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Extracellular Fabrication of Silver Nanoparticles using *Pseudomonas aeruginosa* and its Antimicrobial Assay

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ABSTRACT

Use of microorganisms for the synthesis of nanoparticles is in the limelight of modern nanotechnology. Using Pseudomonas aeruginosa, biosynthesis of silver nanoparticles was investigated. Apart from standardizing the best parameter for the synthesis of silver nanoparticles, efforts were directed towards assessing the reducing agent involved in reduction of silver ion to silver nanoparticles. The involvement of nitrate reductases as reducing agent was confirmed by biochemical assay. The nitrate reductase activity got reduced from 0.9876 µmole/min/ml to 0.3233 µmole/min/ml after bio fabrication of silver nanoparticles. The most influential parameters for the synthesis of silver nanoparticles were found to be 100°C and pH 10 that can very effectively biosynthesize silver nanoparticles from a 100 ppm aqueous solution of $AgNO_3$. The silver nanoparticles exhibited maximum absorbance at 450 nm in UV–Vis spectrum. The XRD spectrum of silver nanoparticles exhibited 2θ values corresponding to the silver nanocrystals. TEM micrographs revealed the formation of well-dispersed silver nanoparticles of 20-50 nm. The silver nanoparticles showed maximum antimicrobial activity against Pseudomonas aeruginosa, followed by Staphylococcus aureus and minimum anti-microbial activity was noted against Escherichia coli. It was interesting to note that Pseudomonas aeruginosa that biosynthesized the silver nano particle was most affected by its anti-bacterial activity.

Keywords: *Pseudomonas aeruginosa*, Biosynthesis, Silver nanoparticles, Surface Plasmon Resonance, Nitrate reductase, antimicrobial activity.

INTRODUCTION

Outbreak of the infectious diseases is caused by different pathogenic bacteria and the development of antibiotic resistance the pharmaceutical companies and the researchers are searching for new antibacterial agents. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties Nanotechnology refers broadly to a field of applied science and technology whose unifying theme is the control of matter on the atomic and molecular scale. The metal microbe interactions have an important role in several biotechnological applications including the fields of bioremediation, biomineralization, bioleaching, and microbial corrosion.[1,2] Recently a few microorganisms have been explored as potential biofactories for synthesis of metallic nanoparticles such as cadmium sulfide, gold, and silver[3-7]. Biosynthesis of nanoparticles has received considerable attention due to the growing need to develop environmentally benign technologies in material synthesis. For instance, a great deal of effort has been put into the biosynthesis of inorganic materials, especially metal nanoparticles using microorganisms. Both live and dead microorganisms are gaining importance by virtue of their facile assembly of nanoparticles. Moreover, the problems

concerning the synthesis of nanoparticles and their stabilization can be solved in tandem and mild conditions. Research in nanotechnology provides reliable, eco-friendly processes for the synthesis of nanoscale materials. Inspiration from nature comes through magnetotactic bacteria synthesizing magnetite nanoparticles, diatoms synthesizing siliceous materials and S-layer bacteria producing gypsum and calcium carbonate layers. Marcetol [8] showed that silver nanoparticles (SNPs), like their bulk counterpart, are an effective antimicrobial agent against various pathogenic microorganisms. Although various chemical and biochemical methods are being explored for production of SNPs, microbes are exceedingly effective in this process. New enzymatic approaches using bacteria and fungi in the synthesis of nanoparticles both intra- and extracellularly have been expected to have a key role in many conventional and emerging technologies. Synthesis of nanoparticles was found to be intracellular in many cases but makes the job of downstream processing difficult. Microorganisms, such as bacteria and fungi, now play an important role in the remediation of toxic metals through the reduction of the metal ions Our group has explored extracellular synthesis of gold nanoparticles using bacterial exudates, algal and plant extracts [5,9,10].

The present work has focused on the development of an extracellular biosynthesis of SNPs using *Pseudomonas aeruginosa and it optimization and brief input on possible mechanism involved in bio-reduction of silver ions.*

MATERIALS AND METHODS

Culturing the microbe: *Pseudomonas aeruginosa* culture was procured from National Collection of Industrial Microorganism (NCIM), Pune. A loopful of *Pseudomonas aeruginosa* culture was inoculated in 250ml conical flask containing 100ml sterile Nutrient Broth. The inoculated medium was incubated at 37^0C in a rotary shaker at 120 rpm for 24 hours. After 24 hours, the culture was centrifuged to separate bacterial cells. Centrifugation was done at 5000 rpm for 10 minutes. Supernatant and pellet were separated. The supernatant obtained after centrifugation was used for nanoparticles synthesis.

