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Effect of Chitosan on Biofilm Formation of Multi-Drug Resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Abstract : This investigation examined chitosan's impact on clinically isolated bacteria's ability to form biofilms. Serial tests were used to create chitosan. At Medical City Hospital, 137 clinical burn swap samples were obtained from patients. *Pseudomonas aeruginosa*: was found in 69 of them, and *Staphylococcus aureus* in 19, according to the Vitek 2 test. When cultured in microtiter plates, only 25 of P. aeruginosa and 5 of S. aureus were capable of forming biofilm. The outcomes demonstrated that at a concentration of 1.4 mg/ml, chitosan was more effective at preventing the production of biofilm. These findings suggest that in the future, this medication may also be utilized to treat MDR bacteria.

Keywords: Chitosan, Biofilm, Multi-drug Resistant. *Pseudomonas aeruginosa*

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Introduction

Multidrug-resistant (MDR) species such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, are often implicated as the cause of acute and chronic infections, leading to suffering, mortality, and higher health care expenditures (1,2). Numerous studies show that human diseases are associated with bacteria's ability to form biofilms (5). Microbial biofilms are groupings of adhering bacterial cells surrounded in an extracellular matrix (6). Adaptation to surface-attached growth inside a biofilm affects gene and protein expression, as well as metabolic activity (7,8), conferring resistance to antimicrobial therapy (9,10) and host clearance (11,12). Many pathogenic and nosocomial bacteria form biofilms in natural settings and infected tissues (13). Biofilm development is essential in chronic human infections (4,16,17). Chitin (_-(1–4)-poly-N-acetyl-D-glucosamine) is nature's second-most abundant polysaccharide (1). The exoskeletons of insects, lobsters, shrimp, and crabs are very rich in chitin (1,2), as are other types of organisms. Chitin (poly-(-1!4)-2-amino-2-deoxy-D-glucopyranose) is the main source of chitosan, which is made by removing the acetyl group (CH3-CO) (1–4). Alongside enzymatic methods, chemical processes are used to extract shellfish minerals and proteins (1–7). due to its biodegradability, biocompatibility, and low toxicity, chitosan has several applications. Chitosan is used as a flocking agent in water treatment (18–

22), an elicitor to activate plant defenses (23), a supplement during food preservation and in food additives .a dehydrating agent in cosmetics and a drug delivery carrier and hydrogel film in pharmaceutical areas. Many articles have proven chitosan's antibacterial and antifungal properties. Demineralization and deproteinization affect the MWs and distribution of deacetylated chitosan (4,5). For example, Long-term, high-temperature deproteinization forms low-molecular-weight, deacetylated chitosan (1–7). Chitosan's anti biofilm action depends on the target microorganism. Physicochemical characteristics contribute to chitosan's antibacterial action.

Materials and methods

Isolation and identification

From 1st September 2021 to 20th November 2021, (178) burn samples (swaps) were collected from individuals in burn center of medical city hospitals (Baghdad) order to lock for any pathogenic bacteria. Then, the swaps were inoculated into BHI and incubated for 24 hrs. at 37C . Samples were transformed into blood and MacConkey agar media and then, Colonies representing different morphologies were isolated from the plates and sub cultured into nutrient agar slants for further use.

Isolation of *Staphylococcus aureus*

Samples that showed growth on blood agar only and no growth on macConky. Gram stain were done and the gram positive colonies subjected to catalase test and showed positive result then transferred into manitol salt agar as a selective and deferential media for *Staphylococcus,* and the manitol fermenter colonies selected for coagulase test with 10% rabbit plasma and the positive samples considered as *S.aureus*

Isolation of *Pseudomonas aeruginosa*

Samples that showed growth on blood and macConky agar and the colonies were non lactose fermenter. Gram stain were done and the gram-negative colonies subjected to oxidase test and then transferred into Citramied agar as a selective media for *Pseudomonas*.

