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Biosynthesis of Different Sizes of Silver Nanoparticles by Bacteria Screened from Cultivated Soil

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Abstract: ***Biosynthesis of metal nanoparticles (NPs) is an emerging area in the field of nano-biotechnology due to their unique properties and potential applications. Bacteria which produce extracellular stable metallic silver NPs were successfully enriched from cultivated soil, Kingdom of Bahrain, in the presence of $AgNO_3$ (100 mM). The toxic Ag^+ ion was reduced to non-toxic Ag^0 NPs through bacterial nitrate reductase. When examined by UV-vis spectrophotometer, the silver NPs showed maximum peak at 421 nm which remained stable for several months. The presence of those highly stable silver NPs were confirmed by XRD analysis, optical microscope, SEM and EDS. The bacterial activity was followed for 25 days in the presence of different concentrations of silver nitrate (1-256 mM). Different sizes of silver NPs ranging between 5 to 30 nm were obtained. It was found that the size of the NPs can be controlled by changing the concentration of $AgNO_3$.***

Keywords: Biosynthesis, Silver Nanoparticles, Bacteria, Uv-vis spectroscopy.

1. Introduction

There is growing interest in the area of nanoparticles (NPs) production due to their unique physical and chemical properties. These properties are unique to materials at the nano scale (1-100 nm) compared to their counterpart at bulk with larger size, which cause morphological and functional differences [1], [2]. NPs have catalytic activity, optical, electronic, mechanical, antimicrobial and magnetic properties [3], [4]. Therefore, metal NPs could be used for catalytic, electronics, environmental, medical and biotechnological purposes [5]. Because of the leading role of metal NPs in industry, various chemical methods were used to reduce the noble metals to NPs of different shapes and size [1], [6]. The synthesis of NPs via chemical methods is not recommended as they are laborious, time consuming, expensive and harmful. In addition, those chemical methods are ineffective in production of NPs at high concentrations of reagents and metal precursors, thus, they are not suitable for large scale production [6]. Therefore, there is a need to use a method superior to those chemical methods for NPs production. Biological methods for NPs production have attracted a wide attention as they are safe (not producing toxic compounds during the process), cost effective, requires minimum time and is close to principles of nature [7]-[9]. Biosynthesis of NPs is a biomineralization process which depends on living organisms such as bacteria, fungi, yeasts, algae and higher plants. These organisms were used to precipitate NPs of Silver, Gold, Platinum, Manganese, Selenium, Iron, etc [8]-[11].

Biosynthesis of metallic NPs especially Ag has been well studied for different applications. Different bacterial strains have been used to produce NPs with reasonable properties [12], [13]. Although metallic silver is rarely available in nature, few amount of nanosilver could be enough to be used for its applications. The property of high surface area to volume ratio of silver NPs can increase its activity even at low concentration [14]. Silver NPs have unique physical and chemical properties which differ from the bulk silver [14]. One of the important properties of metal NPs is the surface plasmon resonance known as SPR phenomenon (the fluctuation of conduction of electrons when they interact with electromagnetic field at certain wavelengths) causing its displacement from the equilibrium position around the positive core, and generating strong electric field on the surface of the NPs, leading to the appearance of a characteristic peak in UV-vis spectroscopy [15]. This feature provides silver with very useful electrical and optical properties for several sensing and imaging applications [14]. Another important property of silver NPs is the antimicrobial activity. The antibacterial activity of silver NPs is widely studied recently as it is more effective than other NPs [16]. It inhibits the growth of microorganisms more effectively than bulk silver due to their nanosize [14]. Silver NPs kill a wide range of Gram positive, Gram negative and antibiotic resistant bacteria. Moreover, they enhance the effectiveness of several antibiotics [14]. The antimicrobial activity of silver NPs can also affect fungal species, such as *Aspergillus* sp., *Candida* sp., *Saccharomyces* sp. [14] and *Penicillium* sp. [17].

