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Original Article

Evaluation of the Biological Activity of Laboratory-Prepared Chitosan from Shrimp Shells against Pathogenic Bacterial Isolates

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Abstract

Chitin is the most substantial natural polysaccharide after cellulose, found in the shells of crabs, shrimps, and other crustaceans. Several medical and environmental applications have been recognized for chitosan. Therefore, the present study aimed to evaluate the biological activity of laboratory-prepared chitosan from shrimp shells against pathogenic bacteria isolates. In the present study, chitosan was extracted from chitin acetate of shrimp shells at different temperatures (room temperature, 65 and 100 ° C) for equal amounts of shells at specified time intervals. The degree of acetylation of different treatments of RT1, RT2, and RT3 reached 71%, 70%, and 65%, respectively. The laboratory-prepared chitosan was examined and antibacterial properties were observed against clinical isolates of bacterial causative agents of urinary tract infections (*E. coli*, *Klebsiella Pneumonia*, *Pseudomonas* spp., *Citrobacter freundii*, and *Enterobacter spp.*). The inhibitory activity of all types of treatments ranged between 12 to 25 mm for all isolates with the highest for *Enterobacter spp.* and the lowest for *Pseudomonas* isolates. The results also indicated a large relative discrepancy between the inhibitory activity of laboratory-prepared chitosan and antibiotics. These results were in the S-R range of the isolates. The similarity of laboratory production conditions and treatments is due to the different proportions of chitin formed in shrimp, environmental conditions, nutrition factors, pH, the extent of heavy metals in the water, and the age of the organism.

Keywords: Antimicrobial Activity, Chitosan, *Enterobacteriaceae*

1. Introduction

Chitosan is a derivative of chitin. The ratio of Glucosamine and N-acetyl-D-glucosamine indicates the degree of deacetylation (DD) in chitosan. Two types of straight-chain copolymers of chitosan and chitin exist (1). Chitosan has high biodegradability and biocompatibility as well as some functional moieties such as N-H and O-H. Therefore, specific physicochemical properties of chitosan that are crucial for drug targeting can be chemically modified and customized (2). Chitin is the second most prevalent polysaccharide in nature after cellulose and is made from (-(1-4)-poly-N-acetyl-D-glucosamine). Fungal spore germination, hyphal elongation, and radial growth are well documented to be inhibited by chitosan (3).

Most studies have examined the effectiveness of Chitosan against food-related yeasts and molds, plant damage, and entomopathogenic fungi (4). Chitosan has antimicrobial properties which prevent germs from growing such as its significant effect on the growth inhibition of the Enterobacteriaceae family as it contains multiple biochemically and genetically related species. *Escherichia coli*, *Shigella sonnei*, *Salmonella sonnei*, *Enterobacter sonnei*, *Proteus sonnei*, and

Yersinia sonnei are all members of the *Enterobacteriaceae* family which may be found in the soil, water, plants, animals, insects, and even humans. *Enterobacteria* are opportunistic pathogens and as a result, this is a clinically significant family (5). Thousands of people die each year due to antibiotic resistance, which h is predicted to become a significant health issue worldwide (6, 7). As many as ten million people could die annually as a result of antibiotic resistance by 2050 (8, 9). The treatment of infectious diseases has significantly changed since the discovery, manufacture, and use of biological materials (10). Therefore, the present study aimed to evaluate the biological activity of laboratory-prepared chitosan from shrimp shells against pathogenic bacterial isolates.

2. Materials and Methods

2.1. Experimental Procedures

2.1.1. Preparation of Chitosan Solutio

Chitosan solution was prepared by dissolving different weights of chitosan in 100 ml of a solution containing acetic acid: distilled water in a ratio of 99:1 with constant stirring until dissolution, with weights of 2, 3, 4, 5, and 10 mg (AOAC, 2006). The scales were obtained from carp after washing and drying with deionized water.

2.1.2. Chitin Preparation

Chitin was extracted from shrimp shells using the method described by Toan, Ng (11) with some modifications.

2.1.3. Deproteinization

Shrimp shells were treated with a solution of 1.2N sodium hydroxide (NaOH) for 24 h at a ratio of 10:1 wt:v at 70-75°C. The sample was then filtered using a Buechner funnel and rinsed several times with distilled water at a high discharge rate to obtain PH7.

