



## **Anti-bacterial and Biogenic Silver Nanoparticles Synthesized using fungus *Aspergillus niger***

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### **ABSTRACT**

*The present study reports eco-friendly and cost effective biosynthesis of silver nanoparticles (Ag-NPs) with the help of cell-free filtrate of fungus *Aspergillus niger*. As a part of characterization, UV-Visible spectrum of aqueous medium showed a peak at 410 nm corresponding to surface plasmon resonance of silver nanoparticles. The spherical shaped silver nanoparticles formed were in the size range of 21.0 – 51.0 nm as observed by SEM-EDAX and TEM analysis. The role of protein as a capping and stabilizing agent was revealed by FTIR study. The XRD study confirmed crystalline silver nanoparticles corresponding to their  $2\theta$  values. The biosynthesized silver nanoparticles showed strong antibacterial action when tested via disc diffusion assay on pathogenic strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The present work provides safe and biogenic silver nanoparticles with potent application as an antibacterial agent in bio-medicine.*

**Keywords:** *Aspergillus niger*, Biogenic silver nanoparticles, Characterization, Antibacterial activity

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### **INTRODUCTION**

Nano-biotechnology is a young and promising area which combines biological principles with physical and chemical procedures to generate nano-sized particles with specific functions. Nanoparticles synthesis is crucial as they hold huge promise in the field of biomedicine, diagnosis, biosensor, therapeutic drug delivery and for the development of many types of novel products with wider applications [1-3].

Silver nanoparticles have several important applications as antimicrobial agents against pathogenic microorganisms which can be easily employed in clothing, environment, biomedical, food preservation, cosmetics and other consumer goods as well as for treatment of diseases [4-6]. Currently, there is a demand for simpler, eco-friendly, reproducible methods for the synthesis of nanoparticles due to growing concern of toxicity of nanoparticles. Biological methods are preferred as alternative route for the synthesis as they are safe than chemical and physical counterparts [7-8]. Different biological materials like various microorganisms, plants and others are known as effective nano-factories but use of fungi has particular advantage as they have high metal tolerance, simpler growth requirements and easier in handling [9-10].

In the present study we have reported extracellular biosynthesis of silver nanoparticles with fungus *A. niger* (MTCC No:-514). The synthesized nanoparticles were characterized using different techniques and studied for anti-bacterial properties. Extracellular approach is significant to the fact that if nanoparticles are synthesized outside of the fungal biomass, they can be directly used in range of applications.<sup>11-12</sup> Several studies have reported the use of fungi and other biological materials for synthesis of nanoparticles with an advantage that different biological entities change the shape and size of nanoparticles after the synthesis. The properties and applications of nanoparticles in various fields are attributed to their size and shape [13-14]. Hence, naturally the nanoparticles obtained from various biological materials like fungal species etc. possess varying biological, physical and chemical characteristics which can be suitably exploited to achieve potent commercial and medical applications.

Thus, we have attempted biosynthesis of silver nanoparticles keeping in mind their clinical, diagnostic, medical therapeutics and antimicrobial applications.

## **MATERIALS AND METHODS**

All chemicals used were of analytical grade. The fungal strain *Aspergillus niger* (MTCC No:-514) was procured from 'Microbial Type Culture Collection and Gene Bank' (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh, India. The fungal isolate was maintained on PDA at 28 °C in BOD incubator for 48-72 hrs and the growth characteristics were noted.

### **Fungal biomass preparation for the synthesis of Ag-NPs**

The fungal biomass used for biosynthesis was grown aerobically in liquid medium containing (g/l)  $\text{KH}_2\text{PO}_4$ -7.0;  $\text{K}_2\text{HPO}_4$  - 2.0;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  - 0.1;  $(\text{NH}_4)_2\text{SO}_4$  -1.0; Yeast extract - 0.6; pH-  $6.5 \pm 0.3$  in 250 ml Erlenmeyer flask along with fungal culture. The inoculated flask was incubated in an environmental shaker at a speed of 150 rpm at 28 °C for 72 hrs. After proper growth, the fungal mycelia were filtered through Whatman filter paper no. 1 and then washed thoroughly with Milli-Q deionized water to remove any medium component. Ten grams of clean and fresh biomass was resuspended in Erlenmeyer flask containing 120 ml of Milli-Q deionized water and agitated at the same conditions as described above. After the incubation the fungal biomass was separated again with the help of Whatman filter paper no. 1 and cell-free filtrate was used for nanoparticles synthesis.

