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Silver nanoparticles: A promising alternative approach to combat bacterial infections

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Abstract
The field of nanotechnology is the most active areas of research in modern material science. The synthesis of Nano biomaterials using biological processes is very fast, environment friendly and non-toxic. In the present study silver nanoparticles were synthesized using *Acremonium* species isolated from spoiled vegetables. They were characterized by UV, SEM, XRD and EDX. The silver nanoparticle synthesized using *Acremonium species* was tested for antibacterial activity against pathogenic microbes *Bacillus subtilis, Klebshiella pneumoniae, Salmonella typhi, staphylococcus aureus, Shigella boydii, Proteus vulgaris, Pseudomonas aurogenosa* and *Escherichia coli.* The AgNPs showed highest antibacterial activity against *Klebsiella pneumonia* followed by *E. coli, S. typhi, Shigella sp., Proteus mirabilis, S. aureus* and *B. subtilis.* It was found that Gram negative bacteria were inhibited to greater extent by silver nanoparticles than Gram positive. The combined use of AgNPs with other antimicrobial agents reduce problem of toxicity and avoid the potential for development of resistance and strongly enhance the microbicidal effect.

Keywords: Silver nanoparticles, *Acremonium sp.*, antibacterial, antifungal, activity

1. Introduction
Nanotechnology is the application of science and technology to control matter at the molecular level. At the Nano scale level, the properties of matter are significantly different from their macroscopic bulk properties. It has recently become one of the most active research fields in technology and engineering. (Bae *et al.*, 2005). The size, shape and elemental distribution of nanoparticles can be characterised by a wide variety of analytical methods like Ultra Violet – Visible (UV-Vis) Infrared (IR) spectroscopy (Amendola & Meneghetti, 2009) and X-ray diffraction can be used to determine nanoparticle size and shape.
Biological synthesis of nanoparticles has gained more attention by the researchers for its potential applications. Nano silver is one of the most thoroughly investigated nano materials and owes its popularity to its bio cidal properties (Vaidyanathan *et al.*, 2009) [21].
Biological systems provide a novel idea for the production of nano-materials Compared with the traditional synthetic methods, (Bansal *et al.*, 2011) [1]. Fungi are the best candidates in the synthesis of metal nanoparticles, because of their ability to secrete a large amount of enzyme (Moharrer *et al*., 2012) [11] and easy to store and isolate from different sources like soil, air, plants, etc. The fungal systems or myco nano factories have been exploited for the synthesis of metal nanoparticles of silver, gold, zirconium, silica, titanium, iron (magnetite) and platinum. A large number of fungal strains are capable to synthesize silver nanoparticles (AgNPs) extracellulary, among which *Fusarium oxysporum Aspergillus fumigatus*, *Aspergillus niger*, *Fusarium semitectum, Penicillium brevicompactum*, *Cladosporium cladosporioides*), and Aspergillus *clavatus. Acremonium* is a cosmopolitan, morphologically simple endophytic fungus. The synthesis and characterizations of silver nanoparticles using Acremonium species have been well documented.
In recent years, the occurrence of antibiotic resistant strains of a number of pathogenic bacteria including *E. coli* has emerged as another health problem over the worldwide. Regular monitoring of antibiotic resistance rates is necessarily required to improve and revise empirical antibiotic therapy protocols (Eshetie *et al*., 2016) [3]. Nanotechnology has paved way to combat the challenges of multi-drug resistance through innate properties of AgNPs which are in limelight since a long time due to their broad spectrum antimicrobial activities against bacteria (Singh *et al*., 2014) [18].
Due to the significant biological properties of silver nanoparticles the present investigation was undertaken to synthesize and characterise silver nanoparticles from fungi and evaluated

