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GREEN SYNTHESIS AND CHARACTERIZATION OF SILVER NANOPARTICLES FROM ASPERGILLUS FLAVUS STRAIN S4

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Abstract

Many methods have been developed for the synthesis of silver nanoparticles (Ag-NPs), yet disadvantages are there to declined their catalytic activity due to the large size with small surface area. Hence, herein, the fungus mediated synthesis of Ag-NPs has been reported. The synthesized Ag-NPs were further characterized by XRD, SEM, EDS, and UV–Vis spectroscopy to study the particle size, surface, crystalline nature, phase formation of Ag-NPs and the produced particles were found to be 41.9 nm. The antibacterial efficiency of synthesized Ag-NPs was examined on various bacteria including *Streptococcus pyrogenes, Escherichia coli, Salmonella typhimurium, Bacillus subtilis.* The Ag-NPs could be considered as excellent broad-spectrum antibacterial agent. More prominently, the Ag-NPs produced by *Aspergillus flavus* exhibited potent antibacterial activity against certain pathogens. *Salmonella typhimurium* exhibited maximum zone of inhibition 29.16±0.76 at 60 µg/mL with respective to the standard antibiotic 32.53±1.33 at 30 µg/mL concentration.

Key Words: Nanotechnology, Silver nanoparticles, *Aspergillus flavus*, Antibacterial activity

Introduction

Nanotechnology provides a platform to modify and develop the important properties of metal in the form of nanoparticles having promising applications in biomarkers, diagnostics, cell labelling, contrast agents for biological imaging, drug delivery system etc. They act as antimicrobial agents and nano drugs for the treatment of various diseases (Sing and Singh, 2011). Nanotechnology is a multidisciplinary field that covers wide range of physical, chemical, electrical, biological and electronics engineering. Nanotechnology is expected to be the basis of many main technological innovations in the 21st century. Research and development in this field is growing rapidly throughout the world. For the synthesis of nanoparticles, a number of chemical

methods exist in the literature, all these protocols involve toxic chemicals, which have been a matter of great concern for environmental reasons. Consequently, researchers in the field of nanoscale material synthesis have been eagerly looking at biological systems for an alternative (Ahmed *et al.,* 2003).

Nanotechnology is the new era of science and technology which deals with matter at atomic or molecular level. It helps to modify or design properties of nanoparticles, which have applications in optical devices, water treatment, production of paints, sensor technology and sunscreen lotions. (Le *et al*., 2011). They are reported to be effective as mosquito repellents (Namitha *et al*., 2013). Nanoparticles have a unique property that they exhibit surface to volume ratio. Due to the increase in surface to volume ratio the properties of the nanoparticles are changed. Earlier, nanoparticles were produced by physical and chemical methods using toxic chemicals which are hazardous. Hence biosynthesis has been considered to be an alternative approach. The metal-microbe interaction has contributed to the progress of bio-nanoparticles synthesis. Mycosynthesis of silver nanoparticles has gained importance because it is a low cost protocol, non-toxic, less labourious and above all it is ecofriendly. Nanoparticles can be synthesized extracellularly or intracellularly. The present study involves the mycosynthesis and characterization of silver nanoparticles from cell free filtrate of filamentous fungi *Aspergillus flavus* strain S4.

Materials and Methods

Synthesis of silver nanoparticles

The *Aspergillus flavus* strain S4 was freshly inoculated on a potato dextrose broth in flask. The flask was incubated in orbital shaker at 30ºC and agitated at 160 rpm for 2 days. The fungal biomass was harvested after 2 days by sieving through Whatman No- 1 filter paper, later thoroughly washed with distilled water to remove the other components in the media from the biomass. Typically 20 gm of fresh and clean biomass was taken into Erlenmeyer flask containing 200 ml of distilled water and the flask was incubated at 28°C for 2 days and agitated at 160 rpm. Later the filtrate was obtained through passage of culture media through Whatman No-1 filter paper. Fifty milliliters of filtrate was taken into 250 ml of Erlenmeyer flask and mixed with 1 mM $AgNO_3$ (0.017 gm $AgNO_3$ /100 ml). The flasks were incubated at 30°C in dark room up to 2 days. Control was maintained (without addition of $AgNO_3$), only cell free filtrate with the experimental flask. In order to use for future experiments the brownish yellow color Ag- NP solution was stored in amber color bottles.

