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RESEARCH ARTICLE

EXTRACTION, PHYSICOCHEMICAL CHARACTERIZATION, STUDY OF HEAVY METAL Mn2+ ADSORPTION AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF CHITOSAN IN SHRIMP SHELL WASTE FROM BENI SAF SEA, ALGERIA AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Antimicrobial properties of chitosan extracted from shrimp processing waste were determined against one gram-positive bacterium methicillin-resistant *Staphylococcus aureus in vitro.* The antimicrobial activities of chitosan were explored by calculation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in media supplemented with 200, 400, 600, 800and 1000 ppm chitosan solution. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the prepared chitosan was 800 and 1000 ppm for both bacterial strains. These results indicate that chitosan from shrimp processing waste could be used as an effective antibacterial agent in the food industry. The ability of chitosan as an adsorbent for Mn (II) ions in aqueous solution was studied. Our results show that the adsorption process is concentration-driven with high capacity of chitosan for the adsorption of these metal ions. At initial manganese concentrations of 3, 6 and 9 mg/L, the adsorbed manganese ion concentrations are 2.4122, 4. 9544, and 7.7667mg/L, respectively. chitosan produced was also characterized with Fourier Transform Infrared Spectroscopy (FTIR).

INTRODUCTION

Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. Currently, chitosan has received extensive attention for its variable applications in the biomedical, food, and chemical industries (Mohanasrinivasan *et al.,* 2013). Chitosan can be obtained by N-deacytlation of chitin and it is a co-polymer of glucosamine and N-acetylyglucosamine unit linked by 1-4 glucostatic bond (Figure 1) (Mohanasrinivasan *et al*., 2013). Much of the commercial interest in chitosan and its derivatives during the last two decades arises from the fact that they combine several favorable biological characteristics, including biodegradability, biocompatibility and non-toxicity; properties which render natural polymers superior over present-day synthetic polymers (Russell and Shar, 2013).

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The current research is to prepare chitosan from shrimp shell waste and to study the physiochemical parameters and characterized by biological activities such as antimicrobial properties of chitosan, obtained from shrimp shell waste. FTIR spectra were also established for chitosan. The affinity of chitosan for manganese was studied using $MnCl_2.4H_2O$ solution as the heavy metal solution containing Mn (II) ions.

MATERIALS AND METHODS

Chemicals acetic acid, hydro chloric acid and sodium hydroxide and all the other chemicals and reagents are purchased from Sigma Chemical Co.

Chitin and chitosan preparation

Sourcing and collection of samples

Shrimp shells were collected at BENI SAF fish market at Tlemcen in Algeria. For this research, shells were collected, in a stomacher bag and transported to the laboratory for preparation. The shells were scraped free of loose tissue, washed and dried at room temperature, for 10 days until it was

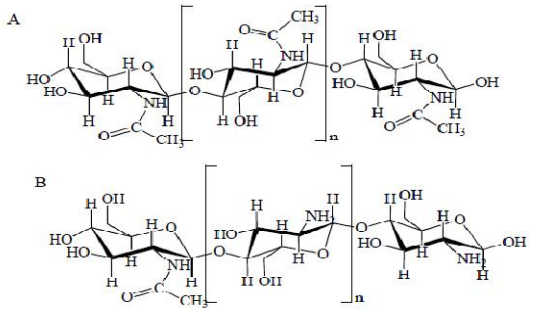

Figure 1. Chemical structure of 100% acetylated chitin (A) and chitosan (B)

well dried and crispy, grounded to pass through a 0. 5-0.8 mm sieve. Then they were subjected to demineralization, deproteinization and deacetylation

Demineralization

Demineralization was carried out in 2M HCl, at ratio 1:15 (w/v)), at room temperature, stirred constantly overnight It was observed that the emission of CO_2 gas depends upon the mineral content. The sample was then washed thoroughly with tap water several times to neutrality. The chitin was dried at ambient temperature (30 ± 2°C). The dried chitin was pulverised into powder using a dry Grinder

Deproteinization

Dried shell waste was washed with tap water and deproteinised by boiling in 3% aqueous sodium hydroxide for 15 min, after chitin was carried out using 2M NaOH at ratio 1:10 NaOH at 60 °C. The treatment was repeated several times. The absence of proteins was indicated by the absence of color of the medium at the last treatment, which was left overnight. Then the resulting solution was washed with water to neutrality. The purified chitin was dried at 50 °C to constant weight.

