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Biocatalysts Of Silver Nanoparticles Synthesized Using An Environmental Isolate, Serratia marcescens S01

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Abstract: Recent advancement in the field of nanotechnology has brought about innumerable developments in sculpturing and assembling materials to nanometer range with varied synthesis protocols. Despite physico-chemical and optical properties of nanomaterials, the application toward biomedical and clinical fields is limited as for the use of toxic chemicals in fabrication of the materials concerned. In this study, an eco-friendly method for the synthesis of silver bio nanoparticles (AgNps) has been investigated using a bacterial isolate, *Serratia marcescens* S01 and its antimicrobial assessment toward *Staphylococcus aureus* and *Klebsiella pneumoniae*. The bio-reduction of silver ion was accomplished using the extra cellular filtrate upon screening for nitrate reductase activity. The synthesized AgNps were characterized using UV-visible spectrum, XRD, FTIR, SEM and EDS. The results revealed a change in color to yellowish brown in the reaction mixture due to the surface plasmon resonance of silver nanoparticles with λ_{max} 428 nm after 96 h of incubation. XRD pattern showed diffraction peaks with 2 θ values indexed to (111), (200) characteristic of silver with a face-centered cubic symmetry. The FTIR peaks were observed at 489, 669, 1541, 2361, 2923 and 3619 cm^{-1} attributing to the involvement of protein. The SEM result showed the uniform distribution of spherical to ovate AgNps ranging from 30 to 70 nm. The interaction of protein with nanoparticles offered stability and behaves as a capping agent in avoiding agglomeration of the particles. The antimicrobial susceptibility testing of AgNps revealed significant bactericidal activity toward *S. aureus* with 11 mm zone size with respect to *K. pneumoniae* showing 9 mm zone size at 20 µL. The positively charged AgNps exhibit a greater affinity toward the negatively charged bacterial cells resulting in the microbicidal activity.
Keywords: Bio-nanoparticles, *Serratia marcescens*, Nitrate reductase, Microbial synthesis.

Introduction

Nanotechnology, an emerging interdisciplinary science provides the ability to engineer the properties of materials by controlling their size, shape etc. has driven research collaboration among chemists, physicists, biologists and engineers for the development of *nano* in biotechnology. This cutting edge technology concerns the development of sustainable experimental protocols probably the top down and bottom up[1] for the synthesis of nanomaterials with fascinating physico-chemical and optical properties. The synthesis process involves a

wide variety of techniques including chemical reduction which use reductants such as $NaBH_4$, N_2H_4, NH_2OH, C_2H_5OH, Ethylene glycol and N, N-dimethylformamide (DMF)[2]. The use of toxic chemical substances and non-polar solvents during synthesis restrict their applications in biomedical and clinical fields and ecological considerations as well. Hence, there is a need for the development of nanotechnology-enabled, cheap, safe, clean and sustainable environment friendly method for synthesizing metal nanoparticles. With the implications of green chemistry principles, metal nanoparticles are being synthesized using microbes[3,4], sea weeds[5] and plant extracts[6]. This biogenic synthesis involving micro organisms have emerged as a simple and feasible alternative to chemical reduction and physical methods. The natural phenomenon of metal-microbe interaction that forms the basis of bioremediation process has contributed to the development of a novel and unexplored area of research on bio-nanoparticles synthesis. The biological synthesis of nanoparticles has evolved from the experiments on bioreduction and biosorption of metals using unicellular[7] and multicellular[8] organisms which reduce and/or assimilate inorganic materials either intra- or extra-cellularly. Reduction of heavy metals has been demonstrated by fungi and bacteria as well where it needs little detailed characterization of bacterial system. The application of bacteria in the synthesis of nanoparticles is exciting since they are relatively easy to maintain, multiplies rapidly under ambient temperature and pressure conditions, secrete large amount of enzymes and metabolites and ease of handling in the laboratory. A very small inoculum size, luxuriant growth characteristics on ordinary media within a short span of time (doubling time) is more advantageous compared to fungi and plant materials. Synthesis of nanoparticles extracellulary (outside the cell) has many applications as the downstream processing is made easier. In addition to gold and silver nanoparticles synthesis, bacteria are also employed in the synthesis of semiconductors (quantum dots) such as CdS, ZnS and PbS for biological detection and cell imaging[9]. Nanocrystal formation varies greatly depending upon the type of bacterium and its growth phase. The stationary phase *E. coli* yields 20 fold increase in CdS nanocrystal formation than in the late logarithmic phase[10]. The present study focuses on the application of an environmental isolate *Serratia marcescens* S01 for the extra cellular synthesis of silver nanoparticles as this bacteria was not explored neither for synthesis of AgNps nor for stability. Characterization of the synthesized bio-nanoparticles has been monitored using UV-visible spectrum, biological interaction examined by FT−IR, the crystal nature studied by X-ray diffraction and nanocomposition analyzed using SEM.

