Research article Available online www.ijsrr.org ISSN: 2279–0543

International Journal of Scientific Research and Reviews

Biofabrication of Silver Nanoparticles by Marine Fungus *Aspergillus fumigatus:* Synthesis, Characterization and Antibacterial Activity

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ABSTRACT

In the present study, we synthesized Silver nanoparticles (AgNPs) from fungal biomass of *Aspergillus fumigatus* using $AgNO_3$ as reducing agent. The synthesized AgNPs were characterized by UV-visible spectroscopy, Fourier transform infrared (FT-IR), Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), X-Ray Diffraction (XRD) and Selected Area Electron Diffraction (SAED). The UV-Visible range showed an absorption band at 446 nm, which corresponded to the surface plasmon absorbance of AgNPs. X-ray diffraction and transmission electron microscopy demonstrated that the biosynthesized AgNPs were crystalline in nature with an average diameter of 5 - 30 nm were noticed. XRD reveals that (111), (200), (220), (311), and (222) crystal planes of the face-centered cubic (FCC) which are the lattice, indicating the crystalline nature of the AgNPs. SAED pattern of AgNPs showed five circular fringes, which were in accordance with XRD data. FT-IR measurements indicated the peaks at 3416, 2926 and 2398 and 473 cm^{-1} corresponding to different organic functional groups possibly involved in the synthesis and stabilization of AgNPs. SEM studies showed formation of well-dispersed nanoparticles in the range of 20 - 50 nm and the shape of nanoparticles was spherical. Further, biosynthesized AgNPs was evaluated for antibacterial activity against *Klebsiella pneumoniae* (MCC 2716), *Escherichia coli* (MCC 2412), *Pseudomonas aeruginosa* (MCC 2408), *Enterobacter aerogenes* (MCC 3092) and *Bacillus subtilis* (MCC 2183) using disc diffusion method. These results revealed that the synthesized AgNPs was found to have significant broad spectrum of antimicrobial activity.

KEYWORDS: Biofabrication; silver nanoparticles; *Aspergillus fumigatus*; antibacterial activity

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INTRODUCTION

Silver is a prehistoric naturally occurring precious metal, most often in the form of a mineral ore in association with other elements[1]. Due to their unique properties, silver has been used for several applications, including as antibacterial agents, household and healthcare-related products, medical device coatings, optical sensors, and drug delivery system[2, 3]. Due to extensive applications of Silver nanoparticles (AgNPs) have gained an increasing interest due to their unique properties, which can be tailored for a specific application by controlling the shape, size, and morphology of the nano-sized particles[4]. Therefore, silver nanoparticles synthesis with defined size and shape are at the leading edge of nanoscience and nanotechnology.

Fabrication of AgNPs is accomplished by several physical, chemical and biological methods using top-down or bottom-up approaches[5, 6]. The advantages of biogenic AgNPs are three-fold; the bio-species can act as a template, a reducing or capping agent for nanoparticles[7]. There are volumes of literature proven that silver AgNPs could be successfully synthesized by bio-nano factories such as algae, fungi, yeast, bacteria, actinobacteria, and plants is a novel environmental friendly science for producing nanoparticles of definite size and shape with unique physical and chemical properties[8,9,10]

Among the microbes, fungi are one among the most important eco-friendly nanoparticle synthesizers of AgNPs. Fungal strains such as *Fusarium*[11], Aspergillus sp[12], *Acinetobacter* sp[13], *Penicillium* sp.[14], *Dictyota* sp.[15], *Duddingtonia* sp.[16], *Rhizopus* sp.[17], and *Raphanu* sp.[18] have been reported to synthesize AgNPs. Among the genus, *Aspergillus* was widely used in many industries for the production of biochemical substances and are therefore well-known for industrial manufacturing purposes. The filamentous components from *Aspergillus fumigatus* were used to synthesize AgNPs[19]and also other species of this genus was utilized for the rapid synthesis of AgNPs[20]. Since to date, there are very few reports available on the use of marine-derived fungus *A. fumigatus* for the synthesis of AgNPs. Therefore, the aim of the study is to synthesize and characterize the AgNPs using *A. fumigatus* and their evaluation of antibacterial activity. Human pathogenic bacteria such as *Klebsiella pneumoniae* (MCC 2716), *Escherichia coli* (MCC 2412), *Pseudomonas aeruginosa* (MCC 2408), *Enterobacter aerogenes* (MCC 3092) and *Bacillus subtilis* (MCC 2183) were used for the antimicrobial activity by agar well diffusion assay in the presence of AgNPs.