### **Extracellular fungal mediated biosynthesis of Ag-NPs**

For the synthesis of silver nanoparticles, 50 ml of cell free filtrate was challenged with 50 ml aqueous silver nitrate solution at the concentration of 1 mM in a 250 ml Erlenmeyer flask and incubated at 28 °C in absence of light. The positive control (cell-free filtrate, without the silver ions) and a negative control (1mM  $\text{AgNO}_3$ , without addition of cell filtrate) were also run simultaneously along with the experimental flask under same conditions. All the procedures were carried out thrice.

### **Characterization of silver nanoparticles**

#### **UV-Visible spectroscopic analysis**

Visual observation was made to measure reduction of silver nitrate by monitoring change in color of the solution incubated with *A. niger*. Synthesized silver nanoparticles were examined by UV-VIS spectrophotometer [ELICO, SL 210], for that 5 ml aliquots were periodically sampled at 24 hrs interval followed by centrifugation before measuring absorbance. The measurement was taken at wavelengths ranging from 200 to 500 nm against negative control.

#### **Particle size measurement**

Particle size distribution of silver nanoparticles was evaluated by ZETA-sizer [Helos-BF, SYMPATEC, Germany] between 0-90°C and in the size range of 0.5 nm to 5 µm. Data generated was analyzed using Zeta-sizer software.

#### **TEM, SEM-EDAX analysis**

Transmission electron microscopy (TEM) [Tecnai 20, Philips, Holland] was employed for measuring size of synthesized nanoparticles using low voltage (100kV). Silver nanoparticles solution was placed on carbon coated grids of specimen holder followed by vacuum desiccation for 12 hrs before analysis.

For SEM-EDAX, freeze-dried samples were mounted on carbon coated grids and gold coated by sputter coater followed by examination in Scanning Electron Microscope equipped EDAX [ESEM EDAX XL-30, Philips, Netherlands] at 30 kV.

#### **Fourier Transform Infrared (FT-IR) spectroscopic analysis**

The aqueous solution containing silver nanoparticles was dried using freeze-drier and the dried powder was diluted with potassium bromide in the ratio of 1:100 to record spectra using FT-IR Spectrometer [Perkin Elmer Spectrum GX, USA] using transmittance mode in the range of 400-4000  $\text{cm}^{-1}$  at a resolution of 4  $\text{cm}^{-1}$ .

#### **X-Ray Diffraction (XRD) analysis**

The XRD spectra were recorded in XRD Diffractometer [Xpert MPD, Philips, Holland] operating at 2 kV from Cu target X-ray tube using freeze-dried powder of silver nanoparticles. The diffracted frequency were measured from 30° to 136° 2θ angles and matched to JCPDF database for qualitative analysis.

#### **Anti-bacterial activity of silver nanoparticles**

The disc diffusion assay on Muller-Hinton agar (MHA) plates was performed to investigate antibacterial activity of silver nanoparticles against pathogenic bacterial strains like *E. coli* and *P. aeruginosa* and also on *S. aureus*. The bacterial strains were procured from IMTECH, Chandigarh, India and streaked on Muller-Hinton agar plates. Further, standard disks separately impregnated with antibiotic Penicillin G (20 µg/disk), silver nanoparticles (20 µg/disk) and a combination of silver nanoparticles and Penicillin G were placed on the MHA plate to study comparative as well as synergistic activity of standard antibiotics

and silver nanoparticles. The zone of inhibition surrounding each disk was measured after 24 hrs incubation at 37 °C. All the procedures were performed thrice.

## RESULTS AND DISCUSSION

Fungal strains procured from MTCC were studied for their morphological characteristics on plates as well as using microscope and were confirmed to be *A. niger* [Figure-1]. The prepared biomass of *A. niger* that was used to synthesize silver nanoparticles is shown in Figure-2.

