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Research Article

The Effect of Ag Content of the Chitosan-Silver Nanoparticle Composite Material on the Structure and Antibacterial Activity

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The aim of this study is to investigate the antibacterial properties and characterization of chitosan-silver nanoparticle composite materials. Chitosan-silver nanoparticle composite material was synthesized by adding $AgNO_3$ and NaOH solutions to chitosan solution at 95°C. Different concentrations (0,02 M, 0,04 M, and 0,06 M) of $AgNO_3$ were used for synthesis. Chitosan-silver nanoparticle composite materials were characterized by Transmission electron microscopy (TEM), X-ray diffraction (XRD), ultraviolet (UV) spectrophotometer, and Fourier transform infrared (FTIR) spectrometer techniques. *Escherichia coli*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* were used to test the bactericidal efficiency of synthesized chitosan-Ag nanoparticle composite materials. The biological activity was determined by the minimum bacterial concentration (MBC) of the materials. Antibacterial effect of chitosan-silver nanoparticle materials was increased by increasing Ag amount of the composite materials. The presence of small amount of metal nanoparticles in the composite was enough to significantly enhance antibacterial activity as compared with pure chitosan.

1. Introduction

Chitosan obtained from a natural polymer chitin has antibacterial feature. As a polycationic polymer, chitosan is an environmental friendly material because of its biodegradability. Nontoxic and antibacterial features of chitosan make it usable for many areas related to human health [1–5]. Due to the functional groups as NH_2 and OH in the chitosan structure, chitosan is used as an excellent chelating agent [6].

Silver (Ag) ion has been used for a long time as antibacterial agent due to its strong inhibiting effect on bacteria. Recently, nanoparticle Ag has taken considerable attention to provide maximum bactericidal effect with minimum amount of Ag [7–10]. Generally chemical reducing agents such as $NaBH_4$, ascorbic acid are used for preparation of Ag nanoparticle [7–11].

Chitosan is used as metal nanoparticle-chitosan material in biomedical applications because of its advantages of biodegradability, antibacterial properties, and excellent chelating agent. Both of Ag and chitosan are antibacterial agents so chitosan-Ag nanoparticle composite material has more antibacterial effect.

Comparative studies showed that chitosan-Ag nanoparticle composite is much more effective against bacteria than pure chitosan [12]. Chitosan is also used as a stabilizer instead of chemical reducing agent for protecting Ag nanoparticles from agglomeration. Because of these specialties of chitosan and Ag, chitosan-Ag nanoparticle composite has taken attention in the recent years [12–18]. In these studies, characterization of chitosan-Ag nanoparticles by spectroscopic methods and antibacterial effects of these materials was investigated. However chitosan [3–5, 8, 19] and Ag nanoparticle separately have been studied extensively [7, 9, 11, 20–24], but further investigations about chitosan-Ag nanoparticles are only rarely carried out. Different techniques were preferred to synthesize chitosan-Ag nanoparticle composites by researchers [8, 12–18]. Chen et al. use γ-ray irradiation to synthesize Ag nanoparticle in chitosan solution [8]. The chitosan-Ag nanoparticles were prepared using chitosan in aqueous solution of acetic acid by Twu et al. [15]. Murugadoss and

Chattopadhyay developed a mild method for synthesizing a chitosan-Ag nanoparticle material in the medium of aqueous sodium hydroxide [14]. They studied the catalytic activity of chitosan-Ag nanoparticles photometrically.

Transmission electron microscopy (TEM), X-ray powder diffraction (XRD), UV spectrophotometer, and Fourier transform infrared (FTIR) spectrophotometer techniques were used for characterization of the size and structure of chitosan-Ag nanoparticles [12–16]. The antibacterial effectiveness was determined by measuring the minimum bacterial concentration (MBC) against usually *Escherichia coli* [13, 16].

