

Characterization And Bioactivity Study of Chitosan Based Nanomaterial From Exoskeleton Waste of *Parapaeneopsis Stylifera*

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Abstract

Chitosan based biomaterial have superior physical and chemical properties such as high surface area, porosity, tensile strength, conductivity as well as increased mechanical properties. Present study discusses about preparation of chitosan from shrimp (*Parapenaeopsis styliifera*) shell waste and its bioactivity effect. Demineralization, deproteinization and deacetylation was done to obtain chitosan and characterized by infrared spectroscopy and were plotted on all specimen over the frequency range of $4000-400\text{cm}^{-1}$ at a resolution of 4cm^{-1} . Degree of deacetylation of chitosan was estimated with the produced spectra of sample. The DDA of extracted chitosan was found to be 89.91 % while the reference standard showed 81.27 %. The FTIR spectrum shows absorption band at 3450cm^{-1} ($-\text{OH}$ stretching) because OH has highly intense absorption band and at about 2870cm^{-1} for the $-\text{C}-\text{H}$ stretching because the intensity of peak is significant and the band does not involve in hydrogen bonding. It also has CH_2 bending at 1420cm^{-1} as well as the absorption band at about 1660cm^{-1} for $\text{C}=\text{O}$ in amide group. Apart from these bands, it shows absorption bands at 1030cm^{-1} and 1070cm^{-1} [$\text{C}-\text{O}$ stretching]. It also contains NH group- stretching vibration at about 3360cm^{-1} . The absorption band at about 1730cm^{-1} is for carbonyl group vibration. Many peaks of chitosan were observed, which shows a broad-OH stretching absorption band between 3450cm^{-1} . Another major absorption band is between 1220 and 1020cm^{-1} which represents the free amino group ($-\text{NH}_2$) at C_2 position of glucosamine, a major peak present in chitosan. Freshwater *Chlorella*, *Chlorella marina* and *Pavlova lutheri* showed maximum flocculation rate of 89%, 71% and 84% at pH 4.0 at a chitosan concentration level of 1% respectively. The results of synthesis of chitosan based nanoparticles by ionic gelation technique showed spherical morphology of the synthesized nanoparticles and the nature of functional groups present in chitosan nanoparticles were also confirmed by FT-IR and FE-SEM. Antibacterial study revealed that chitosan nanoparticles possess strong activity against Gram negative bacteria compared to Gram positive bacteria. Hence this investigation clearly suggests shellfish waste based chitosan could be used as an effective biomaterial in pharmaceutical as well as industrial applications.

Introduction

Chitosan is a linear copolymer composed of β (1 \rightarrow 4)- linked 2-acetamido-2-deoxy- β -d-glucopyranose and 2-amino-2-deoxy- β -d-glucopyranose units and generally found in the shells of crustaceans, particularly crabs and shrimps (Cheng et al. 2011). It is a biocompatible, biodegradable and antimicrobial polymer (Pradeep 2004). Chitosan has a broad range application on several fields such as water treatment, spill oil removal, drug delivery, tissue engineering, wound healing, food preservation and enzyme immobilization (Kofuji et al. 2005). Chitosan has great potential in the fields like drug delivery, gene delivery, cell imaging, sensors, food, agriculture and water treatment as well as diagnosis of some diseases like cancer (Al Sagheer and Muslim 2009). Chitin is a copolymer of *N*-acetyl-d-glucosamine and deacetylated form of chitin refers to chitosan. Chitin and chitosan can be found as supporting materials in many aquatic organisms (Tokura and Tamura 2007).

Nanotechnology may be able to create many new materials with a vast range of applications (Rabindra et al. 2012). The interesting properties of nanoparticles are largely due to the huge surface area of the material accompanied usually by an increase in stability and improved functionality. Chitosan nanoparticles can be formed by incorporating a polyanion such as tripolyphosphate (TPP) into a chitosan solution under constant stirring. Chitosan based nanomaterial have superior physical and chemical properties such as high surface area, porosity, tensile strength, conductivity, photo-luminescent as well as increased mechanical properties. Ana Grenha (2012) has been reported that chitosan nanoparticles have an excellent capacity for associating proteins successfully in drug delivery systems to control the releasing process of the drug.

Chitosan nanoparticles have many applications in medical and pharmaceutical uses. Chitosan has also proved to prevent infections in wounds and enhance the wound-healing process by the growth of skin cells. It can also used as an additive in antimicrobial textiles for producing clothes for healthcare and other professionals. Nanoparticles improve antigen uptake by mucosal lymphoid tissues and induce strong immune responses against antigens and chitosan nanoparticles showed effective antimicrobial activity against *Staphylococcus saprophyticus* and *Escherichia coli* (Yang et al. 2005).

Antibiotic resistant microorganisms have attracted much attention recently. It has been reported that there is no single antimicrobial agent that has not exhibited resistance by microorganisms. This new development has forced scientists to formulate novel antimicrobial agents that show an effect against the continually increasing antibiotic resistant pathogenic microorganisms (Roy et al. 1999). Chitosan, a versatile hydrophilic polysaccharide derived from chitin, has a broad antimicrobial spectrum to which gram-negative and gram-positive bacteria are highly susceptible. There are several mechanisms which could be suggested to explain this activity and one of them was related to the basic nature of the polymer and the amine content (Zamani et al. 2007). Chitosan is a positively charged molecule and the target of its antimicrobial action is the negatively charged cell wall of bacteria, where it binds and disrupts the normal functions of the membrane, e.g. by promoting the leakage of intracellular components and also by inhibiting the transport of nutrients into the cells. Antimicrobial activity of chitosan is due to its polycationic nature. Chitosan may be suitable for incorporating other antipyretic for the preparation of long-acting antibacterial wound dressing (Li-Ming Zhao 2011).

Flocculation, on the other hand takes advantage of aggregating microalgae cells to form larger biomass bulks through interaction of flocculant with the surface charges of suspended solids that creates large particles (Ozacarand 2003). Several flocculants have been developed to induce flocculation of microalgae cells (Chen 2011). Chitosan has emerged as a favourable flocculating agent in harvesting microalgae (Irin 2011). Chitosan is increasingly important as a natural biopolymer due to its unique combination of properties like biodegradability, biocompatibility, renewability, bioactivity, and ecological acceptability (Varma et al. 2004). It has variety of current and potential applications in wastewater treatment (Hu et al. 2013), biomedical engineering, food processing and so forth (Ganguly et al. 2011). Special inorganic metal salts and some synthetic organic polymers are widely used as flocculants for separation of microalgae or other microorganisms (Schenk 2008). Chitosan is a biodegradable and non-toxic flocculant with

interesting polyelectrolytic and adsorption properties (Okuda et al. 1997). Additionally, the high cationic charge density of chitosan allows it to strongly adsorb negative regions on other particles and effectively destabilize them (Renault et al. 2009). As the overall charge of microalgae cells is negative, the positively charged chitosan is strongly adsorbed on microalgae cells and effectively destabilize the microalgae (Wu et al. 2007). Hence this study aimed to extract, characterize and purify chitosan from shrimp shell biowaste by chemical process of demineralization, deproteinisation and deacetylation and synthesis its nanoparticles to analyse its bioactivity.