Deacetylation of chitin

The chitin (10 g) was put into 50% NaOH at ratio 1:20 at 60°C for 8 h to prepare crude chitosan. After filtration, the residue was drained off and washed with tap water to neutrality. The crude chitosan was obtained by drying in an air oven at 50°C overnight. The chitosan was derived as a white powder.

Measurement of degree of N-deacetylation

The samples of chitin and chitosan produced were characterized by Fourier transformed infrared (FTIR) spectroscopy (Bruker Alpha-T) in the range of 400 to 4000 per cm. Samples of chitin and chitosan (10g) were mixed with 100 g of dried potassium bromide (KBr) and compressed to prepare a salt discs (10 mm diameter). The disks were conditioned in a desiccator placed in an oven at 80°C for 16 hr before analysis for reading the spectrum. The absorbances at 1655 and 3450 cm^{-1} were used to calculate the DD according to the following equation (Md Rabiul Hussain, *et al.,* 1978)

$$DD = 100 - \frac{\frac{A1655cm^{-1}}{A3450cm^{-1}}}{1,33} \times 100 \quad (a).$$

Where DD is deacetylation degree; *A* 1655cm^{-1} and *A* 3450cm^{-1} are absolute heights of absorption bands of amide and hydroxyl groups. The factor 1.33 denoted the value of the ratio of A1655 / A3450 for fully Nacetylated chitosan.

Micro organisms

Bacterial culture of methicillin-resistant *Staphylococcus aureus* used in the present studies which is commonly associated with food product as a result of human handling (Kunin, 1987) was obtained from local hospitals. Methicillin-resistant *Staphylococcus aureus* was pre-cultured into trypticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 0.6 % (w/v) yeast extract (TSBYE) (Difco) at 37 °C for 24 h. The culture was kept refrigerated on tryptose soy agar slants during the experiment.

Determination of antibacterial activity

Bacterial inoculums were prepared by Clinical and Laboratory Standards Institute (CLSI) guideline. Bacterial cultures were emulsified in normal saline and turbidity was matched with 0.5 McFarland turbidity standards. The agar cup method (Barry, 1980) was followed to investigate the antibacterial activity of the preparations. 0.1 mL of TSBYE broth culture of the test organism were firmly seeded over the Mueller-Hinton Agar (MHA) plates. Wells of 6 mm diameter was punched over the agar plates using a sterile cork borer. The bottoms of the wells were sealed by pouring 80 µL of molten MHA into the scooped out wells. Five different concentrations (200, 400, 600, 800 and 1000 ppm) were used to evaluate the effects of concentration of chitosan against Methicillin-resistant *Staphylococcus aureus*. The same volume of water was added to the control group. These samples were added at initial of cultivation. Using a micropipette, solutions was added to different wells in the plate. These plates were then kept at low temperature (4ºC) for 2-4 hours and incubated at 37 ºC for 24 hours. After the incubation period formation of zones around the wells, confirms the antibacterial activity of the respective preparations.