In this study, chitosan-Ag nanoparticle composite was synthesized by mild method in the aqueous sodium hydroxide [13, 14]. Both of the structural characterization and antibacterial effectiveness of chitosan-Ag nanoparticles against three Gram-positive and three Gram-negative bacteria were investigated. The effect of Ag concentration of the composite material on the structure and antibacterial activity was also investigated.

2. Materials and Methods

2.1. Materials. Chitosan (>75 deacetylated), silver nitrate, and glacial acetic acid were purchased from Sigma Aldrich chemical Co. Ltd. Sodium hydroxide was obtained from Merck.

Escherichia coli (*E. coli* ATCC 25922), *Acinetobacter baumannii* (*A. baumannii* ATCC 19606), *Staphylococcus aureus* (*S. aureus* ATCC 25923), *Enterococcus faecalis* (*E. faecalis* ATCC 29212), *Pseudomonas aeruginosa* (*P. aeruginosa* ATCC 27853), and *Streptococcus pneumoniae* (*S. pneumoniae* ATCC 49619) were supplied by Microbiologics. Mueller Hinton Broth (Merck microbiology), Chrome agar (Himedia), and Blood agar (%5, Biopen) were used for MBC measurements. Millipore water purification system was used for providing deionized water.

2.2. Preparation of Chitosan-Ag Nanoparticle Composite Materials. Approximately 100 mg of chitosan was placed in a beaker with 50 mL water at 95°C. Chitosan-Ag nanoparticles were prepared by mixing freshly prepared 1 mL 0.02 M $AgNO_3$ and then 100 μL 0.3 M NaOH with homogenizer (18000 rpm, Art Miccra D-1). The color of mixture turned yellow in about one minute after addition of NaOH solution due to the formation of Ag nanoparticles. Then the mixture was stirred for 10 min. Finally, the resulting suspension was filtered and the reddish yellow material was washed until neutral with distilled water. Chitosan-Ag nanoparticle material was dried for structural characterization and bacterial tests [13, 14].

In order to investigate the effect of Ag content on the antibacterial activity, chitosan-Ag nanoparticle materials with different amount of Ag metal were synthesized by using 0.04 M and 0.06 M $AgNO_3$ solution by keeping other conditions unchanged.

The effect of temperature on the formation of Ag nanoparticle was also investigated at 50°C and 75°C.

2.3. Characterization of Chitosan-Ag Nanoparticle Composite Materials. Ag nanoparticles of chitosan-Ag nanoparticle composite materials in 0.1% (v/v) acetic acid were investigated by HP-8473 UV spectrophotometer. Maximum absorption peak of Ag nanoparticle was observed at 413 nm.

Structure analysis of the samples were carried out by X-ray powder diffraction (XRD) on Rigaku D/Max-2200/PC diffractometer with monochromatic Cu resource (A4 1L-Cu/60 kV, 2.0 kW), the 2-theta ranging from 10° to 80° with Cu Kα radiation (λ = 1.5404 Å).

All infrared measurements were performed on a Perkin Elmer Fourier transform infrared (FTIR) spectrophotometer using the attenuated total reflection (ATR) technique and the spectral range is from 650 to 2000 cm^{-1}.

The size of Ag nanoparticles in chitosan-Ag nanoparticle materials was determined by transmission electron microscopy (TEM). After dissolving in ethanol with ultrasonic mixer, samples were dropped onto carbon support film coated with copper TEM grids (200 mesh). Transmission electron microscopy measurements were obtained on JEOL JEM 2100 HRTEM operating at 200 kV (LaB6 filament). Images were taken by Gatan Model 694 Slow Scan CCD Camera.