Nitrate dependent reductase (NR) is responsible for the reduction of Ag^+ ions and the subsequent formation of silver NPs (Figure 1) [18], [19]. NR (EC 1.6.6.1-3) catalyzes NAD(P)H reduction of nitrate to nitrite. Extracellular NR and NR in the periplasmic space are responsible for the reduction of Ag into metallic NPs [20]. Boopathi et al [20] have found spherical silver NPs were surrounded by protein and were accumulated in the periplasmic space, indicating

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the involvement of the periplasmic nitrate reductase in the biosynthesis of silver NPs.

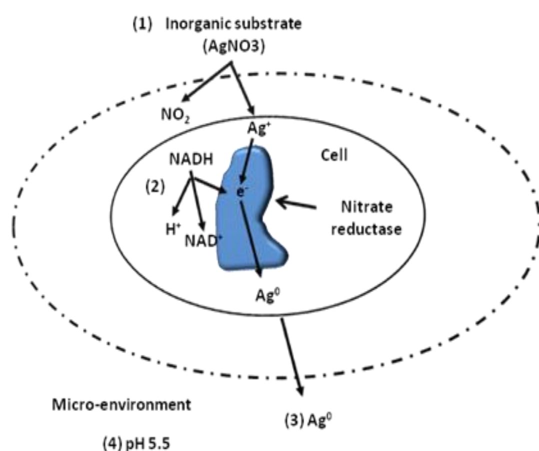

Figure 1: Possible mechanism for silver NPs synthesis using *Bacillus licheniformis,* modified from Kalimuthu et al. [18] and Durán et al. [19].

The present study aims to (1) design a rapid, non-toxic and cost effective method to enrich for bacteria produce Ag NPs from cultivated soil, (2) examine the biosynthesized Ag NPs by UV-vis spectroscopy, (3) confirm the formation as well as characterize the size and shape of Ag NPs by SEM, EDS and XRD and (4) Follow up the activity of the bacteria producing Ag NPs via UV-vis spectroscopy at different concentrations of the precursor $AgNO_3$.

2. Materials and Methods

2.1 Enrichment of Bacteria that synthesize silver NPs

Potential microbial Ag NPs precipitating bacteria with high stability were enriched from cultivated soil (Sakhir, Kingdom of Bahrain). One gram of soil was placed in 100 ml of a growth medium (250 ml shaking flasks, at 35°C, for 72 h). The enrichment medium consisted of 10 $g.L^{-1}$ Yeast extract (YE), 100 mM $AgNO_3$, 152 mM ammonium sulphate and 100 mM sodium acetate.

2.2 Silver NPs Precipitation by Bacteria

The bacterial culture was mixed with $AgNO_3$ to a final concentration of 50 mM $AgNO_3$. Immediately, 20 µl of this mix was placed on a microscopic slide and then covered with a coverslip. To avoid the dryness of the sample on the microscopic slide, the edges of the coverslip were sealed with nail polish. Silver crystals formation was examined (immediately and after 24 hours) by a compound microscope (BX51) fitted with a DP70 Digital Camera.

2.3 UV-vis spectrophotometer, SEM and XRD Examinations

Bacterial culture grown on 100 mM $AgNO_3$ (3 ml) was examined by UV-vis spectrophotometer (Spectronic Unicam) after being well mixed. The experiment was repeated three times against a control in the absence of $AgNO_3$.

Subsequent to growing the bacteria to produce silver NPs, 10 ml of suspension was dried in a desiccator for one week, and then the dried NPs were placed on aluminium stubs using "Carbon Tabs" (Agar Scientific). The stubs were then placed in the device and were dried by vacuum. The samples were subjected to SEM examination (SEM-Zeiss EVO LS 10, at Central Labs of University of Bahrain). Energy Dispersive X-ray Microanalysis of Ag NPs was carried out using micro-analyser (EDS Bruker AXS Microanalyzer).

Dried $AgNO_3$ samples were analyzed by powder X-ray diffraction (XRD) using a Rigaku Ultima-IV diffractometer equipped with Cu α-radiation ($\lambda = 1.5418$ Å) from $2\theta = 30°$ up 80°, with step size of 0.04°, voltage of 40 kV, current of 40 mA, power of 1.6 kW and counting time of 1.0 seconds.