Materials And Methods

Materials

Silver nitrate ($AgNO_3$) was purchased from Qualigens, India. Nutrient Agar (NA) and Luria Bertani (LB) medium were supplied by HiMedia, India. All the other reagents which were of analytical grade were obtained from Fisher Scientific, India and used without further purification.

Isolation and Screening

Rhizosphere soil from agricultural area in FIPPAT, Kancheepuram District, Tamilnadu was collected for the isolation of *Serratia marcescens* S01 and identified according to Holt et al[11]. Samples collected were plated onto modified nitrate agar plates and broth respectively and incubated at 37°C for 24 h. A clear zone around the colonies indicated the reduction of nitrate. Different colonies from the plates were purified through repeated streaking on fresh agar plates. The purified colonies were finally streaked onto NA plates and the isolates that formed a clear zone were selected and S01 was retained for this experiment.

Nitrate reductase assay

Qualitative assessment of the enzyme was determined using Nitrate reductase assay[12]. One hundred mL Nitrate broth (modified) was prepared in 250 mL Erlenmeyer flasks and sterilized. One millilitre of 24 h grown bacterial isolate was used as inoculum and incubated on a rotatory shaker at 150 rpm for 96 h at 37ºC. Assay reagents : equal volumes of sulphanilic acid and α-naphthylamine in 5N α-acetic acid were prepared freshly and 0.1 mL was added to the culture filtrate and observed for colour change.

Inoculum preparation for nanoparticle synthesis

The AgNps were prepared according to the procedure described in the literature[13]. In brief, AgNps were synthesized extracellularly using the culture supernatant obtained from a silver resistant soil isolate. Equal volume of culture supernatant and aqueous $AgNO_3$ (10^{-3} M) prepared using deionised milli-Q H_2O (Milli-Q Integra 3, Millipore, MA) were taken in 250 mL Erlenmeyer flask and incubated at 30 ºC on a shaking incubator

until color change was observed. The solution containing silver nanoparticles were separated and concentrated by repeated washing and centrifugation at 10,000 x g for 15 min and the final suspension lyophilized and stored as powder for further experimental.

Further, the synthesized bio-nanoparticles were characterized by Fourier transform infrared spectroscopy in the region of 4000 − 500 cm^{-1} (Perkin Elmer, USA), morphology and elemental composition were determined using Scanning Electron Microscope (SEM, Model S-3000H, Hitachi, Japan) and X-ray energy dispersive spectroscopy (EDS) to obtain the nanostructural information.

Evaluation of microbicidal effect – Disc diffusion

The antimicrobial efficacy of the silver nanoparticles at different concentrations was determined by performing Antibiotic Susceptibility test using disc diffusion method. Two bacterial test organisms, a gram positive *Staphylococcus aureus* and gram negative *Klebsiella pneumoniae* were used and their respective inoculum was prepared and turbidity adjusted to ~0.5 McFarland standard. The antimicrobial assay was performed using Mueller Hinton Agar (MHA, Qualigens, India) using 3 h broth culture uniformly spread as a lawn. The plates were incubated at 37 °C for 24 h and the diameter of zone of inhibition was measured.

