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Extraction, Characterization and Antibacterial Activity of Chitosan from Mud Crab *Scylla serrata*

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Authors' contributions

This work was carried out in collaboration among all authors. Author AAlagiri conceptualized, did the methodology, did formal analysis, did investigation, did validation, did the resources, wrote the manuscript, wrote, reviewed and edited the manuscript. Authors JP and AAshok did data curation, did formal analysis, wrote, reviewed and edited the manuscript. Author MV supervised the study, conceptualized, did the methodology, did formal analysis, did validation, wrote, reviewed and edited the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The shellfish waste accumulated on a huge scale at seashores and in the seafood industry and very low quantities were used to extract chitosan. In this study, chitosan was extracted from the mud crab *Scylla serrata* shell using conventional chemical methods. The extracted chitosan and commercial chitosan were verified through Field Emission Scanning Electron Microscopy (FE-SEM), Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction (XRD), and an elemental analyzer. ATR-FTIR spectrum confirmed the presence of

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characteristics compounds of chitosan and XRD patterns exhibited crystallinity of chitosan having centered peaks at 10º and 20º in 2θ. Moreover, 46.24% of the degree of deacetylation was obtained for the extracted chitosan altogether enhancing the quality of extracted chitosan. In addition to this, the extracted chitosan was tested for its antibacterial activity against two Gram-negative (*E. coli* and *Pseudomonas*) and two Gram-positive bacteria (*Staphylococcus* and *Bacillus*) in vitro. The antimicrobial activity of extracted chitosan was evident with the greater zone of inhibition against *E. coli* (26±0.32mm) and the least against *Staphylococcus* (18mm). Hence the present study gives insight into the biological properties of extracted chitosan from *Scylla serrata* thereby paving the way for its further use in biomedical science and microbiology.

Keywords: Scylla serrata; chitosan extraction; elemental analysis; ATR-FTIR; XRD; antibacterial activity.

1. INTRODUCTION

Decapod crustaceans are a significant part of the aquatic biota and are a substantial category of seafood worldwide (Krishnaja et al. 1987). The mud crab *Scylla serrata* is the typical highly cultivable crab species along the Indian sea shore and coastal regions, which has enriched nutrition and high meat quality (M.Kathirvel, S.Kulasekarapandain 2004). Moreover, predator consumption and utilization in the seafood industry are consequently accumulating a large number of carapaces and shell particles, finally leading to environmental pollution. Proper bio-management is crucial for protecting the environment from these shell wastes and ensuring a better tomorrow.

Currently, the scientific community has become interested in chitosan poly-β-(1-4)-2-amino-2-deoxy-D-glucopyranose is a deacetylated product of chitin β-(1-4)-2-acetamido-2-deoxy-D-glucan and bio chitosan were extracted from several animals like crustacean's exoskeleton, the cuticle of the insects, fungi cell wall, and annelids (Almanza, et al. 2021). The functional qualities of chitosan include adsorption, food preservation in agriculture, and medical field, mineral binding properties, hypolipidemic activity, biodegradability, antimicrobial activity, immunoadjuvant activity, speeding up the healing of wounds, and inducing phytoalexins (No et al. 2007; Wang et al. 2020). These multiple applications of chitosan in various fields demand large-scale chitosan production all over the world.

Conventional chemical and biological methods are the two major categories of chitosan preparation practices worldwide. The chemical production of chitosan from crab shells goes through the stages of demineralization and deproteinization to produce chitin, which is followed by deacetylation to yield chitosan (Arpi et al. 2021). Several techniques were used for chitosan extraction including conventional chemical methods (Iber et al. 2022); (Pratiwi et al. 2023) enzyme assisted (Nadhem Sayari et al. 2016); (Bellé et al. 2018) microbial fermentation (A. Khanafari et al. 2008) microwave assisted (Sebastian et al. 2019), electrochemical (Nowacki et al. 2020) natural eutectic solvent (Saravana et al. 2018; Morgan et al. 2021). Based on several studies chemical methods have more advantages than biological approaches for chitosan extraction due to the simpler and quicker process, and the products having a lower molecular weight and a high degree of deacetylation which improve its biological activities (Kou et al. 2020).

Chitosan is one of the biochemical substances that scientists extensively study. Several studies have been focused on the antibacterial activity of unmodified chitosan extracted from various sources (Mekahlia and Bouzid 2009); (Teli and Sheikh 2012); (Abdel-Rahman et al. 2015); (Nadhem Sayari et al. 2016); (Chang et al. 2018); (Etemadi et al. 2021); (Mohammadi et al. 2023); (Tamer et al. 2023), due to its intrinsic and extrinsic factors influencing antibacterial effects such as pH, molecular weight, concentration, source of chitosan, degree of polymerization, sources, etc (Ardean et al. 2021). In light of this, we extracted chitosan from the *S. serrata* exoskeleton by conventional chemical methods and evaluated it as an antimicrobial agent as a function of polymer concentration against two Gram-negative and two Gram-positive bacterial strains.