For their applicability in the antimicrobial activity against bacteria

2. Materials and Methods

2.1 Sample collection and Processing

Spoiled vegetables were collected and processed for the isolation of *Acremonium* species (Udoh *et al*., 2015) [19]. The excised infected portions were then plated on to Sabouraud Dextrose Agar (SDA) slants supplemented with 50 mg chloramphenicol and 5 mg gentamicin per litre. It was then incubated at room temperature for 5 days. The isolated fungi were observed for their colony morphology and carefully sub cultured on Sabouraud Dextrose Agar plates and slants. The plates and slants were grown for seven days in an incubator at 28°C. Representative colony types were purified by sub-culturing on fresh SDA plates. The isolated fungi were identified based on the isolate's colony characteristics on culture plates and microscopic observation. Detailed morphological characteristics of the fungi such as hyphae (septation), reproductive structure (sporangia/conidia) in chain or single; the type of spore were observed and recorded.

2.2 Extracellular Synthesis of Silver Nanoparticles by *Acremonium Species*

Acremonium species isolated from spoiled vegetables was used for the biosynthesis of silver nanoparticles.

a. Production of Biomass (Sherif, 2015) [16].

Pure isolates were inoculated in 100 ml of Czepak-dox broth, taken in a 250 ml conical flask. The flask was kept on rotator orbital shaker for seven days at 120 rpm. The cultured material was sieved by funnel separating media content. The biomass, thus obtained was inoculated in 250 ml conical flask containing 100 ml sterilized distilled water and kept for 3 days on rotator shaker for agitation at the speed of 150 rpm. After incubation, the cell filtrate was collected and used for the synthesis of nanoparticle.

b. Synthesis of Silver Nanoparticles (Ravindra & Rajasab, 2015) [14].

Cell filtrate (10 ml) of the fungi was mixed with 50ml of 1 mM silver nitrate Solution in 250ml conical flask and agitated at room temperature. Control (without silver nitrate, only biomass) was also run along with an experimental flask. After 72 hours of incubation, reduction of silver nitrate in to silver ions were observed and recorded.

2.3 Characterization of Silver Nanoparticles

The physical characterisation like structure and size of synthesized AgNPs were assessed by UV-Visible Spectrometry scanning electron microscope (Devi *et al*., 2012) [2] Energy Dispersive Analysis of X-ray (EDX) and the crystallographic structure characterized by X-Ray Diffraction (XRD) (Ravindra & Rajasab, 2015) [14].

2.4 Antimicrobial Activity of Silver Nanoparticles

a. Standardization of Microorganism

The test clinical isolates of bacteria was cultured in nutrient broth and incubated at 37˚C for 24 hours to yield culture density equivalent to 0.5 McFarland standards.

b. Antibacterial activity of silver nanoparticles (Roy et al., 2015)

The silver nanoparticle synthesized using *Acremonium species* was tested for antimicrobial activity by agar well diffusion method against pathogenic bacteria. The pure cultures of bacteria were sub cultured on nutrient broth. Each strain was swapped homogeneously onto the individual plates using sterile cotton swabs. Wells of 10 mm diameter were made on Muller Hinton agar using gel puncture. Different concentration of silver nanoparticle 20, 30, 40 and 50µl was poured on each well. After 24 hours incubation the various levels of zone of inhibition was measured. Three replicates of experiments were carried out.

3. Results

Spoiled vegetables samples were collected and processed for the isolation of fungi. Based on the microscopic and macroscopic morphology Colony 1 and 2 were identified as *Aspergillus Niger and Acremonium species (*Plate-1).

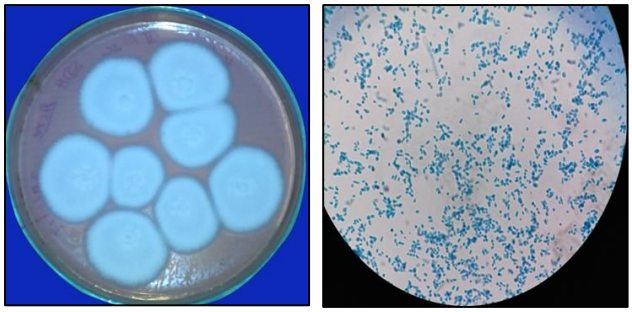


Plate 1: Identification of fungi A. Colony morphology (*Acremonium sp.*) B. Microscopic Morphology (LPCB)

