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ORIGINAL RESEARCH ARTICLE

Extracellular Biosynthesis of Silver Nanoparticles by Endophytic Fungus *Aspergillus terreus* and its Anti-dermatophytic Activity

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ABSTRACT

The emerging of nanoparticles into the living system especially for medical purposes has opened up a new challenge of synthesizing them in a benign fashion. In the present study, 5 endophytic fungi were isolated from the mangrove leaves of *Rhizophora annamalayanna* and among that *A. terreus* was produced the AgNPs extracellularly and the presence of AgNPs was confirmed by XRD, SEM with EDX, TEM and FTIR. Crystalline nature of AgNPs observed in XRD. SEM with EDX and TEM confirmed the presence of AgNPs, reveals that are predominantly spherical in the size of 100nm. FT-IR analysis confirmed the presence of proteins over the AgNPs which acts as a capping agent involved in the reduction of Ag^{+} to Ag^{0}. *In vitro* antidermatophytic activity of synthesized AgNPs also analyzed against *Trichophyton rubrum, Epidermophyton floccosum and Trichophyton mentagrophytes.* The synthesis of AgNPs by the fungus *A. terreus* may therefore serve as a clean, inexpensive, eco-friendly, reliable and safe method to produce an antifungal compounds.

Key words: Silver nanoparticles; *Aspergillus terreus*; Antidermatophytic activity.

1. INTRODUCTION

Nanotechnology has now started leaving the confines of laboratories; and conquering new applications to change our lives. [1] The worldwide nano product market is estimated to reach $1 trillion by the year 2015.[2] As an important metal silver nanoparticle (AgNPs) have a number of applications, from electronics, [3] catalysis, [4] infection prevention, [5] medical diagnosis, [6] sensor technology,[7] biological labeling [8] and treatment of some cancers. [9] Among various metals, silver has been known since ancient times as effective antimicrobial agent for the treatment of diseases, for food preservation, to keep water safe, cosmetics, clothing and numerous consumer products. [10] AgNPs could be used as substrates for Surface Enhanced Raman Scattering (SERS) to probe single molecules [11] and also useful catalysts for the oxidation of methanol to formaldehyde. [12] In last decay, application of nano material has been extensively increased in high demand leads to the bulk production of the nanomaterial. Classically, the nanoparticles are produced by physical method needs more energy [13] and chemical methods are toxic [14] but biological methods are clean, safe and cost effective. [15] Biological methods of nanoparticles synthesis using microorganism [16,17,18] enzyme [19] and plant or plant extract [20] have been suggested as possible ecofriendly alternative to chemical and physical methods. It can also be suitable for large-scale synthesis of nanoparticles. There are several microorganisms from bacteria to fungi have been reported to synthesize inorganic materials either intra- or extracellularly, and thus to be potentially utilized as eco-friendly nanofactories. [21, 22, 23]

However, marine microbes remain relatively unexplored; the marine realm is an exceptional reservoir of micro biota. [24] Most of the researches are restricted to terrestrial microbes, but not with the marine microbes especially from mangrove habitats of extreme environmental conditions. In order to tolerate the extremities, microorganisms from the mangrove environment may produce novel chemicals of unique biological activities. [25] Mangrove is recognized as a highly productive ecosystem, which consists a diverse