2. MATERIALS AND METHODS

2.1 Materials Collection

Mud crab (*Scylla serrata*) shells were collected from the Beypore fish market in Kerala, India, the crabs were identified using taxonomic

identification keys Marine Species Identification Portal (Forskal et al., 1998). The crab shells were completely separated free of loose tissue, washed with cold water, and dried under the sun. They were then crushed and powdered to a small powder size.

2.2 Extraction of Chitosan

2.2.1 Demineralization

The crab shells were demineralized by using 2.5% (w/v) of hydrochloric acid (HCl) to the ratio of 1:2 (w/v) with the ground shell to the solution at room temperature (36°C) up to 6h. The samples were filtered and washed with tap water for 30 minutes until neutral pH. The final demineralized shell powder was dried in a Hot air oven at 60°C for 24h and weighed. (Fereidoon Shahidi' and Jozef Synowieckit 1991).

2.2.2 Deproteinization

Deproteinization was carried out by adding 1M sodium hydroxide solution (NaOH) with demineralized shell powder at a temperature of 70ºC for 3h. The final precipitant was filtered and washed with distilled water and hot ethanol (10 ml/gm). The precipitant was boiled in acetone for 1h to remove impurities. The deproteinized precipitant was dried at 70ºC for 24h and weighed.

2.2.3 Decolouration and dewatering

Decolourizing was conducted by treating the demineralized shell powder acetone for 10 minutes and drying in room temperature for 2h. The final decolorized shells were washed with running tap water, filtered, and dried at 60°C for 24h in a hot air oven, the final product is chitin. (Fereidoon Shahidi' and Jozef Synowieckit 1991).

2.2.4 Deacetylation of chitin

The deacetylation of chitin was done according to the method by Yen (Yen et al. 2009). The chitin was treated with 40% (w/w) aqueous sodium hydroxide (NaOH) with a ratio of 1:15 (w/v) to the solution at 105°C for 2h. The chitin was filtered and washed with deionized water until pH 7. The final powder was dried at 60°C for 24h and weighed.

2.3 Characterisation of Chitosan and Commercial Chitosan

The commercial chitosan was purchased from Sisco Research Laboratories (medium molecular weight, catalog number 18824), and all chemicals used were analytical grade.

2.3.1 Yield, moisture and ash contents

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 (Abideen et al. 2020). The yield of chitosan was determined by using the equation (1).

$$\text{Yield of chitosan } (\%) = \frac{\text{Dried chitosan weight}}{\text{Dried crab shell powder weight}} \times 100 \quad (1)$$

The moisture content was measured by the gravimetric method for both commercial chitosan and extracted chitosan (Olaosebikan et al. 2021). The moisture mass was calculated by drying the chitosan sample to a constant weight and determining the weight of the sample before and after drying. Moisture content was determined using the formula (2) based on the study reported by Abideen (Abideen et al. 2020).

$$\text{Moisture content } (\%) = \frac{(\text{wet chitosan weight (g)} - \text{dry chitosan weight (g)})}{\text{wet chitosan weight (g)}} \times 100 \quad (2)$$

The ash contents were determined according to standard AOAC procedure (AOAC,1990).

2.3.2 Degree of deacetylation

The direct titration method was used to determine the degree of deacetylation of chitosan extracted from mud crabs, which was conducted according to the method by Kjartansson (Gunnar Thor Kjartasson 2008) with some modifications. Chitosan samples (0.1g) were dissolved in 25ml of 0.06M HCl for 1h at room temperature. The solutions were diluted to 50 ml before being titrated with 0.1N NaOH to pH 3.75 under constant stirring. The volume of NaOH at 3.75 pH was acquired and recorded (V1). Titration was continued to 8 pH and the total volume of NaOH (0.1M) was recorded (V2). The degree of deacetylation was calculated using the following equation (3).

$$\text{DD} = \frac{161.16(V2-V1)N}{W1} \quad (3)$$

Where, 161.16 is the mass of the chitosan monomer, V1 and V2 are the volumes of NaOH solution used, N is the strength of the NaOH

solution (0.1M) and W1 is the mass of the sample after correction for moisture. The degree of deacetylation (DD) of the samples was determined in triplicate.

2.3.3 Elemental analysis

Elementor- Vario EL III, the elemental analyser was used to analyse elements in chitosan sample to estimate the contents of Carbon, Hydrogen, Nitrogen and Sulphur.

2.3.4 Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy analysis (ATR-FTIR)

The ATR- FTIR analysis spectroscopy of the extracted chitosan was determined according to the method Muhammad (Mussarar H Mohammed 2013). The FTIR spectra were recorded using a Shimadzu irspirit 2018 version over the frequency range 400-4000cm^{-1}.