Materials And Methods

Preparation of Raw Material

Prawn shell residues of 1 Kg *Parapaeneopsis stylifera* (Fig.1) was collected from 'The Kerala State Co-operative Federation for Fisheries Development Ltd.' (*Matsyafed*) Neendakara, Kollam and immediately placed all the wastes into zip loc bags. The collected samples were washed with tap water to remove the associated debris and other extraneous matter. After draining off the water, measured the weight of crushed wet samples placing them on foil paper. Sun dried the samples for a period of 48 h or until they are brittle easily by hand.

Preparation of extraction

Extraction was made by the methods described by Abdou et al. (2008). The exoskeleton of the shrimp were crushed and treated for de-mineralization with hydrochloric acid (4%v/v) (for 100ml stock solution, 11.2 ml HCl is measured and the volume made up to 100 ml with distilled water) at room temperature (25°C) for 12 h (Fig. 2). It was then washed with distilled water repeatedly unless the pH drops to neutral. To eliminate the proteins of the residue, the de-mineralized shell was then deproteinised by 4% aqueous NaOH (4gm NaOH per 100 ml distilled water) at room temperature for 24 h. The alkali was drained off and washed with distilled water and dried the chitin at temperature 30±2°C. Subsequently, chitosan was prepared at 40°C by deacetylation of chitin by treated with 50% aqueous sodium hydroxide (1:20; w/ v) (50gm NaOH per 100 ml distilled water) for 3 days. After deacetylation, alkali was drained off and washed with distilled water thoroughly up to pH less than 7.5 and dried the chitosan at ambient temperature (37±2°C).

Quality test with 1% acetic acid

Quality of extracted chitosan was checked by a solubility test with 1% acetic acid (1ml acetic acid per 100 ml distilled water). For the estimation of chitosan produced, took the sample out of the storage and weighed few flakes of prawn shells. Then the samples were put inside a clean beaker and 10 to 20 ml of 1% acetic acid was added. After this, the solution was kept in BOD shaker for 30 to 40 minutes and finally the sample weight was determined.

Determination of the Degree of Deacetylation (DDA %).

Properties of chitosan to a varying extent are strongly dependent on degree of N- deacetylation of chitin. Degree of deacetylation of chitosan was determined by FT-IR spectroscopy, IR 22, applying the band A_{1360}/A_{1420} (Moore and Roberts 1980).

Percentage of degree of deacetylation (DDA) was calculated as follows:

$$\%DDA = 100 - [A_{1320}/A_{1420}] \times 0.03822 / 0.3133$$

A1320: Absorption of band at 1320 cm^{-1}

A1420: Absorption of band at 1420 cm^{-1}

Preparation of chitosan nanoparticles (CNPs)

Chitosan nanoparticles were prepared based on ionotropic gelation method with slight modification (Qi et al. 2004; Du et al. 2009; Lu et al. 2009). Extracted chitosan with an appropriate degree of deacetylation were used for formulating chitosan nanoparticles. The chitosan nanoparticles were spontaneously synthesized by drop wise addition of 2.8% solution of TPP (Penta sodium tripolyphosphate) aqueous basic solution (2.8g TPP in 100ml of distilled water) to 2.8% of the chitosan (2.8 g chitosan in 100 ml of 1% acetic acid) acidic solution respectively (ratio of TPP to chitosan was 1:1). The entire experiment was carried out under vigorous magnetic stirring and the reaction was stopped after the formation of milky white suspension and the pellet containing chitosan nanoparticles was collected and freeze-dried for further investigation.

Yield of Nanoparticles

Yield of nanoparticles was calculated using:

$$\% \text{ Yield} = \frac{\text{weight of dried CS} \times 100}{\text{Initial dry weight of starting material}}$$

Screening of antibacterial activity on the extracts of chitosan and chitosan nanoparticle

Disc diffusion method (Bauer et al. 1996) was followed on Muller Hinton agar plates (Hi-media) to determine the *in vitro* antibacterial activity of chitosan and chitosan nanoparticle extracts against pathogenic bacterial isolates. The type cultures were used as test bacterial strains (Table 1). The MTCC and ATCC Type Cultures were initially activated in nutrient broth and subsequently purified by agar streak plate method. Muller-Hinton agar plates were prepared and allowed to solidify for 15 minutes. After solidification, 0.1 ml of 18 h bacterial culture were surface inoculated using sterile swabs and allowed to set for 5 minutes. Sterile discs (6 mm diameter, Hi-media) which were impregnated with suitable aliquots of desired concentration of compound @ $30 \mu\text{l}$ were placed on the inoculated agar surface using sterilized forceps. Assays were carried out in triplicate sets with suitable controls. Cefuroxime (30mg) was used as positive controls while DMSO (dimethyl sulphoxide) disc were used as negative controls. After incubation

at 37°C in incubator (ROTEK) for 24 h, the area of inhibition zone (mm) was determined using a Hi Antibiotic Zone Scale-C (PW-297, Hi-media).

