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# Biosynthesis of silver nanoparticles using *Stenotrophomonas rhizophila* and its application as a disinfectant agent of water

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## ABSTRACT

*This study aimed to examine the capability of Stenotrophomonas rhizophilastrain 39M isolated from lake water to reduce silver to silver nanoparticles. The production of silver nanoparticles by strain 39M was investigated by UV–visible spectroscopy, X-ray diffraction, transmission electron microscope, scanning electron microscopy and energy dispersive X-ray spectroscopy. The results of UV–visibledemonstrated a peak at 420 nm corresponding to the Plasmon absorbance of silver nanoparticles. SEM micrograph showed a formation of silver nanoparticles in the range of 5–50 nm. XRD-spectrumexhibited 2Θ values corresponding to the silver nanocrystal that produces in hexagonal and cubic crystal configurations with different plane of orientation. In addition, the signals of the silver atoms were observed by EDS-spectrum analysis that confirms the presence of silver nanoparticles. The results demonstrated that after 7 days of incubation culture broth produced the highest yield of silver nanoparticles followed by the cell- free supernatant. The antimicrobial activity of silver nanoparticles against Gram-positive and negative bacteria was assayed. In conclusion, the silver nanoparticles produced in the culture broth of strain M39 could be used successfully as an environmentally friendly disinfector of water and wastewater against pathogenic microorganisms.*

**Key words:** Nanosilver, *Stenotrophomonas*, SEM, TEM, XRD

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## INTRODUCTION

Nanotechnology is the technologies that developed and continues to develop milestones in the life of man[1]. The importance of nanotechnology appeared strongly in the field of metal reduction, which can happen in different ways such as physical or chemical synthesis but these ways entirety is expensive as well as it has a harmful environmental impact. So, scientists around the world were resorted to biological methods to create nanoparticles[2]. Synthesis of AgNPs biologically has opened the road for “greener synthesis” of nanoparticles and these have confirmed to be better than both physical and chemical methods due to their slower kinetics, they offer better manipulation and control over crystal growth and their stabilization [3].

Many nanomaterials have been developed such as copper, zinc, titanium magnesium, gold, alginate and silver[3, 4, 5]. However, silver nanoparticles (AgNPs) outperformed their counterparts in the ability to work as antimicrobial against bacteria, viruses, as well as other eukaryotic microorganisms.

There are many microorganisms, which recorded the ability to synthesize AgNPs, which have been applied to a wide range of healthcare products, such as burn dressings, scaffold, water purification systems, and medical devices [6]. Silver nanoparticles have been widely used as a novel therapeutic agent extending its use as antibacterial, antifungal, antiviral and anti-inflammatory agent [3].

Here we report a newly isolated bacterial strain of *Stenotrophomonas rhizophila* that form crystalline silver nanoparticles. The produced silver nanoparticles were characterized using UV–visible spectrometry, transmission electron microscope (TEM), scanning electron microscope (SEM) and X-ray diffraction (XRD) techniques, and also tested as disinfectant agent of water.

## MATERIALS AND METHODS

### 1.1. Sample collection andisolation of silver resistant bacteria

Water samples were collected from Mariout Lake (Alexandria governorate, Egypt) and stored in sterilized bottles in a refrigerator at 4°C during 2011until use. After that water samples were serially diluted in sterile 0.8% NaCl solution and cultured on nutrient agar containing; 2% agar, 1% peptone, 0.5% yeast extract, 0.5% beef extract and 1% NaCl, then supplemented with 3.5 Mm $AgNO_3$ and incubated for 4 days at 30°C. Colonies with dark brown color on the plate were evaluated as $AgNO_3$ resistant.

### 1.2. Classical and molecular identification of the silver resistant bacteria

Morphological examination of individual colonies including colony and cell characterization, Gram-staining test, shape, size and acid fast staining were carried out as described in Bergey's Manual of Systematic Bacteriology [7]. Molecular identification of the selected isolate was performed by the amplification of 16S rDNA with eubacterial universal primers 27F and 1492R[8]. Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with Ampli Taq DNA polymerase and an Applied Biosystems 373 DNA Sequencer (Perkin-Elmer, Foster City, Calif.). The sequence was analyzed using the checkchimera and the similarity rank programs of the ribosomal database project also analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences [9].

### 1.3. Characterization of silver nanoparticles

Different characterization techniques have been employed to identify bio-formed nano clusters of bacteria that loaded with nanosilver. In order to study the formation of silver nanoparticles, samples of 1 ml were withdrawn. To obtain cell-free supernatants, culture broths of the selected strains were centrifuged to remove cells by centrifugal separation (14000 g for 10 min). Subsequently, the absorbance of cell-free supernatants was measured at a resolution of 1 nm using UV–visible spectrophotometer (Labomed. model UV–Vis Double beam spectrophotometer).

