

Silver Nanoparticles Extracellularly Produced by *Serratia* sp. NBL1001 Have Antibacterial Properties

Mary Faith Y. Adan¹, Zyne K. Baybay², Nacita B. Lantican¹, Lilia M. Fernando², Erlinda S. Paterno³, Lucille C. Villegas^{*1}, Leodevico L. Ilag⁴, Andrew D. Montecillo¹

¹Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, Laguna 4031, Philippines

²Nanobiotechnology Laboratory, National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños, College, Laguna 4031, Philippines

³Division of Soil Science, Agricultural Systems Institute, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna 4031, Philippines

⁴Xerion Limited, 246 Esplanade, Brighton, Victoria 3186, Australia

The emergence of multidrug resistant microorganisms ignited interest in silver nanoparticles (AgNPs) and their application as antimicrobial particles. In this study, the synthesis of AgNPs by a wildtype isolate, *Serratia* sp. NBL1001, and preliminary characterization and antibacterial activity of the produced AgNPs were investigated. Extracellular biosynthesis of AgNPs from silver nitrate (AgNO₃) was observed by visual inspection of the crude cell-free NBL1001 supernatant, showing color changes from pale yellow to orange-brown. UV/Vis scanning spectroscopy of the AgNO₃-NBL1001 supernatant solution, upon incubation overnight at 35°C, showed peaks at 430-440 nm, typical for AgNPs. Scanning electron microscopy and energy dispersive x-ray confirmed the presence of AgNPs in the solution, with size range of 15.29-61.78 nm and mean size of 28.80 nm (n=30). Agar-well diffusion assay showed that the AgNPs exhibited antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*. The mean value of the antimicrobial indices exhibited by the AgNPs was highest against *B. cereus* at 1.29, followed by

those of *E. coli* at 1.19, then *S. aureus* at 1.10, and the least was that of the *P. aeruginosa* at 1.0. The results demonstrated that *Serratia* sp. NBL1001 conditioned media can mediate the extracellular synthesis of AgNPs with antibacterial activities against both Gram-positive and Gram-negative bacteria.

KEYWORDS

Microbiology, Silver nanoparticles, *Serratia* sp., biological synthesis, extracellular, antibacterial, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus*

INTRODUCTION

Nanotechnology is referred to as a general purpose technology which has an essential effect on almost all industries and areas of the society (Kaur et al. 2012). It encompasses the engineering of functional systems at the molecular level, which is developing at a very fast pace. The rapid development of the field is attributed to the desire to produce materials with novel and promising properties by controlling and manipulating structures from the atomic and molecular level (Ramsden 2009; Ramsden

*Corresponding author

Email Address: lcvillegas1@up.edu.ph

Date received: August 04, 2018

Date revised: October 22, 2018

Date accepted: October 30, 2018

2011). The materials' unique properties result to a wide range of applications in different fields.

Production of nanoparticles is one essential component of nanotechnology, as nanoparticles hold commercial significance. According to Poole and Owens (2003), particles that measure 1-100 nm in size are called nanoparticles. Nanoparticles demonstrate useful characteristics, and even the functional systems consisting of nanoparticles, exhibit different, and often, superior properties compared to the conventional ones. Thus, nanoparticles are applied to a great extent in the fields of medicine, environmental remediation, renewable energies, electronics, biomedical devices as well as cosmetics and material production (Lu et al. 2007; De et al. 2008; Ghosh-Chaudhuri and Paria 2012). With the advancement of nanotechnology, many types of nanoparticles including gold nanoparticles, silver nanoparticles, copper nanoparticles among others, have been widely synthesized.

Silver nanoparticles (AgNPs) have been increasingly getting attention due to their distinct physical, chemical and biological properties, which include high electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic ability and non-linear optical property (Krut'akov et al. 2008). Due to these properties, AgNPs are widely utilized in the production of ink, microelectronics, and in medical imaging. In addition, AgNPs were found to exhibit promising antimicrobial activity (Ahamed et al. 2010; Kim et al. 2011; Guzman et al. 2012; Hsueh et al. 2015) that led to their use in different consumer products such as plastics, soaps, pastes, food, and textiles. Similarly, the increasing concerns on antibiotic resistance by health organizations around the world are pushing researchers and pharmaceuticals to find other ways to combat microorganisms, either through development of new antibiotics or other substances that can inhibit them. This then, ignited interest in AgNPs and their application as antimicrobial particles (Sharma et al. 2009).

The broad commercialization of products containing AgNPs led to an increase in AgNPs market value (Garcia-Barrasa et al. 2011; Fabrega et al. 2011; Dallas et al. 2011). Thus, chemical and physical methods that result to high yields are being utilized for mass production of AgNPs. However, these methods are associated with high operational costs and energy needs, aside from the extensive use of toxic chemicals. Accordingly, alternative eco-friendly and cost-efficient methods are being sought (Srikanth et al. 2016). Therefore, this study explored an alternative method for extracellular synthesis of AgNPs mediated by microorganisms. Previous studies (Malarkodi et al., 2013; Krithika et al. 2014; El Batal et al. 2016) have reported the extracellular biosynthesis of AgNPs by *Serratia* species. *Serratia* sp. NBL1001, a wildtype isolate obtained from the culture collection of the Microbiology Division, Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Baños, was used to extracellularly mediate the production of AgNPs from silver nitrate. The AgNPs were characterized using UV/Vis spectroscopy and Scanning Electron Microscopy with Energy Dispersive X-ray (SEM-EDX) analysis. The antibacterial activity of the AgNPs was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*.

MATERIALS AND METHODS

Partial characterization of the bacterial isolate

The *Serratia* sp. NBL1001 isolate was characterized while in culture (colony appearance, pigmentation) and morphologically (Gram reaction, cell shape and arrangement, motility, formation of endospore, oxygen and temperature requirement, and growth

factor requirement) following conventional microbiological techniques (Raymundo et al. 2015). Furthermore, the physiological characteristics were determined using the Analytical Profile Index 20E (API20E) strips (bioMérieux, USA).