Determination of MIC and MBC

Serial two-fold dilutions of the antimicrobial agent were prepared in the appropriate culture medium in sterile 96-well round-bottom polystyrene microtiter plates (Greiner Bio-One GmbH). Liquid culture of the test strain methicillin-resistant *Staphylococcus aureus* was inoculated from an overnight culture (5% [vol/vol]), and allowed to grow in the respective broth in a rotary incubator at 37°C. The MIC was read as the least concentration of the antimicrobial agent resulting in the complete inhibition of visible bacterial growth after 24 - 48 h of incubation at 37°C. Antibacterial activities were also expressed as the MBC (minimum bactericidal concentration), defined as the lowest concentration of the antimicrobial agent reducing the bacterial inoculum by ≥ 99.9% within 24 h. The MBC was assigned by estimating the viable count in 20-µl aliquots from each well of the microtiter plates showing absence of growth.

Sorption of manganese ions on chitosan

20mg/L manganese solution was prepared by dissolving 8.99 mg analytical grade $MnCl_2.4H_2O$ powder in distilled water, contaminated water for treatment using chitosan.

This solution was kept as stock solution and 3, 6 and 9 mg/L solutions were prepared by diluting stock solution. 50ml of 3mg/L $MnCl_2$ solution was taken and 50mg of chitosan was added. Then the mixture was continuously stirred using magnetic stirrer for 1 hours at room temperature (28^oC), after that solution was filtered using Whatman filter paper no 2 and 3mg/l $MnCl_2$ solution were analyzed using atomic adsorption spectroscopy to determine amount of manganese absorbed by chitosan. The same steps were repeated to the other dilute solutions.

Statistical analysis

Statistical analysis was conducted using ANOVA analysis (StatBox logiciel, GrimmerSoft; version 6.4, France). Comparisons were made using Student–Newman–Keuls test for multiple comparisons. A $P<0.05$ was considered statistically significant. All data presented are mean values of triplicates obtained from three separate runs (n = 5).

RESULTS AND DISCUSSION

Degree of Deacetylation (DD)

The degree of deacetylation (DD) was calculated by using the equation (a) and FT-IR (infrared spectroscopic analysis) of the prepared chitosan**.** The DD is an important parameter affecting solubility, chemical reactivity, and biodegradability. Depending on the source and preparation procedure, DD may range from 30% to 95% (Abdulwadud Abdulkarim1 *et al*., 2013)**.** This study revealed that, DD of the prepared chitosan is 75%. It is rare that the production of chitosan with 100% degree of deacetylation is achievable. Therefore, commercial chitosan with various degree of deacetylation in the range of 75–85% is commonly found.

FT-IR spectral analysis

The FT-IR spectrum of the chitosan sample from the shell recorded 16 peaks in the range of 689.40/cm and 3430.02 /cm (Figure 2). The FT-IR spectra of shell extracted chitosan showing the absorbance band at 3430.02cm- 3258.97/cm, 3100.03/cm,2877.11/cm,2325.01/cm, 1652.30/cm,1629.99/cm, 1556.30/cm,1377.02/cm,1380.11/cm,1258.98/cm,1155.03/ cm, 1168.38/cm,1009.11/cm, 951.78/cm, and 689.40/cm.

The region between 3000/cm and 3500/cm indicates the hydroxyl stretching vibration. This band is broad because of the hydrogen bonds.The peak at 2877.11/cm represents the characteristic -CH- stretching vibrations. The OH band overlaps the stretching band of NH. Another significant change is observed in the region from 1000 cm-1 to 1200cm-1.

In this region chitosan presents a broad band centered it 1155.03/cm associated with the stretching of C=O. It is also observed It is interesting that the absorption peak of chitosan at1629.99cm-1 corresponding to the chitosan NH_2 band , and the band at 1652.30 cm-1 corresponds to the amide I stretching of C = O. The extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the global increase of nosocomial infections making it necessary to embark on a continued search for new antimicrobial compounds. The antimicrobial activity of chitosan has been recognized and is considered to be one of the most important properties, corresponding directly to their possible biological applications. Chitosan had broad spectrum antimicrobial effects. This study has been conducted to assess inhibitory effects of chitosan in terms of MIC and MBC. The Minimum Bactericidal Concentration (MBC) is the lowest concentration of antibiotic required to kill 99% of the germ.

