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Research Paper

Bio-Processing and Prophylactic Efficacy of Silver Nanoparticles

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In modern world of science, the interaction between inorganic molecules and biological system is one of the most exciting areas of research and there is a growing need to develop an environment friendly benign nanoparticle synthesis process. In recent years, microorganisms have been explored as potential nano-biofactories for the development of experimental synthesis of metallic nanoparticles. In this respect, many unicellular and multicellular microorganisms were viewed as ecofriendly nano-biofactories to produce inorganic materials either on intra or extracellular level. The present work has focused on the study of biogenic synthesis of silver nanoparticles using a soil bacterium. Extra cellular method of synthesis using 1 mM $AgNO_3$ and the culture filtrate was employed for reduction. The reduction of $AgNO_3$ to nanoparticles was observed by the change in color to brown. Characterization studies were performed using UV vis-spectroscopy, X-ray diffraction and SEM imaging. The result revealed a maximum absorbance (λ_{max}) at 421 nm with a face centered cubic (fcc) symmetry comprising spherical particles in the size 30 ~ 70 nm. The mean size of the biogenic nanoparticles was found to be 176.4 nm attributing to the protein that offered stability and behaved as capping agent. Further, the culture filtrate when subjected to enzyme assay gave a cherry red color indicating the presence of the enzyme and characterized by SDS-PAGE. The synthesized silver nanoparticles $\overline{x}$ = 176.4 nm) inhibited the growth of pathogens tested and toxicity could be compared and checked with different mean size of particles in further studies for improving its prophylactic efficiency.

Key Words: Silver Nanoparticles; UV Vis-Spec; XRD; SEM; SDS PAGE; Prophylactic Activity

Introduction

Metal nanoparticles have huge applications in the manufacture of both commercial and personal products. These nanoparticles have profound implications such as gas sensors, catalysts, superconductors, and ceramic pigments [1]. In recent years, nanotechnology is being applied to biology and medicine to bring about revolutionary advances in diagnostics and therapeutics, molecular biology and bioengineering. This has resulted in the production of functional nanoparticles that can covalently bind to biological molecules like peptides, proteins and nucleic acids. Nanoparticles can be used as nano-carriers in drug delivery and open up vast new possibilities for drug synthesis and utilization, cancer treatment, nano-structured films or scaffolds for medical implants, artificial bones and tissues. The analysis and understanding of the fundamental relation between particle size and specific properties, together with the application perspectives has resulted in the elaboration of different nanoparticle synthesis strategies such as chemolithotrophy for energy production, use of these particles for special functions and detoxification for survival in toxic environments [2]. It is well known that many organisms can provide inorganic materials either intra or extra-cellularly and their implications in the remediation of toxic metals [3] is recognized as a worthwhile goal with regard to ecological impact. Micro organisms such as bacteria and fungi are recently found as possible eco-friendly nanofactories in the development of a relatively new and largely unexplored area of research [4]. Among the

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microorganisms, prokaryotic bacteria have received the most attention in the area of biosynthesis of nanoparticles. In this study, an attempt was made on the extracellular synthesis of silver nanoparticles and its influence on the prophylactic efficiency against pathogenic bacterial isolates.

Materials and Methods

1. 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.

2. Isolation and Screening

Five different samples of rhizosphere soil in and around FIPPAT, Chennai, Tamilnadu were collected for the isolation of *Bacillus* and identified according to Holt *et al*. [5]. 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 isolate that formed a clear zone was selected and B01 was retained for this experiment.

3. Nitrate Reductase Assay

Qualitative assessment of the enzyme was determined using Nitrate reductase assay [6]. 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. Further, characterization of the enzyme was performed using SDS-PAGE (10 % gel) with Bovine V fraction (m.w. 66 kDa) as control.