To perform the X-ray diffraction assay, after 7 days of incubation, bacterial biomass was collected by centrifugation 10000 rpm for 10 min, dried in an oven at 60 °C for overnight. Subsequently, the powdered samples were analyzed using X-ray Diffractometer (Schimadzu-7000, USA). Through packing the dried samples into a flat aluminum sample holder, where the X-ray source was a rotating anode operating at 30 kV and 30 mA with a copper target. Data were collected between 108 and 808 in $2\theta$.

For SEM studies, samples were cast on glass slides and dried at ambient temperature, and then the glass slides were fixed on copper supports. The samples were covered with a thin layer of gold, by sputtering. The coated surface was examined using the scanning electron microscope SEM (JEOL JSM 6360LA, Japan) that operate at 20–30 kV in order to identify the morphology of the produced nanosilver on the bacteria and determine their mean grain sizes. The scanning electron microscope is equipped with an energy-dispersive spectroscopy (EDS) unit for qualitative and quantitative analyses and elemental mapping for the produced nanoclusters.

Transmission electron microscope TEM (JEOL JEM-1230, Japan) was utilized to confirms and proofs the previously detected morphology of nanosilver particles using SEM under an accelerating voltage of 100 kV, samples were prepared by placing a drop of hydrophobic nanosilver colloid or its aqueous coordinate on carbon-coated copper grids and dried at room temperature.

### 1.4. Antibacterial assay of produced Ag-NPs

Plate count method was used to compare the antibacterial activity of produced AgNPs. All the glassware, media and reagents used were sterilized in autoclave at 121°C for 20 min. For studying the antibacterial assays, *Pseudomonas aeruginosa* P1, *Salmonellatyphi* S1, *E. coli* E1 [10] and *Klebsiella* were used as a model test strains for Gram negative bacteria, and *Microbacterium* sp. strain Pla1,*Bacillus* sp. strain S5 [11]and *Lactobacillus* sp.were used as a

model test strains for Gram-positive bacteria [10-12]. Plates were poured with bacterial suspension after incubation with different concentration of nanosilver for 1 hour at 30°C. Control plates were maintained with bacterial strains only poured in plate. Then, plates were incubated at 37 °C for 24 h and the total plate count was counted. All the assays were carried out in triplicate. Experimental results were expressed as means standard deviation of three parallel measurements. This statistical analysis was carried out using the SPSS version 12 programmer.

In addition, TEM and SEM microscopy were used as a complementary technique to examine sections and morphology of the treated *E. coli* strain E1 (as a model of Gram negative bacteria) and *Bacillus* sp. as a model of Gram positive bacteria , using procedures for fixing and embedding sensitive biological samples, which are described elsewhere [13]. Prior to microscopic analysis, treated ($10^7$ CFU in 100 ml of LB supplemented with 50 µg/ml of silver nanoparticles for 1 h) bacterial cells were deposited on a Millipore filter and washed with deionized water.

## RESULTS AND DISCUSSION

### 1.5. Isolation and identification of AgNPs producing bacteria

The bacterial isolate 39M that gave the darkest brown color during screening assay was subjected for biochemical and molecular identification. It is well known that bacterial taxonomy was traditionally based on morphological and biochemical features. However, molecular techniques exhibit high sensitivity and specificity for identifying microorganisms and can be used for classifying microbial strains at diverse hierarchical taxonomic levels [12].Morphologically, the colonies of strain 39M were slightly curved rods and yellowish in colour and its biochemical characteristics are presented in Table 1. The homology search values of 16S rDNA of isolate 39M confirmed the biochemical characteristics and showed similarity >98% with *Stenotrophomonas rhizophila*. The Gen Bank accession number of isolate 39M isKJ396343, and it gives the name*Stenotrophomonas rhizophila* strain 39M.