Antibiotic susceptibility testing

MHA preparation from a dehydrated was according to manufacturer company instructions. The medium was refrigerated to -20 °C and placed into the dishes. The plates were dried for immediate use at 35 °C for 30 min when the agar was solidified by placing them in the upright position in the incubator, with the lids tilted. Inoculums were prepared by transferring 3-5 colonies into a tube of 5ml of normal saline (NaCl 0.85%) mixed well and compared with McFarland tube to obtain culture with 1.5×108 CFU/ml, and adjusting to turbidity standard of McFarland 0.5, suspensions were used within 30 minutes. of preparation. Plates were inoculated *via* orienting steril swab into the bacterial inoculums. Excess has been removed by compression and revolving the swab promptly above liquid

levels on the side of the test tube. The chosen antibiotic according to CLSI M100 2020 was placed on the inoculated plate (each plate contains four to five discs) by forceps. Disks must be warm at room temp. Then, under the condition that they must be moved slowly downward with forceps, they were administered on the agar's surface.At 37 °C, inverted incubation of inoculated plates was performed overnight.

Biofilm formation assay (quantitative)

The microliter plate technique was used to identify biofilm for pathogenic isolates (3). Pathogenic isolates were grown overnight in tryptic soy broth (TSB) supplemented with 1% glucose (37).Following that, bacterial cultures were diluted into 5 ml of sterile normal saline (NaCl 0.85%), thoroughly mixed, and then compared to McFarland tubes. Then, 20 µl of bacterial cultures from each isolate were added to three wells (trials were done in triplicate) of a polystyrene microtiter plate that contained 8µl of TSB supplemented with 1% glucose. The plate was then incubated under constant conditions at 37℃ for 18-24hrs. After incubation, the microtiter plate content was removed by aspiration and wells were washed three times with PBS (pH7) in order to remove loosely adherent cells. Then, the remaining bacterial attachment was fixed at 40℃ for 15min. Wells were then stained for 5 minutes at room temperature with 125 µl of a 0.1% crystal violate solution. Next, wells were washed with PBS (pH7), and the addition of 95% ethanol for 15 minutes was used to produce the biofilm (quantitative analysis). By using an ELISA microtiter plate reader, the absorbance of the crystal violate presence was determined at 630 nm. The control was performed with crystal violate binding to the wells exposed only to the culture medium without bacteria. The adherence of biofilm formation was calculated according to the following equation.

$$\text{Adherence of biofilm Formation} = \text{ODs/ODc}$$

Where:
ODs = Mean of OD630 of bacterial samples
ODc = Mean OD630 of control

Recovery and extraction of Chitosan

The presence of chitosan was measured as follows: the solid-state biomass as a whole was first homogenized with 1M NaOH (1:30 w/v) in an autoclave at 121Co, 15 pressure for 15 minutes. Then, alkali insoluble materials (AIMs) were collected using cotton gauze and rinsed with distilled water until the pH reached a neutral state. The washed AIMs were placed in glass petri dishes, weighed, and dried in an oven at 60°C overnight. Chitosan was extracted from AIM with 2% acetic acid (1:40 w/v), then autoclaves for 15 min followed by centrifugation at 10000rpm for 15 min. AIMs were discarded and pH was adjusted to 10 with 4M NaOH (2.1.4.4). Repeating the centrifuge after an overnight period allowed for the collection, washing, and weighing of precipitates. Once again, it was cleaned with acetone (1:20) (w/v) and 95%

ethanol (1:20) (w/v).Chitosan precipitate was dried at 60Cº and measured as (mg chitosan/g chitin) (38).

Characterizations of chitosan

Determination *of degree of deacetylation (DDA)*

The following N-acetylglucosamine standard curve was created to estimate the DDA of chitosan: we showed details the preparation of N-acetylglucosamine serial concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml from a 0.1 mg/ml stock solution as the table (2-1) . The blank was prepared from 0.1 ml of HCL-solution (0.001M) without N-acetylglucosamine. After 5min, the absorbance for each concentration was read at wavelength 199nm and the standard curve of N-acetyl glucosamine was plotted between N-acetyl glucosamine concentrations (mg/ml) and the corresponding absorbance.

