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Biological Synthesis of Silver Nano Particles using *Pseudomonas aeruginosa*

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The field of nanotechnology is observing a remarkable progress over the past few years. Nanomaterials are of interest because they have novel properties and functions attributable to their small size and which may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine, and water treatment. A number of synthesis techniques have been developed including chemical reduction of silver ions in the aqueous solutions, thermal decomposition, and chemical and photo reduction. Chemical reduction is the most frequently applied method for the preparation of silver nanoparticles (AgNPs). Most of these methods are extremely expensive and they also involve the use of toxic, hazardous chemicals which may pose potential environmental and biological risks. Various chemical and physical methods are used for the synthesis of silver nanoparticles but biological methods using micro-organisms have several advantages. Extracellular synthesis could be achieved which is beneficial over intracellular synthesis. One major advantage of having prokaryotes as nanoparticle synthesizers is that they can be easily modified using genetic engineering techniques for the over expression of specific enzymes and also are easy to handle. Thus, the aim of the present study was to synthesize silver nanoparticles biologically from silver resistant species of *Pseudomonas aeruginoas* isolated from rhizosphere flora of the cactus plant. The isolate was identified, studied for its silver resistance and ability to synthesize AgNPs extracellularly. The production parameters were optimised for maximum yield.

Key words: Nanoparticles, Biological synthesis, Silver resistant, Bacterial synthesis.

Nanotechnology is an emerging field in the area of interdisciplinary research, especially in biotechnology. Since prehistoric times, among all inorganic antimicrobial agents, silver has been extensively used to resist infections. Silver nanoparticles are undoubtedly the most widely used nanomaterials amongst all; hence synthesis of silver nanoparticles nowadays is of great importance.

The synthesis of silver nanoparticles is extensively studied by using chemical and physical methods, but the development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. Currently, there is a growing need to develop environmentally safe processes that do not use toxic chemicals in the synthesis protocol. Biological synthesis process provides a wide range of environmentally acceptable methodology, low cost production and minimum time required.

A number of microorganisms including algae, bacteria and fungi have been reported for the green synthesis of silver nanoparticles. Silver nanoparticles can be synthesized from bacteria as bacterial systems are easy to handle and can be manipulated genetically without much difficulty. The nano particles produced by bacteria are extracellular, thus making them more effective.

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A quest for an environmentally sustainable synthesis process has led to few biomimetic approaches. Biomimetics refers to applying biological principles in material formation. One of the fundamental processes in the biomimetic synthesis involved bioreduction (Forough *et al.*, 2010). Biomimetic approaches involve generation of nanocrystals of silver through reduction of silver ions using bacteria and unicellular organisms. The reduction is mediated by the presence of the enzyme in the organisms and has been found to be responsible for the synthesis of nano particles. The use of specific enzyme a NADPH-dependent nitrate reductase present in the bacteria is important in the in vitro synthesis of nanoparticles.

MATERIALS AND METHODS

Enrichment and isolation of silver resistant organisms

The Nutrient Broth and Nutrient Agar devoid of NaCl were used for the enrichment and isolation of silver resistant organisms.

Sample collection

Soil sample from the rhizosphere of cactus plant was obtained. The soil sample (5g) was suspended in 10ml of sterile distilled water and the supernatant obtained was used as the sample. The sample was streaked on sterile Nutrient Agar plates containing different concentrations of $AgNO_3$ (LOBA Chemicals, AR grade), i.e 0.5mM, 1Mm and 1.5mM. The plates were incubated at RT and 37°C for 2days in dark.

Enrichment of bacteria

The isolate obtained on Nutrient Agar plate containing 1.5mM $AgNO_3$ incubated at 37°C was inoculated in the sterile Nutrient Broth containing 1.5mM $AgNO_3$ and the flask was incubated at 37°C for 24 h in dark.

Isolation of bacteria

The enriched sample was then isolated on Nutrient Agar with 1.5mM $AgNO_3$ and the plate was incubated at 37°C for 2days in dark.

Screening for producer of silver nanoparticles

- The 24hr old culture of the isolate (O.D adjusted to 0.1 units at 530nm) was inoculated in the Nutrient broth without NaCl and incubated at 37°C for 24 hrs.
- After incubation, the stock of 5 mM $AgNO_3$ was added to obtain the final concentration of 1 mM and 1.5 mM and observed for formation of brown colouration which indicates the formation of silver nanoparticles.