3.1 Extracellular Synthesis

Pure culture of *Acremonium species* were maintained on SDA slants for biomass production and synthesis of silver nanoparticles. (Plate-1) Pure isolates of *Acremonium species* was inoculated in SDA broth and incubated for 7 days for biomass production. After incubation period the biomass was recovered by sieving through a funnel separating the media contents. The biomass recovered from the medium was found to be wet and colour.

a. Synthesis of silver nanoparticles (Plate-2 & 3)

The fungal wet biomass was mixed with 1mM silver nitrate solution and the synthesis of silver nanoparticles was observed after 72 hours of incubation. Initially the fungal cells were pale yellow in colour. It was observed that the previous pale yellow colour of the reaction mixture is changed to brownish colour after 72 hours of reaction. Appearance of yellowish brown colour in the medium is a clear indication of the formation of silver nanoparticles in the reaction mixture.

Plate 2: production of biomass plate

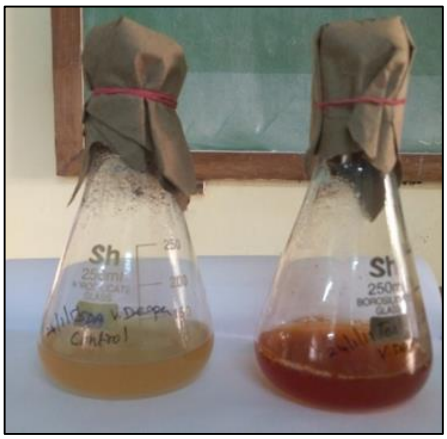


Plate 3: synthesis of silver nano particles

3.2 Characterisation of Silver Nanoparticles

a. Electron microscopy

The AgNPs obtained from *Acremonium species* were predominantly spherical with diameters ranging from a few nanoparticles to well above 50-71nm and also individual nanoparticles were aggregated showing large nanoparticles (Fig. 1).

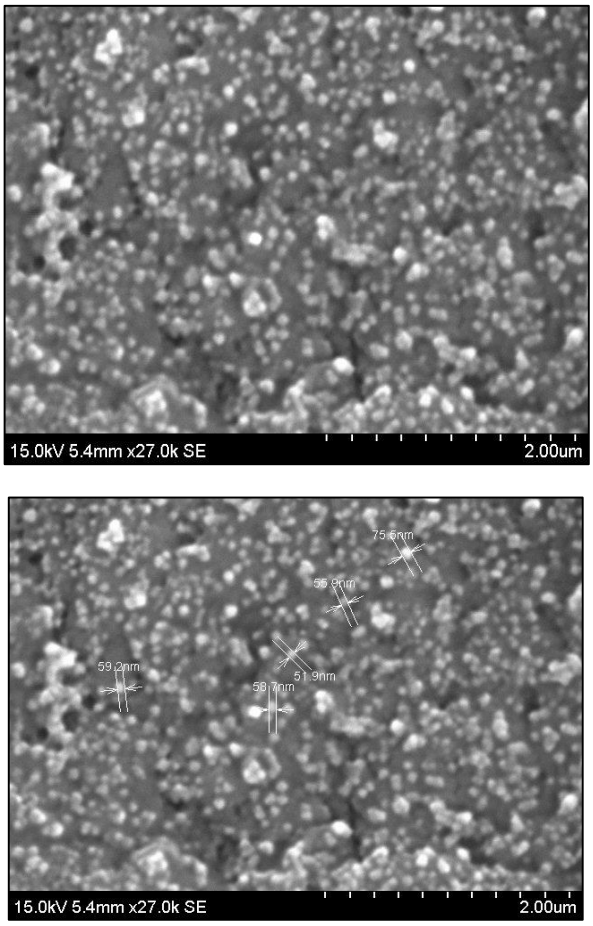


Fig 1: Electron Microscopy

b. Energy Dispersive Analysis of X-ray (EDX) (Fig. 2 & 3) (Table 1)

The EDX spectrum recorded in the spot-profile mode. The optical absorption peak is observed at 2.13 KeV, which is typical for the absorption of metallic silver nanoparticles. From the EDX spectrum it is clear that silver nanoparticles reduced by *Acremonium sp* have the weight percentage of silver as 33.2%.

