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Bactericidal Application of Biosynthesized Silver Nanoparticles

Hala Ezzat Abou El-Hassayeb*

Department of Marine Microbiology, National Institute of Oceanography and Fisheries, Alexandria, Egypt
**Corresponding author*

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A B S T R A C T

Silver bionanoparticles have been known to have inhibitory and bacterial effects. Apart from standardizing the best parameter for the synthesis of silver nanoparticles involved in reduction of silver ion to silver nanoparticles. The involvement of nitrate reductases as reducing agent was confirmed by biochemical assay. The nitrate reductase activity got reduced from 0.9876 mmole/min/ml to 0.3233 mmole/min/ml after biofabrication of silver nanoparticles. In the present study, *Pseudomonas aeruginosa* ATCC 9027 has been used for the biosynthesis of silver nanoparticles (SNPs). It was found that aqueous Ag^2 ions in solution when exposed to Pseudomonas aeruginosa ATCC 9027 get reduced, thereby leading to the formation of silver nanoparticles. The formation of silver nanoparticles was confirmed by the change in colour of the culture filtrate from yellow to brown after the addition of silver nitrate. The morphology and uniformity of silver nanoparticles were investigated by UV–Vis spectrum, X-ray diffraction and scanning electron microscope (TEM). These biosynthesized silver nanoparticles were also evaluated for their antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 2592 and *Escherichia coli* ATCC 8739. It was interesting to note that Pseudomonas aeruginosa ATCC 9027 that biosynthesized the silver nanoparticle was most affected by its antibacterial activity applications.

Introduction

Nowadays, nanotechnology has been developing rapidly as an important field of modern research with potential effects in medicine, agriculture, textiles, electronics, and energy, etc. Nanotechnology involves the development of efficient systems at the molecular scale. Nanotechnology is responsible for the synthesis and design of structures ranging in size between 1 and 100 nm (Rai *et al*., 2014). The synthesis of silver nanoparticles is an important aspect of nanotechnology. Presently, different techniques are available for the synthesis of silver nanoparticles, such as: chemical, physical, and biological. In the past decade, a great attention has been paid to microbial production of nanoparticles. Biosynthesis of silver nanoparticles (AgNPs) is an eco-friendly approach by using different biological sources; for example, plants and microorganisms such as bacteria, fungi, and actinobacteria (Tsibakhashvili *et al*., 2011; v

et al., 2013; Gupta *et al*., 2014; Rai *et al*., 2015). Outbreak of the infectious diseases is caused by different pathogenic bacteria and the development of antibiotic resistance the pharmaceutical companies and the researchers are searching for new antibacterial agents. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties Nanotechnology refers broadly to a field of applied science and technology whose unifying theme is the control of matter on the atomic and molecular scale.

The metal microbe interactions have an important role in several biotechnological applications including the fields of bioremediation, biomineralization, bioleaching, and microbial corrosion (Bruins *et al*., 2000. Recently a few microorganisms have been explored as potential biofactories for synthesis of metallic nanoparticles such as cadmium sulfide, gold, and silver (Sastry *et al*., 2003; Tillmann, 2004; Ahmad *et al*., 2005; Shah *et al*., 2012).Research in nanotechnology provides reliable, eco-friendly processes for the synthesis of nanoscale materials. Inspiration from nature comes through magnetotactic bacteria synthesizing magnetite nanoparticles, diatoms synthesizing siliceous materials and S-layer bacteria producing gypsum and calcium carbonate layers. Marcetol (Duran *et al*., 2003) showed that silver nanoparticles (SNPs), like their bulk counterpart, are an effective antimicrobial agent against various pathogenic microorganisms.

The present work has focused on the development of an extracellular biosynthesis of SNPs using *Pseudomonas aeruginosa* and characterization of these nanoparticles have been carried out by UV-Vis Spectroscopy, XRD and TEM techniques. Moreover, *in vitro* antimicrobial activity of AgNPs synthesized from *Pseudomonas aeruginosa* was evaluated against *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 2592 and *Escherichia coli* ATCC 8739.