2.4. Determination of Antibacterial Activity of Chitosan-Ag Nanoparticle Composite Materials. The biological activity was determined by the minimum bacterial concentration (MBC) of the composites. Three Gram-negative bacteria (*E. coli* (ATCC 25922), *A. baumannii* (ATCC19606), and *P. aeruginosa* (ATCC27853)) and three Gram-positive bacteria (*S. aureus* (ATCC25923), *E. faecalis* (ATCC29212), and *S. pneumoniae* (ATCC49619)) were used for measurement of MBC of chitosan-Ag nanoparticle materials. Bacteria except *S. pneumoniae* were cultured on Chromo agar plates and incubated at 37°C for 24 hours. *S. pneumoniae* was grown on blood agar plate and incubated at 37°C for 24 hours.

Chitosan-Ag nanoparticle materials synthesized by using 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ solution and chitosan without Ag nanoparticle (pure chitosan) were dissolved in 0.1% (v/v) acetic acid solution.

2.5. Determination of Minimal Bacterial Concentrations (MBC) of Chitosan-Ag Nanoparticle Composite Materials. 1 mL Mueller hinton broth dilution was added to each test tube. Test tubes were plugged by hydrophobic cotton and were sterilized at 121°C for 20 min.

Each of chitosan-Ag nanoparticle material solutions and pure chitosan solution in 0.1% (v/v) acetic acid was diluted to obtain four different concentrations of the solutions.

Diluted solutions were added to the sterilized Mueller Hinton broth dilutions. Subsequently 1 mL freshly prepared bacteria suspension was added to each tube so as to obtain final concentration of approximately 10^5 CFU/mL in test tubes. Final concentrations of material solutions were 25 ppm, 50 ppm, 100 ppm, and 200 ppm for chitosan-Ag nanoparticle materials and 50 ppm, 100 ppm, 200 ppm, and 400 ppm for pure chitosan in the suspension. After that, prepared test tubes were incubated at 37°C for 24 hours in the incubator. Finally, incubated test tube suspensions were

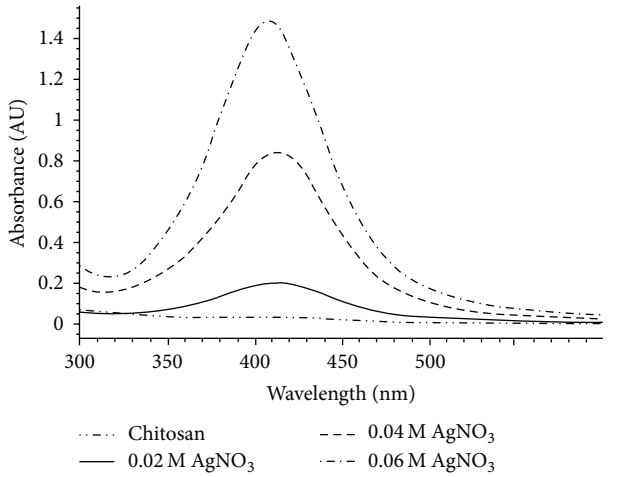

Figure 1: UV-vis absorption spectra of chitosan-Ag nanoparticle materials synthesized with 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ and pure chitosan.

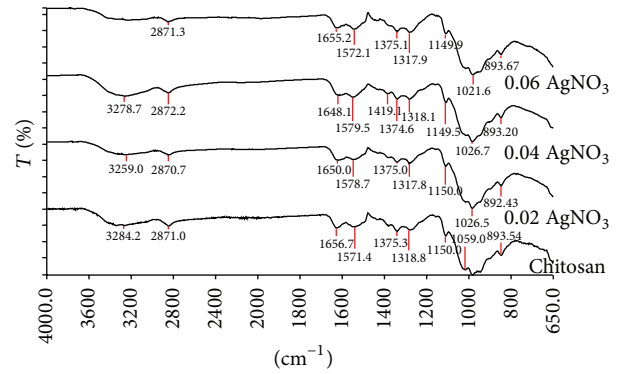

Figure 2: FTIR spectra of chitosan-Ag nanoparticle materials synthesized with 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ and pure chitosan.

