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Enhanced antibacterial and anti-biofilm activities of biosynthesized silver nanoparticles against pathogenic bacteria

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Abstract

The nano-sciences and nanotechnology have brought to fore the nanosized inorganic and organic particles, which are finding the increasing of applications as amendments in industrial, medicine and therapeutics. Sixty isolates of *E. coli* collected from patients in Baghdad. The source of isolates distributed as; 16 (26.6%) isolates from urinary tract infection, 16(26.6%) isolates from stool, 10(16.6 %) isolates from blood, 10(16.6%) isolates from burn patients and 8(13.3%) isolates from wound. The color of the reaction mixture of *E. coli* culture supernatant with silver nitrate and 10mM glucose, changes from light yellow to dark brown after 24hrs of incubation. UV-Vis spectra of Ag NPS showed the peak of spectra at 403.43nm with value about 0.92, while SEM analysis showed particles being mostly spherical in shape, and the size of Ag-Nps ranged from (14.2 to 67.8)nm in diameter with a mean 34.8 nm. The antibacterial effect of Ag-Nps show a higher antibacterial effect against *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* and *Staph. aureus* at concentration of 10µm with inhibition zone of 18, 14, 18 and 16µm, respectively. The effect of Ag-Nps on biofilm formed by pathogenic bacteria showed pink colonies in the presence of Ag-Nps indicating a loss of biofilm formation ability, these isolates included K. pneumonia, *Pseudumonas*, *E. coli* and *Staph. aureus*.

Keywords: Biosynthesis, Ag-Nps, *E. coli*, SEM, UV-Vis, Antibacterial, Antibiofilm.

Introduction

Nanotechnology refers broadly to the synthesis of nanoparticles and the analysis of their applicability in the physical, chemical, and biological fields (Kogan *et al.,* 2007). Nanoparticles have been well applied in the medical, clinical, and biological fields as biosensors, for photo imaging, genes and drugs delivery vehicles, and photo thermal therapy, etc. (Mody *et al.,* 2010).

Silver nanoparticles have attracted significant interest among the emerging nano-products because of their unique properties and increasing use for various applications in nano medicine (Gurunathan *et al.,* 2014). Biological synthesis of nanoparticles is thus an effective and economic approach, which even can control the size and shape of the nanoparticles (Sharma *et al.,* 2015).

The increasing prevalence of microbial resistance has made the management of public health an important issue in the modern world. Although several new antibiotics developed in the last few decades, none has improved activity against multidrug-resistant bacteria. Therefore, it is important to develop alternate and more effective therapeutic strategies to treat Gram negative and Gram-positive pathogens (Mohanty *et al.,* 2012).

Previous studies have investigated the antibacterial effect of Ag-NPs on both Gram-positive and gram- negative bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Rai *et al.,* 2012)

Antimicrobial action of silver illustrating three major pathways: (a) attachment to cell membrane, (b) absorption or trans membrane diffusion into the cell, (c) coagulation and denaturation of bacterial enzymes and proteins (Lansdown, 2010)

In the current search, we have targeted our effort to synthesis Ag-NPs from supernatant of *E. coli* bacteria and inhibited microbial biofilm formation, so that the extent of pathogenesis can controlled.

Materials and Methods

Isolation and characterization of bacterial isolates: Samples collected from different sources (urine, stool, blood, burns and wounds) from patients of many hospitals in Baghdad included: (Al-Zahra, Al-Kendy, Al-Karh and Teaching laboratory in Medical City). These samples isolated

and identified on the cultures of MacConkey, nutrient and blood agar, the results read after 24h of incubation at 37°C.

Morphological characterization (colony color, shape and size) recorded on media and biochemical tests (catalase test, indole production, methyl red, oxidase test, voges proskauer, and citrate production) were tested according to (Forbes *et al.,* 2007). Conventional API 20E system and Vitek2 system used to confirm the characterization of isolates.

Preparation of bacterial supernatant: The bacterial strain cultured in nutrient broth, and incubation in rotary shaker overnight at 37°C. After 24hrs, the supernatant collected and centrifuged at 10,000rpm for 10min, then the supernatant collected for synthesis of silver nanoparticles (Behera *et al.,* 2013).