Materials and Methods

Culturing the microbe

The bacterium *Pseudomonas aeruginosa* ATCC 9027 (Saitou *et al*., 1987) was obtained from Marine Microbiology department, National Institute of oceanography and fisheries, Alexandria, Egypt (NIOF). A loopful of *Pseudomonas aeruginosa* culture was inoculated in 250ml conical flask containing 100ml sterile Nutrient Broth. The inoculated medium was incubated at 37^0C in a rotary shaker at 120 rpm for 24 hours. After 24 hours, the culture was centrifuged to separate bacterial cells. Centrifugation was done at 5000 rpm for 10 minutes. Supernatant and pellet were separated. The supernatant obtained after centrifugation was used for nanoparticles synthesis.

Synthesis of Nanoparticles

Nutrient broth was prepared, sterilized and incubated with a fresh growth of test strain *Pseudomonas aeruginosa* ATCC 9027. The cultured flasks at 150 rpm. After the incubation period, the culture was centrifuged at 12,000 rpm for10 min and the supernatant was used for the synthesis of silver nanoparticles (AgNPs). The supernatant of *Pseudomonas aeruginosa* ATCC 9027 culture was separately added to the reaction vessels containing silver nitrate at a concentration of 0.1 g/L.

The reaction between these supernatant and silver ions was carried out in bright conditions for 72 h. The bioreduction of the silver ions in the solution was monitored by sampling the aqueous solution (2 mL) and

measuring the absorption spectrum of the solution using (Beckman-Du-50) UV-Visible spectrophoto-meter at a resolution of 1 nm.

Characterization of silver nanoparticles (AgNPs)

Visual detection

After 4 h of incubation, the preliminary detection of silver nanoparticles was carried out by visual observation of colour change from yellow to brown. The change in colour clearly indicates the formation of AgNPs.

UV-Vis Measurements

The optical density of these nanoparticles suspended in distilled water was measured by UV-Visible spectrophotometer (Systronics 2202 double beam model) from wavelengths 200-700nm.. The SPR peaks were assessed for size and distribution of Silver nanoparticles.

Transmission Electron Microscopic, TEM Examination of the Nanoparticle

A scanning electron microscope (HRTEM) was used to record the micrograph images of synthesized silver nanoparticles.

XRD Measurements

The formation and quality of nanoparticles were checked by XRD technique. X-ray Diffraction (XRD) measurements of drop-coated films of synthesized nanoparticles on glass substrate were recorded in a wide range of Bragg angles 2θ at a scanning rate of 20min-1, carried out on a Philips PW 1830 instrument that was operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation (λ=1.5405 Å).

Elimination of untreated Ag^+ ions

To eliminate the unreacted Ag^+ from the nanoparticle suspension, the reaction sample was treated with 1% NaCl solution. The precipitate of AgC l was removed by centrifugation at 4,500*g* for 15 min followed by filtration with Whatman filter paper No. 1. Then the AgNPs were centrifuged at 11,000*g* for 30 min and dried at 50 °C. The dried powder of AgNPs was used for antibacterial activity (200 μg ml^{-1}) and minimum inhibitory concentration (MIC) in a concentration range of 5–200 μg ml^{-1} after dissolving in deionized water.

Nitrate Reductase Assay

For extraction of Nitrate Reductase from *Pseudomonas aeruginosa*, the supernatant obtained from the above procedure was homogenized with Tris-HCl buffer (pH 8.0) and then centrifuged at 2000 rpm for 15 min. The supernatant was used as enzyme source. Nitrate Reductase activity was measured by Vega and Cardenas method. The standard graph was calibrated using 50 μM working standard of Sodium nitrite. To 0.1 ml supernatant known amount of 0.1 M KNO_3 was added and incubated for 24 hours. Then 1ml of diazo coupling reagent (1% Sulphanilamide in 3 ml HCl and 0.02% N-(1-naphthyl) ethylenediamine hydrochloride) was added to 3 ml reaction mixture and diluted 10 folds to detect the remaining NO_2. After 30 min of incubation in dark at 37^0C for development of color; O.D. was recorded at 540 nm. The result was calculated against the standard graph of nitrite.

