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Synergistic antibacterial activity of mycosynthesized AgNPs with antibiotics against multidrug resistant *Pseudomonas aeruginosa* and investigation of protein profile

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Silver nanoparticles (AgNPs) have been identified as promising bactericidal agents especially towards multidrug resistant bacteria (MDR). Fungi are excellent biogenic sources for synthesizing AgNPs with antibacterial activity. In the present study, AgNPs were synthesized using *Aspergillus niger* extract and characterized. Individual and synergistic antibacterial activities of AgNPs were tested against isolated hospital strain of MDR *Pseudomonas aeruginosa* by disc diffusion assay. SDS-PAGE analysis was performed to analyse the protein expression before and after treatment with AgNPs. Results revealed the successful synthesis of AgNPs using *A. niger*by 24 hrs and the average particle size was measured as 18.72±0.2 nm. AgNPs were predominantly spherical and monodispersed. 18±0.05 mm zone of inhibition was observed for *P. aeruginosa* when treated with AgNPs alone whereas synergistic treatment of synthesized AgNPs along with Tetracycline showed impressive antibacterial activity (27.32±2 mm). Protein expression of *P. aeruginosa* varied before and after treatment which was evident from the results. This indicated the effective breakdown of cell wall and cellular proteins on the synergistic treatment of AgNPs and antibiotics. Thus the study demonstrates a feasible and eco-friendly approach to synthesize AgNPs with enhanced antibacterial activity against MDR bacterial strains.

Keywords: Antibacterial activity, Protein expression, Silver nanoparticles, Synergistic activity

The persistent emergence of antibiotic resistant bacteria is occurring globally, threatening the efficacy of antibiotics that have changed conventional medicine and saved millions of lives. Antibiotic resistance has become one of the most challenging healthcare problems in recent decades. The antibiotic resistance crisis has thrived due to the overuse and misuse of antibiotics. Nevertheless it is also attributed to the lack of new drug discovery and development by pharmaceutical industries[1]. Several strains and variants of bacteria have become less vulnerable towards antibiotic treatment leading to the growth of highly infectious strains[2]. Theranostics to the newly developed resistant and stronger strains is highly expensive and complicates. Over 70% of the bacterial strains have become resistant to one or more antibiotics that are regularly used to eliminate them. Humans infected with MDR organisms tend to require prolonged treatment at hospitals and are in need of a combination of three or more antibiotics to put down the infection[3]. However, the treatment with regular antibiotics has become less efficient, toxic and expensive over time. Nanoparticles have acquired important status in the field of medicine in the recent decades due to their unique characteristics[4]. They usually are clusters of atoms ranging between 1-100 nm in size and size, shape, composition of the nanoparticles play a major role in determining the property of the particles. Green synthesis of metallic nanoparticles has gained attention owing to its simple procedures of synthesis, eco-friendly nature and cost-effectiveness[5]. Among the metal nanoparticles, green synthesized Silver nanoparticles (AgNPs) have several applications in the field of medicine, electronics, effluent treatment, cosmetics and optics[6-10]. AgNPs synthesized in green routes have demonstrated excellent antibacterial, anticandidal, antifungal, antioxidant, anti-inflammatory and anticancer properties[11,12]. In the past years, numerous physical and chemical methods were utilized to synthesize metallic nanoparticles. However, compared with the conventional methods, biological systems provide unique arena for production of nanoparticles.

Fortunately, several plants, plant parts, microorganisms from bacteria to fungi have showed the