**Table (1): Biochemical characterization of isolate 39M**

| Biochemical test | isolate 39M |
|---|---|
| Mannitol salt agar | -ve |
| Simmons citrate agar | +ve |
| Gelatin test | +ve |
| Lactose fermentation | +ve, gas |
| Eosin methelene blue agar | +ve |
| Lysine iron agar | +ve |
| Mackoncy agar | -ve |
| Peroxidase test | -ve |

It is known that members of the Gram-negative bacterial genus *Stenotrophomonas* were found in a wide variety of environments and geographical regions, as well as associated with humans as a nosocomial pathogen [14]. Out of this genus, species *Stenotrophomonas maltophilia* was used as a living factory for synthesis of gold and silver nanoparticles[13, 15]. However, *Stenotrophomonas maltophilia* is a human pathogen and its infection is difficult to treat [16]. In contrast, the species *S. rhizophila* includes non-pathogenic strains isolated from the rhizosphere of plants [17]. Interestingly, our knowledge this the first report about the use of a strain belonging to the species *S. rhizophila* to produce AgNPs. Exploring the work to test strain M39 as a potential plant growth promoter is currently underway in our lab.

### 1.6. Characterization of silver nanoparticles

The production of silver nanoparticles can determine by using UV-visible spectrophotometry. Figure (1) shows the UV–Visible spectra of silver nanoparticles after 7 days of incubation in the range 250–650 nm. A typical silver nanoparticles absorption band in the visible region between 350 and 460 nm was showed an absorbance peak around 420 nm which is characteristic of silver nanoparticles, due to its surface Plasmon resonance absorption band, this result shows the amount of produced silver nanoparticles[18-20].

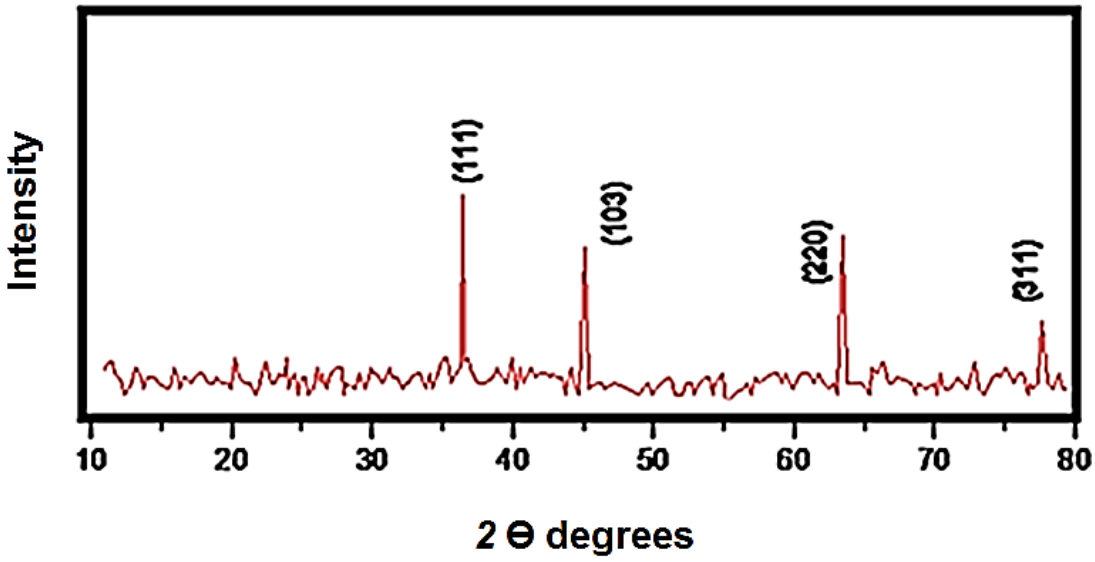


**Figure 1: UV–visible absorption spectrum of aqueous media containing silver ion (3.5 mM) and cell filtrate of strain 39M**

As shown in Figure 2,XRD measurements displayed that the diffraction pattern utilizing strain 39M recorded four strong diffraction peaks [38.11° (111), 45.2° (103), 64.42° (220), and 77.47° (311)] that can be assigned to both hexagonal and face-centered-cubic phases of Ag (JCPDS 00-004-0783 and No. 00-041-1402) [21-23]. However, this result predicts that strain 39M has the ability to produce silver in biphasic structures, where the crystal structure of produced metallic silver has hexagonal and cubic crystal configurations, and the cubic silver phase is the most demonstrated phase. A few weak additional and yet unassigned peaks were also noticed in vicinity of the characteristic peaks of silver. These blunt Bragg peaks might have resulted from some bioorganic compounds/proteins present in the media broth. The intensity of the Bragg reflections suggests strong X-ray scattering centers in the crystalline phase and could possibly arise from metalloproteins in the broth [24].