Table (2-1): Preparation of N-acetyl glucosamine Concentrations

Tube number	Volume of stock solution N-acetyl glucosamine (ml)	Volume of 0.001 M HCl-solution (ml)	Total volume (ml)	N-acetyl glucosamine concentrations (mg/ml)
1	0.00	0.1	0.1	0.00
2	0.01	0.09	0.1	0.01
3	0.02	0.08	0.1	0.02
4	0.03	0.07	0.1	0.03
5	0.04	0.06	0.1	0.04
6	0.05	0.05	0.1	0.05

Infrared spectroscopy (FTIR)

Both commercial and synthesized chitosan were subjected to FTIR spectrum and then compared between them .In order to create 0.5 mm thick disks, a 2 mg sample of chitosan that had been dried overnight at 60°C at reduced pressure was thoroughly combined with 100 mg of KBr.Following that, the disk was dried for 24 hours at 110°C under reduced pressure.With the Bruker 66 Spectrometer and a blank 100 mg KBr disk, infrared spectrometer measurements were taken amd compaired with *Aspergillus flavus* F1.

Anti-biofilm activity of chitosan

To determine the antibiofilm activity of chitosan we selected the strongest biofilm formatting isolate of each bacterial species pseudomonas ,and S.aureus. three replicas were made for each species. the biofilm was observed with the chitosan and without it spontaneously to measure its direct effect by adding the sub inhibition concentration for each bacterium and the results were measured

Results and discussion

Out of the 178 wound swab samples,41 had no bacterial growth. Numerous factors, such as unknown agents like viruses and anaerobic bacteria, which were excluded from our experiment, could be the cause for these events. A total of 137 isolates were discovered and identified using biochemical testing., *S. aureus* made up 13.8% of 19 cases, *Pseudomonas* aeruginosa made up 50.3 % of 32 cases , other gram-positive bacteria made up 7.2%, and gram-negative bacteria made up 28.7 %. As shows in figure 1.