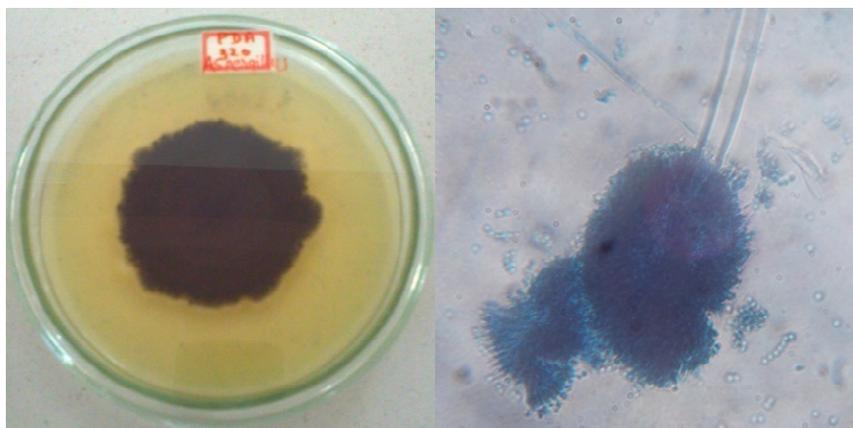


Figure 1: Growth of *Aspergillus niger* and Staining by Lactophenol Cotton Blue

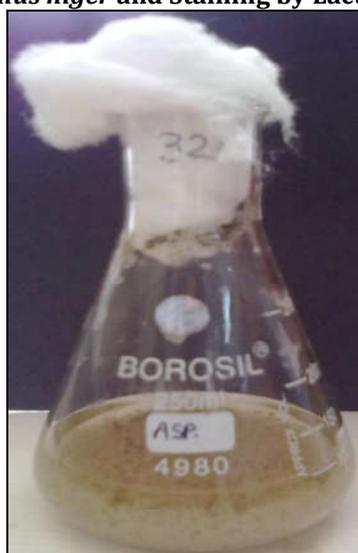


Figure 2: Biomass preparation for synthesis of Ag-NPs by *A. niger*

### Characterization of silver nanoparticles

#### Visual Observation

The change in color was visually observed after 72 hrs when *A. niger* was challenged with  $\text{AgNO}_3$ . The color changed from pale yellow to brown in a flask which was incubated with fungus [Figure-3]. The brown color intensity in experimental flask increased with increase of incubation period while positive control containing only silver nitrate did not show any color change. Brown color is an indication of silver nanoparticles as proved by Duran *et al*<sup>15</sup> working with fungus *Fusarium oxysporum* and by Thirumurugan *et al* [16] using fungal strain *Phytophthora infestans*.

Basavaraja *et al* [17] and Minaeian *et al*<sup>18</sup> reported that extracellular synthesis of silver nanoparticles using microbes appeared brown colored due to reduction of silver ions by the reducing agents secreted by the microbes.

#### UV-Visible spectral analysis

Various metal nanoparticles in the size range of 2-100 nm can be characterized spectrophotometrically using light wavelength in the range of 300-800 nm [19]. Silver nanoparticles can also be characterized using spectrophotometric measurements in the wavelength range 400-500 nm [20].

The presence of silver nanoparticles can be ascertained by UV-Visible spectroscopy based on metal ions exhibiting specific surface plasmon resonance [17, 21-22]. In the current investigation the spectra of *A.*

*niger* fungal filtrate silver nanoparticles exhibited a significant peak nearer to 410 nm [Figure-4] which are in the range for silver nanoparticles production but the positive control did not show any characteristic significant peak. Reduction of silver began within 24 hrs and maximum intensity was observed at 72 hrs which indicated complete reduction of silver ions.

Shahverdi *et al* [23] observed the peak nearer to 430 nm when silver nanoparticles were synthesized using *Enterobacteriaceae* family and filamentous fungi.



Figure 3: Ag-NPs cell filtrates of *A. niger* with AgNO<sub>3</sub> (Right) and Positive control (Left)