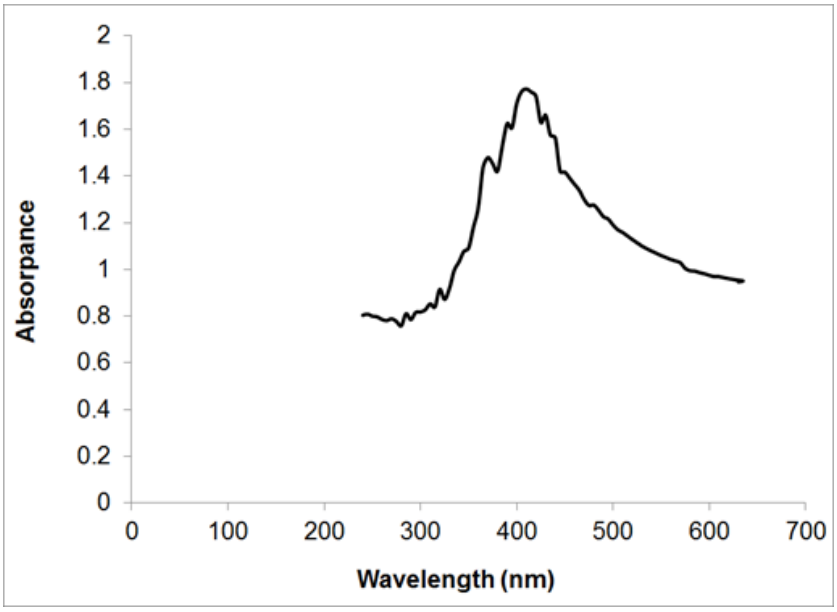


**Figure 2: XRD patterns for the produced by strain 39M**

The SEM image illustrated in Figure 3 shows Ag-NPs in a size range from 15 to 50 nm. Ag-NPs appeared adhesive in the body of the bacterial cell and take the form of one size and tight. These results were consistent with [13,25]. Furthermore, TEM technique was employed to visualize the size and shape of the formed Ag-NPs. As shown in Figure 4A, TEM image shows the Ag-NPsas very small pieces of bark, these nanoparticles are roughly circular in shape with smooth edges [26]. On the other hand, in spite of the SEM images illustrate the surface distribution of

Ag-NPs on the surface of bacterial cells; however they do not mean that all nanoparticles are bound to the cell surfaces, because those dispersing in the solution may also deposit onto the surface of the cells during the drying process which is a necessary step before SEM observation. The extracellular formation of Ag-NPs was predicted from the TEM images, where Figure 4A suggests that the Ag-NPs are free. Nevertheless, the possible mechanism for the nanoparticles biosynthesis has been reported by Ahmad et al. They suggested the possible role of NADH-dependent nitrate reductase in the reduction of silver ion to metallic silver. However, the lack of fundamental understanding of the underlying mechanisms of biologically synthesis of nanoparticles is currently an issue. It is both desirable and foreseeable that a great deal of effort will be put towards the elucidation of the synthesis mechanism. To this extent, a systematic investigation of the role that each component of the biological synthesis has in NPs synthesis would be an important undertaking, in order to provide a set of rules that will assist in the synthesis of NPs inFigure4Bshow cross section of strain 39Mand it show accumulating of Ag-NPs in the body of bacterial cell. It is indicate also that Ag-NPs may be formed outside the cell and penetrated bacteria after formation or Ag-NPs formed inside the cell and split cell wall and then went out to the outside [27].