Synthesis of silver nanoparticles: Ten milliliter (10ml) of supernatant mixed with 10ml of silver nitrate ($AgNO_3$) solution to each concentration (1, 5 and10) mM, and another tube with reaction mixture without silver nitrate served as control. pH of reaction was (8.5), the solution were incubation at 30°C for 24hrs and kept in dark to avoid any reaction during testing. After 24hrs the solution change to brown from yellow solution and indicted the synthesis of nanoparticles, the silver nanoparticle ($AgNO_3$) purified by centrifugation at 10.000 rpm for 5min twice, and collected for further characterization (Chaudhari *et al.,* 2012).

Synthesis of silver nanoparticles using culture supernatant of *E. coli* long with glucose: Bacterial supernatant with each different concentration (1, 5 and 10) mM of $AgNO_3$, and 1mM glucose was mixed in 1:1:1. The result solution kept in rotary shaker (200rpm) at 37°C for 24hrs (Chaudhari *et al.,* 2012).

Nanoparticles characterization: Identification of silver nanoparticles done in Nano center in Technology University by using the following Methods:

Scanning electron microscopy (SEM): A scanning electron microscope model (TESCAN-VEGA/USA) with resolution 3nm at 30 kV takes Ag Nps. images. The assembly attached with a computer software programming to analyze the mean size of the particles in sample.

UV-VIS spectroscopy: UV-Visible Spectrophotometer (Metertech SP 8001), in the range of 300-800nm, were used to study optical properties.

Particle size distribution analysis: Zeta plus particle sizing software (Version 5.34), used to determine Ag-nanoparticles size diameter, zetapoteintial, motility and frequency.

Determination of antimicrobial activity by agar well diffusion: Antimicrobial activity of the synthesized silver nanoparticles tested against different pathogenic bacteria isolates (*E. coli*, *Staph. aureus*, *Pseudomonas aeruginosa* and *Kebsiella*) which resistant to all kinds of antibiotics applied, and determined by modified Kirby-Bauer disc diffusion method. In brief, bacterial culture was prepared by spreading 100µL of bacterial suspension (10^6 CFU/mL of each test organism) on solid Muller Hinton agar. The plates allowed standing for 10-15min to allow culture absorption. The 8mm size wells punched into the agar with the head of sterile micropipette tips. The wells in each plate loaded with 100µL of different concentrations (1, 5 and 10)mM of nanoparticles suspension. After incubation at 37°C for 24hrs, the size of the inhibition zone around each well measured (Joa *et al.,* 2015).

Biofilm production by congo red method: Single colony for *E. coli* cultured on congo red agar plates and incubated aerobically for 24-48hrs at 37°C. Positive result indicated by black colonies with a dry crystalline consistency (Nivedith *et al.,* 2012).

Effect of silver nanoparticles on biofilm formation: One ml of silver nanoparticles biosynthesized by *E. coli*, with different concentration (1, 5 and 10)mM added to Congo red agar medium, left in room temperature to dry completely, then plates were inoculated with clinical isolates, and Incubated aerobically for 24 to 48hrs at 37C^{o} (Blanco *et al.,* 2005).

Result and Discussion

Sixty isolates of *E. coli* collected from patients in Baghdad (Figure 1) summarized type of specimens, numbers and percentages of these isolates. The source of isolates distributed as; 16(26.6%) isolates from urinary tract infection, 16(26.6%) isolates from stool, 10(16.6 %) isolates from blood, 10(16.6%) isolates from burn patients, and 8(13.3%) isolates from wound.