Table 1. Bacterial cultures used for the antibacterial screening

Bacterial species	Source
<i>Escherichia coli</i>	ATCC - 25922
<i>Bacillus cereus</i>	ATCC - 21366
<i>Staphylococcus aureus</i>	MTCC-740
<i>Klebsiella pneumoniae</i>	MTCC-4030
<i>Pseudomonas aeruginosa</i>	MTCC-424

Determination of micro algal flocculation rate

The flocculating rate of chitosan material was measured using different microalgae suspension. For this, starter cultures of *Chlorella marina*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Nannochloropsis salina* and *Pavlova lutheri* were received from the Central Marine Fisheries Research Institute, Vizhinjam and maintained in Walne's medium at a proportion of 1 ml l⁻¹ Walne's A, 0.5 ml l⁻¹ Walne's B and 0.05 ml of Vitamin B1 and B12 (Walne, 1974). The initial cell density of microalgae noted for *Chlorella marina*, *Chlorella vulgaris*, *Nannochloropsis oculata*, *Nannochloropsis salina* and *Pavlova lutheri* are 1.7x10⁷ cells ml⁻¹, 3.8x10⁷ cells ml⁻¹, 1.7x10⁷ cells ml⁻¹, 2.5x10⁷ cells ml⁻¹ and 2.6 x10⁷ cells ml⁻¹ respectively and pH of the above algal samples were adjusted to 4, 7 and 9 using 1 M NaOH and 1 M HCl. Different concentrations of chitosan (1%, 2% and 4%) were added drop by drop to 10 mL aliquots of different microalgal suspension along with continuous stirring over a magnetic stirrer. The flocculating rate of different microalgae in different pH using different concentration of chitosan was observed. The mixture of algal culture and 1ml of the flocculent which is added drop by drop was stirred in a magnetic stirrer at 150 rpm for 1 min, slowly stirred at 80 rpm for 5 min, and allowed to settled for 5 min. Optical density (OD) of the clarified solution was measured with spectrophotometer (Systronics AU 2701) at 550 nm and flocculating rate was calculated according to the following equation:

$$\text{Flocculating rate \%} = (A - B)/A \times 100\%$$

Where, A and B are the OD at 550 nm of the control and sample supernatant, respectively.

Characterization

Chitosan and chitosan nanoparticles were characterized by FT-IR and FESEM measurements.

Characterization by Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR spectra of the samples were recorded by using a Thermo Fisher Scientific Nicolet is50 FT-IR spectrophotometer (Sophisticated Instrumentation & Computation Centre (SICC), University of Kerala, Trivandrum) in the region of $4000\text{-}400\text{cm}^{-1}$ with spectra resolution of 4 cm^{-1} . As for the dissolved chitosan and the composite solution of chitosan/TPP nanoparticles, the spectra were recorded after a drop of the both solution was placed on the diamond reflector.

Surface morphology determination by FE-SEM

The morphological study of nanoparticles was performed by Field emission scanning electron microscope (FE-SEM) (Hitachi, Model-S4800 type II). Nanoparticles were dispersed in water (5 ml) and sonicated for 3 min (E-Chom Tech, Taipei, Taiwan). Few drops of the prepared samples were put on double sided adhesive tape fixed on metal stub. After drying, gold coating was performed for 80 second under vacuum.

Results

Extraction of chitosan

Chitosan was extracted from 1 Kg waste residues of *Parapaeneopsis styliifera* (Fig. 3 & Fig. 4). Chitin obtained after the chemical process of demineralization and deproteinisation was further deacetylated to extract chitosan. In this process, 330 g of dried residues were used to extract about 9.0 g dry-1 weight chitosan. Quality test with acetic acid also indicated that chitosan was readily soluble in 1% acetic acid.

Determination of degree of deacetylation

Present study indicated that different concentrations of NaOH and demineralization with hydrochloric acid and acetic acid influenced the yield of the extraction process. It was also proved that the methods used also had an effect on the degree of deacetylation (Table 2) and to confirm that the biopolymer was chitosan, the product obtained was characterized and compared by infrared spectrometry. The degree of deacetylation of chitosan was calculated from its FT-IR spectra using the absorbance bands of 1320 and 1420. The degree of acetylation of the extracted sample chitosan is found to be 19.27, which is calculated from the Fourier Transform Infrared Spectrograph of the extracted sample. This degree of acetylation indicates that the purity of the extracted sample chitosan which is represented by its degree of deacetylation as 89.91%. The reference standard used for the whole process showed a degree of acetylation of about 11.91 and degree of deacetylation of 81.27%. The prepared chitosan nanoparticle exhibited degree of acetylation and decetylation as 9.57 and 90.42% respectively (Table 2). Hence the degree of deacetylation of extracted chitosan and chitosan nanoparticle were found to be 89.91% and 90.42% compared to 81.27% of commercial chitosan as determined by elemental analysis.

Table 2. Percentage DDA of chitosan samples

Samples	Absorbtion band	transmittance	absorbance	%DDA
Commercial chitosan	1320	98.1	0.0083	81.27
	1420	99.5	0.0022	
Sample chitosan	1320	98.2	0.0079	89.91
	1420	99.7	0.0013	
Chitosan nanoparticle	1320	98.2	0.0079	90.42
	1420	99.4	0.0026	

Chitosan nanoparticles

At the initial stage of the experiment, little amount of CNPs in the solution and the system showed the property as a clear solution. Gradually the amount of CNPs in the solution increased and the system changed from a clear solution to an opalescent emulsion, indicating the formation of CNPs (Fig. 5). Preparation of chitosan nanoparticles is based on an ionic gelation interaction between positively charged chitosan and negatively charged tripolyphosphate at room temperature. Interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution and ultra-sonication time. There are many factors have effect on the size of chitosan nanoparticles such as chitosan concentration, sodium tri polyphosphate concentration, pH of solution and ultrasonication time. In the present study, kept the pH of solution and ultrasoincation time constant at pH 5.5 and 45 min respectively and changing the concentration of both chitosan and TPP. FESEM images of the biosynthesized nanoparticles are displayed in Fig.6. Spherical nanoparticles are seen that appear to be well separated and stable over the steps of the preparation process. FT-IR spectra of pure chitosan, reaction solution of chitosan with including CNPs are showed in Fig 5. Band at 3448.61 cm^{-1} corresponds to the combined peak of the NH_2 and OH groups stretching vibration in chitosan. For reaction solution, the intensities of amide bands at 1560.77 cm^{-1} , which also observed clearly in pure chitosan and decrease dramatically. One new absorption band at 1629.68 cm^{-1} , which can be assigned to the absorption peak of the NH_3^+ absorption of chitosan is also observed.