MATERIALS AND METHODS

Materials

All the chemicals used in this study including silver nitrate ($AgNO_3$) were of analytical grade and purchased from Himedia Ltd, India. Deionized water was used as solvent throughout the experiments.

Synthesis of silver nanoparticle

Aspergillus fumigatus was originally collected from the marine sediment of Appa Island, Gulf of Mannar, Tamilnadu, India. The isolated fungi (*A. fumigatus*) was identified on the basis of their morphological characteristics and microscopic examination. For the synthesis of AgNPs, *A. fumigatus* was grown in a 250 mL Erlenmeyer flask that contained Potato dextrose broth (100 mL) at 37ºC in a shaker incubator set at 140 rpm for 5 days. After incubation, the biomass of was separated by filtration, washed with sterile distilled water to remove the traces of culture media components, resuspended in 100 mL distilled water, incubated at 28°C for 24 hours and then filtered. 100 mL cell free filtrate solution of *A. fumigatus* was taken in a clean 250 mL conical flask; to which 10mL of aqueous solution of $AgNO_3$(1mM) was added and incubated in the dark condition at 37ºC in shaker incubator and the experimental reaction was carried out for 24 hours.

Characterization of silver nanoparticle

UV-Visible spectroscopy

Biosynthesis of AgNPs using *A.fumigatus* was analyzed and monitored by employing specific LSPR peak using UV–Vis spectroscopy (50 ANALYTIKJENA). UV-Visible spectra were recorded in the range from 250 to700 nm using deionized water as the blank.

FT-IR analysis

After the incubation period of 24 hours, the AgNPs were characterized by FT-IR and XRD. The FI-TR spectrum was taken in the mid IR region of 400-4000 cm^{-1}. The spectrum was recorded using ATR (attenuated total reflectance) method. The sample was directly placed in the KBr crystal and the spectrum was recorded in the transmittance mode.

X-RAY DIFFRACTION ANALYSIS

The silver nanoparticles prepared as described above were analyzed by X'pert PRO PAN analyzed X-ray diffractometer with Syn Master 793 software to identify the crystal phase of nanoparticles. The XRD pattern was recorded using computer controlled XRD-system, JEOL, and Model: JPX-8030 with CuK radiation (Ni filtered = 13418 Ao) at the range of 40kV, 20A. The ‘peak search' and ‘search match' program built-in software was used to detect the peak table and ultimately for the identification of XRD peak.

MORPHOLOGICAL ANALYSIS OF SILVER NANOPARTICLES

The previously detected morphology of nanomaterials was confirmed by Scanning Electron Microscopy (JEOL JSM 6360LA, Japan) and Transmission Electron Microscopy (TEM), Techai 20G2-FEI. Samples were prepared by placing a drop of hydrophobic nano-material colloid or its aqueous coordinate on carbon-coated copper grids and dried at room temperature. The dried silver nanoparticles sample was placed into a flat aluminum sample holder, where the X-ray source was a rotating anode operating at 30 kV and 30 mA with a copper target. Data was collected between 10º and 90º.

Particles were dispersed in water by ultrasonicator for 15 minutes and cast onto a copper grid by dropping method to determine the size and shape of the particles. Transmission Electron Microscopy (TEM), with an acceleration voltage of 200Kv was used to characterize the AgNPs.

