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PRODUCTION OF SILVER NANOPARTICLES BY A PHYTOPATHOGENIC FUNGUS *BIPOLARIS NODULOSA* AND ITS ANTIMICROBIAL ACTIVITY

S. SAHA[1], J. SARKAR[1], D. CHATTOPADHYAY[2], S. PATRA[3], A. CHAKRABORTY[4], K. ACHARYA[1*]

[1]*Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, Kolkata-700019*

[2]*Department of Polymer Science & Technology, University College of Science & Technology, University of Calcutta, Kolkata-700009*

[3]*Govt. College of Engineering and Ceramic Technology, 73 A.C. Banerjee Lane, Kolkata-700010*

[4]*Centre for Research in Nanoscience and Nanotechnology, University of Calcutta, Kolkata-700009*

In this work we reported the potentiality of a phytopathogenic fungus *Bipolaris nodulosa* to produce anisotropic silver nanoparticles using its mycelia free media (MFM). These nanoparticles were characterized by UV-Vis spectroscopy, Dynamic light scattering (DLS) and X-ray diffraction (XRD). For morphological details Transmission electron microscopy (TEM) and Atomic force microscopy (AFM) were carried out. Fourier transform infrared spectroscopy (FTIR) was also performed to find the groups of the capping materials which were responsible for the stability of the nanoparticles. The effects of these nanoparticles against different microorganisms were also determined.

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1. Introduction

Recently, nanotechnology research is emerging as the cutting-edge technology, interdisciplinary with physics, chemistry, biology, material science and medicine. Since prehistoric times, among all inorganic antimicrobial agents, silver has been extensively used to resist infections. As silver salts, having an antimicrobial effect [1], are used in a variety of applications including dental work, catheters and burn wounds [2, 3], synthesis of silver nanoparticles now a days are of great desire. Although an array of physical and chemical methods have been used to synthesize silver nanoparticles, the use of bio-compatible, non-toxic, cost-effective and eco-friendly methods predominate the others. The rich diversity of microorganisms such as bacteria, fungi, etc. and their potentiality to control the synthesis of various metallic nanoparticles, should be taken into consideration which is yet to be fully explored. Moreover, biologically synthesized silver nanoparticles have many applications in areas of non-linear optics, spectrally selective coating for solar energy absorption and intercalation materials for electric batteries, optical receptors, catalysts in chemical reactions, biolabelling and antibacterials [4].

A number of microorganisms including algae [5], bacteria [6,7] and fungi have been reported for the green synthesis of silver nanoparticles. But, filamentous fungi are more advantageous over the bacteria and algae in having fungal mycelial mesh which can withstand flow pressure and agitation and other conditions in the bioreactors or other chambers. Nevertheless, due to their fastidious growth, easy handling property and easy fabrication property,

*Corresponding author: krish_paper@yahoo.com

the process could be scaled-up. Moreover, their extracellular secretions of reductive proteins are more which helps in outside precipitation of nanoparticles and minimizes the unnecessary cellular components, aiding direct use in various applications. The initial approach of mycosynthesis of silver nanoparticles was carried out by challenging an acidophilic pathogenic fungus of *Taxas* plant, *Verticillium* sp. with silver nitrate ($AgNO_3$) leading to the reduction and accumulation of Ag-nanoparticles of about 25 nm in diameter intracellularly within the biomass [8]. Beside this other silver tolerant fungi like *Fusarium oxysporum* [9], *F. solani* USM 3799 [10], *Aspergillus niger* [11], *Coriolus versicolor* [12], etc. can produce silver nanoparticles (Ag-nanoparticles) of different sizes but with spherical shapes. But, *A. fumigatus* can produce both spherical and triangular shaped silver nanoparticles of size 5-25 nm [13]. Later on a number of fungi have been investigated by scientists and were found to be capable of biosynthesizing Ag-nanoparticles having different particle size and shape, both extra and intracellularly [14]. (Review paper)

The antibacterial property of silver nanoparticles has been studied by a number of microbiologists. Sondi and Salopek-Sondi [15] evaluated the antimicrobial activity of silver nanoparticles against *Escherichia coli.* The results confirmed that the treated *E. coli* cells were damaged, showing pit formation on bacterial cell walls. In another work, Jain and co-workers [16] tested the bacterial action of silver nanoparticles-coated polyurethane foam and suggested its use as antibacterial water filter. Recently, Hu et al [17] treated cotton fabrics with suspension of silver oxide in chitosan and studied antibacterial actions against *Staphylococcus aureus*.