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ability to successfully support the synthesis of metal nanoparticles either intracellularly or extracellularly[13]. Microbes are considered to be efficient biological nano-factories to synthesize AgNPs. Silver nanoparticles have been produced using bacteria such as *Pseudomonas stutzeri* AG259[14] and *Bacillus licheniformis*[15]. Silver resistant bacteria *Morganellaapsychrotolerans* are capables of producing nanospheres and nanoprisms of AgNPs on successful control of the bacterial growth kinetics[16]. However, fungal species are appreciated for the secretion of bioactive substances in higher amounts that are suitable for the large scale synthesis of AgNPs. 5-15 nm quasi spherical nanoparticles were efficiently synthesized using *Fusarium oxysporum*[17]. Employing fungi as reducing and capping agents in the green synthesis of AgNPs is attractive due to high yield, low toxicity and improved stability. The fungal metabolites associate with the AgNPs that plausibly provides them with stability, prevents agglomeration and also enhance their antimicrobial activity[18]. At present, the development of eco-friendly antimicrobial agents do not focus only on immediate efficacy but also on their ability to reduce antibiotic resistance. Therefore, research on nanoparticles derived from natural sources to act as antimicrobial agents has gained the spotlight among researchers. The present study highlights the synthesis of AgNPs using *Aspergillus niger* fungal extract; a natural source and its demonstrates its efficient antibacterial activity against multidrug resistant hospital isolated strain of *Pseudomonas aeruginosa*.

Materials and Methods

Synthesis of AgNPs using *A. niger*

Various concentrations of silver nitrate ($AgNO_3$) solutions of 0.5M, 0.25M, and 0.125M were prepared in double distilled water and membrane filtered. About 100mL Sabouraud's broth media was sterilized and inoculated with *A. niger*as well as Silver nitrate solutions in different flasks. Flasks were incubated at room temperature until colour change and appearance of mycelium[19].The mat of fungal mycelium was separated from the broth by filtration. The solution was centrifuged at 12,000 rpm for 15 min, after which the supernatant was discarded and the pellet was redisposed in deionized water and passed through membrane filter. The obtained nanoparticles were stored for further analysis.

Characterization of Silver nanoparticles

The characterization of the synthesized AgNPs is necessary to obtain information on their composition, functional groups, nature and morphology of the particles. Characterization of the AgNPs was performed using various analytical techniques such as UV-Visible spectroscopy, Fourier Transform Infrared Spectroscopy (FT-IR), X-ray powder diffraction (XRD), Energy Dispersive Analysis of X-Ray Diffraction Spectroscopy (EDAX), Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)[20]

Antibacterial activity

Antibacterial activity of the synthesized AgNPs was analyzed using discdiffusion method with slight modifications as mentioned by Vaishavi *et al.*[21]. Sterilized plates of Muller-Hillton Agar were prepared to which multiple antibiotic resistant *P. aeruginosa* was swabbed onto the surface of the plates. To the discs 50µL of AgNPs synthesized from 0.5M Silver nitrate solutions were added and incubated at 37°C for about 24 h. About 50µL of Silver nitrate solution (0.5 M) was used as the control. For synergistic activity the overnight culture was inoculated on Muller- Hinton agar medium along with antibiotic discs and AgNPs. The zone of inhibition was measured after overnight incubation at 37°C. To observe for synergistic effects, the nanoparticles and antibiotics were treated together against *P.aeruginosa* using antibiotics and nanoparticles only as control[22].

Protein Profile and SDS-PAGE analysis of *P. aeruginosa*

P. aeruginosa culture was grown for 24 h in Luria-Bertani broth and broth containing AgNPs. The contents were centrifuged and lysed using lysis buffer. Protein estimation of the lysed sample was done by Folin-Ciacalteau method[23]. The whole cell protein for SDS-PAGE analysis was prepared and the Vertical anionic electrophoresis was carried out in the discontinuous sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% (w/v) separating gel and 5% (w/v) stacking gel. Voltage was set at 50V to run the sample through the stacking gel, after which the voltage was increased to 200 V till bromophenol blue tracking dye reached bottom of gel, approximately 3 h.

Results

Synthesis of Silver nanoparticles using *A. niger*

The flask containing various concentrations of $AgNO_3$ and *A. niger* showed colour changes from 24 hrs. By 48 hrs the colour of the solution in the flask changed from pale yellow to brown and their colour remained stable for several months without any stabilizing agent (Fig. 1).