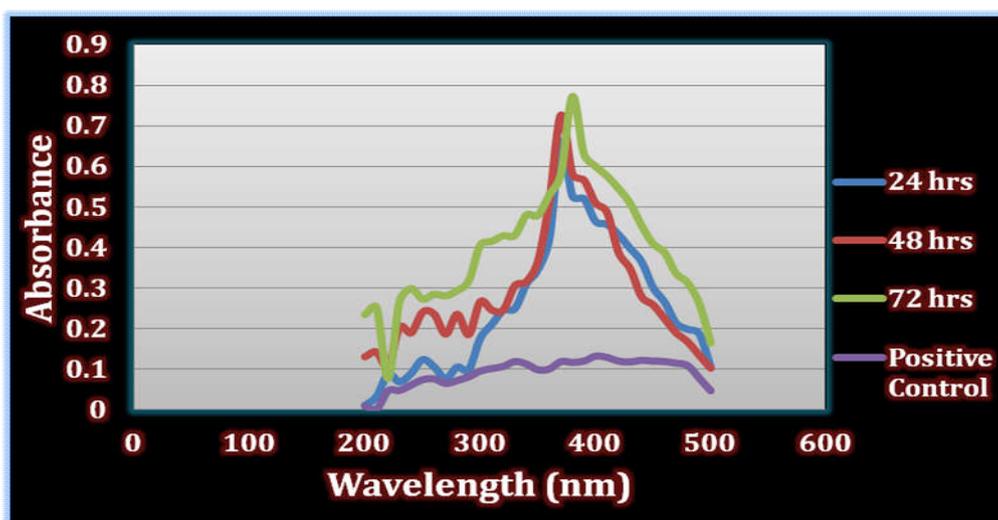
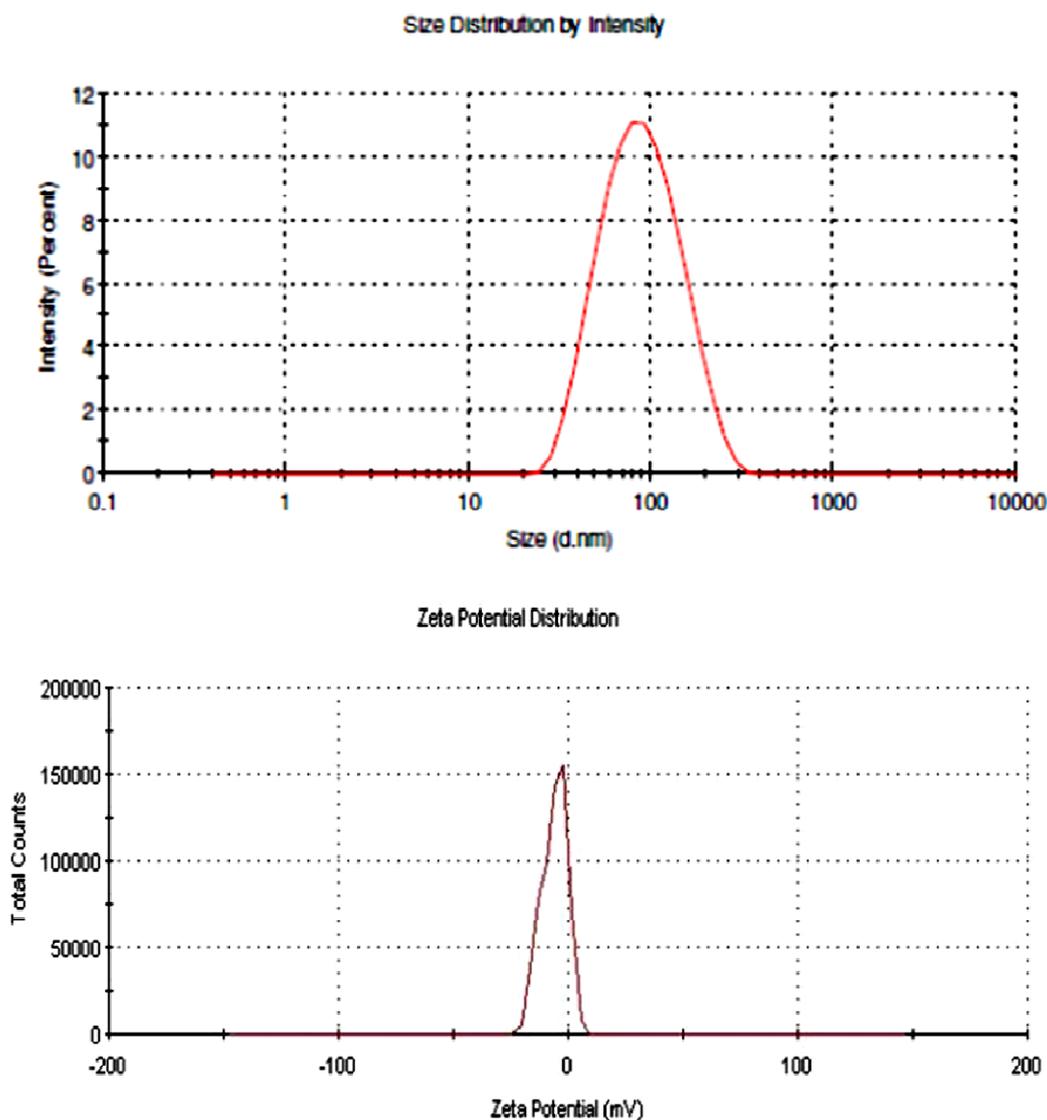