Determination of antimicrobial activity by well-diffusion method

The SNPs synthesized from *Pseudomonas aeruginosa* ATCC 9027 were tested for

antimicrobial activity by well-diffusion method against pathogenic organisms such as *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 2592 and *Pseudomonas aeruginosa* ATCC 9027. The pure cultures of organisms were subcultured on Nutrient broth at 37°C on a rotary shaker at 200 rpm. Wells of 6-mm diameter were made on Nutrient agar plates using gel puncture. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, 20 µL (0.002 mg) of the sample of nanoparticles solution was poured onto each of two wells and the third one as a control on all plates. After incubation at 37°C for 18 hours, the different levels of zone of inhibition were measured.

Results and Discussion

Characterization of Synthesized Silver Nanoparticles (AgNPs)

In the present study, the biosynthesis of silver nanoparticles by *Pseudomonas aeruginosa* ATCC 9027 was studied. Visual observations showed a change of colour in silver nitrate solution from yellow to brown(Fig. 1), whereas no colour change was observed in the culture supernatant without silver nitrate or in media with silver nitrate alone. The appearance of of yellowish brown colour in silver nitrate treated culture supernatant suggested the formation of silver nanoparticles (Sastry *et al*., 2003; Vighneshwaan *et al*., 2000; Konish *et al*., 2004).

The synthesized AgNPs were characterized by UV-Vis spectroscopy (Tillmann *et al*., 2004). In the UV-Vis absorption spectrum, the absorbance scan taken by UV-VIS spectrophotometer showed a sharp plasmon peak at ~ 435 nm confirming the presence of silver (Figure 2). Observation of this peak, assigned to a surface Plasmon, is well documented for various metal nanoparticles with sizes ranging from 2-100 nm.

X- ray diffraction (XRD) was carried out to confirm the crystalline nature of the particles and the XRD pattern obtained is shown in Figure 3. A comparison of the XRD data with the standard (joint committee in powder diffraction standards file no:040783)confirmed that the particles formed in our experiments were silver nanocrystals which can be depicted by the peaks at 2θ values of 38.45 ,44.48,64,69 and 77.62 corresponding to (111), (200), (220), and (311) planes for silver respectively. This XRD pattern confirms the crystallinity of SNPs. The mean particle diameters of SNP were calculated from the XRD data which can be derived by Debye Scherrer equation.

$$D = K \lambda / \beta ½ \cos\theta$$

This equation exploits the reference peak width at angle θ, where λ is the x-ray wavelength (1.5418), β ½ is the width of the XRD peak at half height and K is the shape factor. The calculated average particle size of SNP was 20-50 nm which can also be confirmed by TEM results.

Transmission electron microscopy (TEM) images of nanoparticles that were synthesized by *Pseudomonas aeruginosa* indicated that the nanoparticles were in the size range of 10 to 20nm (Figure 4) which is in close agreement with the particle size calculated from the XRD profile.

Nitrate Reductase Assay

Studies have indicated that NADH and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles. *Pseudomonas aeruginosa* is known to secrete the cofactor NADH and NADH-dependent enzymes,

especially nitrate reductase, which may be acting as a scaffold or nucleating agent and might be responsible for the bioreduction of Ag+ to Ag^0 and the subsequent formation of Silver nanoparticles. The same enzyme later then acts as a capping agent, thus ensuring complete formation of thermodynamically stable nanostructures. The molecular activity of nitrate reductase in the bacterial exudates of *P.aeruginosa* was found to be 0.9876 mmole/min/ml; which got reduced to 0.6546 mmole/min/ml when it was subjected to 100^0C (Fig 5). After the formation of silver nanoparticles the nitrate reductase activity was again assayed in the reactant mixture which showed a substantial decrease with 0.3233µmole/min/ml in the solutions (bacterial exudates) having Silver nanoparticles as compared to nitrate reductase activity in bacterial exudates without silver nanoparticles. This result confirms the involvement of nitrate reductase in the reduction of silver ion to silver nano particles. The fact that which capping proteins are involved in stabilizing the particle is yet to be explored.

