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Synthesis and characterization of chitosan prepared from shrimp shell

Nalini Singh[1], SunitaChahar[2]
N.E.S Ratnam College of Arts, Science and Commerce, Bhandup, Mumbai, Maharashtra, India

Abstract
The present study deals with synthesis and characterization of Chitosan from the Shrimp shell waste from the local market in Mumbai, Maharashtra. In order to obtain Chitosan, shrimp shell waste undergoes chemical extraction process which involves demineralization, deproteinization and deacetylation. Chitosan, a soluble biopolymer in any solvent with pH less than 6.5, was extracted by deacetylation of chitin (deprotenized product) with 60% NaOH for 24 hours at 60°C. Chitosan solubility was analysed with two solvents i.e acetic acid and ascorbic acid of 1% concentration. The degree of deacetylation was calculated by the acid-base titration method and was found to be 77.90%, the Chitosan obtained were characterized by Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM).

Keywords: chitosan, FTIR, SEM, shrimp shell, degree of deacetylation, solubility

Introduction
Worldwide tons of shrimps are produced and consumed as a protein rich sea food every year. The external shells of shrimp comprise about half of its body weight and are usually discarded. This shrimp shell waste is considered as huge source of chitin (15-25% of dry weight), protein (35-50%) and inorganic compound such as calcium carbonate which cause huge problems for environmental pollution, mainly due to its odour and trivial solubility in water (Muley et al. 2018 and Nargis et al., 2007, Islam et al. 2017 and Rengga et al. 2018) [18, 17]. Chitin is a naturally abundant polymer and is highly insoluble product used in many foods, cosmetics and pharmaceutical products (Islam et al. 2017 and Islam et al., 2011) [17]. Chitosan owns eco-friendly characteristics such as being biodegradable, non-toxic and biocompatible. It is a natural biopolymer, and copolymer of glucosamine and N-acetylglucosamine prepared by deacetylation of chitin which is mostly found in the exoskeleton of crustaceans, fungi and insects (Zahedi et al., 2018, Hossain et al., 2014, Islam et al., 2011, Arafat et al., 2015) [17, 23]. Chitosan is insoluble in water and in any alkaline solvent whereas it is highly soluble in any organic or inorganic solvent under acidic condition due to the protonation of its amino groups (Raafat et al. 2009 & De Queiroz et al. 2017) [6]. Chitosan has application in various fields such as in agriculture, pharmaceuticals, cosmetics, biomedical, paper industry, food and textile and in water purification (Muzzarelli, 1985 and Al-Manhel et al., 2018) [29, 30]. The main objective of the present study was to extract Chitosan from shrimp shell and study its properties.

Materials and Methods
Fresh shrimp shells (*Penaeus monodon*) from a single species of shrimp were collected from the local fish market in Mumbai. The shrimp shells were properly washed with water and kept for sun drying for 2 days followed by oven drying for 2hrs at 60^0 C. After drying, the shells were crispy enough to be grounded into powder and to be passed through a sieve of 250 µm.

The dried shrimp shell powder were then stored in plastic bottle at room temperature. All the chemical reagents (i.e NaOH and HCL) required for the synthesis were of a highly pure grade and double distilled water was used for the preparation of the solution.

Synthesis of Chitosan: This includes three steps i.e Demineralization, Deproteinization and Deacetylation.

- **Demineralization:** In this step, the dried shrimp shell powder is treated with 5 % HCL solution for 24 hrs at ambient temperature (30 ± 2^0C) .The solid to solvent ratio was 1:6 (w/v) as mentioned by Arafat et al., 2015 [2]. After 24 hrs the residue becomes quite soft, and was washed and soaked in water to remove acid until the pH is neutral. The residue obtained was then oven dried at 60^0 C.
- **Deproteinization:** Deproteinization of Demineralized shell was carried out by using 5% NaOH solution for 48 hrs at ambient temperature (30 ± 2^0C) by keeping solid to solvent ratio 1: 10 (w/v) as described by Arafat et al., 2015 [2]. After processing, the residue was washed and soaked in water until neutral pH. Then the residue was dried until it become crispy. The product obtained is called as chitin. This Chitin flakes was grounded into fine particle to undergo deacetylation.
- **Deacetylation:** In this step, removal of acetyl group from chitin was experimented by using 60% NaOH solution at 65^0 C temperature with a solid to solvent ratio 1:10 (w/v) for 3 hours. The residue was washed with water until neutral pH. The resulting Chitosan was then dried at 60^0 C for 4 hours and was ready for characterization.