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microbial community. [26] Over the past decades, mangrove endophytic fungi have attracted increasing attention among taxonomists, mycologists, ecologists, chemists, and evolutionary biologists. [27] While a number of reports are available on the biological synthesis of AgNPs, the potential of endophytic microorganisms, microbes that colonize in living internal tissues of plants without causing any immediate, overt negative effects [28] has not yet been tapped. Very few reports are available where in endophytic fungi were used for the synthesis of nanoparticles. One such study employed an endophytic fungus *Colletotrichum* sp. isolated from geranium leaves of *Pelargonium graveolens* for the extracellular synthesis of gold nanoparticles. [29] Another study revealed that *Aspergillus clavatus* (AzS-275), an endophytic fungus isolated from sterilized stem tissues of *Azadirachta indica* and reported the AgNPs antibacterial effect. [30] Recently one such report on endophytic *Aspergillus concius, Penicillium janthinellum* and *Phomosis* sp. isolated from *Avicennia marina, Suaeda monica and Rhizophora mucronata* mangrove plant leafs and used for extracellular biosynthesis of AgNPs. [31] Based on the endophytic bacterium, *Bacillus cereus* was isolated from the *Adhatoda beddomei* used to synthesize the AgNPs. [32] Despite these impressive results, the origin of fungi have the ability to synthesis AgNPs were still limited, and the detailed mechanism was not well elucidated. Therefore, it is a great significance to explore novel fungal strain for synthesizing AgNPs based on the biodiversity. Moreover, it could also facilitate the deeper understanding of molecular mechanism for AgNPs biosynthesis. However, there are restricted, investigations on the mangrove endophytic fungi for nanoparticle synthesis. So, the present study was concentrated on the synthesis and characterization of AgNPs from the endophytic *Aspergillus terreus* which was isolated from mangrove leaves of *Rhizophora annamalayanna.*

2. MATERIAL AND METHODS

2.1: Isolation and Identification of Endophytic Fungi:

Mangrove leaves of *Rhizophora annamalayana* were collected from Vellar estuary, South east coast of India. The leaves were transported to the laboratory in sterile polythene bags, washed thoroughly in running water and processed within 24 h. The leaf segments (about 0.5mm) were cut from the middle portion and surface sterilized by immersion in 75% ethanol for 30 sec, followed by immersion in 4% sodium chloride for 90 sec and then rinsed with sterile distilled water for 10 sec. [33, 34] The surface sterilized leaf segments were evenly spaced in petri dishes (9mm) containing Potato Dextrose Agar medium (amended with chloramphenicol 150 mg /1). The endophytes were distinguished from one another by their cultural characteristics and Lactophenol Cotton Blue mounting method. Further, the identification was confirmed by the Agharkar Research Institute (ARI), Pune.

2.2: Mangrove Endophytic Fungal Biomass Preparation:

After the identification, the *A. terreus* was selected for the production of AgNPs due to the rapid color change. *A. terreus* was inoculated in liquid media containing (g/l) KH_2PO_4, 7.0: K_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)2SO_4$, 1.0; yeast extract, 0.6; and glucose, 10.0. The flasks were incubated at 25°C for 3 days in a rotary orbital shaker at a speed of 150 rpm. The biomass was harvested after 72 h of growth by sieving through a plastic sieve. The biomass was washed with sterilized distilled water to remove any medium component. 20 g of biomass (fresh weight) was mixed with 200 ml of deionized water in a 500 ml Erlenmeyer flask and agitated in the same condition for 72 h at 27° C. After the incubation, the cell filtrate was obtained by passing it through Whatman filter paper No. 1. Filtrate was collected and used for further nanoparticles synthesis.

2.3: Biosynthesis of AgNPs:

For the synthesis of AgNPs, 50 ml of 1mM $AgNO_3$ solution was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 25°C in dark with pH 6. Control (only biomass) was also run along with the experimental flask.

2.4: Characterization of synthesized AgNPs:

AgNPs were collected for the determination of the formation of AgNPs by X'Pert Pro X-ray diffractometer operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation in a 0-90 degree 2θ value configuration. The SEM (JEOL, Model JFC-1600) analysis was performed in selected point locations on the nanoparticle sample ranging from approximately 1 cm to 5 microns in width were imaged in a scanning mode using conventional SEM techniques (magnification ranging from 20x to approximately 30,000xs, spatial resolution of 50 to 100 nm). EDX analysis was carried out by the same

instrument and employed to confirm the presence of silver in the particles. Samples for TEM (JEOL model 1200EX) analysis were prepared on carbon-coated copper grids operated at an accelerating voltage of 120 kV. Dry powder of nanoparticles was made by under hot air over then it was subjected to FTIR spectroscopy analysis carried out on a Perkin-Elmer Spectrum instrument in the diffuse reflectance mode at a resolution of 4 cm^{-1}in KBr pellets. All the characterization study was conducted by sophisticated analytical instrument facility, Cochin.