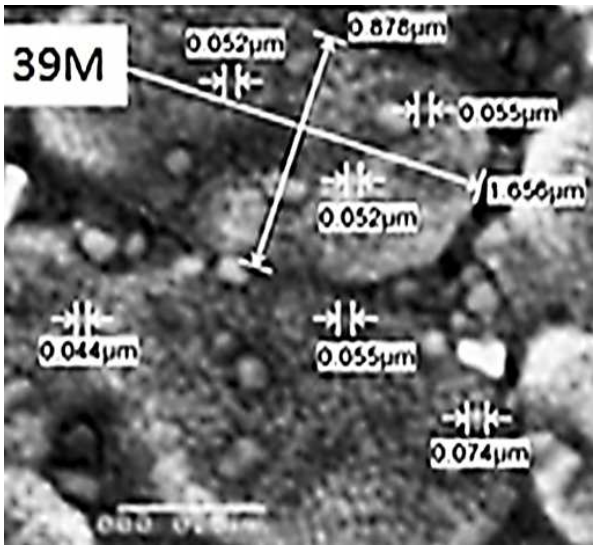


**Figure 3: Scanning electron micrograph of strain 39M**

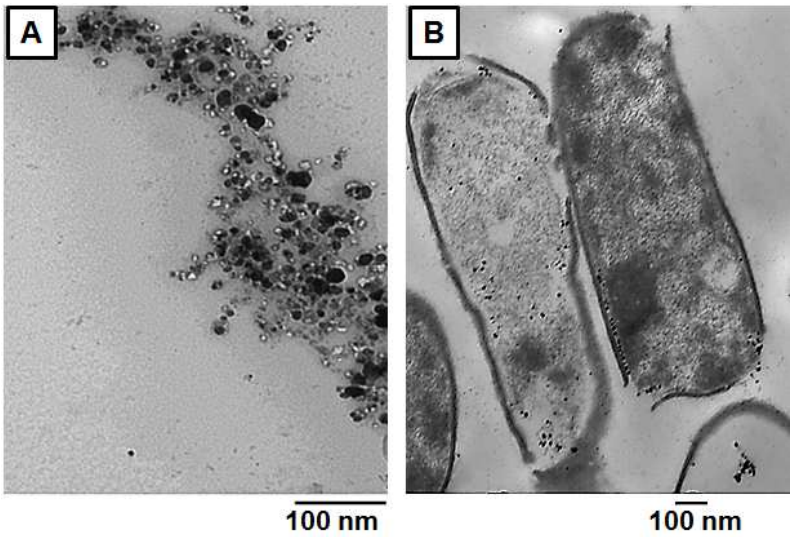


**Figure 4: Transmission electron microscope image of Ag-NPs in culture broth (A), cross sections of strain 39M grown in 3.5 Mm $AgNO_3$ (B)**

Energy dispersive X-ray spectroscopy (EDS) illustrates the patterns of the produced Ag-NPs. The signals from the silver atoms are observed with strain 39M which confirm the presence of Ag-NPs. The recorded signals of C, O and Na atoms are may be due to X-ray emission from the organism and remaining media in agreement with the XRD observation. As presented in Table 2, the atomic percentage of strain also supports this deduction.

**Table 2: EDS analysis of nanosilver produced by strain 39M**

| Elements (%atomic) | | | | |
|---|---|---|---|---|
| Ag | C | N | O | Na |
| 9.87 | 39.14 | 12.41 | 36.55 | 2.1 |

### 1.7. Antibacterial assays

The bacterial strains, *E.coli*, *Salmonella* sp., *Mycobacterium* sp., *Lactobacillus* sp., *Klebsiella* sp, *Pseudomonas aeruginosa* and *bacillus* sp. as a model of Gram negative and Gram positive bacteria, were cultured in a 50 ml LB broth at 37°C for overnight. Different volume of this grown culture approximately $10^3$CFU were incubated again with 4 concentrations of Ag-NPs and incubated on 37°C four 1 hours. After that the culture was poured and speededon LB agar plates. The plates were incubated for 24 h at 37°C and the numbers of grown colonies were counted as illustrated in Table (3).

**Table 3: The antimicrobial effect of different concentrations from Ag-NPs with different pathogenic bacteria strains**

| Strain | Ag-NPs Concentrations (µl) | | | | |
|---|---|---|---|---|---|
| | Control | 10µl | 15 µl | 20 µl | 25 µl |
| *E.coli* | 3* 103 | 900 | 500 | 145 | 0 |
| *Bacillus* sp. | 1 * 103 | 310 | 201 | 122 | 0 |
| *Mycrobacterium* sp. | 2 * 103 | 80 | 54 | 26 | 0 |
| *Lactobacillus* sp. | 2.8 * 103 | 1095 | 967 | 10 | 0 |
| *Klebsiella* sp. | 2 * 103 | 0 | 0 | 0 | 0 |
| *Pseudomonas aeruginosa* | 8 * 103 | 350 | 201 | 96 | 0 |
| *Salmonella* sp. | 2 * 103 | 2* 103 | 1001 | 0 | 0 |