Figure 4: UV-Visible spectrum of Ag-NPs by *A. niger* (Mean of three replicates)

#### ZETA sizer analysis

Size of Ag-NPs synthesized using *A. niger* was measured with Zeta sizer having average diameter of 81 nm (100% intensity) and Zeta-Potential of -6.12 mV [Figure-5]. Zeta-Potential analysis showed that silver nanoparticles possess some charge on the surface which contributes to their stability. Results also indicate the particles were finely distributed inside the solution and also very well dispersed due to negative charge which repelled each other.

Solanki *et al*<sup>24</sup> attempted biosynthesis of silver nanoparticles with *P. brevicompactum* and found average diameter of 52nm (100% intensity). Navin *et al*<sup>25</sup> used *Aspergillus flavus* NJP08 for extracellular biosynthesis and characterization of silver nanoparticles and observed 10 to 35 nm sized silver nanoparticles with average size  $17 \pm 5.9$  nm and almost 80 % of the particles in the 10 to 25 nm range.



**Figure 5: Particle Size analysis by intensity and Zeta-Potential measurement with ZETA Sizer for *A. niger***

#### TEM, SEM-EDAX analysis

TEM image of silver nanoparticles by *A. niger* indicated that silver nanoparticles were spherical shaped and in size range of 21.0 – 51.0 nm [Figure-6]. Vigneshwaran *et al* [26<sup>26</sup> performed biological synthesis of silver nanoparticles using the fungus *Aspergillus flavus* and observed that nanoparticles were well separated without agglomeration with an average size of  $8.92 \pm 1.61$  nm.

Ag-NPs obtained using *A. niger* were scanned both randomly and individually using SEM. The SEM micrograph of single nanoparticle scanned randomly showed smooth morphology and spherical shape [Figure-7]. The investigation study of Navin *et al* [25] under low magnification revealed that the particles were spherical in shape and uniformly distributed (monodispersed) without significant agglomeration when they utilized *Aspergillus flavus* NJP08 for extracellular biosynthesis and characterization of silver nanoparticles. The fungal species *Aspergillus terreus* was utilized by Guangquan *et al* [27] for biogenic synthesis to get 1-20 nm size and spherical shape with polydispersity.

The EDAX profile of silver nanoparticles synthesized by *A. niger* [Figure-8] showed a strong signal at  $\sim 3$ eV indicating the existence of silver atoms and no other metal element. The EDAX profile also confirmed the presence of biomolecule as shown by peaks of C, O and N elements. Seema *et al*<sup>28</sup> performed microbial synthesis of silver nanoparticles and assigned EDAX spectra for Cu and C due to carbon coated copper grid and peaks of N, P and S represented proteins as a capping agent. Jain *et al*<sup>29</sup> reported 59% silver with small elemental composition of 8.99% Nb and weaker signal of C, O, and N from proteins.