Results

Influence of incubation time

The culture supernatant of *Serratia marcescens* S01 was used for the synthesis of silver nanoparticles. The reducing potential of the bacterium was determined by nitrate reductase assay using culture supernatant. Maximum reduction was found after 72 h as indicated by development of deep cherry red color upon addition of assay reagents with the increase in cell growth as judged by OD 600.

Effect on $AgNO_3$ reaction

The presentation color of the $AgNO_3$-cell free extract mixture indicated the start of the reaction within 4 h of incubation which was analyzed periodically every 6 h. The change in color to yellowish brown indicated the reduction and growth of silver nanoparticles. UV-visible spectrum analysis showed maximum adsorption peak between 420 and 430 nm after 96 h of incubation (Figure 1).

FTIR spectroscopy is a useful tool for quantifying secondary structure in the metal nanoparticles – protein interaction by the absorption of infra red (IR) radiation through resonance of non-centro symmetric (IR active) modes of vibration. The FTIR spectrum (Figure 2) displayed a peak at 3619 cm^{-1} for free hydroxyl O−H stretch, small peak at 2923 cm^{-1} corresponds to the stretching vibrations of primary and secondary amines between P−H was reported at 2361 cm^{-1} with a bending vibration peak at 1541 cm^{-1} with N−H vibration. The peaks at 669 cm^{-1}, 618 cm^{-1} and 573 cm^{-1} correspond to C−S disulfide stretching vibration. Similarly, the peak at 489 cm^{-1} showed −S−S (polysulfide) stretching vibration indicating the frequent occurrence of thiols and its substituted compounds constituting the backbone of the interacting protein.

The typical XRD pattern (Figure 3) of the reduced metal ie., $AgNO_3$ shows diffraction peaks at 2θ = 38º, 45º that can be indexed to (111), (200) planes of silver (PDF No.04-0783) that confirmed the main composition of the nanoparticles was silver. In addition there were also some little peaks corresponding to oxides and carbonates of silver (PDF No.26-0339) which may be attributed to oxygen and carbon dioxide present in the atmosphere. SEM was employed to observe the topography and size of the nanopartices as shown in Figure 4. The appearance of silver nanoparticles was round to ovate in a regular fashion and size ranging between 30 and 70nm and also can be seen from the EDS spectrum (Figure 5) of the silver nanoparticles with strong signals from Ag, C, O and N.

The antibacterial efficacy of biogenic silver nanoparticles tested against bacterial cultures showed significant inhibitory effect. Among the organisms tested, *Staphylococcus aureus* showed the maximum susceptibility of zone size 11 mm followed by *Klebsiella pneumoniae* with 9 mm as its zone size. This clearly indicated the toxic nature of silver nanoparticles as against cell filtrate (control) having a broad spectrum target which could bring about an inhibitory effect on the bacterial cultures (Figure 6).