4. Silver Nanoparticle Synthesis

The AgNps were prepared according to the procedure described in the literature [7]. In brief, AgNps were synthesized extracellulary using *Bacillus* B01 grown in LB broth. The cell free extract, 100 mL was mixed with aqueous solution of 1 mM $AgNO_3$ prepared using Deionised Milli-Q water (Milli-Q Integra 3, Millipore, MA) in a 500 mL Erlenmeyer flask. Control flask was prepared by the addition of 1 mM $AgNO_3$ to sterile broth and the flasks incubated on a rotatory shaker at 150 rpm in dark condition at 30°C and observed for a color change. Regular monitoring was done by sampling 2 mL aliquot of the reaction mixture at intervals observing the maximum absorbance in UV-visible spectrophotometer (UV-1800, Shimadzu, Japan) in the range 200-800 nm at a resolution of 1 nm. The 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.

5. Characterization of Nanoparticles

The biogenic nanoparticles were characterized using Rigaku multiflex diffractometer (Rigaku Americas Incorporation, Houston, TX) and the morphology and elemental composition were determined using Scanning Electron Microscope (SEM, Model S-3000H, Hitachi, Japan) and Energy dispersive X-ray Spectroscopy (EDAX) to obtain the nanostructural information. The particle size distribution and zeta potential was analyzed providing the refractive indices of silver and the dispersant (Malvern Zetasizer Nanoseries) and the average mean size with respect to intensity was recorded.

6. Prophylactic Efficacy of AgNps

The efficacy of the AgNps synthesized by the bacterial isolate was determined by performing Antibiotic Susceptibility test using well diffusion method. Five bacterial test organisms were used and their respective inoculum was prepared in peptone water and turbidity adjusted to ~0.5 McFarland standard. The antimicrobial assay was performed using the silver nanoparticles along with antibiotics such as Cefixime and Cefotaxime (1 mg/mL) as control on MHA. 20 µL each of silver nanoparticles and control was added to wells and the plates were incubated at 37°C for 16-18 h and the diameter of

zone of inhibition was measured.

Results

1. Bio-processing

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 brown (Fig. 1) indicated the reduction and growth of silver nanoparticles. UV-visible spectrum analysis showed maximum adsorption peak between 420 and 430 nm and remained constant at 421 nm after 72 h of incubation (Fig. 2).

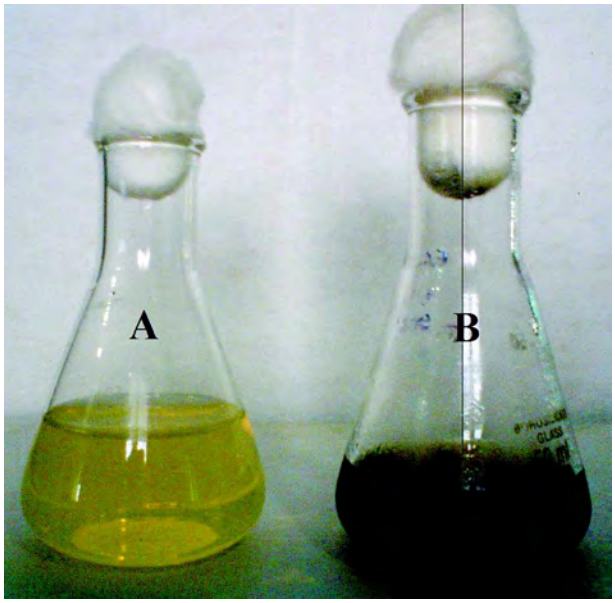

Fig. 1: Synthesis of AgNps with brown colour (B) change from golden yellow colour (A)

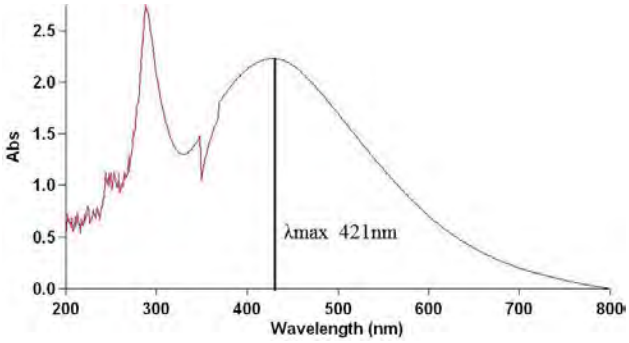

Fig. 2: UV-Vis spectrum of AgNps with maximum absorbance at 421 nm

2. Nitrate Reductase Assay

The assay confirmed the presence of the enzyme and the conversion of nitrate to nitrite upon addition of reagents giving a cherry red color appearance (Fig. 3). Further characterization done using SDS-PAGE showed a distinct band in proximity to the 66 kDa band of Bovine V fraction. The molecular weight was found approximately in the range of 40-50 kDa (Fig. 4).

