

Mycosynthesized Silver Nanoparticles from marine derived *Aspergillus terreus* and its bactericidal activity against Clinical Pathogens

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ABSTRACT

The green synthesis of nanoparticles has received increasing attention due to the growing need to develop safe, cost-effective and eco-friendly technologies for nano-material synthesis. In the present study, silver nanoparticles (AgNPs) were synthesized using reduction of aqueous Ag⁺ ion with the culture supernatants of *Aspergillus terreus*. Bioreduction of AgNPs was characterized by ultraviolet-visible spectroscopy, X-ray Diffraction (XRD), Zeta potential, Fourier Transmission Infra Red spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and Transmission Electron microscopy (TEM). The synthesized AgNPs were polydispersed spherical particles ranging in size from 50 to 100nm stabilized in the solution. Furthermore the antimicrobial potential of AgNPs was studied. The synthesized AgNPs could efficiently inhibit the human pathogenic bacterial strains.

Keywords: Mycosynthesis, Silver nanoparticles, *Aspergillus terreus*, Antibacterial activity, Clinical pathogens.

I. INTRODUCTION

Nanotechnology is one of the most fascinating research areas in modern material science and the synthesis of nanoparticles is gaining importance all over the world (Liu *et al.*, 2009). The nanoparticles have significantly enriched physical, chemical, and biological properties due to their size (1-100 nm) (Emory and Nie, 1998). The synthesis of nanoparticles has a significant breakthrough in the field of nanotechnology in the last 15 years and proves its potentials by new and varied applications (Bursten *et al.*, 2016). Nanoparticles have delivered solution to various problems like climate change and pollution control, clean water technology, energy generation, information storage and biomedical applications (Ganaie *et al.*, 2014; Patil *et al.*, 2016; Nalwa, 2014). Among the different types of nanoparticles, metal

nanoparticles have an eye-catching role owing to their innumerable physical and chemical properties. They are synthesized by different physical, chemical and biological methods (Iravani *et al.*, 2014).

Silver has been considered as oligodynamic because of its ability to exert a bactericidal effect at low concentrations. It has long been known for its powerful antimicrobial agent and has the potential to kill approximately 650 varieties of microorganisms (Marx and Barillo, 2014). The synthesis of silver nanoparticles (AgNPs) has received enormous interest due to their capability programs as catalysts, plasmonics, optoelectronics, biosensors and antimicrobial activities (Shanmuganathan *et al.*, 2017). AgNPs are effective bactericides with a demonstrated ability in inhibiting biofilm formation and reducing microbial concentration and development of

antibiotic resistance. Even at low concentrations, AgNPs are able to completely inhibit the growth of Gram-negative bacteria and to strongly reduce that of Gram-positive bacteria (Kumar *et al.*, 2018; Rudakiya and Pawar, 2017; Maruthai *et al.*, 2017). In addition, they can enhance the activity of antibiotics themselves when combined with commercial antibiotics like penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin (Satapathy *et al.*, 2017; Ahmed *et al.*, 2018). Some of the numerous nanoparticles like silver nanoparticles have obtained an awful lot deserved interest due to their effective antimicrobial homes and occasional toxicity closer to mammalian cells.

For the last two decades; extensive work has been done to develop new drugs from natural biological sources because of the resistance of micro-organisms to the existing drugs. Using microorganisms can doubtlessly dispose of the problem of environmental contamination; consequently microorganisms are continuing to be investigated inside the synthesis of metal nanoparticles (Ahmad *et al.* 2003). The biosynthesis of nanoparticles from bacteria, fungi and plants is environmental friendly and sustainable, economical technique which provides an alternative method for nanoparticles synthesis (Zhang *et al.*, 2003). Fungi has many advantages over other microorganisms as they require simple nutrients to grow, the process of biosynthesis using fungi is simple, easy, cheap, covers huge surface areas by suitable growth of the fungi (Sastry *et al.*, 2003). Despite these facts, during biosynthesis of nanoparticles using fungi, they offer trouble-free downstream processing than bacteria and it has higher tolerance and metal bioaccumulation abilities (Mandal *et al.* 2006) as a result, considered as obviously fungi are called us nano-factories. Several literatures were documented the intra and extracellular synthesis of silver nanoparticles by the fungi (AbdelRahim *et al.*, 2017; Mohanpuria *et al.*, 2008; Bhainsa *et al.*, 2006). However, marine fungi continue to be tremendously