Phylogenetic analysis and identification of the bacterial isolate

Identification based on the *16S ribosomal RNA* gene amplicon sequencing was done. Genomic DNA isolation using QIAamp DNA Mini kit (Qiagen), and *16S rRNA* gene amplification via polymerase chain reaction using universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGGTTACCTTGTTACGACTT 3') (Lane 1991, Stackebrandt & Liesack 1993). PCR products were sent to 1st BASE Laboratories SdnBhd, Malaysia for *16S rRNA* gene sequencing. The resulting *16S rRNA* gene sequences and chromatograms were analyzed using MEGA 6 (v. 6.0) (Tamura et al. 2013) and were aligned using the BLAST software v. 2.6.1. (Zhang et al. 2000) of the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), in comparison with 16S ribosomal RNA sequences (Bacteria and Archaea) Database.

Test for extracellular silver nanoparticle synthesis

The ability of *Serratia* sp. NBL1001 to mediate the extracellular synthesis of silver nanoparticles (AgNPs) was determined following the methods of Malarkodi et al. (2013) and Cariño et al. (2017) with some modifications. A loopful of the bacterial isolate was inoculated in 10 mL of nutrient broth and was incubated at 35 °C with shaking (150 rpm) for 24 hours. Ten mL of negative control (nutrient broth only) was also subjected to the same incubation conditions. After incubation, the broth containing the isolate was centrifuged at 2000 xg for 15 minutes to pellet the cells and obtain the supernatant. The supernatant was filtered using 0.22-micron pore size membrane filter to ensure that it was cell-free. Then, silver nitrate (AgNO₃) aqueous solution was added at a final concentration of 2 mM to the vials with the supernatant only and negative control (nutrient broth). The AgNO₃-supernatant mixtures were incubated with shaking (150 rpm) at 35 °C for 18 to 24 h in the dark, and the control solution (AgNO₃-nutrient broth) was also prepared and subjected to the same incubation conditions. Visual inspection was performed for all the set-ups, relative to color changes in the control, to determine whether AgNPs were formed.

Preliminary characterization of AgNPs formed

The UV-visible spectra of the incubated AgNO₃-supernatant mixtures were measured using GENESYS 10S UV-Vis spectrophotometer (v4.003) (Thermo Scientific, USA) which was set at scanning mode with fast scan speed at 2.0 nm intervals. The formation of the AgNPs in the aqueous mixtures was ascertained by measuring the UV-Vis spectra of the mixtures from 300 to 700 nm. After which, the mixtures of the AgNO₃-supernatants of *Serratia* sp. NBL1001 were subjected to high-speed centrifugation at 9000 xg and washed with sterile HPLC-grade water to obtain concentrated AgNPs, which were resuspended in HPLC-grade water. The solution was then submitted to the Advanced Device and Materials Testing Laboratory (ADMATEL) - Industrial Technology Development Institute (DOST, Taguig City) for Field Emission Scanning Electron Microscopy (FESEM) and Energy Dispersive X-ray analysis (EDX) (using Dual Beam Helios Nanolab 600i, FEI Company, United States).

Determination of the antibacterial activity of the AgNPs using agar-well diffusion assay

To concentrate and prepare the AgNPs for the assay, 15 mL of the AgNO₃-supernatant mixture (positive for AgNPs) was subjected to high-speed centrifugation at 9000 xg for 30 minutes,

Table 1: Sequence similarities of *Serratia* sp. NBL1001 with *Serratia* group using BLAST

Genbank Accession No.	% Similarity	E value	Nearest Phylogenetic Affiliation
NR_044385.1	100%	0	<i>Serratia nematodiphila</i> KCTC
NR_036886.1	100%	0	<i>Serratia marcescens</i> subsp. <i>sakuensis</i> DSMZ
NR_114043.1	99%	0	<i>Serratia marcescens</i> subsp. <i>marcescens</i> NBRC
NR_113236.1	99%	0	<i>Serratia marcescens</i> subsp. <i>marcescens</i> JCM
NR_114716.1	99%	0	<i>Serratia rubidaea</i> DSMZ
NR_024644.1	99%	0	<i>Serratia rubidaea</i> JCM
NR_114157.1	99%	0	<i>Serratia odorifera</i> NBRC
NR_114155.1	99%	0	<i>Serratia ficaria</i> NBRC
NR_037110.1	99%	0	<i>Serratia odorifera</i> DSMZ
NR_041979.1	99%	0	<i>Serratia ficaria</i> DSMZ

and the pellet obtained was washed with HPLC-grade water.

After washing, the pellet was resuspended in 1 mL sterile HPLC-grade water. This was stored at 4 °C until further use. About 200 µL of the concentrated AgNPs was diluted with 800 µL HPLC-grade water when used in the antibacterial assay.

Following the method of Balouiri et al. (2016) with some modifications, agar-well diffusion assay was performed to determine if the AgNPs have antibacterial activity. The test organisms used for the assay were *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*. Each of the 24-h culture of the test organisms was diluted using 0.85% saline solution, and adjusted to match 0.5 McFarland Standard (bioMérieux, USA). Then, 100 µL of the diluted test organism was spread plated on Mueller-Hinton agar, after which six agar wells (diameter = 6.5 mm) were bored on the plate. A volume of 20 µL of each of the negative control (water), positive controls (29.4 mM AgNO₃ and 100 ppm kanamycin) and triplicates of the prepared AgNPs were placed on the agar wells on the bacterial lawn. All the plates were prepared in triplicates and incubated in dark condition at 37°C for 24 hours. After incubation, the diameters of the zones of inhibition were observed and measured (in mm) using Vernier caliper. The Antimicrobial Index (AI) was computed based on the formula used by Balela and Amores (2015).

Statistical analysis

The antimicrobial index (AI) values of the treatments against each test microorganism after 24 h incubation, were subjected to F-test using ANOVA. Any significant differences in the mean antimicrobial indices of each substance were grouped and compared. Pairwise comparisons using Tukey's test were employed to compare the mean AI of the AgNPs to the positive (100 ppm kanamycin, 29.4 mM AgNO₃) and negative (water) controls. Similar statistical analysis was performed to compare the mean AI of AgNPs among each test organism. All statistical tests were computed using Minitab 17 (Minitab 17 Statistical Software 2010).