Characterization of silver nanoparticles from *Aspergillus flavus* strain S4

The synthesized Ag-NPs were first characterized by UV-Visible spectrophotometer in the range of 320 - 560 nm with control as the reference. Further the Ag-NPs kept at room temperature for three months to test their stability. The biologically synthesized silver nanoparticles were freeze dried on lyophilzer using ERIDAE refrigerated air dryer machine (Zhejiang, China) and the powdered sample was used for X-ray diffraction (XRD) analysis. The XRD analysis was

performed by X'Pert Pro A Analytical X–ray diffractometer instrument using Analytical X-Ray Systems Co. Ltd. (Netherlands) CuKα radiation (k = 1.54056 Å) in the range of 20-80 at 40 keV. Scanning Electron Microscopic (SEM) analysis was carried out using JEOL-JSM-6610 SEM, Japan with 1µm resolution. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid was allowed to dry by keeping it under a mercury lamp for 5 min. The morphological analysis of the Ag-NPs was done with Scanning electron microscopy (SEM) at different magnifications.

Antibacterial activity of silver nanoparticles

Antibacterial property was performed by Agar well diffusion method. Potato dextrose agar medium was placed in 100 ml conical flask and was sterilized. The media was poured in to sterilized petri plates. Chloramphenicol was taken as a positive control and DMSO was taken as negative control for antibacterial activity. Inoculum was spread over the surface of agar plates with sterile glass spreader. Four wells were made at equal distance using sterile cork borer. To test the antibacterial activity of Ag-NPs, sample was made to a final concentration of 100 mg/ml. Aliquots of (60 µg/ml, 80 µg/ml) the silver nanoparticles were poured on each well. After completion of incubation period at 30^{o}C and 24 h the susceptibility was measured by considering the inhibition zone diameter around each well to the nearest mm.

Statistical analysis

All the experiments were performed in triplicates and the results are expressed as mean ± standard deviation calculated using Microsoft Excel.

Results and Discussion

Extracellular synthesis of Ag-NPs

A comprehensive study of extracellular synthesis of Ag-NPs was carried out in this work. The fungal biomass after 48 h incubation was filtered and the filtrate was subjected to $AgNO_3$. The reaction was started after 24 h incubation in dark condition, the pale-yellow color of the cell free filtrate (CFF) changed to dark brownish yellow color indicating the formation of Ag-NPs (Fig. 1) which correlates with the results obtained by Ingle *et al.,* (2008) who reported mycosynthesis of silver nanoparticles using the fungus *Fusarium acuminatum* and its activity against some human pathogenic bacteria.

In the present study silver nanoparticles were synthesized at room temperature. Green synthesis method provides at low cost, environment friendly, easily scale up for large scale synthesis. The previous reports by Sivakumar *et al.,* (2011) show a green synthesis method where there is no need to use high pressure, energy, temperature and toxic chemicals. The color change was caused by the surface plasmon resonance (SPR) of Ag-NPs in the visible region was also reported by Afreen and Ranganath, (2011) using the fungus *R. stolonifer* and also by Singh *et al.,* (2014) using endophytic fungus, *Penicillium* sp.

Fig. 1 Synthesized silver nanoparticles

Scanning Electron Microscope Analysis

The shape and size of the nanoparticles were elucidated with the SEM. The silver nanoparticles synthesized by *Aspergillus flavus* strain S4 extract were scanned using SEM. The average size of the silver nanoparticles was 50 nm, spherical in shape with a small percentage of elongated particles (Fig. 2).