unexplored. Therefore, in the present study, our attempt was to synthesize AgNPs extracellularly using *Aspergillus terreus* isolated from the marine sediment. The properties of obtained AgNPs were characterized by ultraviolet-visible spectroscopy, XRD, Zeta potential, FT-IR, SEM and TEM. Furthermore, the synthesized AgNPs were subjected for their antibacterial efficacy against human pathogenic bacterial strains.

II. MATERIALS AND METHODS

Chemicals

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

Collection of fungi

The fungal strains used in the present investigation were originally isolated from marine sediments of Appa Island at the Gulf of Mannar. The isolated fungus (*Aspergillus terreus*) was identified on the basis of their morphological characteristics and microscopic examination. The identified strains were sub cultured time to time to maintain the viability of cultures in the Laboratory at Indian Biotrack Research Centre, Thanjore, South India during the study period. The culture was grown and maintained on Potato Dextrose Agar (PDA) plates at 28°C and preserved as agar slants at 4°C respectively.

Biomass Preparation

To prepare biomass for biosynthesis of silver nanoparticles, the fungal strains were grown in a liquid medium containing (g/l): KH_2PO_4 7.0g; K_2HPO_4 2.0g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1g; $(\text{NH}_4)_2\text{SO}_4$ 1.0g; yeast extract 0.6g; and glucose 10.0g. The Erlenmeyer flasks containing 250mL liquid media were inoculated with fungal spores and incubated at 28°C on a rotary shaker at 200rpm for 5days. After incubation the biomass was filtered using Whatman No.1 filter paper and then

extensively washed with sterile distilled water to remove any medium component that may interact with silver. Approximately 20g of fresh and clean biomass was taken in the Erlenmeyer flasks containing 100 mL of sterile Milli-Q deionized water. The flasks were incubated under the same conditions described above for 24hr. The biomass was then filtered and the cell free filtrate was collected for subsequent experiments.

Biosynthesis of AgNPs

AgNPs were synthesized using 50mL of cell free filtrate mixed with 10mL (10mmol/L) 0.1N AgNO₃ solution in a 250 mL Erlenmeyer flask incubated at 28°C in the dark for 24hrs. A flask with no addition of silver ion was used as control. AgNPs were concentrated by centrifugation of the reaction mixture at 10,000rpm for 10 min twice, and then were collected for further characterization.

Characterization of silver nanoparticles

The different parameters were optimized for the synthesis of AgNPs including concentration of silver nitrate, concentration ratio of fungal extract and silver nitrate, time, temperature and pH which has been identified as factors which affect the productivity of AgNPs. The pH was maintained with help of 0.1N HCl and 0.1N NaOH.

UV-Vis studies

The colour change was observed in the silver nitrate solution incubated with the fungal cell free extract. The reduction of nanosilver was monitored by measuring the UV-Vis spectrum of the reaction medium after 24hours of incubation by drawing 1cm³ of the sample and the absorbance was monitored at a resolution of 1nm and a wavelength of 300–700nm using a T80 UV-Vis spectrophotometer, PG Instruments Limited, UK.

Fourier Transmission Infra Red spectroscopy (FT-IR) studies

FT-IR spectroscopy is frequently used to find out whether biomolecules are involved in the synthesis of nanoparticles. FT-IR is a suitable, valuable, non-invasive, cost effective, and simple technique to identify the role of biological molecules in the reduction of silver nitrate to silver. The FT-IR spectrum of AgNPs was obtained from Thermo Scientific Nicolet (iS50, India) FT-IR with resolution at 4 cm⁻¹ and the wavelength ranging from 400 to 4000 cm⁻¹.