Chemicals and Glassware - Chemicals used for the synthesis of Silver nanoparticles were Silver nitrate ($AgNO_3$) (Sigma-Aldrich). 100mL of 1mM aqueous $AgNO_3$ solution was taken in 500mL of Erlenmeyer flask for synthesis of Silver nanoparticles.

Fabrication of Silver nanoparticle – The supernatant obtained from the above procedure was added to $AgNO_3$ to make its concentration to 100 ppm. The desired pH of the reaction medium was adjusted by adding 1 M NaOH solution or 1 M HCl solution. In order to optimize the nanoparticle formation, the impact of different temperatures RT (30°C) and boiling temperature (100^0C) was assessed. These temperatures were used for studying the effect of pH (2, 6, 8, 9, 10 & inherent pH) on synthesis of SNPs. The best temperature and pH was kept constant to study the most effective concentration of $AgNO_3$ that can be reduced under selected conditions to silver nano particles. The parameters obtained from the above two experiment were kept constant to comprehend the optical as well as morphological features of SNPs.

Nitrate Reductase Assay: For extraction of Nitrate Reductase from *Pseudomonas aeruginosa*, the supernatant obtained from the above procedure was homogenized with Tris-HCl buffer (pH 8.0) and then centrifuged at 0^0C at 2000 rpm for 15 min. The supernatant was used as enzyme source. Nitrate Reductase activity was measured by Vega and Cardenas method [11].The standard graph was calibrated using 50 µM working standard of Sodium nitrite. To 0.1 ml supernatant known amount of 0.1 M KNO_3 was added and incubated for 24 hours. Then 1ml of diazo coupling reagent (1% Sulphanilamide in 3 ml HCl and 0.02% N-(1-naphthyl) ethylenediamine hydrochloride) was added to 3 ml reaction mixture and diluted 10 folds to detect the remaining NO_2. After 30 min of incubation in dark at 30^0C for development of color; O.D. was recorded at 540 nm. The result was calculated against the standard graph of nitrite.

Determination of antimicrobial activity by well-diffusion method: The SNPs synthesized from *Pseudomonas aeruginosa* were tested for antimicrobial activity by well-diffusion method against pathogenic organisms such as *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*. The pure cultures of organisms were subcultured on Müller-Hinton broth at 35°C on a rotary shaker at 200 rpm. Wells of 6-mm diameter were made on Müller-Hinton agar plates using gel puncture. Each strain was swabbed uniformly onto the individual plates using sterile cotton swabs. Using a micropipette, 20 µL (0.002 mg) of the sample of nanoparticles solution was poured onto each of three wells on all plates. After incubation at 35°C for 18 hours, the different levels of zone of inhibition were measured.

***Characterization of Nanoparticles* –** was done by following methods:
UV-Vis Measurements - was carried out on a dual beam spectroscope Lambda 25 Perkin Elmer, USA using deionized water as the reference. The colloidal solution was then added into a quartz cuvette cell followed by immediate spectral measurements. The SPR peaks were assessed for size and distribution of Silver nanoparticles.

Transmission Electron Microscopic Examination of the Nanoparticle – was done to know the morphology of SNP, using high-resolution analytical transmission electron microscope (HRTEM) Carl Zeiss Micro imaging, GmbH, Germany with an electron kinetic energy of 200 kV. For sample preparation, 2-3 drops of the colloidal gold solution were dispensed onto a carbon-coated 200-mesh copper grid and dried under ambient condition before examination.

XRD Measurements: was done to know the crystallographic information of SNP. X-ray diffraction (XRD) patterns were recorded by a (PANalytical, Philips PW 1830, The Netherlands) operating at 40 kV and a current of 30 mA with Cu Kα radiation (λ = 1.5404 Å) and the 2θ scanning range was of 30-80° at 2° min^{-1}. The colloidal suspension containing metal nanoparticles was dried on a small glass slab.

