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Biosynthesis, Characterization and Antimicrobial Activity of Silver Nanoparticles by *Aspergillus niger* Isolated from the Rotten Onion

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A B S T R A C T

Present day, researchers turn to natural processes such as using biological microorganisms in order to develop reliable and ecofriendly methods for the synthesis of metallic nanoparticles. In this research, we had investigated extracellular biosynthesis of silver nanoparticles using *A. niger*, isolated from the rotten onion. The formation of silver nanoparticles in the cell filtrates was confirmed by change in the color of filtrates of cell, absorption peak between 300-600 nm in UV-VIS spectra. There was a logical relationship in the efficiencies of studied *A. niger* in the production of silver nanoparticles and their nitrate reductase activity. The microscopic analysis of the colonies of *A. niger* was done by using 0.1% congo red dye. It effectively showed the morphological structure of the fungus with *Conidia* and *Conidiophores.* Hence, 0.1% congo red dye was used to stain the fungal smear and study it under the microscope. The present study showed that *A. niger* have potential for the biosynthesis of silver nanoparticles depending on their nitrate reductase activity.

Introduction

Nanotechnology deals with the stabilization and synthesis of matters at the nanoscale ranging from 1 to 100 nm (1). Nanomaterials have size-related and novel physic chemical properties which are significantly different from their macroscopic properties (2). Nanoparticles as the cornerstone of nanomaterials are the starting points for preparing many nanostructures, so their synthesis is considered as an important part of the research attempts in nanoengineering and nanoscience (3). One of the most widely used nanoparticles are sliver nanoparticles (4). In recent years, silver nanoparticles have found enormous applications in various fields such as antimicrobial agents, filters,

microelectronics, biolabeling, sensors, and catalysis, because of their specific physiochemical and biological properties (5–10). These nanoparticles have no toxic effects on humans; however, have inhibitory effects on the growth of bacteria, virus and other eukaryotic microorganisms (11). In addition to their distinctive properties, their production cost is relatively low (12).

Fungi secrete large amounts of enzymes and are easy growing on every medium so they are considered as a proper selection for the biosynthesis of nanoparticles (18, 19). Many studies have been done so far utilizing various species of fungi for the biosynthesis of sliver nanoparticles such as *Aspergillus* (8, 20, 21), *Fusarium* (22, 23), *Penicillium* (24, 25), *Trichoderma* (26, 27), and *Cladosporium* (28). Among minerals, fungi required nitrogen in the largest amounts, nitrogen can be considered as the limiting factor for their growth can be accounted. Unlike bacteria, fungi cannot fix atmospheric nitrogen, but they are able to use many other forms of nitrogen namely, amino acids, ammonium, and nitrate. Several fungi can convert nitrate as sole source of nitrogen to ammonium by the enzyme nitrite reductase and nitrate reductase (29).

Currently, scientists focus on biosynthesis of nanoparticles using bacteria (16), fungus (8), and plants (17). These biogenic processes are of low cost, safe and high yield, and environment-friendly in comparison with other synthetic procedures (3). Traditionally chemical and physical methods have been utilized for the synthesis of nanoparticles (13, 14). Basically, the chemical methods have harmful effects and the physical methods have low yields on the environment due to use of toxic solvents and the generation of hazardous by-products (15). Previous studies proposed the probable role of the reduced form of nicotinamide adenine dinucleotide (NADH) and NADH-dependent nitrate reductase in the reduction of silver ion to metallic silver (18, 30). The precise reaction mechanism leading to the biosynthesis of sliver nanoparticles is yet to be clarified. In the present study, we have investigated the extracellular biosynthesis of sliver nanoparticles using *Aspergillus niger*. In order to determine the probable role of nitrate reductase in the formation of sliver nanoparticles, we have analysed the relationship between the quality and quantity of biosynthesized sliver nanoparticles by the studied *Aspergillus niger* species and their nitrate reductase activity.