RESULTS AND DISCUSSION

Characteristics and identity of *Serratia* sp. NBL1001

Serratia sp. NBL1001 exhibited opaque, mucoid, round, entire, umbonate, red with white margin colonies on Nutrient Agar plate. It was found to be Gram-negative small rod occurring singly, catalase positive, oxidase negative, facultative anaerobe,

mesophilic, non-spore former, and it did not require growth factors. It was also found to be motile and positive for the aerobic and anaerobic oxidation/fermentation of glucose. These characteristics and the production of red pigment and characteristic growth on MacConkey agar, are all of the genus *Serratia* (Dworkin et al. 2006).

Molecular identification of the isolate using *16S rRNA* gene sequencing and BLAST analysis revealed that *Serratia* sp. NBL1001 has 100% sequence similarity to *Serratia marcescens* and *Serratia nematodiphila* (Table 1). It was also found to be 99% similar to different strains of the *Serratia* group which include *Serratia marcescens* subsp. *marcescens*, *Serratia rubidaea*, *Serratia odorifera*, and *Serratia ficaria*. Further confirmation of its identity was by biochemical tests using API20E to differentiate *Serratia* sp. NBL1001 from other *Serratia* species. The tests were done using BioMérieux Analytical Profile Index 20E (API20E), an identification system used for Enterobacteriaceae and other non-fastidious Gram-negative rods which utilize biochemical tests, and database to identify a limited number of bacteria (Smith et al. 1972). The results obtained were similar to the published literature describing *Serratia marcescens* (Farmer et al. 1985; Holt et al. 1994). In addition, considering that *Serratia* sp. NBL1001 was unable to ferment arabinose which is a differentiating characteristic of *S. marcescens* from *S. liquefaciens* (Hejazi and Falkner 1997) and *S. nematodiphila* (Zhang et al. 2009), it can be concluded that the identity of *Serratia* sp. NBL1001 may be *S. marcescens*. However, it is noteworthy that *Serratia* NBL1001 was positive for melibiose oxidation/fermentation when most *S. marcescens* strains would test negative for this carbohydrate source.

Characteristics of the synthesized silver nanoparticles

To examine the extracellular synthesis of AgNPs, removal of the bacterial cells before addition of the silver (Ag) ion source was by centrifugation, and subsequent membrane filtration of the *Serratia* sp. NBL1001 spent broth culture. The assay procedure for the extracellular synthesis of nanoparticles was adapted from the methods of He et al. (2006) and Saifuddin et al. (2009). The crude *Serratia* sp. NBL1001 cell-free supernatants were used for the assay. Since AgNO₃ can undergo photoreduction (Hada et al. 1976), all solutions were incubated under dark condition. The shaking allowed for an increased interaction of the molecules in the solution, while the temperature was set at 35°C to maintain smaller AgNP size in accordance with Schmid (1992) who stated that AgNPs are smaller when synthesized at higher temperatures. Preliminary detection of AgNP production is usually by visual observation of color change (Singh et al. 2015).

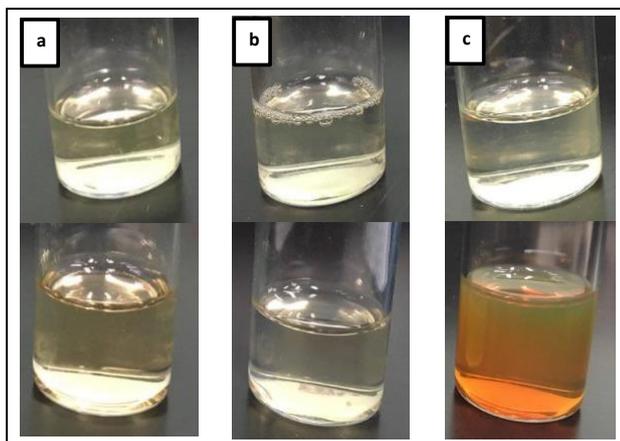


Figure 1: Color changes of nutrient broth (top) and *Serratia* sp. NBL1001 supernatant (bottom) in Nutrient broth (a) before addition of AgNO_3 , (b) after addition of AgNO_3 , and (c) after 24 h of incubation

Nutrient broth did not exhibit color change whereas the supernatant solution showed color changes after addition of AgNO_3 and after incubation for 24 h at 35°C with shaking at 150 rpm in the dark; specifically, formation of orange-brown solution was observed (Figure 1). A color change to reddish-brown to brown of the reaction solution implies AgNP production (Shahverdi et al. 2007; Singh et al. 2013). This color change likely indicated the formation of silver nanoparticles. This would also indicate the reduction of AgNO_3 into AgNPs mediated by the *Serratia* sp. NBL1001 cell-free supernatant. The control solution, nutrient broth with AgNO_3 , showed no color change upon incubation in the same condition as the cell-free supernatant- AgNO_3 . This may indicate that there was no AgNPs produced in the control solution.

The solution containing AgNPs turned orange-brown in color due to the AgNPs' unique optical property called surface plasmon resonance (SPR). SPR is where the free electrons in the metal nanoparticle are driven into oscillation due to a strong coupling with a specific wavelength of incident light resulting to high absorption and scattering intensity of silver nanoparticles as compared to its individual and bulk material (Patching 2014). Removal of the bacterial cells was effected by centrifugation and subsequent membrane filtration of the *Serratia* sp. NBL1001 broth culture before addition of AgNO_3 . Only the bacterial metabolites, secreted proteins, shed cell membrane components along with the spent media components were present in the supernatant solution; thus, the synthesis of AgNPs from AgNO_3 was implied to be extracellular or cell-free. According to Singh et al. (2015), the only proposed mechanism of AgNPs synthesis using culture supernatant would be the presence of the bacterial metabolites or by-products and other media components remaining in the solution upon filtration, that could have reduced silver ions to AgNPs. The supernatant solution may be an ideal source of reductants that would reduce silver from Ag^{1+} ions to elemental Ag (Ag^0) as nanoparticles. The reductants may include enzymes, reducing sugars along with other proteins that are highly negative and must lose their electrons.

