Insight into eco-friendly fabrication of silver nanoparticles by *Pseudomonas aeruginosa* and its potential impacts

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Abstract. Although green synthesis of nanoparticles (NPs) has replaced conventional physicochemical methods owing to eco-friendly and cost effective nature but molecular mechanism is not known completely. Elucidation of the mechanism is needed to enhance the production of control size synthesis and for understanding the biomineralization process. Here we report the facile, extracellular biosynthesis of silver nanoparticles (AgNPs) by *Pseudomonas aeruginosa* JP1 through nitrate reductase mediated mechanism. $AgNO_3$ was reduced to AgNPs by cell filtrate exposure. UV-visible spectrum of the reaction mixture depicted reduction of ionic silver (Ag^+) to atomic silver (Ag^0) by a progressive upsurge in surface plasmon resonance (SPR) band range 435-450 nm. X-ray diffraction analysis showed the 2θ values at 38.08°, 44.52°, 64.42° and 77.44° confirming the crystalline nature and mean diameter [6.5-27.88nm (Ave = 13.44 nm)] of AgNPs. Transmission electron microscopy analysis demonstrated the spherical AgNPs with size range 5-45 nm. Stabilizing proteins and rhamnolipids were recognized by Fourier transform infrared spectroscopy. Nitrate reductase was purified and characterized (molecular weight 65 kDa and specific activity = 5.6 U/mg). To probe the plausible mechanism purified enzyme was retreated with $AgNO_3$. Characteristic SPR bands range (435-450 nm) and Particle-induced x-ray emission results also confirmed the synthesis of AgNPs (59679.5 ppm) in solution. These results demonstrated that, nitrate reductase as a principal reducing agent in the mechanistic pathway of AgNPs synthesis, which leads to the understanding of metal transformation and biomineralization processes for controlling the biogeochemical cycles of silver and other heavy metals.

Key words: Eco-friendly, Pseudomonas aeruginosa, Silver nanoparticles, Nitrate reductase, Biomineralization

1. Introduction

Biological synthesis of nanoscale materials is a limelight of modern nanotechnology. Silver nanoparticles (AgNPs) have garnered much attention due to the wide range of applications in catalysis[1], membrane bioreactors[2], DNA sequencing [3] and cancer treatment[4]. Several physical and chemical strategies have been employed for the production of nanoparticles (NPs)[5]. Conventional synthesis procedures are becoming obsolete due to the high cost[6], hazardous nature[7], and low yield[8]. Since there is growing need to explore the alternative synthesis protocols which are facile, eco-friendly and cost effective. As inspired by the bioreduction of silver ion (Ag^+) by *Pseudomonas stutzeri* [9], green synthesis of AgNPs using microorganisms has become a hot topic[10]. Extracellular bacteriogenic synthesis is preferred due to rapid growth and simplified downstream processing. Although biogenic synthesis has been also demonstrated by fungus[11], plants extracts like starch[12] and enzymes[13] but molecular mechanism yet to be elucidated for enhanced and controlled size synthesis [14].

Complete understanding of the synthesis pathway will be helpful in bio-mineralization and biotransformation of heavy metals. Basic insights of enzymes-metal interactions are also essential to overcome bottlenecks associated with bioremediation strategies[15]. Several studies have suggested the involvement of nitrate reductase in bio-reduction of metal ions[16]. Fungal mediated *invitro* synthesis of AgNPs was reported from *Fusarium oxysporum* based α-NADPH dependent nitrate reductase acted as electron shuttle [17]. Moreover, biological reduction of Ag^+ was partially inhibited by piperitone for enterobacteria emphasizing the critical role of specific enzyme[18]. Remarkably periplasmic nitrate reductase (NapC) have been linked to the intracellular AgNPs formation by metal reducing *E.coli* [18]. Jain *et al* speculated the possible mechanism of extracellular AgNPs synthesis in *Aspergillus flavus* NJP08 [19].

Another study demonstrated superoxide mediated synthesis of AgNPs by a fungus and indirectly linked nitrate reductase participation [20]. Recently AgNPs synthesis has been investigated with immobilized NADH-dependent nitrate reductase hence substantiating the enzymatic (Nitrate reductase) reduction in underlying mechanism of AgNPs synthesis [21]. Numerous studies have purified the nitrate reductase as prime reducing agent but some additional evidence and validations are still needed to support the mechanistic theory. We assume that resynthesizing the AgNPs from purified enzyme may corroborate the proposed pathway. In our previous study nitrate reductase was probed as the principal reducing agent through positive correlation between enzymatic specific activity and AgNPs synthesis [22].

In present study, efforts were directed to validate the enzymatic pathway of extracellular AgNPs synthesis. *Pseudomonas aerogenosa* JP1 isolated from a metal contaminated soil was used a source of

nitrate reductase. Nitrate reductase activity of cell filtrate was evaluated for reduction of silver nitrate to AgNPs. Extracellularly synthesized AgNPs were demonstrated by UV-vis XRD, FTIR, TEM along with purification of nitrate reductase. Purified enzyme retreatment with $AgNO_3$ resynthesized the AgNPs which symmetrically depicted the role of nitrate reductase in mechanistic pathway. Efficient metal transformation strategies will improve geo-microbiological processes in metal contaminated environments.