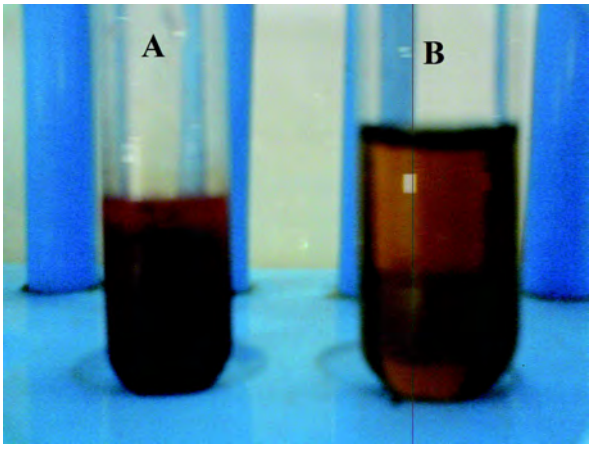

Fig. 3: Nitrate reductase assay showing cherry red colour change upon reduction (A) with no colour change (B) in control

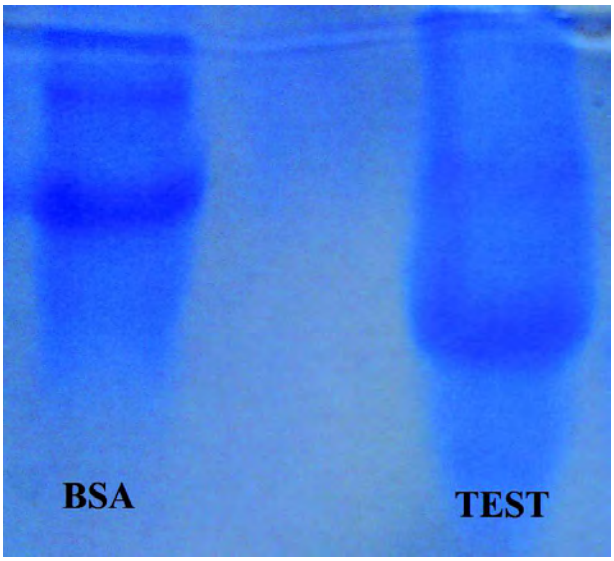

Fig. 4: SDS-PAGE showing distinct band of test protein (~40-50 kDa) in close proximity of BSA (66 kDa)

3. Characterization of AgNps

The typical XRD pattern (Fig. 5) of the reduced metal ie., $AgNO_3$ shows diffraction peaks at 2θ = 38º, 44º that can be indexed to (111), (200) planes of silver (PDF No.04-0783) that confirmed the main composition of the nanoparticles was silver with a face centric cubic symmetry. SEM was employed to observe the topography and size of the nanoparticles as shown in Fig. 6. The appearance of silver nanoparticles was spherical to ovate in a regular fashion and size ranging between 30 and 70 nm with an average size of 40-45 nm. It was also inferred from the EDAX spectrum (Fig. 7) of the silver nanoparticles exhibiting strong signals from Ag, C, O and N.