2.3.5 X-ray diffraction analysis (XRD)

X-ray diffraction analysis (XRD) was carried out for the commercial chitosan and extracted chitosan samples. Empyrean, Malvern Panalytical the instrument was operating at a voltage of 40 kV and a current of 30 mA. The XRD pattern was recorded in the 2θ range of 5°C to 80ºC in a fixed time mode at room temperatures.

2.3.6 Field emission- scanning electron microscope (FE-SEM)

The external morphology of extracted and commercial chitosan was characterized using a Field emission- scanning electron microscope (FE-SEM). The samples were dried using a freeze dryer at 105ºC coated with a thin layer of gold and analysed using FE-SEM.

2.4 Preparation of Chitosan Solution

Chitosan solutions are prepared by dissolving 2.5% (w/v) chitosan with 1.0% (v/v) acetic acid solution. The pH was adjusted to 5.8 with 10M NaOH because the most suitable pH for solubilizing chitosan is 5.8 (Ardean et al. 2021). After stirring overnight, the solutions were autoclaved at 120ºC for 15 min.

2.5 Microorganisms Preparation

The antibacterial activity of the extracted chitosan of the *S. serrata* shell was tested against two Gram-positive and two Gram-negative bacterial strains. They were *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Escherichia coli* are Gram-positive and Gram-negative bacteria respectively collected from a microbiology laboratory, Coimbatore.

2.6 Antimicrobial Activity

Different concentrations of the sample (50µg/mL,100 µg/mL, and 200µg/mL) were prepared from the solution (5mg/ml) prepared from stock solution (1mg/ml), chitosan was mixed in the appropriate amount of 0.1% acetic acid. 5.3g of Muller Hinton agar was dissolved in 100 ml of distilled water and autoclaved to sterilization. The sterilized medium was poured on the sterilized plates. To assess the antibacterial activity of the sample against *Escherichia-coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa,* an appropriate quantity of these bacteria was swabbed gently on the Muller Hinton agar plate and equitized wells were created. The plates were kept overnight for incubation at 32°C. After the incubation time, the zone of inhibition was measured in mm and three replicate plates were used for each concentration.

2.7 Statistical Analysis

All data were evaluated using an independent t-test for both commercial and extracted chitosan and the significance level was calculated at $p<0.05$.

3. RESULTS AND DISCUSSION

Chitosan is a natural biopolymer obtained from chitin and one of the most abundant bio-polysaccharides in nature (Ardean et al. 2021). Among all other invertebrates chitosan prepared from crab shells had significantly improved antibacterial activity, degree of deacetylation, nitrogen content, etc (Rao-chi Chien, Ming-Tsung Yen 1996). In this present study chitosan was extracted from *S. serrata* shell by using the cheap, quicker and eco-friendly conventional acid-alkali method (Table 1) and (Fig. 1).

3.1 Yield

The yield of extracted chitosan and moisture content extracted from *S. serrata* is presented in Table 2. In this study, chitosan yield from *S. serrata is* 44.2±3.4% almost similar to the 39% yield reported in mud crabs by Ali et al., (2019). An earlier study by Parthiban (Parthiban et al.

2017) indicated that the yield of chitosan from shrimps is 15%, from crab is 13%, and 12.55 from squillas**.** Sreelekshmi (Sreelekshmi et al. 2022) produced chitosan up to 18-20% from shrimps. High yield was obtained which justifies the high potential of mud crab chitosan usage as an economic production of chitosan on an industrial scale due to the availability of mud crabs and the low cost of the source.

3.2 Moisture

In the present study, the moisture content of *S. serrata's* chitosan is 7.43% which is significantly higher than the commercial chitosan (Max 5%) (Table 2). The hygroscopic nature of chitosan is around 7% (Anand M et al. 2014). The moisture content from *S. serrata's* chitosan obtained was 7.43±0.20 %. Previously the moisture contents of chitosan were reported in the range of 2.37-5.4% from crayfish by Adyinka et al. (2020) whereas, Sajomsang and Gonils (2010) reported 8.7% in chitin collected from Cicada. Standard chitosan moisture content for usage in applications ranged from 5.0% to 15.0%, depending on humidity and chitosan form regardless of whether it is flakes or powder (Struszczyk 2006).