Identification of organisms producing silver nanoparticles

The bacterial isolate was studied for the morphological and physiological studies. The colony characteristics of the isolate were studied. Various biochemical tests were performed to identify the isolate (Bergeys Manual).

Morphological characteristic

Gram staining was performed to study the gram nature and morphology of the isolate.

Physiological characteristic

Further study was carried out by using identification flow chart by using Bergey's Manual of Determinitative Bacteriology.

Tube No.	Ag Conc (mM)	Stock (ml)	Nutrient Broth (ml)	T.V (ml)	Culture (ml)
Blank 1.0mM	1.0	1.0	4.0	5	-
Test 1.0mM	1.0	1.0	4.0	5	0.1
Blank 1.5mM	1.5	1.5	3.5	5	-
Test 1.5mM	1.5	1.5	3.5	5	0.1

Oxidase test was performed by adding a few drop of oxidase reagent (tetramethyl paraphenyl diamine dihyrocholride) to a filter paperstrip and smearing a loopful of culture on the reagent zone.

Glucose fermentation test was performed by growing the culture isolate in sterile 1% glucose in peptone water base with inverted Durham's tube and Andrade's indicator.

Pigment production and resistance to cetrimide was studied by isolating the culture on cetrimide agar plate.

Production of silver nanoparticles

Media and production conditions

- Nutrient broth (without NaCl) was used for inoculum preparation.
- 24 hr old culture O.D adjusted to 0.1 units at 530 nm in sterile Distilled water was used for inoculation.
- The cultures were grown in this broth for 24 hrs at 37°C.
- Stock of $AgNO_3$ to obtain the final concentration of 2mM and 5mM is then added to this inoculum.
- Thus, production was performed in 250 ml Erlenmeyer flask containing 100ml medium at 37°C for 4 days.

Medium optimization

The medium optimization was conducted in a series of experiments changing one variable at a time, keeping the other factors fixed at specific time conditions.

Different factors were chosen for aiming to obtain higher productivity of the AgNPs

- Concentration of $AgNO_3$: 1mM - 10mM
- Time of production: 0 h, 24 h, 48 h, 72 h, 96 h
- Temperature for production: 37°C and Room Temperature

Characterization of silver nanoparticles

The production of silver nanoparticle in the production flask was characterized by using following instruments: UV-Visible Spectroscopy and Diffraction Light Scattering Microscope.The sample is centrifuged at 10,000 rpm for 15 mins before analyzing the sample in these instruments. Clear brown supernantant was analysed.

UV-Visible Spectroscopy (EQUIP-TRONICS, Model No. EQ-825)

The silver nanoparticles were characterized by UV-Visible Spectroscopy. After 24 hrs interval the clear supernatant was analysed under UV-Visible spectroscopy subsequently measuring UV-Visible spectra, at the wavelength of 350nm to 500nm.

Diffraction Light Scattering Microscope

The clear supernatant after 24 hrs interval was analysed to check the particle size under DLS. Before analyzing, the supernatant was passed from 0.2 micron Syringe filter to separate the cell debris if present.

RESULTS AND DISCUSSION

Enrichment and isolation of silver resistant organisms

The Nutrient Agar plate containing 1.5 mM $AgNO_3$ incubated at RT showed 1 isolate and the plate incubated at 37°C showed almost 2 different isolates.

The nitrate reductase is essential property for metallic reduction. The isolate which was showing this property was selected for further enrichment. Thus, isolate 1 obtained on plate incubated at 37°C which was dark brown in colour and showing metallic sheen was selected.

The selected isolate, inoculated in the enrichment medium showed brown colour in the enrichment flask. Further the enriched medium was isolated on the Nutrient agar plate containing 1.5 mM $AgNO_3$ to obtain pure culture. After incubation at 37°C for 2 days, pure colonies were obtained on Nutrient Agar plate containing 1.5mM $AgNO_3$.

Brown colouration was observed in the test tubes Test 1.0mM and Test 1.5mM indicating the formation of silver nanoparticles (AgNPs). The characteristic brown color of the solution is due to the excitation of Surface Plasmon vibrations (essentially the vibration of the group conduction electrons) in the silver nanoparticles (Vahabi K et.