EVALUATION OF ANTIBACTERIAL ACTIVITY

The biosynthesized silver nanoparticles were tested for their antibacterial activity against bacterial pathogens using well diffusion method. The bacterial strain such as *Klebsiella pneumoniae* (MCC 2716), *Escherichia coli* (MCC 2412), *Pseudomonas aeruginosa* (MCC 2408), *Enterobacter aerogenes* (MCC 3092) and *Bacillus subtilis* (MCC 2183) was procured from the National Center for Microbial Resource at National Centre for Cell Science, Pune, India. Bacterial strains were cultured on Mueller-Hinton agar. Bacterial inoculants were prepared in nutrient broth at 0.5 McFarland standards. Each test organism (100µL) was mixed with cooled Mueller-Hinton agar and poured into 80 mm Petri dishes. Wells (4 mm) were cut out, and 30µL of AgNPs were added to the wells. The plates were incubated at 37°C for 24 hours and the diameters of the zones of inhibition were measured. All experiments were performed in triplicates.

RESULTS AND DISCUSSION

In recent years, development eco-friendly metal nanoparticles have gained special attention due to the wide range of biomedical and physiochemical applications. Since ancient times, silver has been broadly studied and used to treat many bacterial infections, improve wound healing without scarring and in medicinal device coatings[21]. In the present study, silver nanoparticles have been synthesized from fungal biomass of *A. fumigatus* which acts as reducing, stabilizing as well as capping agent. On addition of silver ion to the cell free fungal filtrate, it showed a gradual change in color from pale yellow to dark brown which indicates the synthesis of silver nanoparticles (Fig.1).

Fig 1.Biogenesis of AgNPs; a) Cell filtrate of *Aspergillus fumigatus*
b) Cell filtrate of *Aspergillus fumigatus* with 0.1N $AgNO_3$ after 24 hrs of incubation

Controls (without silver ion) exhibited no change in color of the cell filtrate in the same experimental condition and there was no precipitation or aggregation of silver nanoparticles was observed even after the incubation for two to three weeks.

UV-Visible spectral analysis

The formation and stability of AgNPs from fungal extract was monitored by UV-Vis spectroscopy. The surface Plasmon resonance absorption peak was centered near 446 nm, indicating the presence of AgNPs. In order to examine the stability of silver NPs, the color changes were monitored periodically after the synthesis. There was no visible change in color up to three months after synthesis which is indicating stability of nanoparticles. In this study, 24 hours were taken for the synthesis of silver nanoparticles and the bioreduction of (silver ions) Ag+ to $Ag^{(0)}$. The synthesis reaction was started with the introduction of aqueous silver nitrate solution into cell free fungal filtrate. Silver nanoparticles exhibit yellowish brown color due to excitation of Surface Plasmon Resonance (SPR) vibrations, so the color from colorless to dark brown confirmed the form of colloidal silver nanoparticles from silver nitrate after incubation and it was confirmed by UV-Visible spectroscopy (Fig. 2).

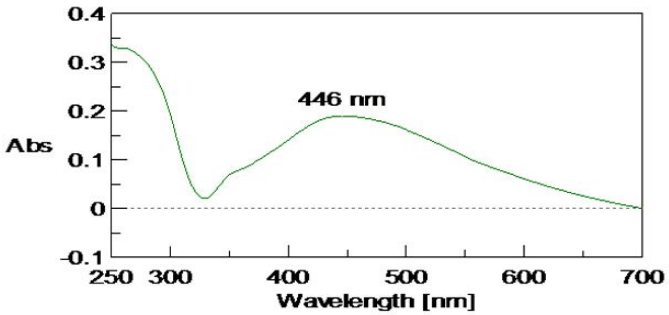

Fig .2. UV-Visible spectra of biogenic silver nanoparticles

This absorption peak value is similar to silver nanoparticles from biologically synthesized by microbial and plant extraction method[22, 23].

FT-IR ANALYSIS

The FT-IR spectrum of the AgNPs was used to identify the organic functional groups in the fungal biomass responsible for the bioreduction process. The FT-IR spectrums of AgNPs are shown in (Fig. 3) which recorded from the powdered sample.