In the present work we explore the potentiality of a 'nodulosa' group of phytopathogenic fungus, *Bipolaris nodulosa*, causing seedling blight and leaf stripes of *Eleusine indica*, for the first time to synthesize Ag-nanoparticles when its mycelia-free media was seeded with silver nitrate solution. Furthermore, we also investigated the antimicrobial effects of the synthesized silver nanoparticles against representative microorganisms of public concern.

2. Experimental details

2.1. Pathogen isolation and production of mycelia free media

The infected leaves of *Eleusine indica* were brought to the laboratory and from the infected lesions, pathogen was isolated on Potato Dextrose Agar (PDA) slants following Davies' method. The pathogen was characterized and identified according to Subramanium and Ellis [18, 19, 20]. The fungus was grown aerobically in liquid medium containing malt (0.3%), glucose (1%), yeast extract (0.3%), peptone (0.5%) (purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India) and distilled water. The Erlenmeyer flasks of 250 ml capacity were inoculated with fungal mycelia and incubated at 30 ± 2˚C with shaking at 150 rpm. After 10 days of incubation, mycelia were separated from the culture broth by filtration through Whatman filter paper No.1.

2.2. Synthesis of silver nanoparticles by mycelia free media

The chemical silver nitrate ($AgNO_3$) was purchased from Sigma, St. Louis, MO, USA. In 250 ml Erlenmeyer flasks 100 ml of mycelia free media and 1mM silver nitrate ($AgNO_3$) (final concentration) solution was added and placed on a rotary shaker at 150 rpm at room temperature. Simultaneously, a positive control of the mycelia free media and a negative control of only silver nitrate solution were maintained under the same conditions (Fig. 1). The silver nanoparticles were separated out by centrifugation (at 12000 g for 10 min) and the settled nanoparticles were washed with deionized water (three times). The purified silver nanoparticles were re-dispersed in water by ultrasonication (Piezo-u-sonic ultrasonic cleaner, Pus-60w).

Fig. 1. Mycelia free medium with $AgNO_3$ (1mM) and controlled sets (negative and positive) at room temperature.

2.3. UV-Vis spectroscopic studies

To monitor the fungus dependent bioreduction of Ag^+ in aqueous solution of 1mM $AgNO_3$ to silver nanoparticles (Ag^0), the samples were scanned in a UV-Vis Spectrophotometer (HITACHI-1130 Spectrophotometer) at room temperature.

2.4. Measuring the size by Dynamic Light Scattering Test

Particle size was measured by laser diffractometry using a Nano Size Particle Analyzer (ZEN 1600 MALVERN USA) in the range between 0.6 nm to 6.0 μm, under the following conditions: particle refractive index 1.590, particle absorption coefficient 0.01, water refractive index 1.33, viscosity –cP, temperature- 25ºC and general calculation model for irregular particles. Thirteen measurement cycles of 10 seconds each were taken and the average was done by using software (DTS, Ver. 5.00 from Malvern).

2.5. XRD measurement

The liquid reaction mixture after bioreduction was dried at 45ºC in a vacuum drying oven. Then the dried mixture was collected for the determination of the formation of silver nanoparticles. The vacuum-dried silver nanoparticles were used for powder X-ray diffraction (XRD) analysis. The spectra were recorded in a PW. 3040/60 PANalytical X-ray Diffractometer (Cu Kα radiation, λ 1.54443) running at 45 kV and 30 mA. The diffracted intensities were recorded from 2 degrees to 90 degrees 2θ angles.

2.6. FTIR analysis

For Fourier transform infrared spectroscopy (FTIR) analysis, the vacuum dried silver nanoparticles were mixed with Potassium Bromide (KBr) at a ratio of 1:100 and the spectra were recorded with a SHIMADZU 8400S Fourier Transform Infrared Spectrophotometer using a diffuse reflectance accessory. The scanning data were obtained from the average of 49 scans in the range between 4000 to 400 cm^{-1}.