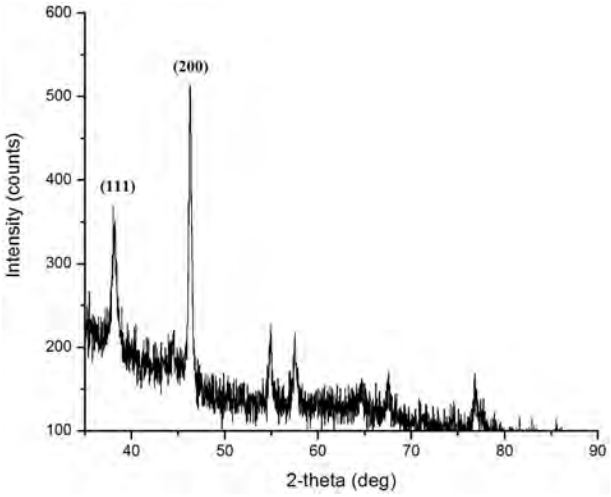

Fig. 5: X-Ray Diffraction patterns of AgNps showing peak indices and 2θ positions

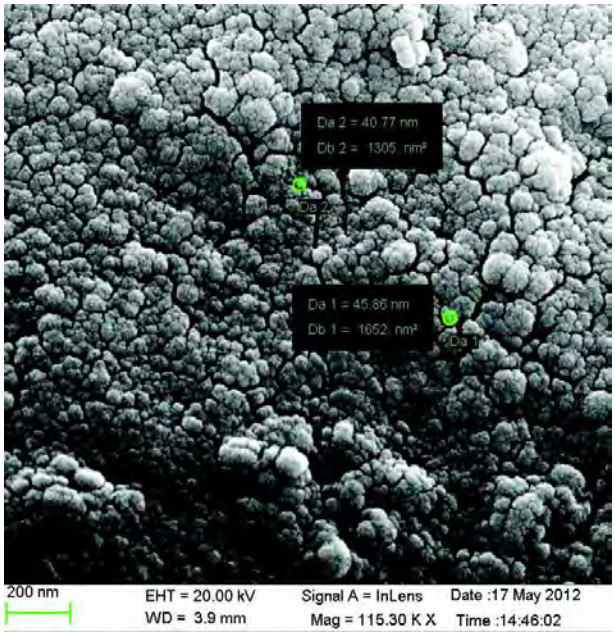

Fig. 6: SEM image of silver nanoparticles of average size of 40-45nm (scale bar corresponds to 200 nm)

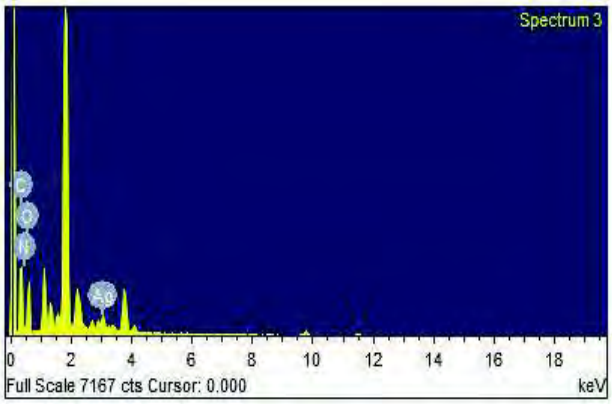

Fig. 7: EDS spectrum of silver nanoparticles (AgNps)

4. Mean Size of AgNps

The mean size of the synthesized AgNps was determined to be 176.4 nm as shown in Fig. 8 and the zeta potential was calculated as 0.34 mV with a positive polarity (data not shown). The increase in size of the particle was due to the capping of the protein present in the medium thus offering stability to the AgNps.

5. Antimicrobial Susceptibility Testing of AgNps

The antibacterial efficacy of biogenic silver nanoparticles was tested against isolated bacterial cultures. 20 µL of AgNps was loaded onto wells on MHA medium along with antibiotics. It showed good antimicrobial activity against all the organisms tested (100%) i.e., all the isolates were more susceptible toward AgNps whereas only one organism was found sensitive to antibiotics used. Among the five organisms, gram positive *Staphylococcus aureus* showed the maximum susceptibility to antibiotics and silver nanoparticles followed by *Acinetobacter, Serratia, Pseudomonas* and *Candida* sp. (Table 1).