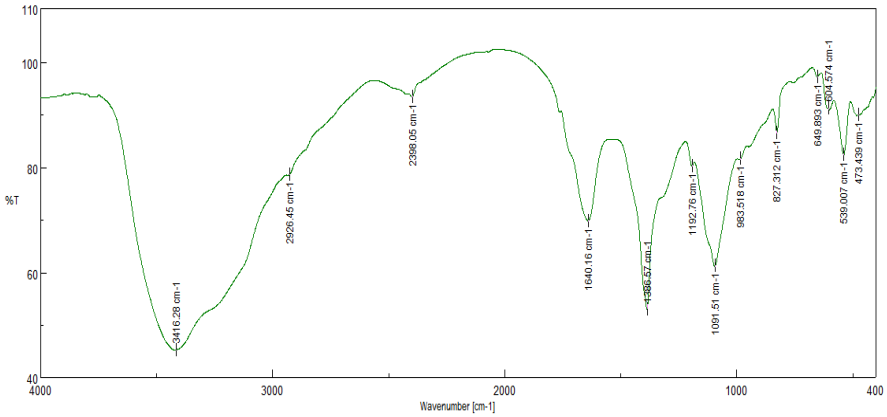

Fig. 3. The FT-IR spectrum of the biogenic silver nanoparticles

FT-IR spectra clearly represent various characteristic peaks between 3416 and 473 cm^{-1}. The peaks at 3416, 2926 and 2398 cm^{-1} were corresponding to protein, enzymes or polysaccharide components and assigned to the stretching vibrations of primary and secondary amines. A band at 3416 cm^{-1} is the bending vibration of primary amine stretching.

The bands observed at 2926 assigned to strong C-H stretching and 1386 cm^{-1} indicates the presence of –C-H bending. The band at 2398 cm^{-1} indicates the presence of C-C stretching and peaks at 1192, 1091 cm^{-1} were corresponding to alkoxy C=O stretching. Additionally, a sharp absorption peak at 1640 cm^{-1} demonstrated stretching vibration due to the carbonyl group (C = O). Absorption peaks at 1384 cm^{-1} have originated due to C–N stretching vibrations of the amino group. The band at 827 cm^{-1} indicates the presence of aromatic C-H stretching In addition, the peaks at 649, 604, 539 and 473 cm^{-1} correspond to metal binding carboxylic (M↔C≡O) groups.

In the previous studies, the carboxylic groups are known to coordinate metal ions which may act as a nucleation site for nanoparticle formation[24]. The overall peaks from FT-IR observation confirm the presence of a functional group of the protein i.e. amino and the carboxyl group in the samples of silver nanoparticles. It can be assumed that silver nitrate reducing fungi produce extracellular protein molecules or peptide chains which may act as template nucleation site of silver

atom clusters and may reduce silver ions to form the silver nanoparticles. These nanoparticles are bound to the functional organic groups (carboxyl and amine) from the fungal content of protein. This functional group may acts template, reducing and capping of nanocrystals. The present result was supported by TEM observation (Fig. 5).

X-ray diffraction analysis

The crystalline structure of the fabricated AgNPs was investigated by XRD analysis and the obtained X-ray diffraction pattern is shown in Fig.4.

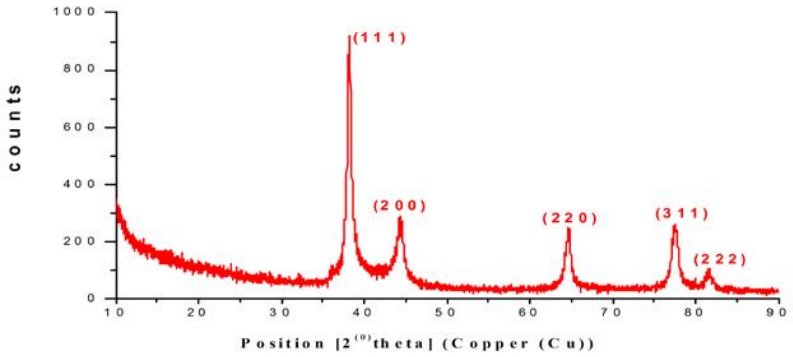

Fig. 4. The XRD patterns of biogenic silver nanoparticles from *Aspergillus fumigatus*

The peaks were assigned to diffraction signals of (111), (200), (220), and (311) plane for face cantered cubic (FCC) silver. The lattice constant calculated from this pattern was 4.0869Aº a value in agreement with literature report (4.0855 Aº) JSPCDS file no 89-3722, which indicates that the formation of silver crystalline nature of NPs synthesized from *A. fumigatus*. Our results are in good agreement with crystalline silver nanoparticles reported by Sarsar *et al.*, [25].