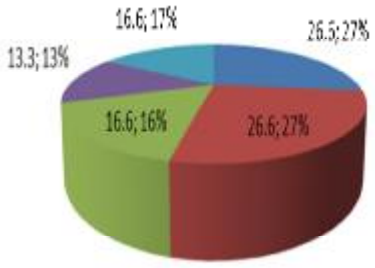

Figure (1): Distribution numbers of *E. coli* isolates according to the source of samples

Synthesis of silver nano particles using culture supernatant of *E. coli*: The color of the reaction

mixture of E. coli culture supernatant with aqueous solution of silver nitrate (conc.1, 5 and10mM), changes from light yellow to brown after 24h of incubation, this confirmation the synthesis of nanoparticles in the medium. Control mixture (without silver nitrate) showed no color change (Figure 2). The color increased with period of incubation due to the reduction in Ag^{o} and accumulation of Ag+ ions in the supernatant (Mahanty *et al.,* 2013).

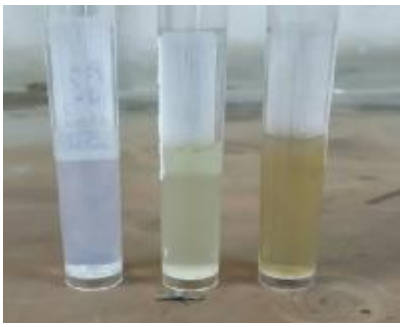

Figure (2): Synthesis of silver nanoparticles by supernatant of E. coli

Synthesis of silver nanoparticles using culture supernatant of E .coli long with glucose: The color of the reaction mixture of *E. coli* culture supernatant with aqueous solution of silver nitrate (Conc.1, 5 and 10)mM; and 10mM glucose (V 1:1:4), changes from light yellow to dark brown after 24hrs of incubation confirmation the synthesis of nanoparticles in the medium. Control (without silver nitrate) showed no color (Figure 3).

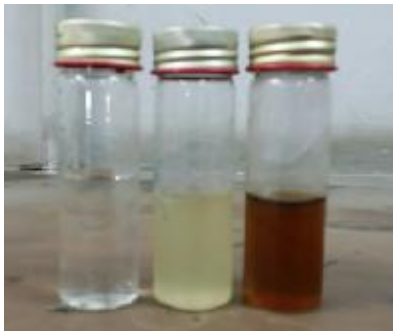

Figure (3): Synthesis of silver nanoparticles by supernatant of *E. coli* with glucose

The strong color change suggested that the synthesis of silver nanoparticles could be better in addition of glucose (Chaudhari *et al.,* 2012; Masurkar *et al.,* 2012) Glucose used as reducing agent because of the encapsulation effect of glucose and trapping the Ag-NPs inside in glucose (Kushwahal *et al.,* 2015). The result of Peng *et al.* 2016) suggest that elevation initial glucose concentration exposed more aldehyde group to electro statically interact with Ag+ which resulted in high reduction rate Ag+ to Ag° by glucose.

Characterization of synthesized silver nanoparticles:

UV-Vis spectroscopy Analysis: Figure (4) shows the UV-Vis spectra of silver nanoparticles synthesized by E. coli. The peak of the above spectra found at 403.43nm with peak value about 0.92, and this peak is due to surface plasmon resonance (SPR) property of silver nanoparticles.

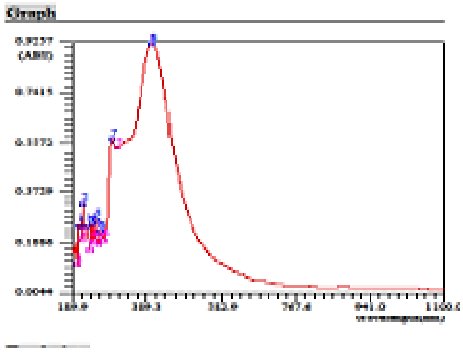

Figure (4): UV-Vis spectra of silver nanoparticles synthesized by *E. coli*

Various reports have established that the resonance peak of silver nanoparticles appears around this region. Samples exposed to the silver nitrate solution shows the wide spectrum range around 400nm. The presence of the broad resonance indicates the aggregation of the silver nanoparticles in the solution (Sunkar and Nachiya, 2012). Ramalingam *et al.* (2013) proved strong and broad surface plasmon peak observed between 415 and 425nm for silver nanoparticles intensity of the peak indicates the narrow size distribution of AgNPs in supernatant of *Pseudomonas aeruginosa*. While (Dosh, 2013) shows the UV-Vis spectra of silver nanoparticles synthesized by *B. thuringiensis*, and peak found at 421nm. (Kulkarni *et al.,* 2015) Represents the UV/vis spectrum of culture supernatant of *Deinococcus radiodurans* containing AgNPs indicating the surface Plasmon resonance centered at approximately 426nm, confirming the extracellular accumulation of AgNPs in the solution.

