is to study the thermal stability; elemental analysis is to calculate the degree of deacetylation. Finally, a scanning electron microscope is to demonstrate the morphology of the product. (A. Rajalakshmi, 2013)

5.2 FTIR Spectroscopy

The IR spectral data for the produced chitosan [Cs1] [Cs2] [Cs3] [and Cs4] revealed the following peaks: peaks at 3440.9 cm−1, 3396.1 cm−1, 3438.7 cm−1, 3441.5 cm−1 is assigned to -OH and -NH stretching vibrations, while the peaks at 2960.8 - 2890.4 cm−1, 2971.3 cm−1, 2959.7 - 2890.6 cm−1, 2961.4 - 2890.5 cm−1 are assigned to the aliphatic C-H stretching vibration in the -CH and -CH2 groups. The amide frequencies consist of the -C-O bond stretch of the remaining acetamido groups and the N-H bending vibrations of the -NH2 groups are observed at 1663.5 and 1559.9 cm−1, 1754.1, and 1664.7 cm−1, 1659.5 and 1561.7 cm−1,1656.3 and 1562.5 cm−1 respectively. The peak at 1429.9 cm−1, 1451.1 cm−1, 1418.2 cm−1, 1419.1 cm−1 is assigned to -NH2 deformation. Further bending vibrations are observed at 1379.4 cm−1, 1409.5 cm−1, 1380.5 cm−1, 1381.8 cm−1 for the C-C-H symmetric bending vibration in the alcohol. Stretching vibrations are also observed at 1317.2 and 1156.9 cm−1, 1154.5 cm−1, 1316.0 and 1156.8 cm−1, 1316.9 and 1156.9 cm−1 for the C-N stretching vibration and at 1072.7 and 1032.3 cm−1 for the -CO stretching vibration of the alcohol groups as shown in Figure 1. (Majid Pakizeh, 2021)

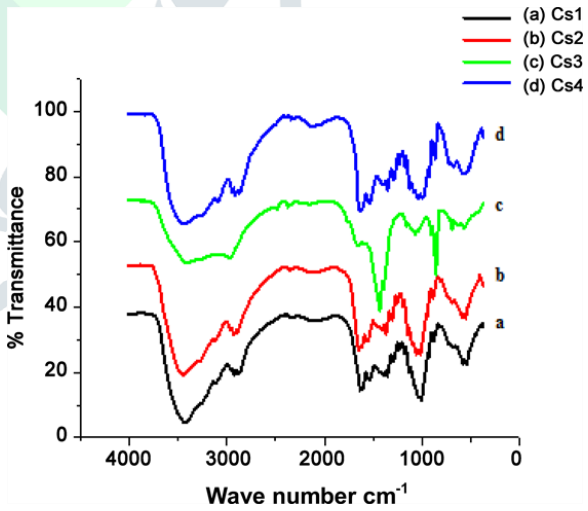


Figure 1. Infrared spectra of (a) Cs1, (b) Cs2, (c) Cs3 and (d) Cs4.

5.3 X-Ray Diffraction (XRD)

The X-ray diffraction is used in the characterization of crystalline materials. By studying the X-ray diffraction of the extracted chitosan from the four methods, it can be concluded that the order of crystallinity is of different chitosan samples: [Cs2 >

Cs3 > Cs1> Cs4], so the highest crystallinity is shown by chitosan produced from method 2 [Cs2] as shown in Figure 2. (Khanafari A., 2008)

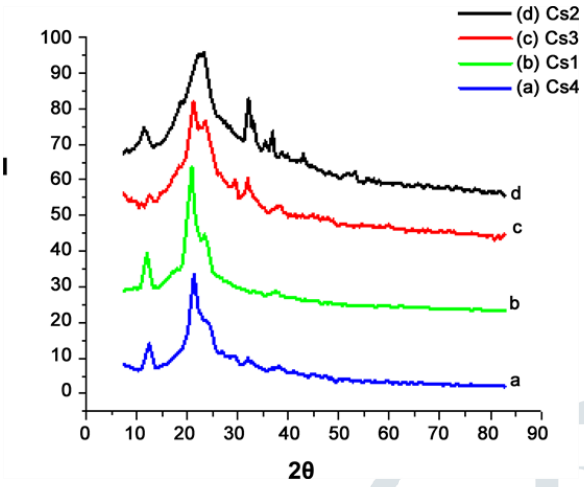


Figure 2. X-ray diffraction pattern for (a) Cs4, (b) Cs1, (c) Cs3 and (d) Cs2.