2 Experimental

2.1 Biosynthesis of silver nanoparticles

In this study, extracellular AgNPs were synthesized using the cell filtrate of a *Pseudomonas aerogenosa* JP1 isolated from the metal contaminated soil. Purified bacterial culture was aerobically cultivated in the slightly modified MGYP media containing glucose 10g/L, peptone 5g/L, malt extract 3g/L and yeast extract 3g/L for 100 mL growth medium at 37°C on a rotary shaker (12 x g) for 24 h. Cell free extract was obtained by harvesting the bacterial culture after centrifugation at 13416 x g at 4°C for 15 min (centrifuge Model H-251, Kokusan Co., Ltd., Tokyo, Japan). Equal volumes of the supernatant were mixed with the aqueous solution (10 mM) of silver nitrate in Erlenmeyer flask (150 mL) and incubated at 37°C in the rotary shaker (12 x g) for 8h. Subsequently, AgNPs were concentrated and washed with chilled ethanol to remove media components. Air dried AgNPs were subjected to further characterization.

2.2 Characterization of silver nanoparticles

Preliminary characterization for AgNPs was done by noticing the visible colour change. Enzymatic reduction of silver ions (Ag^+) was also perceived by measuring the optical density of the reaction mixture via UV-Vis spectrophotometer (Agilent 8453) at different time intervals along with symmetrically noticing the visible colour change. X-ray diffraction (XRD) analysis was done to confirm crystalline nature and mean diameter. Silica powder-coated film of AgNPs were subjected to XRD analysis operating at 30 kV, 20 mA with CuKα radiation in a transmission mode, (X'pert PRO XRD, PANalytical BV, Almelo, and The Netherlands) A carbon-coated copper transmission electron microscopic (TEM) grid was prepared containing a film of AgNPs and examined by TEM at an accelerating voltage of 80 kV (JEM-1010, JEOL Ltd, Tokyo, Japan). Fourier transform infrared spectroscopy (FTIR) is a sensitive technique to quantify the secondary structure of proteins through the resonance of non-centrosymmetric mode of vibrations[23]. To identify the capping molecule nature and interaction with metal NPs was analysed by FTIR machine (Model 200-VT, Perkin-Elmer, Shelton, CT).

2.3 Enzyme Characterization for Elucidation of molecular mechanism

Enzyme characterization was done by protein precipitation of bacterial cell filtrate with 70% ammonium sulphate saturation. Protein precipitates were concentrated by centrifugation at 4830 x g and 4°C for 20 min (centrifuge Model H-251, Kokusan Co., Ltd., Tokyo, Japan). Crude protein pellet was suspended in phosphate buffer (50 mM) with pH 7.4 to estimate the protein content (Bradford assay)[24] and nitrate reductase activity as described in literature[25]. Crude protein was further purified by size exclusion chromatography with Sephadex G-100 and phosphate buffer (pH 7.4). Size exclusion chromatography is useful technique to separate the protein molecules on size basis[26]. The molecular size of purified nitrate reductase was determined by SDS-PAGE and proteins were visualized by staining with Coomassie brilliant blue R-250.

Molecular Size was determined by comparison with standard protein marker (Bio-Rad, USA) [27]. To Probe the molecular mechanism of NPs synthesis purified nitrate reductase was retreated with $AgNO_3$ solution (10mM). Heat Inactivated purified enzyme was also given the same treatment as a control and test tubes were incubated at 37°C for 8h. The reaction mixture was characterized by UV-vis spectroscopy and Particle induced x-ray emission analysis (PIXE) after the visible colour change. Previously, PIXE analysis has been used to determine the elemental composition with minimal sample preparation and higher sensitivity [28]. For PIXE analysis pelletized samples were irradiated with the 3MeV proton beam from the 5MV Pelletron Tandem accelerator. The emitted x-rays were detected by a $30mm^2$ Si (Li) detector and energy resolution of 138 eV (FWHM) at 5:9 keV of Mn. GUPIXWIN v 2.2.3 software [29] was used to process the PIXE data.

3 Results and discussion

3.1. UV-Vis spectrophotometric analysis

Primary detection of AgNPs synthesis was done by the visible colour change from light yellow to dark brown (Figure.1A). The gradual colour change was the clear indication of $AgNO_3$ reduction through a catalytic component present in cell filtrate (Figure 1A). The specific colour change was due to the excitation of Surface Plasmon Resonance (SPR) in the production of AgNPs. The spectra of AgNPs showed the strong absorption (SPR) in range of 435-450 nm (Figure 1B). Progressive colour change and corresponding increasing intensities of UV-vis spectra were perceived up to 8 hours. The high intensity of SPR band was probably due to increased concentration of AgNPs in reaction mixture [30]. Later on, there was no increase in SPR band indicating the completion of the synthesis reaction. Previously, biosynthesis of AgNPs has been reported by Bacillus sp CS 11 with SPR at 450 nm [31].