2.1.4. Demineralization

The sample was treated with 0.7 N hydrochloric acid (HCl) at room temperature for 15 min at a ratio of 1:10 wt:v. Then the sample was washed well with water to remove acid and calcium carbonate to obtain pH7 and dried in an air oven at 60 °C for 24 h.

2.1.5. Discoloration

The sample was treated with acetone at a ratio of 1:10 wt:v for 10 min. The sample was filtered and dried for 2 h, and then shortened with sodium hypochlorite 32% solution at a ratio of 5:1 wt:v for 15 min at room temperature with constant stirring. Then it was washed with distilled water and dried in an air oven at 60 °C for 24 h.

2.2. Methods

Three different temperatures were used for extracting chitosan from shrimp shells:

2.2.1. Preparation of Chitosan

According to Toan, Ng (11), chitosan was made in four distinct methods by treating laboratory-prepared chitin with a 50% NaOH solution at a ratio of 1:13 wt:v:

- First treatment (RT1): Primary treatment for 48 h at room temperature.

The sample was immediately filtered and washed with distilled water under vacuum after treatment for 48 h at room temperature, then dried in a hot air oven at 60° C for 24 h.

- Second treatment (RT2): This is considered the starting point.

After the first treatment, the sample was quickly filtered and washed with distilled water under vacuum to obtain pH7, then dried in an oven at 61°C for 24 h. Then the precipitate was dried at 55°C in a vacuum oven.

-Third treatment (RT3): A 20-hour base treatment at 100 °C.

The sample was treated for 20 h at 100 °C and then filtered and washed several times with distilled water to obtain pH7, and then dried in a hot air oven at 60 °C for 24 h.

2.2.2. Detection of Chitosan by Fourier Transform Infrared Spectroscopy (FTIR)

Chitosan prepared from carp scales was detected at the Faculty of Pharmacy, University of Kufa, Najaf, Iraq. The dried chitosan was mixed with dry potassium bromide at a ratio of 1:5 wt:v with a ceramic mortar for 10 min and compressed by a hydraulic press at a

pressure of 8 bar for 60 s before being analyzed by FTIR (Biotech. Engineering Co.Ltd).

2.2.2.1. Degree of Deacetylation (DD%)

The degree of removal of acetyl groups (DD) was estimated based on the FTIR results. The absorbance at wavelength A 1655 (1655) represents the amine group compared to that at wavelength A 3450 (3450), representing the hydroxyl group and serving as an internal standard. It does not decompose and is unaffected by the transactions that occur during the extraction of Chitosan. The absorbance was calculated based on Beer-Lambert law according to the equation: (A: absorbance, T: permeability)

A%= 2-log T%

The degree of removal of acetylcholine groups was calculated as mentioned by Maghsoudi, Razavi (12).

2.2.2.2. Specimens Collection

In this study, 33 samples were collected from patients with urinary tract infections at Al-Sadr Teaching Hospital, Najaf, Iraq, and cultured on agar plates. The plate was incubated for 18-24 h at 37 ° C.

Amount of absorbed water (ml/g) = amount of added water (10ml) - the amount of water after separation

2.2.2.3. Preparation of the Bacterial Suspension

Each bacterial suspension was produced to a turbidity of 0.5 McFarland standard (1.5x108 CFU / ml). Turbidity was determined using the Kirby-Bauer method by a spectrophotometer at 625 nm in turbid suspension (13).

2.2.2.4. Determination of Antimicrobial Activity

The Vitek 2 system isolated and identified *E. coli, Klebsiella pneumonia, Pseudomonas spp, Citrobacter freundii, and Enterobacter spp*. Then, 0.1 ml of culture was spread on Mueller Hinton Agar using a sterile brush and dried at room temperature for 10-15 min. The agar well diffusion technique (13) was employed. Then, three wells with a diameter of 10 mm were created on the surface of the culture medium after sterilizing with the cork borer, and 50 L was added to each well of prepared chitosan. The plate was incubated at 37 °C for 18-24 h. The diameter of the zone of inhibition was measured.

