



Research Article

Novel Preparation of Chitosan from Crab Shell using Probe Sonicator and its Antibacterial activity

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Abstract

Chitosan is the significant derivative from the crab shell and also used for numerous applications including domestic, agriculture and biopharmaceutical purpose. In practice, chitosan was extracted from crab shell waste by chemical method involving demineralization, deproteinization and deacetylation whereas deacetylation process was toughest, costly and time consuming process with utilization of 50% NaOH repeatedly. The aim of the present study is to minimize the chemical utilization by using probe sonicator. Resulted chitosan had high degree of deacetylation which was confirmed by FT-IR and also the evaluation of the antibacterial activity against clinical isolates such as *Escherichia coli* and *Staphylococcus aureus* were performed. This study showed that probe sonicator mediated preparation of chitosan has high degree of deacetylation and potential antimicrobial agent.

Keywords: Chitosan, crab shell, chemical utilization, probe sonicator, antimicrobial agent.

Introduction

Chitosan, a biodegradable and biocompatible polymer is a modified natural carbohydrate and the second most abundant polysaccharide in nature. It can be synthesized by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. It consists of repeating units of glucosamine and N-acetyl-glucosamine, the proportions of which determine the degree of deacetylation of the polymer. Chitosan is available in a wide range of molecular weights and deacetylation degrees. Chitosan is natural and linear polysaccharides made from chitin by a chemical process involving decalcification, deproteinization, decolourization and deacetylation has received extensive consideration because of its unique properties. Prevention of growth of pathogens is habitually achieved by using chemical preservatives which act as antimicrobial compounds that inhibit the growth of unwanted microorganisms. The chemical preservatives having potential toxicity draw the interest to find an alternatives source for antimicrobial compounds. Naturally occurring marine compounds possess antimicrobial activity against diverse microorganisms which causes diseases to human beings. Crab Shell is made up of three basic components namely chitin, protein and calcium salts of which chitin are most important for scientific studies. Chitin is a white porous polysaccharide and biopolymer.

Deacetylation of chitin produces non-toxic biodegradable polymer of D-glucosamine known as chitosan. Chitosan is the significant derivative from crab shell extract used for several application such as domestic, agriculture and biopharmaceutical. It has been used as a compound in all types of dressing, surgical sutures, dental implants, rebuilding bones and gums. It is also helpful in kidney failure and it binds with toxins in the digestive tract thereby leading to their excretion (Kim et al., 2009; Ahamed and Sastry, 2011a). Considering the above points in view, the present study represents the extraction of chitosan by novel method using probe sonicator and evaluation of its antibacterial activity by broth dilution and disc diffusion methods.

Materials and methods

Crab shell: Crab shells were collected from nearby fish market (Tirupattur, India). The chemicals (Analytical grade) used in the study was purchased from Sigma-Aldrich Chemical Co., USA.

Preparation of chitosan: Chitosan was obtained by deacetylation of chitin from crab shell by modified method of Ahamed et al. (2015). Crab shells were washed thoroughly to remove sand and other impurities and dried well and soaked in 3N hydrochloric acid solution overnight to remove calcium carbonate.

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The shells were treated with acetone for discoloration. The discolored and decalcified shells were then poured in 5% sodium hydroxide solution for deproteinization. The resultant chitin was deacetylated using the same sodium hydroxide solution to give chitosan. This step was achieved by probe sonicator. Complete deacetylation was confirmed by its solubility in 0.1N hydrochloric acid. Chitosan formed was washed with distilled water, dried at 30°C and stored until further use.

Film preparation: Chitosan films were prepared by casting. About 1% chitosan solution was prepared in 1% acetic acid solution. After 15 min under continuous stirring, glycerol was added. Stirring was continued for 30 min until total homogenization of the mixture was obtained. After that, the final pH (pH = 4.0 ± 0.1) was measured with a digital pH-meter (Crison pH-meter BASIC 20+). Finally, the solutions were filtered and 25 mL of the solution were poured into 90 mm diameter Petri dishes (Ahamed et al., 2015).

Characterization studies

Determination of the degree of deacetylation of crab shell chitosan: The first derivative UV-Spectrophotometry method was used after slight modification for the determination of the degree of deacetylation of chitosan (Ahamed et al., 2015). The degree of deacetylation (DD) of the chitosan was determined using the following formula:

$$DD = 100 - \left(\frac{A}{(W - 204A) / 161 + A} \right) \times 100$$

Where A is the amount of N-acetylamino determined/204 and W is the mass of chitosan used.

Determination of molecular weight of crab shell chitosan: The molecular weight was calculated using Mark-Houwink equation relating to intrinsic viscosity.

$$[\eta] = K_m M_v^a$$

Where $K_m = 1.81 \times 10^{-3}$ and $a = 0.93$ are the empirical viscometric constant that are specific for a given polymer, solvent and temperature.