RESULTS AND DISCUSSION

Impact of different pH on formation of Silver nano particles at 30 & 100ºC fabricated using *Pseudomonas aeruginosa* exudates are presented in Table-1, which shows that pH 10 has yielded the best results at 100°C.

Table 1: Impact of pH and Temperature on the Biosynthesis of silver nano particles using 100 ppm silver nitrate and *Pseudomonas aeruginosa* exudates

pH	Observations Made at					
	30^{0}C			100^{0}C		
	Time Taken for Change in color	UV-Vis Spectra	XRD data	Time Taken for Change in color	UV-Vis Spectra	XRD data
2	24 h	Broad peak at 480 nm	Crystalline structure	< 25 sec	Broad peak at 533 nm	Crystalline structure
6	24 h	No peak	Crystalline structure	< 25 sec	Sharp peak at 520 nm	Crystalline structure
8	24 h	Broad hump at 492 nm	Crystalline structure	< 25 sec	Broad hump at 495 nm	Crystalline structure
9	24 h	Broad hump at 487 nm	Crystalline structure	<25 sec	Sharp peak at 487 nm	Crystalline structure
10	24 h	No peak	Crystalline structure	< 25 sec	Sharp peak at 450 nm	Crystalline structure
pH of exudate	24 h	Broad hump at 477 nm	Crystalline structure	< 25 sec	Sharp peak at 479 nm	Crystalline structure

Pseudomonas aeruginosa exudate was used for fabrication of Silver Nanoparticles(SNPs).Silver nitrate was added to the exudate at 30 and 100°C as seen in Table 1. Table 1 gives a brief overview on the Impact of pH on fabrication of SNPs at both lower and higher temperture.Figure 1 shows the UV-Visible spectra of the SNPs synthesized using *Pseudomonas aeruginosa* exudate at different temperatures. The bacterial exudate which was pale yellowish in colour, became pale grey; when Silver nitrate was added. Further change in color was observed i.e. from pale grey to light brown and then brown after □24 of storage at 30°C and □25 sec at 100°C, indicating the formation of SNPs(Table 1).This color arises due to the surface plasmon vibrations in the metal nanoparticles [12] [13] [14].UV-Vis spectra of SNPs when synthesized at 30°C showed broad hump at all the pH.(Fig 1a)The area under the hump might have large sized polydispersed SNPs which can be confirmed with TEM .The reason behind formation of large sized anoparticles may be lack of sufficient activation energy to the reducing agents or improper balance between the dielectric constant which may lead to agglomeration of the SNPs and thereby giving broder humps.On the contrary ,when SNPs were synthesized at 100°C remarkable sharp peaks were witnessed at alkaline pH 9,10 and inherent pH with Surface Plasmon resonance bands centered at 487 ,450 & 479 nm respectively.broad humps were seen in rest of the pH. High ionic strength coupled with high temperature (100º C) might be instrumental in enhancing the synthesis of nanoparticles. This may also be due to the activation of enzymes present in the bacterial exudates which are responsible for reducing and capping of the nanoparticles.A remarakable property of biologically synthesized metal nanoparticles at 100°C (i.e. capping of SNP) to prevent agglomeration of the particles and stabilizing in the medium. This evidence suggests that the biological molecules could possibly perform the function for the formation and stabilization of the SNP in aqueous medium. It is well known that proteins can bind to SNP

either through free amine groups in the proteins [15] and therefore, stabilization of the SNP by surface-bound proteins is a possibility.