2.7. AFM observation of silver nanoparticles

Size and the surface topography of the drop coated film of the silver nanoparticles was investigated with Atomic Force Microscope (AFM) [NANOSCOPE (R) 111a Veeco multimode,

USA] and high resolution surface images were produced. In AFM characterization, the tapping mode (NP10) with a silicon probe over scan sizes of 10 μm was used.

2.8. EDX observation of silver nanoparticles

Energy-dispersive X-ray (EDX) analysis was carried out by the same instrument and employed to confirm the presence of silver in the particles as well as to detect the other elementary compositions of the particles.

2.9. TEM observation of silver nanoparticles

Samples of the aqueous suspension of silver nanoparticles were prepared by placing a drop of the suspension on carbon-coated copper grids and allowing the water to evaporate. TEM observations were performed on Tecnai G^2 spirit Biotwin (FP 5018/40) operated at an accelerating voltage at 80 kV.

2.10. Assay for antimicrobial activity of silver nanoparticles

The silver nanoparticles in deionized water were tested for their antibacterial activity by the agar diffusion method. Six bacterial strains, *Bacillus subtilis* [MTCC 736], *Bacillus cereus* [MTCC 306], *Pseudomonas aeruginosa* [MTCC 8158], *Proteus vulgarius* [MTCC 426], *Escherichia coli* [MTCC 68] and *Micrococcus luteus* [MTCC 1538] were used for this analysis. These bacteria were grown on nutrient broth (NB) media (purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India.) for 24 hours prior to the experiment, seeded in agar plates by the pour plate technique. Cavities were made using a cork borer (5 mm diameter) at an equal distance and were filled with the silver nanoparticle solution and then incubated at 37°C for 24 hours.

3. Results and discussion

After addition of aqueous $AgNO_3$ (1mM), the mycelia free media showed a gradual change in colour at room temperature with time from yellowish to light pink, reddish brown and finally to dark brown within 24 hours. The later color is characteristic of the surface plasmon resonance (SPR) of silver nanoparticles [12]. The control sets (positive and negative) showed no change in colour under the same experimental conditions. The reduction of silver was subjected to spectral analysis by using the UV-Vis spectrophotometer. This showed an absorbance peak around 420 nm which was specific for silver nanoparticles (Fig. 2). It might arise from the excitation of a longitudinal plasmon vibration of silver nanoparticles in the solution [21].

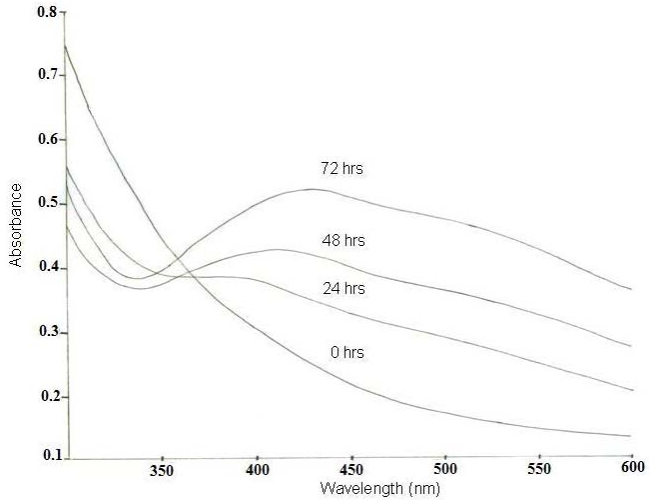

Fig. 2. UV-Vis spectra of the Ag-mycelium free media at room temperature.

Particle size was determined by dynamic light scattering measurement. Laser diffraction studies revealed that particle size obtained from DLS were monodisperse in nature and are in the range of 10 to 60 nm (Fig. 3).

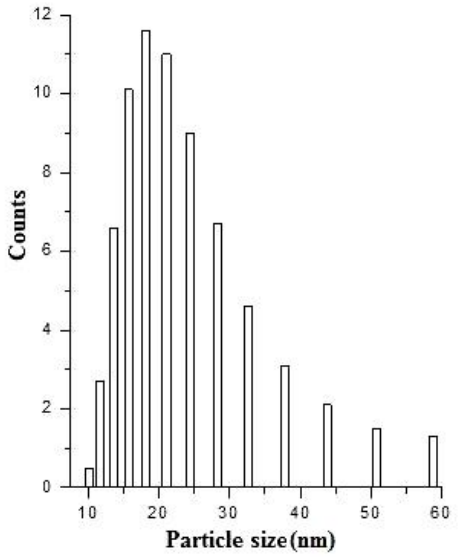

Fig. 3. DLS data plotted graphically indicating particle size range.