5.4 Thermal Stability (Thermogravimetric Analysis) (TGA)

The thermograph of the produced chitosan's [Cs1] [Cs2] [Cs3] [Cs4] were evaluated by using TGA in air at heating rate 10˚C/min and recorded in Figure 3 and Table 1: It shows the following data: the weight loss of the extracted chitosan by the four methods at the beginning may be due to the ease of degradation of the amide groups; however the weight loss in the high temperature range is attributed to the degradation of the main chain. (Suneeta Kumari P. R., 2016)

The data reported in Table 1 showed that chitosan (Cs2) possesses the highest thermal stability.

5.5 Degree of Deacetylation for Chitosan

By using the elemental analysis, the percentage of free amino groups on the chitosan can be determined by using the following equation:

$$DD = \left(1 - \frac{(C/N) - 5.145}{6.186 - 5.145}\right) \times 100$$

The location of 5.145 is related to completely N-deacetylated chitosan ($C_6H_{11}O_4N$ repeat unit) and 6.186 is the fully N-acetylated polymer ($C_8H_{13}O_5N$ repeat unit). The value of degree of deacetylation of chitosan samples was calculated and reported in Table 2. The data indicated that the highest degree of deacetylation (DD) shown by the Cs2, Cs3. It can be concluded that the degree of deacetylation of chitosan increased by increasing the concentration of the NaOH used in. The elemental analysis and the degree of deacetylation are shown in Table 2.

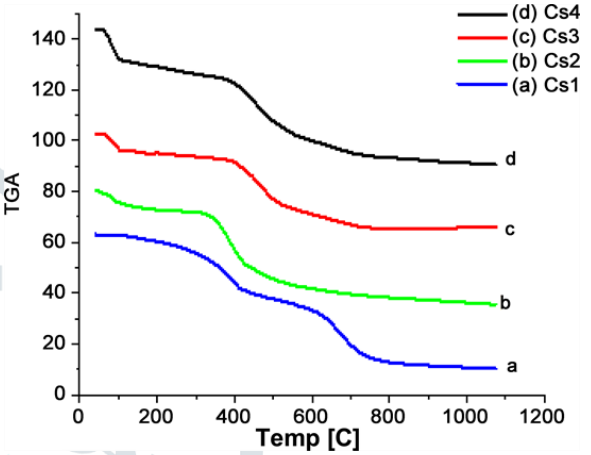


Figure 3. TGA for (a) Cs1, (b) Cs2, (c) Cs3 and (d) Cs4.

Cpd.No	Temp.	wt. Loss%	Temp	Wt. loss%	Temp	Wt. loss%
Cs1	230.0	13.63	690.0	25.17	-	-
Cs2	250.0	10.82	283.33	5.105	550.0	27.84
Cs3	225.0	10.89	704.16	53.33	-	-
Cs4	195.83	9.14	391.66	31.60	629.16	32.60

Table 1. Thermal properties of the extracted chitosan by the four methods. (MS Hossain, 2014)

Cpd.No	% C	% H	% N	% DD	% NaOH
Cs1	40.66	5.75	6.65	6.917	35
Cs2	28.90	3.70	5.51	90.4	50
Cs3	31.80	2.80	6.01	85.95	55
Cs4	27.6	3.82	4.30	21.0	70

Table 2. The elemental analysis, and the degree of deacetylation of chitosan.

6. Conclusions

Crab (C. sapidus) is a widely distributed species throughout the world and can be commercially cultivated. The shell of this seafood should be evaluated as an alternative biological source of chitosan, as it is a waste product after the consumption of the crab as a food source. The results of the present study revealed that synthesized chitosan can be a good alternative to synthetic antioxidants. The chitosan from crab shell waste showed antimicrobial activity against common pathogenic microorganisms tested in the present study. It can be suggested that the characterized chitosan might be served as a natural alternative to commercial chitosan and to the synthetic antioxidants/ antimicrobials. The chitosan has been characterized as to its degree of deacetylation. Chitosan's different degrees of deacetylation can be obtained from the deacetylation of chitin in strong sodium hydroxide solution at different periods of time after extraction from crab shells, which waste crab shells in Bangladesh and this is a preliminary study to evaluate various levels of deacetylated chitin for various applications. Chitosan (Cs2) possesses the highest thermal stability, crystallinity, and degree of deacetylation which is attributed to the increase of the sodium hydroxide concentration (70%), and its morphology shows crystals on its smooth surface.

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