Morphological analysis of silver nanoparticles

Scanning Electron Microscopy

The structure was further confirmed by SEM images. A representative SEM micrograph of silver nanoparticles obtained after 24 hrs of incubation is presented in Figure 5.

The micrograph showed nanoparticles with spherical shape. The size of the particle ranged from 20- 50nm. Majority of the silver nanoparticles were showing aggregates of varying sizes and few of them were scattered as observed under SEM.

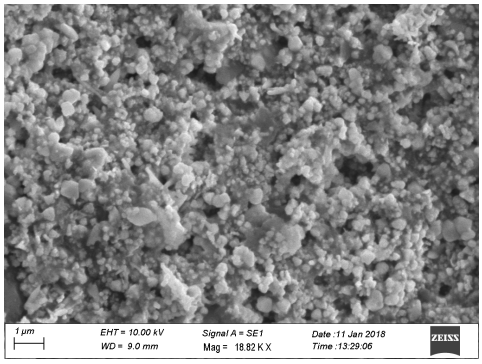

Fig. 5. SEM micrograph of silver nanoparticles synthesized by *Aspergillus fumigatus*

TEM and Selected area electron diffraction pattern (SAED)

The Tranmission Electron Microscopy micrograph of the biosynthesized silver nanoparticle derived from *Aspergillus fumigatus* (Fig. 6) was observed.

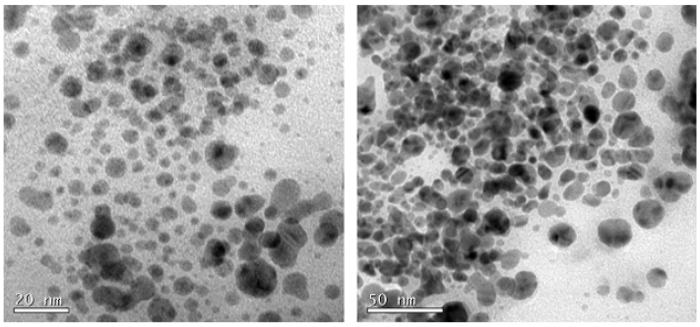

Fig. 6 TEM micrograph of the biogenically synthesized silver nanoparticles

According to the obtained TEM micrographs, AgNPs were quasi-spherical, well separated from each other and minor polydispersity could be observed in the range between of 5-30 nm were noticed. The scale bar corresponds to 20 nm and 50 nm. Selected area electron diffraction pattern form the single spherical shaped silver nanoparticles were shown in Fig. 7.

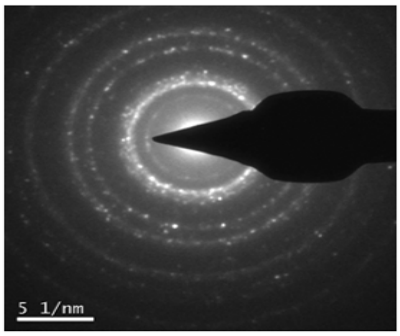

Fig. 7 Selective Area Electron Diffraction spectra for the silver nanoparticles

The set of spots with the strongest intensity could be indexed to 111 and 110 reflections which indicate that Ag is single crystals with a (111) lattice plane as the basal plane. The SAED pattern indicates that silver nanoparticles are spherical shaped mostly (111) lattice plane for the face-centered cubic crystal structures was conformed. The microbial and enzymes mediated synthesized Ag $^{(0)}$) were mostly observed in FCC crystals type[26]. The similar result was observed by fungal species-mediated synthesized silver nanocrystals. The crystal phase analyses of face-centered cubic (FCC) and then the high intensity of 111 plane structure. It supported to the SAED observation results (Fig. 7). Mandal *et al.* (2006) proposed that the proteins can bind with nanoparticles either through free amine groups or crystalline residues in the proteins [27].