Sadowski *et al.,* (2008) reported that scanning electron microscopy has provided morphology and size details of the synthesized particles. Silver nanoparticles synthesized by *Aspergillus niger* were in the range of 100 nm. The shape of the particles can be affected due to drying (Kalaiselvan *et al.,* 2009). They have reported that the nanoparticles were in the size range of 50-100 nm. Nithya and Ragunathan, (2014) reported that the SEM micrograph of Ag-NPs being formed using *Aspergillus niger* cell free filtrate. The micrograph clearly illustrates the needle shaped nanoparticles with the size range of 70-200 nm. *Fusarium oxysporum* silver nanoparticles were almost spherical in shape of size 25-50 nm, and attached to the surface of fungal cell (Atef *et al.,* 2013). Hemashekhar *et al.,* (2017) reported that Scanning Electron Microscopy analysis was performed to study size and surface morphology of the silver nanoparticles synthesized from endophyte *Aspergillus niger* extracts. The average particle size was 41.9 nm calculated by using Debye- Scherrer equation.

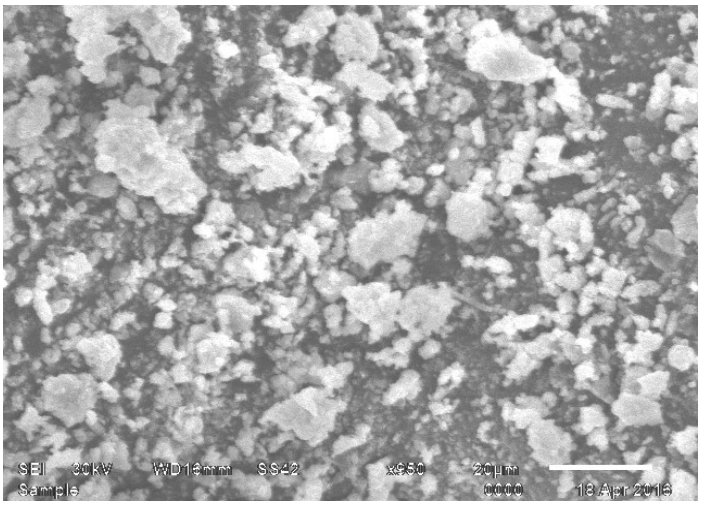

Fig. 2 SEM image of Silver nanoparticles at x950 magnification

UV- visible spectra of Ag-NPs

Synthesized Ag-NPs absorption capacity was observed at every 24 h of incubation. Fig. 3 shows the absorption maxima (0.72) at 450 nm after 72 h of incubation which is surface plasmon resonance, monitored by ultraviolet-visible spectroscopy (UV-2450, Shimadzu) of colloidal Ag-NPs solution. Up to some extent the $AgNO_3$ intensity was increased with time and was clearly recorded in the spectra. Bioactive compounds are responsible for the reduction of metal ions for synthesis of nanoparticles (Wiley *et al.,* 2006). Moharrer *et al.,* (2012) reported the synthesis of silver nanoparticles using *Aspergillus flavus* which shows the absorption maximum at 425 nm. Whereas *A. clavatus* synthesized Ag-NPs exhibited the maximum absorbance at 420 nm (Saravanan and Nanda, 2010).