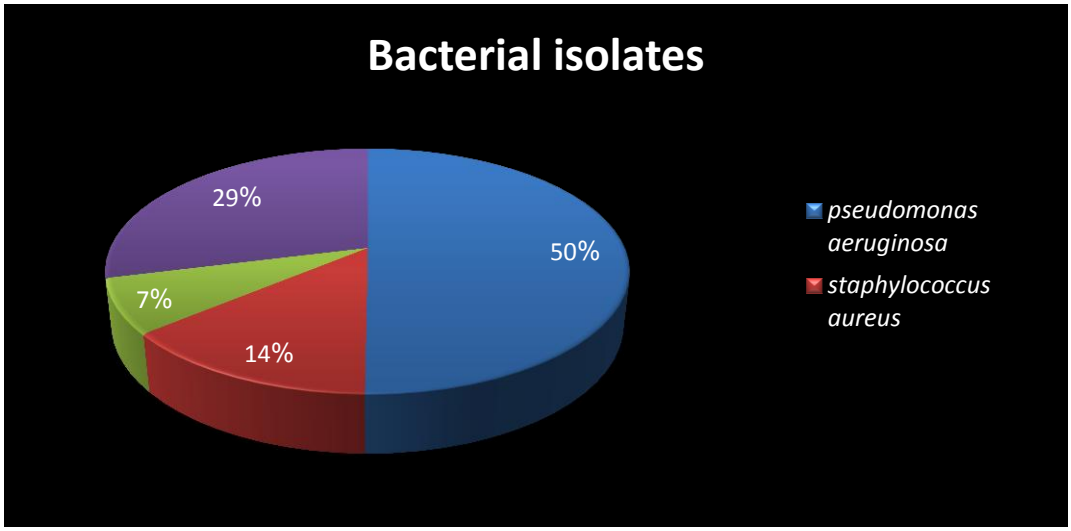

Figure 1: Ratio of bacterial isolates among burn samples

Antibiotic Susceptibility Testing

The majority of the bacterial isolates were found in this study were antibiotic-resistant. *S. aureus* showed resistant as the following: 80% penicillin, 40% cifipem, 100% gentamycin, 80% tobramycin, 20% amikacin, 80% levofloxacin, 60% chloramphenicol, 60% nitrofurantoin, 100% tetracyclin. (14) Bacterial targets of antibiotics in methicillin-resistant *S. aureus*. *(14)* (as figure 2). and *P. airginosa* showed resistant as the following: 86% ciftazidime, 48% cefipem, 79% gentamycin, 41% amikacin, 20% tobramycin, 65% levofloxacin, 62% pipracillin, 86% colstin, 68% imipenem, 68% aztreonam (19)). Correlation between bacterial growth and antibiotic resistance in *Pseudomonas aeruginosa*: a meta-analysis. (21) (figure 3). In addition to the increasing rates of reported antimicrobial resistance among clinical strains, antimicrobial resistance is an inherent characteristic of bacterial biofilms that may make treating patients even more challenging (20). High biofilm production by invasive multiresistantstaphylococci (22).

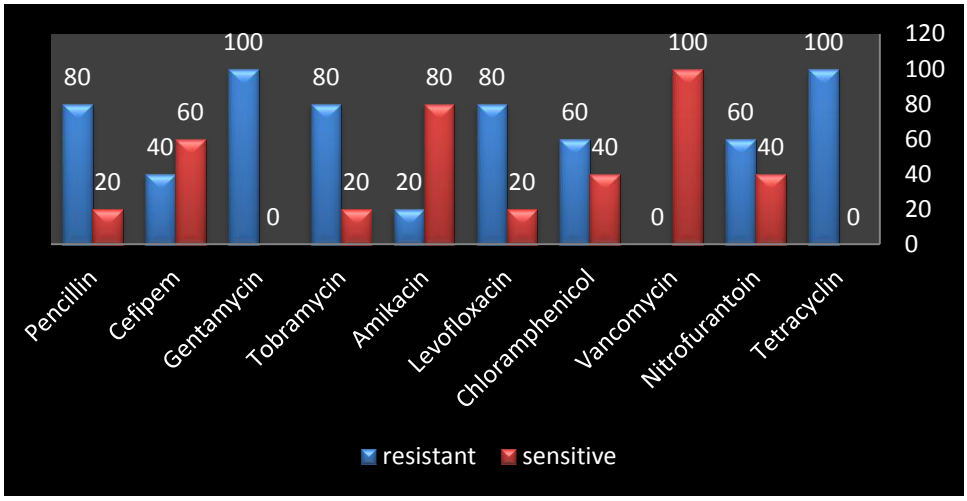

Figure 2: Antibiotic Susceptibility of *Staphylococcus aureus*

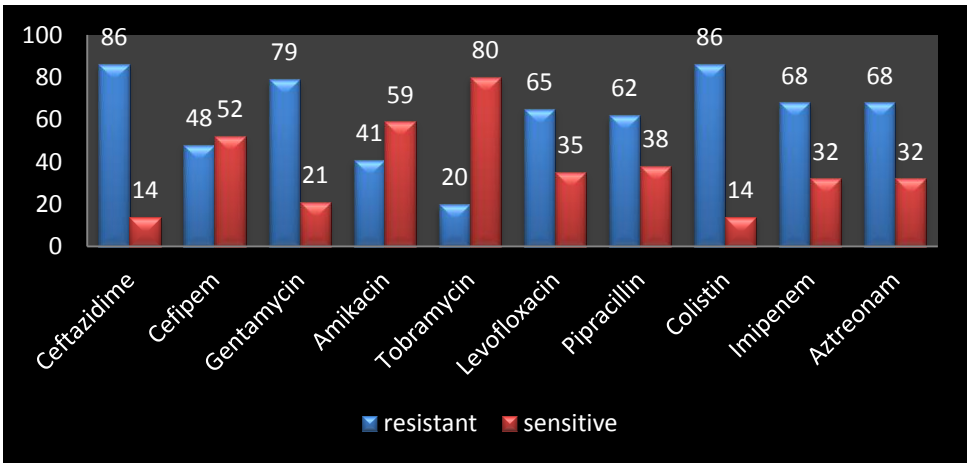

Figure 3: Antibiotic Susceptibility of *pseudomonas aeruginosa*

Biofilm formation

The traditional method of biofilm formation pinpointing with microtiter plates produced the following results for S. aureus: weak 20%, moderate 20%, and strong 60% (as figure 4). Pseudomonas aeruginosa were: weak 24%, moderate 26%, strong 50% (figure 5). Biofilm formation by clinical isolates and the implications in chronic infections. Comparing antimicrobial resistance to each strain's capacity to produce biofilms, that found that MDR phenotypes were more frequently seen in bacteria that could form biofilms.