The Effect of different concentrations of $AgNO_3$ on bacterial activity and size of silver NPs formation.

The bacteria were grown on different concentrations of AgNO3 from 1 to 256 mM. Each sample (3 ml) was examined by UV-vis spectroscopy (340-600 nm). The samples were mixed well to avoid precipitation of NPs. Replicates were applied.

3. Results and Discussion

3.1 Enrichment and silver NPs precipitation

The enrichment culture with 100 mM $AgNO_3$ showed a change in color from yellow to dark brown (Figure 2, A and B), suggesting that bacteria which reduce the silver into silver NPs were grown in the medium. This change in color due to Ag NPs was confirmed by Zaki et al. [21]. They have found this change in the color of the medium when silver NPs were produced using E. coli, Bacillus megaterium, Acinetobacter sp., and Stenotrophomonas maltophilia. The dark brownish color is considered as the primary indication of silver NPs synthesis [21].

The appearance of this dark color due to bacterial silver reduction to silver NPs was confirmed by Gurunathan et al. [22] and Priya et al. [23]. The intense color of silver NPs is due to their surface plasmon resonance (SPR) which differs from the bulk silver or silver ions.

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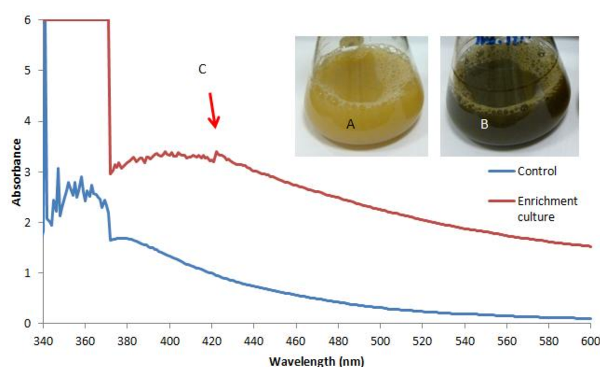

Figure 2: UV-vis spectrum for the enrichment culture in the presence of $AgNO_3$ (100 mM)

Inset: The enrichment culture at 0 time (A) and after 24 h (B) in the presence of $AgNO_3$ (100 mM). The color was changed from yellow to dark brown (C) The SPR peak of Ag NPs at 421 nm.

Selection of bacteria producing rapid metallic Ag NPs in the presence of silver nitrate (100 mM) and pH 5.5 was successfully established. The current simple selection method is eco-friendly in terms of not producing toxic chemicals during the biosynthesis process, low cost and time saving approach to produce Ag NPs. Therefore the bacteria could be considered as potential factories for silver NPs. The bacteria were subcultured successfully in the presence of $AgNO_3$. These bacteria failed to grow in the absence of $AgNO_3$ suggesting that $AgNO_3$ is one of the limiting factors for the growth of Ag NPs producing bacteria.

3.1 UV-vis examination of Ag NPs

UV-vis spectrum for this sample shows a peak at 421 nm which proves that metallic Ag NPs were produced (Figure 2C). As another indicator for the formation of Ag NPs, a peak at 421 nm was shown. This peak is assigned to SPR peak for Ag NPs. When silver NPs interact with light at certain wavelength, they will give intense color which can be detected by UV-vis spectroscopy [14]. Similar result was obtained in another study aimed at producing silver NPs by aquatic weeds (*Eichornia crassipes*) [24] and *Klebsiella pneumonia* [25]. Another surface Plasmon resonance peak at 390 nm was shown for Ag NPs produced by *Bacillus megaterium* [16]. A broader peak (420-430 nm) was reported for extracellular synthesis of Ag NPs produced by P. aeruginosa [26].