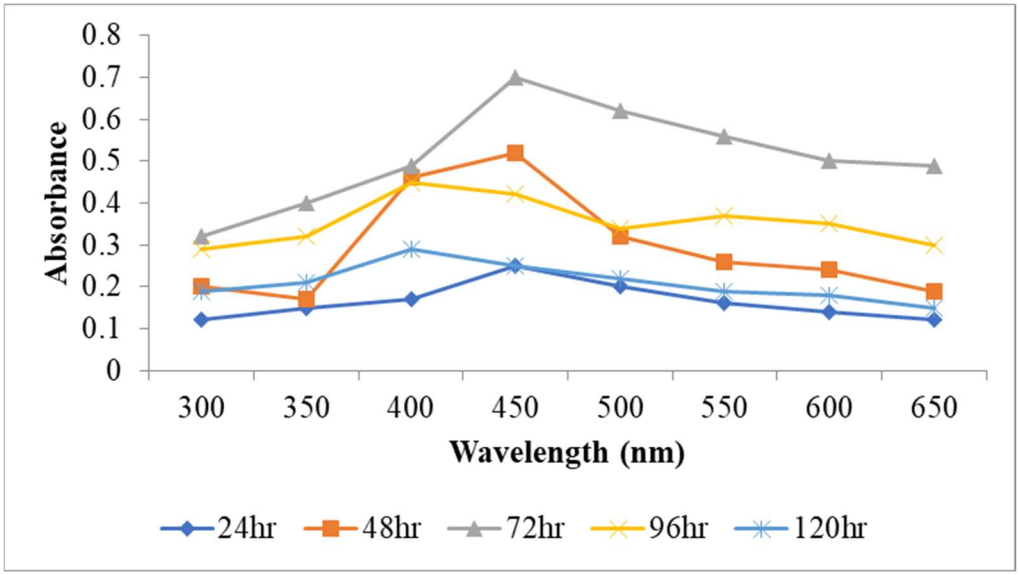

Fig. 3 UV-visible spectra of Ag-NPs

X-ray diffraction analysis of Ag-NPs

The crystalline nature of the synthesized nanoparticles depicted with Bragg's peaks at 38.2º, 44.4º, 64.5º, 77.4º. The X-ray diffraction of silver nanoparticles using *Aspergillus flavus* strain S4 is given in Fig. 4. XRD analysis showed three clear diffraction peaks corresponding to the (111), (200), (220), (311) planes confirmed the formation of Ag-NPs. Liangwei Du *et al.,*(2015) synthesized silver nanoparticles using *Penicillium oxalicum* at two different pH 8.0 and pH 12. Irrespective of the values of pH the Ag-NPs showed four characteristics diffraction peaks at 38.2º, 44.4º, 64.7º, 77.7º. These are corresponding at (111), (200), (220) and (311) Bragg's reflections respectively. Ag-NPs synthesized from *A. terreus* were examined by the XRD pattern showed 2θ values at 32.3º, 45.1º, 75.9º assigned to the planes of (111), (200), (311) correspond to face centered cubic structure of Ag-NPs.

Prema and Raju (2009) reported the X-ray diffraction pattern which showed the presence of sharp reflections at 111, 200, 220 and 311. Our results correlate with them at facets 111 and 200. Li *et al.,* (2012) also revealed the intense XRD peaks corresponding to the (111). (200), (220), (311) planes at 2θ angles of 38.28°, 44.38°, 64.54°, and 77.64° respectively. Hemashekhar *et al.,* (2017) reported that the silver nanoparticles from *Aspergillus niger* confirmed by X ray diffraction analysis, the lattice plane indexed to the (111), (200), (220), (311) with the Bragg reflections with 2θ values of 380, 440, 640, 770 (JCPDS file no. 4-783).

