Fascinating approach for using metabolites products of living microorganisms as reducing agents for preparing silver nanoparticles

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Abstract
A crucial area of research in nanotechnology is the formation of environmentally benign nanoparticles. Both unicellular and multicellular play an important role in synthesis nanoparticles through the production of inorganic materials either intracellularly or extracellularly. The agents (pigments, siderophores, cell extracted metabolites and reducing compounds) were used to prepare silver nanparticles with different sizes and shapes. The color variations (dark yellow, slightly dark yellow and golden yellow) arising from changes in the composition, size, and shape of nanoparticles, surrounding medium can be monitored using UV-visible spectrophotometer. These effects are due to the phenomena called surface plasmon resonance. The silver nanoparticles have Plasmon resonances ranged between (390, 383 and 365) nm which they are among the limitation of silver nanoparticles (360 – 420 nm). AFM analysis of Ag NP's showed partially purified big triangular Ag NP having edge length around ~1. µm. Hexagonal particles on the background of a matrix made up of some molecules which may be metabolites products are found. Small spherical nanoparticles embedded in some kind of matrix indicate that this molecule acts as capping agent, which inhibits further growth of nanoparticles. Also ribbons like structures of width around 50 nm which are intertwined are the noble and rare structures which are synthesized by this method. MIC of silver Nanoparticles for. E coli, ranges between 80-90 µg/ml.., Serrtia, ranges between 50-60 µg/ml and Shagilla, ranges between 90-100 µg/ml.

Keywords: Metabolites product of microorganisms, silver nanoparticles

Introduction

Nanotechnology indicates as many processes which are design, creation, synthesis, manipulation of matter at the nanometer scale (1–100 nanometers) and the exploitation of novel phenomena and properties of matter at that scale.[1,2] A new dimension of particles can be created by the field of nanotechnology with varied physical, chemical and biochemical properties.[4, 14, 21] There are two approach mainly used for preparation nanoparticles which are top–down and bottom–up. Slicing the bulk material into pieces known as top-down.[3, 5] whereas controlling condensation of solute molecules that get formed during a chemical reaction defined as bottom-up.[6, 12]

Many applications are affiliated with using the technology of nanoparticles which are drug delivery.[7] monitoring live cell interactions, and cell imaging .[8] and as biosensors.[9]

goals of research

1. The use of bacteria for synthesis of metal nanoparticles is not new for nanotechnology field, but synthesis using bacterial metabolites like pigments, siderophores, and cell extracted metabolites is novel. The pigments from Serritia marcescens, P. aeruginosa and fungi are a secondary metabolite compounds and are associated with stationary phase of growth. Pigments are a chloroform-soluble compound that reacts with molecular oxygen to form superoxide, hydrogen peroxide and hydroxyl radicals. Because of its redox cyclic nature, pigments have a broad range of biological activity, including bactericidal properties. Due to its structural and functional properties we speculated that it can also be useful in reduction of metals.

2. Synthesis by using different metabolites and reducing agents functional groups of metabolites product can act as reducing agents, and used to reduce Ag+ ions to synthesize stable silver nanoparticles in water. We are thus reporting facile synthesis of nanoparticles capped with different metabolites and reducing agents where those metabolites product acts as capping as well as reducing agent. Since these nanoparticles are synthesized in aqueous medium and thus can have various biological applications.

3. Nanobiotechnology provides different approaches for synthesis of nanomaterials, which are simpler than physical and chemical methods. Chemical methods for preparing nanoparticles are energy intensive, requires stringent synthesis protocols. In contrast biological synthesis require simple biological system (e.g. microorganisms or their products) as well as it occurs at room temperature and neutral pH.