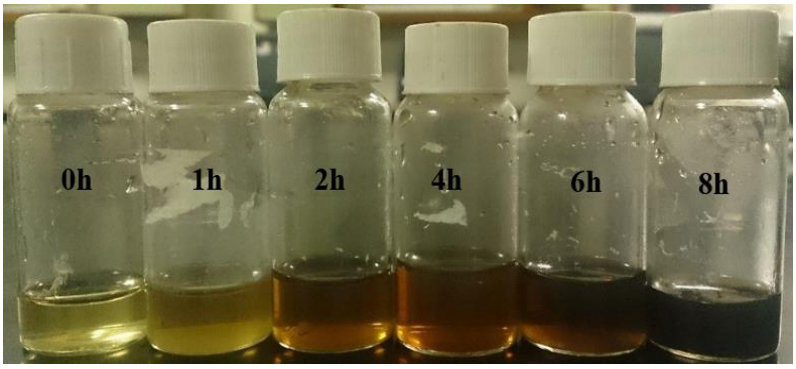

Figure. 1(A). Visible Colour change of the reaction mixture at different interval

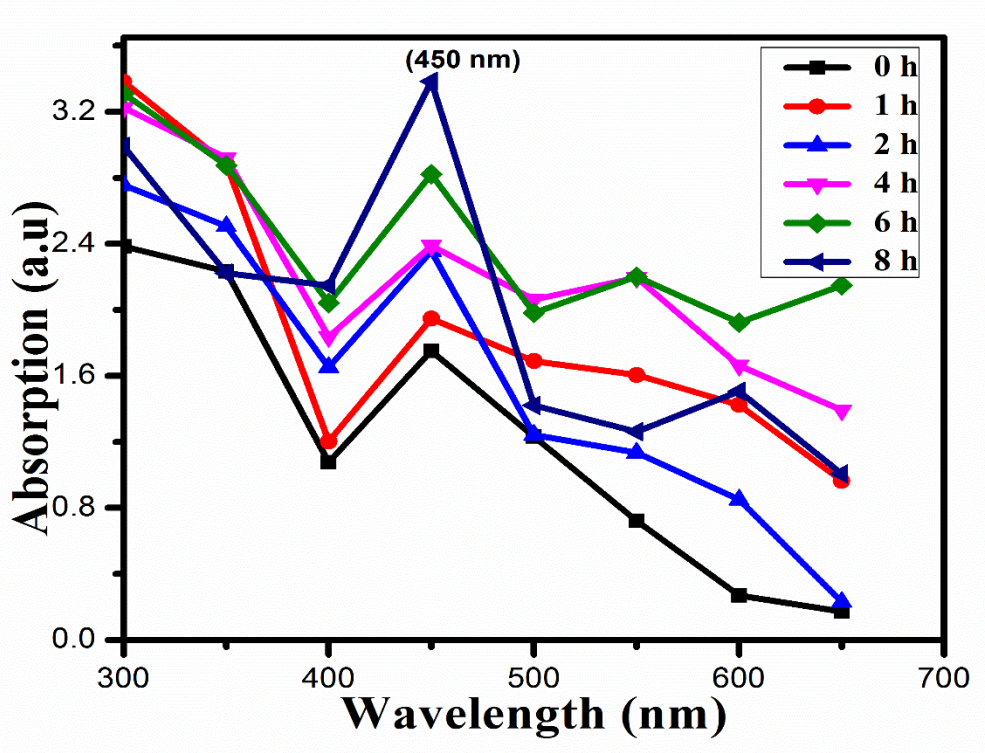

Figure. 1(B): UV–Visible absorption spectrum of AgNPs at different time intervals.

3.2. X-ray diffraction (XRD) analysis

X-ray diffraction analysis was performed to determine the crystalline structure of AgNPs. Figure.3 illustrates the XRD pattern of biosynthesized AgNPs by using *Pseudomonas aeruginosa* JP1 extract. XRD pattern exhibited specific Bragg peaks at 2θ values of 38.08°, 44.52°, 64.42° and 77.44° which are indexed by hkl planes 111, 200, 220 and 311 of the face- centered cubic (fcc) crystal structure (JCPDS card no.04- 00783). XRD results clearly displayed that pure AgNPs were produced by enzymatic reduction (Figure 2A). Debye-Scherer equation was used to determine the size range of AgNPs 6.5-27.88nm with mean diameter 13.44nm (Figure 2B). Our result corroborates the previous

findings of extracellular synthesis of AgNPs (13nm) by *Pseudomonas aeruginosa*[32]. Small insignificant peaks were also observed in XRD pattern showing some organic impurities present in the reaction mixture[33].