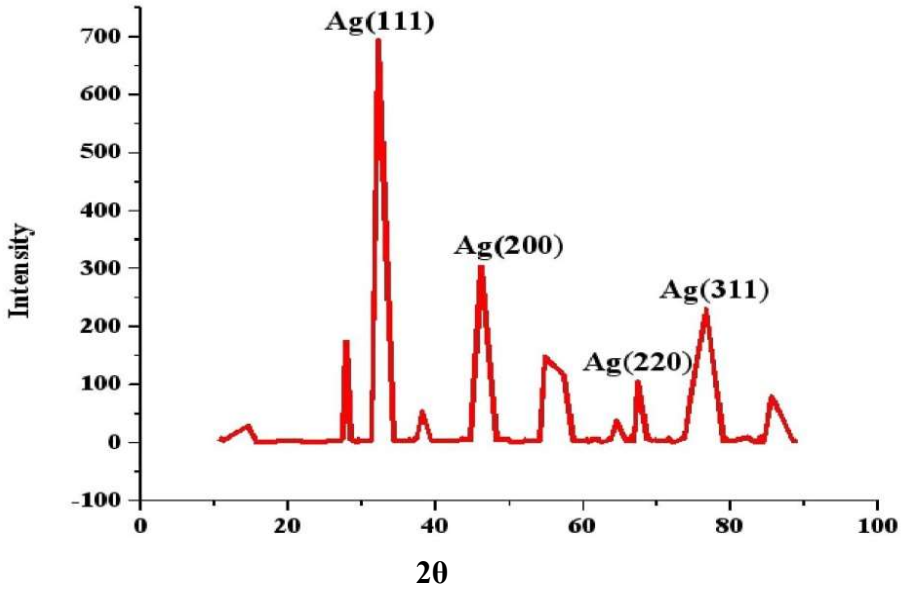

Fig. 4 X-ray diffraction analysis of Ag-NPs

Antibacterial activity of Ag-NPs

The antibacterial activity of Ag-NPs against various pathogenic microorganisms was investigated. Compared with the control, the diameter of inhibition zones increased for all the test pathogens (Table 1). The Ag-NPs produced from *Aspergillus flavus* strain S4 could inhibit the growth of four different typical pathogenic bacteria, including *Streptococcus pyogenes,*

Salmonella typhimurium, Escherichia coli, Bacillus subtilis. Thus, Ag-NPs could be considered as excellent broad-spectrum antibacterial agents. More importantly, the Ag-NPs produced by *Aspergillus flavus* strain S4 exhibited potent antibacterial activity against certain pathogens. *Salmonella typhimurium* exhibited maximum zone of inhibition 29.16±0.76 at 60 µg/ml (Fig. 7) when compared to the standard antibiotic 32.53±1.33 at 30µg/ml concentration. Since the biosynthesized Ag-NPs showed considerable antibacterial activity they could be potential to be widely used in clinical applications.

Recent works revealed that the biosynthesized Ag-NPs showed promising activity independently and also in combination with antibiotics (Ingle *et al.,* 2008). Similar type of work was also presented by Lara *et al.,* (2010) where they showed the excellent antibacterial activity of Ag-NPs against multidrug resistant *Pseudomonas aeruginosa, E. coli, Streptococcus* sp., and *S. pyogenes.* In recent years, the nanoparticle synthesis has been considered as an interesting alternative technique to antibiotics and appears to have a high potential in solving bacterial multidrug resistance in human pathogenic bacteria (Rai *et al.,* 2012). Hoda Erjaee *et al.,* (2017) synthesized silver nanoparticles from *Chamaemelum nobile* showed the zones of inhibition for *E. coli*, *S. typhimurium*, *S. aureus* and *B. subtilis* (15.1 ± 0.2, 14.3 ± 0.3, 13.0 ± 0.1 and 14.3 ± 0.2 mm, respectively). In general, Ag-NPs showed more antibacterial activity compared with the $AgNO_3$ solution.

S.No	Test organism	Zone of inhibition (mm)			
		Ag-NPs (µg/mL)			
		20	40	60	Positive control (Chloramphenicol) 30 µg/mL
1	*Streptococcus pyogenes* (MTCC-442)	18.16±0.76	22.76±1.25	28.23±0.83	30.83±2.02
2	*Escherichia coli* (MTCC-3160)	10.43±1.42	15.23±0.83	21.86±0.41	28.86±1.89
3	*Salmonella typhimurium* (MTCC-2745)	20.86±0.41	25.33±0.83	29.16±0.76	32.53±1.33
4	*Bacillus subtilis* (MTCC-5856)	21.1±0.9	25.16±0.80	28.43±1.42	29.66±1.22