To confirm the presence of AgNPs, the solutions were subjected to UV/Vis spectroscopy by scanning at 300-700 nm. The supernatant with AgNO_3 exhibited single broad surface plasmon resonance (SPR) band with maximum absorbance at 410-420 nm and indicative of spherical silver nanoparticles (Figure 2). The concentrated AgNPs were also found to exhibit a single SPR band with maximum absorbance peak at 430-440 nm (Figure 3), still indicative of AgNPs but may have exhibited some degree of aggregation as evidenced by the shift in max absorbance peak. UV/Vis spectrophotometric analysis is a fundamental analytical technique used to determine the production and stabilization of

nanoparticles in aqueous solution (Baia et al. 2007). The maximum absorbance peak indicates the relative sizes of the nanoparticles in a solution where a higher number corresponds to a larger particle size (Saion et al. 2013). The increase in the max absorbance peak may likely be caused by the aggregation of the AgNPs and loss of some stabilizing agents present in the media components upon centrifugation and washing. It is known that the increase in the size of the particles formed due to aggregation leads to an increase in maximum absorption peak (Natsuki et al. 2015). In addition, spherical silver nanoparticles are expected to exhibit a single SPR band whereas anisotropic particles may demonstrate multiple SPR bands depending on the particle morphology as per Mie's theory (Herguth and Nadeau 2004). Based on these published studies, the broad and single SPR bands exhibited by the AgNP solutions likely indicate that spherical AgNPs were synthesized.

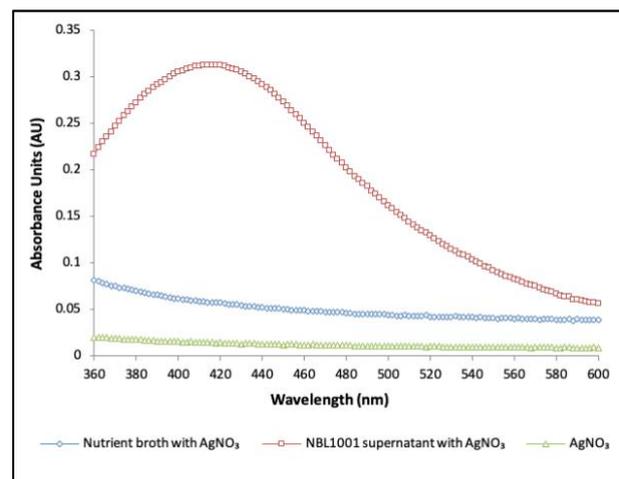


Figure 2: Absorption spectra of the crude *Serratia* sp. NBL1001 supernatant- AgNO_3 mixture after 24 hours of incubation showing single plasmon resonance band with maximum absorbance peak at 410-420 nm

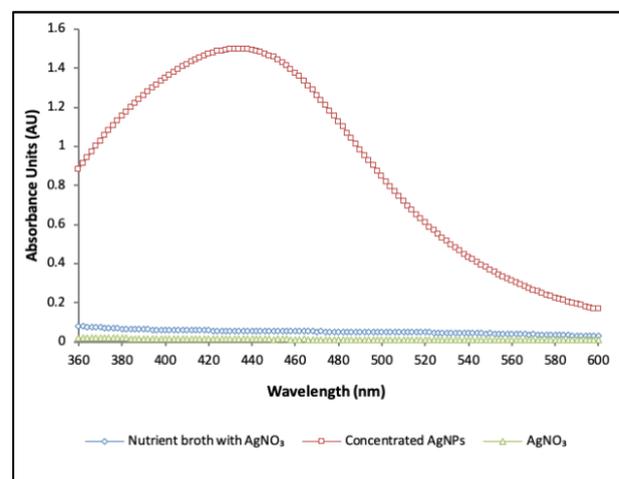


Figure 3: Absorption spectra of the concentrated AgNPs extracellularly produced by isolate *Serratia* sp. NBL1001 showing single plasmon resonance band with maximum absorbance peak at 430-440 nm

To further characterize the AgNPs, SEM-EDX analysis was performed. The nanoparticles were observed to be spherical and occurred in either cluster or monodispersed with size ranging from 15.29–61.78 nm with mean nanoparticle size of 28.80 nm (Figure 4). The effect of incubation temperature on the size of resulting AgNPs can also be investigated on a separate study. The production and presence of silver nanoparticles in the solution was also further supported by EDX spectroscopy analysis, which is used for the elemental analysis and chemical

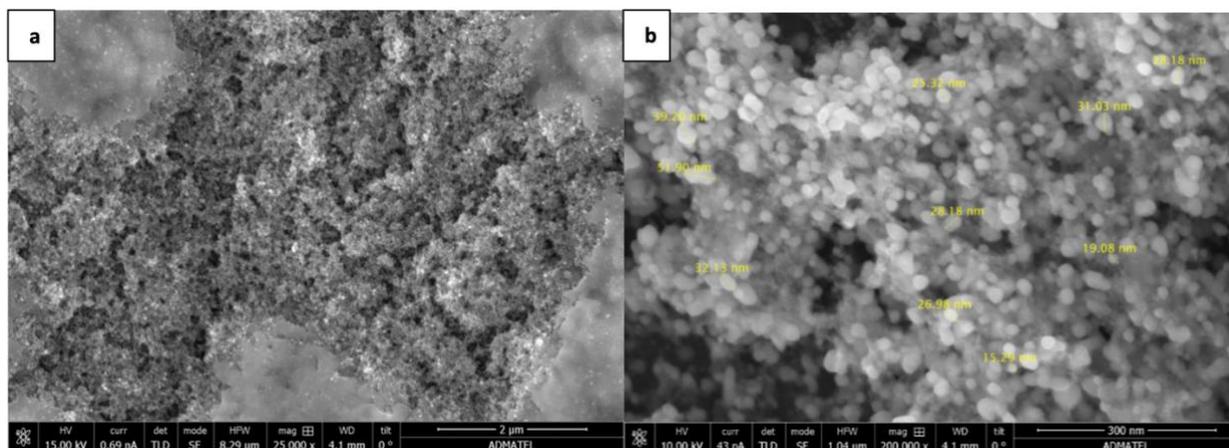


Figure 4: Scanning electron microscopy (SEM) images of the concentrated spherical AgNPs, with sizes ranging from 15.29–61.78 nm and mean size of 28.80 nm (n=30), extracellularly produced by *Serratia* sp. NBL1001 after 24 h incubation with shaking at 150 rpm and temperature of 35°C taken at (a) 25, 000x and (b) 200, 000x magnification