The absorption peak of the produced silver NPs in this study showed no shifting of its position for several months, indicating their high stability. Stable NPs show the plasmon resonance peak fixed on a certain wavelength for long time, while samples which change the peak position indicate changes in its characteristics with time, which is undesirable [27]. Saileikaite et al. have synthesized silver NPs by chemical reduction method, and demonstrated the stability of the produced NPs by observing the plasmon peak position with time [27]. It was close to 445 nm and with no shifting of its position [27]. Saifuddin et al. attributed the extreme stability of the silver NPs produced by *Bacillus subtilis*, to "capping agents" which could be proteins secreted by the bacterial cells [8].

The absorption peak of the produced silver NPs showed narrow appearance with no broadening of the peak with time. The peaks wideness can be associated with the size distribution of the produced NPs. The broader the peak indicates that the produced NPs are of variable sizes. The narrower the peak, the less size variability the NPs are [28]. Therefore, silver NPs which were produced in this study were of less size distribution as they showed narrow plasmon peak, as mentioned previously.

3.2 Light microscopic examination of Ag NPs aggregates

Examination under the light microscope showed the precipitation of Ag aggregates of crystals (Figure 3). To be sure that those aggregates were produced due to bacterial activity, precipitation of those particles were followed up by light microscope for 24 h. When bacterial culture was mixed in the presence of $AgNO_3$ (final concentration of 50 mM) (Figure 3A), dark aggregates of crystals were observed after 24 h (Figure 3B). The precipitation of those NPs was not observed in the absence of $AgNO_3$ or bacteria when were run under similar duration and conditions. This crystals formation in the presence of both bacteria and $AgNO_3$ indicates that the precipitation of Ag NPs was due to the bacterial activity in the presence of $AgNO_3$.

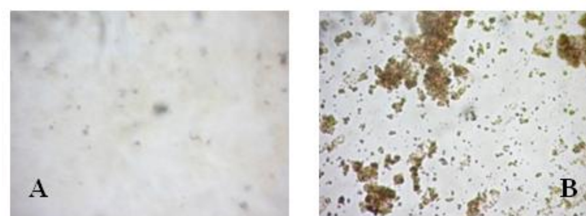

Figure 3: Light microscopic images of Ag NPs aggregates which were precipitated after mixing the bacterial cells with $AgNO_3$ (final concentration of 50 mM) at (A) 0 h and (B) 24 h.

3.3 Examination of Ag NPs by SEM, EDS and XRD

To confirm the structure, shape, and size of Ag NPs which were produced by enrichment bacteria, SEM, EDS and XRD analyses were performed. Almost spherical monodispersed and aggregates of NPs were precipitated as shown by SEM micrograph (Figure 4A).

The metallic silver NPs appeared brighter in color than the impurities (residue of media) in the sample which were of darker color, due to the high molecular weight of the metallic silver as explained by Reed, 2005 [29]. The produced silver NPs in this study were of more or less spherical shape and with size in the range of 5-30 nm. Different sizes of biosynthesized Ag NPs were reported in literature. 50-100 nm of Ag NPs were produced by bacteria [25] whereas 5-55 nm of Ag NPs were produced by nitrate reductase extracted from the leaf of *Dalbergia sisso* [30].

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The EDS analysis of some of the shiny spots which were observed using backscattered electron detector (BSD, sensible to the atomic number of elements) showed the highest composition of silver element (Figure 4B) confirming the presence of silver NPs. The other elements detected such as nitrogen, oxygen, sodium, sulfur and carbon were detected due to the presence of the medium and bacterial cells in the sample.