Materials and Methods

Isolation of fungus

Aspergillus niger was isolated from the rotten onion. The rotten onion was homogenized and added to the distilled water to make suspension of it. The suspension was diluted, spread on SD agar and incubated for 4-5 days at 28˚c. After 5 days loopful of colonies were spread on another SD agar plate and again incubated for 4-5 days at room temperature. After incubation colony was checked in microscope by using 0.1% congo red dye for confirmation of *Aspergillus niger.*

Biomass preparation

Aspergillus niger colony was transferred to SD broth by making suspension and kept in shaker for 4-5 days at 120 rpm. After 5 days, the biomass was filtered using Whatman filter paper and remaining media removed by washing this biomass with distilled water. Then, kept it in the shaker with distilled water for 1-2 days at 120 rpm.

Biosynthesis of AgNPs

The biomass was filtered and kept with silver nitrate solution (1mM and 10mM) ratio was

1:1. All flasks were then kept in dark for 1-2 days. All solutions were kept in dark to avoid any photochemical reactions during the experiment. Then, changes in the color were observed from colorless to brown color.

Characterization of AgNPs

The preliminary characterization of AgNPs was done by observing the color change from colorless to brown. The bio reduction of Ag^+ in aqueous solution was monitored using an ultraviolet-visible spectrophotometer from 300-600nm, at a resolution of 20nm. The peak of the result was observed to confirmed the bio reduction of Ag^+.

Purification of AgNPs

The sample was centrifuged at 10,000 rpm for 15 min and pellets were collected. The pellets were dried in the hot air oven then, weighed and stored for further use.

Antimicrobial activity analysis

Both antibacterial and antifungal activity analyzed for the antimicrobial activity of biosynthesized AgNPs. Both activities were performed by disc diffusion method. *Pseudomonas aeruginosa*, *Enterococcus* and *Staphylococcus aureus* strain of bacteria were investigated. Bacterial colonies were spread on the Muller Hilton agar plate and with the help of the cup borer a well was created and AgNPs solution poured into this well . It allowed to diffused overnight in the incubator at 37℃. The zone of inhibition was checked in the next day and the results were recorded. For the antifungal activity *Fusarium* and *Alternaria* spp. of fungus were investigated. Antifungal activity also performed by disc diffusion method as same as for the antibacterial activity. The zone of inhibition and results were also recorded.

Results and Discussion

Fungus isolation

The colonies of *Aspergillus niger* on SDA agar plate were grown within 5 days at the room temperature. First it looks white in color but later it completely turned into black color.

The first picture gives detail about 3rd day appearance of colonies and the second picture shows that it completely turned into black color.

Then, the microscopic analysis of these colonies had been done for the conformation of *Aspergillus niger* spp. The dye used to visualize the fungus is 0.1% congo red. It effectively showed the morphological structure of the fungus with conidia and conidiophores. Thus, 0.1% congo red dye can be used to stain the fungal smear and study it under the microscope

Biomass preparation

For the biomass production this colonies are transfer to SD broth and put it in shaker for 4-5 days at 120 rpm. It gives visible growth of biomass of the fungus *Aspergillus niger*.

Biosynthesis of silver nanoparticles (AgNPs)

Aspergillus niger isolate was successfully incorporated in biosynthesis of silver nanoparticles by reducing silver nitrate. The biomass was filtered out from the broth and kept in silver nitrate solution in dark conditions. This biomass then reacts with silver nitrate solution and after 1-2 days it gives a visible color change which is colorless to yellowish brown. This color change indicates that the silver nanoparticle has been formed.