Zeta potential measurement

The Zeta potential of biosynthesized AgNPs in the solution were analyzed using a 90 Plus Particle Size Analyzer, Brookhaven Instruments Corporation, using Zeta Plus software. Three milliliter of solution was placed in zeta cell for measuring zeta potential and the measurement is based on the direction and velocity of particles under the influence of known electric field.

X-ray Diffraction Analysis (XRD)

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 freeze-dried silver nanoparticles were used for powder XRD analysis. 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.

Scanning electron microscopy (SEM)

Characterization of synthesized nanoparticles was carried out to determine the size, shape, and conformity. For which the oven dried nanoparticle powder was subjected to SEM. Scanning electron microscope (JEOL Model JSM-6390 LV) with secondary electron detectors at an operating voltage of

20 kV was used to record the images of synthesized AgNPs using dry powder of the sample.

Transmission electron microscopy (TEM)

For Transmission electron microscope (TEM) measurements a drop of 10 fold diluted sample of the synthesized SNPs was placed on the carbon coated copper grids and allowed the water to evaporate before loading them onto a specimen holder. TEM observations were performed on an H-7500 electron microscope (Hitachi, Japan) operated at an accelerating voltage of 120 kV.

Bactericidal activity of mycosynthesized silver nanoparticles

The antimicrobial activities of biosynthesized AgNPs were determined by using the agar well diffusion method against one gram positive and four gram negative bacteria. All the test cultures *Escheritia coli*, *Bacillus substilis*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* were procured from the Microbial Type Culture Collection Center (MTCC), Chandigarh, India and were maintained at 4°C on nutrient agar until use. The test cultures were inoculated into nutrient broth and incubated overnight. The Muller Hinton agar media was prepared and 25mL of MHA was poured on to the petriplates and allowed to solidify. 1% of each bacterial inoculum was swabbed on the agar plates and allowed to dry for 10 minutes before making wells. Then AgNPs with different concentrations (100µL, 150µL and 200µL) was poured into each well with the help of micropipette. The plates were incubated at 37°C for 24hrs in upright position and zone of inhibition was measured.

III. RESULTS AND DISCUSSION

In present study, silver nanoparticles were formed by reduction of Ag⁺ into Ag⁰ with the addition of cell free filtrate of *Aspergillus terreus* to the solution of 1mM AgNO₃. With the addition of AgNO₃ solution, the

cell free filtrate changed from light yellow to brown colour, while the control showed no colour change under the same experimental conditions (Fig.1). Appearance of brown colour is due to the surface plasmon resonance (SPR) of silver nanoparticles and thus the colour change is the indicative of the formation of AgNPs. It could be noticed that the earlier reports the reaction mixtures required 24-48hrs for the reduction of the silver ions in the aqueous solution into nanoparticles (Savithramma et al., 2011). In the present study, the colour change was initially noticed from 2 hours and it took 12 hrs for the complete synthesis of AgNPs and the bioreduction of silver ions. The intensity of the colour change was increased which was directly proportional to the incubation period of nanoparticle synthesis. It may be due to the excitation of SPR and reduction of AgNO₃. It was exactly correlated with the earlier report of Vigneshwaran *et al.*, (2007) who indicate the colour change occurred because of the several proteins, free amino acids and enzymes present in the fungal extract reduced the silver metal ions into silver nanoparticles. Vanaraja *et al.*, (2015) reported that the reductase enzyme was released by the *A. niger* into the solution which is involved in the reduction of silver ions into nanoparticles.

Bioreduction of silver ions into nanoparticles was further confirmed by UV-visible spectral data. The concentrated dark brown colour of the *A.terreus* occurred after the addition of AgNO₃, after 12hrs of reaction as seen in UV absorption spectra (Fig. 2). After 12 hr, the maximum absorption band at 430 nm which is a typical Plasmon band corresponding to the spherical silver nanoparticles. Ramar *et al.* (2015) mentioned in his previous studies that spherical silver nanoparticles contribute to the absorption band at around 450nm in UV-Vis spectra. So the band at 430 nm showed in fig. 2 may corresponds to the silver nanoparticles in spherical shape. The possible mechanism proposed the reduction of silver ions is mainly due to a conjugation between the electrons-

shuttle with the fungal derived reductase enzyme. This has been previously described as being common for biomolecules (Gopinath and Velusamy 2013; Fayaz *et al.*, 2010).