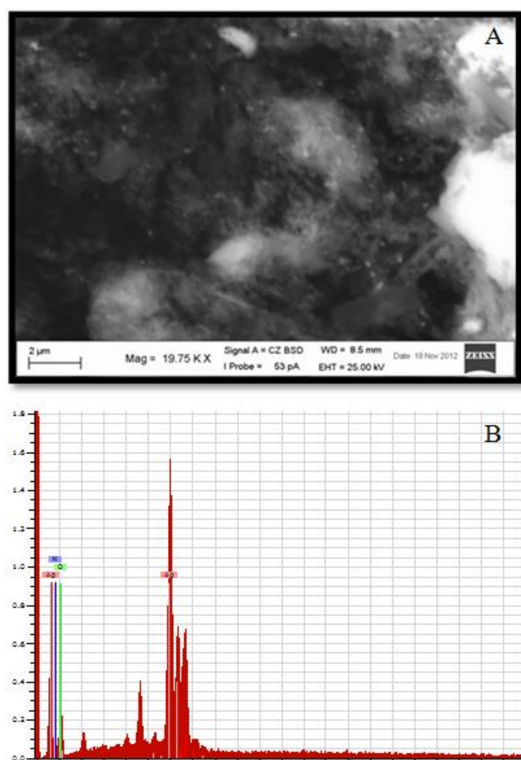

Figure 4: (A) The SEM micrograph of the produced silver NPs (10 to 20 nm) in enrichment culture

(B) EDS analysis of the silver NPs from the enrichment culture. Other peaks were found as impurities from the presence of bacteria and medium in the examined sample.

The biosynthesized silver NPs using bacterial isolates were precipitated and characterized by Zaki et al. using EDS analysis [21]. Their results showed that the highest composition of the sample was silver, and other observed signals were due to the presence of impurities of bacteria and the remaining media in the sample.

X-Ray diffraction is one of the methods widely used for the characterization of the crystal structure, phase composition, checking the presence of impurities, determination of the crystalline size of powdered NPs. The crystal structure differs from the morphological shape of the NPs, in which the crystal structure is the arrangement of the atoms in a particular way, generating unique pattern of planes and facets that characterize the crystal structure of a material. The diffraction of X-ray beam from the planes in specific directions and at specific angles determines the crystal structure of the material, whereas the peak shape and broadening are associated with crystallite size and microstrains. The combination of the atomic crystals of the NPs gives their morphological shape, which can be observed by SEM.

Peaks of Ag NPs are obvious in the XRD which ascertain the precipitation of Ag NPs. The pattern of XRD showed four strong peaks (Figure 5) located at 2θ values of 38.2˚, 44.4˚, 64.6˚, and 77.6˚. These values matched well with the cubic crystal lattice planes, (111), (200), (220) and (311) respectively of silver metal using the Joint Committee on Powder Diffraction Standards (JCPDS), data card 01-071-4613. The average crystallite size was estimated to be around 8 nm, which is known as quantum dots (NPs in the range of 1-10 nm are called QDs) (Fig 5). The other peaks are attributed to the impurities present in the sample such as the medium and bacterial cells.

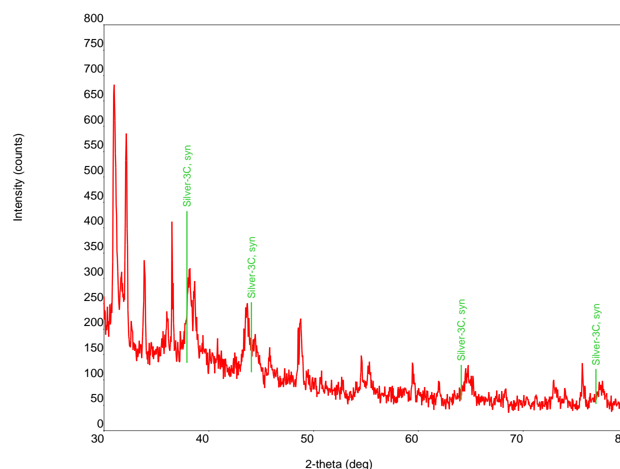

Figure 5: X-ray diffraction (XRD) of Ag NPs deposited on a Si(111) wafer at room temperature for the enrichment culture in the presence of $AgNO_3$ (100 mM).

3.4 Effect of different concentrations of $AgNO_3$ on bacterial activity, growth and size of Ag NPs

The activity of the bacteria which were subcultured from the enrichment culture was tested at different concentrations during 25 days. All samples showed precipitation of Ag NPs which increased with time (Figure 6). Data for other concentration was not shown.