Table.1 Optical density of sample solution at different wavelength

Nanometer	O.D.
300	0.123
320	0.124
340	0.128
360	0.163
380	0.236
400	0.270
420	0.277
440	0.275
460	0.266
480	0.242
500	0.217
520	0.200
540	0.164
560	0.159
580	0.147
600	0.135

Table.2 Size of zone of inhibition for Bacterial species

Tested organisms	Size of inhibition (mm) Control	Size of inhibition (mm) Test
Pseudomonas aeruginosa	23	11
Enterococcus	22	13
Staphylococcus aureus	37	5

Table.3 Size of zone of inhibition for fungal species

Tested organism	Size of inhibition zone (mm) Control	Size of inhibition zone (mm) Test
Fusarium	40	11
Alternaria	45	1

Image.1 Growth of the colony: *A. niger* colony 3rd day (left side) and 5th day (right side).

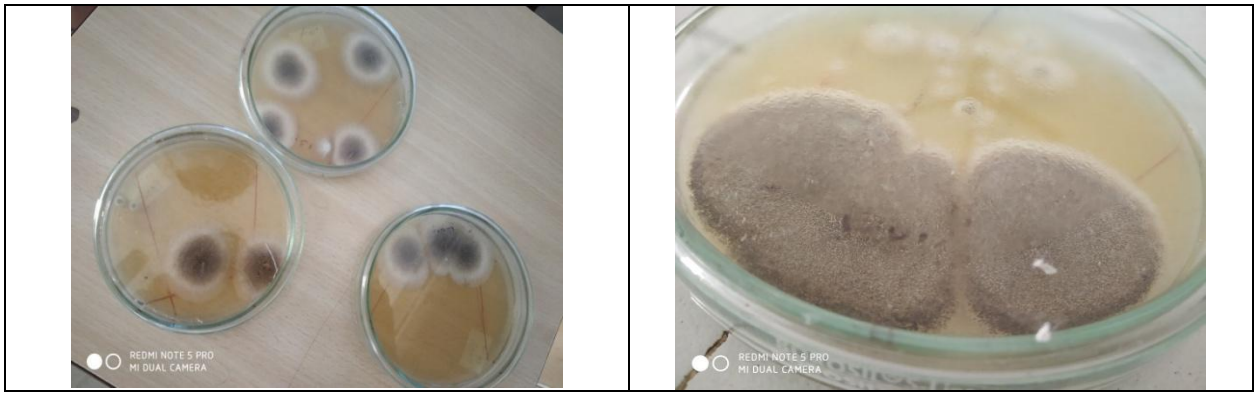


Image.2 Microscopic views of *A. niger*colony.

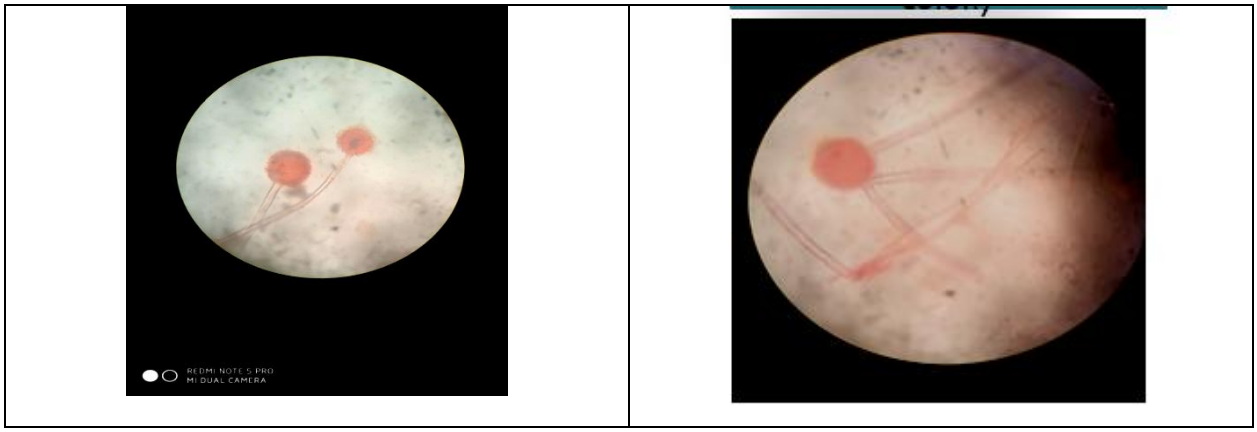


Image.3 Fungal biomass.