(26)Characterization of biofilm formed by multidrug resistant *Pseudomonas aeruginosa* DC-17 isolated from dental caries (27).

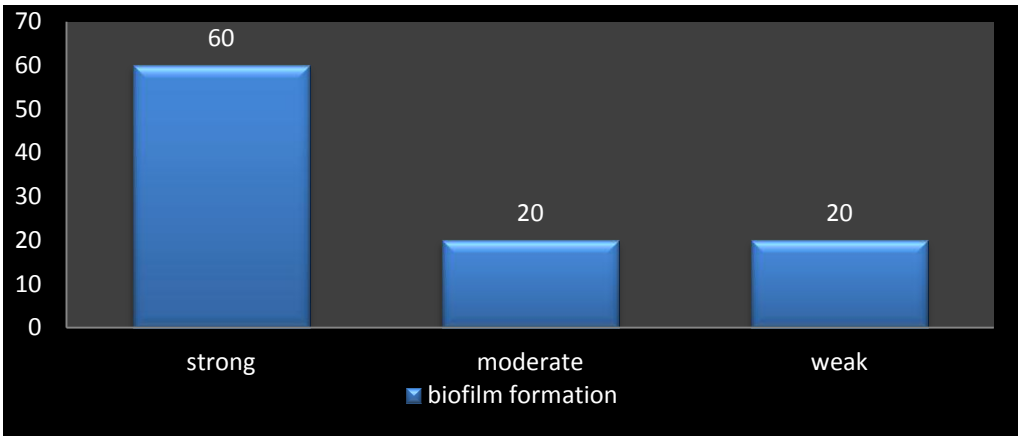

Figure 4: Biofilm formation *Staphylococcus aureus*

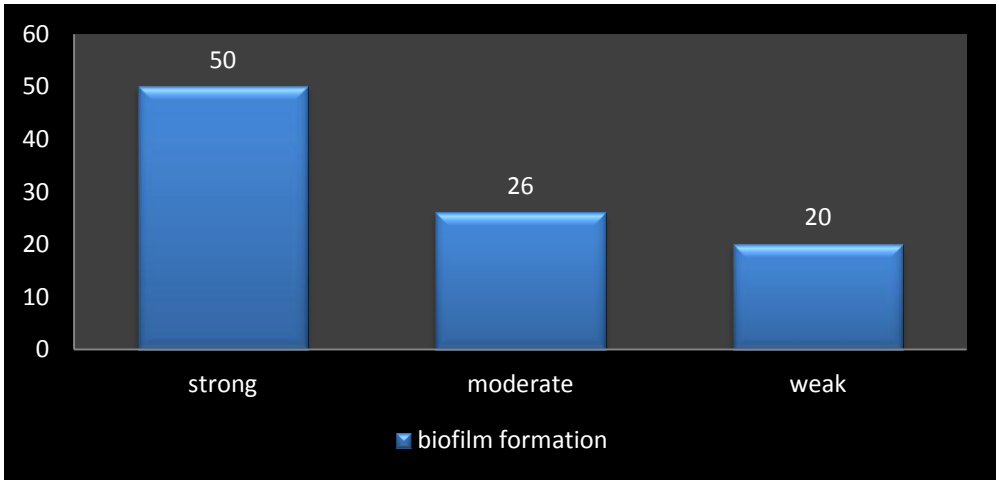

Figure 5: Biofilm formation of *Pseudomonas aeruginosa*

Extraction of chitosan

Traditional commercial chitin preparation from crustacean shell (exoskeleton) involves two processes. protein separation by alkali treatment (ii) calcium carbonate (and calcium phosphate) separation by acidic treatment at high temperature (28) . The shrimp shells were first treated with NaOH and HCl to remove protein and minerals. The conversion of shrimp shells to chitin removes protein and other nitrogenous components, reducing nitrogen content (29) Chitin molecules are organized into helicoidal, microfibrillar structures in shell proteins (30). Chitin binds proteins, minerals, lipids, and pigments (31) .Harsh acid treatments induce polymer hydrolysis, variable chitin characteristics,

and pollution (32). High NaOH and deproteinization temperatures can deacetylate and depolymerize chitin (33). To test the indicated optimum conditions and model correctness, chitin from shrimp shell sources was used in repeated experiments.