FT-IR analysis was commonly carried out to identify the functional group involved in reduction of silver ion to metallic AgNPs. FT-IR measurements of the dried and powdered samples of Ag-NPs showed the presence of four bands at 3357.46, 2923.45, 1745.2675 and 712.56. The peak at 3357.46 indicates the strong stretching vibrations of hydroxyl functional group and the peak at 1459.05 indicates the bending of -O-H group. The peak at 1401.19 indicates strong S=O vibrations. The peak at 2926.45 and 2857.02 may be related to medium -CH- stretching is the characteristic of the carboxylic acid group. The 1648.77 and 1542.77 correspond to the binding vibrations of amide I and amide II due to carbonyl stretch and N-H stretch vibrations in the amide linkages of the proteins. The peaks 1234.22 may be represent the residual nitrate (CN) and a peak at 1035.59 indicates CO-C-CO stretching vibrations anhydride linkages of proteins.

Zeta potential (ZP) values reveal the information regarding the surface charges and stability of the synthesized AgNPs. In the present study, the zeta potential value for AgNPs fabricated from *A.terreus* was (Fig. 4) indicating the stability of the synthesized nanoparticles. The hydroxyl groups from the *A.terreus* may have a stronger ability to bind metal ion, so that these compounds could form a coat over the metal nanoparticle to prevent the gathering of nano materials and exist as negatively charged.

The X-ray diffraction pattern of biosynthesized silver nanoparticles produced by *A.terreus* was further demonstrated and confirmed by characteristic peak observed in XRD image (Fig. 5). The XRD patterns showed an intense peaks at $2\theta = 32.81^\circ$, 46.17° , 57.65° and 76.71° , which may be indexed to the planes

(111), (200), (220) and (3111) respectively. The presence of four prominent Bragg's reflection, corresponding to the (111), (200), (220) and (311) orientations agree with those reported for silver nanoparticles. The XRD spectrum of biosynthesized silver nanoparticles was matched well with the JCPDS file no.87-0720, which indicates the crystalline nature of face-centered cubic silver. The broadening of Bragg's peak indicates formation of nanoparticles and XRD patterns obtained are consistent with earlier reports. These results were in good agreement with the recent report of Krishnaraj *et al.*, (2014).

The appliance of fabricated AgNPs was highly dependent on the chemical composition, shape, size and monodispersity of particles (Daniel and Astruc, 2004). To augment the application scope, the AgNPs obtained were systematically characterized using SEM, TEM and XRD analysis. SEM used to characterize physical properties such as morphology, shape, size or size distribution of materials at the microscale and nanoscale (Brodowski *et al.*, 2005). The SEM images of biosynthesized AgNPs from *A.terreus* was shown in Fig.6. A typical SEM image resulting that the biosynthesized AgNPs are formed in nanoscale range approximately 50-100nm and the SEM image clearly show the AgNPs may be distributed in spherical shape. Similar phenomenon was reported by Rani *et al.* (2017). Further, the nanocrystalline nature of AgNPs was confirmed by TEM (Fig 7). It revealed that AgNPs are spherical in shape, uniformly distributed without significant agglomeration. Size and shape of the AgNPs depends on the type of the microbes and factors such as the temperature and pH of the medium. Consequently, considerable size variability can be seen of AgNPs produced by different fungal species. In our study the AgNPs are may be in size about 50-100nm and spherical in shape. AgNPs generally show a typical absorption peak at approximately 3keV due to the surface plasma resonance phenomenon (Prasad *et al.*, 2012). The variation in the nanoparticles size

range in SEM, TEM and XRD studies may due to the aggregation of particles during drying process.