2.2.2.5. Statistical Analysis

The data were obtained and transferred to a Microsoft Excel spreadsheet and descriptive statistics were calculated. SAS software (version 9.1) was used to analyze the data. A two-way ANOVA was used to investigate whether an interaction was observed between the effect of extract concentration and the pathogenic bacteria. In both tests, a *P*-value less than 0.05 is considered to be statistically significant (Tukey's test). In addition, an analysis was performed to determine the difference between the means. One-way ANOVA was performed to reveal statistical differences using various zones of inhibition when chitosan extracts were used against the isolates in this study.

3. Results and Discussion

3.1. Diagnosis of Chitosan

Chitosan was prepared in the laboratory using shrimp shells purchased from local markets in Najaf and Basra, Iraq, and then exposed to three different temperature and time treatments. Figure 1 illustrates the chitosan yield obtained from the three treatments (RT3, RT2, RT1) of shrimp depending on the extraction procedure. Statistically significant differences (P= 0.05) were observed between the treatments (17.5, 13.5, and 11.25%, respectively, based on the dry weight of shells). The yield variation might be due to differences in base treatments, extraction temperature, and duration since higher extraction temperatures and periods result in lower yields than lower extraction times (14). According to Hosseinnejad and Jafari (15), chitosan from shrimp residues varied from 17.36 to 13.12%. Also, they discovered that the temperature and time required to eliminate acetylcholine aggregates significantly affected yield, and the yield decreases by increasing temperature.

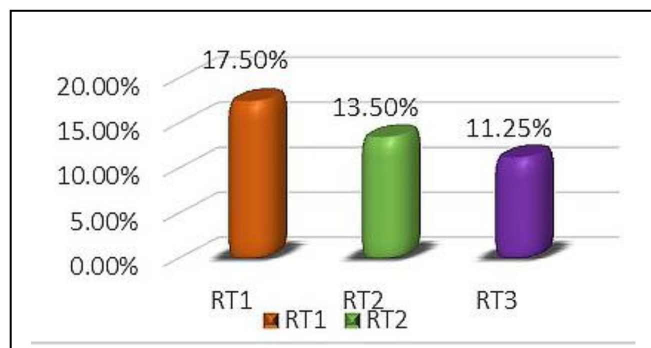
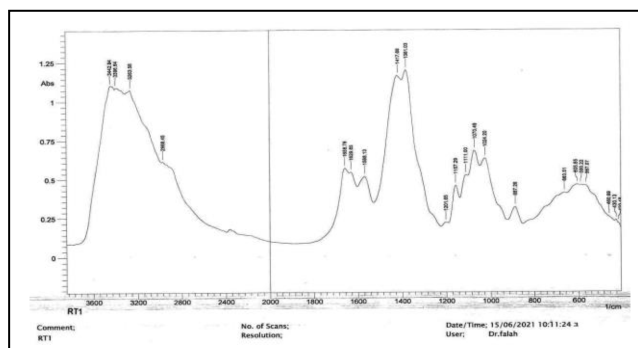


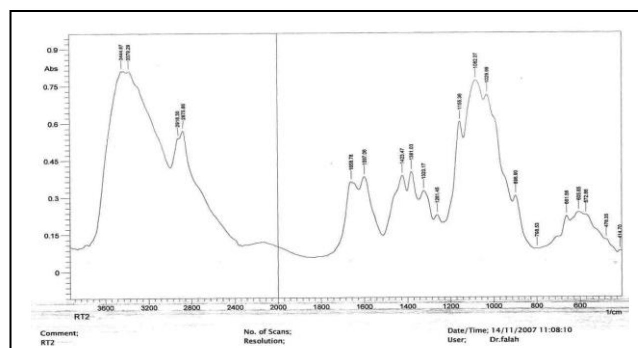
Figure 1. Percentage of chitosan prepared from shrimps

Many variables contribute to the variance in shrimp yield percentages, including the age of the shrimp, the pH of the environment, and the presence of heavy metal ions in the water, which inhibit the development of chitin in the shells.