Material and Method
Pigment

1. **prodigiosin pigment:** Serritia marcescens was grown in Luria broth (0.5% glucose, 0.3% tryptone, 0.1% yeast extract, 0.1% NaCl, pH6) for 48 hours at room temperature on shaker (150rpm). The culture suspension was then centrifuged at 9000 rpm for 15 minutes to separate biomass. The supernatant was used

for further experimentation. The supernatant was centrifuged at 3000 rpm for 20 min. then, the supernatant was decanted and digested with double volume of 1 N NaOH then was kept in boiling water bath for 1 hours. Absolute ethanol was added and centrifuged at 3000 rpm for 20 min. The supernatant was decanted and was dissolved with equal volume of petroleum ether then was allowed to settle for 10 min. Finally the upper layer was collected in evaporating dish then was heated till dry residue remain and the residue was dissolved in 5 ml of acidified ethanol The methanol extract was then reacted with 1mM AgNO3.

2. **Pyocyanin pigment:** Pseudomonas aeruginosa was grown in Luria broth (0.5% glucose, 0.3% tryptone, 0.1% yeast extract, 0.1% NaCl, pH6) for 48 hours at room temperature on shaker (150rpm). The culture suspension was then centrifuged at 9000 rpm for 15 minutes to separate biomass. The supernatant was used for further experimentation. Chloroform is added to the supernatant in 1:1 proportion. This mixture was then kept on shaker, overnight to allow extraction of pigment from aqueous phase to organic phase. The two phases were separated using separating funnel. Chloroform was evaporated and the residue was dissolved minimum quantity of methanol. The methanol extract was then reacted with 1mM AgNO3.

Siderophore

Pseudomonas aeruginosa was grown in King's B medium (2% Peptone, 1%Glycerol, 0.15 %Magnesium Sulfate, 0.15% Dipotassium hydrogen orthophosphate) for 48 hours at room temperature and on shaker (150 rpm). The culture suspension was then centrifuged at 9000 rpm for 15 minutes to separate biomass. The supernatant was then used for further experimentation.

The pH of the supernatant was checked. This supernatant without any processing was then mixed with 1mM AgNO3. To confirm that the supernatant is having some molecule or molecules that are responsible for reducing silver, control reaction was carried out. The reaction of Sterile Luria broth at pH 10 (same as that of supernatant), with 1mM AgNO3 was performed.

Then the supernatant was used to extract siderophore. The pH of the supernatant was made 2 by adding concentrated HCl to it. Then absolute ethyl acetate was added in 1:1 ratio to the acidified supernatant. This mixture was then kept on shaker overnight to allow extraction of siderophore from aqueous phase to organic phase. The two phases were separated using separating funnel. Ethyl acetate was evaporated and the residue was dissolved in minimum quantity of methanol.

Synthesis of silver nanoparticles

1. 10 ml of aqueous AgNO3 (10 – 3 M), 200 ML of (Pigments extracted or siderophore or cell extacted metabolites or reducing compounds - like Amp -.) were mixed

2. 10 ml of aqueous AgNO3 (10 – 3 M), 10 ml of aqueous Amp. (10 –3 M) and 200 ML of (Pigments extracted or siderophore or cell extracted metabolites -.) were mixed

3. 10 ml of aqueous AgNO3 (10 – 3 M), 200 ML of (different metabolites products) were mixed.

The color of the solution changed to golden yellow, indicates the formation of silver Nanoparticles. The solution was allowed to cool to room temperature.[12, 13]

Chemical characterization

1. **Changing the color of solution:** changing color of solution to dark yellow means silver Nanoparticles are formed

2. **UV-Visible spectroscopy studies:** The reduction of AgNO3 to silver Nanoparticles was monitored by UV-NIR spectroscopy.[7, 19, 20, 21]

Biological characterization

Concentration dependent antimicrobial studies were performed by checking Minimum Inhibitory Concentration (MIC) for organisms using nanoparticles. MIC is defined as a lowest concentration of an antimicrobial compound that will inhibit visible growth of microorganisms after overnight incubation.[18]

Here, MIC was determined using broth dilution test which employs serial two fold dilutions between selected maximum and minimum concentration of antimicrobials that will prevent growth of microorganisms after subculture onto antibiotic free media.