Image.4 Figure 8 Color changes in flask: fungal filtrate with 1mM silver nitrate (leftside) control (middle) and fungal filtrate with 10mM silver nitrate.

Image.5 Purified silver nanoparticles.

Image.6 Zone of inhibition against *Staphylococcus aureus*: Test (left side) and control (right side)

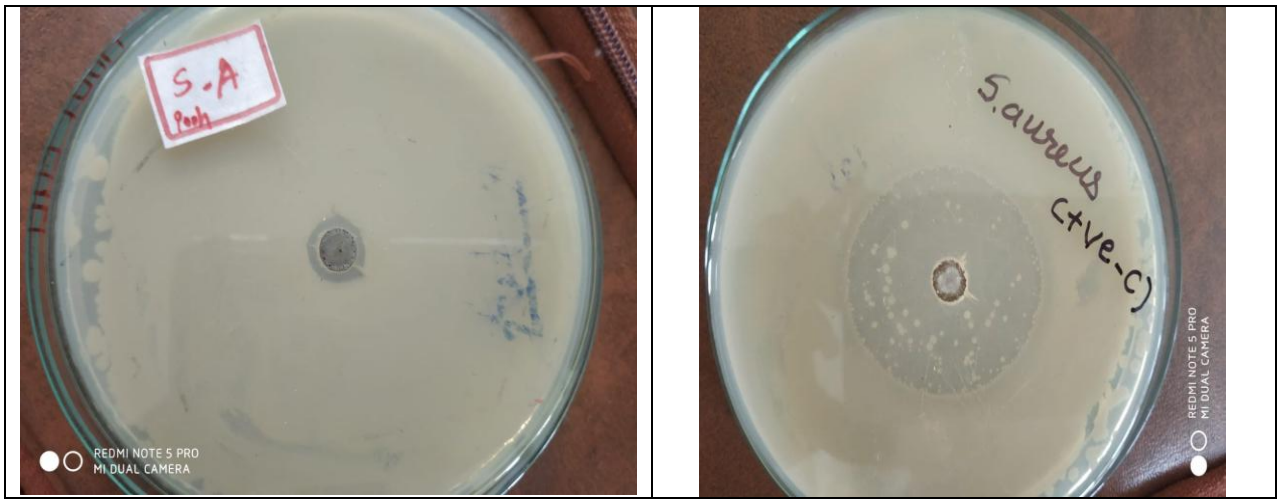


Image.7 Zone of inhibition against *Pseudomonas aeruginosa*: Test (left side) and control (right side)

