

ENDOPHYTIC FUNGAL ASSISTED SYNTHESIS OF SILVER NANOPARTICLES, CHARACTERIZATION, AND ANTIMICROBIAL ACTIVITY

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Received: 03 January 2015, Revised and Accepted: 31 January 2015

ABSTRACT

Objective: The objective was to investigate the biosynthesis of silver nanoparticles (SNPs) using extracellular fungal filtrate of *Aspergillus niger* and check their antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*.

Methods: 10 ml of extracellular fungal filtrate of *A. niger* was added to 50 ml of 1 mM silver nitrate and incubated at room temperature for 24 hrs. SNPs were characterized using ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), atomic force microscopy (AFM), and X-ray diffraction analysis (XRD). Antimicrobial activity was checked against *S. aureus* and *E. coli* by employing disc diffusion method.

Results: The color change of the solution from light yellow to dark brown indicated the formation of SNPs. The formation of SNPs was further confirmed by UV-Vis spectroscopy, which showed the characteristic peak between 400 and 460 nm. TEM and AFM analysis showed that the size of SNPs were between 10 and 50 nm with roughly spherical in shape. XRD analysis confirmed the crystalline nature of SNPs synthesized by showing the Bragg's peaks which could be indexed to (111) and (220) of face cubic crystal phase of silver. FTIR showed the peaks at 1026, 1215, 1348, 1632, and 2928/cm, which were responsible for the different functional groups possibly involved in the synthesis and stabilization of SNPs. The SNPs formed the inhibition zones of 14.0 and 12.5 mm against *S. aureus* and *E. coli*, respectively.

Conclusion: It is concluded that the biosynthesis of SNPs using extracellular fungal filtrate of *A. niger* was simple, eco-friendly, and robust. The SNPs synthesized were well-dispersed, crystalline in nature and also proved to be excellent antimicrobial agents.

Keywords: Silver nanoparticles, *Aspergillus niger*, Transmission electron microscopy, X-ray diffraction, Antimicrobial activity.

INTRODUCTION

Nanobiotechnology is the most promising area in the emerging trends as it involves simple, cost effective, and an eco-friendly synthesis of nanoparticles and their wide variety of applications in the pharmaceutical and biomedical field [1]. Owing to their small size (<100 nm), high surface area to volume ratio, reduced imperfections and spatial confinement, metal nanoparticles particularly silver nanoparticles (SNPs) possess characteristic physicochemical properties, which includes optical, electronic, thermal, magnetic, catalytic, and biological properties [2-4]. Due to these properties, SNPs find many applications in different fields including optical receptors [4,5], sensors [6], catalysts in chemical reactions [7], sporting equipment [8], anticancer [9], and antimicrobial agents [10-13].

Various physical and chemical methods which including radiation assisted [14], thermal decomposition [15], laser ablation [16], sonochemical [17], photochemical [18], and polyaniline synthesis [19] have been reported to synthesize SNPs. However, all of these methods involve the use of toxic materials possess health and environmental risks. Biological approaches including plant extract mediated approaches [10-12], microbial methods [13,20] have been reported. Biosynthesis of SNPs using these extracts does not involve the use of any toxic material and this approach is cost effective and very simple process. However, fungal mediated synthesis of SNPs has advantage over plant and bacterial approaches due to the secretion of extracellular proteins present in the fungal filtrate involve in capping and stabilization of SNPs synthesized [29,30]. In this study, we report the synthesis of SNPs from an endophytic fungi *Aspergillus niger* isolated from the leaves of *Centella asiatica* L. an important medicinal plant belongs to Umbelliferae family. The synthesized SNPs were characterized by

ultraviolet-visible (UV-Vis) spectroscopy, Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), atomic force microscopy (AFM), and X-ray diffraction (XRD). Antimicrobial activity of the synthesized SNPs was checked against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) by employing disc diffusion method.

METHODS

Collection and sterilization of plant material

Leaves of the *C. asiatica* L. were collected from medicinal plants garden and authenticated with taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. The voucher specimen was deposited (VM-0912) in the herbarium. Leaves were washed under running tap water and then with sterilized double distilled water (SDDW). The leaves then surface sterilized using 70% alcohol for 60 seconds, and finally rinsed with SDDW for 3-5 minutes.

Isolation of endophytic fungi

Potato dextrose agar (PDA) medium was used to isolate the endophytic fungi. The leaf explants were cut into 0.6 cm × 0.8 cm fragments and inoculated on PDA plates. The plates were incubated at 28°C for 5-7 days for the growth of endophytic fungi. The pure culture was identified as *A. niger* by cotton blue staining based on colony morphology, growth, and hyphae. Pure cultures of *A. niger* were transferred to fresh potato dextrose broth (PDB). The PDB flasks were incubated at 28°C under continuous shaking for 5-7 days, and the extracellular filtrate was collected after the incubation. The extracellular filtrate was used for the synthesis of SNPs.