Anti-microbial assay

The antibiotic activity of SNPs was investigated against various pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using well-diffusion method (Fig 6). The mean of three replicates of the diameter of inhibition zones (in millimeters) around each well with SNPs solution is represented in (Table1). The highest antimicrobial activity was observed against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* and *Escherichia coli*.

Table.1 Zone of inhibition of SNPs against various pathogenic bacteria Pathogenic bacteria Diameter of Zone of inhibition (mean of 4 replicates)

Pathogenic bacteria	Diameters of zone of inhibition (mean of 3 replicates)
Pseudomonas aeruginosa	24mm
Staphylococcus aureus	18mm
Escherichia coli	15mm

Fig.1 Conical flasks containing *Pseudomonas aeruginosa* culture supernatant in aqueous AgNO3 solution: (A) At the beginning of reaction showing no colour change;&(B) After 72 h of reaction showing brown colour.

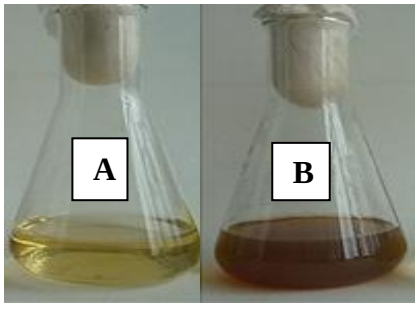


Fig.2 UV-Vis absorbance of AgNPs

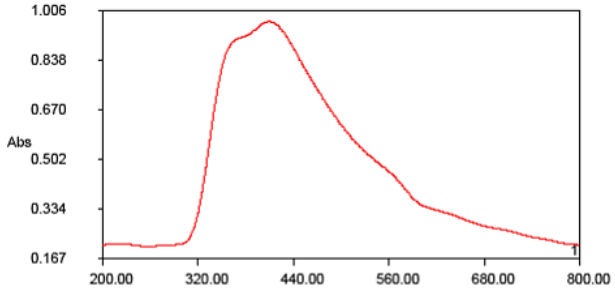


Fig.3 XRD pattern of silver nano particles synthesized using bacterial exudates of *Pseudomonas aeruginosa*

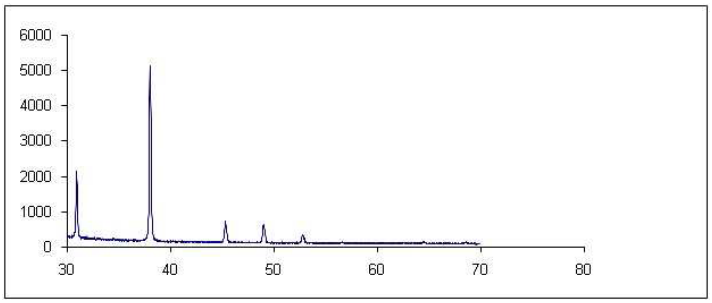


Fig.4 TEM image of Silver nanoparticles synthesized using *Pseudomonas aeruginosa*