Characterizations of chitosan

Determinat*i*on of degree of deacetylation (DDA)

The amount of deacetylation for the generated chitosan was examined during the bioconversion process; however, only a small amount (3.8 mg/g) was created after 5 days of incubation. Since the discovery of the CDA enzyme, the bioconversion of chitin to chitosan has been studied. However, a significant degree of deacetylation is still challenging to achieve, mostly due to the crystalline and insoluble character of chitin(34). As a result, different methods were utilized to enhance chitin's capabilities by diminishing its crystallinity and so affecting the amorphous structure of chitin, which CDA needs in order to access and generate chitosan (35).

FTIR spectroscopy

FT-IR spectrum (4000 to 400) cm-1was used to identify and confirmed the characteristic functional groups of the produced chitosan. The isolated fractions gave IR spectra similar to that of the commercial chitosan from shrimp shells. As can be seen from the FT-IR spectra presented in (figure 6), a broad absorption band in the range of 3000cm-1 – 3500cm-1 is found which is attributed to O–H stretching vibrations and at 1400-1650 cm-1 refer to C=O bond(36). In addition, the peaks around 2885, 1650, 1589,1326 and 1080 cm -1 are assigned to the stretching vibrations of aliphatic C– H, Amide I (-NH deformation of –NHCOCH3), Amide II, Amide III and C–O–C, bonds respectively. According to IR spectra results, the basic molecular structure of both produced and commercial chitosan is significantly similar.