Characterization of Silver nanoparticles

UV-Visible spectroscopy

The absorption spectrum of the AgNPs solution prepared in various concentrations showed a Surface Plasmon Band at around 409- 448nm, indicating the presence of Ag nanoparticles. Fig. 2 shows the UV-vis spectra of the nanoparticles obtained at different concentrations of the silver nitrate solution (0.5M, 0.25M, 0.125M, and 0.062M) after 24hrs reaction time. The absorption spectra remained stable even 3 hrs after the reduction reaction had occurred. The maximum biomass was obtained in mid exponential phase (starting range of stationary phase) of culture and also the maximum synthesis of nanoparticles was obtained in the same incubation period. The higher the concentration of the extract being added, the higher was the possibility of the silver nitrate reduction to formation of well-defined and stable silver nanoparticles. Thus AgNPs synthesized from 0.5 M AgNO3 solution is chosen for further characterization.

Fourier Transform Infrared Spectroscopy (*FTIR*)

The FTIR spectra provided information about the local molecular environment of the organic molecules

Fig. 1 — Synthesis of AgNPs using *Aspergillusniger* filtrate

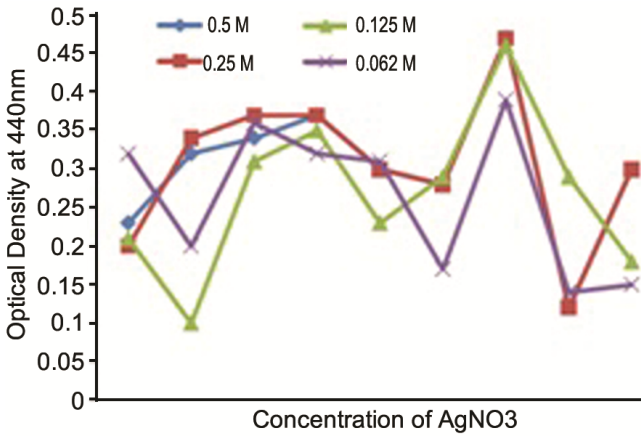

Fig. 2 — Comparison of AgNPs absorption spectra synthesized using different concentrations of $AgNO_3$

on the surface of the nanoparticle. FTIR spectra of silver nanoparticles exhibited prominent peaks at 3483, 3219, 1309 and 1635 cm−1 (Fig. 3). The broad band appearing at 3483 & 3219.19 cm−1 assigned for O−H stretching vibration indicates the presence of hydroxyl groups in the reducing agent. The strong intense peaks at 1309.67 and 1635.64cm−1 corresponds to C−N stretch vibrations for amide I bands of proteins in the extract. The result of this FTIR spectroscopic study confirmed that the extract has the ability to perform dual functions of reduction and stabilization of silver nanoparticles. The peak near 800.46 cm−1 assigned to C=CH2 and the peaks near 725.23 cm−1 and 561.29 cm−1 assigned to CH out of plane bending vibrations are substituted ethylene systems –CH=CH (cis). The FTIR results thus interpret the groups that may belong to biomolecules responsible for the reduction of the silver ions and capping of the bioreduced AgNPs synthesized from fungus.

X-ray powder diffraction (*XRD*)

X-ray diffraction was carried out to confirm the crystalline nature of the particles. The XRD pattern showed four intense peaks in the whole spectrum of 2 theta values ranging from 20 to 80. The four diffraction peaks 38.930, 46.970, 65.340, and 77.540 were observed. A comparison of the obtained XRD spectrum with the standard confirmed that the silver particles formed in the experiments were in the form of nanocrystals, corresponding to [111], [200], [220] and [311], respectively, the mean size of nanoparticles was calculated using Debye-scherrer's equation by determining the width of the peaks. The size of the nanoparticles synthesized was found to be 5-12 nm.

Energy Dispersive Analysis of X-Ray Diffraction Spectroscopy (*EDAX*)

Elemental silver in the prepared nanoparticles was recognized by EDAX study. Spectroscopy of AgNPs synthesized from *A. niger* showed peak at 3.0 keV. The percentage of Ag significantly showed the production of AgNPs (Fig. 4)

Scanning Electron Microscopy (*SEM*)

The scanning electron microscopic (SEM) image showed high density silver nano particles synthesized from the fungus. The silver nanoparticles were observed at different magnifications. SEM image showed individual silver nanoparticles as well as a few aggregates. The observed morphology of the silver nanoparticles was predominantly spherical. The size of the nanoparticles falls within the range of 18–20 nm (Fig. 5).