Fourier Transform Infrared Spectroscopy (FTIR) analysis: The Fourier Transform Infrared Spectroscopy of the crab shell chitosan was taken by Perkin Elmer Spectrum 2 Fourier Transform Infrared Spectrometer using a 500 mg KBr pellet containing 2 mg of the sample.

Scanning Electron Microscopy (SEM): Dried samples were coated with solid ions using an ion coater (fisons sputter coater) under the following conditions. 0.1 Torr pressure, 200 mA current and 70s coating time.

Surface structure was visualized by scanning electron microscope (SEM model LEICA stereo scan 440) using a 15 KV accelerating voltage.

Antibacterial activity: The antimicrobial activity of chitosan film was tested against the growth of two typical pathogenic bacterial isolates namely *Escherichia coli* and *Staphylococcus aureus*. Two methods were used to assess the antimicrobial activity. On one hand, disc diffusion method adapted from Khanafari et al. (2008) was performed. Agar plates were spread with 0.1 mL of inoculums containing approximately 105–106 CFU/mL of bacteria. Films (sterilized with UV light) were cut into a disc shape of 15 mm diameter and then placed on the surface of the medium. After incubation for 24 h at 37°C, the plates were optically examined for the zone of inhibition in the contact area. The same procedure was applied for the films dried in an air-circulating oven at 105°C for 24 h. The total area was used to evaluate the antimicrobial potential of films. On the other hand, the antimicrobial effect of chitosan films in the bacterial growth was also evaluated by the liquid culture medium. The liquid culture assay was conducted in nutrient broths with film pieces (sterilized with UV light). Cell growth was observed by reading the medium turbidity at 620 nm. All the results are the mean of three replicate assays.

Results and discussion

During the past few years, significant research has been carried out in the field of chitosan chemistry with the aim of improving its productivity and reduced utilization of chemicals. In the present study, crab shell chitosan was prepared in a novel approach by the utilization sound waves in the form of probe sonicator. The chitosan was made in the form of sheet can be easily handled by the clinicians and these material are expected to adhere to the applications (Ahamed, 2016).

Degree of deacetylation of crab shell chitosan: The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound namely chitosan with a high degree chemical reactive amino group (-NH₂). This makes the degree of deacetylation (DD) an important property in chitosan production as it affects the physicochemical properties, hence determines its appropriate applications (No and Meyers, 1995). The degree of deacetylation of chitosan ranges from 56–99% with an average of 80%, depending on the crustacean species and the preparation methods (Li et al., 1992). Chitin with a degree of deacetylation of 75% or above is generally known as chitosan. The degree of deacetylation of crab shell chitosan of this study was found to be 72.05±0.15%. These results are in agreement with earlier studies (Meenakshi et al., 2002).

Fig. 1. FT-IR spectroscopy analysis of crab shell chitosan.

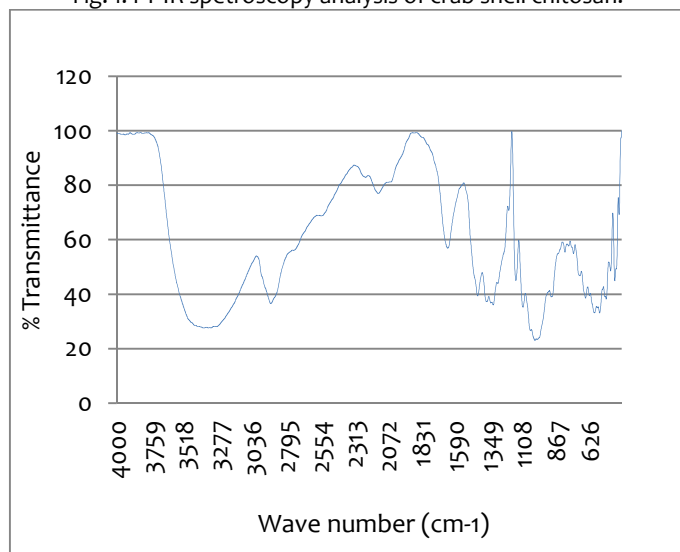
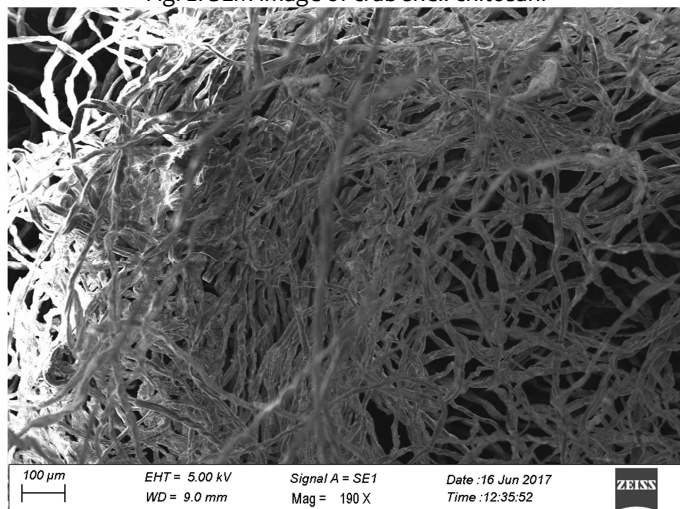


Fig. 2. SEM image of crab shell chitosan.