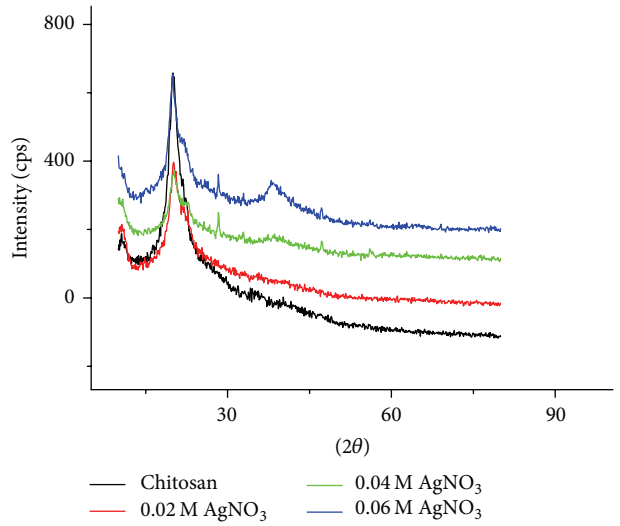

Figure 3: XRD patterns of chitosan-Ag nanoparticle materials synthesized with 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ and pure chitosan.

inoculated on Chromo agar plates to control the effectiveness of diluted four different concentrations of solutions for each bacterium except *S. pneumoniae*. The suspensions with *S. pneumoniae* were grown on blood agar plate and then all of the plates were incubated at 37°C for 24 hours. The absence of bacteria on the inoculated agar plates with minimum concentration of solutions indicated minimum bactericidal concentration (MBC).

3. Results and Discussion

3.1. Structural Analysis of Chitosan-Ag Nanoparticle Composite Materials. Ag nanoparticle-containing chitosan composite materials were investigated to understand the interactions between Ag and chitosan and the formation of Ag nanoparticles by increasing $AgNO_3$ concentration. XRD patterns indicated the existence of Ag nanoparticles in chitosan matrix, FTIR spectra provided information of structural change, UV spectra gave Ag concentration in chitosan, and TEM images showed that the size of the Ag nanoparticles well penetrated into chitosan.

UV-vis absorption spectra of chitosan-Ag nanoparticle materials synthesized by using 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ and pure chitosan are shown in Figure 1.

The maximum absorption peak was observed at about 413 nm which is the characteristic absorption peak for Ag nanoparticles. Also UV absorption peak of chitosan-Ag nanoparticles prepared by other researchers was recorded in the range 410–420 nm [8, 13–16]. Figure 1 also displays that the intensity of the absorption peak of Ag nanoparticles increased by increasing the concentration of $AgNO_3$ used for preparing chitosan composite material. So this result showed that the amount of Ag nanoparticles of chitosan composite materials increased by increasing $AgNO_3$ concentration.

Figure 2 illustrates FTIR spectra of chitosan and chitosan-Ag nanoparticle materials synthesized with 0.02 M, 0.04 M, and 0.06 M $AgNO_3$. The broad absorption peak at 3700–3100 cm^{-1} is the merged characteristic bands for OH and NH_2 groups [14] and $CONH_2$ absorption band is also observed at near 1657 cm^{-1} in the FTIR spectrum of chitosan [3]. The shift was observed from 1657 cm^{-1} in the Ag loaded chitosan spectra. This shift may indicate the binding of Ag nanoparticles to N–H bond of chitosan [3, 12, 14, 16].

XRD patterns of chitosan and Ag-loaded chitosan are shown in Figure 3. XRD pattern of pure chitosan exhibits a strong characteristic peak at about $2\theta = 20^\circ$ for chitosan. XRD patterns of chitosan-Ag nanoparticle materials synthesized with 0.04 M and 0.06 M $AgNO_3$ showed another peak at about 38° corresponding to Ag nanoparticle in addition to the chitosan at 20° [8, 11, 12]. The peak at $2\theta = 38^\circ$ associated with the crystal face of (111) of Ag [11] became more obvious upon increasing concentration of $AgNO_3$ used for preparing chitosan composite material.