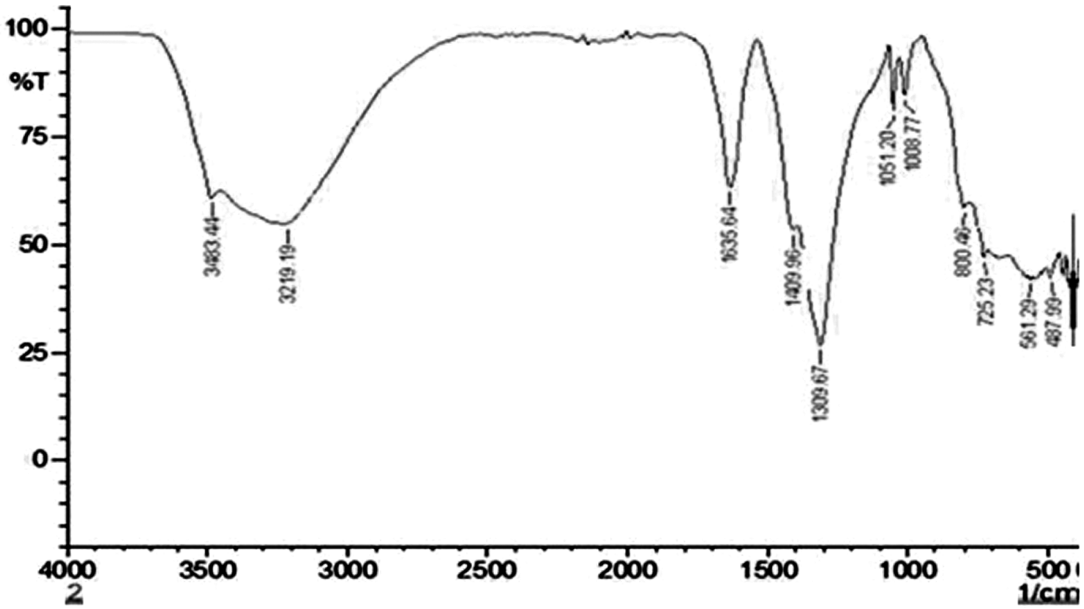

Fig. 3 — FT-IR spectrum of AgNPs

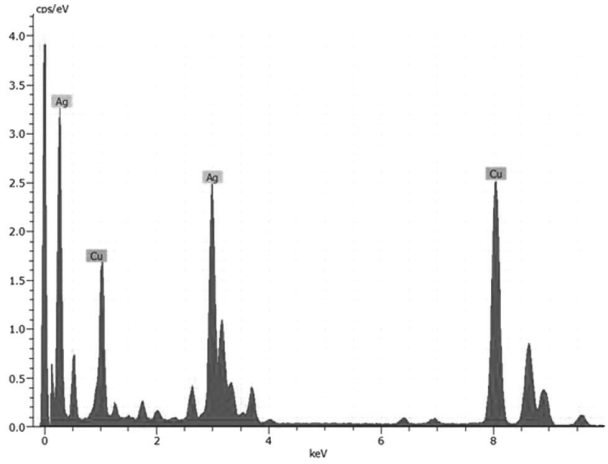

Fig. 4 — EDAX analysis of AgNPs

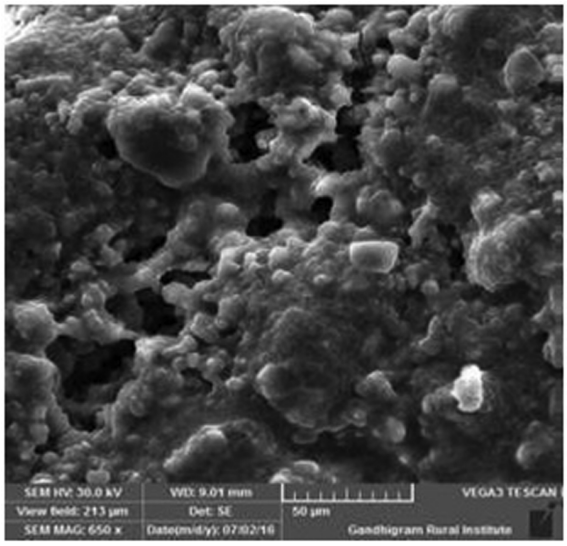

Fig. 5 — SEM micrograph of AgNPs

Transmission Electron Microscopy (*TEM*)