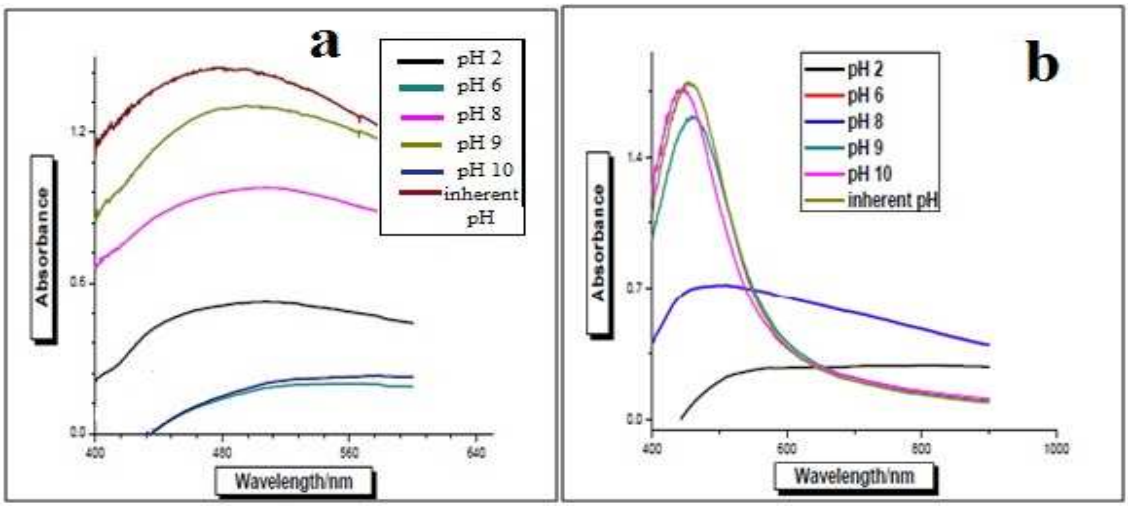

Figure 1 : UV-vis spectra showing Impact of pH and Temperature on biosynthesis of Silver nanoparticles(a)At 30°C (b) At 100 °C using 100 ppm Silver nitrate and *pseudomonas aeruginosa* exudates

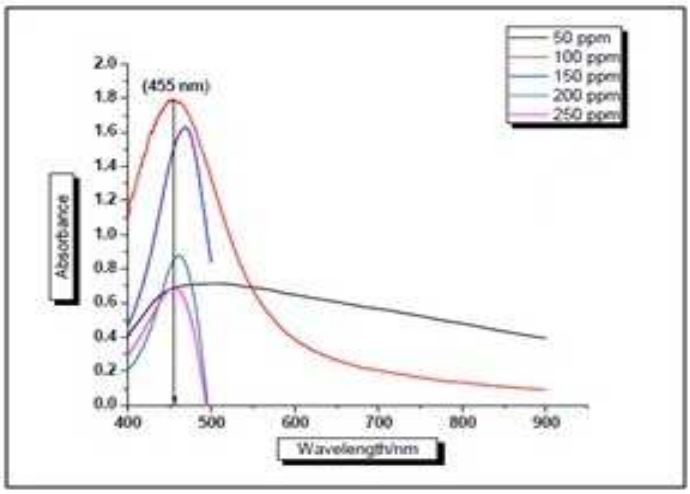

Figure 2 : UV-Vis spectra showing Impact of Concentration on biosynthesis of Silver nanoparticles using *pseudomonas aeruginosa* exudates at 100 °C and pH 10.

Table 2: Impact of Concentration on the Biosynthesis of silver nano particles using *pseudomonas aeruginosa* exudates at 100°C with pH 10

Conc. of $AgNO_3$	Observations		
	Time taken for change in color	UV-Vis Spetra	XRD
50 ppm	24 hr to light brown	Broad hump at 490 nm	crystalline structure
100 ppm	24 hr to brown	Sharp peak at 455 nm	crystalline structure
150 ppm	24 hr to brown	Sharp peak at 464 nm	crystalline structure
200 ppm	24 hr to dark brown	Medium intensity peak at 462 nm	crystalline structure
250 ppm	24 hr to dark brown	Medium intensity peak at 462 nm	crystalline structure

Inorder to study the impact of silver concentration on SNP fabrication.silver concentration (silver nitrate) was varied keeping the temperature and pH constant which is summerized in Table 2.The biosynthesized SNP has different

crystallite shapes which are dependent on the concentration of the Ag^+ ion in solution, the enzymes released by *Pseudomonas aeruginosa* strains, and pH of the solution. However, it must be pointed out that the crystallite shape is not the only important factor influencing the properties of nanometal, but the particle size is also a crucial factor for optoelectronics and other applications of the nanomaterials. Hence, Silver ion concentration was varied inorder to get desired sized SNPs. As observed in Fig 2, 100 ppm Silver nitrate was found to be the most influential concentration which gave a sharp SPR band at 455 nm. Other concentrations viz:50 ppm,150 ppm,200 ppm & 250 ppm gave SPR bands at around 460 nm and above indicating formation of larger sized SNPs.