3.1.1. Detection of Chitosan by Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is one of the most important and fastest techniques used to qualitatively detect chitin and chitosan due to sensitivity and no need for high purification or dissolution in specific solvents. However, one of its disadvantages is the difference in the percentage of DD according to the equation used to calculate it. Determining the active groups in organic compounds and calculating the optical transmittance are important in the FTIR technique (16). The results indicated that the studied chitosan samples had different characteristics than the chitin produced from it and amino groups are among the essential active groups with an absorption peak appearing at a frequency of 1658 cm-1 of this spectrum as the presence of this group indicates the existence of chitin. Additionally, chitosan represents the absorbance for limited wave numbers between 1157-1024, 1155-1020, and 1155-1024 cm-1 for the RT1, RT3, and RT2 chitosan models, respectively. The primary group in chitosan is one of its stable properties and a guide to the formation of acetylation as presented in figure 2. The results of the present study are consistent with previously published work by Sini, Santhosh (17).





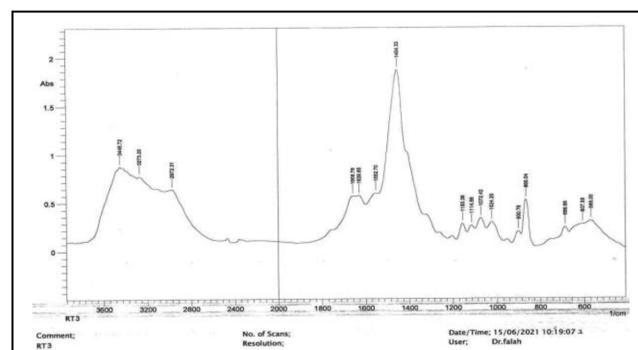


Figure 2. Detection of chitosan extracted from shrimp shells (FTIR)

3.1.2. Degree of Deacetylation (DD %)

Chitosan treatments RT1 to RT3 were evaluated using the FTIR technique to determine how much of the acetyl groups had been removed and the results are shown in table 1. The amide group was used to measure the content of N-acetyl groups, while the frequency 3455 cm-1 was used as a scale for hydroxyl groups.

Table 1. The degree of acetylation in chitosan treatments

Chitosan Treatments	Treatment Details	Deacetylation DD%
RT1	Base treatment at room temperature for 48 h	71%
RT2	Base treatment at 65°C for 20 h	70%
RT3	Base treatment at 100°C for 20 h	65%

The results revealed a difference in the percentage of DD for chitosan treatments, as the removal values for the treatments were 71, 70, and 65%, respectively. Increasing the temperature leads to a decrease in the percentage of acetylcholine groups for chitosan treatments. RT3 treatment performed weakly in removing and distributing acetyl groups in chitosan compared to other treatments. Alves, Furman (18) report that when performing the adaptation for this simplified method and stirring at room temperature, the DD values in the range of 77 to 80% demonstrate the viability of this method for the intended purpose of the produced chitosan.

3.1.3. Analysis of the Biological Activity of Chitosan

According to the results, chitosan prepared from shrimp shells showed biological activity with high and various rates in different concentrations (2,3,4,5,10 mg/ml) and treatments against the growth of gram-negative bacteria isolated from patients with urinary tract infection. Increased concentration of chitosan has a significant inhibitory effect on the growth of pathogenic bacteria, as the zone of inhibition ranged from 18-24, 22-12, 15-25, and 14-21 mm in *E. coli*, *Proteus*, *Enterobacter*, and *Klebsiella,* respectively in different treatments of shrimps (Table 2). The laboratory-prepared chitosan from shrimp shells had a great inhibitory activity against gram-negative pathogenic bacteria.

Table 2. The zone of inhibition for chitosan extracted from shrimp shells

Zone of Inhibition of RT	Type of Bacteria
18 – 24 mm	*E. coli*
12 – 22 mm	*Proteus*
12 – 22 mm	*pseudomonas*
15 – 25 mm	*Enterobacter*
14 – 21 mm	*Klebsiella*

The antibacterial ingredient is chitosan (AB). Activity increases with concentration until a critical concentration (CC) is reached, which then decreases (19). The discovery of the main peaks of chitosan properties at 1345, 1420, 1560, 1655, and 3290 cm1, respectively, validated the solubility of chitosan suspensions (18). The temperature should be noted to have an essential effect on the activity of chitosan as an antibacterial. As presented in figure 3, chitosan prepared at room temperature showed significant biological activity against pathogenic isolates compared to those of other temperatures.