characterization that measures the energy X-rays or electromagnetic emissions characteristic of different elements from which the X-rays are emitted. The EDX analysis showed signal for elemental silver (Ag), constituting 47.36% of the specimen, along with signals of other elements, O, C, Cl, Si, Na, S, K and Mo, which were possibly contributed by proteins and other organic and inorganic compounds in the solution (Figure 5). The presence of the other elements may imply that further concentration of the solution might be needed to obtain the pure AgNPs. On another note, the presence of other elements in the solution may help in stabilizing the silver nanoparticles (Sperling and Parak 2010).

Antibacterial activity of the silver nanoparticles

The antibacterial activity of the *Serratia* sp. NBL1001-synthesized AgNPs was determined following the agar well diffusion assay method by Perez et al. (1990) with modifications. The method was used in order to prevent adherence of AgNPs to the discs (disk diffusion method) which may interfere in the diffusion of the nanoparticles along the plate. The positive controls used in the study were kanamycin (100 ppm) and aqueous silver nitrate (29.4 mM), which are both known to exhibit antibacterial activity. Kanamycin is an aminoglycoside antibiotic which blocks protein synthesis in bacteria (Madigan et al. 2013). AgNO₃ is the Ag ion source for the reactions, which is a known antiseptic. It is non-toxic at low concentrations, but excessive exposure to AgNO₃ causes toxicity and other conditions such as argyria or formation of black pigmentation in the skin (Young 2004). On the other hand, the negative control used was HPLC-grade water which was the resuspension medium of the AgNPs.

Antibacterial activity assay of the AgNPs against *E. coli*, *P. aeruginosa*, *S. aureus* and *B. cereus* resulted to zones of clearing indicating that the AgNPs exhibited antibacterial activity against the four test organisms (Figure 6). The mean value of the diameter of the zones of inhibition exhibited by the AgNPs was highest against *B. cereus* at 14.89 mm, followed by those of *E. coli* at 14.22 mm, then against *S. aureus* at 13.67 mm, and the least was that of the *P. aeruginosa* at 13.00 mm. A larger zone of inhibition in *S. aureus* as compared to the antibiotic kanamycin (100 ppm) and a larger clearing zone in *B. cereus* as compared to the AgNO₃ were observed using AgNPs. In addition, AgNPs inhibited the kanamycin-resistant *P. aeruginosa* (ZOI dia. = 13.00 mm). The resistance of *P. aeruginosa* to kanamycin is highly attributed to the aminoglycoside phosphoryl transferases commonly found in this bacterium. These phosphoryltransferases act on the 3'-OH target of the

aminoglycosides conferring resistance of the bacteria to not regularly used aminoglycosides (Poole 2011).

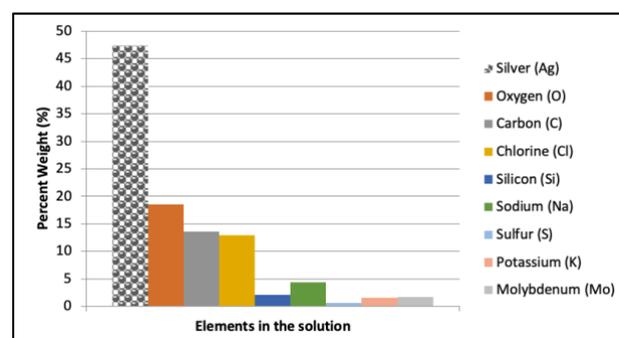


Figure 5: Percent weight (% w/w) of elements detected using EDX analysis in the aqueous solution of *Serratia* sp. NBL1001-produced AgNPs with the highest value of 47.36% w/w of AgNPs.

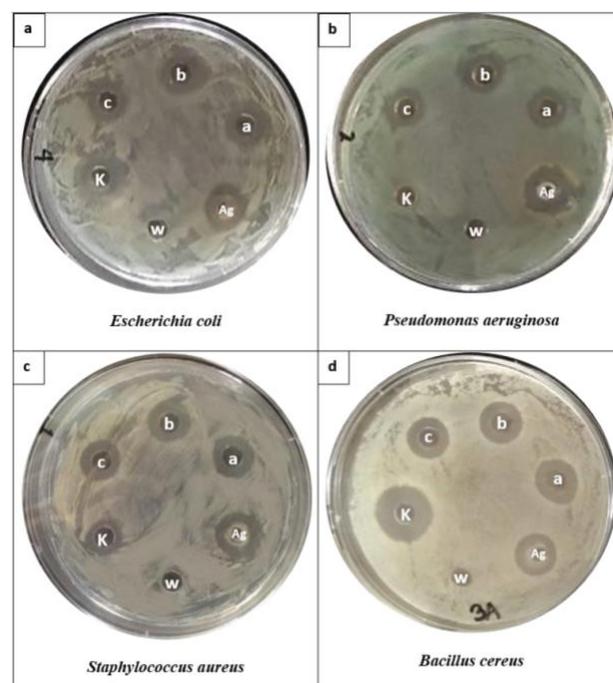


Figure 6: Zones of clearing showing antibacterial activity of 20 uL diluted AgNPs (triplicate) against (a) *Escherichia coli*, (b) *Pseudomonas aeruginosa*, (c) *Staphylococcus aureus*, and (d) *Bacillus cereus*

The standardized analysis of activity using antimicrobial indices (AI) exhibited by AgNPs against the four test organisms was

Table 2: Analysis of Variance (ANOVA) of average antimicrobial indices (AI) exhibited by water, 100 ppm kanamycin, 29.4 mM AgNO₃ and AgNPs against test organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* with the average AI values of AgNPs all greater than or equal to 1.00

Test Organism	P-value	Analysis	Conclusion*
<i>Escherichia coli</i>	0.263	0.263 > 0.05	Fail to reject Ho
<i>Pseudomonas aeruginosa</i>	0	0 < 0.05	Reject Ho
<i>Staphylococcus aureus</i>	0.002	0.002 < 0.05	Reject Ho
<i>Bacillus cereus</i>	0	0 < 0.05	Reject Ho

* Null hypothesis: All means are equal
 Alternative hypothesis: At least one mean is different
 Significance level: $\alpha = 0.05$
 Decision rule: Reject Ho if p-value $\leq \alpha$, otherwise fail to reject Ho

Table 3: Pairwise comparison of average antimicrobial indices (AI) exhibited by water, 100 ppm kanamycin, 29.4 mM AgNO₃ and AgNPs against test organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* with the average AI values of AgNPs all greater than or equal to 1.00.