Biosynthesis of SNPs

Silver nitrate (AgNO_3) was purchased from Molychem, India. 10 ml of fungal filtrate was added to 50 ml of 1 mM AgNO_3 and incubated at room temperature for about 24 hrs. After incubation, the color change of the solution was observed from light yellow to dark brown color for the detection of synthesis of SNPs.

Characterization of SNPs

UV-Vis spectrum was recorded between 200 and 800 nm for the confirmation of the synthesis using UV-Vis spectroscopy, Analytical Technologies Ltd, India. The possible mechanism of the synthesis and stabilization of SNPs was checked by FTIR analysis using FTIR, Alpha interferometer, Bruker, Switzerland. TEM analysis was performed to know the size and shape of the SNPs synthesized. AFM analysis was carried out to reveal the surface topology using NT-MDT, Solver Next, Russia. XRD characterization was carried out to reveal the crystalline nature of the SNPs.

Antimicrobial activity of SNPs

The synthesized SNPs were evaluated for their antimicrobial activity against *S. aureus* and *E. coli*. Antimicrobial activity was carried out on nutrient agar (NA) media by employing disc diffusion method [10,11]. 200 μl of bacterial inoculum was swabbed on NA plates. Each NA plate comprises of three paper discs. One treated disc impregnated with 20 μl of SNP solution. Second disc impregnated with 20 μl of streptomycin, a standard antibiotic. The plates were incubated for about 24 hrs at 38°C to observe the inhibition of the bacterial growth. Minimum inhibitory concentrations (MICs) of AgNPs were determined by agar micro dilution method with slight modifications. Overnight culture of each test organisms (approximately 10^6 CFU) was inoculated into the wells and the AgNPs was tested at a concentration from 25 to 5 μl . The plates were incubated for 24 hrs at 38°C. MIC was determined as the least concentration of the AgNPs that inhibited the growth of the test organisms.

RESULTS AND DISCUSSION

In the present study, we have carried out the biosynthesis of SNPs using fungal filtrate of *A. niger*, an endophytic fungi isolated from *C. asiatica* L., an important medicinal plant, which harbors many endophytic fungi. Initially, the formation of SNPs was detected by the color change of the solution from light yellow to dark brown. The color change indicated the reduction of silver ions (Ag^+) into SNPs (Ag^0) [1]. The color change is due to surface plasmon resonance (SPR) excited by electromagnetic waves in the visible region. Metal nanoparticles with fascinating colors related with surface plasmon effect and strong optical absorbance offer a wide variety of applications.

UV-Vis analysis

The synthesis of SNPs was further confirmed by the UV-Vis spectrum which showed the peak between 400 and 460 nm (Fig. 1). The UV-Vis peak is the characteristic of SNPs due to SPR exhibited by SNPs [13]. The SPR peak and different compounds secreted in the fungal filtrate could be responsible for biosynthesis and capping of SNPs.

FTIR analysis

FTIR spectrum showed the peaks at 1026, 1215, 1348, 1632, and 2928/cm (Fig. 2). The peak at 1026/cm could be indexed to O-H group of the phenols and clearly indicated the involvement of polyphenols or flavonoids in the bioreduction of silver ions into SNPs [10]. The peak at 1215/cm responsible for correspond to C-O stretching from carboxylic acid of the proteins [21]. The peak at 1348/cm responsible for C-N absorption band of the proteins. The peak at 1632/cm responsible for the amide groups of the proteins [22]. The peak at 2928/cm could be indexed to the methylene group of the proteins. The peaks 1215, 1348, 1632, and 2928/cm clearly revealed that the proteins present in the fungal filtrate played a main role both in the bioreduction and stabilization of SNPs.

TEM analysis

TEM analysis was carried out to reveal the morphology and size of the SNPs synthesized. TEM micrograph indicated that the SNPs synthesized

were roughly spherical in their morphology and with size ranging between 10 and 50 nm. In the solution, SNPs were aggregated and stable (Fig. 3). TEM results are consistent with many reports for the fungal mediated synthesis of SNPs [13].