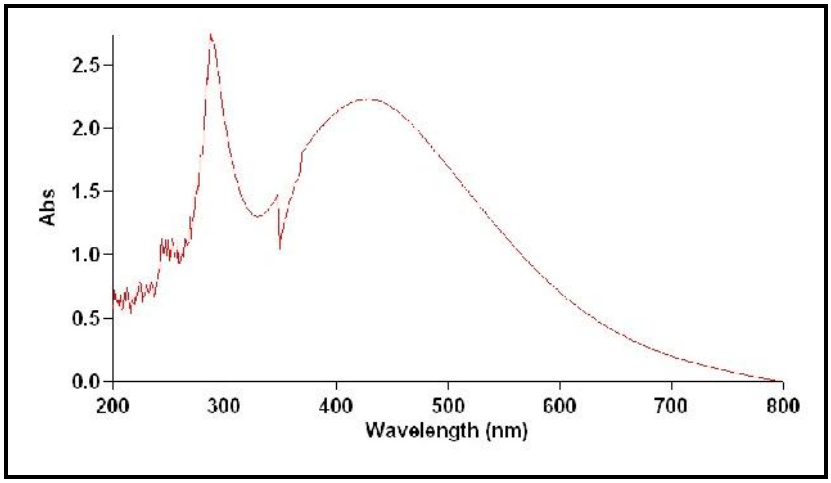

Figure 1. UV-visible spectrum of silver nanoparticles showing λmax at 428 nm

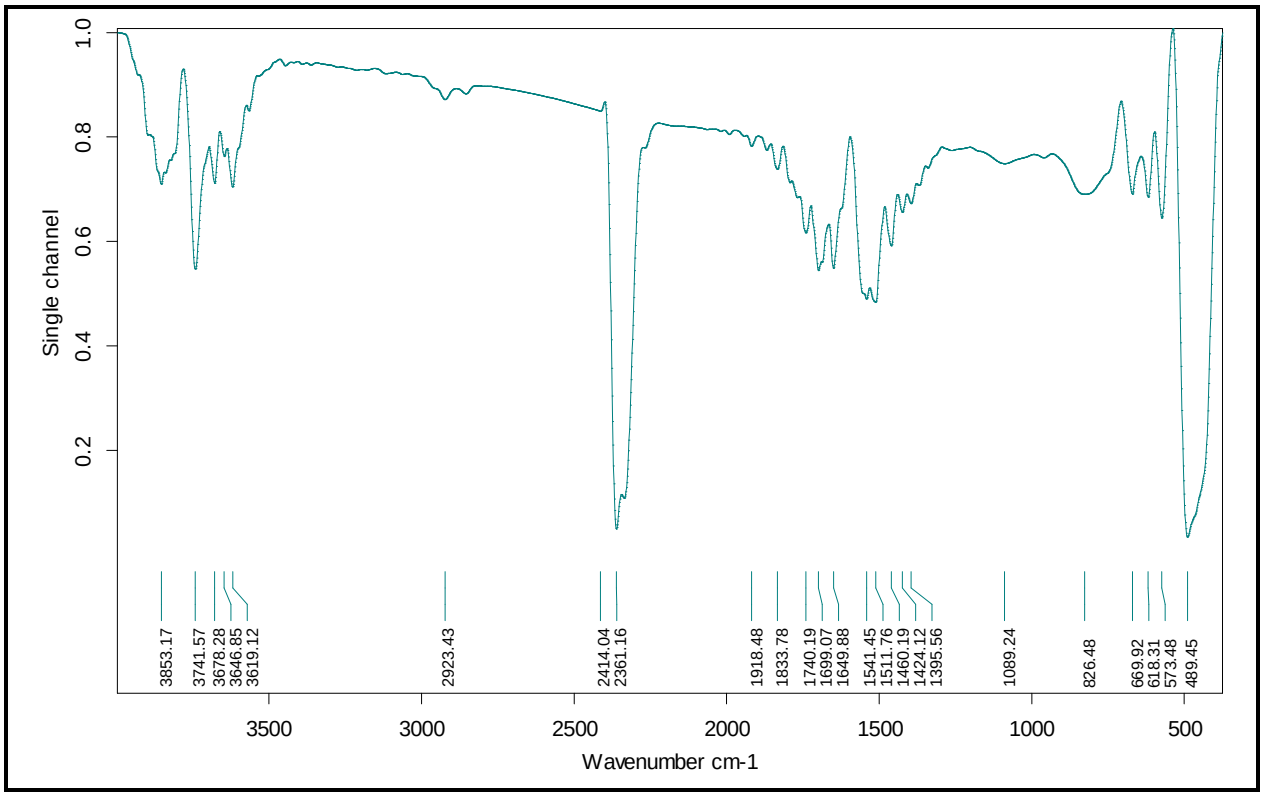

Figure 2. FTIR spectra of AgNps synthesized using the extracellular filtrate of *Serratia marcescens* S01