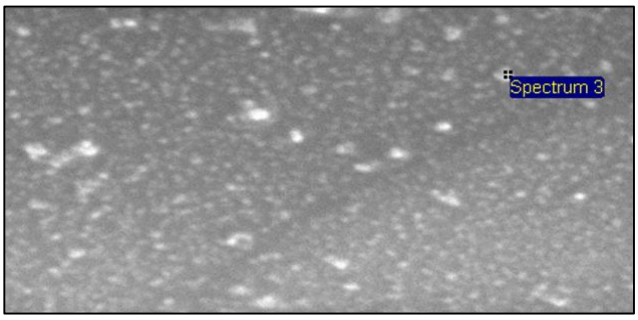


Fig 2: Energy Dispersive Analysis

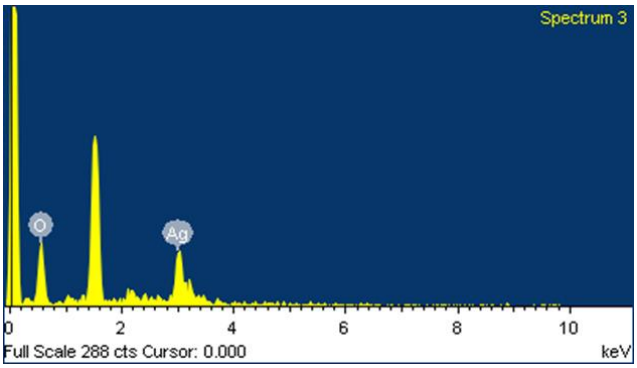


Fig 3: EDX Graph of Silver Nanoparticles

c. UV-Visible Spectrometry

The absorption spectrum of the pale yellow-brown silver colloids showed a surface Plasmon absorption band with a maximum at 254nm-474 nm indicating the presence of spherical or roughly spherical Ag nanoparticles.

d. X-ray Diffraction (Figure-4)

The XRD patterns of AgNPs indicated that the structure of silver nanoparticles is Face Cubic Centre (FCC) The pattern of the sample matched well with the standard patterns of silver (JCPDS file No. 04-0783). All of the peaks of the patterns of the samples can be readily indexed to face cantered-cubic silver (JCPDS file No. 04-0783), where the diffraction peaks at 2θ values of 38.24, 44.42, 64.44, 77.40° can be ascribed to the reflection of (111), (200), (220), (311) planes of the face-centred cubic silver, respectively. No peaks from other phases were detected, indicating high purity of the products.

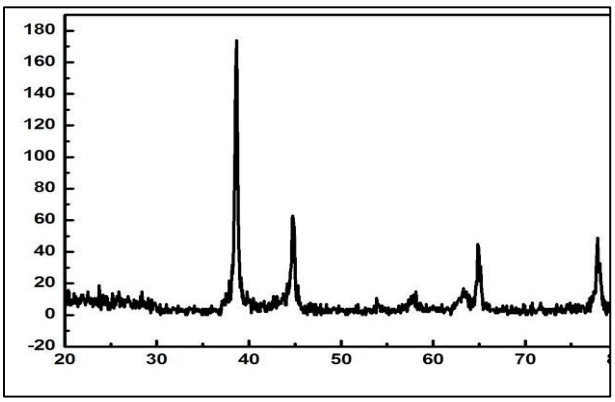


Fig 4: X-Ray Diffraction

3.3 Antimicrobial Activity of Silver Nanoparticles

The antimicrobial activity of synthesized silver nanoparticles against various pathogenic organisms including bacteria and fungi was investigated.