2.5: Evaluation of antidermatophytic activity:

The antidermatophytic activity of AgNPs was tested against the dermatophytes such as *T. rubrum, E. floccosum and T. mentagrophytes* by well diffusion method. [35] The PDA plates were inoculated by swabbing the test dermatophytic suspensions to create a confluent lawn of dermatophytic growth and four wells were made on the swabbed PDA plates. The synthesized AgNPs (5µl, 10µl, and 15µl) were loaded on each well and wells with commercial antifungal agent of Griseofulvin (10 µl) were maintained as positive control. After 48–72 h of incubation at 35°C room temperature, the susceptibility of the test organisms was determined by measuring the diameter of the zone of inhibition around each well.

3. RESULTS AND DISCUSSION

The silver nanoparticles are used widely in technology, medicine and consumer products, but there are limited data from the mangrove sources. Among the nanoparticles, silver nanoparticles have been widely used in bio-labeling, antimicrobial agents, catalysts, [36] and sensors. [37] Due to its unique optical, [38] electrical, [39] and magnetic properties, they mainly find their application in optics, electronics and catalysis.

In the present study, 5 different fungal colonies were isolated from the mangrove plant leaf sample and identified as *Aspergillus flavus, Aspergillus* sp., *Aspergillus terreus, Penicillium* sp., *Fusarium moniliforme* at ARI, Pune. Among the identified isolates, only one fungal culture namely *A. terreus* was selected for the biosynthesis of AgNPs due to the rapid reaction. After the addition of the 1mM silver ion solution the color of yellow was changed to brown. Which indicates the formation of colloidal silver particles and the color change was due to the excitation of surface plasma vibrations, typical of the AgNPs. Control did not showed any change in the color of the mixture. So, this was selected for the production of AgNPs and characterization studies. A report showed that, by using the marine fungal strain of *Penicillium fellutanum* isolated from rizosphric soil, it was possible to obtain silver nanoparticles at a faster rate by extra-cellular means. Silver nanoparticles were synthesized within 10 min of silver ions being exposed to the culture filtrate and proved that the increase in colour intensity of culture filtrate was due to increasing number of nanoparticles formed by reduction of silver ions. The particles obtained had a good monodispersity. [40]

3.1: Characterization studies of synthesized AgNPs:

Further confirmation of synthesized AgNPs was examined by the XRD pattern of diffraction peaks showed at 2θ values of 32.32°, 45.99°, 66.72° and 75.76° assigned to the planes of (111), (200), (220) and (311) faced centre cubic (fcc) of silver were obtained ranging from 10 to 80 (**Fig 1**). The values agree well with those reported for silver (face centric cubic) by Joint Committee on Powder Diffraction Standards File No. 04-0783. The SEM examination showed AgNPs on the surface of the fungus with the aggregation with granular, spherical structures (**Fig 2**). In EDX peak of Ag at 2 to 3 KeV confirms the formations of 'silver' nanoparticles (**Fig 3**). TEM image showed the synthesized AgNPs are variable shapes, most of them present in spherical in nature well dispersed in water with size range of 100 nm and be stable for at least three month (**Fig 4**).

The FT-IR spectrum recorded from the freeze-dried powder of AgNPs, formed after 72 hrs of incubation with the fungus filtrate. The amide linkages between amino acid residues in proteins give rise to the well-known signatures in the infrared region of the electromagnetic spectrum. The bands seen at 3454 cm^{-1} and 2884 cm^{-1} were assigned to the stretching vibrations of primary and secondary amines, respectively; while their corresponding bending vibrations were seen at 1624 and 1385 cm^{-1}, respectively. The two bands observed at 1108 and 957 cm^{-1} can be assigned to the C-N stretching vibrations of aromatic and aliphatic amines (**Fig 5**). Overall observation confirms the presence of protein in the samples of AgNPs which act as a stabilizing agent.