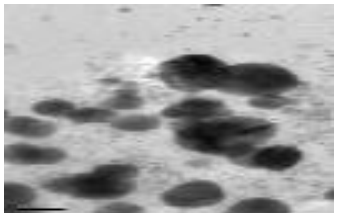
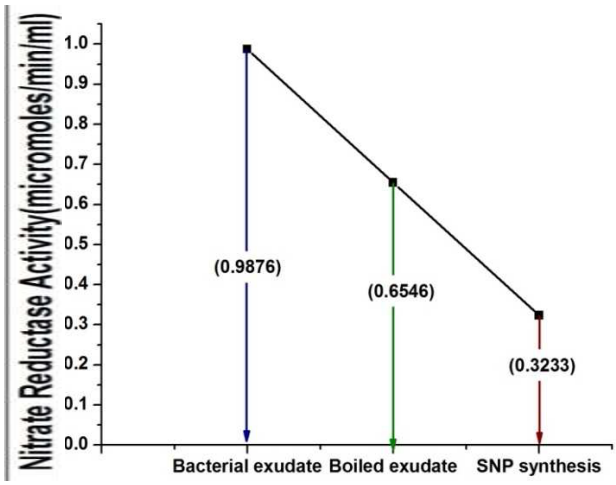
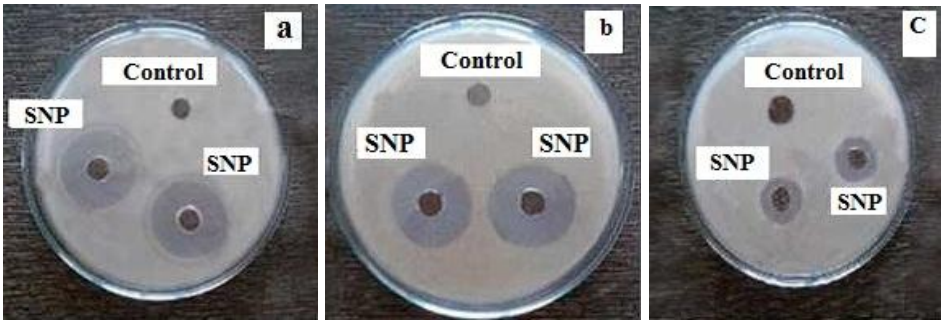


Fig.5 Nitrate reductase activity of *Pseudomonas aeruginosa* exudates, Boiled bacterial exudates and silver nanoparticles respectively in µmoles/min/ml



Pseudomonas aeruginosa

Fig.6 Antimicrobial activity of silver nanoparticles against various pathogenic bacterial strains. (a)*Pseudomonas aeruginosa* (b)*Staphylococcus aureus* & (c) *Escherichia coli* shown by well-diffusion method.



In the present study, biosynthesis of AgNPs by *Pseudomonas aeruginosa* was observed by the colour change of culture filtrate from yellow to brown, after addition of 1 mM solution of silver nitrate. The change in colour clearly indicated that there was formation of AgNPs in the reaction mixture (Shanmugaiah *et al*., 2015). The UV-Vis spectrophotometer analysis revealed that the absorbance peaks at ~ 435 nm, which are very specific for the synthesis of AgNPs. The XRD pattern thus obtained clearly shows (111), (200), (220), and (311) planes, and exhibit that the synthesized AgNPs by the *Pseudomonas aeruginosa* were crystalline in nature (Konish *et al*., 2004).

The results of TEM analysis clearly showed that bacteria have the potential to form smaller size particles with higher surface area. (Sukanya *et al*., 2013; Manivasagan *et al*., 2013; Birla *et al*., 2009). Antibacterial activity of biogenically synthesized AgNPs was evaluated against various pathogenic organisms such as *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* using well-diffusion method. The AgNPs synthesized from *Pseudomonas aeruginosa* strain showed the highest antibacterial activity against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* and *Escherichia coli* (Birla *et al*., 2009). The thrust to develop eco-friendly procedures for the production of nanoparticles arises from the extremely recent nanotechnology research. Extracellular biosynthesis of silver nanoparticles was achieved by an easy biological procedure using *Pseudomonas aeruginosa* as the reducing agent. The method exploits a cheap and easily available biomaterial not explored so far for the synthesis of metallic nanoparticles.

In conclusion, biological synthesis of metal nanoparticles using bacteria is a reliable and with ecofriendly protocol Bacteria. The mechanism of synthesis of metal nanoparticles by microbes is not clearly explored. *Pseudomonas aeruginosa* was capable of producing silver nanoparticles extracellularly and were quite stable in the solution .Applications of such eco-friendly nanoparticles in bactericidal applications, makes this method potentially exciting for the large-scale synthesis of other nanomaterials.

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