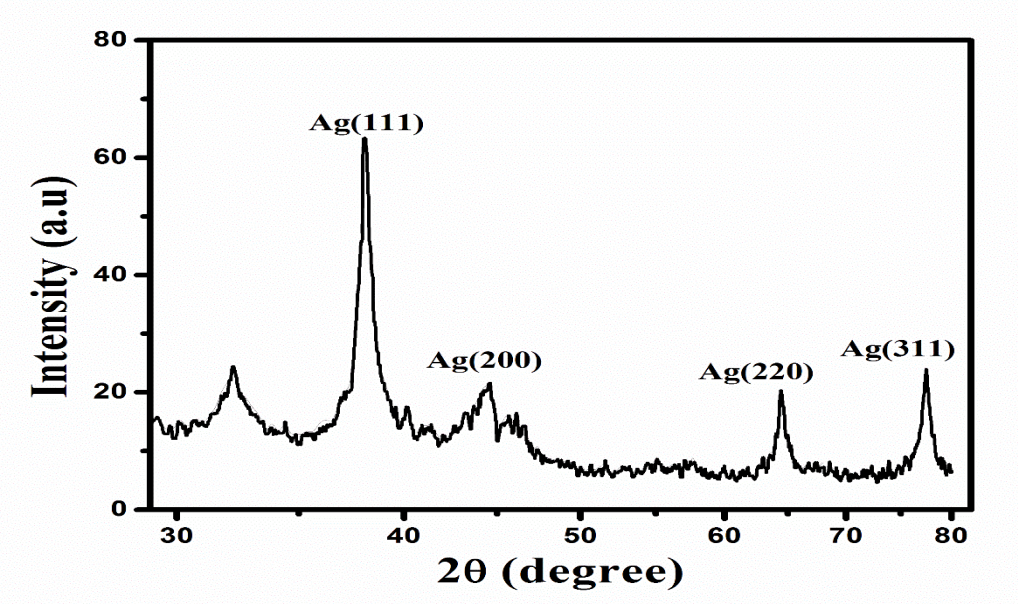

Figure 2(A): XRD Pattern of AgNPs synthesized by *Pseudomonas aeruginosa* JP1.

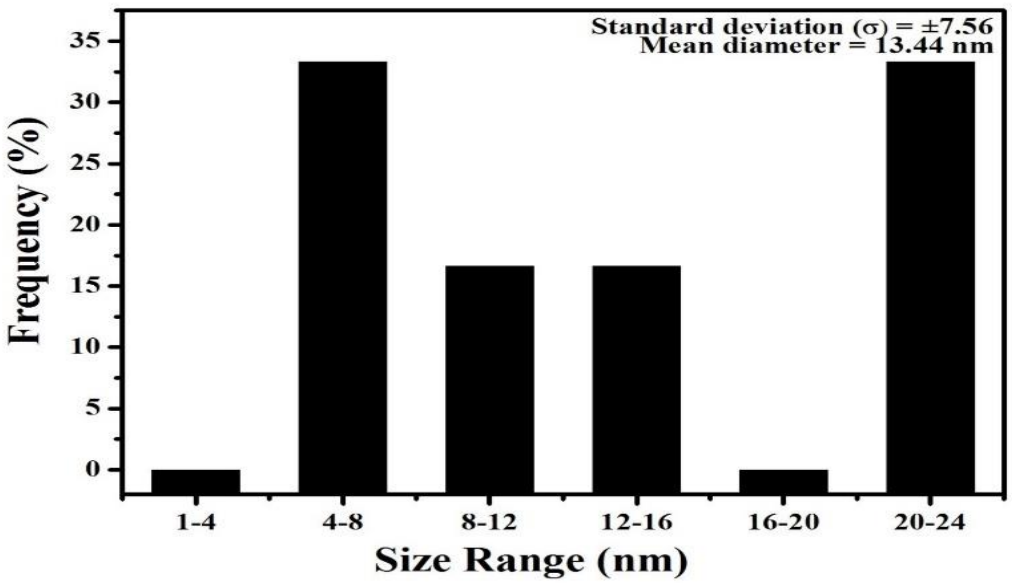

Figure.2 (B): Histogram showing the AgNPs Size distribution.

3.3. Transmission Electron Microscopy (TEM)

Transmission electron micrographs clearly showed synthesized distinct and spherical AgNPs (Figure. 3). TEM analysis provided the size distribution in range 5-45nm for AgNPs (figure. 3) which is in agreement with XRD results (Figure.2). Despite some aggregates majority of AgNPs was monodispersed

and stabilized by capping molecules (Figure. 3). Monodispersity can be linked with capping proteins present on the surface of AgNPs. Zaki *et al* (2011) have reported similar results for AgNPs synthesis (15-50nm) by different bacterial isolate[34].