SEM analysis of the nonmaterial: The morphological features of synthesized silver nanoparticles studied by SEM analysis shown in Figure (5). SEM analysis showed less aggregation of Ag-NPs with particles being mostly spherical in shape. The average size is 34.8nm. The SEM analysis showed that the synthesized AgNPs were spherical in shape for *E. coli* and other types of bacteria *Pseudomonas aeruginosa* (Ramalingam *et al.,* 2013), *B. thuringiensis* (Dosh, 2013), and *Deinococcus radiodurans* (Kulkarni *et al.,* 2015).

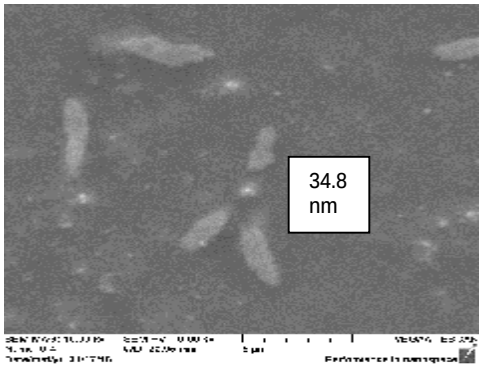

Figure (5): SEM image of silver nanoparticles synthesize by *E. coli*

Particle size analysis: The size of Ag-nanoparticles ranged from (14.2 to 67.8)nm in diameter with a mean diameter is 34.8nm (Figure 6). The size of Ag-NPs synthesized by *Bacillus thuringensis* (45–100nm), *Deinococcus radiodurans* (25–46nm), and *P. aeruginosa* (13–76nm) could be attributed to different cell growth and metal incubation conditions (Dosh, 2013; Kulkarni *et al.,* 2015; Ramalingam *et al.,* 2013).

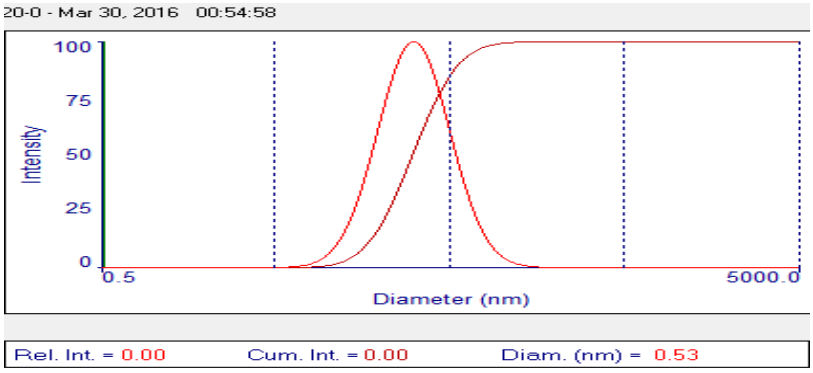

Figure (6): Particle size histogram of silver nanoparticles synthesize by *E. coli*

Antibacterial activity of silver nanoparticles by agar well diffusion method: Figure (7) shows a clear inhibition zone treated with Ag nanoparticles against *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia* and *Staph. aureus*. There are no antibacterial activity against *Staph. aureus* and klebsiella pneumonia at concentration of (1µM), while show antibacterial effect against *Pseudmanus*, and *E. coli* with inhibition zone of 10mm, and 11mm respectively. It show antibacterial effect against *E. coli*, *P. aeruginosa*, *Klebsiella pneumonia* and *Staph. aureus* at concentration of 5µm, with inhibition zone of 11, 12, 14 and 11mm, respectively, while show a higher antibacterial effect against *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* and *Staph. aureus* at concentration of 10µm with inhibition zone of 18, 14, 18 and 16µm, respectively.