Test Organism	Average Antimicrobial Indices (Tukey's Grouping*)			
	HPLC-grade water	Kanamycin (100 ppm)	AgNO ₃ (29.4 mM)	Silver nanoparticle (AgNPs)
<i>Escherichia coli</i>	0	1.36 (A)	1.18 (A)	1.19 (A)
<i>Pseudomonas aeruginosa</i>	0	0	1.46 (A)	1.00 (B)
<i>Staphylococcus aureus</i>	0	0.95 (B)	1.31 (A)	1.10 (B)
<i>Bacillus cereus</i>	0	1.90 (A)	1.26 (B)	1.29 (B)

* Tukey's grouping presented per row (test organism); mean AI values with the same letters have no significant differences

reflective of the zones of inhibition (Table 2). The mean values of antimicrobial indices by the AgNPs were all greater than or equal to 1.00. It was highest against *B. cereus* at 1.29, followed by those of *E. coli* at 1.19, then against *S. aureus* at 1.10, and the least activity was against *P. aeruginosa* at 1.00. The mean AI values for each of the four test microorganisms were subjected to F test using ANOVA. The results (Table 2) showed that at $\alpha=5\%$, there was no significant difference between the mean values of the AI exhibited by AgNPs, kanamycin (100 ppm) and AgNO₃ (29.4 mM) against *E. coli*, thus it was regarded that the effect of AgNPs against *E. coli* is comparable to the effect of the positive controls. On the other hand, the results showed that at least one of the mean AI values of the assay against *P. aeruginosa*, *S. aureus*, and *B. cereus* is significantly different from the others. Thus, Tukey's post-hoc test was used for pairwise comparison of the mean AI values. The pairwise comparison (Table 3) showed that the mean AI of the AgNO₃ was significantly larger than that of the AgNPs, implying that AgNO₃ is more efficient in inhibiting the growth of *P. aeruginosa*. However, the development of ZOI of the AgNPs indicates that AgNPs are more efficient than kanamycin, which did not exhibit any activity against *P. aeruginosa*. Tukey's grouping also showed that the antibacterial action of AgNPs is comparable with that of the antibiotic, kanamycin, but inferior to that of AgNO₃ in inhibiting *S. aureus*. Lastly, the mean AI of the treatments against *B. cereus* showed that AgNPs and AgNO₃ had AIs that are comparable. On the other hand, kanamycin had

a significantly higher mean AI resulting in the most efficient antibacterial activity against *B. cereus* when compared to AgNO₃ and AgNPs. It must be noted, however, that it is not the intention of the study to compare the potency of AgNPs with that of AgNO₃. It is just to show that the synthesized AgNPs have antibacterial properties comparable to other known substances included in the test.

The mean AIs exhibited by the AgNPs for each of the four test microorganisms were also subjected to F test using ANOVA. The results showed that at $\alpha=5\%$, there was significant difference between the mean values of the AIs exhibited by AgNPs against the four test organisms, thus Tukey's post-hoc test was used for pairwise comparison of the mean AI values. The pairwise comparison (Table 4) showed that the mean AI of the AgNPs against *B. cereus* was significantly higher than *S. aureus* and *P. aeruginosa* but was comparable to the mean AI exhibited against *E. coli*. The mean AI of *E. coli* was also found to be comparable to mean AIs exhibited against both *B. cereus* and *S. aureus* but had a significantly higher mean AI as compared to the AIs exhibited against *P. aeruginosa*. As mentioned, the mean AI exhibited by AgNPs against *S. aureus* was not significantly different to that against *E. coli* but was significantly higher than that against *P. aeruginosa* and significantly lower compared to the mean AIs exhibited against *B. cereus*. Lastly, the mean AIs exhibited against *P. aeruginosa*

Table 4: Pairwise comparison of average antimicrobial indices (AI) exhibited by the synthesized silver nanoparticles (AgNPs) against test organisms *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus* with the average AI values of AgNPs all greater than or equal to 1.00

Test Organism	Average AI of AgNPs against the test organism	Tukey's Grouping*	
<i>Escherichia coli</i>	1.19	A	B
<i>Pseudomonas aeruginosa</i>	1.00		C
<i>Staphylococcus aureus</i>	1.10		B C
<i>Bacillus cereus</i>	1.29	A	

* Average AI values with the same letters have no significant differences

was found to be significantly lower among the mean AIs exhibited by AgNPs against *B. cereus*, *E. coli* and *S. aureus*. For future studies, it would be interesting to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBCs) of the AgNPs produced by the wildtype isolate *Serratia* sp. NBL1001.

Based on these, it can be concluded that the wildtype isolate *Serratia* sp. NBL1001 with most likely identity of *Serratia marcescens* can produce supernatants which mediate extracellular synthesis of AgNPs having great antibacterial potential against both Gram-positive and Gram-negative bacteria. However, further studies on its mechanism of inhibiting the growth of the test organisms must be done.