AFM analysis

AFM analysis was carried out to reveal the surface topology of the synthesized SNPs. AFM analysis revealed that the synthesized SNPs

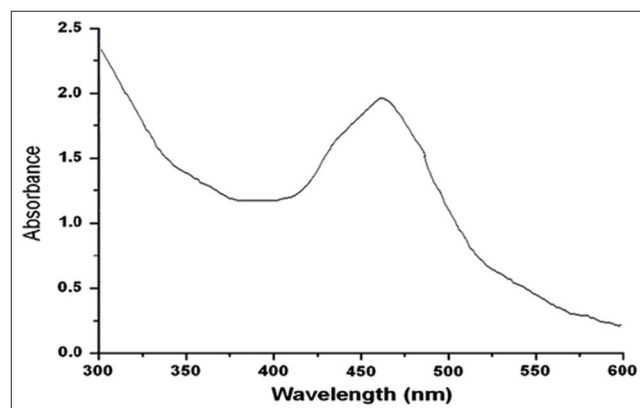


Fig. 1: Ultraviolet-visible spectrum of the silver nanoparticles synthesized using culture filtrate of *Aspergillus niger*

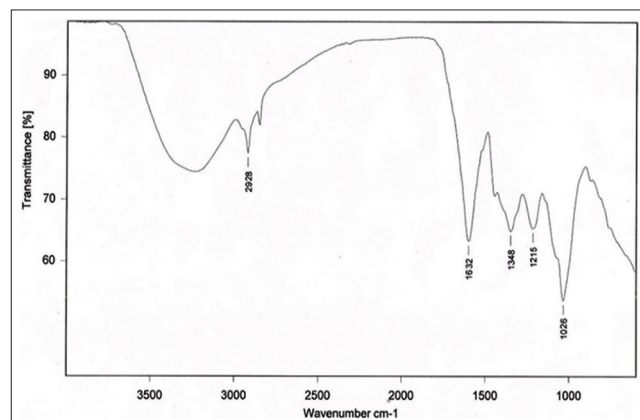


Fig. 2: Fourier transforms infrared spectroscopy spectrum of the silver nanoparticles synthesized using culture filtrate of *Aspergillus niger*

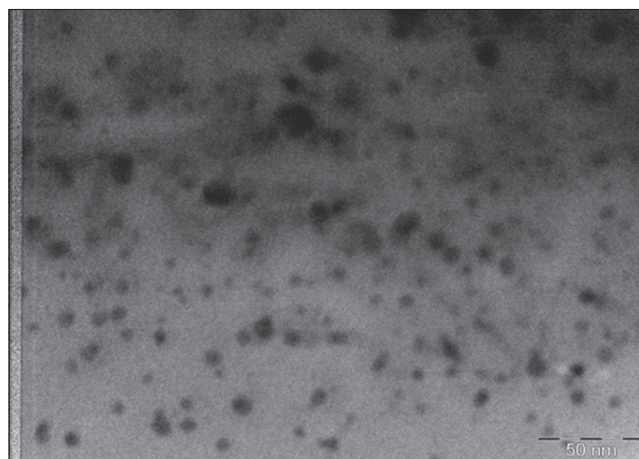


Fig. 3: Transmission electron microscopy micrograph of synthesized silver nanoparticles. Scale bar represents 50 nm

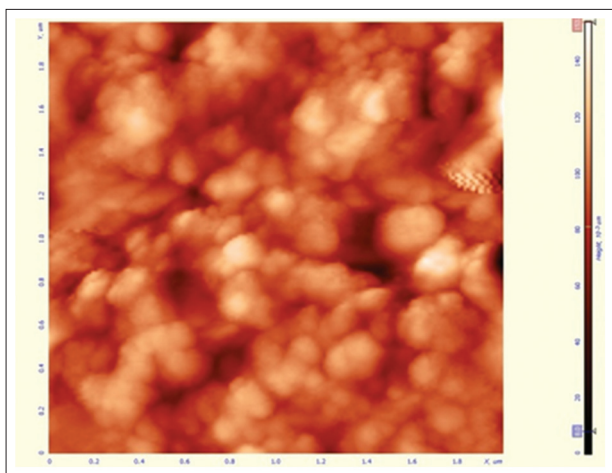


Fig. 4: Atomic force microscopy micrograph showing spherical shaped silver nanoparticles aggregated in the form of grains