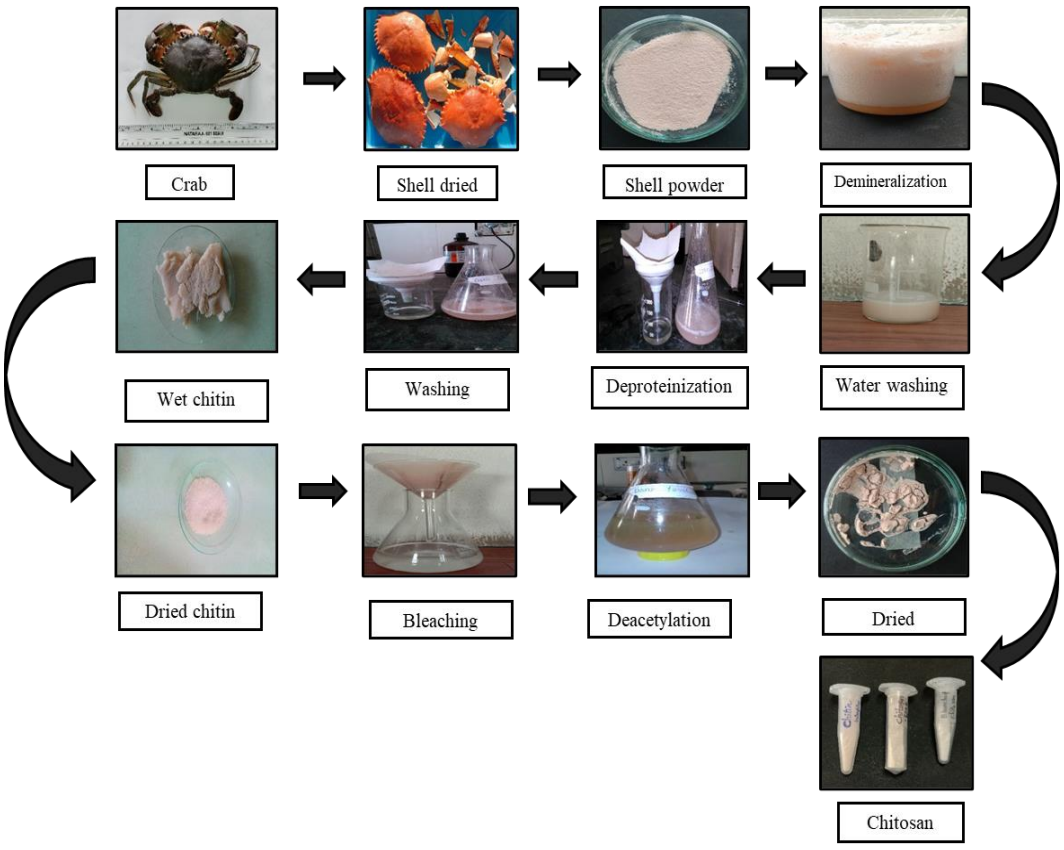


Fig. 1. The chemical extraction steps of chitosan from the *S. serrata* shell

Table 1. Details of the chitosan yield obtained from *Scylla serrata* in the acid-alkali extraction method

Sl. No	Content	Weight in g
	Preparation of chitosan	
1.	Dry shell weight	100g
2.	Ground shell weight	90.12±0.5g
3.	Demineralized shell	86.92±0.6g
4.	Deproteinized shell (raw chitin)	82.24±0.41g
5.	Decolorized shells (chitin)	82.12±0.21g
6.	De-acetalized shells (chitosan)	44.2± 3.4g

**Values are given as mean ± SD of three replicates*

Table 2 The yield of extracted chitosan, moisture content extracted from mud crab (*Scylla serrata*) shells

Sl. No	Characteristics (%)	Commercial chitosan	Extracted Chitosan
1.	Yield	-	44.2± 3.4 %
2.	Moisture content	Max 5%	7.43±0.20 %*
3.	Ash content	5.23%	7.63 ± 0.52%*
4.	Degree of deacetylation	Approx. 90%	46.24 %*
5.	Colour	White to pale yellow	White to pale yellow

*Values are given as mean ± SD of three replicates, and * indicates significant difference between commercial chitosan and extracted chitosan is statistically significant p<0.05*

3.3 Ash Content

The ash content of extracted chitin was 7.63±0.52%, according to Kumari et al. (2017) which indicates the complete demineralization process during the extraction process. The ash content values for both commercial chitosan and extracted chitosan which are significantly varied each other (Table 2).

3.4 Degree of Deacetylation

The deacetylation is the removal of acetyl groups from the chitin and the resulting compound chitosan has the chemically reactive amino group ($-NH_2$) (Fernandez-Kim 2004). In this study, the degree of deacetylation of extracted chitosan was found to be 46.24% which is significantly different from the commercial chitosan (90%) (Table 2). The degree of deacetylation may vary a range from 30% to 95% depending on the source and preparation procedure (Di Martino et al. 2005). According to Asrahwi et al. (2023), the highest chitosan yield obtained from the chitin of *S. serrata* was 93% at a 3: 1 ratio of NaOH and chitin.

3.5 Elemental Analysis

The elemental analysis was used to determine the percentage of carbon, nitrogen, and hydrogen that was presented in the extracted chitosan (Karnkowska 2005). The elemental studies of extracted chitosan show the carbon percentage is high (40.2%) and that of nitrogen, and hydrogen were 8.2% and 7.1% respectively, similarly Yen et al., (2009) also reported a higher percentage of elements in chitosan extracted from crab shells. According to Riccardo Muzzarelli, (1985), after deacetylation chitosan with a nitrogen content is more than 7% is one of the features of chitosan, and it confirmed the extracted product is a qualified chitosan in elemental analysis.