Table 1. Morphological and cultural characteristics of the isolates

Characters	Isolate 1 (37°C)	Isolate 2 (37°C)	Isolate 1 (RT)
Size	3mm	5mm	1mm
Shape	Circular	Circular	Circular
Colour	Dark brown	Cremish brown	White
Margin	Irregular	Irregular	Irregular
Elevation	Convex	Low convex	Flat
Opacity	Opaque	Opaque	Opaque
Consistency	Sticky	Butyrous	Butyrous
Gram nature	Gram negative coccobacilli	Gram positive bacilli	Gram positive cocci

al, 2011). On the other, the blank tubes without the addition of culture showed no formation of brown colour, which indicates that the colour change, is due to presence of organisms.

Identification of organisms producing silver nanoparticles

The isolate obtained was identified and characterized by morphological and physiological characteristic, by using Bergey's Manual of Determinative Bacteriology.

Physiological characters of isolate were obtained based on the Gram nature and morphology.

Oxidase test

As the isolate is gram negative coccobacilli, it was observed for the Oxidase test. If the Oxidase test is positive it is an indicative of *Aeromonas, Vibrio* or *Pseudomonas* species. If the test is negative then it belongs to Enterobacteriacea and then further processed depending on the results.

As Oxidase test showed the deep blue colour development at the inoculation site within 10 seconds, indicating the positive test, the isolate obtained may belong to either of three *Aeromonas, Vibrio* or *Pseudomonas* species.

Sugar fermentation test

Sugar fermentation test with glucose is performed to further differentiate between the 3 species. If glucose fermentation gives the positive results then the isolate belongs to *Aeromonas* or *Vibrio* species. If the test is negative then the isolate belongs to *Pseudomonas* species.

Glucose fermentation test showed negative results which indicates that the isolate belong to the *Pseudomonas* species.

Table 2. Screening for producer of silver nanoparticles

Tube No.	Observations
Blank 1.0mM	No brown colour
Test 1.0mM	Brown colour
Blank 1.5mM	No brown colour
Test 1.5mM	Brown colour

Table 3. Biochemical tests for *P. aeruginosa*

Test	Results
Glucose formentation	-
Indole test	-
Methyl Red test	-
Voges Proskauer test	-
Citratase test	+
Urease test	+
Nitrate peptone test	+
Gelatinase	+
Phenyl pyruvic acid test	-
TSI:	
Butt	Alkaline
Slant	Alkaline
H_2S	-
Gas	-
Oxidative fermentation test	+

+Positive test, - negative

Fig. 1. Isolates obtained on Nutrient agar plate containing 1.5 mM $AgNO_3$ incubated at 37°C

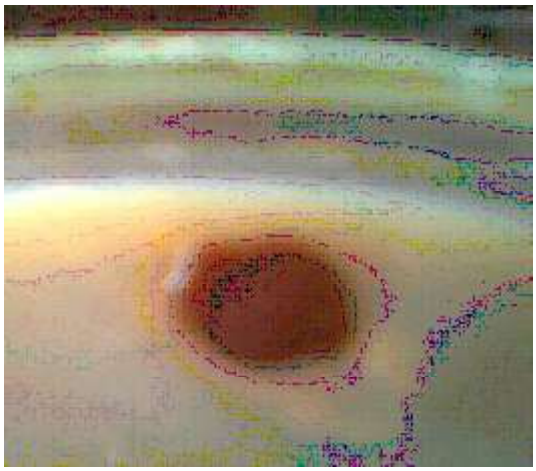

Fig. 2. Selected isolate

Fig. 3. Pure colonies obtained on Nutrient agar plate containing 1.5 mM $AgNO_3$

Pigment production

The isolate belonging to *Pseudomonas* species was further checked for pigment production. The isolate was isolated on Cetrimide Agar. It was found that the isolate showed production of pyocyanin pigment (bluish green pigment).

Biochemicals for *Pseudomonas aeruginosa*

Production of silver nanoparticles

During the production period of 4 days, the colour change from pale yellow to brown indicates reduction of metallic silver.