Table 1 Antibacterial activity of Ag-NPs

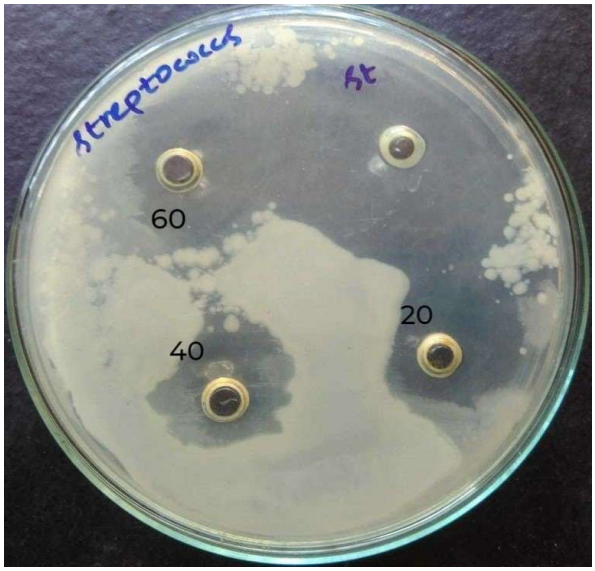

Fig. 5 Antibacterial activity of Ag-NPs against *Streptococcus pyogenes*

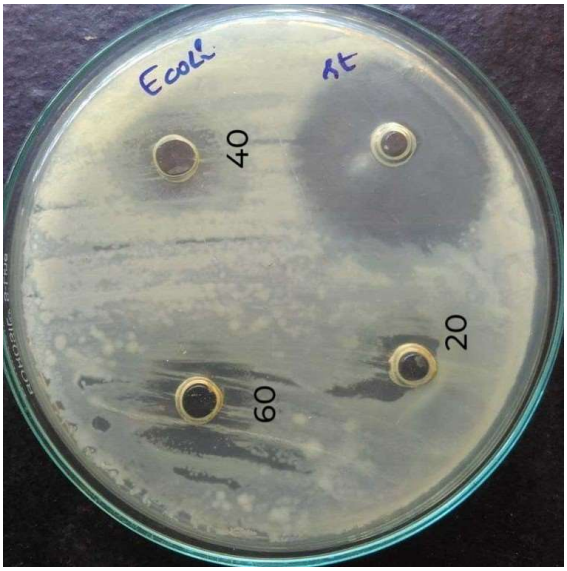

Fig. 6 Antibacterial activity of Ag-NPs against *Escherichia coli*

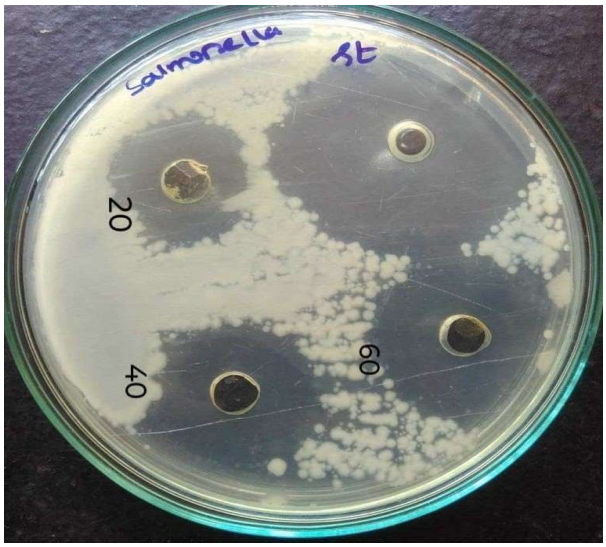

Fig. 7 Antibacterial activity of Ag-NPs against *Salmonella typhimurium*

Fig. 8 Antibacterial activity of Ag-NPs against *Bacillus subtilis*

Conclusion

In the field of nanotechnology, development of reliable and eco-friendly processes for the synthesis of metallic nanoparticles is prime need. The present study fulfills the objective of 'Green' synthesis of silver nanoparticles by a simple method. We have developed a fast, eco-friendly, simple and economical approach for preparation of stable silver nanoparticles by reduction of silver nitrate solution with a bio-reduction method using *Datura stramonium* leaves extract. The

characteristics of the obtained silver nanoparticles were studied using UV-Vis, FTIR, XRD, SEM and zeta potential analysis techniques. The experimental results showed that the synthesized silver nanoparticles are stable with an average size of about 15-30 nm. However, further studies are needed for the isolation and identification of active components or antimicrobial compounds present in medicinal plant and also in vitro as well as in vivo studies are required for better understanding of toxicological aspects that will be related to Ag-NPs.

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