3.6 ATR- FTIR Spectrum Analysis

Fourier transform Infrared spectroscopy is the easiest and fastest method for identifying allomorphic forms of chitosan. ATR-FTIR spectra analysis of commercial chitosan shows stronger characteristics peaks such as 1643.12 cm^{-1} representing CO stretching, and the peak at 1024.43 $cm^{-1}$is the stretching vibrations, these observations supported the previous studies (Si Trung and Bao 2015) (Fig. 2A). According to (Kumirska et al. 2010) and (J. Pradhan et al. 2024) the absorption peaks at 892.14 cm^{-1} indicating the ring stretching of 1-4 glycosidic linkage in chitosan.

As shown in Table 3 the extracted chitosan, the O-H stretching band was depicted at 3638.47 cm^{-1}, which shows the alcohol group of chitosan. A band identified as the alcohol group (O-H band) was between 3650-3200 cm^{-1}. The stretching band for C-O was at 1026.70 cm^{-1}, and the stretching band for N-H in the extracted chitosan was at 3426.94 cm^{-1}. In addition, the bending band of N-H in the extracted chitosan was in the 1626.08 cm^{-1}, the band identified as the amine group (N-H bending band), which absorbs infrared between 1640-1550 cm^{-1} by Pavia et al., (2009). Mohammed (2013) reports that different degrees of deacetylation showed different peaks in the spectra of FTIR due to the absorption band of the N-H group and O-H group at different IR, which depends on the deacetylation process (Fig. 2B). The peaks were in agreement with previously extracted chitosan from *Scylla olivacea* (Sarbon et al. 2015) and from *Pinna bicolor* (Sudatta et al. 2020), which shows characteristic peaks of chitosan.

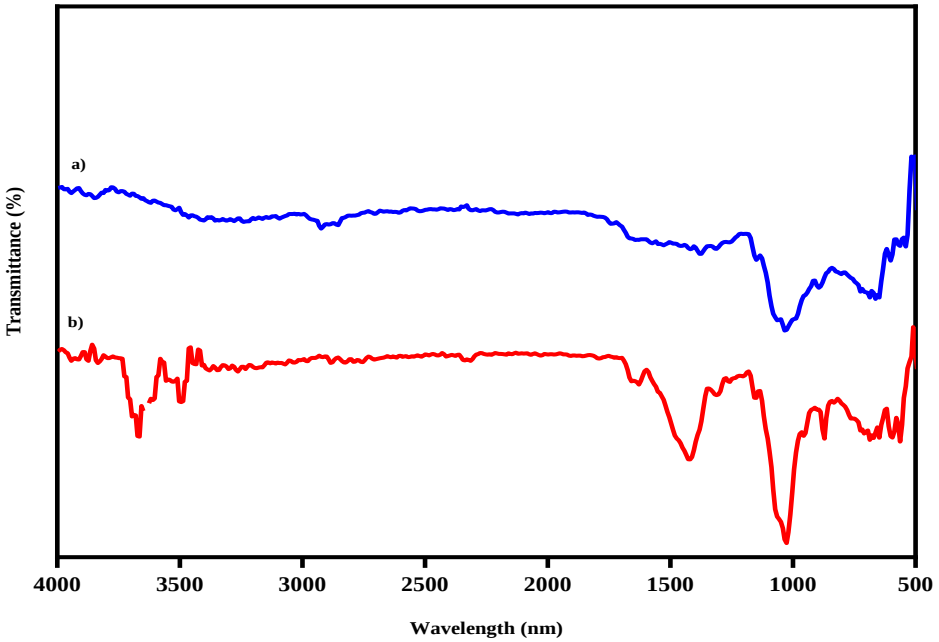


Fig. 2. FTIR spectrum data of a) commercial chitosan and b) extracted chitosan

Table 3. Characteristics IR absorption frequencies of extracted chitosan

Sl No	Functional Group	Types of vibration	Characteristics absorption(cm^{-1})
1	OH alcohol group	Stretch	3638.47
2	NH amide group	Stretch	3426.94
3	NH amine group	Bending	1626.08
4	CH alkene group	Bending	1311.81
5	CO alcohol group	Stretch	1026.70