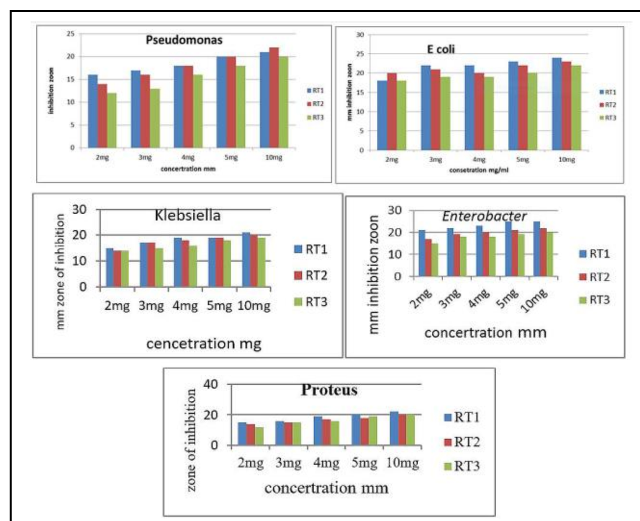


Figure 3. Biological activity of pathogenic isolates of different chitosan treatments prepared from shrimp shells

Furthermore, AB activity of chitosan is highly dependent on incubation temperature which is significantly boosted at 37 °C (20). As a result, AB activity of chitosan is nevertheless promising in refrigerated conditions despite the limiting effect of temperature, and 100% bacterial suppression was not achieved. Similar findings have been recently published, suggesting that when bacteria are exposed to high temperatures, they become vulnerable to the action of chitosan nanoparticles (21). Low temperatures might alter the structure of bacteria by reducing the number of surface binding sites (or electronegativity). As a result, fewer protonated chitosan amino groups

may interact with negatively charged sites on the bacterial surface and reduce the AB activity of chitosan. The data of the present study demonstrated that the AB activity of both chitosan significantly but not intensely decreased as the temperature decreased.

3.1.4. Effect of Bacterial Species

Figure 4 presents the percentage survival and reduction of five species of bacteria when in contact with chitosan. Chitosan is more effective against *E. coli* than other germs. The chitosan solution is more sensitive to gram-negative bacteria (22, 23). Gram type, hydrophilicity, negative charge density, and adsorptive capacity are different. A higher electrostatic interaction between positively charged chitosan amino groups and negatively charged bacterial surfaces may occur in gram-negative bacteria (24). Gram-negative bacteria have higher hydrophilicity and chitosan adsorption on their cell walls than those of gram-positive bacteria, which may contribute to the A.B. effect (3, 21). Also, the structural arrangement of envelope/membrane components in gram-positive and gram-negative organisms. Gram-negative bacteria have a bilayered phospholipid membrane with a single phospholipid layer and a thin layer of peptidoglycan on the outside. This difference in peptidoglycan layer thickness may render gram-negative bacteria more vulnerable to chitosan action (25, 26). This might explain why different authors came to conflicting conclusions when comparing the effect of chitosan. Data in the present study indicate that partial solubilization is required for chitosan to have an A.B. action. Consequently, reduced M.W. (including low-MW species or chitooligosaccharides, even in trace levels) and increased DDA are preferred (27-29). Also, reducing the size of chitosan particles was found to increase its antibacterial properties. Additionally, chitosan has antibacterial, wound healing, and mucoadhesive properties which makes it an ideal drug carrier (30).