Yield of chitosan and chitosan nanoparticle

The yield of chitosan was obtained by comparing the weight of the raw material to the weight of chitosan, which was obtained after the treatment, the weight of the dry shells taken for chitosan extraction was 780gm. After the process of demineralization, deproteinisation and deacetylation obtained 8.89 g of chitosan. The yield of chitosan nanoparticle was also calculated with the following equation.

$$\% \text{ Yield} = \frac{\text{weight of dried CS} \times 100}{\text{Initial dry weight of starting material}} = \frac{2.8\text{g} \times 100}{5.6\text{g}} = 50\%$$

Screening of antibacterial activity

The antibacterial screening of CNPs and extracted chitosan (1 mg/ml) were performed on pathogenic bacterial strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Klebsiella pneumonia* and *Escherichia coli*. The results indicated that CNPs inhibited most of the bacterial isolates to various degrees in agar disc diffusion method. According to the obtained results, Gram +ve strains exhibited most sensitivity compared to other Gram -ve isolates. However, most of the Gram -ve strains, did not exhibit a higher range of inhibition. The standard chitosan produced an inhibition zone of 12 mm towards Gram -ve *Pseudomonas aeruginosa*, 8 mm towards *Klebsiella pneumonia* and 9 mm towards *Escherichia coli*. The moderate level of antibacterial activity was produced by Gram -ve *Klebsiella pneumonia* against standard chitosan. It was also noted that among the Gram -ve bacteria, not all the target strains tested were equally susceptible to the antimicrobial metabolites against extracted chitosan and CNPs. Both Gram-positive and Gram-negative bacteria were susceptible to chitosan and chitosan nanoparticle greatly when compared with cefotaxime (30mg, USP). The most susceptible organisms was Gram +ve *Bacillus subtilis* against CNPs and the inhibition zone recorded was 23 mm (Table 3). It was also found that the inhibition zone size obtained by the disc containing chitosan powder solution around the bacteria is low compared to the disc containing biosynthesized CNPs. *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Escherichia coli* was found to be inhibited by chitosan. It was also observed that extracted chitosan, which had a higher degree of deacetylation showed enhanced antimicrobial activity. The study highlights the need for standardized methods to be used in evaluating chitosan's antimicrobial properties in future studies. Antibacterial screening of commercial antibiotics are depicted in Table 4.

Table 3 Results of Antibacterial screening

Samples	Bacterial pathogens (Inhibition zone in mm)				
	<i>Staphylococcus aureus</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus subtilis</i>	<i>Klebsiella pneumonia</i>	<i>Escherichia coli</i>
Standard chitosan	10	12	11	8	9
Extracted chitosan	13	19	20	15	13
Chitosan nanoparticle	16	20	23	17	14

Table 4. Antibacterial screening of commercial antibiotics

Test microorganism	Cefotaxime (30 mg), usp	Ampicilin (10 mcg)
<i>Staphylococcus Aureus</i>	16	11
<i>Pseudomonas aeruginosa</i>	14	-
<i>Bacillus Subtilis</i>	18	-
<i>Klebsiella pneumonia</i>	14	-
<i>Escherichia coli</i>	15	12

Determination of micro algal flocculation efficiencies

Table 5 to Table 9 showing the flocculation efficiencies (calculated using OD 550) microalgae such as *Pavlova lutheri*, *Nannochloropsis salina*, *Nannochloropsis oculata*, *Chlorella marina* and *Chlorella pyrenoidosa* in different pH. The results indicated that the flocculation rate of chitosan depended both on the pH as well as the concentration of chitosan. Freshwater *Chlorella*, *Chlorella marina* and *Pavlova lutheri* showed maximum flocculation rate of 89%, 71% and 84% at pH 4.0 at a chitosan concentration level of 1% respectively. While *Nannochloropsis salina* showed maximum flocculation of 83% (pH 4) at a chitosan concentration of 4%. *Nannochloropsis oculata* exhibited maximum flocculation rate of 99% (pH 9.0) at the chitosan concentration of 1%. Among the microalgae *Nannochloropsis oculata* showed higher flocculation rate compared to other species.

The marine algae, *Chlorella marina* showed comparatively lower flocculation rate. Flocculation efficiency was not high for acidic pH values nevertheless; a great recovery was obtained at alkaline pH values. When pH value exceeds 9, turbidity of culture increases. In alkaline solutions, chitosan is able to produce large and dense flocks. However, in acidic solutions, it produces dispersed and small flocks. The results of flocculation tests at pH 4 in *Nannochloropsis oculata* (acidic solution) showed relatively low separation. The low separation efficiency is due to the changes in conformation of polymer chains. Relatively high results (99%) of flocculation at pH 9 also noted in the case of *Nannochloropsis oculata*. The results indicate that, given enough time, flocculation in alkaline solutions reaches the same figure regardless of the presence of flocculants. Therefore, pH 7 was the ideal solution for further analysis on the performance of chitosan. It was found that the flocculation rate of chitosan depended both on the pH as well as the concentration of chitosan. The results of conducted experiments also indicated the superior potential of magnetic chitosan in comparison to pure chitosan in separating microalgae cells from algal culture in a relatively shorter time. The flocculant dosage and solution pH proved to have significant effects on flocculation process. Thus, adjusting pH and dosage plays a major role in separation optimization. Practical application of chitosan as a water treatment coagulant is examined in the study presented here. From these results, it is suggested that chitosan aided flocculation could be used as an effective method with immense potential in water quality management.

Table 5. Rate of flocculation of freshwater *Chlorella*

Algae	pH	Concentration	OD ₁	OD ₂	Flocculation Efficiency
Freshwater <i>Chlorella</i>	4	1%	0.802	0.086	89%
		2%		0.211	72%
		4%		0.265	66%
	7	1%		0.128	84%
		2%		0.177	77%
		4%		0.227	71%
	9	1%		0.115	85%
		2%		0.308	61%
		4%		0.233	70%

Table 6. Rate of flocculation of *Chlorella marina*

Algae	pH	Concentration	OD ₁	OD ₂	Flocculation Efficiency
<i>Chlorella marina</i>	4	1%	0.982	0.281	71%
		2%		0.329	66%
		4%		0.76	22%
	7	1%		0.465	52%
		2%		0.337	65%
		4%		0.728	25%
	9	1%		0.333	66%
		2%		0.379	61%
		4%		0.83	15%

Table 7. Rate of flocculation efficiency of *Nanochloropsis salina*

Algae	pH	Concentration	OD ₁	OD ₂	Flocculation Efficiency
<i>Nanochloropsis salina</i>	4	1%	0.879	0.26	73%
		2%		0.33	66%
		4%		0.166	83%
	7	1%		0.251	74%
		2%		0.201	79%
		4%		0.176	82%
	9	1%		0.288	71%
		2%		0.272	72%
		4%		0.23	76%

Table 8. Rate of flocculation efficiency of *Nanochloropsis occulata*

Algae	pH	Concentration	OD ₁	OD ₂	Flocculation Efficiency
<i>Nanochloropsis occulata</i>	4	1%	0.998	0.099	88%
		2%		0.147	83%
		4%		0.115	86%
	7	1%		0.086	90%
		2%		0.145	83%
		4%		0.162	81%
	9	1%		0.113	99%
		2%		0.083	90%
		4%		0.088	89%