MIC studies for *E.coli*, *shiglla* and *Serria* were performed in nutrient broth. Stock solution of Nanoparticles was made. dilutions were made by adding sterilized nanoparticles to

sterile broth. Concentration of Nanoparticles in each tube was differed. 100ul of inoculum was added to each tube & was incubated on shakes at 37C. After 24 hours incubation, turbidity was checked and MIC end point was read as lowest concentration of nanoparticles at which there is no visible growth.[14]

Results
Characterization studies
[A] Chemical Characterization

After reduction reaction took place in the above mentioned reactions their chemical characterization was done as following:

Changing the color of solution

The colour of the solution changed to yellow after few minutes (but the intensity of color increased with increasing the time of reaction), indicates the formation of silver

nanoparticles. The indication for production of silver nanoparticles was by detection change in color of solution from white to (slightly dark yellow) (golden yellow) (dark yellow)

The difference in the color of reaction leads to form silver nanoparticles with different sizes and shapes as elucidate in fig. (1, 2, 3,).
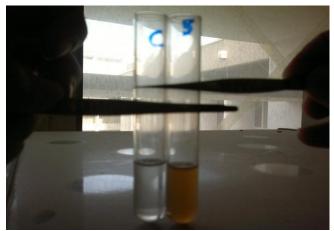

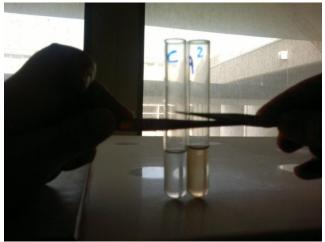**Fig. 1: Showing change in color of solution from white to (dark yellow) as indication of formation of Nanoparticles**

Fig. 2: Showing change in color of solution from white to (golden yellow) as indication of formation of Nanoparticles.
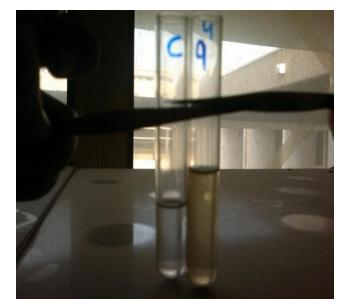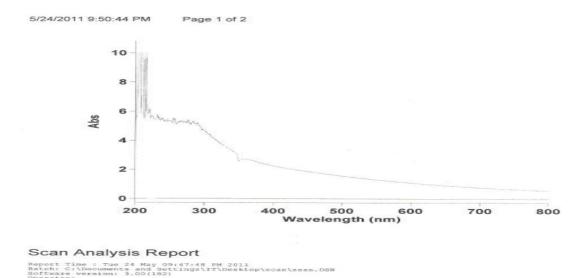

Fig. 3: Showing change in color of solution from white to (slightly dark yellow) as indication of formation of Nanoparticles

UV-Visible spectroscopy analysis:
The reduction of AgNO3 to silver Nanoparticles was monitored by UV-NIR spectroscopy

Through the observing that Resonance is observed when the dielectric constant of metal is equal to twice the dielectric constant of the medium. Silver particles surface plasmon resonance in the visible range was around 390 nm., 383 nm. and 365 as elucidate in the figure (4, 5, 6,)

Meanwhile the. UV-NIR spectroscopy to (AgNO3 alone) as control was elucidate as in fig. (7) absence of peak around 360 -420 nm

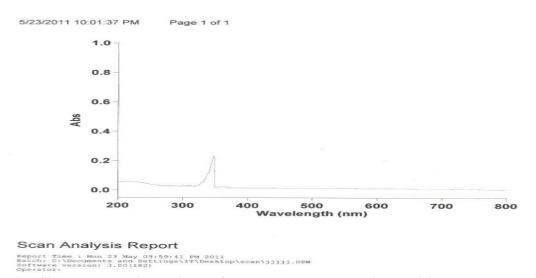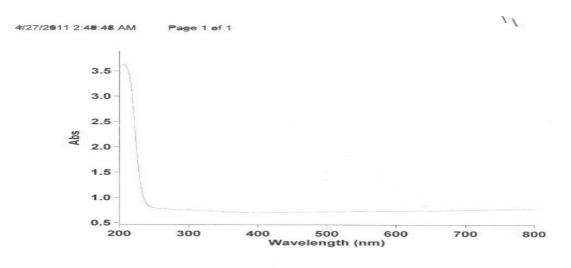5/24/2011 9:50:44 PM   Page 1 of 2