TEM photographs of chitosan-Ag nanoparticle materials are presented in Figures 4–6. The size of Ag particles showed that loaded Ag particles on chitosan were achieved to be nanosized. Ag particles were well dispersed in chitosan

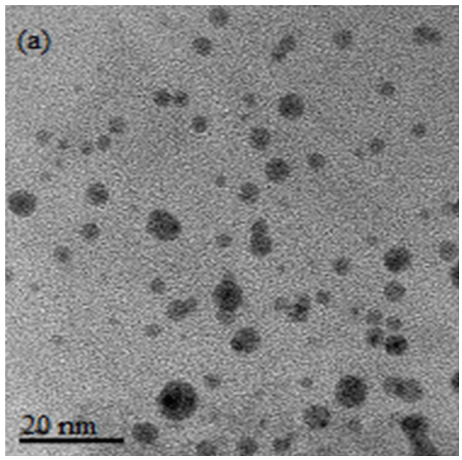

Figure 4: TEM image of chitosan-Ag nanoparticle materials synthesized with 0.02 M $AgNO_3$.

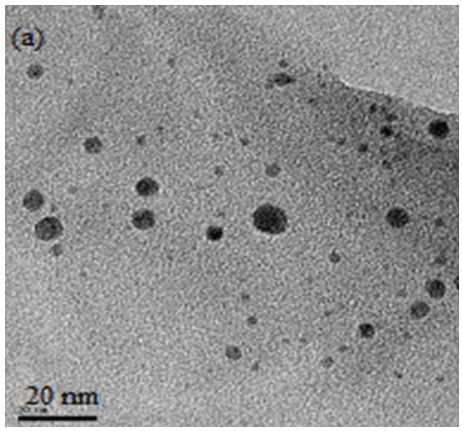

Figure 5: TEM image of chitosan-Ag nanoparticle materials synthesized with 0.04 M $AgNO_3$.

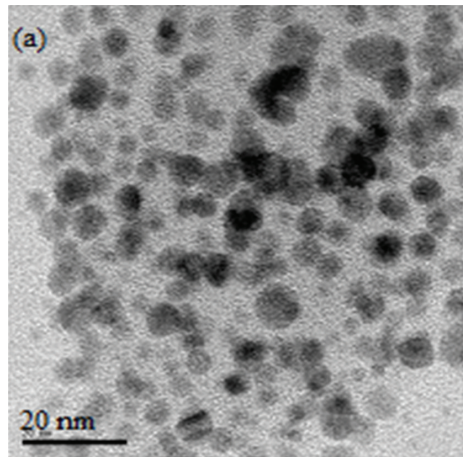

Figure 6: TEM image of chitosan-Ag nanoparticle materials synthesized with 0.06 M $AgNO_3$.

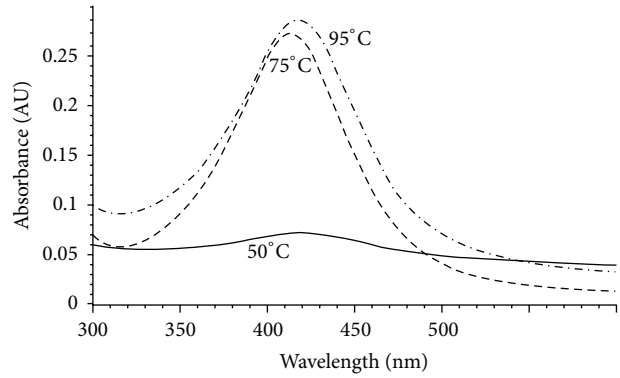

Figure 7: UV-vis absorption spectra chitosan-Ag nanoparticle materials synthesized with 0.02 M $AgNO_3$ at 50°C, 75°C, and 95°C.

matrix with the average diameter of around 3–8 nm. Compared with Figures 6 and 4, Figure 5 showed that Ag particles synthesized with 0.06 M $AgNO_3$ are more densely and more than 50% of the particles are in the range of 5–8 nm in diameter in chitosan matrix.