a. Antibacterial activity of silver nanoparticles (Plate-4) (Fig. 5)

Antibacterial activity of synthesised silver nanoparticles against clinical isolates was assayed by well diffusion method. Silver nanoparticles were able to inhibit *Klebsiella pneumoniae* with maximum zone of inhibition (12mm) at a concentration of 50µl. *Escherichia coli*, *Salmonella typhi* and *Shigella boydii* were equally inhibited by silver nanoparticles with inhibition zone (10mm) at the concentration of 50 µl least zone of inhibition (6mm) was shown by *Bacillus subtilis* (Fig. 6) (Table-3).

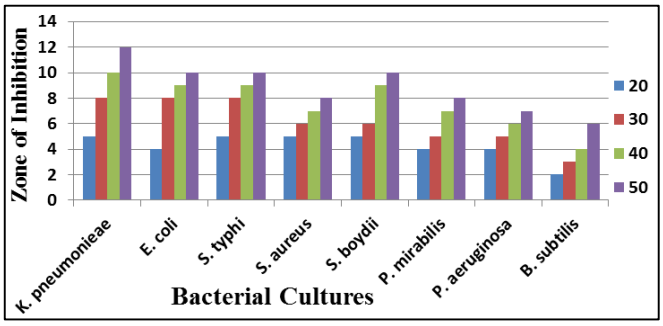
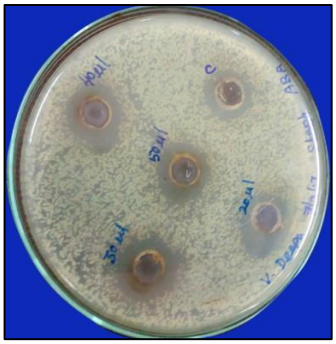


Fig 5: Antibacterial Activity of Silver Nanoparticles

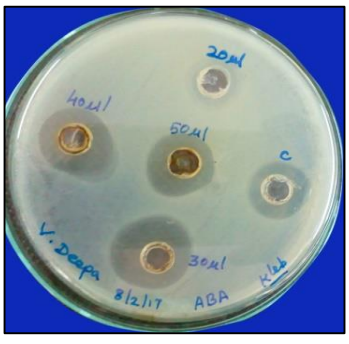
1. *B. subtilis*

2. *E. coli*

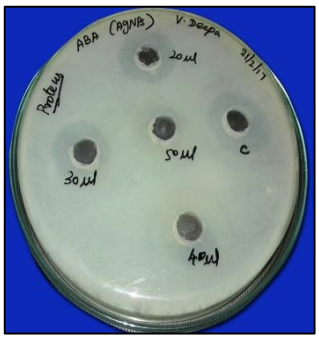


3. *S. aereus*

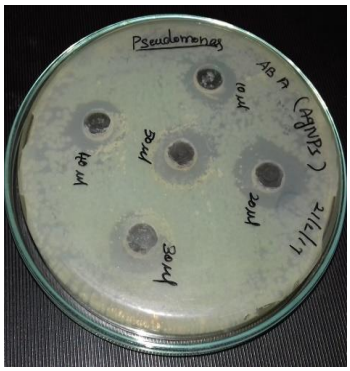
4. *S. typhi*



5. *K. pneumonea*



6. *Proteus mirabilis*



7. *P. aerogenosa*

Plate 4: Antibacterial activity of silver nanoparticles

4. Discussions

There is a pressing demand to discover novel strategies and identify new antimicrobial agents from natural and inorganic substances to develop the next generation of drugs or agents to control microbial infections. In recent times, the advances in the field of nanosciences and nanotechnology has brought to fore the nano sized inorganic and organic particles which are finding increasing applications as amendments in industrial, medicine and therapeutics, synthetic textiles and food packaging products. AgNPs were considered, in recent years, particularly attractive for the production of a new class of antimicrobials opening up a completely new way to combat a wide range of pathogens.