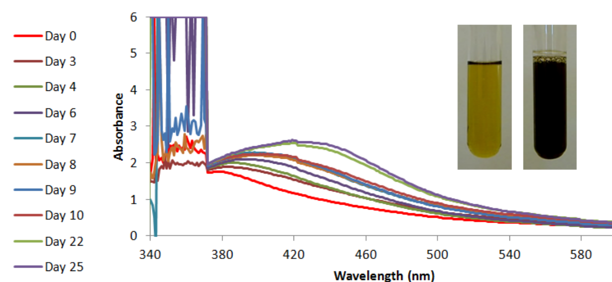

Figure 6: UV-vis spectra recorded from the aqueous 1mM $AgNO_3$ solution as a function of time (in days) of addition of the bacterial biomass. The inset shows test tubes containing $AgNO_3$ solution before (test tube on the left) and after reaction with the bacterial biomass for 25 days.

The bacterial activity increases with increasing concentrations in the range 1-16 mM after which an obvious decrease in the activity was observed; thereby inhibition

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concentration of $AgNO_3$ was above 16 mM (Figure 7).

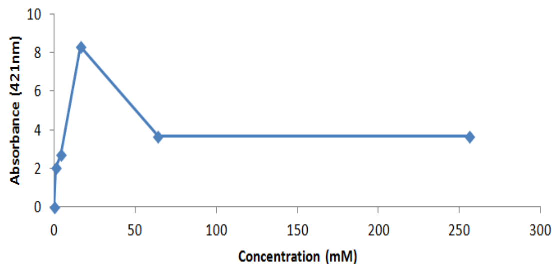
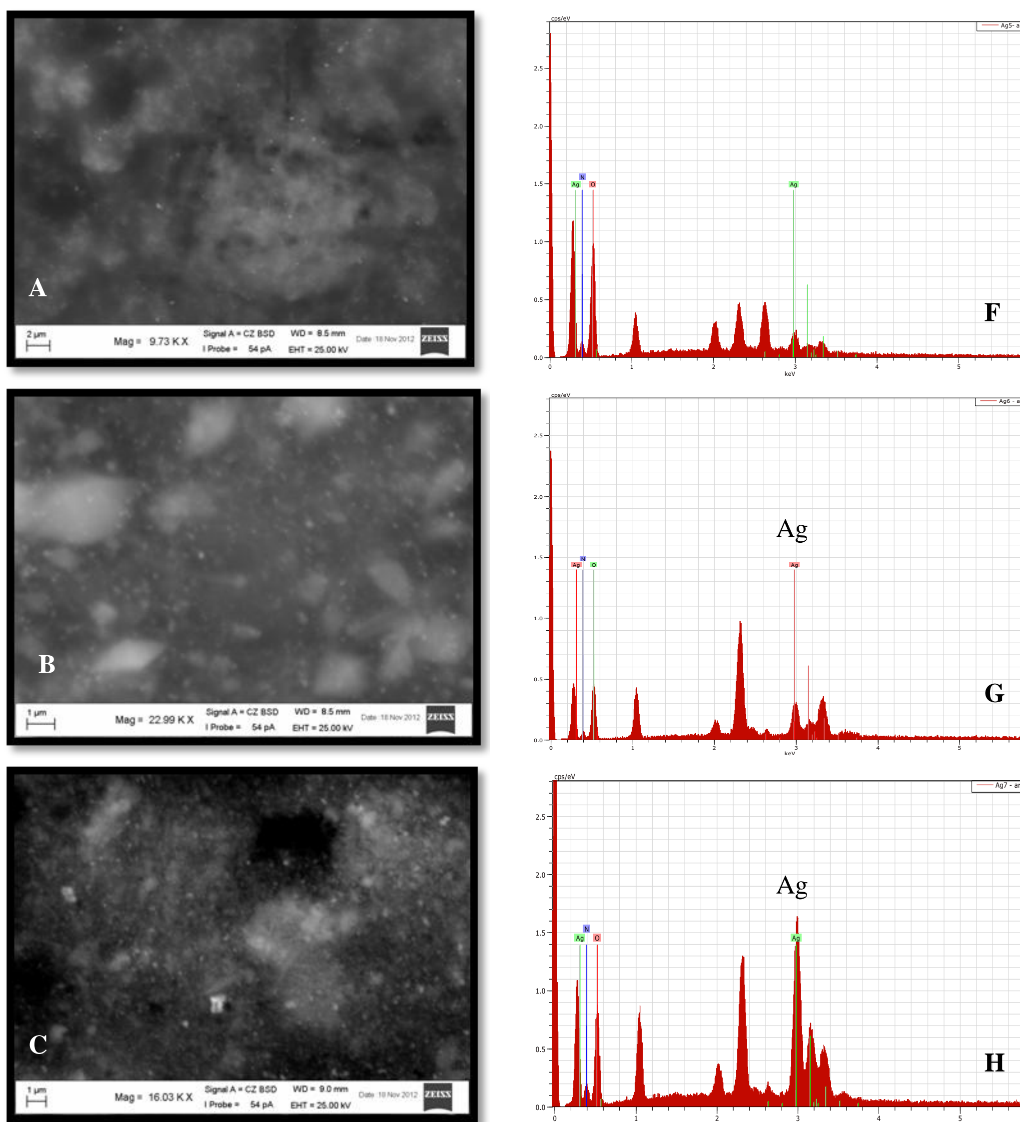