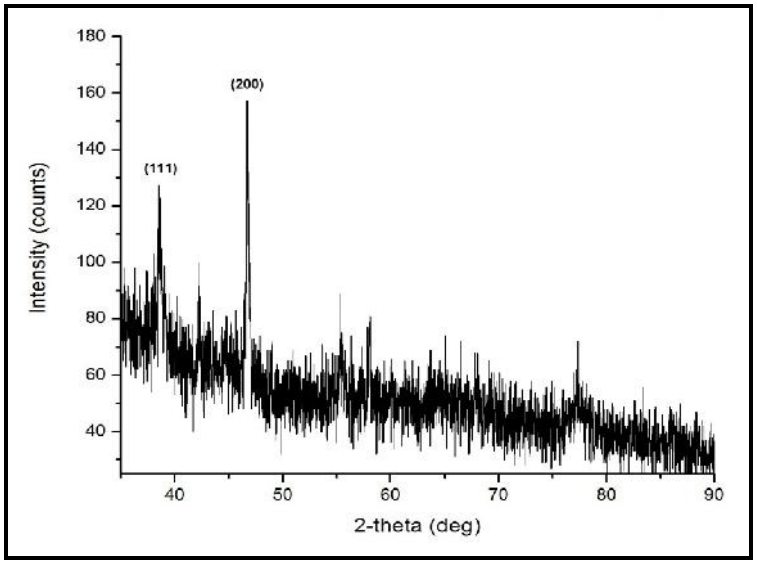

Figure 3. X-ray diffraction showing peak indices and 2θ positions

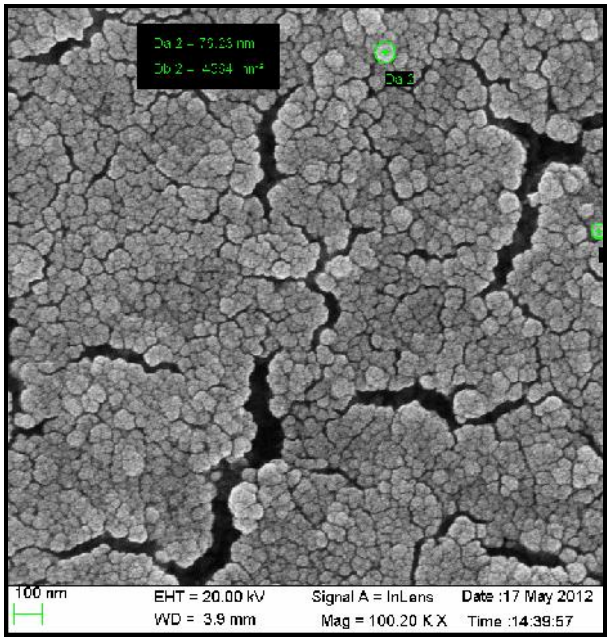

Figure 4. SEM picture showing silver nanoparticles (scale bar corresponds to 200 nm)

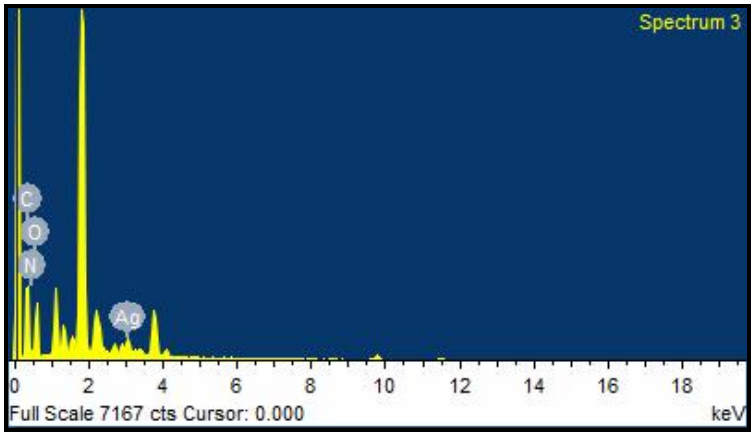

Figure 5. X-ray Energy Dispersive spectra (EDS) of prepared silver nanoparticles. Strong signals from atoms in the nanoparticles are observed in spectrum and confirm the reduction