Transmission Electron Microscopy (TEM): A typical TEM image is presented in Fig 3; shows agglomerated Silver nano particles at 30°C as seen in fig 3a and monodispersed spherical shaped SNPS of a maximum of 20 nm sizes were formed at 100°C as observed in Fig 3b. Agglomeration of SNPs may be due destabilization of electric double layer of silver ions .The microscopic observation is in agreement with the UV-Vis spectroscopic studies. Silver Nanoparticles synthesied were highly uniform in size ranging 20-50nm .

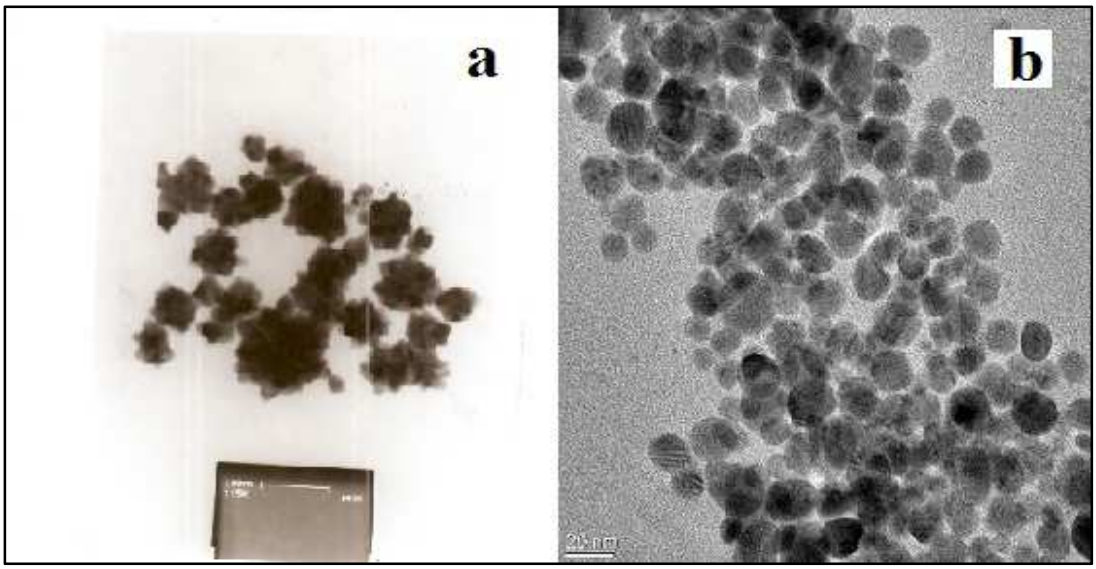

Figure 3: TEM image of Silver nanoparticles synthesized using *Pseudomonas aeruginosa* showing the impact of pH and temperature (a)at 30°C with pH 8 and 100 ppm silver nitrate (b) at 100°C with pH 10 and 100 ppm silver nitrate.

X -Ray Diffraction: A comparison of the XRD data with the standard (joint committee in powder diffraction standards file no:04-0783)confirmed that the particles formed in our experiments were silver nanocrystals which can be depicted by the peaks at 2θ values of 38.45 ,44.48,64,69 and 77.62 corresponding to 111 , 200 , 220 and 311 planes for silver respectively.This XRD pattern confirms the crystallinity of SNPs. The mean particle diameters of SNP were calculated from the XRD data which can be derived from by Debye Scherrer equation.

$$D = \frac{K\lambda}{\beta_{1/2}\cos\theta}$$

This equation exploits the reference peak width at angle θ, where λ is the x-ray wavelength (1.5418), $\beta_{1/2}$ is the width of the XRD peak at half height and K is the shape factor. The calculated average particle size of SNP was 20-50 nm which can also be confirmed by TEM results.