Previous studies reported successful application of different AgNPs shapes and concentrations against fungi such as *Fusarium*, *Aspergillus* and *Alternaria alternata* showed high inhibition of pathogenic bacteria (Gao et al., 2012). The antibacterial activities of AgNPs were showed in both gram positive and gram negative bacteria. The different concentrations 100 μ L, 150 μ L and 200 μ L of AgNPs were used for antimicrobial activity assay, which disturbs the surface of the bacterial cell membrane therefore disturbing the permeability and respiration functions. The maximum antibacterial activity in 200 μ L/mL concentration of the synthesized AgNPs was 23mm, followed by 22mm 20mm, 16mm and 8mm for *Enterobacter aerogenes*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* receptively. The zone of inhibition produced by biosynthesized AgNPs in each bacterium was shown in Table1. *Enterobacter aerogenes* and *Escherichia coli* show highest antibacterial activity for synthesized AgNPs compared to other bacteria. This confirms that AgNPs from *A. terreus* exhibited good antibacterial potential against gram-negative bacterial strains when compared to gram positive strain. Whereas the gram negative *Klebsiella pneumonia* shows no zone of inhibition at lower concentration of AgNPs which indicates its resistance against biosynthesized AgNPs at lower concentrations. The result also shows that the zone of

inhibition was increased when the concentration of AgNPs is increased. The result of the antibacterial assay is depicted in Table1. These results confirm previous work carried out by Li et al. (2011) and Bindhu and Uma Devi (2013). The application result indicated and confirmed that AgNPs have good antimicrobial activity against different microorganisms and hence it could have great impact on eco-friendly mode of drug treatment for bacterial diseases. More than that, this method could be useful for different field of science like medicine and drug delivery in a safer way.

IV. CONCLUSION

In the present study, Silver nanoparticles have been synthesized using fungal extract of marine origin and tested against one gram positive and four gram negative bacterial pathogens. The varying inhibitory effects of the AgNPs against *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes* and *Bacillus subtilis* was observed. According to the reports, the inhibitory effect of AgNPs fluctuates in different bacteria due to the variation in their capsular and cell wall composition, thickness of the S-layer or a combination of these. Likewise, the bacterial growth rate and its byproducts may also influence the inhibitory activity of AgNPs. Thus we conclude that, an increasing concentration of AgNPs is required to achieve a higher inhibitory effect.

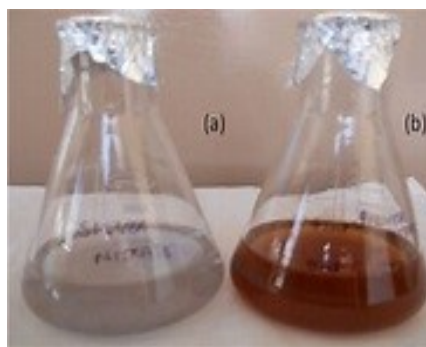


Figure 1. Biosynthesis of AgNPs from *Aspergillus terreus*
a) Cell free filtrate without AgNO₃ b) Cell free filtrate with AgNO₃