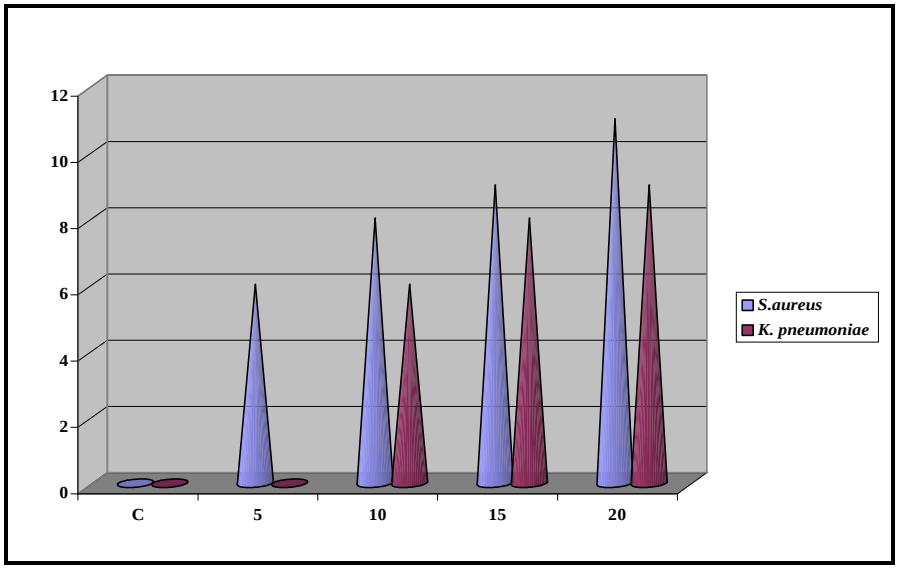

Figure 6. Antimicrobial activity of silver nanoparticles at different concentrations toward *Staphylococcus aureus* and *Klebsiella pneumoniae*

Discussion

In the present study, extra cellular synthesis of silver nanoparticles and its interaction with protein moiety was performed using an environmental isolate *Serratia marcescens* S01 altogether assessing its disinfecting property. Similar synthesis protocol for silver nanoparticles has been adopted various bacterial strains[13,14]. The bio-reduction of silver ion to silver nanoparticles with the appearance of brown color was observed after 96 h of incubation. The brown color formed would be due to the excitations of surface plasmon resonance of silver with its characteristic absorbance at 428 nm[14,16]. The appearance of only a single surface plasmon resonance band appeals that the spectra belong to isotropic and spherical nanoparticles which was further confirmed by SEM. This investigation is in agreement with early reports on the adsorption peak sites and their basic relatedness to the particle size[17,18]. Mainly protein is involved in the bioreduction process[18] as observed from Nitrate reductase assay and FTIR studies. The mechanism involved in the transformation of silver ion might attribute to the involvement of an enzyme probably nitrate reductase as previously reported[14,20]. The catalytic activity of NADH-dependent reductase and the role of NADH as an electron carrier might have attributed in the silver ion reduction process. The free amine groups of cysteine residues in the proteins bind to silver nanoparticles offering stability[21]. However, the carbonyl group and peptides behave as a capping agent[22] over the nanoparticles thereby avoiding aggregation of particles.

The antimicrobial study elucidates that silver nanoparticles brought about significant inhibitory effect on the gram positive (*Staphylococcus aureus*) compared to gram negative (*Klebsiella pneumoniae*) bacteria. The surface modified AgNps with a positive charge has a greater affinity toward the negatively charged bacterial cells.

Conclusion

In this study, a simple, spontaneous, microbial extra cellular synthesis of silver nanoparticles has been achieved using a soil isolate *Serratia marcescens* S01. This is an environment friendly approach in the synthesis protocol where the experiment is carried out at ambient temperature, neutral pH and non-toxic solvent, water. The functional reducing agents and enzymes released by the bacterium are responsible for the conversion of harmful ions into non-harmful matters in the nanoparticles synthesis. The nanoparticles with its physico-chemical and optical properties transformed using micro biota will have a role in remediation of toxic products piled up in the environment. Secondly, those organisms that show resistance toward antibiotics can be effectively targeted by these AgNps unveiling its broad spectrum pharmacological property.

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