Table 9. Rate of flocculation efficiency of *Pavalova lutheri*

Algae	pH	Concentration	OD ₁	OD ₂	Flocculation Efficiency
<i>Pavalova lutheri</i>	4	1%		0.091	84%
		2%		0.185	69%
		4%		0.147	75%
	7	1%		0.1	83%
		2%	0.601	0.122	79%
		4%		0.255	57%
		1%		0.138	77%
		2%		0.131	78%
		4%		0.231	61%

FT-IR spectroscopy

All the Infrared spectra were plotted on all specimen over the frequency range 4000-400cm⁻¹ at resolution of 4cm⁻¹. Degree of deacetylation (DDA) of chitosan was estimated with the produced spectra of sample. Properties of chitosan to a varying extent are strongly dependent on degree of N- deacetylation of chitin. The FTIR spectra of commercial chitosan are presented in Fig. 7. The graph represents wave number (cm⁻¹) along the x-axis and percentage transmittance along y-axis and has several absorption bands. The graph shows the absorption band at 3450 cm⁻¹ (-OH stretching) because OH has highly intense absorption band and at about 2870 cm⁻¹ for the -C-H stretching because the intensity of peak is significant and the band does not involve in hydrogen bond. It also has CH₂ bending at 1420cm⁻¹ as well as the absorption band at about 1660cm⁻¹ for the C=O in amide group (amide1 band). Rather than these bands, it shows absorption bands at 1030cm⁻¹ and 1070cm⁻¹[C-O stretching]. The absorption bands also occur in 897cm⁻¹ (glycoside linkage) and at 1660cm⁻¹(asymmetric C-O stretching). It also contains NH group- stretching vibration at about 3360cm⁻¹ and 1380 absorption band for the CH₃ in amide group. The absorption band at about 1660cm⁻¹ for C=O in amide group (amide 1 band) and the absorption band at about 1730cm⁻¹ for carbonyl group vibration. The peaks of absorption band at about 1275cm⁻¹ for carbonyl group vibration. Another absorption band occurred at 1030cm⁻¹ for C-O stretching.

Many peaks of chitosan were observed which shows a broad-OH stretching absorption band between 3450cm⁻¹. Another major absorption band is between 1220 and 1020 cm⁻¹ which represents the free amino group (-NH₂) at C₂ position of glucosamine, a major peak present in chitosan. Peak at 1384 cm⁻¹

represents the –C-O stretching of primary alcoholic group. Symmetric or asymmetric CH₂ in both absorption bands at about 2920 and 2880cm⁻¹. In the case of chitosan, TPP nanoparticles the tip of the peak of 3438 cm⁻¹ has a shift to 3320 cm⁻¹ and becomes wider with increased relative intensity indicating an enhancement of hydrogen bonding. In nano particles, the peaks for N-H bending vibration of amine I at 1600 cm⁻¹ and amide II carbonyl stretch at 1650cm⁻¹ shifted to 1540 cm⁻¹ and 1630 cm⁻¹. The cross linked chitosan also showed a P=O peak at 1170 cm⁻¹. These results also attributed to the linkage between phosphoric and ammonium ion. It is also noted that the tripolyphosphoric groups of TPP are linked with ammonium groups of chitosan. The terminal phosphate group of TPP binds with amine (NH₂) group of chitosan by ionic bond. In chitosan nanoparticles the peak was shifted to 1564.03cm⁻¹ due to the wagging of NH₂ bond. The ionic interaction with the phosphate group of TPP indicated the conversion of chitosan polymer in the nano form, that forms a cross link with TPP. The strong and sharp peak of phosphate at 1092cm⁻¹ in chitosan nanoparticles confirmed the involvement of TPP while making the nanoparticles.

In the FTIR spectra of chitosan nanoparticles, a strong wide peak in the 3500-3300 area shows hydrogen bonded (O-H) stretching vibrations. The peak for asymmetric stretching C-O-C was found at 1150cm⁻¹. In the spectra, the tip of the peak of 3488 cm⁻¹ has a shift to 3320 cm⁻¹ and becomes wider with increased relative intensity showed enhancement of H-bonding. The cross linked chitosan also shows a P=O peak at 1170cm⁻¹. It is because of the linkage between phosphoric and ammonium ions. So we can understand that the tripolyphosphoric groups of TPP are linked with ammonium groups of chitosan. In the FTIR spectra of chitosan the C-N stretching is shown between 1250-1375cm⁻¹. But in C-N, the peak shifted to 1564 cm⁻¹ due to wagging of NH₂ bond. The strong and sharp peak of phosphate at 1092 cm⁻¹ confirmed the involvement of TPP while making nanoparticle. The presence of P=O and P-O groups are at a frequency of 1180 cm⁻¹. Results of preliminary investigations on the experimental conditions for the formation of chitosan nanoparticles showed that nanoparticles could be obtained by varying the concentrations of chitosan with concentration of TPP respectively (Fig. 5).

Field Emission Scanning electron microscopic (FE-SEM) studies of chitosan nanoparticles

The FE-SEM micrograph details of the pure chitosan nanoparticles was represented in Fig. 8. The pure chitosan texture is plain without pores having smooth, compact and homogeneous even surface structure with no gross effects, while the SEM micrograph of chitosan nanoparticles. Figure 6 revealed the rough surface morphology, with a solid dense cubical or rectangular structure and not aggregated. The spheres exhibited mean diameters around 500 nm. The nanoparticles dry powder consists of individual nanoparticles, which touched each other, but retained their original size and shape. The size variation also related to different conditions of sample preparation for SEM. The synthesized nanoparticles were found to be nearly spherical shape with size in the range of 135-729 nm. The synthesized nanoparticles showed potent antibacterial activity against Gram positive and Gram negative bacteria and the results revealed that natural sources of materials such as shell wastes could be used for preparation of CNPs instead of use of chemical substances.

SEM micrograph of pure chitosan revealed that the texture is plain without pores having smooth, compact and homogeneous even surface structure with no gross effects. The SEM micrograph of chitosan nanoparticles (Fig. 6) revealed the rough surface morphology, has a solid dense cubical or rectangular structure and not aggregated. The spheres showed mean diameters around 500 nm. Nanoparticles dry powder consists of individual nanoparticles, which touched each other, but retained their original size and shape. The size variation also related to different conditions of sample preparation for SEM.