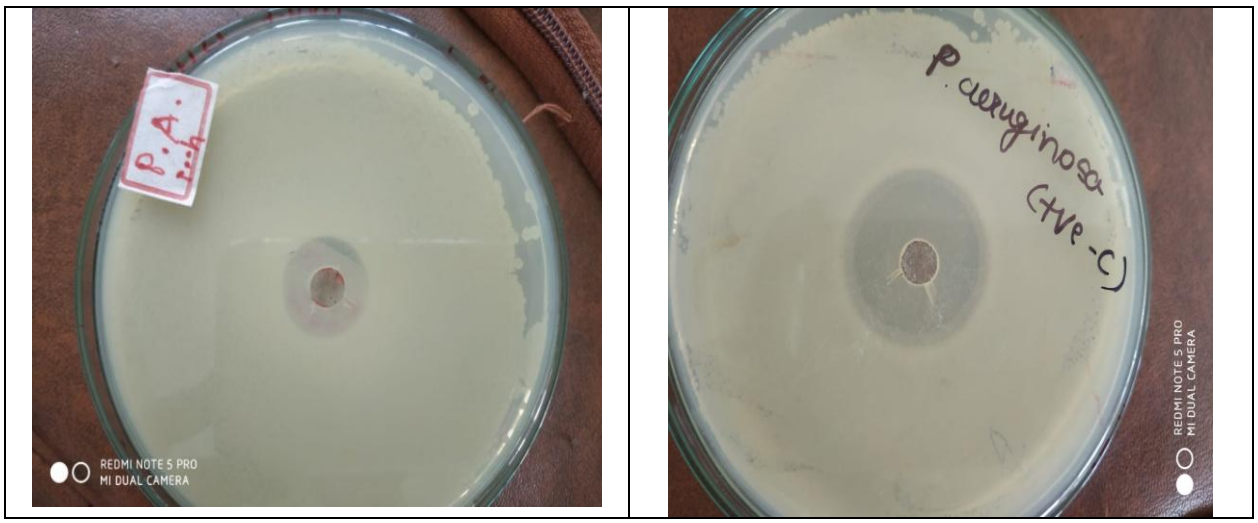


Image.8 Zone of inhibition against *Enterococcus*: Test (left side) and control (right side)

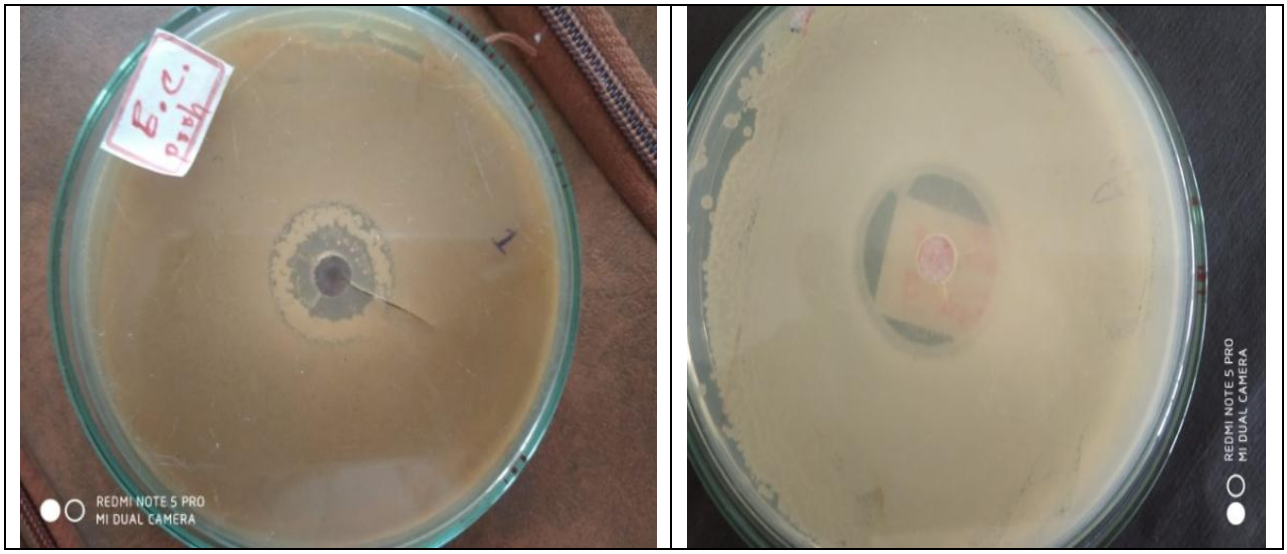


Image.9 Zone of inhibition (Test) against *Fusarium* (left side) and *Alternaria* (right side)