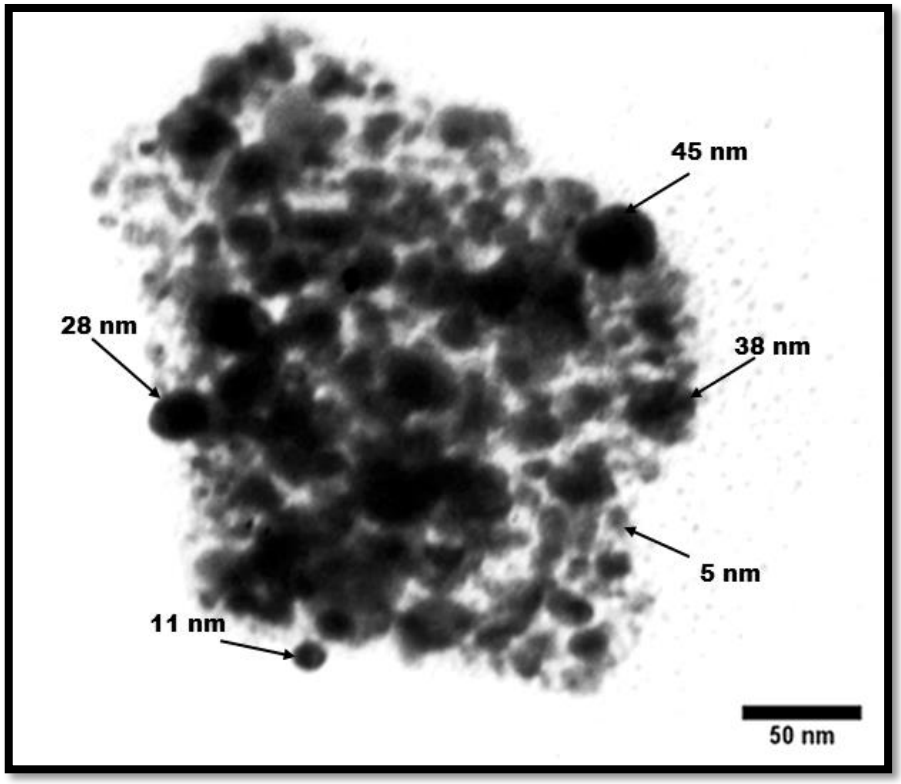

Figure. 3: TEM images of AgNPs synthesized by Pseudomonas *aeruginosa* JP1.

3.4. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infra-red (FTIR) analysis was performed to identify the nature of stabilizing molecules. FTIR spectrum showed the major absorption bands at 782, 1022,1055,1149,1368, 1599, 1632, 2869 and 2978 cm^{-1} (Figure.4). The band 782 cm^{-1} can be linked to C–H group of proteins. The absorption band 1632 cm^{-1} was due to carbonyl stretch vibrations in amide linkages and was recognised as amide I[4]. Bending vibration of N-H bond from primary amines was indicated by 1599 cm^{-1} (Figure.4). Absorption bands at 1055 cm^{-1} & 1149 cm^{-1} may represents the C–O–H bending vibrations and C–O stretching vibrations due to the proteins and rhamnolipids respectively[32]. Stretching vibrations of C–O–C bond in rhamnose sugar was evidenced by the absorption band at 1022 cm^{-1} (Figure.4). Wave number 1736 cm^{-1} indicated the stretching vibration of carbonyl (C=O) group in rhamnolipids [35].

C–H stretching symmetric and anti-symmetric modes were noticed in the range 2869-2978 cm^{-1} which represents the aliphatic and aromatic compounds respectively[36]. Remarkably, strong absorption

band 1368 cm^{-1} from bending vibrations of carboxylic acid functional group confirmed the presence of rhamnolipids on AgNPs[35]. The functional group (C–H) has been identified in the characterization of purified rhamnolipids molecules[37]. FTIR results showed the presence of capping proteins and rhamnolipids on the surface of AgNPs. This could be inferred that proteins along with rhamnolipids might be acting as stabilizing agent (Figure.4). Current results corroborate with our previous findings of stabilizing proteins in biogenic AgNPs [22]. Protein molecules might be attached with AgNPs by cysteine residues which prevent aggregations (Figure.3). FTIR result was also in close agreement with TEM results.

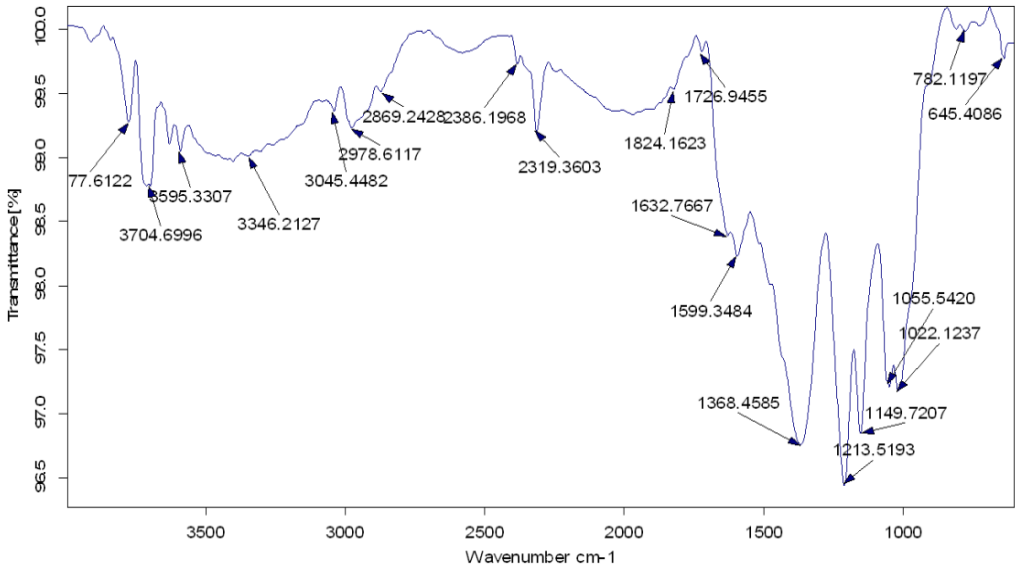

Figure. 4: FTIR spectrum of AgNPs synthesized by *Pseudomonas aeruginosa* JP1.