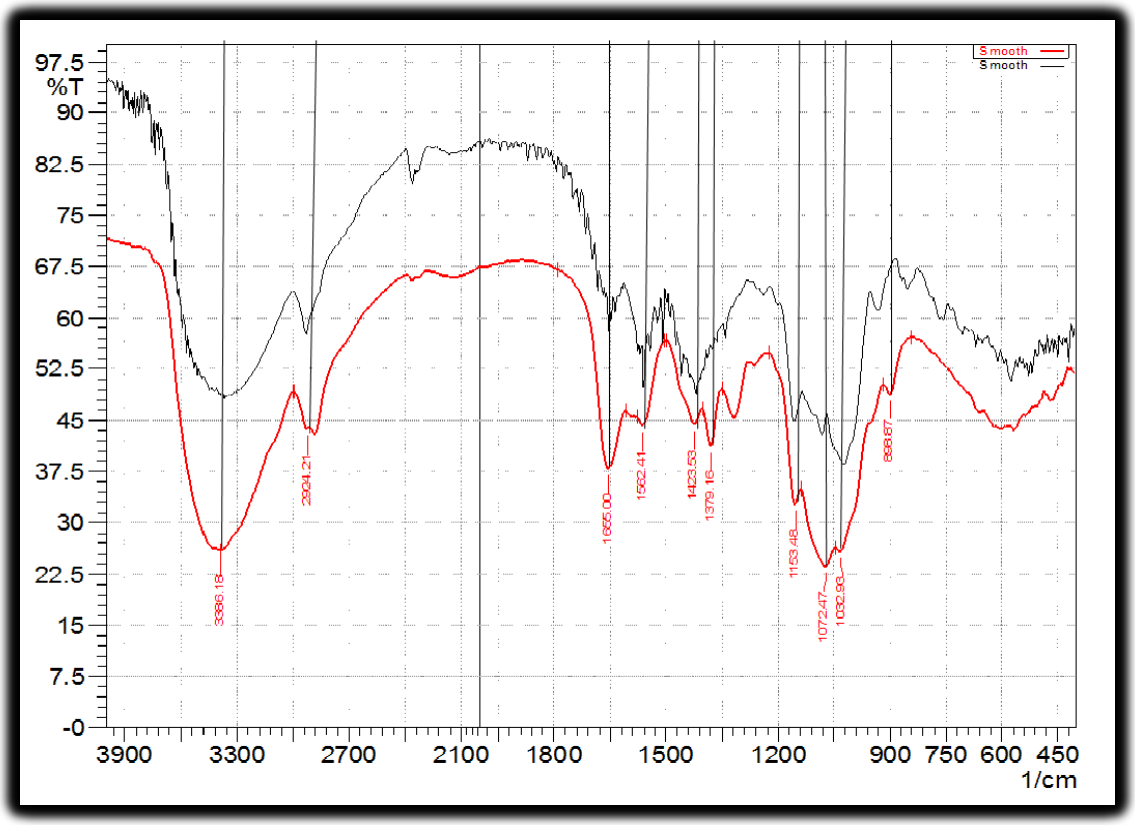

Figure 6: FTIR spectra of commercial chitosan (red color); andchitosan produced by *Aspergillus flavus* F1 in SSF (black color)

Anti-biofilm activity of chitosan

The recent research used a microtiter plate assay and the crystal violet staining method to determine the biofilm formation by *Staphylococcus aureus* and *pseudomonas aeruginosa*in response to MICs of chitosan (1.6mg/ml). The responses were measured using an ELISA reader to measure the absorbance of stained biofilms at 590 nm. The findings demonstrated that *Staphylococcus aureus* and *pseudomonas aeruginosa*in are sensitive to the antibiofilm effects of chitosan compounds. When chitosan was used at a concentration of 1.4 mg/ml compared to biofilm formation without chitosan, a considerable ratio of biofilm formation was inhibited. (Figures7) depicted how chitosan affected the development of biofilm.

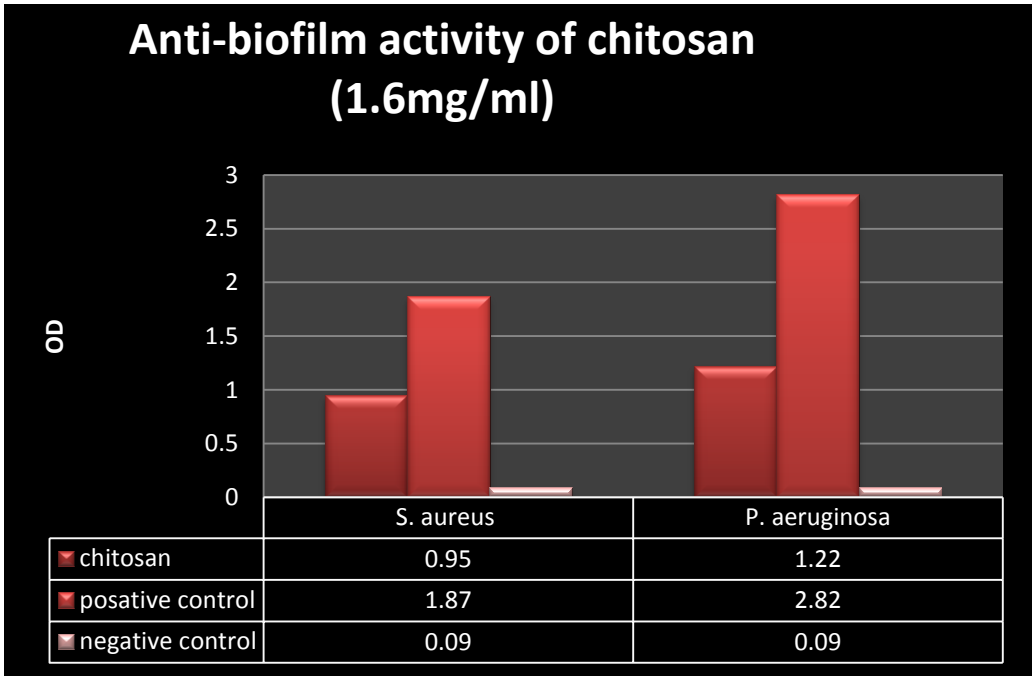

Figure 7: Anti-biofilm activity of chitosan against *Staphylococcus aureus and pseudomonas aeruginosa*in biofilm.

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