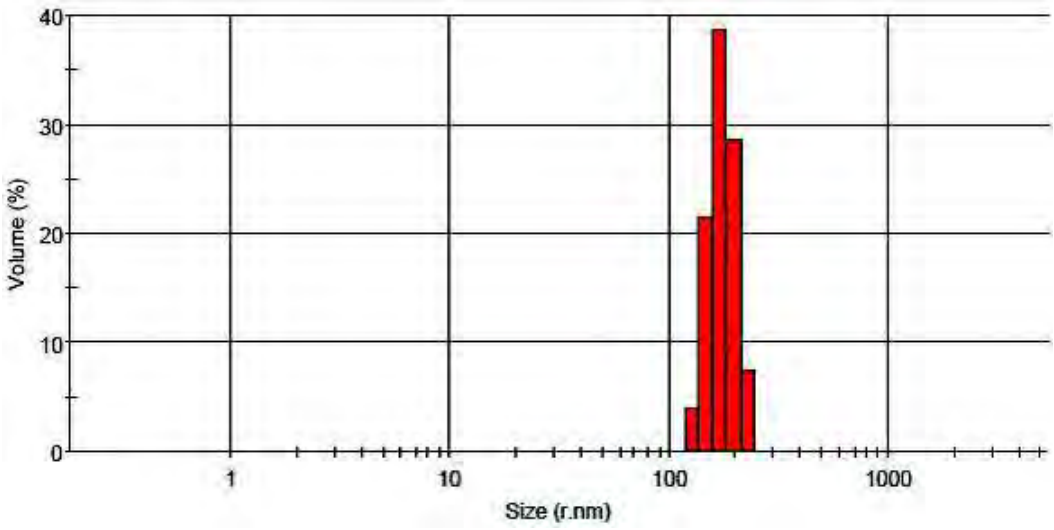

Fig. 8: Particle size distribution of AgNps with an average size of 176.4 nm

Table 1: Prophylactic efficacy of synthesized silver nanoparticles (AgNps) *vs* antibiotics

Test organisms	Diameter of zone of inhibition (in mm)			
	Culture filtrate	AgNps (10µg/mL)	Control Cefixime (1µg/mL)	Cefotaxime (1µg/mL)
S. aureus	NZ	11	17	22
Acenitobacter sp.	NZ	9	NZ	NZ
Serratia sp.	NZ	9	NZ	NZ
Pseudomonas sp.	NZ	8	NZ	NZ
Candida sp.	NZ	7	NZ	NZ

NZ – No Zone

Discussion

In the present study, extra cellular synthesis of silver nanoparticles was performed using an environmental isolate *Bacillus* B01. This bacterium was employed in the nanoparticles synthesis considering its diversified growth at extreme temperatures, salt tolerance, metal resistance and ubiquitous nature was found to be more advantageous compared to fungi and plant materials. Similar synthesis protocol for silver nanoparticles has been adopted using ATCC strains [8, 9, 10]. The nitrate reducing potency of the bacterium was applied in nanoparticle synthesis

