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Research Article

Antibacterial Activity of Crab-Chitosan against *Staphylococcus aureus and Escherichia coli*

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ABSTRACT

The effect of crab-chitosan on the susceptibility of *Staphylococcus aureus* and *Escherichia coli* was studied. Chitosan which is known to own multiple functional properties has created significant interest among the researchers due to their biological activities and impending applications in the food, pharmaceutical and agricultural industries. The antimicrobial activities of chitosan against *Staphylococcus aureus* and *Escherichia coli* were explored by calculation of the Minimum Inhibitory Concentration (MIC) in media supplemented with 600, 800, 1000, 1200, 1300 and 1400 ppm chitosan adjusted to pH 6 or 7. The Minimum Inhibitory Concentration (MIC) of the prepared chitosan was 1200 and 1300 ppm for *Staphylococcus aureus* and *Escherichia coli* respectively.

Keywords: Chitosan, antibacterial activity, *S. aureus, E. coli,* crab shell

1. 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 [1].

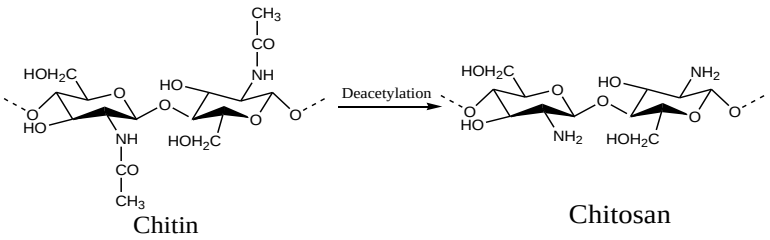

Fig.1: Structure of chitin and chitosan

Recent research has focused on the possibility of developing chitosan as a natural disinfectant [2]. Chen *et al* applied chitosan as a natural disinfectant against waterborne pathogens and proved it to be promising [3]. Much of the interest in the antimicrobial properties of chitosan has focused on the prospect of plant protection [4]. Some biochemical activities in chitosan have been attempted to control plant diseases [5]. Treating tomato plants with chitosan solution reduced mycelial growth, sporangial production, and release of zoospores and germination of cysts of *Phytophthora infestans* which resulted in significant disease protection [6]. Numerous studies on bactericidal activity of chitosan have been carried out [7-8] and reviewed [9]. It has also been used in the removal of waterborne pathogens in waste water and as a food preservative by applying a coat on the exterior of vegetable and fruit products [10].The aim of this study was to evaluate antimicrobial properties of crab chitosan, obtained from crab shell.

2. MATERIALS AND METHODS

2.1. Materials

Crab-shells were collected from Khulna, Bangladesh. Crab-shells were scraped free of loose tissue, washed with cold water and dried in the sun. Chemicals used in processing crab-shells were of analytical grade.

2.2. Preparation of chitosan and Chitosan solution

Chitosan (DD 65%) was prepared from crab shell via chitin as reported data [11]. The typical production of chitosan from crustacean shell generally consists of three basic steps: demineralization, deproteinization and deacetylation. The shells were demineralized by agitating continuously with 5% HCl at the ratio of 1:15 (w/v, shell to solution) 36 hours at room temperature. The demineralized shells were treated with 5% NaOH solution at the ratio of shell to solution of 1:10 (w/v) at 90-95°C for 6 hours. The deproteinized shells were filtered and washed with tap water until NaOH was removed completely, then dried overnight in an oven at 55-60°C. The shells were filtered and washed with tap water until became

neutral. Then deacetylation of chitosan was carried out by hydrolyzing with 80% NaOH at the ratio of 1:20 (w/v, chitin to solvent) at 90-95°C for 5 hours. This product was washed with tap water until it became neutral and dried overnight at 55- 60°C. In the preparation of chitosan solutions, 1.0% (w/v) chitosans were dispersed in a 1.0% (v/v) acetic acid solution. Chitosan solution that obtained was agitated over night with a stirrer and autoclaved at 120°C for 15 minutes at 15 PSI pressure.

2.3. Micro organisms

The antibacterial activity of the prepared chitosan from crab shell was tested against two strains. They were *Staphylococcus aureus* (ATCC 25922) and *Escherichia coli (*ATCC 25923*). Staphylococcus aureus* is a gram positive bacterium which is commonly associated with food product as a result of human handling. *Escherichia coli* is a gram negative bacterium and was chosen because it is responsible for more infections than all other genera combined [12].