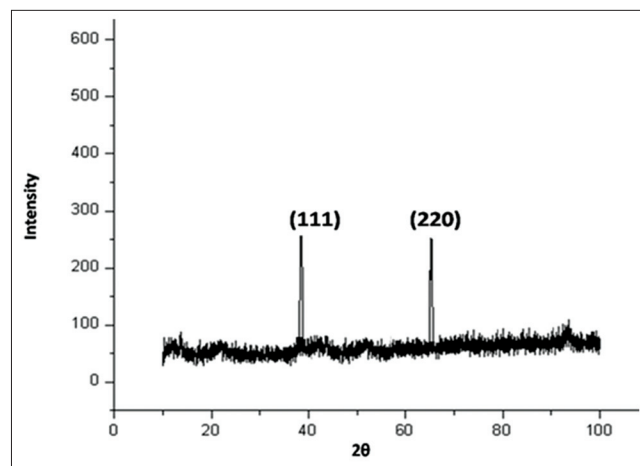


Fig. 6: X-ray diffraction pattern obtained for silver nanoparticles synthesized using culture filtrate of *Aspergillus niger*

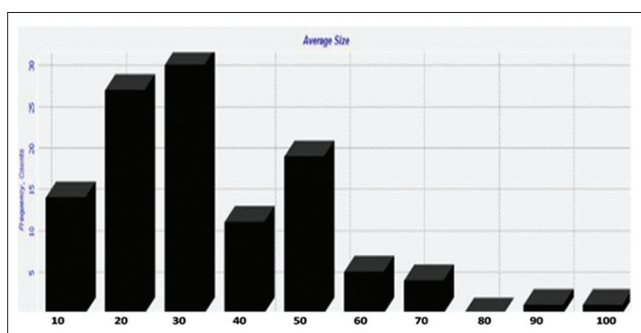


Fig. 5: Atomic force microscopy grain analysis showing silver nanoparticles with 10-50 nm in size

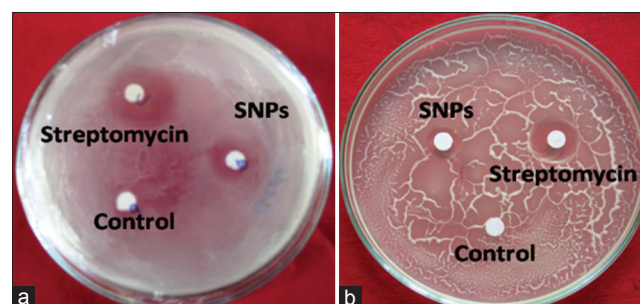


Fig. 7: Antimicrobial activity of silver nanoparticles against *Staphylococcus aureus* (a) and *Escherichia coli* (b)

Table 1: Antimicrobial activity of SNPs synthesized (mean values±SD of three replicates)

S. no.	Tested organism	Diameter of inhibition zone (mm)	
		Streptomycin	SNPs
1	<i>Staphylococcus aureus</i>	18.0±0.4	14.0±0.2
2	<i>Escherichia coli</i>	16.5±0.3	12.5±0.1

SNPs: Silver nanoparticles, SD: Standard deviation

were spherical in shape and aggregated in the form of grains (Fig. 4). AFM grain analysis revealed that most of the synthesized SNPs were between 10 and 50 nm in size represented in the graph by taking average size on X-axis and frequency counts on the Y-axis (Fig. 5).

XRD analysis

XRD analysis was carried out to reveal the crystalline nature of SNPs. XRD pattern (Fig. 6) showed the peaks at 38.36° and 64.66° responsible for (111) and (220) planes of face cubic crystal of silver [JCPDS 89-3722]. XRD results confirmed that synthesized SNPs were crystalline in nature and consisted with earlier reports for the fungal mediated synthesis of SNPs [20].

Antimicrobial activity

Antimicrobial activity of the SNPs was checked against *S. aureus* and *E. coli*. The SNPs formed the inhibition zones against *S. aureus* and *E. coli* of 14.0 mm and 12.5 mm, respectively (Fig. 7). The results of the SNPs compared with streptomycin against these microbes were

showed in the Table 1. The SNPs synthesized from fungal filtrate of *A. niger* showed very good antimicrobial activity against both Gram-positive and Gram-negative bacteria and thus proved their biomedical importance. Different concentrations of AgNPs extract ranging from 5 to 25 µl were assessed against the test microorganisms. The AgNPs were most effective against the Gram-positive bacteria *S. aureus* with a MIC of 5 µl, whereas MIC of 10 µl was reported against *E. coli*. The molecular mechanism behind the antimicrobial activity was elucidated by many scientists. SNPs initially bind the bacterial membrane and thus change the PMF of the membrane. As a result, SNPs enter inside the while the important ions and the biomolecules enter outside the cell. SNPs can interrupt the important processes like bacterial DNA replication and protein synthesis and thus bacterial division is stopped.

ACKNOWLEDGMENTS

The authors are thankful to NABTD, DAE-BRNS, BARC, Mumbai, MH, India for financial support. The authors are also thankful to CSIR-IIT, Hyderabad, AP, India for technical support.

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