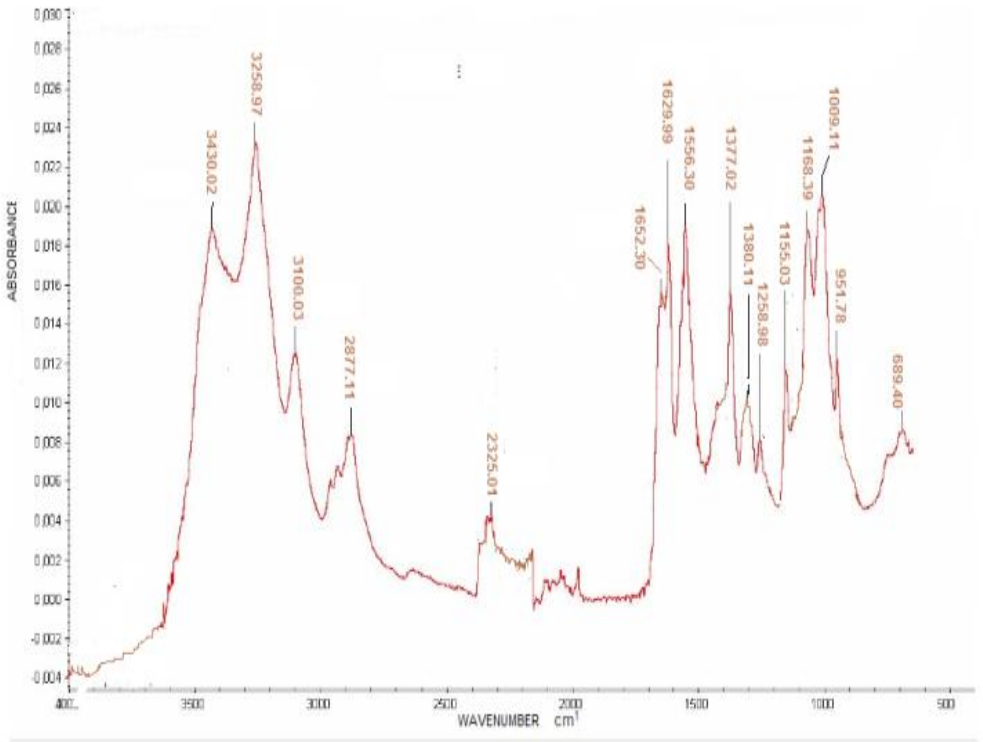

Figure 2. FTIR of prepared chitosan

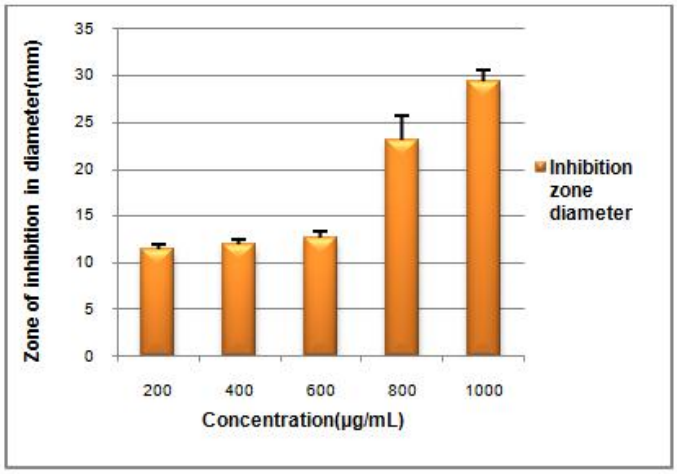

Figure 3. Antimicrobial activity and zone of inhibition of chitosan (mm) SD (n=5)

Effect of chitosan obtained from the shrimp shells was evaluated against methicillin-resistant *Staphylococcus aureus* and the results are presented in Table 1.