Molecular weight of crab shell chitosan: Molecular weight of chitosan varied with the sources and also the methods of preparation. The molecular weight of commercial chitosan fell between 100 to 1200 kDa Daltons. In the present study, molecular weight of crab shell chitosan was found to be 600 ± 10 kDa.

FTIR Spectroscopy and SEM image: The IR spectrum of crab shell chitosan (Fig. 1) of this study is similar to the one reported earlier (Shipra et al., 2011). The characteristic -OH stretching vibration with strong intensity broad band around $3200-3600 \text{ cm}^{-1}$, the -OH in plane deformation vibration from $1450-1300 \text{ cm}^{-1}$ and C-O stretching vibration band from $1114-1025 \text{ cm}^{-1}$ are seen. The finger print region and the pattern of bands below 1250 cm^{-1} confirm the nature of the cellulose. The infrared spectrum of chitosan shows the characteristic peaks assignment at 3362 cm^{-1} representing O-H stretch overlapped with N-H stretch,

peaks at 2929 cm^{-1} showing C-H stretch, 1929 cm^{-1} representing amide II band and C-O stretch of acetyl group, 1583 cm^{-1} corresponds amide II band and peaks between $1480-1432 \text{ cm}^{-1}$ corresponds to asymmetric C-H bending of CH_2 group. These results are agreement with earlier studies (Shipra et al., 2011). Figure 2 shows the scanning electron microscopy (SEM) image of the probe sonicator mediated chitosan. The fibrous nature of chitosan is clearly visible with the coating of one fiber and another along with incorporated with individual and aggregated fibers. The diameter of the chitosan fiber was in the range of $10-20 \mu\text{m}$ which is in agreement with reported literature (Ahamed and Sastry, 2011b).

Antibacterial activity: From this study, the chitosan act as an antibacterial agent against the clinical isolates such as *Escherichia coli* and *Staphylococcus aureus*. The chitosan had an ability to inhibit the growth of microbes such as *Escherichia coli* and *Staphylococcus aureus* compared to that of the commercially available chitosan. The kill percentage (Liquid culture test) was represented in Table 1 and 2 and the zone of inhibition (Disc diffusion method) of antibacterial activity was represented in Table 3.

Table 1. Liquid culture test: Antibacterial activity against *E. coli*.

Sample ID	Composition	Kill %
B _E	<i>Escherichia coli</i> (Broth)	0
C _{E1}	Chitosan + <i>Escherichia coli</i>	78
T _{E1}	Chitosan + <i>Escherichia coli</i>	95

Table 2. Liquid culture test: Antibacterial activity against *S. aureus*.

Sample ID	Composition	Kill %
B _E	<i>Staphylococcus aureus</i> (Broth)	0
C _{S1}	Chitosan + <i>Staphylococcus aureus</i>	75
T _{S1}	Chitosan + <i>Staphylococcus aureus</i>	93

Table 3. Zone of Inhibition against pathogens.

Bacterial strains	Zone of inhibition (mm)		
	C	S ₁	T ₁
<i>Escherichia coli</i>	0	10	14
<i>Staphylococcus aureus</i>	0	9	12

C- Control, S₁- Commercially Available Chitosan, T₁- Synthesized Chitosan.

Table 1 and 2 showed that the kill percentage of the clinical isolates was high compared to that of the commercially available chitosan. So it was concluded that the newly synthesized chitosan using probe sonicator has a high potentiality to inhibit the growth of the pathogens compared to that of the commercially available chitosan. In this, the clinical isolates *Escherichia coli* shows high degree of kill percentage when compared to *Staphylococcus aureus*.



In this method, the maximum zone of inhibition was resulted in *Escherichia coli* compared to that of the *Staphylococcus aureus*. The zone of inhibition of *Escherichia coli* and *Staphylococcus aureus* was observed for commercially available chitosan is 10 mm and 9 mm, the newly synthesized chitosan was found to be 14 mm and 12 mm respectively. Azad et al. (2004) reported that the chitosan had an ability to kill the bacterial strains such as *Escherichia coli*. The kill percentage was reported as 92%. So, the results were parallel with the reported literature. So, the present investigation concluded that the newly synthesized chitosan using probe sonicator was a novel approach and it had an ability to inhibit the growth of the microbes such as *Escherichia coli* and *Staphylococcus aureus*. The kill percentage of the synthesized chitosan film showed significant results and toxicity against studied microbes *Escherichia coli* and *Staphylococcus aureus*.

Conclusion

From the study it was concluded that novel preparation of crab shell chitosan was a successful method with easiest, non-costly and controlled process with little utilization of 50% NaOH. In the other hand, the prepared crab shell chitosan exhibited good antibacterial activity against the tested microbes.

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