$$\text{Yield (\%)} = \frac{weight\ of\ chitosan\ obtained}{weight\ of\ the\ raw\ material} \text{ x } 100$$

Determination of yield of Chitosan:
The yield of the Chitosan was obtained by comparing the weight of the raw material used for the preparation of

Chitosan (g) to the weight of the Chitosan obtained after the treatment using following formula (Mohanasrinivasan et al. 2013).

Determination of degree of deacetylation:
The degree of deacetylation (DD) was assessed by the acid-base titration method (Hossain and Iqbal 2014). Dried Chitosan (0.1 g) was dissolved in 30 ml of 0.1 M HCl solution at room temperature and mixed properly unless the Chitosan was totally dissolved. Then 5–6 drops of methyl orange was added. The red coloured Chitosan solution was titrated with 0.1 M NaOH solution until it turned orange. The DD was calculated by the formula:

$$\text{DD (\%)} = \frac{C1V1 - C2V2}{\text{M} \times 0.0994} \times 0.016$$

Where, C1=concentration of standard HCl aqueous solution (mol·dm-3), C2 = standard NaOH solution (mol·dm-3), V1= volume of HCl aqueous solution used to dissolve Chitosan (ml), V2= volume of NaOH solution consumed during titration (ml), and M= weight of Chitosan (g). The number 0.016 (g) is the equivalent weight of NH2 group in 1 ml of standard 1 mol/l HCl aqueous solution and 0.0994 is the proportion of NH2 group by weight in Chitosan.

Moisture and Ash Content
Moisture content of the shrimp shell Chitosan was determined by the gravimetric method (Black, 1965) whereas Ash content was calculated by standard method (AOAC, 1990). Procedures were as follows.

Moisture content: weighed and recorded, weight of the oven dried crucible, placed 1.0g of Chitosan in triplicate in the crucibles, recorded weight of the crucible with Chitosan, then placed lid over the crucible to avoid contamination in the oven. Adjusted the oven temperature to 60ºC, and dried the sample for 24 hrs. After 24 hrs crucibles with Chitosan were removed from oven and were placed in desiccator until it cools to room temperature. Weighed the sample, and recorded the weight as weight of dry sample. Calculated moisture content as

$$\text{\% of Moisture content} = \frac{(wet\ weight\ (g) - dry\ weight\ (g))}{wet\ weight\ (g)} \text{ x } 100$$

Ash content
Take a tarred crucible which is already ignited, cooled, and weighed. Place 2.0g of Chitosan (triplicate) into the crucible and weigh. The Chitosan was heated in a muffle furnace preheated to 600ºC for 6 hr. The crucibles were allowed to cool in the furnace to less than 200ºC and then placed into desiccators with a stoppered lid. Allowed them to cool and weighed crucible and ash.

$$\text{\% of Ash content} = \frac{(Weight\ of\ crucible + ash\ residue) - (empty\ weight\ of\ crucible)}{(weight\ of\ crucible + sample) - (empty\ weight\ of\ crucible)} \text{ x } 100$$

Solubility: To analyse the solubility of Chitosan two solvents i.e acetic acid and ascorbic acid of 1% concentration were utilized. 0.1g of Chitosan (in triplicate) was placed into a pre-weighed centrifuge tube with 10 ml of acetic acid and ascorbic acid respectively for 60 min and was vigorously shaken with the interval of 5 minutes at room temperature. The centrifuge tube containing solution was then immersed in a boiling water bath for 20 minutes, cooled to room temperature and centrifuged at 10,000 rpm for 20 min. The supernatant was decanted and the undissolved particles were washed in distilled water (20ml) then centrifuged a 10,000 rpm. The supernatant was removed and undissolved pellets were dried at 60^0 C for 24hr. finally, the dried pellets was weighed and percentage solubility was determined by using the following formula. (Sun-Ok Fernandez-Kim, 2004)

$$\text{\% solubility} = \frac{\text{(Initial weight of tube + Chitosan)} - \text{(Final weight of tube + Chitosan)}}{\text{(Initial weight of tube + Chitosan)} - \text{(Initial weight of tube)}} \text{ X } 100$$