3.5 Possible mechanism of nanoparticle synthesis

Elucidation of synthesis mechanism was initiated with enzyme characterization by screening the crude extract for nitrate reductase activity. Later, total protein was precipitated out and fractionated by size exclusion chromatography. The fraction with highest specific activity (5.68 U/mg) was subjected to SDS-PAGE analysis. Molecular size of purified nitrate reductase was 65 kDa (Figure.5), which may belong to respiratory or periplasmic nitrate reductases involved in metal reduction [16]. The molecular size of nitrate reductase was in close range as previously reported (70 kDa)[38]. In order to explore the underlying molecular mechanism of biogenic AgNPs synthesis, purified nitrate reductase was retreated with $AgNO_3$ solution (10 mM).Enzymatic reduction of $AgNO_3$ to AgNPs confirmed the nitrate reductase participation in mechanistic pathway.

Further validation was done by pre-heat treatment of purified nitrate reductase and reacting with $AgNO_3$. Heat inactivated enzyme was unable to reduce the $AgNO_3$ solution in tube B, whereas

noticeable colour change in tube A can attributed to nitrate reductase activity (Figure.6). Moreover, UV-vis spectrum produced SPR band within 435-450 nm range indicating the AgNPs formation and substantiating the participation of nitrate reductase in biosynthesis pathway (Figure.7). Biogenic AgNPs formulation has been demonstrated by characteristic SPR peaks[39]. PIXE analysis also revealed the AgNPs presence (59679.5ppm) in the reaction mixture (Figure.8). Previously, PIXE technique has been successfully used for the detection and quantification of AgNPs in aqueous food matrices[40].

Heat treatment inactivated the nitrate reductase (Figure.6) which provided the additional evidence to the mechanistic theory (Figure.9). Hence emphasizing the involvement of nitrate reductase in extracellular AgNPs synthesis as described in the literature [41]. The role of catalytic protein in AgNPs synthesis has been elaborated in our previous study [22]. In another study NADH-dependent nitrate reductase mediated synthesis of AgNPs have been investigated[21]. Previously, Periplasmic nitrate reductases (Nap) and Respiratory nitrate reductases (Nar) have been associated with the biosynthesis of AgNPs [16]. Recently, a similar mechanism was proposed for superoxide-mediated synthesis of AgNPs [20]. Consequently, it can be anticipated that nitrate reductase is a key player in the plausible mechanism of metal transformation into NPs.

Nitrate reductase enables the electron transfer (electron shuttle) from nitrate molecule to the metal ion for NPs formulation (Figure. 9). Stable and size specific nanoscale materials can be synthesized at large scale by optimizing the enzyme physiology. Current findings are in accordance with Jain *et al* (2011) studies for fungal enzyme mediated synthesis of extracellular AgNPs [19]. The underlying mechanism will provide a breakthrough for synthesizing noble metal nanomaterials with monodispersity and controlled morphologies. Interestingly, microbes have evolved the several mechanisms for metal resistance. The fundamental insight of enzyme-metal interaction is elaborated here which, will enable the biotransformation of toxic heavy metals hence providing the detoxification effect[42].

Nitrate reductase producing microbes can potentially enhance the efficiency of bioremediation strategies[43]. Metal-microbe interaction and role of secreted enzymes still needs further annotation. A better understanding of microbial transformation pathway at genetic level will leads to develop new genetic tools for accelerating the bioremediation.[44]. Moreover, metal reducing microbes and extracellular electron transfer mechanism may have implications in electro-microbiological applications for renewable energy[23].

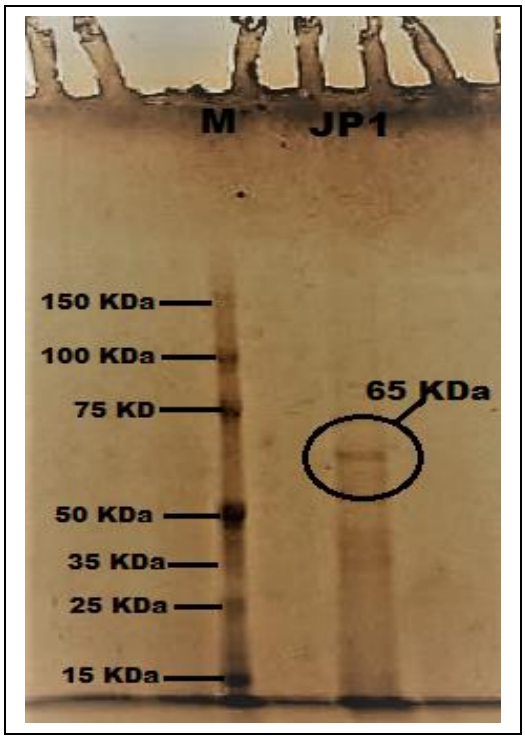

Figure 5: SDS- PAGE of purified nitrate reductase of *Pseudomonas aeruginosa* JP1.