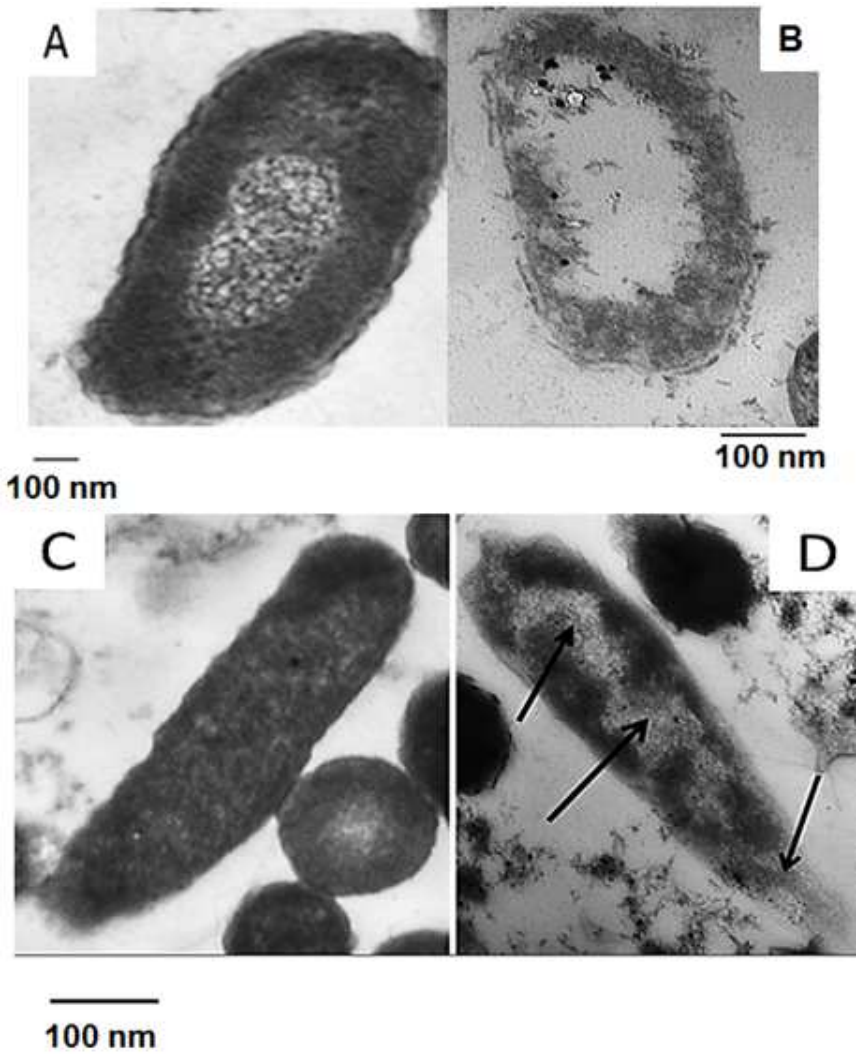


**Figure 5: Internal morphology of pathogenic bacteria observed by TEM. Untreated E.*coli* (A) treated *E.coli* (B) untreated *Bacillus* sp. (C) treated *bacillus* sp. (D)**

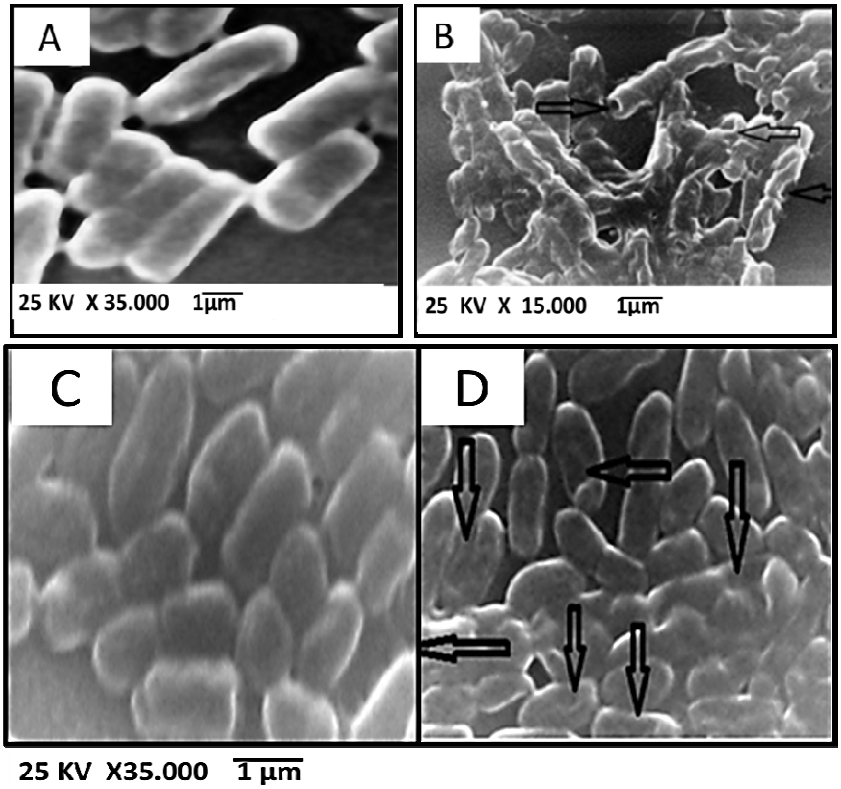


**Figure 6: External morphology of pathogenic bacteria observed by SEM. Untreated *E.coli* (a), treated *E.coli* (b), untreated *Bacillus* (c), treated *Bacillus* (d)**