Water binding capacity: Water binding capacity (WBC) of shrimp shell Chitosan was measured by following modified method of Knorr, (1982). 0.5 g of Chitosan was added in pre-weighed centrifuge tube with 10 ml water and was mixed for 1 min on vortex mixer for the dispersion of sample. The centrifuge tube was kept in room temperature for 30 min with intermittent shaking for 10 seconds every 10 min and centrifuged at 3000 rpm for 30 min. After the supernatant was decanted, the tube was weighed again.
WBC was calculated as follows:

$$\text{WBC (\%)} = \frac{water\ bound\ (g)}{Initial\ weight\ of\ sample\ (g)} \times 100$$

Fat binding capacity: Fat binding capacity (FBC) of Chitosan was measured using a modified method of Knorr, (1982). FBC was initially carried out by weighing a centrifuge tube containing 0.5 g of sample, adding 10 ml of oil (soyabean oil) and mixing on a vortex mixer for 1 min to disperse the sample. The contents were left at ambient temperature for 30 min with shaking for 5 s every 10 min and centrifuged at 3,000 rpm for 25 min. After the supernatant was decanted, the tube was weighed again.
FBC was calculated as follows:

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

Scanning electron microscopy (SEM)
The surface morphology and physical state of Shrimp shell based Chitosan were studied using scanning electron microscopy. Scanning electron microscopy (SEM) having a magnification range of Low: 25X to 10,000X and High: 100X to 1,000,000X at 4x5 photo size and accelerating voltage 30 kV.

Fourier transform infrared spectroscopy (FTIR)
Infra-Red spectra of Chitosan were performed using FTIR spectrophotometer (Bruker, Vertex 80), in the range of 450–4000 cm-1, using ATR mode of operation. Each spectrum is an average of 64 scans with a resolution of 0.2 cm-1.

Results and Discussion
Chitosan was extracted from the shrimp shell wastes (*Penaeus monodon*) by undergoing demineralization, deprotenization and deacetylation. The Physicochemical properties of Chitosan are shown in Table. 1.

Yield: From 200 g of dried shrimp shell 36.24 g of Chitosan was extracted and the yield percentage was 18.12

%. The yield obtained in our study was in accordance with the yield reported by Arafat et al., 2015 [2] (18.97%), Ghannam et al., 2016 (18.50%) and Mohanasrinivasan et al., 2013 (17%). On the other hand the yield obtained is higher than those reported by Hossain and Iqbal, 2014 (15.40%) and Islam et al., 2011 (15.21%). Whereas the amount of Chitosan yielded by Muley et al., 2018 (22.08%) in prawns were comparatively higher as observed in the present study. The decrease in the yield might be due to the loss of sample weight because of excessive removal of acetyl groups from the polymer during deacetylation process, the step in which chitin is converted into Chitosan and the loss of Chitosan particle during washing.

Moisture Content: The moisture content of Chitosan was found to be 1.35±0.07% as shown in the Table 1, which was in agreement to that reported by Mohanasrinivasan et al. 2013, Islam et al. 2011 [17] and Muley et al. 2018 [16]. Moisture content of Chitosan less than 10 % encourages better storage stability and quality (Li et al.1992 and Muley et al. 2017).

Ash Content: The extracted Chitosan had an ash content of 0.80±0.02% as mentioned in Table 1. According to No et al. (1995), the limit of ash content in a high quality Chitosan should be less than 1 %. Ash content directly associate with purity and efficiency of the demineralization step in removing minerals. Occurrence of ash in Chitosan could prominently affects the solubility, molecular weight and viscosity in different solvents (Mohanasrinivasan et al. 2013).