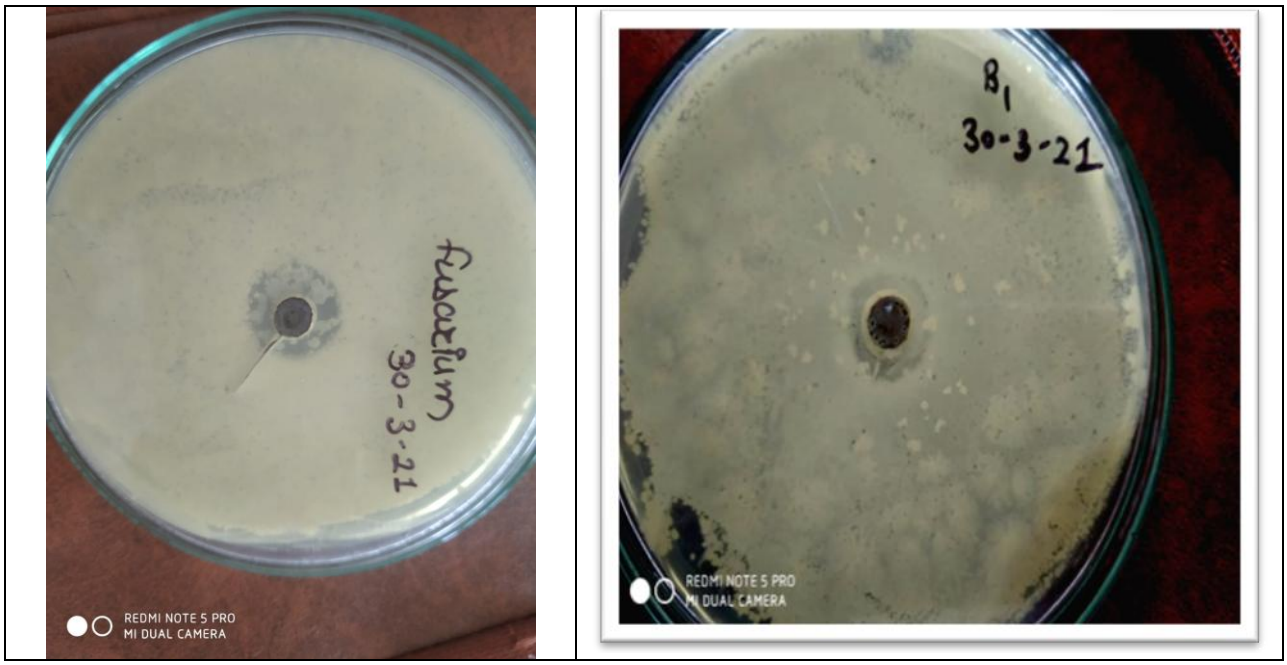
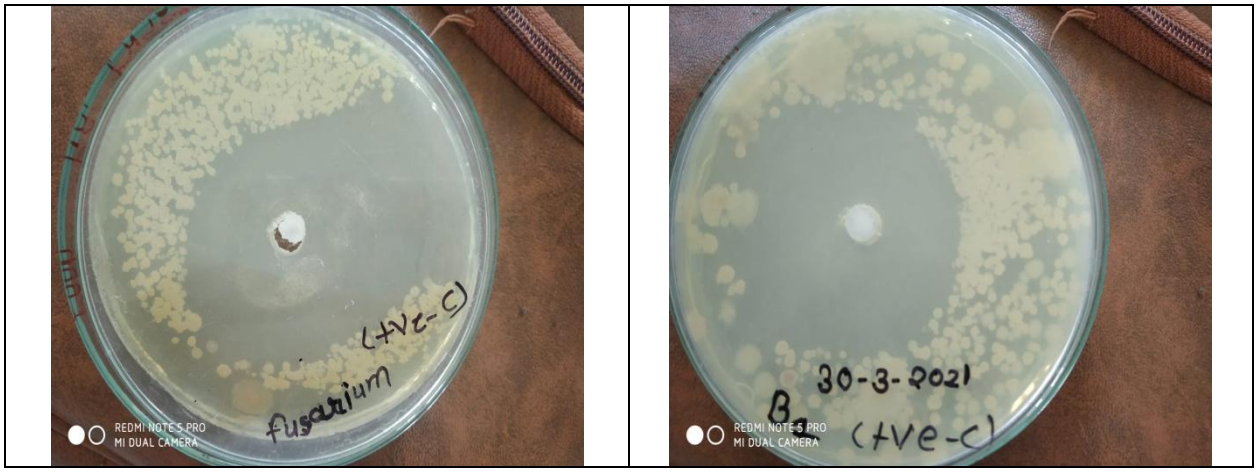