This electrostatic interaction results in twofold interference: by promoting changes in the properties of membrane wall permeability, thus incite internal osmotic imbalances and consequently restrain the growth of microorganisms (Shahidi *et al*., 1999) and by the hydrolysis of the peptidoglycans in the microorganism wall, leading to the leakage of intracellular electrolytes such as potassium ions and other low molecular weight proteinaceous constituents (e.g. proteins, nucleic acids, glucose, and lactate dehydrogenase) (Chen *et al*., 1998), (Papineau, 1991). Visual confirmation of an effective membrane lyses been also reported on gram-negative and gram-positive bacteria (Chung and Chen, 2008). Since such mechanism is based on electrostatic interaction, it suggests that the greater the number of catonized amines, the higher will be the antibacterial action (Másson *et al*., 2008).

Sorption of manganese ions on chitosan

During recent years, heavy metal pollution of the aquatic environment has become a worldwide problem because most of them have toxic effects on organisms. Both essential and non-essential heavy metals have a particular significance in human health when used for drinking purpose.

Table 1. MIC and MBC of prepared chitosan against methicillin-resistant *staphylococcus aureus*

	Concentration(ppm) growth in peptone broth							
	1200	1000	800	600	400	200	MIC (ppm)	MBC (ppm)
methicillin-resistant *staphylococcus aureus*	-	-	+	+	+	+	800	1000

Table 2. amount of manganese adsorbed after adding 50mg of chitosan with 28º C and pH=5.4 (atomic absorption method)

Total Mn 2+ in original solution (ppm)	Total Mn 2+ after addition of chitosan (ppm) (adsorb)
3	2.4122
6	4. 9544
9	7.7667

As shown in Figure 1 chitosan markedly inhibited the growth of methicillin-resistant *Staphylococcus aureus*,. The activity increased with increasing concentration of chitosan. The inhibition zone diameter for methicillin-resistant *Staphylococcus aureus* was in the range of 11-29mm, the highest antibacterial activity against the strain of methicillin-resistant *Staphylococcus aureus* was 29 mm with 1000 µg/mL chitosan. The MBC and MIC values of chitosan was measured by macro and micro broth dilution techniques and results are presented in Table 1. The Minimum Bactericidal Concentration (MBC) is the lowest concentration of antibiotic required to kill 99% of the germ. Not as commonly seen as the Minimum inhibitory Concentration (MIC). It can be determined from broth dilution MIC tests by sub culturing to agar media without antibiotics. Antimicrobials are usually regarded as bactericidal if the MBC is no more than four times the MIC (Jones *et al*., 1985). According to previous studies (Blois Jia *et al*., 2001) the antibacterial activity of chitosanmay result from its polycationic structure due to the protonation of –NH2 on the C-2 position of the Dglucosamine repeat unit. Positively charged chitosan can bind to bacterial cell surface which is negatively charged and disrupt the normal functions of the membrane by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells (Helander *et al*., 2001), (Rhoades and Roller, 2000).

The concentration of manganese was recorded in in original solution ranging from 3,6 and 9 ppm. After Chitosan–sand treatment, metal concentration ranged from 2.4122, 4.9544 and 7.7667ppm respectively. The efficiency of Mn 2+ removal was 80.40, 82.57and 86.29 %. The highest efficiency rate was found with concentration of manganese 3 ppm (Table 2).

Conclusion

Conclusion In this study chitosan has been successfully prepared from shrimp shell waste. By employing FTIR spestroscopy, all functional groups in chitosan macromolecules are elucidated characterization of the prepared chitosan showed that it can be used commercially. chitosan from shrimp shell waste have excellent antibacterial activity against gram- gram-positive bacterium methicillin-resistant *Staphylococcus aureus*.

Chitosan could be a good source of drugs that may be used against bacterial infection. The study indicated that Removal rate of metal is excellent Chitosan based adsorbent may offer an alternative to traditional treatment methods. The unique properties of Chitosan together with availability, makes Chitosan an exciting and promising agent for the purification of surface water for household drinking purpose

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