Figure. 6: Tube A showing visible colour change in reaction mixture of purified enzyme and $AgNO_3$ and tube B shows no colour change by inactivated enzyme and $AgNO_3$.

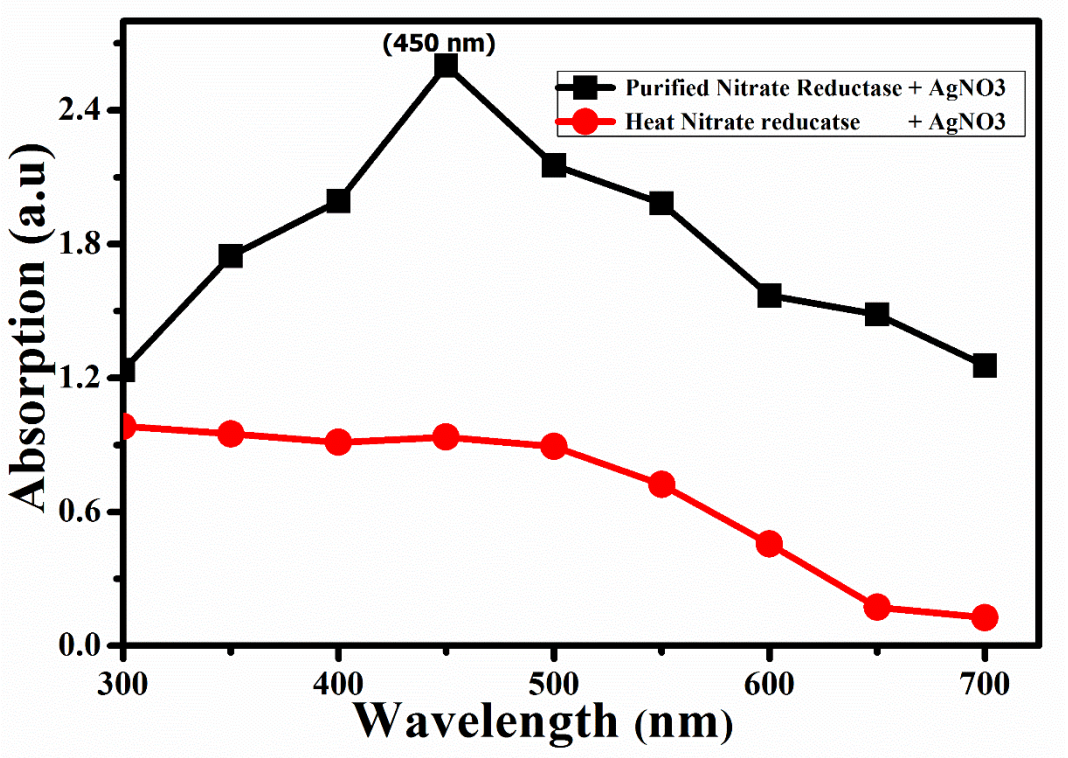

Figure.7 .UV–Visible spectrum of reaction mixtures containing the purified enzyme and AgNO3 solution.