Discussion

Chitosan based nanomaterial have superior physical and chemical properties such as high surface area, porosity, tensile strength, conductivity, photo-luminescent as well as increased mechanical properties. Present study discusses about preparation of chitosan from shrimp shell waste and its bioactivity effect. This study also focuses on the synthesis of chitosan nanoparticles by ionic gelation technique using sodium tripolyphosphate (TPP). (Santos et al. 2011). The results obtained showed a higher yield than that found for the chitin extracted from shrimp *Penaeus brasiliensis*. In our study, the maximum chitosan obtained from chitin deacetylation (89.91%) was similar to the reported value of shrimp *Macrobrachium rosenbergii* (65%). Results obtained by Battisti and Campana-Filho (2014) showed that 80% of chitin was transformed into chitosan.

There is an emergence in the development of antibiotic resistance among bacteria which necessitates several researches to discover novel antimicrobial agents with more potential (Friedman and Juneja 2010). In the present study, chitosan nanoparticles exhibited excellent antibacterial activity which can be further augmented for various applications. Although reports are available on antibacterial activity of chitosan, very few reports are available regarding the application of chitosan nanoparticles on bacterial pathogens for the enhanced antibacterial activity. As our results revealed that chitosan exhibits higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. This may be due to the property of chitosan nanoparticles polycation nature with high surface charge density which interact with bacteria and tightly absorbed onto the surface of bacterial membrane to disrupt the membrane of both Gram-positive bacteria than Gram-negative bacteria (Avadi et al. 2004; Abou-Zeid et al. 2011).

According to the obtained results, it was found that Gram-positive bacteria (*B. cereus*) more sensitive to CNPs compared to Gram-negative bacteria (*K. pneumoniae*). It was also found that the inhibition zone size created by the disc containing chitosan powder solution around the bacteria is minimum compared to biosynthesized CNPs. The present study also explored that spherical and oval shapes of CNPs were obtained by reacting chitosan with negatively charged functional groups such as [- OH] and [- COO] anions.

The results are consistent with those of previous investigations about synthesis of CNPs by using water-soluble linkages such as phosphates, sulfates, cyanates and other agents. It also seems that biocompatible chitosan has been successfully modified by condensation reaction using natural compounds of chitosan extract as cross-linking agents to form nanoparticles. Because of the high efficiency of CNPs on bacteria, it also seems that the biosynthesized CNPs are positively charged and the polycationic CNPs with higher surface charge density interact with the bacteria to a greater degree than

chitosan itself. The study highlights the need for standardized methods to be used in evaluating chitosan's antimicrobial properties in future studies. The antimicrobial activity of chitosan becomes notable at pH 5–6 and this is likely due to the fact that the amino groups of chitosan become positively ionized below pH 6.

Unmodified chitosan is not antimicrobially active at pH 7, likely due to lack of dissolution and lack of positive charges on the amino groups. Numerous works claim that chitosan is involved in a dual mechanism including coagulation by charge neutralization and flocculation by bridging mechanisms (Renault et al. 2009). High cationic charge density is a characteristic of chitosan, while the overall charge of microalgae cells is negative. Chitosan has strong charge-neutralization ability because of the fast adsorption that originates from its large positive charge (Wu et al. 2007). The positively charged chitosan chain is strongly adsorbed on the negatively charged microalgae cells due to the electrostatic attraction. This helps chitosan quickly neutralize the cell charges, with most of the charged groups being close to the surface of the cells (Farid et al. 2013). In order to investigate the influential range of pH, three distant pH points (4, 7, and 9) were selected. Flocculation efficiency was not high for acidic pH values nevertheless; a great recovery was obtained at alkaline pH values. In alkaline solutions, chitosan is able to produce large and dense flocks. However, in acidic solutions, it produces dispersed and small flocks. In our study, chitosan with a degree of deacetylation of 89% was obtained by chemical method involving demineralization, deproteinisation and deacetylation of shrimp shell biowaste. From these results, it is suggested that chitosan aided flocculation could be used as an effective method with immense potential in water quality management. The results of flocculation tests at pH 9 in *Chlorella marina* (acidic solution) showed relatively low separation (less than 15%). The low separation efficiency is due to the changes in conformation of polymer chains. It is previously reported, in acidic solutions chitosan chains become more extended and therefore produce smaller, looser flocs. The results of *Nanochloropsis oculata* exhibited relatively high results (47–99%) of flocculation even at pH 9. However, it has been reported that in alkaline solutions, flocculation is mainly due to chemical precipitation caused by the presence of Mg²⁺ particles (called auto-flocculation) (Guanyi et al. 2014). The results also indicate that, given enough time, flocculation in alkaline solutions reaches the same figure regardless of the presence of flocculant. Moreover, it is indicated that in neutral solutions, due to the more coiled structure, chitosan is able to produce larger and denser flocs (Aranaz et al. 2009). Therefore, pH 7 was the ideal solution for further analysis on the performance of chitosan. The results of conducted experiments indicated the superior potential of pure chitosan in separating microalgae cells from algal culture in a relatively shorter time. The flocculant dosage and pH proved to have significant effects on flocculation process. Thus, adjusting pH and dosage plays a major role in separation optimization.

Scanning Electron Microscopic (SEM) study was carried out to determine the topography, surfaces, structures, morphologies and composition of the chitosan nanoparticles (Khanmohammadi et al. 2015). In the present study also successfully synthesized and optimized chitosan nanoparticle. On comparing the SEM micrograph details, it was observed that the chitosan nanoparticles have a relatively rougher surface with uneven structure which exhibited highly amorphous feature. Ghadi et al. (2014) stated that chitosan nanoparticles prepared from chitosan were found to be having the spherical shape. Particle size has an important role in obtaining optimal *in vitro* efficacy and also a crucial impact on the *in vivo* fate of a drug

delivery system. It is also observed that decreasing particle size could increase the dissolution and thus increase the bioavailability of poorly water soluble molecules. In the present study observed the chitosan nanoparticles mean diameters around 500 nm (Sudheesh et al. 2013). Smaller size particles have efficient interfacial interaction with the cell membrane compared to larger size particles due to the endocytosis and these particles could improve the efficacy of the particle-based oral drug delivery systems. Small particle size can also increase the bioavailability and prolong the blood half-life and increases the efficacy of drugs also.

Conclusion

The results revealed that chitosan nanoparticles synthesized by the ionic gelation method possess strong antibacterial activity against Gram negative bacteria when compared to Gram positive bacteria. Algal flocculation study indicated that the flocculation rate was found to be dependent on pH as well as the concentration of chitosan. Freshwater *Chlorella*, *Chlorella marina* and *Pavlova lutheri* showed maximum flocculation rate of 89%, 71% and 84% at pH 4.0 at a chitosan concentration level of 1% respectively. The spherical morphology of the synthesized nanoparticles and the nature of functional groups present in chitosan nanoparticles were confirmed by FT-IR and FE-SEM elemental analysis. It is also suggested that chitosan aided flocculation could be used as an effective method with immense potential in water quality management. The findings from this research revealed promising anti-microbial activity extracted chitosan from shrimp shell waste with high degree of deacetylation and have the definite potential to develop further into antibacterial drugs in future.