2.4. 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 [13] was followed to investigate the antibacterial activity of the extracts. 0.1 ml of TSB broth culture of the test organisms were firmly seeded over the Mueller-Hinton Agar (Lab M, UK) 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 50-100 µl of molten MHA into the scooped out wells. Using a micropipette, solution of chitosan was added to different wells in the plate. These plates were then kept at low temperature (4°C) for 2-4 hours and incubated at 37°C for 24 hours. After the incubation period formation of zones around the wells, confirms the antibacterial activity of the respective extracts. All the results were compared with the standard antibiotic disc of Doxycyclin Hydrchloride 30µg.

2.5. Determination of MIC and MBC

Minimal inhibitory concentrations (MIC) and Minimal bactericidal Concentration (MBC) of chitosan were determined in Muller–Hinton (M–H) broth (Lab M, UK): based on the method of Ruparelia *et al*. [14], the strains were inoculated into M–H broth and incubated to the logarithmic growth phase at 37°C. MIC values of chitosan against the test pathogens were determined by micro and macrodillution broth technique [15] using Mueller-Hinton medium (Table 2).

3. RESULTS AND DISCUSSION

Effect of chitosan obtained from the crab-shell was evaluated against *Staphylococcus aureus* and *Escherichia coli* and the results are presented in Table 1. *S. aureus* can cause skin infections, such as pimples, impetigo, boils, cellulitis folliculitis, carbuncles, scalded skin syndrome, and abscesses, life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It is still one of the five most common causes of nosocomial infections [16]. *Escherichia coli* is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteremia, cholangitis, urinary tract infection (UTI), and traveler's diarrhoea, and other clinical infections such as neonatal meningitis and pneumonia [17].The chitosan was obtained from the crab shell as in reported data [11]. The MIC and MBC values of chitosan were determined against pathogenic *S. aureus* and *E. coli* by macro and micro broth dilution techniques and results are presented in table 2.

Table 1: Antimicrobial activity and zone of inhibition of chitosan (mm)

Test organism	Concentration(µg/mL) growth in peptone broth				Doxycycline HCl (30µg)
	1000	800	600	400	
	Zone of inhibition in diameter(mm)				
S. aureus	13	13	12	10	25
E. coli	10	10	8	8	23

As shown in Table 2, MIC values of chitosans varied depending on bacteria strains. 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 [18]. The highest zone of inhibition against S. *aureus* and *E. coli* were found 13 mm and 10 mm at the dose of 1000 and 800 ppm/ well respectively . Many hypotheses have been proposed to elucidate the mechanism of antibacterial activity of chitosan. Chitosan contains three types of reactive functional groups, an amino/acetamido group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively.

Table 2: MIC and MBC of prepared chitosan against S.aureus and E.coli

	Concentration(ppm) growth in peptone broth						MIC	MBC
Test organism	1400	1300	1200	1000	800	600	(ppm)	(ppm)
S. aureus	-	-	+	+	+	+	1200	1300
E. coli	-	+	+	+	+	+	1300	1400

The amino contents are the main reason for the differences between their structures and physicochemical properties as well as are correlated with their chelation, flocculation and biological functions [19]. Three models have been proposed, the most acceptable being the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes. In this model the interaction is mediated by the electrostatic forces between the protonated NH_3^+ groups and the negative residues [20], most probably by competing with Ca^{2+} for electronegative sites on the membrane surface [21]. This electrostatic interaction results in twofold interference: i) by promoting changes in the properties of membrane wall permeability, thus incite internal osmotic imbalances and consequently restrain the growth of microorganisms [22] and ii) 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)[23-24]. This model was investigated in a current work by Raafat *et al.* [25], who observed under transmission electron microscope the ultrastructural changes of *S. simulans 22* cells upon exposure to positively charged chitosan. It was possible to observe and identify chitosan molecules attached on bacteria cell surfaces. In the interacting sites it was registered that the cell membrane became locally separated from the cell wall, giving rise to "vacuole-like" structures beneath the wall. The detachment generates ions and water efflux, provoking decreases on the inside bacteria pressure [25]. Visual confirmation of an effective membrane lyses been also reported on gram-negative and gram-positive bacteria [26]. 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 [27].

4. CONCLUSION

This study revealed that crab chitosan could be used to inhibit *S. aureus* and *E. coli* in food products involving human handling where there is possibility of contamination of *S. aureus* and *E. coli*.

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