Figure 7: The relationship between the concentration of $AgNO_3$ and OD at 421 nm.

To relate the size of silver NPs to the concentration of $AgNO_3$, the subcultured was grown on different concentrations $AgNO_3$ (1 to 256 mM). The size of Ag NPs produced by bacteria was observed to vary with concentration as shown by SEM (Figure 8, 9). The size of $AgNO_3$ decreases with the increase of the concentration of $AgNO_3$. At low concentration the size of Ag NPs (1 and 4 mM) was in the range 20-30 nm, which decreases to 10-20 nm and then up to 2-14 nm at 64 mM and 256 mM respectively. This decrease in size might be attributed to the decrease in the activity of the bacteria at high concentrations as mentioned above, since the toxicity of $AgNO_3$ proven to be above 16 mM. This result has been confirmed by earlier work by Safekordi et al. [28] who found that NPs size decreases with the increase of $AgNO_3$ concentration using *E. coli.* Their study showed broader surface Plasmon resonances of samples with higher concentration, and thus suggested that the less concentration of $AgNO_3$ used, the narrower the size distribution of the silver NPs. The decrease in size with increase of $AgNO_3$ was further confirmed by Gurunthan et al. [22] where *E. coli* was used to precipitate Ag NPs. The authors opined that with high concentration of $AgNO_3$, a coat is formed around the growing silver NPs preventing its aggregation, thus NPs appear smaller than those produced in lower concentrations of $AgNO_3$.



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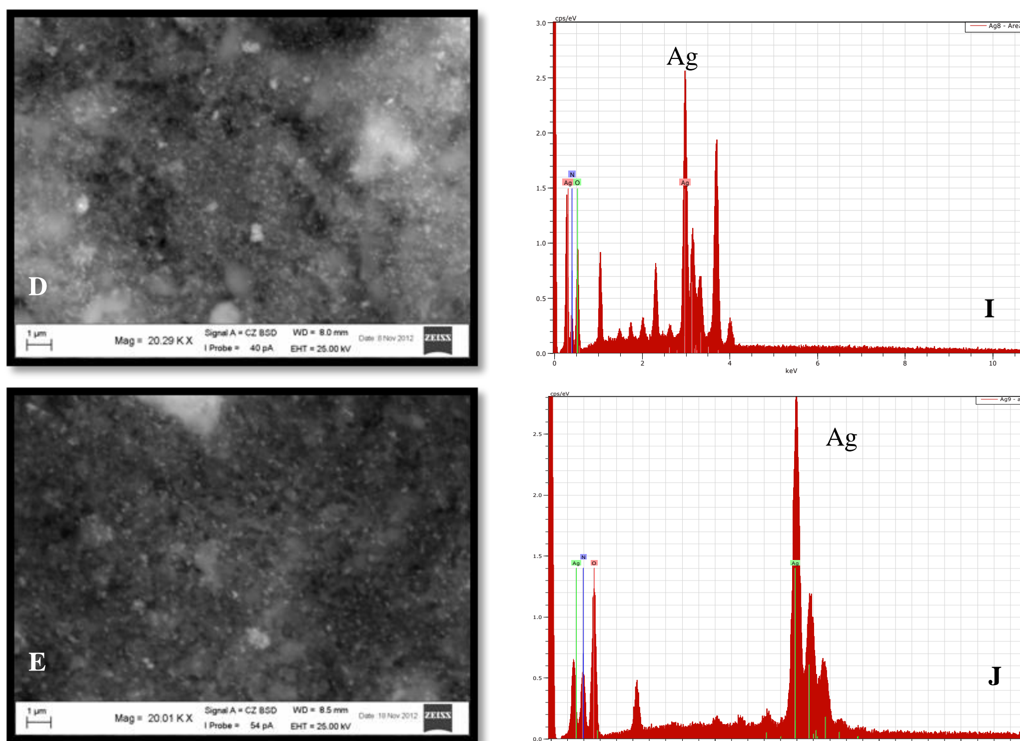