Declarations

Ethical approval

For this type of study, ethical approval is not required.

Consent for publication

Not applicable

Availability of data and materials

All data and materials are availed in the manuscript and no additional input is required.

Competing interests

The authors declare that they have no competing interests

Funding

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Authors' contributions

VSP, HA, SS, AA, JAF and SSB of this paper have directly participated in the planning, execution, or analysis of this study with the due role of the corresponding author in overall coordination as well. VSP guided the research work, performed editing, critical revision and supervised the findings of the study. All authors read and approved the final manuscript.

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Figures



Figure 1

Prawn shell residues of *Parapaeneopsis stylifera*

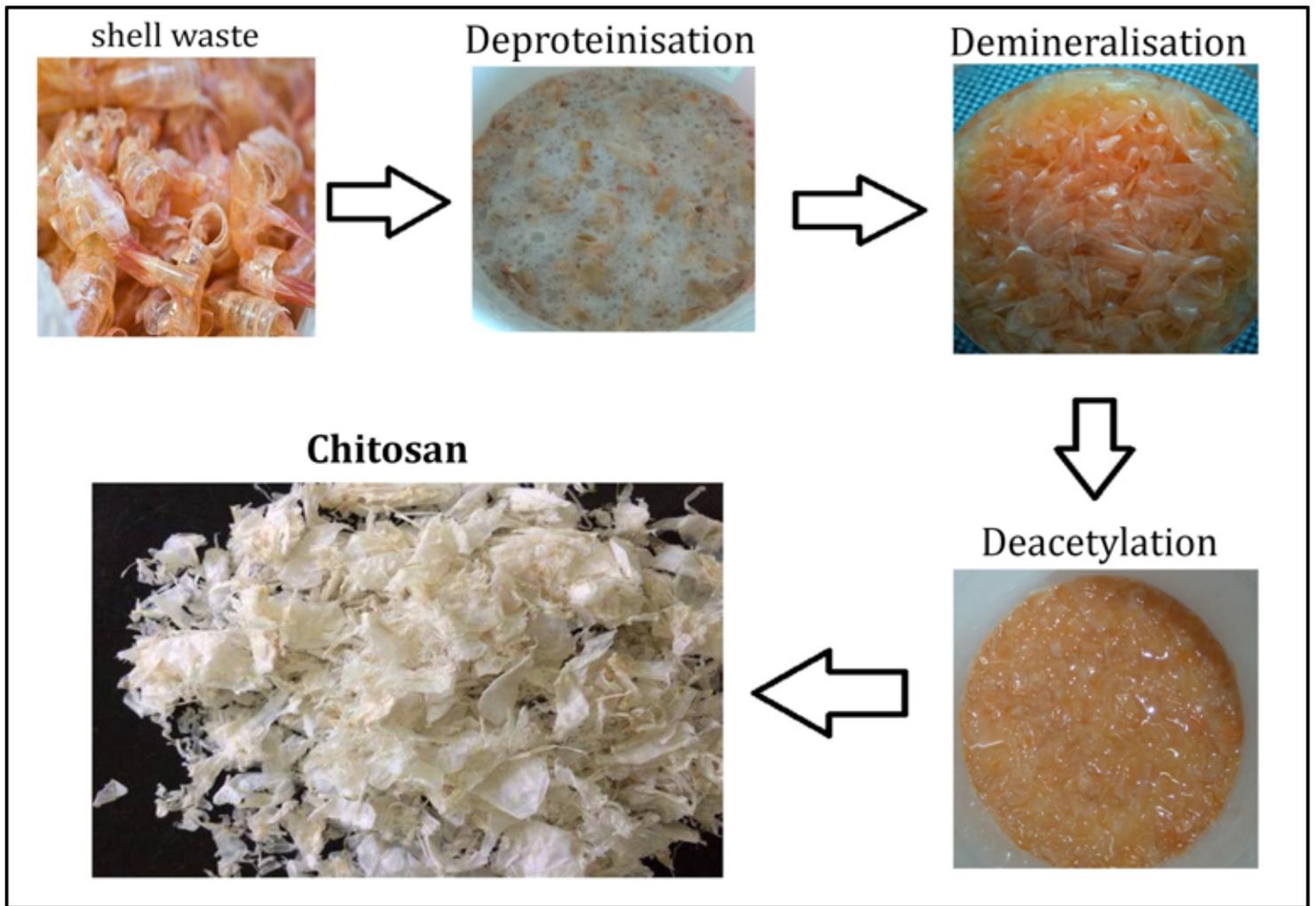


Figure 2

Different stages of chitosan extraction



Figure 3

Standard chitosan



Figure 4

Extracted chitosan



Figure 5

Extracted chitosan nanoparticle

Image not available with this version

Figure 6

Spherical nanoparticles are seen that appear to be well separated and stable over the steps of the preparation process.