The appearance of brown color indicates that the formation of silver nanoparticles in the reaction mixture and the efficient reduction of the Ag ions occurs extracellularly than the intracellular

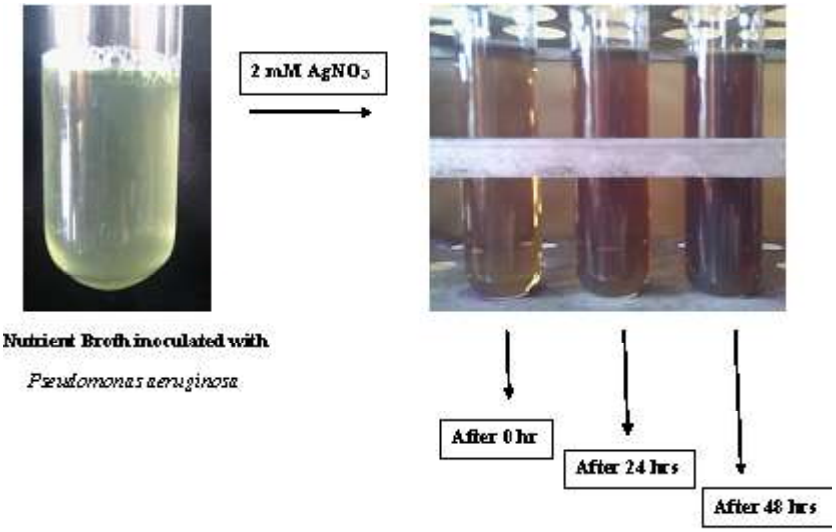

Fig. 4. Production of AgNPs, colour change observed during the production period

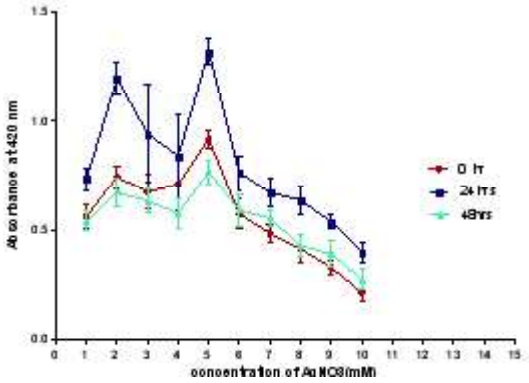

Fig. 5. Graph of concentration of $AgNO_3$ in AgNPs production v/s Absorbance at 420 nm