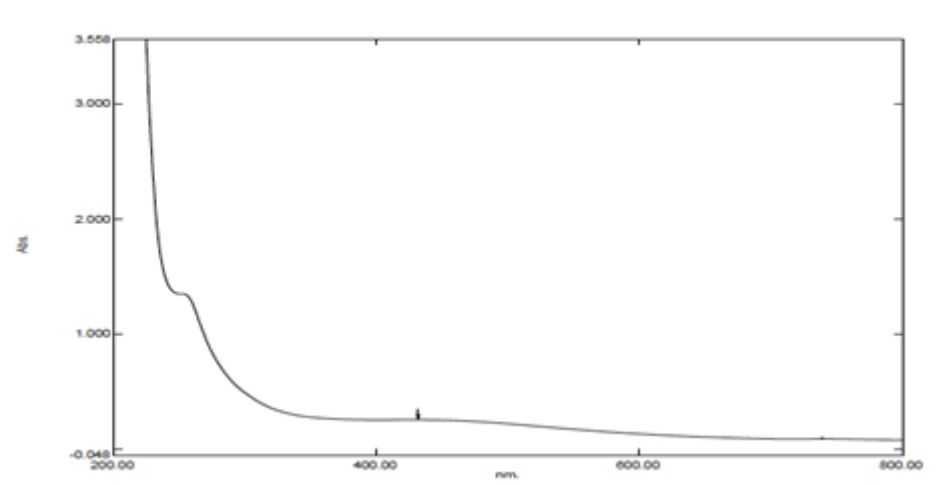


Figure 2. UV-visible spectra of synthesized AgNPs from *A. terreus*

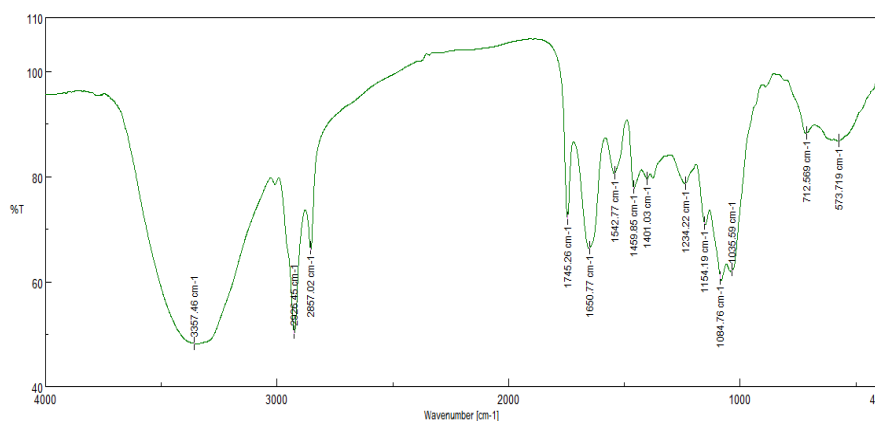


Figure 3. FT-IR analysis of synthesized AgNPs from *A. terreus*

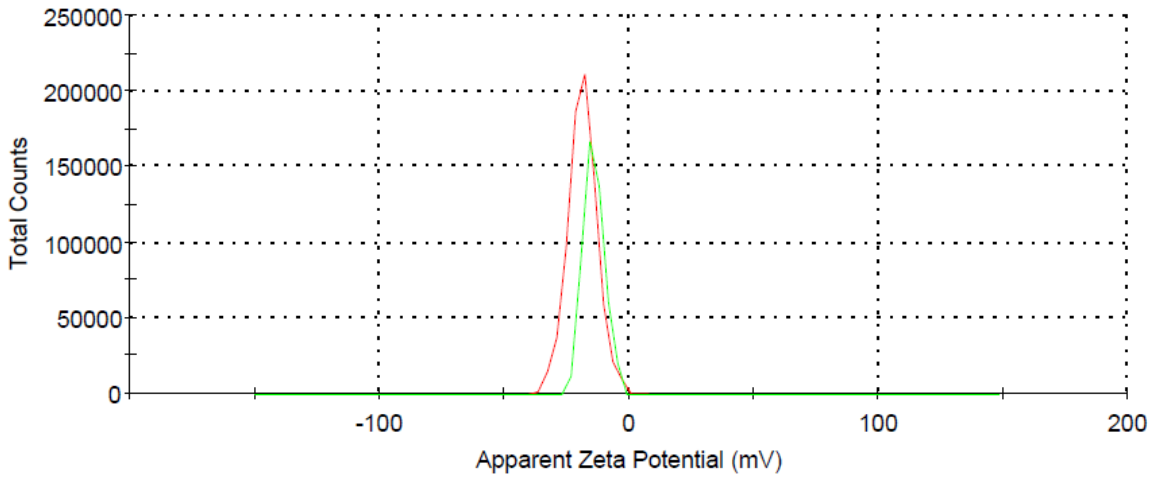


Figure 4. Zeta potential analysis of synthesized AgNPs from *A. terreus*

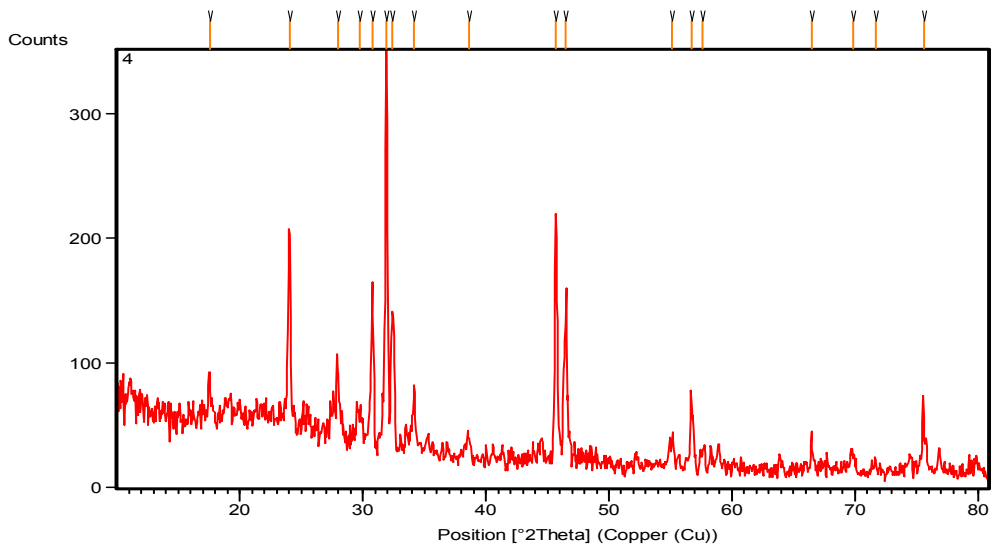


Figure 5. XRD pattern of biosynthesized AgNPs

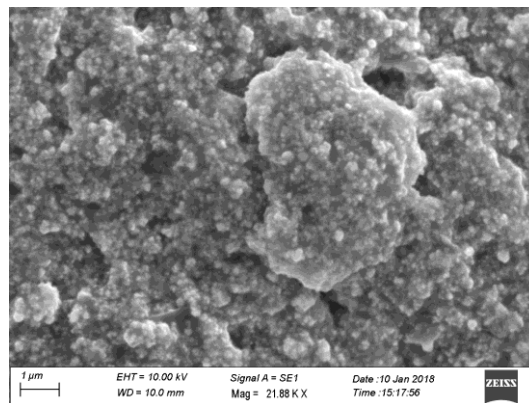