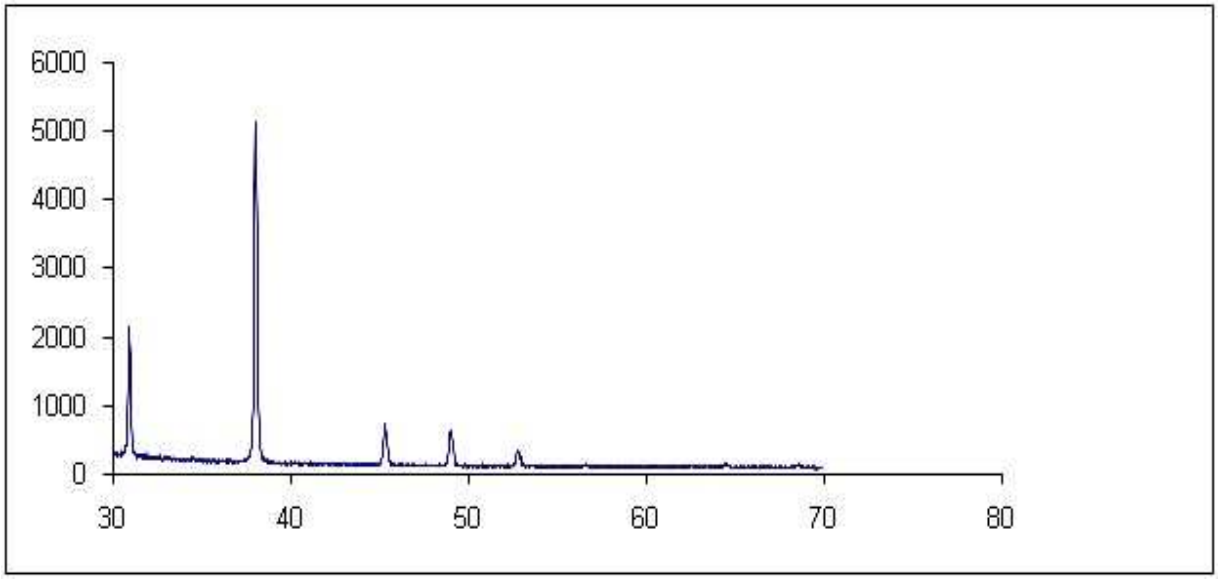

Figure 4: XRD pattern of gold nano particles synthesized using bacterial exudates of *Pseudomonas aeruginosa*, showing typical Bragg reflections for Silver nanoparticles

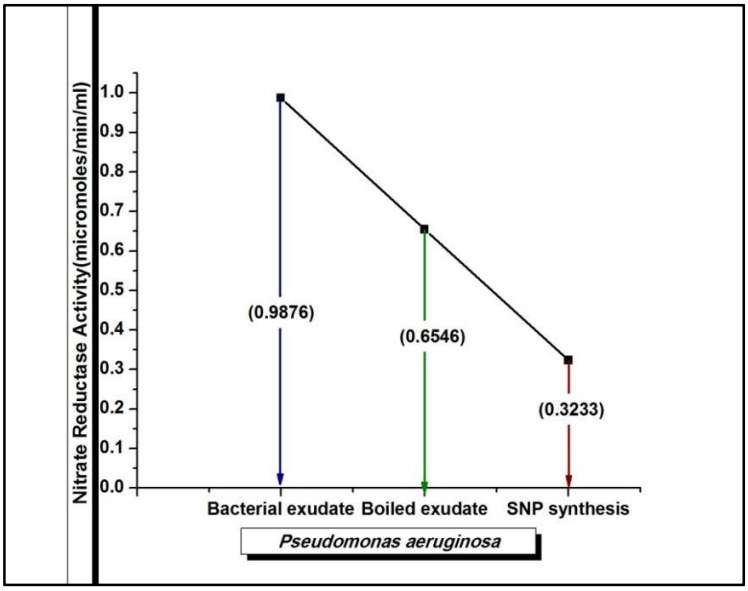

Figure 5: Nitrate reductase activity of *Pseudomonas aeruginosa* exudates, Boiled bacterial exudates and gold nanoparticles respectively in µmoles/min/ml