Water Binding and Fat Binding Capacity

Water binding capacity (WBC) and Fat binding capacity (FBC) of Chitosan derived from shrimp shell was found to be 562.2±0.6% and 391.46±0.80% respectively. The result observed in the present study are in agreement with the studies reported by Hossain et al. 2014, Mohanasrinivasan et al. 2013, Islam et al. 2011 and Muley et al. 2018) [16]. According to Mohanasrinivasan et al. 2013 WBC and FBC are the functional properties of Chitosan that differs with the method of preparation. Changing the arrangement of steps such as demineralization and deproteinization causes a noticeable influence on WBC and FBC (Rout, 2001) [22].

Solubility: To determine the quality of Chitosan, solubility plays an important role, where higher solubility refers to a superior quality Chitosan. In the present study the solubility of the synthesized Chitosan was found to be 82±0.1% and 80.06±0.9% in acetic acid and ascorbic acid respectively. According to Hossain and Iqbal, 2014 solubility of Chitosan is been affected by various factors which includes temperature and time taken for deacetylation, NaOH concentration, earlier treatments applied for chitin isolation, ratio of deprotenized product (chitin) of shrimp shell to alkali solution, and particle size of Chitosan, out of which the solubility is mostly influenced by degree of deacetylation. Brine and Austin, 1981 noted that poor solubility of Chitosan indicates partial removal of protein and acetyl group. Since the solubility of Chitosan depends on the elimination of acetyl group therefore the lower degree of deacetylation could certainly affect the results.

Degree of Deactylation: Degree of deacetylation (DD) is the ratio between glucosamine and N-acetyl glucosamine, which is predominantly been influenced by NaOH concentration and temperature. The process of converting chitin into Chitosan was determined by the formation of glucosamine. Consequently, higher the glucosamine content higher the degree deacetylation of Chitosan (Vijay Kumar et al., 2019, Hossain and Iqbal 2014, Hargono et al., 2003). In the present case, that DD of the Chitosan using acid-base titration method was 77.90%. As soon as the degree of deacetylation reaches 50 %, Chitosan is found to be dissolved in weak acids. As per the earlier studies, the DD of Chitosan may varies from 30% to 99% depends on the species and the method used for the isolation of Chitosan. It is unusual to achieve the 100% DD (No et al. 1995, Islam et al., 2011 and Vijay Kumar et al., 2019).

Scanning Electron Microscopy: The morphology of Chitosan was studied by scanning electron microscopy (SEM). In Figure 2 shows the SEM images of Chitosan synthesized from shrimp shell was observed to have layers of flakes, non-homogenous and non-smooth surface with strips and shrinkage and porous surface could be seen on specific areas.

Crumbling flakes along with fibril structures were observed at high magnification (Yen et al. 2009 & Kucukgulmez et al. 2011).

FTIR: The FTIR spectra of the Chitosan isolated from shrimp shell confirms its structures and is shown in Fig1. Chitosan presented some characteristic peaks, the peak in the region of 3,446.26 cm^{-1} that corresponds to OH and NH stretching vibrations of free amino groups and intermolecular hydrogen bonding (Mohanasrinivasan et al. 2013, Arafat et al., 2015 and Dahmane et al., 2014) [2]. The band observed at 2,923 and 2,851 cm-1 corresponds to asymmetric stretching of CH3 and CH2 in the present sample (Mohanasrinivasan et al. 2013 and Guo et al. 2005). Peaks at 1661 and 1560 cm-1 were due to -C=O stretching (amide I) and NH stretching (amide II) of NH-COCH3 group. NH primary, secondary and tertiary bonds vibration was observed at 1380 cm-1.The band of saccharide around 1116 cm−1 was due to the antisymmetric stretching of the C–O–C bridge. C–O stretching of the structure was observed at 1027 cm−1 and 1073 cm−1. The band located at 896 cm−1 was due to the C–H β-glycosidic bond out of plane vibration (Martín-López et al. 2020 & El Knidri et al. 2015 & Ghannam et al., 2016). Fourier Transform Infrared spectrum shows characteristic peaks of carbonyl at 1,661.25 cm-1 and amide at 3,446.26 cm-1 which ensures that this is the FT-IR of Chitosan.

Conclusion

Chitosan was successfully synthesized from the shrimp shell waste by using chemical method. The present study indicates that the prepared Chitosan is soluble in both 1% acetic acid and ascorbic acid solutions. The FTIR and SEM of the synthesized Chitosan confirmed that the obtained material is Chitosan. The extraction of Chitosan has potential to be used in biofertilizers, controlling root knot nematode infection and as a soil amendment in the field of agriculture.