Figure 6. SEM images biosynthesized AgNPs from *A. terreus*

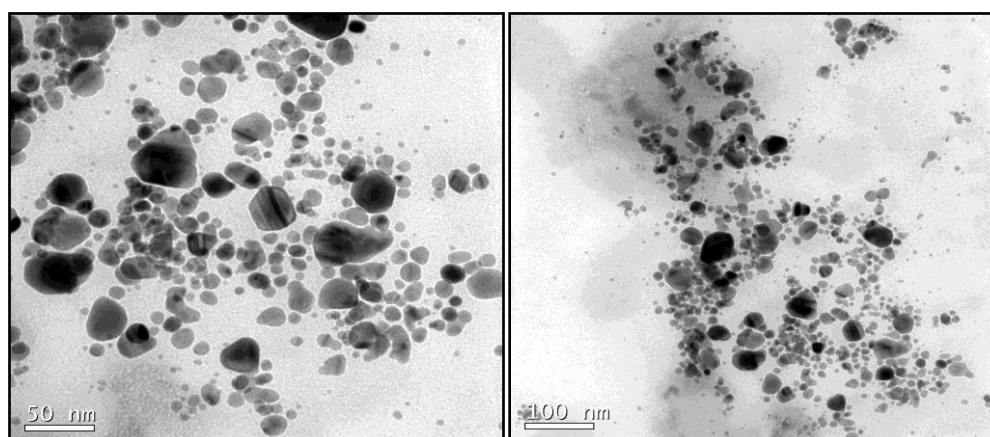


Figure 7. TEM images showing the size and shape of biosynthesized AgNPs

Table1. Zone of inhibition produced by AgNPs against pathogenic bacteria

S. No	AgNP Concentration $\mu\text{L/mL}$	Escherichia coli mm	Klebsiella Pneumoniae mm	Bacillus subtilis mm	Enterobacteraerogens mm	Pseudomonas aeruginosa mm
1.	100	12	-	9	16	11
2	150	17	4	13	19	16
3	200	22	8	16	23	20

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