An elemental composition analysis employing EDX showed the presence of a strong signal from silver atoms (74.63%) (Fig. 4). Moreover, the presence of sharp optical absorption peak in the range of 3 to 4 keV which is typical for the absorption of metallic silver nanocrystallites [22]. This analysis indicated that the nano-structures were composed solely of silver. However, there were other EDX peaks for Si, P, Cl and Ca, suggesting that they were mixed precipitates from the centrifuged MFM. X-ray diffraction (XRD) further confirmed the generation of Ag^0. Inspection of the XRD patterns of vacuum dried silver nanoparticles reveal the existence of sharp diffraction lines at low angles (2° to 99°). The silver nanoparticles exhibited peaks of silver at 2θ=38˚, 44˚, 64˚ and 78˚ that can be indexed to the (111), (200), (220) and (311) facets of silver, respectively (Fig. 5) which agree with the values reported for face centered cubic (fcc) silver nanocrystals (JCPDS card file no. 4-783).

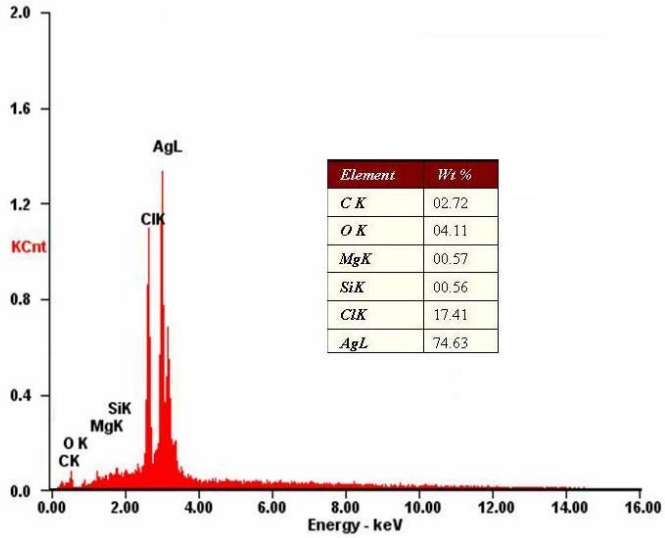

Fig. 4. EDX spectrum recorded showing sharp peak between 3 and 4 keV confirming the presence of silver.

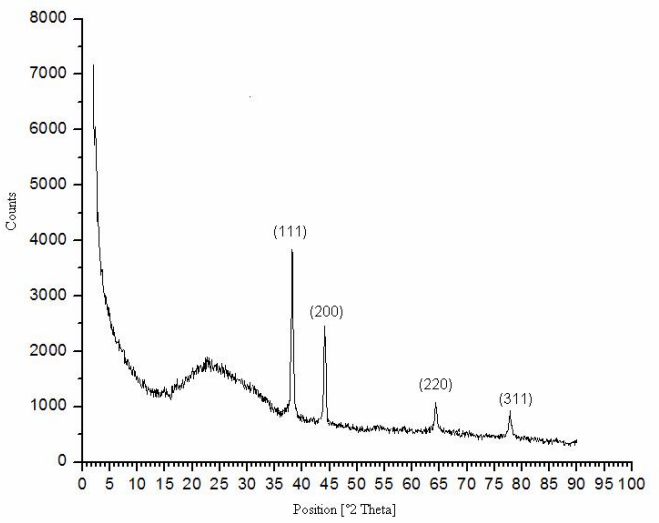

Fig. 5.XRD patterns recorded showing 4 sharp peaks corresponding to the diffraction from 111, 200, 220 and 311 planes of silver with fcc lattice.