Figure 1: TEM micrographs of Ag-NPs by *A. niger* on the scale of 100

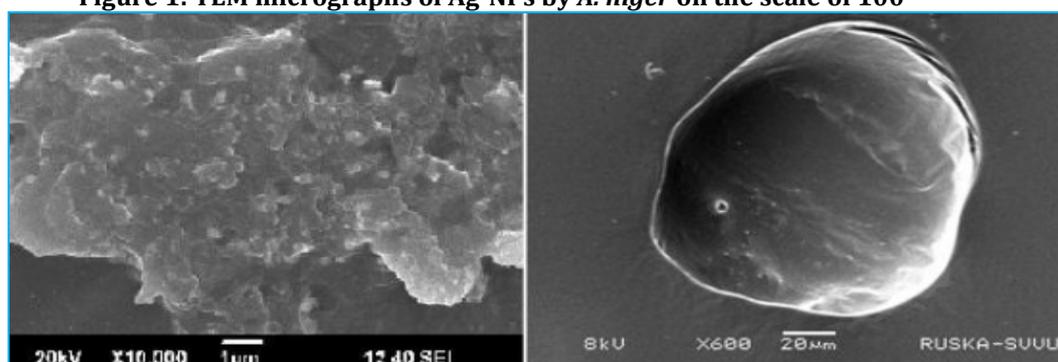


Figure 7: SEM micrograph of Ag-NPs for *A. niger*

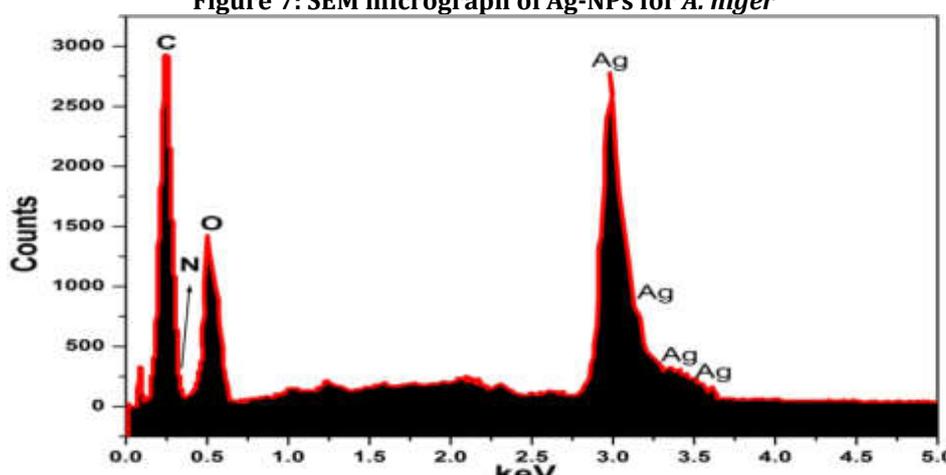


Figure 8: EDAX spectrum of Ag-NPs by *A. niger*

#### Fourier Transform Infrared (FT-IR) spectroscopic analysis

FTIR analysis of nanoparticles synthesized by *A. niger* was carried in the range of 450 to 3000  $\text{cm}^{-1}$  [Figure-9]. The spectra revealed the presence of different functional groups. The strongest and broadest peak at 3290.73  $\text{cm}^{-1}$  indicates stretching of secondary amide N-H functional group and bonding of -H. The intense peak at 2928.01  $\text{cm}^{-1}$  corresponds to alkane with respect to C-H stretching. The C $\equiv$ C stretching was indicated by broad peaks at 2161.23  $\text{cm}^{-1}$ . The various peaks observed at 1772.02 correlated with -anhydride (C=O stretching), 1613.81  $\text{cm}^{-1}$  corresponded to-alkene (C=C stretching), 1538.60 matched with -aromatic  $\text{cm}^{-1}$  (C-C stretching), while 1386.45  $\text{cm}^{-1}$ , 1313.48  $\text{cm}^{-1}$  and 1080.10  $\text{cm}^{-1}$  corresponded with primary alcohol (C-O stretching) and 528.55-alkene (=C-H bending) respectively suggests strong interaction of Ag-NPs with different functional groups. Thus, above results indicated prevention of agglomeration and stabilization of nanoparticles probably due to the role of protein for the coating of nanoparticles like capping agent [30]. Present investigation was also matched with the FTIR study conducted by Ahmad *et al* [31] in which they had employed *Fusarium oxysporum* for green synthesis of silver nanoparticles.