3.2: Antidermatophytic activity of AgNPs:

It is well known that Ag based compounds have strong antimicrobial effects and they are chemical

antimicrobial agents. [41] The most important problem caused by the chemical antimicrobial agents is multi drug resistance. Therefore, an alternative way to overcome the drug resistance of various microorganisms is needed in medical devices desperately. Ag ions and Ag salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms. [42]

In the present study, efficacy of synthesized nanoparticles was tested against *T. rubrum, E. floccosum* and *T. mentagrophytes* by well diffusion method at different concentration levels. The maximum activity was observed in *E. floccosum* followed by *T. rubrum, T. mentagrophytes* (**Fig 6**). Among the concentration tested, 15µl of bionanoparticles showed maximum activity against all dermatophytes (**Table 1**). Keuk-Jun *et al* (2009) have reported nano-Ag breaks down the membrane permeability barrier of *C.albicans*, it is possible that nano-Ag perturbs the membrane lipid bilayers, causing the leakage of ions and other materials as well as forming pores and dissipating the electrical potential of the membrane. [43]

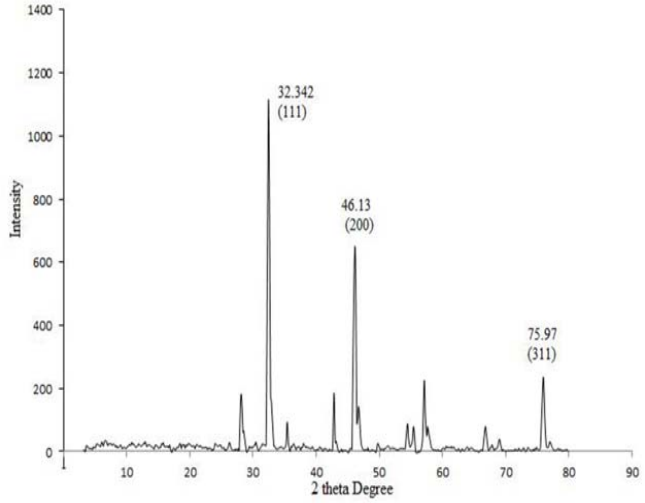

Figure 1: XRD pattern of AgNPs

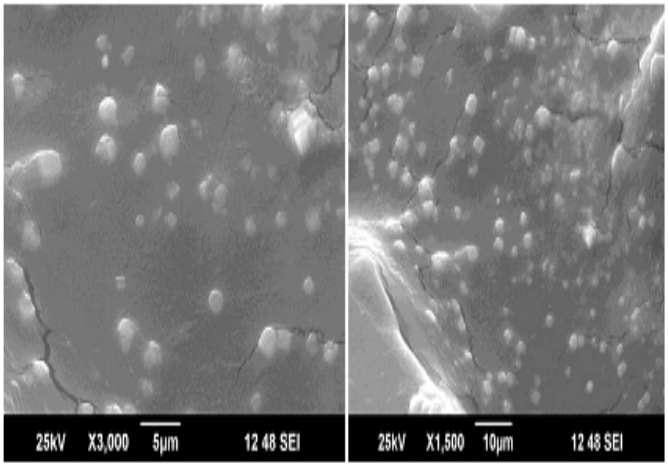

Figure 2: SEM view of silver nanoparticle

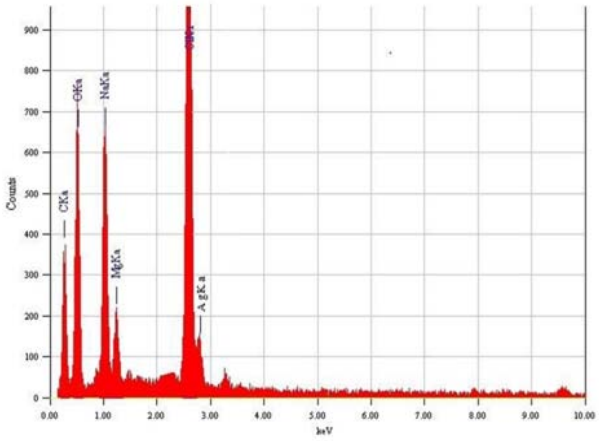

Figure 3: EDX spectrum showing the presence of elemental silver.