Figure 8: SEM micrographs of silver NPs produced by enrichment culture grown on 1-256 mM silver nitrate (A-E respectively) for 25 days. EDS analysis (next to each SEM micrograph) confirmed the formation of the Ag NPs (F-J).

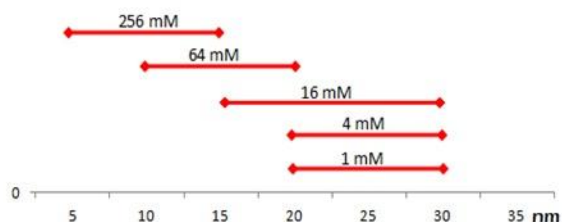

Figure 9: Sketch for the relationship between Ag NPs which were produced by bacteria and concentration of $AgNO_3$.

In this study, bacteria which tolerate high concentration of $AgNO_3$, up to 256 mM, were isolated. Narayanan et al. [12] suggested that microorganisms which are able to grow on such high concentration of silver compounds have a property of detoxification of these compounds, by converting them into stable nontoxic materials (insoluble nanoclusters), either by reduction, biomineralization, biosorption, complexation, precipitation, or intracellular bioaccumulation. Most of the studies in literature use $AgNO_3$ concentrations of 1 mM for biosynthesis of silver NPs [8], [16], [25], [31]. Gurunthan et al. [30] studied the biosynthesis of silver NPs by *E. coli* at $AgNO_3$ concentrations ranging from 1 to10 mM. The authors recorded a decrease in silver NPs production at $AgNO_3$ concentrations higher than 5 mM [31] due to the toxicity effect of silver ions on the bacteria.

4. Conclusion

Selection of bacteria producing rapid metallic Ag NPs in the presence of silver nitrate (100 mM) and pH 5.5 was successfully established. The current simple selection method is eco-friendly in terms of not producing toxic chemicals during the biosynthesis process, low cost and time saving approach to produce Ag NPs. The bacterial production of silver NPs was shown initially by the color change of the culture medium from yellow to dark brown. It was confirmed by the appearance of Plasmon resonance peak at 421 nm, SEM, coupled with EDS and XRD analyses. The size of Ag NPs can be controlled by changing the concentration of $AgNO_3$.

5. Acknowledgements

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6. Future Scope

Biosynthesis of metallic silver NPs (AgPs) are attracting attention due to the positive environmental impact of using microorganisms in NPs production, low cost process, time saving approach and unique physical and chemical properties. These unique properties of Ag NPs enable them to be used for sensing and imaging applications, medical biosensors and antimicrobial agent. There is a potential use of silver NPs in the agricultural, medical and other industries future. Due to the antimicrobial activity of Ag NPs, remarkable advances in agricultural sector could be approached effectively for plant disease management as biopesticides.

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