As shown in Table 3, different action of Ag-NPs on gram negative and gram positive bacteria was observed due to the different peptidogly can layer thickness in both bacterial cell walls. The activity of Ag-NPs against pathogenic bacteria was depending on its concentration where, the Ag-NPs accumulated in membrane making pits in cell wall causing leakage of cytoplasm to outside as observed by TEM with both *E.coli* and *bacillus* sp. (Figure 5). However, previous studies reported that the mechanisms of action of Ag-NPs as antibacterial not yet completely understood. But there are three theories explain these mechanisms. Ag-NPs may release silver ion (Ag+) and it work against respiration of bacteria and make change in dissolved oxygen, Ag-NPs accumulate in bacterial cell and effect on cell permeability, or Ag-NPs direct attack and damage bacterial cell [28-31]. In addition Ag-NPs may be strap DNA inside bacterial cell [32].

TEM and SEM microscopy were used as a complementary technique to examine sections and morphology of the treated E.coli strain (as a model of Gram negative bacteria) and bacillus (as a model of Gram positive bacteria) using procedures for fixing and embedding sensitive biological samples, which are described elsewhere [6]. The action of Ag-NPs on the structure and morphology of pathogenic bacteria was observed. Where, TEM analyses in figure 5A show healthy cells of E.coli. However, Figure 5B showed E.coli cells treated with Ag-NPs and it was collapsed significantly, cell wall disappearance and Ag-NPs penetrate inside the cytoplasmic content [6, 31, 33]. Figure 5C display the untreated cells of bacillus sp. with normal structure and retain the content of cytoplasm and cell wall in good condition. But figure 5D show the disruption of cell wall of treated cells with Ag-NPs with a significantly leakage of cytoplasm out of cell one.

SEM analyses in Figure 6A show the untreated cells with Ag-NPs were regular with a typical rod shape. However, Figure 6B show that the surface of *E.coli* cells after treating by Ag-NPs were distorted and almost lost its original shape and turned from rod shape to unspecified shape with the appearance of holes in some of the cells as indicated by arrows [33-34]. Also Figure 6C show the normal shape and healthy cells of untreated *Bacillus* sp. But, Figure 6D shows that treated *Bacillus* cells with Ag-NPs were damaged and some cells have big pits.

## CONCLUSION

This work demonstrates the ability of *Stenotrophomonas rhizophila* strain 39M in converting silver macromolecules into silver nanoparticles. The synthesized silver nanoparticles were characterized by UV–Vis spectroscopy and confirmed by X-ray, TEM and SEM analysis. The nanoparticles formed by the isolate were found to be act as an

antimicrobial agent. So, Ag-NPS synthesized by strain 39M indicate that the rapid synthesis of nanoparticles would be suitable for developing a "green nanotechnology" biosynthesis process for mass scale production. To our knowledge, this the first report confirming the ability of a strain belonging to the species *Stenotrophomonas rhizophila* able to produce Ag-NPs.