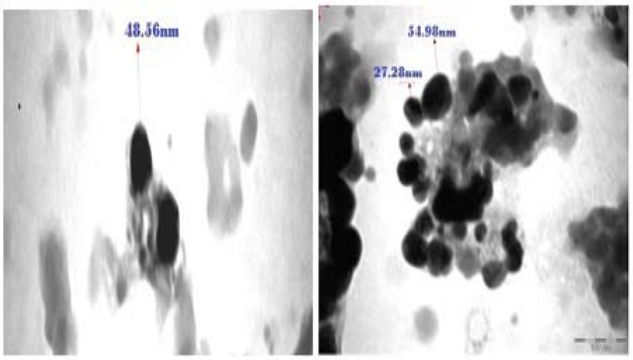

Figure 4: TEM bright field image of the AgNPs depicting spherical structures

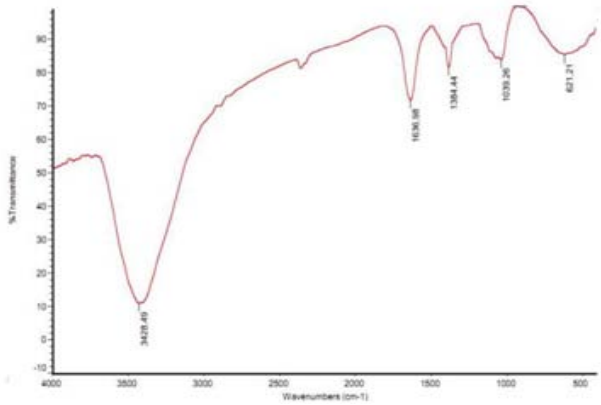

Figure 5: FTIR analyses of AgNPs

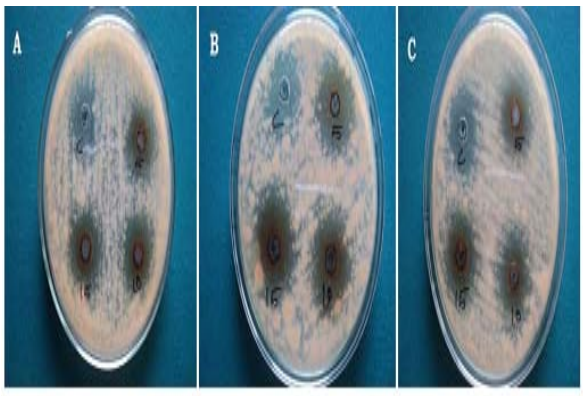

Figure 6: Antidermatophytic activities of synthesized AgNPs against (A) *T. rubrum* (B) *E. floccosum* (C) *T. mentagrophytes*

at different concentration, C- Griseofulvin (10 µl) positive control

Table 1: The antidermatophytic activity of silver naoparticles synthesized by *A. terreus*

Dermatophytic pathogens	Antibiotic Griseofulvin-positive control (10µl)	Different concentration of synthesized silver nanoparticles (µl)	Zone of inhibition (mm)
T. rubrum	6 ±0.04	5	10±0.03
		10	12±0.04
		15	13±0.03
E. floccosum	10±0.02	5	13±0.01
		10	14±0.03
		15	15±0.05
T. mentagrophytes	9±0.13	5	9±0.02
		10	10±0.02
		15	12±0.04

4. CONCLUSION

Moreover, the *A. terreus* as an alternative myco-biosystem for the synthesis of AgNPs, which are simple, cost effective and the synthesized nanoparticles are highly stable and reproducible. The nanoparticles also showed remarkable inhibition against dermatophytic fungi and also suggest their future use in potent broadband antidermatophytic agents/drugs.

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