In this study, the antimicrobial activity of AgNPs using a novel biosynthetic method was evaluated. Silver nanoparticles exhibited remarkable antimicrobial activity against a wide range of bacteria
(Fig. 8)

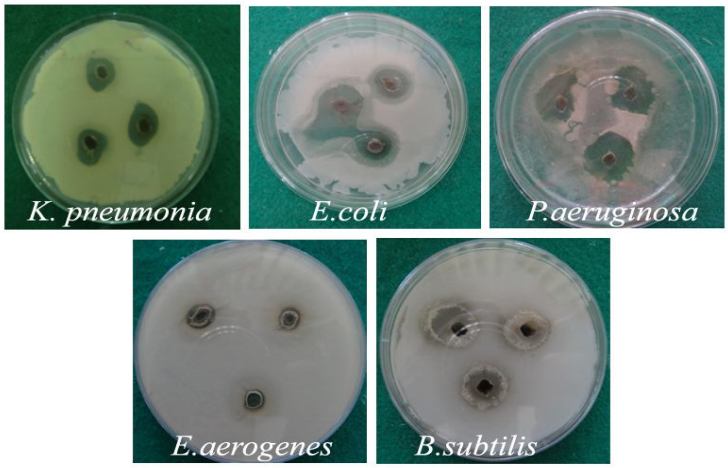

Fig. 8: Antibacterial test results of biogenic silver nanoparticles

The mean of three replicates of the diameter of the zone of inhibition (30μg/mL) for each microorganism was determined to be about 16.8 ± 0.21, 20.5 ± 0.13, 23.8 ± 0.13, 12.4 ± 0.21 and 22.7 ± 0.11mm, respectively, for *Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes* and *Bacillus subtilis*. The highest antimicrobial activity was observed against *Pseudomonas aeruginosa, Bacillus subtilis*, and *Escherichia coli*. These findings are in agreement with previous studies that examined the antimicrobial activity of AgNPs against *Bacillus subtilis* and *Candida albicans*[28, 29]. Several scientific reports have been documented for antibacterial activity of AgNPs including direct damage to the bacterial cell membrane, the release of silver ions and subsequent generation of reactive oxygen species (ROS) which finally lead to the

increased membrane permeability and DNA damage[30, 31, 32]. Smaller AgNPs having the large surface area available for interaction would give better bactericidal effect than the larger AgNPs. Our results were in well agreement with the observations reported by Duncan (2011) which suggested that the interaction of silver nanopareticles to the bacterial cell depends on the availability of the surface area, and the size and shape of the silver nanoparticles could play an important role in the enhancement of antimicrobial activity [33].

CONCLUSION

In conclusion, silver nanoparticles were synthesized using the biomass of marine derived *Aspergillus fumigatus* and highly promising as a green, sustainable, simple, and easily disseminated method. FT-IR reveals that protein molecules can be bound to nanoparticles and XRD result confirms face-centered cubic (FCC) silver crystal structure. TEM results also supported that the *A. fumigatus* synthesized spherical shaped silver nanoparticles. Furthermore, the biosynthesized silver nanoparticles well display a prominent antimicrobial activity against pathogenic microorganisms. Taken together, the data collected in this study suggests that it would be significant to understand the mode of action of the biosynthesized nanoparticles prior to their use in nanomedicine applications.

ACKNOWLEDGMENTS

The authors are thankful to the Management of A.V.VM. Sri Pushpam College (Autonomous), Poondi-613 503, Department of Biotechnology, Thiruvalluvar University, Vellore and Department of Microbiology, Bharathidasan University, Tiruchirappalli for providing necessary facilities and support to carry out this research work.

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