The average size of 18.72 nm was observed from TEM results. Most of the nanoparticles were found to be spherical in shape with smooth edges and monodispersed .The selected-area electron diffraction (SAED) patterns demonstrated the concentric diffraction rings as bright spots corresponding to the presence of AgNPs (Fig. 6).

Antibacterial activity

Individually AgNPs showed significant inhibition activity against *P.aeruginosa*. With respect to Tetracycline and Ampicillin the synergistic effect of AgNPs was high against *P.aeruginosa* followed by Chloramphenicol and Erythromycin. When compared to the individual effects of the antibiotic, the combination of antibiotics and AgNPs showed better results in inhibiting the multi-drug resistant pathogen. The synergistic activity of AgNPs and Tetracycline was more effective when compared to the activity of AgNPs along with the antibiotics such as Ampicillin, Chloramphenicol and Erythromycin.

Protein profile

The AgNPs synthesized from the fungi were tested along with standard antibiotics such as ampicillin, chloramphenicol, erythromycin and tetracycline to study their synergistic effect on the total protein content of *P. aeruginosa*. The total protein content

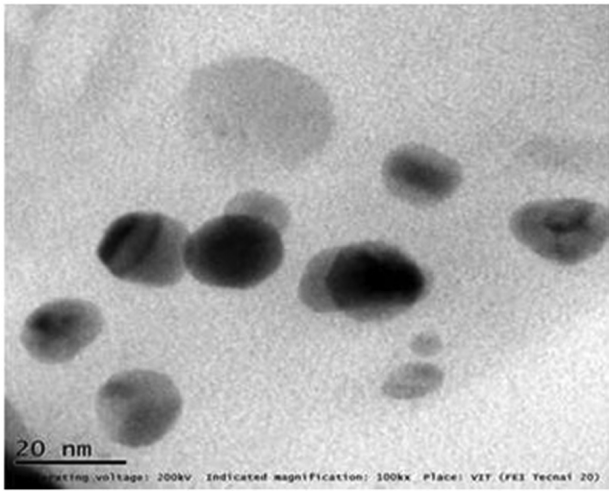

Fig. 6 — TEM micrograph of AgNPs

was estimated by Lowry's method. The control showed a total protein content of 99.12±0.05. There was highest decrease in the protein concentration of about 58% on the addition of Tetracycline along with AgNP (0.5M AgNO3). Chloramphenicol and ampicillin along with AgNP showed a significant decrease of 57.6 and 50.34%, respectively. Erythromycin showed the least reduction in protein content by 32.2% only.

SDS PAGE analysis

SDS-PAGE analysis was done to identify the protein profile of *P. aeruginosa.* The study included the protein expression of *P. aeruginosa* when antibiotics alone was administered and when antibiotics were administered along with AgNPs .The molecular marker contains the following markers in KDa – 205, 97.4, 66.0, 43.0, 29.0, 20.1, 14.3, 16.5, 6.5 and 3.5. It was observed that *P.aeruginosa* expressed two higher molecular weight protein bands which were antibiotic resistant (Fig. 7). These protein bands disappeared after treatment with silver nanoparticles along with antibiotics. The other proteins towards lower molecular weight showed less expression in presence of silver nanoparticles and antibiotics. This revealed that *P. aeruginosa* that expressed the proteins in the presence of antibiotics were highly antibiotic resistant but in the presence of AgNPs and antibiotics these proteins were not expressed indicating that AgNPs have inhibited the growth of *P. aeruginosa* by destroying its protein machinery.