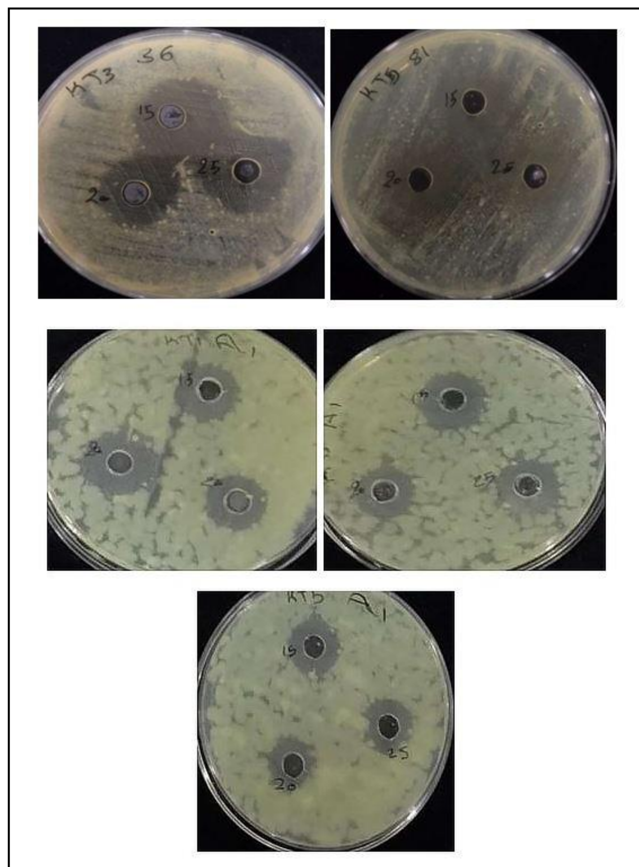


Figure 4. Zones of inhibition formed by the laboratory prepared chitosan

4. Conclusion

The present study demonstrates that chitin extracted from shrimp shells may be employed in various applications, particularly when transformed into the more beneficial component of chitosan. Chitosan is made by mixing several sources and treating them with diluted HCl and NaOH. Chitosan has significant antibacterial activity against *Enterobacteriaceae* when produced at room temperature and this action is influenced by pH, temperature, chitosan content, purity, and bacterial type. Further study is required on chitosan activity to fully understand the methods and variables involved in extracting Chitosan and improving its effectiveness in suppressing and eliminating the growth

of harmful bacteria and promoting its usage as an alternative to the antibiotic. Furthermore, chitosan producers might collect and treat these wastes before donating or selling them to research organizations, especially those focused on nanotechnology. Chitosan production provides businesses with potential for future investments on a national and global scale and generates new sources of profit that may help build the economy.

Authors' Contribution

Study concept and design: S. M. J.
Acquisition of data: S. M. J.
Analysis and interpretation of data: S. M. J.
Drafting of the manuscript: S. M. J.
Critical revision of the manuscript for important intellectual content: S. M. J.
Statistical analysis: S. M. J.
Administrative, technical, and material support: S. M. J.

References

1. Rinaudo M. Chitin and chitosan: Properties and applications. Prog Polym Sci. 2006;31(7):603-32.
2. Inamuddin A, Mohammad A. Applications of nanocomposite materials in drug delivery: Elsevier; 2018.
3. Salman KA, Jawad SM, Chafat NN, Al-Bdery AS. Efficacy of Bark (Juglans regia L.) Extracts Against Periodontitis Bacteria: an in vitro Study. Indian J Forensic Med Toxicol. 2021;15(3):5493.
4. Goy RC, Britto Dd, Assis OB. A review of the antimicrobial activity of chitosan. Polímeros. 2009;19(3):241-7.
5. Brenner DJ, Fanning GR, Knutson JKL, Steigerwalt AG, Krichevsky MI. Attempts to classify Herbicola group-Enterobacter agglomerans strains by deoxyribonucleic acid hybridization and phenotypic tests. Int J Syst Evol Microbiol. 1984;34(1):45-55.
6. Adzitey F. Incidence and antimicrobial susceptibility of Escherichia coli isolated from beef (meat muscle, liver and kidney) samples in Wa Abattoir, Ghana. Cogent Food Agric. 2020;6(1):1718269.
7. Momtaz H, Dehkordi FS, Hosseini MJ, Sarshar M, Heidari M. Serogroups, virulence genes and antibiotic resistance in Shiga toxin-producing Escherichia coli isolated from diarrheic and non-diarrheic pediatric patients in Iran. Gut Pathog. 2013;5(1):1-10.
8. Bengtsson-Palme J, Kristiansson E, Larsson DJ. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol Rev. 2018;42(1):053.
9. Praveenkumarreddy Y, Akiba M, Guruge KS, Balakrishna K, Vandana KE, Kumar V. Occurrence of antimicrobial-resistant Escherichia coli in sewage treatment plants of South India. J Water Sanit Hyg Dev. 2020;10(1):48-55.
10. Stange C, Sidhu J, Tiehm A, Toze S. Antibiotic resistance and virulence genes in coliform water isolates. Int J Hyg Environ Health. 2016;219(8):823-31.
11. Toan NV, Ng CH, Aye KN, Trang TS, Stevens WF. Production of high- quality chitin and chitosan from preconditioned shrimp shells. J Chem Technol Biotechnol. 2006;81(7):1113-8.
12. Maghsoudi V, Razavi J, Yaghmaei S. Solid state fermentation for production of chitosan by aspergillus niger. 2009.
13. Baccer R, Kirby M, Sherris J, Turek M. Antibiotic susceptibility testing by standard single disc diffusion method. Am J Clin Pathol. 1966;45:493-6.
14. Perez C. Antibiotic assay by agar-well diffusion method. Acta Biol Med Exp. 1990;15:113-5.
15. Hosseinnejad M, Jafari SM. Evaluation of different factors affecting antimicrobial properties of chitosan. Int J Biol Macromol. 2016;85:467-75.
16. Liu Y, Xing R, Yang H, Liu S, Qin Y, Li K, et al. Chitin extraction from shrimp (Litopenaeus vannamei) shells by successive two-step fermentation with Lactobacillus rhamnoides and Bacillus amyloliquefaciens. Int J Biol Macromol. 2020;148:424-33.
17. Sini TK, Santhosh S, Mathew PT. Study on the production of chitin and chitosan from shrimp shell by using Bacillus subtilis fermentation. Carbohydr Res. 2007;342(16):2423-9.
18. Alves HJ, Furman M, Kugelmeier CL, Oliveira CRd, Bach VR, Lupatini KN, et al. Effect of shrimp shells milling on the molar mass of chitosan. Polímeros. 2017;27:41-7.
19. Zvezdova D. Synthesis and characterization of chitosan from marine sources in Black Sea. Annual Proceedings," Angel Kanchev" University of Ruse. 2010;49(9.1):65-9.