FTIR results showed nine prominent bands (Fig. 6), of which bands at 1379 cm^{-1} and 1018 cm^{-1} can be assigned for C-N stretching vibrations of aromatic and aliphatic amines respectively, bands at 2921 cm^{-1} and 2852 cm^{-1} attributes to the side chain vibrations consisting of C-H stretching symmetric and antisymmetric modes of aliphatic and aromatic groups respectively and bands at 3433 cm^{-1} and 667 cm^{-1} indicating N-H stretching. The presence of band at about 1743 cm^{-1} corresponding to carbonyl stretch vibrations in ketones, aldehydes and carboxylic acids were noteworthy and infered that the reduction of silver ions were coupled to the oxidation of the hydroxyl groups in fungal hydrolysates released in Ag-fungal myceliafree media. Remaining other two bands at 1625 cm^{-1} and 1461 cm^{-1} signified the presence of amide I group (beta-sheets) and carboxylic groups respectively.

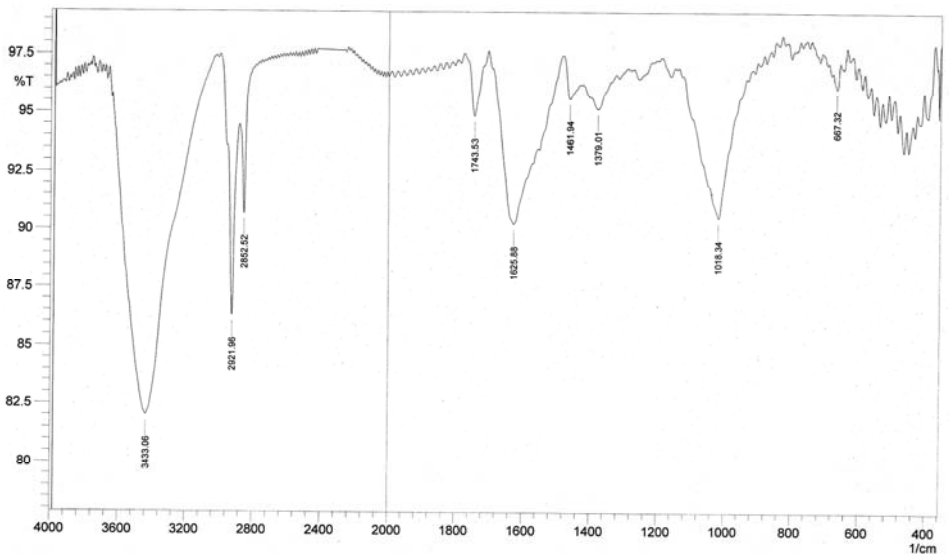

Fig. 6. FTIR spectra of Ag-nanoparticles synthesized by mycelia free media of the fungus.

Results obtained from AFM study represent a clear concept regarding shape (Fig. 7). Majority of the particles were symmetrical and spherical in shape, but some were hexahedral, triangular and semi-pentagonal, well distributed without any aggregation.

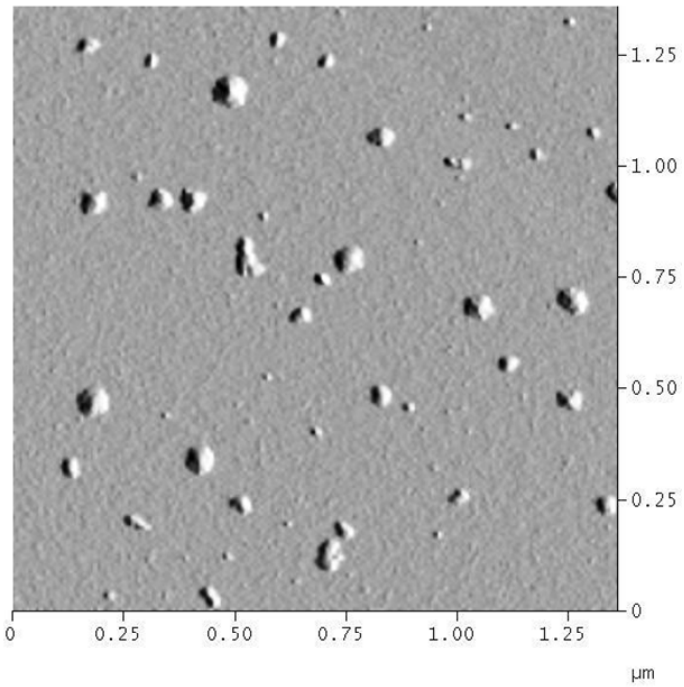

Fig. 7. AFM images showing clear spherical, hexahedral and semi-pentagonal structures of the Ag-nanoparticles.