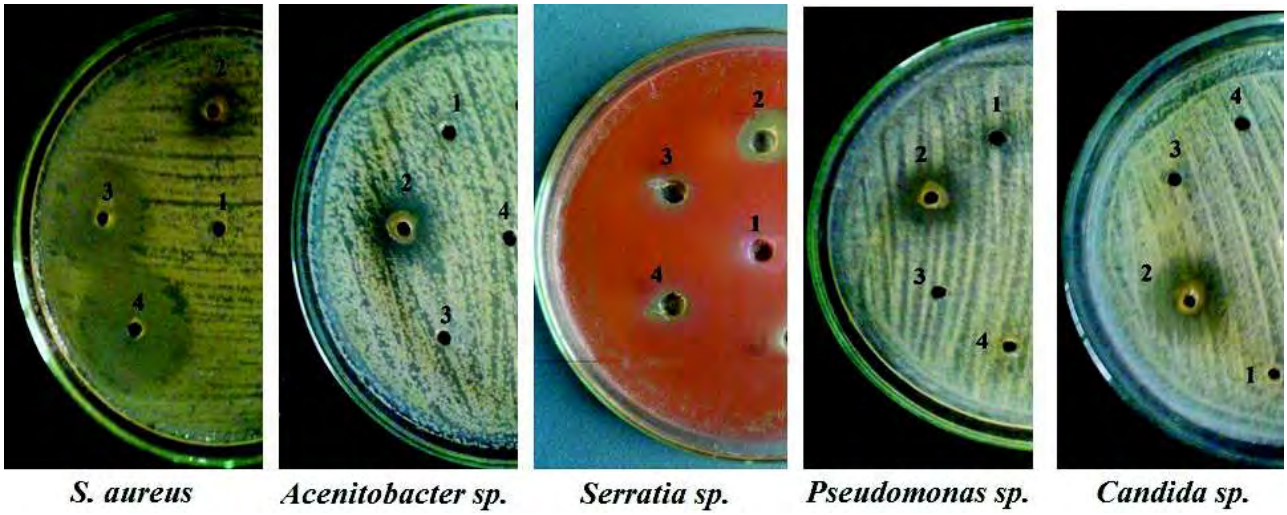

Fig. 9: Prophylactic efficacy of AgNps and Antibiotics : 1–Crude extract; 2–AgNps; 3–Cefixime; 4–Cefotaxime

process at greater efficiency. Nitrate reductase assay result revealed the development of cherry red color [8] within 34 h of incubation attributing to the production of extra cellular enzyme/metabolite in reducing nitrate. In case of metal salt, the reduction process was achieved after 72 h of incubation under aerated condition at 30ºC. There is a direct correlation between the length of time and the reduction process that follows enzyme to substrate concentration. The gradual change in color of the medium to brown indicated the synthesis and growth of silver nanoparticles. The presence of a single resonance band (λ_{max} 421 nm) in the spectra clearly indicated the synthesis of isotropic and spherical nanoparticles as evident from SEM [11].

However, on analyzing the particle size distribution of AgNps, the average size was found to be 176.4 nm. The encapsulation of the AgNps by the protein moiety (extra cellular component) behaved as a capping agent resulting in the increase of average particle size. Further, SDS-PAGE was performed to determine the molecular weight of the unknown protein along with a known protein marker BSA. Distinct band in close proximity to BSA demonstrated the presence of the protein with an approximate molecular weight in the range 40-50 kDa [12], characteristic of reductase.

Furthermore, reports on the silver nanoparticles produced within 28 h of incubation using culture mass of *Fusarium oxysporum* [13] and 1 h using plant extract [14] stating the difference in reducing potential between microbial and higher forms.

The antimicrobial activity of AgNps was performed toward the frequently encountered pathogens of gram positive and gram negative bacterium. There was a significant inhibitory effect against all the tested organisms tested (100%) i.e., all were sensitive. Among them, gram positive *S. aureus* showed a maximum susceptibility toward AgNps and antibiotics (Cefixime and Cefotaxime) with 11, 17 and 22 mm (Fig. 9). The remaining micro organisms which were not responding to the antibiotics were susceptible to AgNps attributing to the toxic nature of the nanoparticles. This study elucidates that 1 mM of reduced silver nanoparticles could bring about an inhibitory effect on the pathogens similar to the work carried out by Nanda *et al.* [7] and contrary to 50 mM and 6.5 mM considered as the maximum and minimum dose for bactericidal activity as previously reported [15]. The surface modified AgNps with a positive charge has a greater affinity toward the negatively charged bacterial cells [16]. It is still unclear how the silver nanoparticles paralyse the bacterial cell and the complexity in understanding the multiple targets it aims at needs further investigations.

Conclusion

The biogenic synthesis of silver nanoparticles was found effective and is advantageous as far as the environmental issues are concerned. One drawback in this method is the control in particle size which is time consuming. Extensive study is required to derive particle size of equal proportions. The efficacy of the silver nanoparticles on par with the antibiotics was comparatively better and would be used as an antibacterial agent after checking its stability and the level of toxicity in further studies.

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