20. Paulino AT, Simionato JI, Garcia JC, Nozaki J. Characterization of chitosan and chitin produced from silkworm crysalides. Carbohydr Polym. 2006;64(1):98-103.
21. Chen Y-L, Chou C-C. Factors affecting the susceptibility of Staphylococcus aureus CCRC 12657 to water soluble lactose chitosan derivative. Food Microbiol. 2005;22(1):29-35.
22. Tsai G, Su W-H, Chen H-C, Pan C-L. Antimicrobial activity of shrimp chitin and chitosan from different treatments. Fish Sci. 2002;68(1):170-7.
23. Eaton P, Fernandes JC, Pereira E, Pintado ME, Malcata FX. Atomic force microscopy study of the antibacterial effects of chitosans on Escherichia coli and Staphylococcus aureus. Ultramicroscopy. 2008;108(10):1128-34.
24. Manni L, Ghorbel-Bellaaj O, Jellouli K, Younes I, Nasri M. Extraction and characterization of chitin, chitosan, and protein hydrolysates prepared from shrimp waste by treatment with crude protease from Bacillus cereus SV1. Appl Biochem Biotechnol. 2010;162(2):345-57.
25. Goy RC, Morais ST, Assis OB. Evaluation of the antimicrobial activity of chitosan and its quaternized derivative on E. coli and S. aureus growth. Rev Bras Farmacogn. 2016;26:122-7.
26. Jawad SM, Salman KA, Hussein HA. TLC Identification of Bacteriocin from Different LAB Clinical Isolates of Najaf Hospitals and in Vitro Evaluation of Its Effectiveness Against Three Pathogenic Bacterial Isolates. Med Legal Update. 2021;21(3).
27. Ardila N, Daigle F, Heuzey M-C, Ajji A. Antibacterial activity of neat chitosan powder and flakes. Molecules. 2017;22(1):100.
28. Ardila N, Medina N, Arkoun M, Heuzey M-C, Ajji A, Panchal CJ. Chitosan–bacterial nanocellulose nanofibrous structures for potential wound dressing applications. Cellulose. 2016;23(5):3089-104.
29. Arkoun M, Daigle F, Heuzey MC, Ajji A. Antibacterial electrospun chitosan- based nanofibers: A bacterial membrane perforator. Food Sci Nutr. 2017;5(4):865-74.
30. Jhaveri J, Raichura Z, Khan T, Momin M, Omri A. Chitosan nanoparticles-insight into properties, functionalization and applications in drug delivery and theranostics. Molecules. 2021;26(2):272.