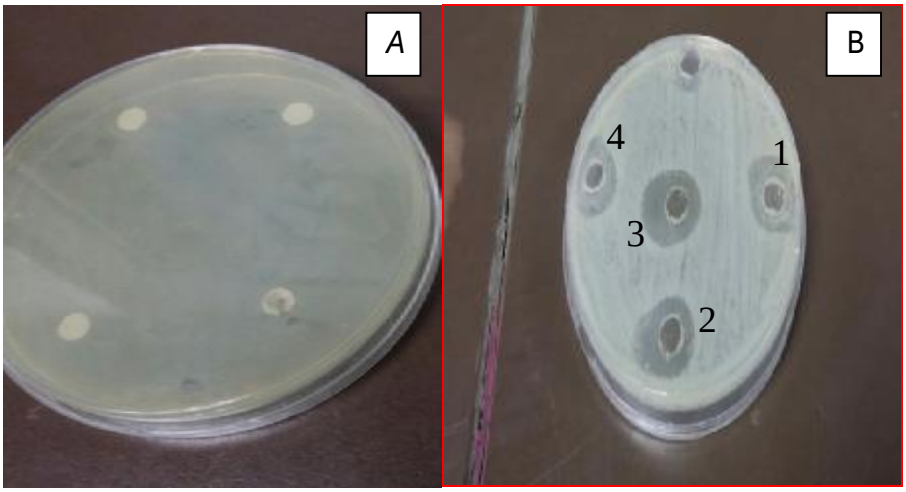

Figure (7): Antibacterial activity of Ag-NPs, A: control, B: inhibition zones of bacterial isolates of 1- *Staph. aureus*, 2- *Klebsiella pneumonia*, 3- *P. aeruginosa*, 4-*E. coli* .

Several mechanisms proposed to explain the inhibitory effect of silver nanoparticles on bacteria. This metal has a broad antimicrobial activity spectrum against both gram-positive and gram-negative bacteria. The activity of silver nanoparticles studied against multiple drug resistant (Chaudhari *et al.,* 2012, Franci *et al.,* 2015). The bactericidal activity of silver nanoparticles is due to their small size and large surface area to volume ratio (Thomas *et al.,* 2014).
Determination of biofilm formation by congo red agar (CRA) method: Results showed that biofilm formed by gram positive bacteria included (3) isolates of *Staph. aureus*, while gram-negative bacteria (5) isolates of *E. coli*, (3) isolates of *K. pneumonia* e, (4) isolates *Psudomanus aeruginusa* by Congo red agar method (Figure 8). Black color colonies inducted for the biofilm formation, while the weak slime producers usually remained pink, though an occasional darkening at the centers of the colonies observed. A darkening of the colonies, with the absence of a dry crystalline colonial morphology indicated an indeterminate result (Thuptimdang *et al.,* 2015).

The congo red dye used directly to recognize the production of exopolysaccharide, which is the necessary requirement for biofilm formation, so CRA test is simple and fast (Wagner and Iglewski, 2008).

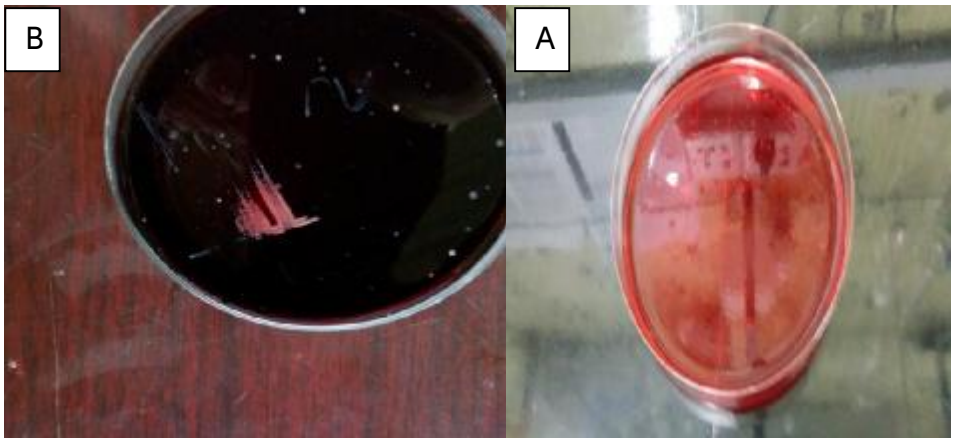

Figure (8): Biofilm activity of *E. coli* on congo red agar, A: CRA media, B: Biofilm