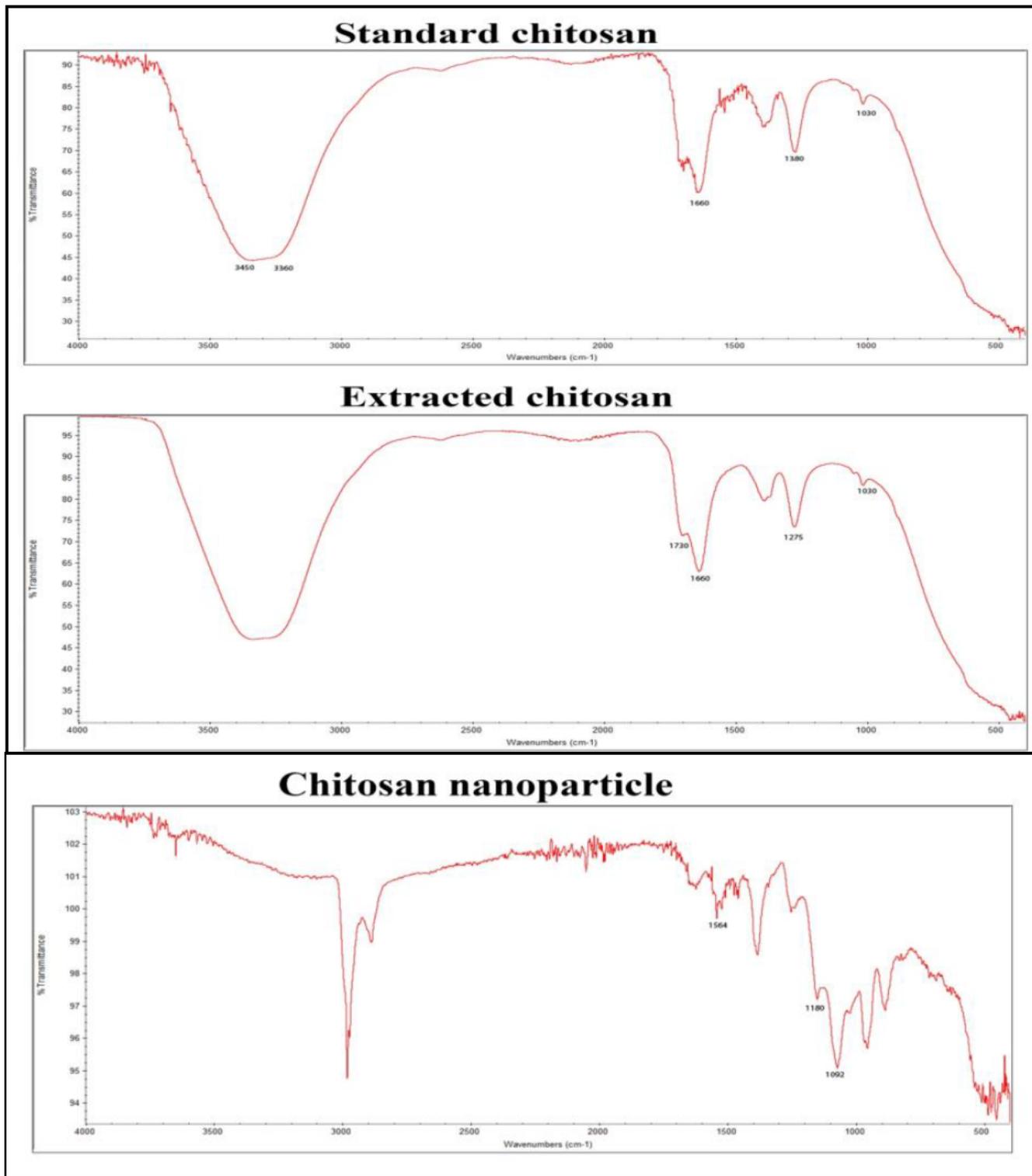


Figure 7

FT-IR spectra of commercial chitosan, extracted chitosan and chitosan nanoparticle

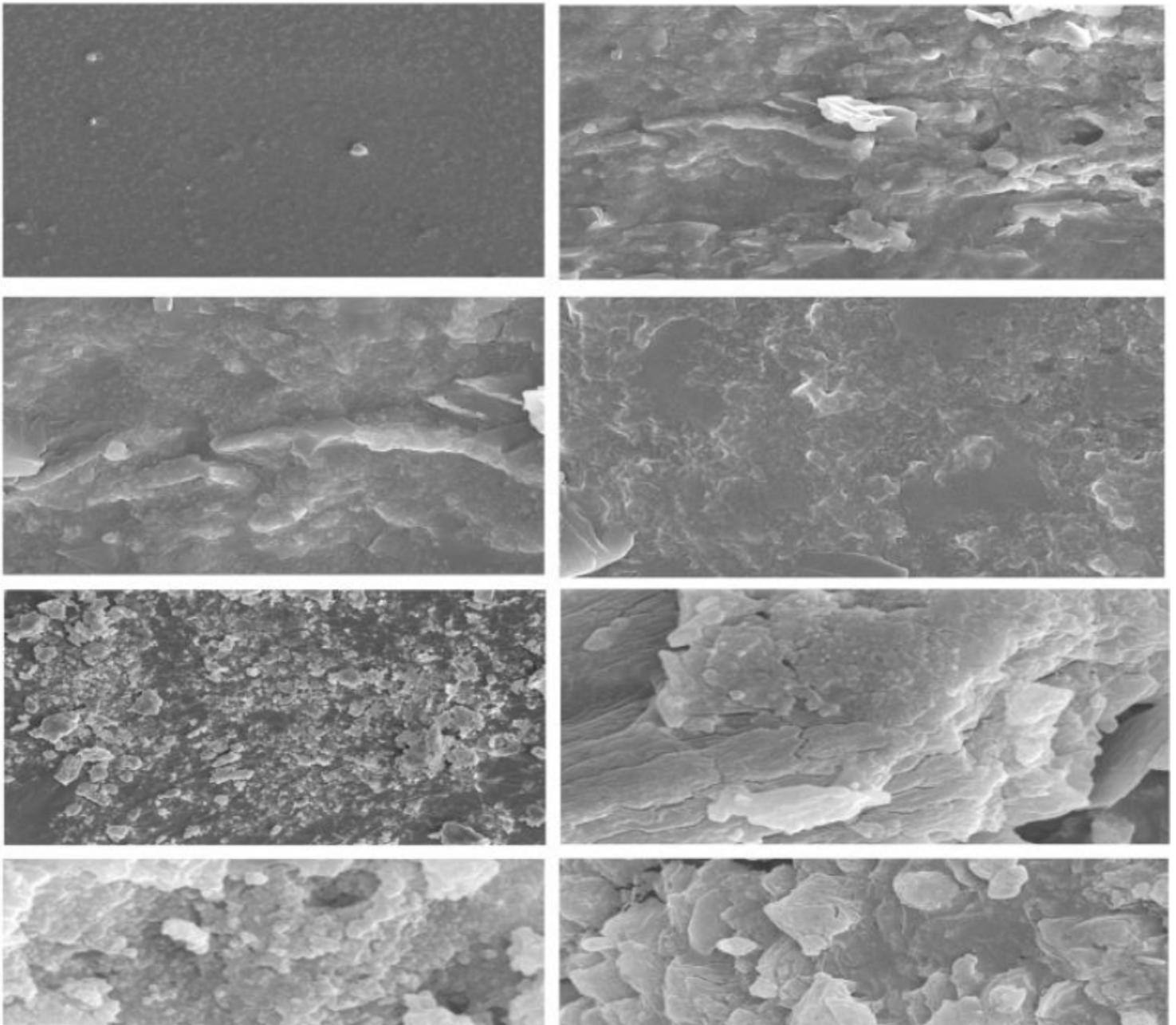


Figure 8

FE-SEM images of chitosan nanoparticle