Hence in this present investigation an attempt was made to synthesise silver nanoparticles from fungi and its antimicrobial activity was explored. *Acremonium sp.* isolated from vegetables samples was used for the synthesis of silver nanoparticles.

Application of fungi for production of silver nanoparticles, is the emphasis of the present investigation. Production of silver nanoparticles through fungi has several advantages over the above mentioned approaches. They include tolerance towards high metal nanoparticle concentration in the medium, easy management in large-scale production of nanoparticles, good dispersion of nanoparticle and much higher amounts of protein expressions. (Vahabi *et al*., 2011) [20].

The biomass recovered was mixed with silver nitrate solution and observed for synthesis of silver nanoparticles after 72hours of incubation. The reduction of silver ions and formation of silver nanoparticles was indicated by appearance of yellowish brown colour in the medium. The colour change from pale yellow to yellowish brown when 1Mm silver nitrate was added to the solution is due to the excitation of surface plasmon vibrations in the metal nanoparticles. Control without silver ions showed no change in colour when incubated under the same conditions. A similar report of silver nanoparticle synthesis induced by fungi was reported by Honary *et al*., 2013 [4].

In the biosynthesis of metal nanoparticles by a fungus, the fungus mycelium is exposed to the metal salt solution, which helps the fungus to produce enzymes and metabolites for its own survival. In this process the toxic metal ions are reduced to the none-toxic metallic solid nanoparticles through the catalytic effect of the extracellular enzyme and metabolites of the fungus.

In the present study silver nanoparticles synthesised by fungi were characterised by different physical methods like Scanning electron microscopy, UV-visible spectrometry, EDX and X-ray diffraction.

UV-visible spectrometry is one of the most widely used technique for the characterization of nanoparticles. It is generally recognised that UV-visible spectrometry could be used to examine size and shape controlled nanoparticles in aqueous suspensions. Absorption spectra of AgNPs formed in the reaction medium has absorbance peak at 254nm (0.95) and 474nm (0.715). These results were consistent with the report of Li *et al*., 2012, stated that strong broad peak at 440nm.

The peak at 290nm is because of organic moieties present in the reaction mixture. This observation matches with the result of EDAX where K & O peak occurred due to organic moieties. The broadening of the peak indicated that the particles are poly dispersed.

EDX (Energy Dispersive Analysis of X-ray) gives qualitative, as well as quantitative status of elements that may be involved in the formation of AgNPs. In the EDAX spectrum the peak around 2.130KeV correspond to the binding energies of Ag, which is typical for the absorption of metallic AgNPs. Stronger signals were also observed from other atoms like O&K. The weight percentage of silver was found to be 33.2%. Magdi *et al* 2014 [10], reported the weight percentage of silver to be 52.9% from EDX spectrum of AgNPs synthesis by fungi.

The size, shape and distribution of synthesized silver nanoparticles were characterised by SEM. It shows particles are spherical in shape with average size ranging from 50-71nm.Individual nanoparticles were aggregated which may be due to the presence of cell components on the surface of nanoparticles acting as capping agent (Vanaja *et al*., 2013) [22].

As silver is a cubic structure, it can be assumed that most of the nanoparticle-like structures are formed by crystallites in cubic faces parallel to the substrate (single crystalline) In addition, the XRD peaks could be attributed to the crystallographic planes. Hence, from the XRD result, it is clear that AgNPs formed from *Acremonium sp* were essentially crystalline (Lanje *et al.*, 2010) [7].

In the present study, the antibacterial activities of AgNPs were investigated. The antibacterial activities of silver nanoparticles were carried out by well diffusion method. The biologically synthesized AgNPs were found to be toxic against pathogenic bacteria and fungi of selected species. The AgNPs showed highest antibacterial activity against *Klebsiella pneumonia* followed by *E. coli, S. typhi, Shigella sp, Proteus mirabilis, S. aureus* and *B. subtilis*. Maximum zone of inhibition was shown by *Klebsiella*. Least inhibition zone was shown by *S. aureus* and *B. subtilis*. Similar results were reported by Savithramma *et al*., 2011 [15].