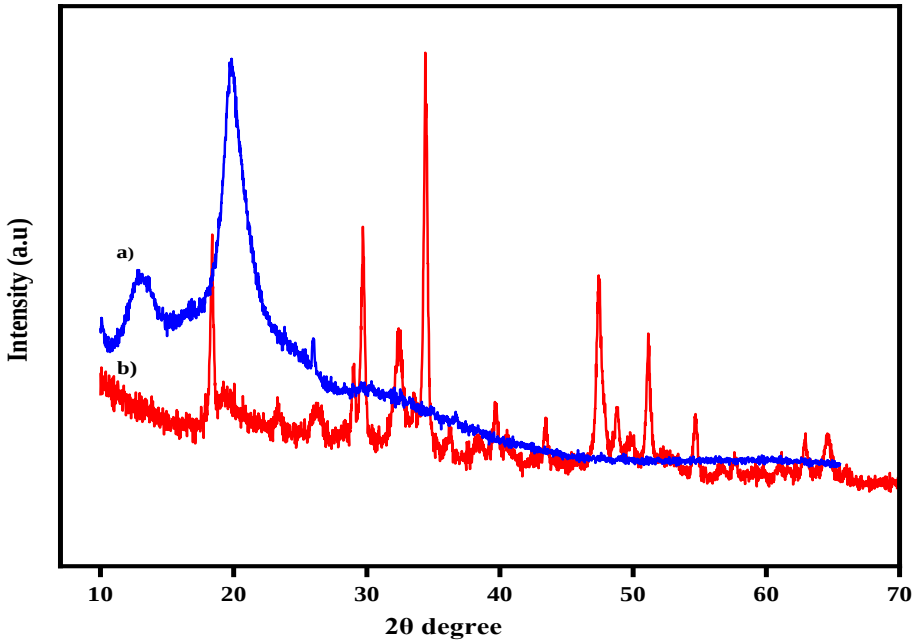
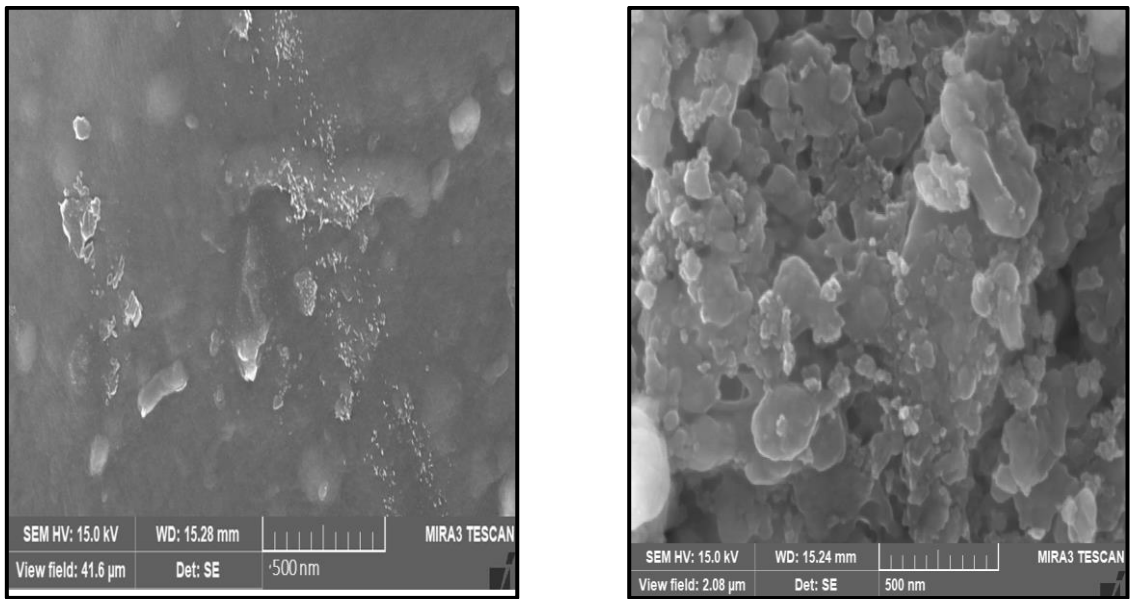


Fig. 3. X-ray diffraction spectra of a) commercial chitosan and b) extracted chitosan



a) **b)**

Fig. 4. The scanning electron microscopy (SEM) images of a) commercial chitosan and b) extracted chitosan

3.7 X-ray Diffraction (XRD) Spectrum Analysis

XRD patterns of both commercial and extracted chitosan are shown in Fig. 3. The XRD pattern of commercial chitosan shows strong characteristic peaks around the 10º and 20º values of chitosan along with many other weak peaks. The extracted chitosan shows a total of 10 strong peaks, among them the sharp peaks were observed between 18º-60º with the strongest peak at 34.38º, and 19.63º at 2θ. The XRD pattern of commercial chitosan exhibited its two characteristic peaks at 2θ = 10.35º, 18.38º, and 29.7º, the tendon polymorph (Qin et al. 2006). The XRD spectrum of the present study has great intensity across the sequences, indicating the changes in the production sequence and some morphological changes in the extracted product.