Other tests for the characterization of silver nanoparticles are also recommended which includes X-ray Diffraction (XRD) analysis, Fourier Transform Infrared (FTIR) Spectroscopy and Atomic Force Microscopy (AFM). XRD analysis may be performed to reconfirm AgNP synthesis and its oxidation state in the solution. FTIR Spectroscopy may be used to determine the existence and nature of encapsulation and Atomic Force Microscopy (AFM) to determine the size distribution of the synthesized AgNPs. *Serratia* sp. NBL1001 supernatant-AgNO₃ solution incubation. UV/Vis spectroscopy utilization may also be increased to observe AgNP stability and further aggregation. For the antibacterial activity assay, other parameters including concentration and oxidation state of AgNPs may also be determined to know how it affects the antibacterial activity. The assay may also be designed for other currently significant microorganisms, such as drug-resistant bacteria. The action of AgNPs against the microorganisms may also be observed using highly sophisticated techniques such as high resolution microscopic (AFM, FE-SEM, TEM, and XRD), spectroscopic (DLS, ESR spectroscopy, Fluorescence spectroscopy, inductively coupled plasma-optical emission spectroscopy, UV-vis), molecular, and biochemical techniques.

ACKNOWLEDGEMENT

The authors would like to thank the following: Dr. Florinda M. Piano; Philippine Society for Microbiology, Microbiology Division & Molecular Biology and Biotechnology Laboratory, Institute of Biological Sciences, College of Arts and Science, University of the Philippines Los Baños; Nano-Biotechnology Laboratory, National Institute of Molecular Biology and Biotechnology, UPLB; Dr. Rina B. Oplencia, and; supported in part by Science, Technology, Research and Innovation for Development (STRIDE) Program of the United States Agency for International Development (USAID) under research grant number: 0213997-G-2016-002-01.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

All authors contributed to the form and content of this paper through data gathering, data analysis, and writing.

REFERENCES

- Ahamed M, Alsalhi MS, Siddiqui MK. Silver nanoparticle applications and human health. *Clin Chim Acta* 2010; 411: 1841-1848.
- Baia L and Simon S. UV-VIS and TEM assessment of morphological features of silver nanoparticles from phosphate glass matrices. In: A. Méndez-Vilas and J. Diaz, eds., *Modern Research and Educational Topics in Microscopy*. Badajoz, Spain: Formatex. 2007: 576-583.
- Balela M and Amores K. Formation of highly antimicrobial copper nanoparticles by electroless deposition in water. *Science Diliman* 2015; 27: 10-20.
- Balouiri M, Sadiki M, Ibsouda KS. Methods for in vitro evaluating antimicrobial activity: A review. *JPA* 2016; 71-79.
- Carino SS, Montecillo AD, Atienza MTJ, Paterno ES, Ilag LL, Fernando LM. Phenotypic characterization and identification of *Lysinibacillus* sp. NBL1a capable of extracellular biogenic synthesis of gold nanoparticles. *Philipp Agric Scientist* 2017; 100: S81-S91.
- Dallas P, Sharma VK and Zboril R. Silver polymeric nanocomposites as advanced antimicrobial agents: classification, synthetic paths, applications, and perspectives. *Adv Colloid Interface Sci* 2011; 166: 119-135.
- De M, Ghosh PS and Rotello VM. Applications of nanoparticles in biology. *Adv Mater* 2008; 20: 4225-4241.
- Dworkin M, Falkow S, Rosenberg E, Schleifer K and Stackebrandt E. *The Prokaryotes. Proteobacteria: Gamma subclass*. 3rd ed. New York: Springer 2006: 197-214.
- El-Batal AI, El-Hendawy HH, Faraag AH. Synthesis and characterization of silver nanoparticles by *Serratia marcescens* strains isolated from different sources in Egypt. *Nature and Science* 2016; 14:12.