#### X-Ray Diffraction (XRD) analysis

XRD analysis was also performed for silver nanoparticles synthesized by *A. niger* [Figure-10]. Prominent Ag-NPs peaks at (111), (200) planes respectively were at 38° and 46° of 2 $\theta$ . Chloride ions were used in

cell free preparations as a result of that the peak at  $32^\circ$  might be corresponding to AgCl. The result was matched with reference to the Bragg's peak position and agreed well with those reported for silver (face centric cubic) by Joint Committee on Powder Diffraction Standards File No. 040783 [32-33]. The crystalline nature of Ag-NPs was confirmed by broad peaks at (111), (200) and (220). The XRD analysis by Mouxing *et al*<sup>34</sup> for green synthesis with the use of *Aeromonas* sp. for silver nanoparticles indicates various peaks at  $2\theta = 7.9^\circ, 11.4^\circ, 17.8^\circ, 30^\circ, 38^\circ$  and  $44^\circ$ .

#### Disk diffusion assay

The disk diffusion assay for nanoparticles synthesized by *A. niger* also revealed significant antibacterial activity in the form of clear zone of inhibition around the disc against pathogenic bacterial strains [Figure-11].

The antibacterial activity was higher in the case of *P. aeruginosa* compared to *E. coli* and *S. aureus*. It was clearly observed that (Table-1) silver nanoparticles show stronger antibacterial activity in contrast to antibiotic Penicillin G at the same concentration for all three bacterial strains. Further, combination of silver nanoparticles and Penicillin G exhibits potent activity due to synergistic effect compared to only antibiotic or silver nanoparticles. The synergistic effect was more lethal for *E. coli* and *S. aureus* comparing *P. aeruginosa*.

The possible interaction of silver nanoparticles with bacteria occurs via electrostatic interactions as nanoparticles being positively charged and bacterial cells have negative charge. Ahmad *et al* [31] stated that chemical substance like antibiotics may be not a potent agent as they can also interact with other living biological entities. Some microorganism also develops resistant metabolism against chemical substances thus biologically synthesized silver nanoparticles are extremely suitable for antimicrobial agents.