Comparing ATR-FTIR and XRD of both commercial chitosan and extracted chitosan implies the chemical acid-alkali extraction process used in this study is effective and able to preserve its chemical structure of the chitosan and it shows the high possibilities of production of qualified chitosan. All the physicochemical characterization and functional properties of extracted chitosan suggest that the effective chitosan extraction from the *S. serrata* shell by chemical acid-alkali methods and having high possibilities of biological activities shown by chitosan.

3.8 FE-SEM Analysis

FE-SEM images give detailed information about the surface morphologies and microstructures of extracted chitosan and commercial chitosan (Fig. 4). The FE-SEM images of extracted chitosan showed densely packed micro globular structures. Previous studies by Kaya Baran, et al. (2014) and Kaya, Baublys, et al. (2014) shows porous chitosan from commercial sources, while the smooth, non-porous commercial chitosan is here. The smooth and regular nanofiber without pore chitosan was obtained from *Penaeus monodon* (Terkula et al. 2022) and the same properties have also been reported in crab chitosan by Kumari et al. (2017).

3.9 Antibacterial Activity

Chitosan is classified according to its level of N-acetylation (DA) which is related to its biological and physicochemical properties (Mahlous et al. 2007). Furthermore, some researchers hypothesized that the cationic character of chitosan contributes to its antimicrobial action against bacteria and fungi (Rabea et al. 2003) *and* (Goy et al. 2016). The electrostatic contact between positively charged R-N$(CH_3)^{3+}$ sites and negatively charged microbial cell membranes

leads to cellular lysis, and this interaction is thought to be the primary antibacterial mechanism of chitosan (Rabea et al. 2003), (Tripathi et al. 2008). Moreover charged chitosan blocks the vital nutrients' entry into the cell and prevents the development of microbes (Zheng and Zhu 2003). Above mentioning implies a polymers with large charge densities were anticipated to have better antibacterial action (Goy et al. 2016).

Previous experimental data proved that water-insoluble chitosan is an antibacterial agent in an acidic medium, here we used 1% acetic acid at pH 5.8 for solubilizing chitosan, which is the appropriate pH for medical purposes (Qin et al. 2006). According to Bakshi et al. (2020) chitosan is a cationic polysaccharide consisting of D-glucosamine and N-acetyl D-glucosamine as basic units in their structure. Due to the cationic nature of chitosan, our results suggest chitosan shows more antibacterial activity against Gram-negative bacteria than Gram-positive (Table 4). Previous studies have supported that unmodified chitosan often has a high antimicrobial effect on Gram-negative compared to Gram-positive strains (Chung and Chen 2008), (Hassan et al. 2018). However, there are several contradictory pieces of studies reported by other researchers as greater antibacterial activity of chitosan on Gram-positive strains is predominant due to the lack of extra outer membrane as in Gram-negative (Kyoon et al. 2002), (Leandro Prezotto da Silva and Mirna Helena Regali Seleghim 2010), (Sudarshan et al. 2009), (Peter Eaton et al. 2008), (Hosseinnejad and Jafari 2016), (Lim and Hudson 2004). It emphasizes the advantages of chitosan as a Gram-positive antibacterial agent as it directly blocks the bacterial cell wall consequently preventing nutrients and essential elements from entering into the intra-cellular space (Hosseinnejad and Jafari 2016) and another possible bactericidal mechanism of chitosan is the direct interaction with nucleic acids and interferes with the normal physiological processes (Amidi et al. 2010).

Although the main antibacterial mechanisms are believed to be the electrostatic interactions between positively charged chitosan groups and negatively charged sites on microbial cells and in contrast the repelling effect in between the positively charged bacterial cells and positively charged chitosan (Rabea et al. 2003). *E. coli* and *Pseudomonas* show greater zone of inhibition such as 26±0.32mm and 25±1.45mm respectively (Fig. 5). In the case of Gram-positive such a *Bacillus subtilis* and *Staphylococcus* the zone of inhibition becomes less (19±0.33mm and 18mm respectively). The peptidoglycans layer thickness is an important factor in the effectiveness of the antibacterial activity of chitosan, a rigid structure can act as a barrier against chitosan interactions (Qin et al. 2003). According to Peter Eaton et al. (2008) generally, *E. coli* have cell wall thickness of 7-8mm which is thinner than that of *Staphylococcus aureus* of 20-80mm, possess difficult of cellular lysis.

Helander et al. (2001) and Kravanja et al. (2019) explained the mechanisms behind the chitosan as an antibacterial agent, even at lower concentrations chitosan binds to the negatively charged cell surface of Gram-negative bacteria and overall coats the cellular membrane and interferes the cell membrane permeability, blocking nutrient entry, and leading to cell death. Increasing the concentrations, more chitosan adsorbed on the cell wall and entered the cell, affecting bacterial physiological processes by binding nucleic acids and leading to enzyme leakage. The hydrophilicity of Gram-negative is significantly higher than Gram-positive, which highlights the more sensitivity to the action of chitosan consequently more morphological changes in the cell wall of Gram-negative compared to Gram-positive (Rinaudo 2006). Along with these, the presence of higher anionic radicals in the cell wall of Gram-negative possess a high affinity to the positive amino ends of chitosan (Hassan et al. 2018).