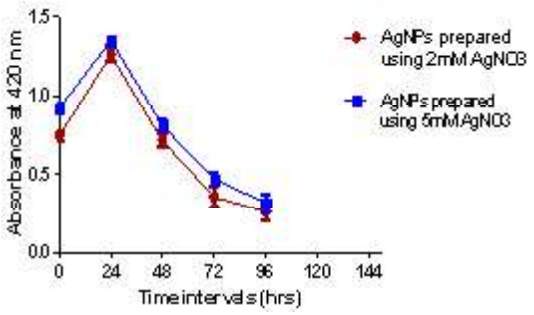

Fig. 6. Time of production and its respective absorbance at 420 nm

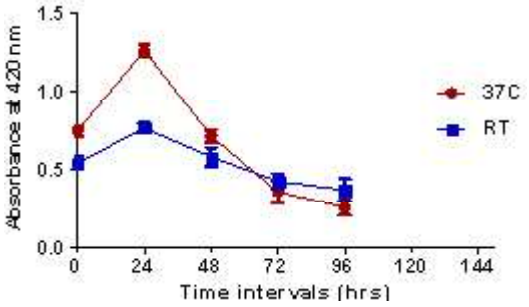

Fig. 7. Effect of temperature on AgNPs production using 2 mM $AgNO_3$

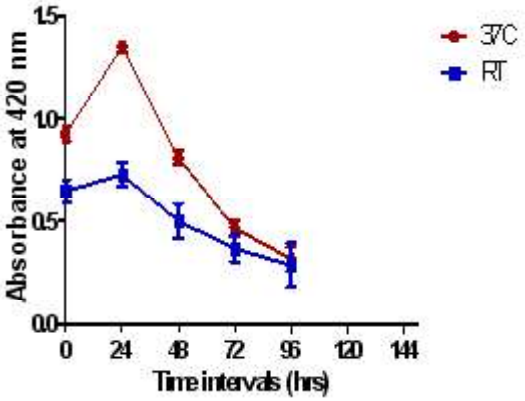

Fig. 8. Effect of temperature on AgNPs production using 5 mM $AgNO_3$

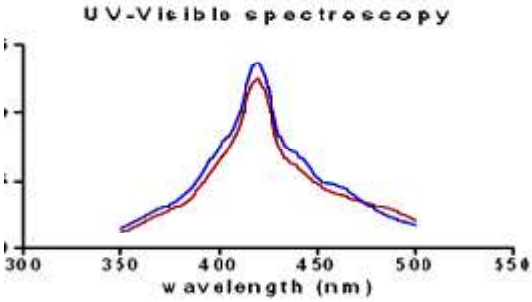

Fig. 9. Graph of the samples prepared using 2 mM and 5 mM of $AgNO_3$ analyzed under UV-Visible spectroscopy (24 hr old sample)

(pellet material) culture (Kannan *et al.,* 2010). Hence, the results obtained shows that *Pseudomonas aeruginosa* produces AgNPs extracellularly which offers a great advantage over an intracellular process of synthesis from application point of view. The nanoparticles formed inside the biomass would have required additional step of processing for the release of the nanoparticles from the biomass by ultrasound treatment or by reaction with suitable detergents.

It is indicated that nicotinamide adenine dinucleotide, reduced form (NADH) and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles (Maliszewska *et al.,* 2009).

Medium optimization

Optimization of various parameters namely the concentration of $AgNO_3$, time of

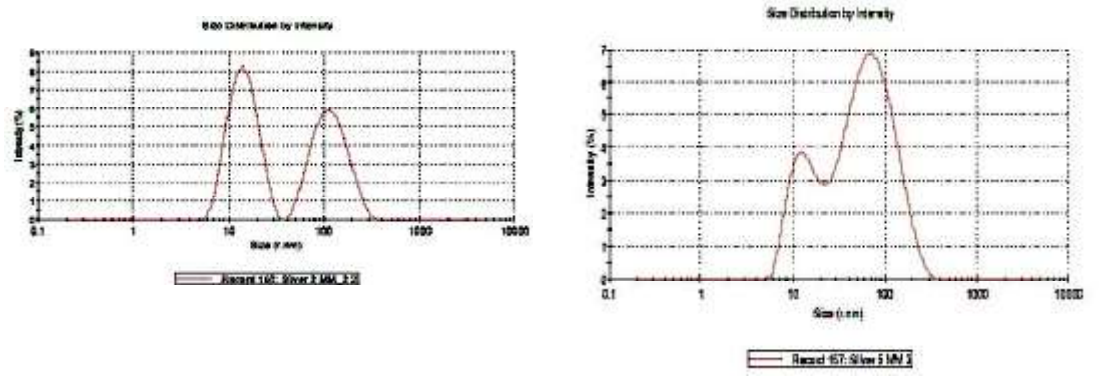

Fig. 10: DLS Spectra

production, temperature for production were studied by conducting a series of experiments changing one variable at a time, keeping the other factors fixed at specific time conditions.

Concentration of $AgNO_3$

From the above result it was concluded that the production of AgNPs increases with the increase in concentration of $AgNO_3$ up to 2 mM. However, with the further increase in concentration of $AgNO_3$, the AgNPs production decreased. But high production of AgNPs was obtained with 5mM concentration of $AgNO_3$.

The results obtained were in accordance with the study (Nayak, 2011), that used *P. purpurogenum* for AgNPs production. It was observed that the AgNPs formation increases with increase in substrate concentration up to 1.5 mM. With further increase in substrate concentration, nanoparticle formation did not increase within 24 h. But appreciable amount of silver nanoparticle was obtained with as high as 5 mM substrate concentration.

Hence, 2mM and 5mM concentration of $AgNO_3$ is selected for further AgNPs production.

Time of production

Fig 10 show that the optimum time of AgNPs production was found to be 24 hours for both AgNPs solution prepared using 2 mM and 5 mM $AgNO_3$. After 24 hrs, there was a decrease in AgNPs production. The decrease in production may be due to aggregation of nanoparticles or may be due to inactivation of the enzyme (Saiffudin *et al.,* 2008).