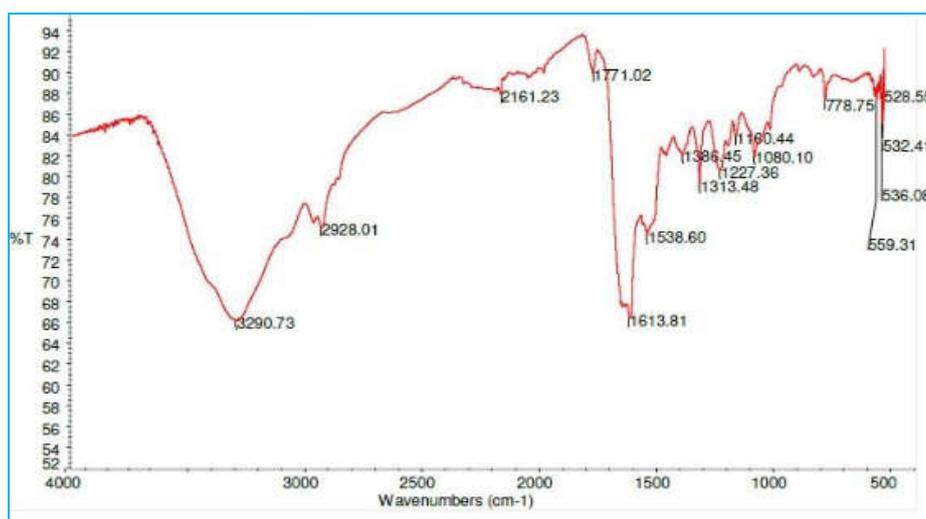


Figure 9: FT-IR Spectrum of Ag-NPs for *A. niger*

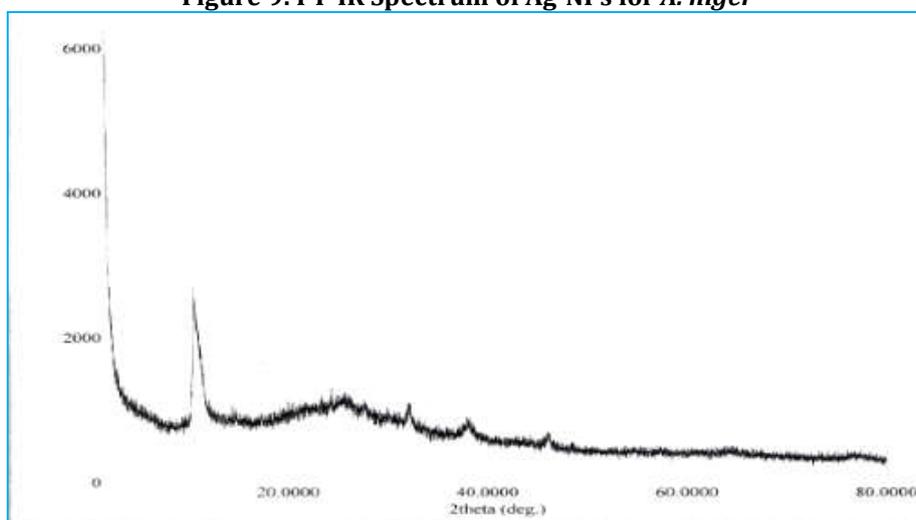
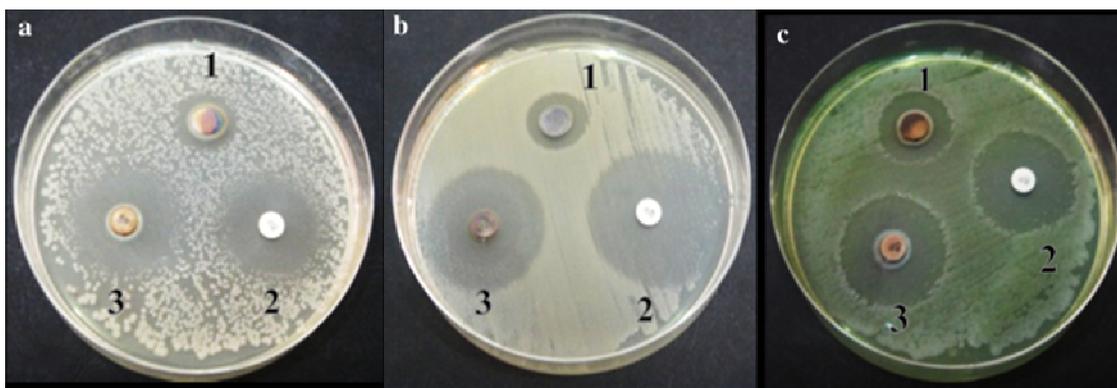


Figure 2: X-Ray Diffraction studies of Ag-NPs for *A. niger*



**Figure 11: Antibacterial Effect of Ag-NPs for *A. niger* on (a) *E. coli* (b) *P. aeruginosa* and (c) *S. Aureus*; 1 Penicillin G (20 µg/disk), 2 Ag-NPs(20 µg), 3 Ag-NPs + Penicillin G**

**Table-1 Antibacterial activity of Ag-NPs (synthesized by *A. niger*) and antibiotic against pathogenic bacteria**

	Zone of Inhibition Diameter in mm (Mean of Three Replicates)		
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>
<b>Penicillin G (20 µg/disk)</b>	7 ± 1 mm	9 ± 1 mm	12 ± 2 mm
<b>Ag-NPs(20 µg)</b>	14 ± 1 mm	23 ± 2 mm	18 ± 3 mm
<b>Ag-NPs + Penicillin G</b>	19 ± 2 mm	26 ± 3 mm	25 ± 2 mm

## CONCLUSION

The fungal strain *A. niger* was employed for the biosynthesis of silver nanoparticles. The UV-Visible spectra showed a characteristic peak at 410nm and FTIR study confirmed the role of protein moiety as a stabilizing and capping agent. The silver nanoparticles were spherical, smooth and with the size in the range of 21.0 – 51.0 nm. The EDAX profile showed a strong signal at ~3eV confirming presence of only silver and no other metal. XRD confirmed that particles were cubical and crystalline in nature. The synthesized nanoparticles showed very efficient anti-bacterial activity in contrast to Penicillin G even at low concentration against human pathogens. Further, synergistic effects of combination of silver nanoparticles and Penicillin G were more potent compared to antibiotic or silver nanoparticles used alone, which can be exploited for clinical purpose. The extracellular biosynthesis of silver nanoparticles mediated by *A. niger* enables us easier handling during downstream processing. The biosynthesized nanoparticles can be directly used for different applications as they are devoid of cellular components. Fungi yields rapid and large scale production of nanoparticles with small biomass which are economically viable. We feel scaling up the production of Ag-NPs using fungi should be explored further to make it more cost effective and easily exploitable for biomedical applications.

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