Representative Transmission electron microscopic (TEM) images recorded different sizes of silver nanoparticles which arose from the bioreduction of silver nitrate by MFM at room temperature for 24 hours (Fig. 8). TEM observations revealed the formation of spherical, semi-pentagonal, hexahedral structures of the silver nanoparticles in the reaction solution. The diameters of these silver nanoparticles were measured and the sizes obtained, were in the range from 10 to 60 nm.

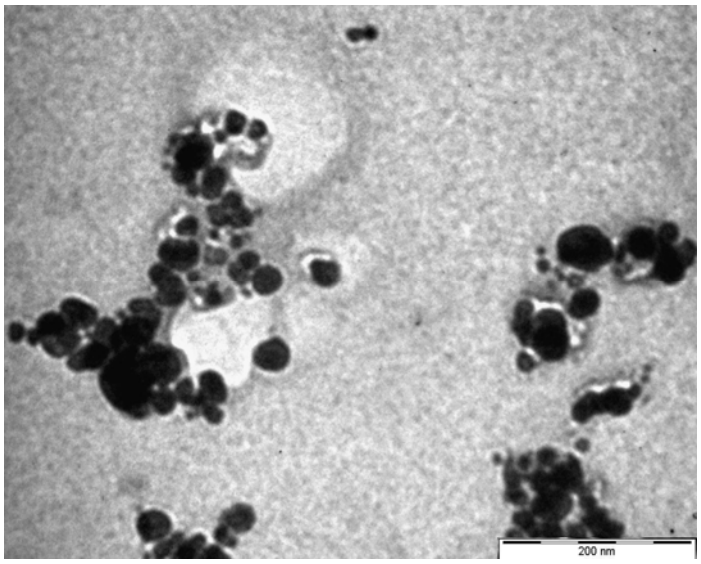

Fig. 8. TEM images of Ag-nanoparticles synthesized by mycelium free spent medium depicting spherical, hexahedral, pentagonal and semi-pentagonal structures.

Antimicrobial tests were performed against *Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Proteus vulgarius, Escherichia coli* and *Micrococcus luteus* on petri

plates by cup plate method. Silver nanoparticles at a concentration of 100 µg/ml showed a range of specificity towards its antimicrobial activity (Fig. 9). The diameter of the inhibition zones for all the tested bacteria were measured and plotted graphically.

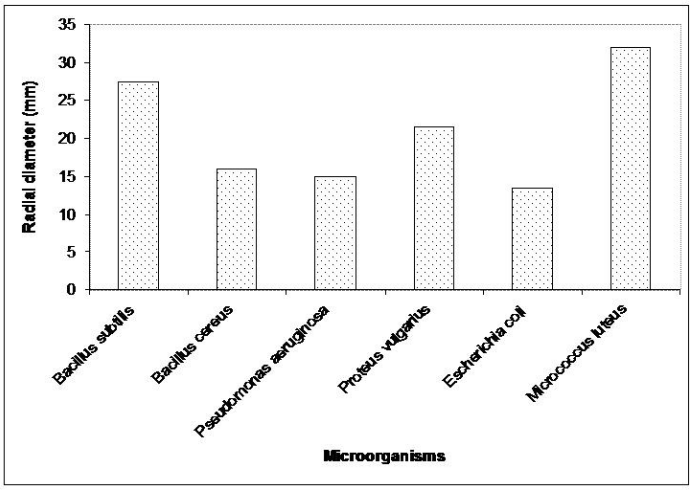

Fig. 9. Bar graph showing the antimicrobial activity of various bacteria against the synthesized silver nanoparticles.

4. Conclusions

The present study demonstrated the extracellular mycosynthesis of anisotropic silver nanoparticles by mycelia free media within 24 hours at room temperature when seeded with 1 mM $AgNO_3$. The nanoparticle characterization and morphology were verified by DLS, TEM, AFM, XRD and EDX. The FTIR spectroscopy report highlighted the coordination behaviors between amino groups in MFM and Ag ions which were accountable for the reduction of Ag^{+1} to Ag^0, its capping and stabilization of Ag-nanoparticles. Furthermore, these MFM mediated silver nanoparticles showed excellent antimicrobial activity against the mentioned six bacterial strains. Therefore, it could be concluded that these protein-conjugated metal nanoparticles can have immense use as water-soluble metallic catalysts in chemical reactions, biolabelling and as antibacterials.

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