Nitrate Reductase Assay: Studies have indicated that NADH and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles. *Pseudomonas aeruginosa* is known to secrete the cofactor NADH and NADH-dependent enzymes, especially nitrate reductase, which may be acting as a scaffold or nucleating agent and might be responsible for the bioreduction of Ag^+ to Ag^0 and the subsequent formation of Silver nanoparticles. The same enzyme later then acts as a capping agent, thus ensuring complete formation of thermodynamically stable nanostructures [17]. The molecular activity of nitrate reductase in the bacterial exudates of *P.aeruginosa* was found to be 0.9876 µmole/min/ml; which got reduced to 0.6546 µmole/min/ml when it was subjected to 100^0C (Fig 5). After the formation of gold nanoparticles the nitrate reductase activity was again assayed

in the reactant mixture which showed a substantial decrease with 0.3233µmole/min/ml in the solutions (bacterial exudates) having Silver nanoparticles as compared to nitrate reductase activity in bacterial exudates without silver nanoparticles. This result confirms the involvement of nitrate reductase in the reduction of silver ion to silver nano particles. ***The fact that which capping proteins are involved in stabilizing the particle is yet to be explored.***

Anti-microbial assay: The antibiotic activity of SNPs was investigated against various pathogenic organisms such as *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa* using well-diffusion method (Fig 6). The mean of four replicates of the diameter of inhibition zones (in millimeters) around each well with SNPs solution is represented in Table 3. The highest antimicrobial activity was observed against *Pseudomonas aeruginosa* followed by *Staphylococcus aureus* and *Escherichia coli*. The Minimum inhibition concentration (MIC) of $AgNo_3$ nanoparticles was 50 ppm.

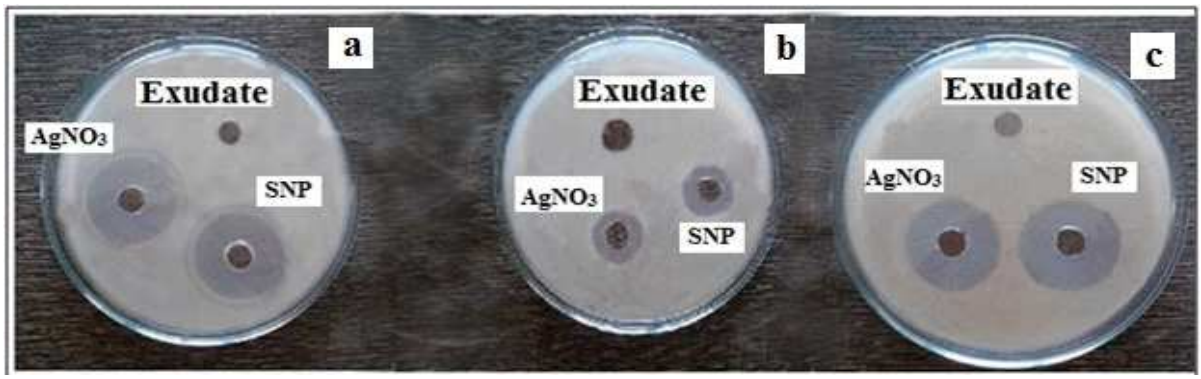

Figure 6: Antimicrobial activity of silver nanoparticles against various pathogenic bacterial strains. (a)*Pseudomonas aeruginosa* (b) *Escherichia coli* & (c) *Staphylococcus aureus* shown by well-diffusion method.

Table 3: Zone of inhibition of SNPs against various pathogenic bacteria

Pathogenic bacteria	Diameter of Zone of inhibition (mean of 4 replicates)
Escherichia coli	15 mm
Staphylococcus aureus	17 mm
Pseudomonas aeruginosa	23 mm

The antimicrobial property of silver is related to the amount of silver and the rate of silver released. Silver in its metallic state is inert but it reacts with the moisture in the skin and the fluid of the wound and gets ionized. The mechanism for the antimicrobial action of silver ions is not properly understood however, the effect of silver ions on bacteria can be observed by the structural and morphological changes. It is suggested that when DNA molecules are in relaxed state the replication of DNA can be effectively conducted. But when he DNA is in condensed form it loses its replication ability hence, when the silver ions penetrate inside the bacterial cell the DNA molecule turns into condensed form and loses its replication ability leading to cell death. Also, it has been reported that heavy metals react with proteins by getting attached with the thiol group and the proteins get inactivated[18,19]. However, the silver nanoparticles show efficient antimicrobial property compared to other salts due to their extremely large surface area, which provides better contact with microorganisms. The nanoparticles get attached to the cell membrane and also penetrate inside the bacteria. The bacterial membrane contains sulfur-containing proteins and the silver nanoparticles interact with these proteins in the cell as well as with the phosphorus containing compounds like DNA. When silver nanoparticles enter the bacterial cell it forms a low molecular weight region in the center of the bacteria to which the bacteria conglomerates thus, protecting the DNA from the silver ions. The nanoparticles preferably attack the respiratory chain, cell division finally leading to cell death. The nanoparticles release silver ions in the bacterial cells, which enhance their bactericidal activity [19,20,21,22]