It was found that Gram negative bacteria were inhibited to a greater extent by silver nanoparticles than Gram positive bacteria.

Silver nanoparticles are very toxic in Gram-negative bacteria than the Gram-positive bacteria due to the difference in their cell wall. The mechanism of its antibacterial activity was not understood clearly. Some of the researchers explained the possible mechanism of antibacterial activity of silver nanoparticles. Silver nanoparticles attach with the cell wall of bacteria by electrostatic attraction and disrupt the cell permeability and respiration due to the generation of the reactive oxygen species. Silver nanoparticles bind with thiol groups of DNA and RNA and affect the protein synthesis of the bacteria (Kumar *et al*., 2014; Muhsin *et al*., 2014) [6, 12].

The antibacterial activity of different metal nanoparticles such as silver colloids is closely related to their size; that is, the smaller the silver nuclei, the higher the antibacterial activity (Khan *et al*., 2018) [5]. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles and since they easily penetrate into the cell (Shrivastava *et al*., 2007) [17]. AgNPs have a surface/volume ratio much greater than the corresponding bulk material; therefore, modalities and amount of the interactions with the bacterial surfaces are facilitated and determine a higher antibacterial activity. It is likely that a combined effect between the activity of the nanoparticles and free ions contributes in different ways to produce a strong antibacterial activity of broad spectrum.

The potential benefits of nanotechnology in biomedical and industrial applications have become widely accepted and are the most promising sector for the generation of new applications in medicine. It is now clear that AgNPs possess a strong antibacterial highlighted by the present study AgNPs have the ability to interact with various microorganisms (such as bacteria and fungi) and also impact both the growth of and mature bacterial biofilms and, therefore, could be used as broad spectrum antimicrobials.

Due to the structural difference in the composition of the cell walls of Gram-positive and Gram-negative AgNPs have significantly less effect on the growth of Gram-positive bacteria. The studies on the combined use of AgNPs with other antimicrobial agents can help reduce the problem of toxicity and to avoid the potential for development of resistance and, above all, strongly enhance the microbicidal effect. The broad spectrum of bioactivity of AgNPs makes them promising agents not only to fight infections, but in many other biomedical area.

Table 1: The Element Composition of the Agnps of Edx Spectra

S. No	Elements	Weight	Atomic%
1.	O K	66.80	93.13
2.	Ag L	33.20	6.87

Table 2: Antibacterial Activity of Silver Nanoparticles against Pathogens

S.NO	Concentration of silver nanoparticles (µl)	Zone of inhibition (mm)						
		K. pneumoniae	***B. subtilis***	***S. typhi***	***S. aureus***	***S. boydii***	***P. aeruoginosa***	***E. coli***
1.	20	5	2	5	5	5	4	4
2.	30	8	3	8	6	6	5	8
3.	40	10	4	9	7	9	6	9
4.	50	12	6	10	8	10	7	10
5.	Control (Chloramphenicol-5mg)	9	8	10	10	11	8	11

5. Conclusion

The fungal mediated green chemistry approach towards the synthesis of nanoparticles has many advantages such as ease with which the process can be scaled up, economic viability, possibility of easily covering large surface areas by suitable growth of the mycelia, etc., AgNPs were considered, in recent

years, particularly attractive for the production of a new class of antimicrobials opening up a completely new way to combat a wide range of bacterial pathogens. Although the highly antibacterial effect of AgNPs has been widely described, their mechanism of action is yet to be fully elucidated. In fact, the potent antibacterial and broad-spectrum activity against morphologically and metabolically different microorganisms seems to be correlated with a multifaceted mechanism by which nanoparticles interact with microbes. Moreover, their particular structure and the different modes of establishing an interaction with bacterial surfaces may offer a unique and under probed antibacterial mechanism to exploit.

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