Image.10 Zone of inhibition (control): *Fusarium* (left side) and *Alternaria* (right side)



This color change is mainly due to the Plasmon resonance surface of deposited silver nanoparticles, i.e., the color of the nanoparticles was due to coherent and collective surface electrons oscillations. It has been reported that AgNPs exhibit a dark brown color in aqueous solution as a result of surface Plasmon resonance. (Link S and Ei-sayed, 2003) (31). When the concentration of silver nitrate solution is 1mM it reacts with fungus and gives dark brown color but when this solution was centrifuged, it did not give pellets of silver nanoparticles. Besides, the pellets were formed when 10 mM $AgNO_3$ solution is used.

Characterization and purification of AgNPs

For the characterization of silver nanoparticles spectroscopic analysis has been done. UV-Visible spectroscopy was used to inspect the color change in the reaction mixture and the indicator of the formation of AgNPs. UV-Vis spectrophotometer was used for scanning the sample at 300-600 nm absorbance spectrum. Fungal cell filtrate treated with silver nitrate solution is known to show peak around 420nm with high absorbance as demonstrated by Ingle *et al.*, 2008 (31). This research also supports this and gives peaks at 420nm that indicates the biosynthesis of nanoparticles by *Aspergillus niger*. After centrifugation process, the AgNPs pellets have been formed in the centrifuge tube which is then discarded and kept in oven for drying. After this whole process, only pure silver nanoparticles were left.

The antimicrobial activity

Antibacterial activity

The antibacterial activities against *Pseudomonas aeruginosa*, *Enterococcus* and *Staphylococcus aureus* were investigated. The antibacterial activity checked against silver nanoparticles and the results shows zone of inhibition of the colonies. Thus, it describes that this AgNPs can be used for effective antibacterial activity for further conditions and it could be considered as excellent broad-spectrum antibacterial agents. The control taken for this analysis was cefuroxime. Since, the biosynthesized AgNPs showed considerable antibacterial activity, they could be potentially widely used in clinical applications.

Antifungal activity

For antifungal activity, *Fusarium* and *Alternata* spp. of fungus were investigated.

The well diffusion method was used to assess the antifungal potential of biosynthesized AgNPs. After 5 days of incubation at room temperature, the zone of inhibition appeared. The control taken for this analysis was fluconazole. The size of the zone is described in the table. This positive result indicates antifungal properties of silver nanoparticles. AgNPs were assumed to have a significant antifungal activity, requiring further investigation for clinical applications.

Biosynthesis of silver nanoparticles can easily be achieved in vitro using *Aspergillus* fungus as the biological system. It provides a simple and ecofriendly way to produce colloidal nanoparticles of silver. *Aspergillus niger* can easily be exploited and maintained under controlled condition and utilizing its biomass and biomass extract for the production of silver nanoparticles. *Aspergillus niger* fungus was found to be effective in the reduction of silver ions into silver nanoparticles. Furthermore, the fungi-mediated AgNPs were utilized as efficient biocontrol agent for suppressing various bacterial and fungal species. The zone of inhibition suggests the effectiveness of biosynthesized silver nanoparticles against bacterial and fungal colonies. In conclusion, the fungal based synthesis of metal nanoparticles is efficient and environment-friendly way to prepare biocontrol agents for bacterial and fungal species.

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