3.2. Effect of Temperature on the Formation of Chitosan-Ag Nanoparticle Composite Materials. The effect of temperature on the formation Ag nanoparticle was also investigated. The materials were prepared by adding 0.02 M $AgNO_3$ at 50°C, 75°C, and 95°C. The color of material did not turn reddish yellow which indicates the formation of Ag nanoparticle after the addition of NaOH solution at 50°C.

UV-vis absorption spectra of chitosan-Ag nanoparticle materials synthesized with 0.02 M $AgNO_3$, at 50°C, 75°C, and 95°C are shown in Figure 7.

The Intensity of the absorption peak of Ag nanoparticles at 413 nm increased by increasing temperature keeping other conditions unchanged. This result showed that the formation of Ag nanoparticles of chitosan composite material is related to the synthetic temperature and the optimum temperature for reduction of Ag ions is about 95°C.

3.3. Antibacterial Effect of Chitosan-Ag Nanoparticle Composite Materials. Antibacterial effectiveness of chitosan-Ag nanoparticle materials was investigated against three Gram-positive and three Gram-negative bacteria. Each of chitosan-Ag nanoparticle materials synthesized with 0.02 M, 0.04 M, and 0.06 M $AgNO_3$ and pure chitosan solutions in 0.1% (v/v) acetic acid were prepared for MBC determination with four final concentrations of 25 ppm, 50 ppm, 100 ppm, and 200 ppm for chitosan-Ag nanoparticle materials and 50 ppm, 100 ppm, 200 ppm, and 400 ppm for chitosan. Incubated test tube suspensions were inoculated on Chromo agar plates, *S. pneumoniae* in the suspensions was grown on blood agar plate to control the effectiveness of diluted four different concentrations of materials in solution, and then all of the plates were incubated at 37°C for 24 hours. The absence of bacteria on the inoculated agar plates with minimum concentration of solutions indicated minimum bactericidal concentration (MBC). MBC of chitosan-Ag nanoparticle composite materials and pure chitosan were listed in Table 1.

Table 1: Antibacterial effect of chitosan-Ag nanoparticle composite materials.

	Minimum Bactericidal Concentration, MBC (ppm)					
Material	*S. Pneumoniae*	*A. Boumanni*	*E. Coli*	*P. Aeruginosa*	*E. Faecalis*	*S. Aureus*
Composite A	100–200	50–100	100–200	50–100	50–100	50–100
Composite B	100–200	50–100	50–100	25–50	50–100	50–100
Composite C	50–100	25–50	50–100	<25	25–50	25–50
Pure chitosan	200–400	>400	>400	>400	200–400	>400

Composite A: Chitosan-Ag nanoparticle material synthesized with 0.02 M $AgNO_3$; Composite B: Chitosan-Ag nanoparticle material synthesized with 0.04 M $AgNO_3$; Composite C: Chitosan-Ag nanoparticle material synthesized with 0.06 M $AgNO_3$.

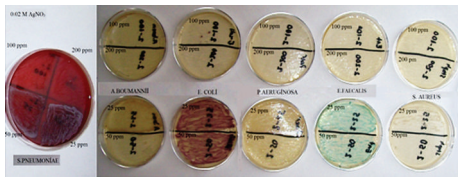

Figure 8: Antibacterial activity of chitosan-Ag nanoparticle materials synthesized with 0.02 M $AgNO_3$.

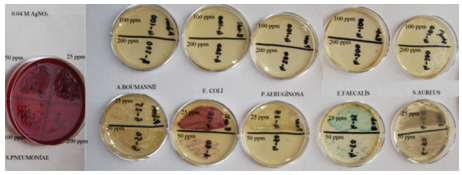

Figure 9: Antibacterial activity of chitosan-Ag nanoparticle materials synthesized with 0.04 M $AgNO_3$.