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## REFERENCES

[1] Jannathul-Firdhouse M, Lalitha P, *J. pharm.Clinic.Res.*, **2013**, 6, 92.
[2] Chaudhari P, Masurkar S, Shidore V, Kamble S, *J. Appl. Pharm. Sci.*, **2012**, 2, 3, 25.
[3] Vaidyanathan R, Kalishwaralal K, Gopalram S, Gurunathan S, *Biotechnol. Advan.*, **2009**, 27, 924.
[4] Ninganagouda S, Rathod V, Jyoti D, Singh k, m-ul-haq, *Inter.J.Pharma. And Bio. Sci.*, **2013**,4, 2, 222.
[5] Das V, Thomas R, Rintu V, Soniya E, Mathew J, Radhakrishnan E, *3Biotech*, **2013**, DOI 10.1007/s13205-013-0130-8.
[6] Li W, Xi X, Shi Q, Zeng H, OU-Yang Y, Chen Y, *Appl.Microb. Cell Physiol*, **2010**, 85, 1115.
[7] Claus D, Berkeley R, Genus *Bacillus* Chon, In: Sneath P H A, Mair N S, Sharpe M E, Holt J G, editors; Sneath P H A, Mair N S, Sharpe M E, Holt J G, editors. *Bergey's manual of systematic bacteriology*, Baltimore, Md: The Williams & Wilkins Co.; **1986**, 2, pp 1105.
[8] Lane D, 16S/23S rRNA sequencing, In: Stackebrandt E, Goodfellow M (eds), *Nucleic Acid Techniques in Bacterial Systematics*, John Wiley & Sons: New York, NY, USA. **1991**,pp 115.
[9] Al-Thani R, Abd-El-Haleem D, Al-Shammri M, *Afr. J.Microbiol. Res.*, **2009**, 3, 761.
[10] Abd-El-Haleem D, Kheiralla Z, Zaki S, Rushdy A, Abd-El-Rahiem W, *J. Environ. Monit.*, **2003**, 5, 865.
[11] Abd-El-Haleem D, Moawad H, Zaki E, Zaki S, *Microb. Ecol*., **2002**, 43, 217.
[12] Abd-El-Haleem D, zaki S, J. *Microbiol. Biotechnol*., **2006**, 16, 1706.
[13] Zaki S, EL.Kady M, Abd-EL-Haleem D, *Mater. Res. Bullet.*, **2011**, 46, 1571.
[14] Denton M, Kerr K, Clin. *Microbiol. Rev*., **1998**, 11, 7.
[15] Nangia Y, Wangoo N, Goyal N, Shekhawat G, Raman C, *Microb. Cell Fact.*, **2002**, 8, 39.
[16] Gilligan P, Lum G, Van-Damme P, Whittier S, Burkholderia, Stenotrophomonas, Ralstonia, Brevundimonas, Comamonas, Delftia, Pandoraea, and Acidivorax. In: *Manual of Clinical Microbiology* (Murray PR, Baron EJ, Jorgensen JH et al., Eds) (8th ed.). ASM Press, Washington, DC.**2003**,pp 729.
[17] Wolf A, Fritze A, Hagemann M, Berg G, *Int. J. Syst. Evol. Microbiol*., **2002**, 52, 1937.
[18] Henglein A, *J. Phys. Chem*., **1993**, 97, 5457.
[19] Sastry M, Mayya K, Bandyopadhyay K, *Colloids Surf*., **1997**, 127, 221.
[20] Sastry M, Patil V, Sainkar S, *J. Phys. Chem*., **1998**, 102, 1404.
[21] Sulaiman G, Mohammed W, Marzoog T, Al-Amiery A, Kadhum A, Mohamad A, Asian Pacific *J.Trop. Biomed.*, **2013**, 3, 1, 58.
[22] Jain D, Kachhwah S, jain R, Srirastava G, kothari S, *Ind.J. Experim. Biol.*, **2010**, 84, 1152.
[23] Lavanya M, Veenavardhini S, Gim G, Kathiravan M, Kim S, *Inter. Res. J. Biol. Sci.*, **2013**, 2, 3, 28.
[24] Shankar S, Ahmad A, Parsricha R, Sastry M, *J. Mater. Chem*., **2003**, 13, 1822.
[25] Malarkodi C, Rajeshkumar S, Paulkumar K, Gnanajobitha G, Vanaja M, Annadurai G, *Nanosci. Nanotechnol.* : An International Journal, **2013**, 3, 2, 26.
[26] Wei X, Luo M, Li W, Yang L, Liang X, Xu L, Kong P, Liu H, *Biores. Technol*., **2012**,103, 273.
[27] Klaus T, Joerger R, Olsson E, Granqvist C, *PNAS*, **1999**, 13611.
[28] Marambio-Jones C, Hoek E, *J. Nanopart. Res*.,**2010**, 12, 1531.
[29] Kim J, Kuk E, Yu K, Kim J,Park S, Lee J, Kim S, Park Y, Park Y, Hwang C, Kim Y, Lee Y, Jeong D, Cho M, *Nanomedicine: Nanotechnol*., *Biol. Med*.**2007**, 3, 95.
[30] KB H, AJ B, *Biochem*. ,**2005**, 44, 13214.
[31] Chen M,Yang Z, Wu H,Pan X, Xie X,Wu C, *Intern. J. Nanomed*,**2011**, 6, 2873.
[32] Elechiguerra J, Burt J, Morones J, Bragado A, Gao X, Lara H, Yacaman M, *J. Nanobiotechnol*., **2005**, 3, 6.
[33] Zaki S,Elkady M,Farag S, Abd-EL-Haleem D, *Mater. Res. Bull*.,**2012**, 47, 4286-4290.
[34] Kim, Hwan S, Lee H, Ryu D, Choi1 S, Lee D, Korean *J. Microbiol. Biotechnol.*, **2011**, 39, 77–85.