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Table 1: Physicochemical properties of Chitosan

Attributes	Chitosan
Yield (%)	18.12%
Moisture content (%)	1.35±0.07%
Ash content (%)	0.80±0.02%
Solubility (%) in 1% acetic acid	82±0.1%
Solubility (%) in 1% ascorbic acid	80.06±0.9%
Degree of Deacetylation (%)	77.90%
Fat binding capacity (%)	391.46±0.80%
Water binding capacity (%)	562.2±0.60%

Mean ± Std. Deviation

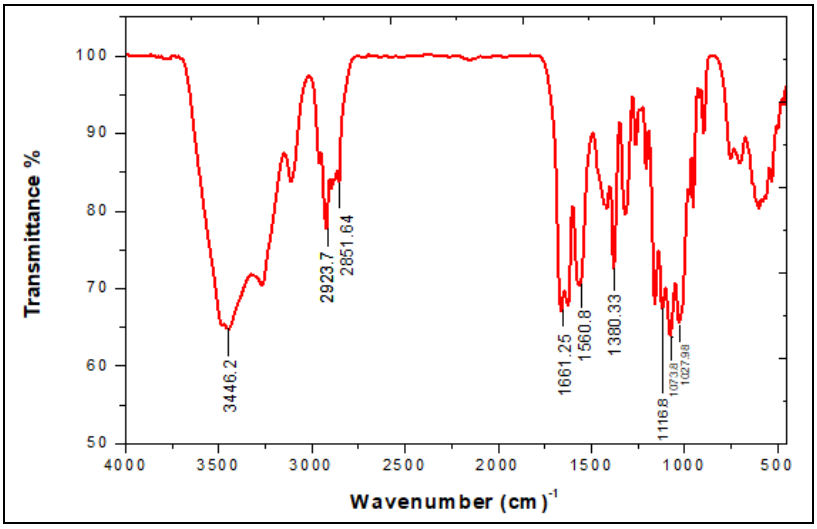

Fig 1: FTIR Spectrum of Chitosan

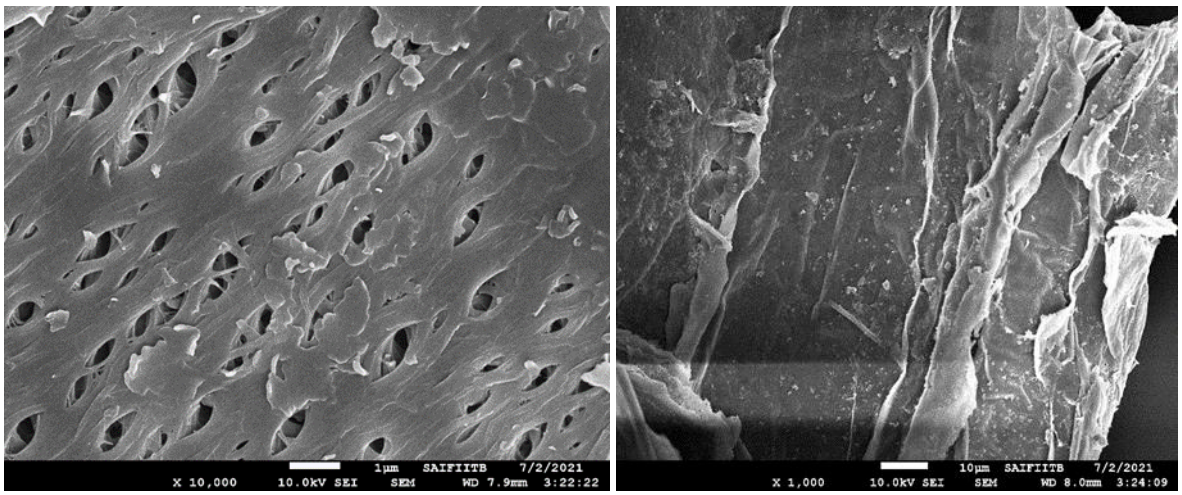

Fig 2: SEM photographs of Extracted Chitosan.

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