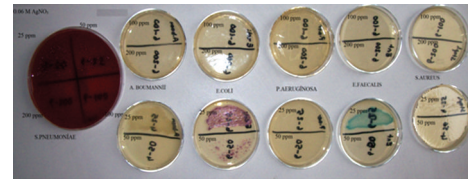

Figure 10: Antibacterial activity of chitosan-Ag nanoparticle materials synthesized with 0.06 M $AgNO_3$.

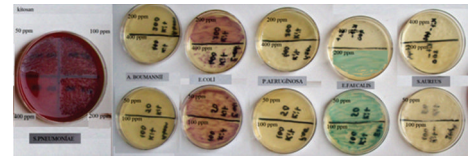

Figure 11: Antibacterial activity of chitosan.

Figure 8 shows antibacterial activity of chitosan-Ag nanoparticle material synthesized with 0.02 M $AgNO_3$. MBC of this chitosan-Ag nanoparticle material is between 100 and 200 ppm against *S. pneumoniae* and *E. coli*, but very little growth appeared on Chromo agar plate with 100 ppm of chitosan-Ag nanoparticle material in suspension towards *E. coli*. MBC is between 50 and 100 ppm against *A. baumannii, E. faecalis, P. aeruginosa*, and *S. aureus*.

Figure 9 indicates antibacterial activity of chitosan-Ag nanoparticle material synthesized with 0.04 M $AgNO_3$. MBC of chitosan-Ag nanoparticle material is between 100 and 200 ppm against *S. pneumonia,* but a little growth appeared on agar plate with 100 ppm of chitosan-Ag nanoparticle material. MBC is between 50 and 100 ppm against *E. coli, A. baumannii, S. aureus,* and *E. faecalis* bacteria, but very little growth appeared on Chromo agar plate with 50 ppm of chitosan-Ag nanoparticle material towards *E. coli* and *E. faecalis*. MBC is between 25 and 50 ppm against *P. aeruginosa*.

Figure 10 shows antibacterial activity of chitosan-Ag nanoparticle materials synthesized with 0.06 M $AgNO_3$. MBC of chitosan-Ag nanoparticle material is between 50 and 100 ppm against *S. pneumonia* and *E. coli* and between 25 and 50 ppm against *E. faecalis, A. baumannii,* and *S. aureus*. *E. coli* was grown a little on Chromo agar plate with 50 ppm of chitosan-Ag nanoparticle material in comparison with 25 ppm of chitosan-Ag nanoparticle material and a little growth appeared on Chromo agar plate with 25 ppm of chitosan-Ag nanoparticle materials towards *S. aureus*. There was no growth for *P. aeruginosa* on Chromo agar plate.

Antibacterial effect of chitosan-Ag nanoparticle materials increased with increasing $AgNO_3$ concentration.

The antibacterial effect of pure chitosan was also investigated and shown in Figure 11. The results showed that MBC is between 200 and 400 ppm of chitosan against *E. faecalis* and *S. pneumoniae* and MBC is bigger than 400 ppm towards *E. coli, A. baumannii, S. aureus,* and *P. aeruginosa*.

4. Conclusion

The antibacterial properties of chitosan-Ag nanoparticle composite materials and the effects of Ag concentration of composite materials on the structure and the antibacterial effectiveness were investigated against three Gram-positive and three Gram-negative bacteria. Antibacterial effect of chitosan-Ag nanoparticle materials increased with increasing Ag concentration of the composite material. The results of this study also suggested that the presence of a small percentage of Ag nanoparticles in the composite was enough to enhance antibacterial activity significantly towards both Gram-negative and Gram-positive bacteria as compared with pure chitosan.

Conflict of Interests

None of the authors have any conflict of interests regarding the publication of this paper.

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