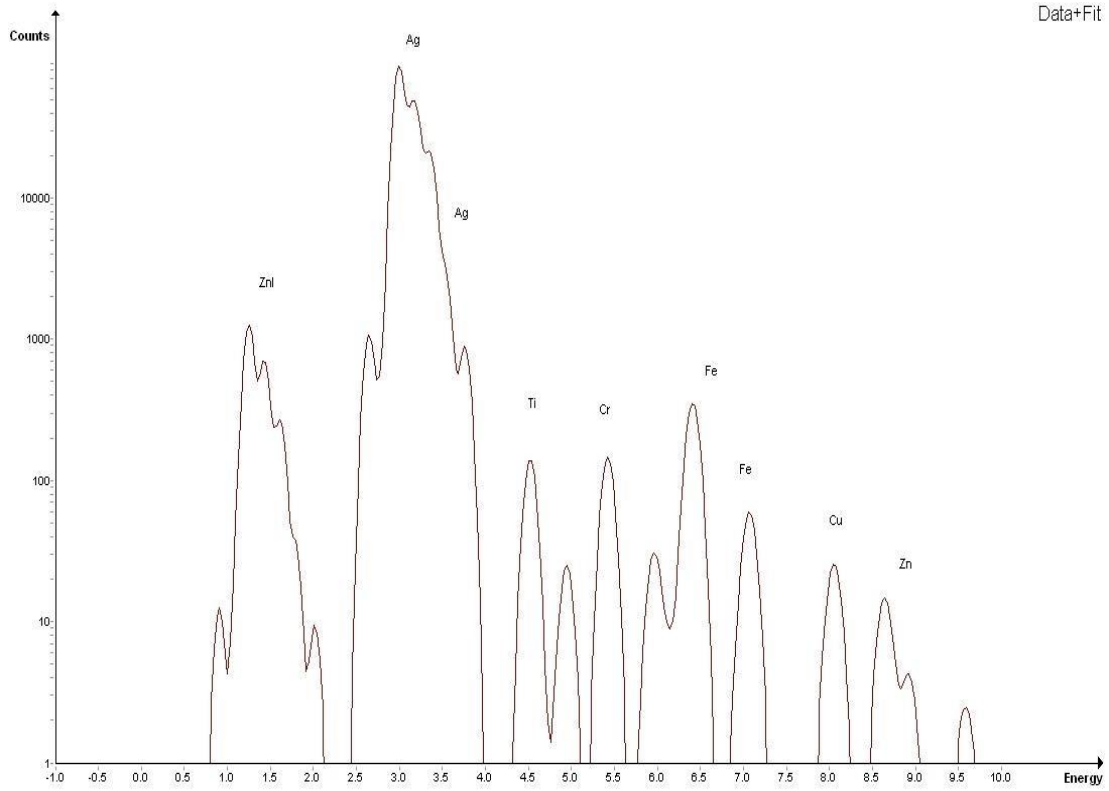

Figure. 8: PIXE Spectrum of AgNPs synthesized by purified nitrate reductase.

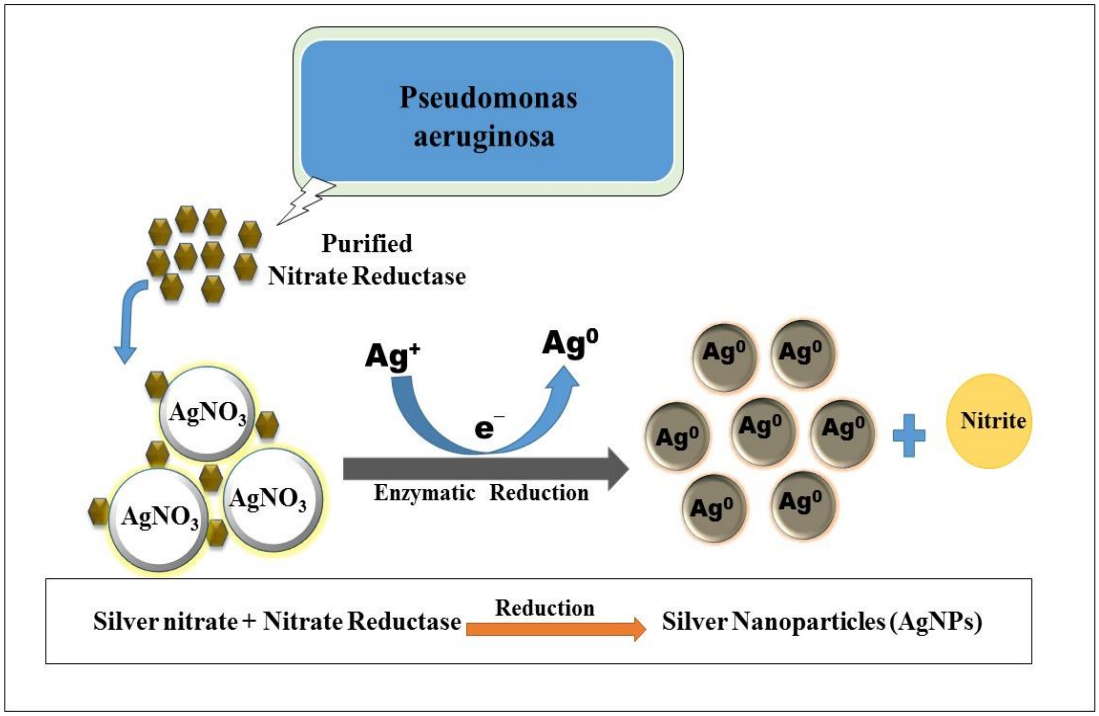

Figure. 9: Possible mechanism for extracellular synthesis of AgNPs

4. Conclusion

Microorganisms can reduce Ag^{+} to AgNPs and potentially can serve as nano- factories. Here we reported that nitrate reductase plays a crucial role in extracellular AgNPs synthesis by *Pseudomonas aeruginosa* JP1. This study is helpful in understanding the mechanism involved green synthesis of AgNPs, which will have many applications regarding the enhanced synthesis of AgNPs with controlled dimensions. Nitrate reductase producing microbes may also have a great implication in electro-microbiology related processes like MFCs for exploring the electron transfer mechanism to electrodes. It is expected that results of present study will also provide a detailed understanding of biomineralization and biotransformation processes and biogeochemical cycles for silver and other heavy metals.

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6. References

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