- Fabrega J, Luoma SN, Tyler CR, Galloway TS and Lead JR. Silver nanoparticles: behaviour and effects in the aquatic environment. *Environ Internat* 2011; 37: 517-531.
- Farmer J, Davis, BR, Hickman-Brenner FW, McWhorter A, Huntley-Carter GP, Asbury MA, Riddle C, Wathen-Grady HG, Elias C, Fanning GR, Steigerwalt AG, O'hara CM, Morris GK, Smith PB and Brenner DJ. Biochemical identification of new species and biogroups of Enterobacteriaceae isolated from clinical specimens. *J Clin Microbiol* 1985; 21: 46-76.
- Garcia-Barrasa J, Lopez-De-Luzuriaga JM and Monge M. Silver nanoparticles: synthesis through chemical methods in solution and biomedical applications. *Cent Eur J Chem* 2011; 9: 7-19.
- Ghosh-Chaudhuri R and Paria S. Core/Shell nanoparticles: classes, properties, synthesis mechanisms, characterization, and applications. *Chem Rev* 2012; 112: 2373-2433.
- Guzman M, Dille J and Godet S. Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria. *Nanomedicine* 2012; 8: 37-45.
- Hada H, Yonezawa Y, Yoshida A and Kurakake A. Photoreduction of silver ion in aqueous and alcoholic solutions. *J Phys Chem* 1976; 80: 2728-2731.
- He Y, Yuan J, Su F, Xing X and Shi G. *Bacillus subtilis* assisted assembly of gold nanoparticles into long conductive nodous ribbons. *J Phys Chem* 2006; B110: 17813-17818.
- Hejazi A and Falkiner FR. *Serratia marcescens*. *J Med Microbiol* 1997; 46: 903-912.
- Herguth WR and Nadeau G. Applications of scanning electron microscopy and energy dispersive spectroscopy (SEM/EDS) to practical tribology problems. Vallejo, CA: SGS Herguth Laboratories. 2004: 1-9.
- Holt JG and Krieg NR. *Bergey's Manual of Determinative Bacteriology*, 9th ed. Washington, DC: Williams & Wilkins, Co. 1994: 787.
- Hsueh Y, Lin K, Ke W, Hsieh C, Chiang C, Tzou D and Liu S. The antimicrobial properties of silver nanoparticles in *Bacillus subtilis* are mediated by released Ag⁺ ions. *PLOS ONE* 2015, [online] 10(12), p. e0144306. Available at: <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0144306>.
- Kaur G, Singh T and Kumar A. Nanotechnology: A review. *IJEAR* 2012; 2: 50-53.
- Kim SH, Lee HS, Ryu DS, Choi SJ and Lee DS. Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus* and *Escherichia coli*. *Korean J Microbiol Biotechnol* 2011; 39: 77-85.
- Krithika K, Sruthi CV, Geetharamani G. Production of silver nanoparticles from *Serratia marcescens* and its application as antibacterial agent. *SIRJ-APBBP* 2014; 1:5.
- Krutyakov YA, Kudrinskii AA, Olenin AY, Dzhurik P and Lisichkin GV. Aggregative stability and polydispersity of silver nanoparticles prepared using two-phase aqueous organic systems. *Nanotechnol Russ* 2008; 3: 303-310.
- Lane DJ. 16s/23s rRNA sequencing in *E.coli*. In: Stackebrandt, E. and M. Goodfellow (Editors). *Nucleic Acid Techniques in Bacterial Systematics*. John Wiley and Sons, New York, NY, USA. 1991:115-148.
- Lu AH, Salabas EL and Ferdi S. Magnetic nanoparticles: synthesis, protection, functionalization, and application. *Angew Chem Int Ed Engl* 2007; 46: 1222-1244.
- Madigan M, Martinko J, Stahl D, and Clark D. *Brock Biology of Microorganisms*. 13th edition. Benjamin Cummings, San Francisco, CA, USA. 2012: 1043.
- Malarkodi C, Rajeshkumar S, Paulkumar K, Vanaja M, Jobitha G and Annadurai G. Bactericidal activity of bio-mediated silver nanoparticles synthesized by *Serratia nematodiphila*. *Drug invention today* 2013; 5: 119-125.
- Minitab 17 Statistical Software. Computer software. State College, PA. 2010. Minitab Inc.
- Natsuki J, Natsuki T and Hashimoto Y. A review of silver nanoparticles: synthesis methods, properties and applications. *IJMSA* 2015; 4: 325-332.
- Patching SG. Surface plasmon resonance spectroscopy or characterization of membrane protein-ligand interactions and its potential for drug discovery. *Biochim Biophys Acta* 2014; 1838: 43-55.
- Perez C, Paul M and Bazerque P. Antibiotic assay by agar-well diffusion method. *Acta Biol Med Exp* 1990; 15: 113-115.
- Poole Jr. C. and Owens F. *Introduction to nanotechnology* 1st ed. USA: Wiley, John & Sons, Inc. 2003: 400.
- Poole K. *Pseudomonas aeruginosa*: resistance to the max. *Front Microbiol* 2011; 2: 1-13.
- Ramsden, J. *Essentials of nanotechnology* 1st ed. Ventus Publishing ApS, 2009: 126.
- Ramsden J. *Nanotechnology: an introduction* 1st ed. Oxford, UK: Elsevier Inc. 2011: 288.
- Raymundo AK, Oplencia RB, Bautista VV. *Laboratory Exercises on Phenotypic Methods in Microbial Identification*. Microbiology Division, IBS-CAS, University of the Philippines Los Banos, College, Laguna. 2015:131
- Saifuddin N, Wong CW and Yasumira AN. Rapid biosynthesis of silver nanoparticles using culture supernatant of bacteria with microwave irradiation. *Electron J Chem* 2009; 6: 61-70.
- Saion E, Gharibshahi E and Naghavi K. Size-controlled and optical properties of monodispersed silver nanoparticles synthesized by the radiolytic reduction method. *Int J Mol Sci* 2013; 14: 7880-7896.
- Schmid G. Large clusters and colloids. *Metals in the embryonic state*. *Chem Rev* 1992; 92: 1709-1727.
- Shahverdi AR, Fakhimi A, Shahverdi HR and Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. *Nanomedicine* 2007; 3: 168-171.
- Sharma VK, Yngard RA and Lin Y. Silver nanoparticles: green synthesis and their antimicrobial activities. *Adv Colloid Interface Sci* 2009; 145: 83-96.

- Singh B, Singh B, Singh A, Khan W, Naqvi A and Singh H. Mycofabricated biosilver nanoparticles interrupt *Pseudomonas aeruginosa* quorum sensing systems. Scientific Reports 2015; [online] 5(1). Available at: <https://www.nature.com/articles/srep13719>.
- Singh R, Wagh P, Wadhvani S, Gaidhani S, Kumbhar A, Bellare J and Chopade BA. Synthesis, optimization, and characterization of silver nanoparticles from *Acinetobacter calcoaceticus* and their enhanced antibacterial activity when combined with antibiotics. Int J Nanomedicine 2013; 8: 4277–4290.
- Smith PB, Tomföhrde KM, Rhoden DL and Balows A. API system: a multitube micromethod for identification of *Enterobacteriaceae*. Appl Microbiol 1972; 24: 449-452.
- Sperling RA and Parak WJ. Surface modification, functionalization and bioconjugation of colloidal inorganic nanoparticles. Phil Trans R Soc Lond A 2010; 368: 1333–1383.
- Srikar SK, Giri DD, Pal DB, Mishra PK and Upadhyay SN. Green synthesis of silver nanoparticles: a review. Curr Opin Green Sustain Chem 2016; 6: 34-56.
- Stackebrandt E and Liesack W. Nucleic acids and classification. In: M. Goodfellow and A.G. O'Donnel (Editors). Handbook of New Bacterial Systematics. Academic Press, London, England, UK. 1993:151-189.
- Tamura K, Stecher G, Peterson D, Filipinski A and Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30: 2725-2729.
- Young JA. Silver Nitrate. Chem. Educ 2004; 81 (9): 1259. DOI: 10.1021/ed081p1259
- Zhang CX, Yang SY, Xu MX, Sun J, Liu H, Liu JR, Liu H, Kan F, Sun J, Lai R and Zhang KY. *Serratia nematodiphila* sp. nov., associated symbiotically with the entomopathogenic nematode symbiotically with the entomopathogenic nematode *Heterorhabditidoides chongmingensis* (Rhabditida: Rhabditidae). Int J of Syst and Evol Microbiol 2009; 59: 1603-1608.
- Zhang Z, Scott S, Lukas W and Webb M. A greedy algorithm for aligning DNA sequences. J Comput Biol 2000; 7: 203-214.