CONCLUSION

Pseudomonas aeruginosa exudates can extracellularly biosynthesize thermodynamically stable desired size and shape of silver nanoparticle by optimizing pH and temperature. The reducing agent involved in reduction of silver

ion to silver nanoparticle was found to be nitrate reductase.TEM micrographs revealed that alkaline pH was optimum for fabrication of spherical SNPs. The SNPs thus synthesized possessed highest antimicrobial activity against *Pseudomonas aeruginosa*.

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REFERENCES

[1] R.M. Bruins, S. Kapil, S.W. Oehme, *Ecotoxicol Environ Saf* ,**2000**,45,198
[2] T.J. Beveridge, M.N. Hughes, H. Lee, K.T. Leung, R.K. .Poole, I. Savvaidis,et al, *Adv Microb Physio*l **1997**,38,177
[3] M. Sastry, A .Ahmad, M.I.Khan, R. Kumar ,*Curr Sci* **2003**,85,162
[4] P. Tillmann, *J Mater Chem* **2004**,4,140
[5] A. Ahmad, S. Senapati, M.I .Khan, R. Kumar, M. Sastry, *J Biomed Nanotechnol*,**2005**,1,47
[6] R. Shah, G. Oza, S. Pandey, Madhuri Sharon,, *Journal of Microbiology and Biotechnlology research*,**2012**, 2(3)**in press**
[7] A. Mewada, G.Oza, S.Pandey, Madhuri Sharon, , *Journal of Microbiology and Biotechnlology research*, **2012**, 2(3)**in press**
[8] N. Duran, G.I.H. De Souza, O.L. Alves, E. Esposito, P.D.Marcato, *J Nanotechnol* ,**2003**,122
[9] G. Oza, S. Pandey, R. Shah, Madhuri Sharon, *Euro. Jour.of experimental biology*, **2012, 2(3)**,**in press**
[10] S. Pandey, G. Oza, R. Shah, Madhuri Sharon, Euro. *Jour. of experimental biology*, **2012, 2(3)**,**in press**
[11] M. J Vega, J. Cardenas, *Methods of Enzymology, Academic Press,* New York, **1972**, 23, 255
[12] P. Mulvaney, *Langmuir*, **1996**, 12,788
[13] S. S. Shankar, A. Ahmad, S. Pasricha, M. Sastry, *J. Material Chem*.,**2003** ,13,1822
[14] S. S. Shankar, A. Rai, A. Ahmad, M .Sastry ,*J.Colloid Interface Sci*. ,**2004,**275,496
[15] A. Gole, C. Dash, V. Ramachandran, A.B. Mandale, S.R. Sainkar, M. Rao, M. Sastry, *Langmuir* ,**2001,** 17 , 1674
[16] D. V. Leff, L. Brandt, J. R. Heath, *Langmuir*, **1996**, 12, 4723.
[17] S. He, Z. Guo, Y. Zhang, S. Zhang, J. Wang and N. Gu, , *Materials Letters* , **2007** ,61, 3984
[18] S.Y. Liau, Read DC, Pugh WJ, Furr JR, Russell AD ,*Lett Appl Microbiol*, **1997**;25:279–83.
[19]Feng QL, J. WuJ, G.Q. Chen, F.Z. Cui, T.N. Kim, J.O. Kim, *J Biomed Mater*,**2000**,52(4),662
[20] I. Sondi, B. Salopek-Sondi, *J Colloid Interface* ,**2007**,275,177.
[21] J.R. Morones, J.L. Elechiguerra, A. Camacho, J.T. Ramirez, *Nanotechnology*, **2005**;16:2346
[22] H.Y.Song, K.K. Ko, L.H. Oh, B.T. Lee, *Eur Cells Mater*, **2006**,11, 58.