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Fig. 4: Showing Silver nanoparticles with surface plasmon resonance in the visible range around 390 nm

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Fig. 5: Showing Silver nanoparticles with surface plasmon resonance in the visible range around 380 nm

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Fig. 6: Showing Silver nanoparticles with surface plasmon resonance in the visible range around 365 nm

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Fig. 7: Showing Silver nitrite as control
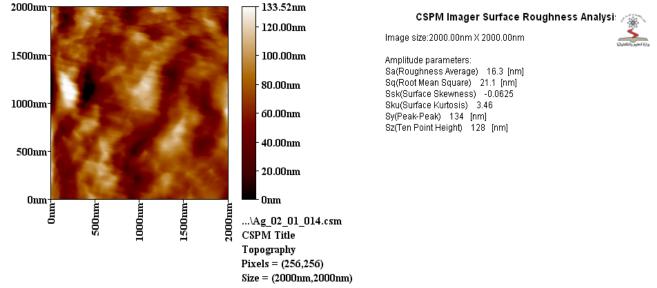
3 - AFM Images

Fig 8: Showing AFM analysis of silver Nanoparticles with partial purified versatile structures like big irregular NP's of edge length 300 – 500 nm and smaller quasispherical NP's. Due to higher magnification, visualization of individual irregular NP of size ~ 200 nm is possible. Ribbons like structures of width around 500 nm which are intertwined are the noble and rare structures which are synthesized by this method. (Fig. one dimension)
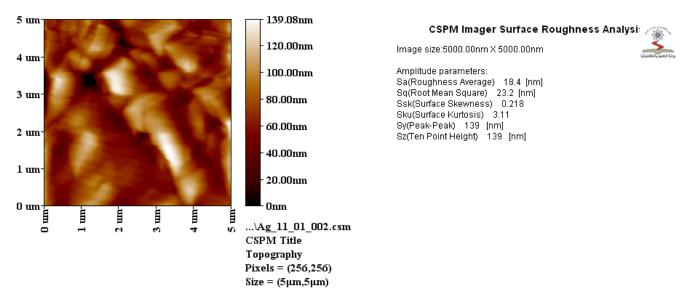
CSPM Imager Surface Roughness Analysi:

Image size:5000.00nm X 5000.00nm

Amplitude parameters:
Sa(Roughness Average)   18.4 [nm]
Sq(Root Mean Square)   23.2 [nm]
Ssk(Surface Skewness)   0.218
Sku(Surface Kurtosis)   3.11
Sy(Peak-Peak)   139 [nm]
Sz(Ten Point Height)   139 [nm]

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CSPM Title
Topography
Pixels = (256,256)
Size = (5μm,5μm)

Fig. 9: showing AFM analysis of Ag NP's with partially purified big triangular Ag NP having edge length around ~1. μm. Hexagonal particles on the background of a matrix made up of some molecules which may be metabolites products are found. Small irregular nanoparticles embedded in some kind of matrix indicate that this molecule acts as capping agent, which inhibits further growth of nanoparticles. (fig. two dimention)
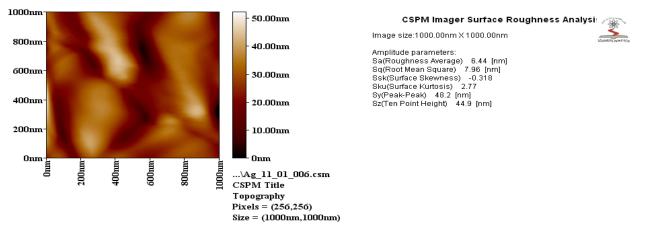

CSPM Imager Surface Roughness Analysi:

Image size:1000.00nm X 1000.00nm

Amplitude parameters:
Sa(Roughness Average)   6.44 [nm]
Sq(Root Mean Square)   7.96 [nm]
Ssk(Surface Skewness)   -0.318
Sku(Surface Kurtosis)   2.77
Sy(Peak-Peak)   48.2 [nm]
Sz(Ten Point Height)   44.9 [nm]

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CSPM Title
Topography
Pixels = (256,256)
Size = (1000nm,1000nm)

Fig. 10: showing AFM analysis of Ag NP's with partially purified big triangular Ag NP having edge length around ~1. μm. Hexagonal particles on the background of a matrix made up of some molecules which may be metabolites products are found. Small irregular nanoparticles embedded in some kind of matrix indicate that this molecule acts as capping agent, which inhibits further growth of Nanoparticles. (fig. one dimention).

Biological Characterization

Minimum Inhibitory Concentration (MIC)
Minimum inhibitory concentration of silver Nanoparticles was determined using *E.coli*, *Serritia*, and *Shagilla*.
Following are the figures of MIC experiments carried out with silver anoparticles

Results

MIC of silver Nanoparticles for *E.coli*, ranges between 80-90 μg/ml.
MIC of silver Nanoparticles for *Serritia*, ranges between 50-60 μg/ml.
MIC of silver Nanoparticles for *Shagilla*., ranges between 90-100 μg/ml.

Discussion

Applications of biology in nanotechnology have led to synthesis of very varied but novel materials. Biological entities like bacteria, fungi have given us a new way to look towards synthesis of nanomaterials. They provide us with a cheaper and better alternative to existing methods. Though a lot is still to be understood especially regarding the exact mechanism of synthesis, it has opened new frontiers integrating all fields of science like molecular biology, genetics, material science and physical chemistry etc. Here we report synthesis of a very important nanomaterial, which is silver Nanoparticles by using (Pigments extracted or siderophore or cell extacted metabolites or reducing compounds - like Amp), as those compounds have ability to work as reducing agent against metal therefore we speculate

to have a role in converting the metal to nano scale through the process of reduction and nucleation then aggregation then forming different size and shape of nanoscale depend on condition of biological forming and concentration of reduction agents and the metals .

This particles also showed very effective way as antimicrobial agent. The characterization of solution was monitored and indicated that silver nanoparticles were produced from mixing AgNO3 and different metabolites products or reducing agents and metabolites products. The color change from white to (slightly dark yellow or dark yellow or golden yellow) as indication of formation of silver Nanoparticles also the Plasmon resonance of particles in solution was monitored and it was 390 nm, 383 nm and 365 nm which refers to silver nanoparticles as the range of Plasmon resonance a range (360 – 420), it depends on shape and size of forming silver nanoparticles .The silver nanparticles were partially purified and it needs more purification work as we visualized that from Plasmon resonance of particles which indicate the solution was contained particles with Plasmon resonance a round (200-300 nm) .

The reason for that is not all bulk metals reduced by (Pigments extracted or siderophore or cell extacted metabolites or reducing compounds - like Amp) and it needs sophisticated purtification technique to separate the nanoparticles from others – AFM analysis of Ag NP's was partially purified big triangular Ag NP having edge length around ~1. μm. Hexagonal particles on the background of a matrix made up of some molecules which may be metabolites products are found. Small spherical nanoparticles embedded in some kind of matrix indicate that this molecule acts as capping agent, which inhibits further growth of Nanoparticles AFM analysis of silver nanoparticles also showed partial purified versatile structures like big irregular NP's of edge length 300 – 500 nm and smaller s quasispherical NP's. Due to higher magnification, visualization of individual irregular NP of size ~ 200 nm is possible. Ribbons like structures of MIC of silver Nanoparticles for *E.coli*, ranges between 80-90 μg/ml.and *Serritia*, ranges between 50-60 μg/ml. *Shagilla*, ranges between 90-100 μg/ml which indicate the efficiency of Nanoparticles as antimicrobial agents

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