Apart from molecular weight, degree of deacetylation, viscosity, pH, concentration, source, and type of bacteria, the solubility of chitosan also influences the antibacterial activity (Ardean et al. 2021). The solid agar medium allows the chitosan molecules to solubilize and dissipate around the wells equally up to a saturation limit beyond the maximum saturation, antibacterial activity becomes a steady line and no more exponential elevation. In agar medium the availability of free protons (H^+) is finite and it makes it difficult to further interact with chitosan amino acids there by the dissolution. Despite higher chitosan concentrations beyond this saturation point, chitosan remains undissolved and aggregated in the medium (Kumar 2000), the pH of the medium also has an important impact on the solubility of chitosan (Rinaudo 2006). This may also be a possibility of almost the same range of zone of inhibition (mm) shared by bacterial strains at higher concentrations (150 µg/µl, 200µg/µl).

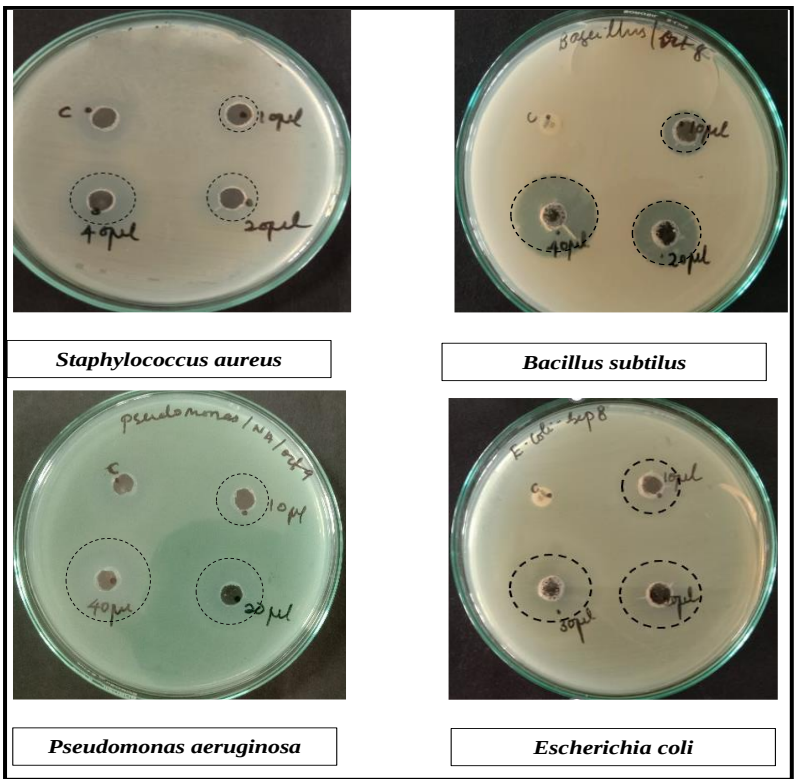


Fig. 5. The antibacterial activities of extracted chitosan in agar well diffusion plates against *Staphylococcus aureus, bacillus subtilus, Psuedomonas aeruginosa, Escherichia coli*

Table 4. The zone of inhibition (mm) showed by Chitosan against 4 different Bacteria

Sl. No	Test organisms	Negative Control	Zone of inhibition in mm The solution (chitosan 5mg/ 1ml)		
		40µl	50µg	100µg	200µg
1	*Pseudomonas aeruginosa*	0	15±0.88	20±0.33	25±1.45
2	*E. coli*	0	16±0.66	21±0.00	26±0.32
3	*Bacillus subtilis*	0	13.6±0.33	15±0.33	19±0.33
4	*Staphylococcus aureus*	0	15±0.00	17±0.00	18±0.00

** Values are given as Mean ± SD*, Each value is taken from three replicates*

4. CONCLUSION

This study investigated the physicochemical and antibacterial activity of extracted chitosan from the mud crab *S. serrata* shells, mostly shells have been discarded as waste without being used, causing environmental pollution in the aquaculture field. The purpose of this study is to corroborate and discuss important attributes of chitosan to analyse its potential in biological applications such as antibacterial activity at different concentrations. Successful antibacterial activity was exhibited mostly against Gram-negative bacterial pathogens like *E. Coli,* and *P. aeruginosa* instead of their thick cell wall. It is also observed that a steady phase in the zone of inhibition at higher chitosan concentrations is due to the reaching the saturation state of dissolution of chitosan in an agar solid medium is also a reason.

DATA AVAILABILITY STATEMENT

Data will be available upon request with the corresponding author.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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