Discussion

Multidrug resistant bacteria have become a major concern globally. Manikandan *et al.*[24]have highlighted the urgent need to explore natural substance to develop novel antimicrobial agents that are non-toxic and environmental friendly to control the upsurge of antimicrobial resistance. The present study demonstrated that the fungal filtrate of *A. niger*is a promising green source for the successful synthesis of AgNPs. Furthermore, AgNPs are fathomed for their antibacterial activity against several bacteria. They are considered as excellent antibacterial agents owning to their ability to destroy the proteins in their cell wall. The cell wall rupture triggered by the AgNPs allows the antibiotics to effortlessly enter the organism and demolish it completely during the synergistic activity of AgNPs and antibiotics[20,24].

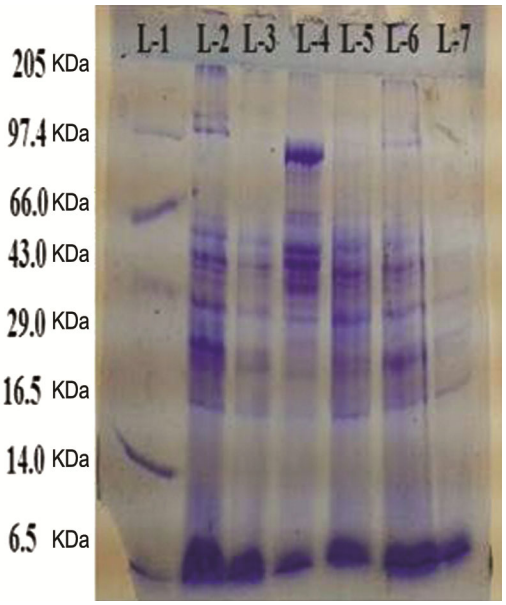

Fig. 7 — SDS-PAGE analysis of *P. aeruginosa* treated with AgNPs and antibiotics (Lane 1: Marker; Lane 2: Chloramphenicol; Lane 3: Tetracycline; Lane 4: Erythromycin; Lane 5: Ampicillin; Lane 6: Tetracycline + AgNPs; Lane 7: Ampicillin + AgNPs

After incubation of the fungal filtrates with $AgNO_3$, the mixture showed a gradual change in colour towards dark brown, which was in accordance with the results of previous studies[25]. The colour of the reaction mixture arises owing to excitation of surface Plasmon Resonance (SPR) vibration in the silver nanoparticles. It is also observed that the absorption peaks increases with increase in the concentration of silver nitrate. The exact mechanism behind the formation of AgNPs is unclear; however literature suggests that trapping and reduction of the silver ions occur due to the electron shuttle process employing nitrate reductase and protein complexes. These enzymes and complexes were detected in fungal filtrate by researchers[26]. The mechanisms of

antibacterial activity of AgNPs are by binding to the membrane of microorganisms through electrostatic interactions, disruption of cell wall and affecting various intracellular processes. AgNPs synthesized using Candida albicans was effective in inhibiting the growth of *Staphylococcus aureus* and *Escherichia coli* showing a 21 mm and 17 mm zones of inhibition, respectively[27]. In another study *A. oryzae* was used to synthesize AgNPs and tested against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus subtilis* which produced satisfying results[28]. This suggests that AgNPs synthesized using green resources showed efficient inhibitory potential against strains that might be resistant to antibiotics. Additionally, the combination of antibiotics along with AgNPs can improve the efficacy and reduce antibiotic resistance.

Conclusion

The present study highlights the use of *Aspergillusniger*as a green resource for synthesis of spherical, small size, crystalline and eco-friendly AgNPs that employing a simple approach. The nanodimensional range of the synthesized AgNPs was between 18-20 nm as confirmed by SEM and TEM analysis. The synthesized AgNPs had a profound inhibitory effect on *P.aeruginosa* individually and also in combination with antibiotics, such as tetracycline and ampicillin. Therefore, the antibacterial potential of AgNPs can plausibly be explored for the control of pathogenic bacteria and to reduce antibiotic resistance.

Conflict of Interest

Authors declare no competing interests.

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