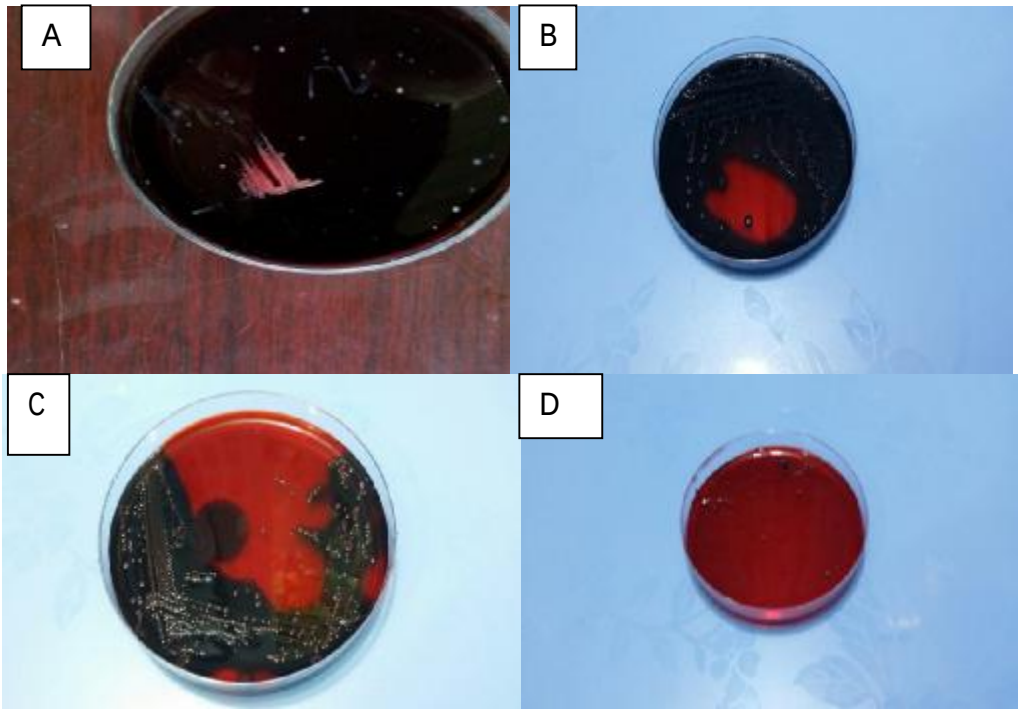

Figure (9): Anti-biofilm activity Ag-Nps synthesized by E. coli (A-biofilm formation, B- antibiofilm(1mM Ag-Nps), C- antibiofilm(5mM Ag-Nps), D- antibiofilm (10mM Ag-Nps)

Effect of Ag NPs on biofilm formation by congo red agar method: The effect of silver nanoparticles synthesized by *E. coli* on biofilm formed by pathogenic bacteria. Pink colonies in the presence of silver nanoparticles indicating a loss of biofilm formation ability, these isolates included *K. pneumonia*, *Pseudumonas*, *E. coli* and *Staph. aureus*, while black colonies formed by other isolates indicated no effect of silver nanoparticle (Ramalingam *et al.,* 2013).

Conclusions

The silver nanoparticles inhibited biofilm production by blocking the formation of exopolysacharides. The inhibitory effect of AgNPs on existing biofilm allocated to the presence of water channels in biofilm. The silver nanopartical move during the water channels used for nutrient moving and spread through exopolysacharide layer (Ramalingam *et al.,* 2013).

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