With increasing reaction time, there is a decrease in absorbance on the UV-visible spectrum. This is mainly due to the fact that the particles grow with time and that longer reaction time promotes the particle aggregation to form larger particles, leading to a decrease in the synthesis of nanoparticles.

Temperature for production

The high reaction temperature resulted in decrease of absorbance, indicating that the size of silver nanoparticles becomes larger with the increase of reaction temperature.

The study (Veerasamy *et al.,* 2011), showed that as the temperature increased, the rate of silver nanoparticles formation also increased. The size is reduced initially due to the reduction in aggregation of the growing nanoparticles. Increasing the temperature beyond a point (75 °C) aids the growth of the crystal around the nucleus which leads to decrease in absorption.

Hence, the results are in accordance with the previous studies and optimum temperature was found to be 37°C for both AgNPs solutions prepared using 2 mM and 5 mM of $AgNO_3$.

Silver nanoparticle characterization

UV-Visible Spectroscopy

Preliminary characterization was done using UV-Visible spectrophotometer. UV-Visible spectra obtained after analyzing clear brown supernantant obtained after the centrifugation of the samples (AgNPs solution prepared using 2 mM and 5 mM $AgNO_3$) at 10,000 rpm for 15 mins, showed a strong peak at 420 nm which is characteristic for Surface Plasmon resonance of silver nanoparticles. The results obtained are in accordance to previous report of biologically synthesized AgNPs using *Bacillus licheniformis* (K. Kalimuthu *et al.,* 2008), *Lactobacillus fermentum* (Sintubin *et al.,* 2009), *Bacillus subtilis* (Saiffudin *et al.,* 2009), *Pseudomonas aeruginosa* (Thirumurugan *et al.,* 2010), *Fusarium oxysporum* (Duran *et al.,* 2007) and *Aspergillus flavus* (Vigneshwaran *et al.,* 2007) where in the absorbance peaks were obtained in the range of 400 to 440.

Diffraction light scattering microscope

The nanoparticles were then analysed for particle size. Size of the nanoparticle was analyzed using Diffraction Light Scattering microscope. The results obtained:

- 2 types of AgNPs were produced using 2 mM of $AgNO_3$: 15nm and 120nm
- 2 types of AgNPs were produced using 5 mM of $AgNO_3$: 15 nm and 80nm

Two kinds of nano particles were obtained, most of the nanoparticles were spherical in shape and had a size of 15 nm and 80nm.

CONCLUSION

Physical and Chemical methods of synthesis of AgNPs are widely used but there has been growing interest in developing biological synthetic protocols for these particles which offers several advantages. This project is focused on the use of bacteria for the synthesis of silver nanoparticles, a low cost approach for reducing silver nirate solution to form nanoparticles.

In the present investigation, the isolation of the silver resistant organisms from the soil sample was carried out and the isolate was enriched in media supplemented with 1.5 mM of silver nirate. The isolate was further isolated on selective media. The isolate showing dark brown colour with metallic sheen and resistance to 1.5 mM of silver nitrate was selected. Identification of the isolate was then carried out by studying morphological and biochemical characteristic like Gram staining, Oxidase test, Sugar fermentation test. The isolate was characterized as *Pseudomonas aeruginosa.*

The isolate was used for nanoparticle production for which medium optimization was carried out. The effect of varying concentration of silver nitrate, time of production and temperature for production was studied for the maximum production of silver nanoparticles.

The concentration of 2 mM and 5 mM of $AgNO_3$ gave maximum production of AgNPs. Effect of time was also analysed for 5 days and the effect of temeperature was studied. The optimum time for production was found to be 24 hrs and temperature for the production was 37°C.

After 24 h interval the sample was centrifuged and the clear supernatant was analyzed under UV-Visible spectroscopy. The visible colour change from pale yellow to brown colour in the reaction flask is an indication of nanoparticle production. Structural characterization of silver nanoparticles was done using UV-Visible spectroscopy and Diffraction Light Scattering microscope. The nanoparticle showed a strong SPR at 420 nm. The particle size was determined by DLS. Size of the particle was analyzed using Diffraction Light Scattering microscope. Two types of AgNPs were produced using 2 mM of $AgNO_3$: 15nm and 120nm and two types of AgNPs were produced using 5 mM of $AgNO_3$: 15 nm and 80nm.

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