



**SYNTHESIS OF NOVEL SILVER NANOPARTICLES (SNPS) USING FUNGAL
ENDOPHYTE *ALTERNARIA ALTERNATA* (MSU-351) AND EVALUATION OF THEIR
ANTIMICROBIAL ACTIVITIES.**

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Article Received on 16/10/2016

Article Revised on 08/11/2016

Article Accepted on 30/11/2016

ABSTRACT

There is an increasing commercial demand for nanoparticles due to their wide applicability in various areas such as electronics, catalysis, chemistry, energy, and medicine. Metallic nanoparticles are traditionally synthesized by wet chemical techniques, where the chemicals used are quite often toxic and flammable. In this work we have investigated extra cellular biosynthesis of novel silver nanoparticles using fungal extract of recently isolated novel endophytic fungus *Alternaria alternata* (MSU-351). The synthesis process was quite fast and silver nanoparticles were formed within minutes of silver ion coming in contact with the cell filtrate. UV–visible spectrum of the aqueous medium containing silver ion showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles. Transmission electron microscopy (TEM) micrograph showed formation of well-dispersed silver nanoparticles in the range of 10–30 nm. The process of reduction being extra cellular and fast may lead to the development of an easy bioprocess for synthesis of novel silver nanoparticles. Development of reliable and eco-friendly process for synthesis of metallic nanoparticles is an important step in the field of application of nanotechnology. Further these biologically synthesized nanoparticles were found to be highly toxic against different bacterial and fungal species. The most important outcome of this work will be the development of cost effective, nanoparticles based medicines from *Alternaria alternata* (MSU-351) for the treatment of microbial diseases. This is for the first time that *A. alternate* fungal extract was used for the synthesis of novel silver nanoparticles.

KEYWORDS: Novel silver nanoparticles (SNPs), Fungal Endophyte, Antimicrobial activity.

INTRODUCTION

Increased industrialization and urbanization has damaged the environment by introducing a number of harmful and unwanted substances. These metal–microbe interactions have important role in several biotechnological applications including the fields of bioremediation, bio mineralization, bioleaching and microbial corrosion. The field of nanotechnology is one of the most active areas of research in modern material sciences. Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. Nanotechnology is a field that is burgeoning day by day, making an impact in all spheres of human life. New applications of nanoparticles and nano materials are emerging rapidly. Nano crystalline silver particles have found tremendous applications in the field of high sensitivity bio molecular detection and diagnostics, antimicrobials and therapeutics, catalysis and micro-electronics. However, there is still need for economic, commercially viable as well environmentally

clean synthesis route to synthesize silver nanoparticles.^[1-2] Though there are several physical and chemical methods for synthesis of metallic nanoparticles, to achieve the objective of developing simple and eco-friendly technology researchers in this field have turned to biological systems.^[3-5]

Biological methods of synthesis have paved way for the “greener synthesis” of nanoparticles and these have proven to be better methods due to slower kinetics, they offer better manipulation and control over crystal growth and their stabilization.^[3-5] This has motivated an upsurge in research on the synthesis routes that allow better control of shape and size for various nano technological applications. Silver has long been recognized as having inhibitory effect on microbes present in medical and industrial process. The most important application of silver and silver nanoparticles is in medical industry such as topical ointments to prevent infection against burn and open wounds.^[3-5]

Holmes et al.^[2] have shown that the bacteria, *Klebsiella aerogenes* when exposed to cadmium ions resulted in intracellular formation of CdS particles in the range of 20–200 nm. Sastry et al.^[6] have reported that fungus *Verticillium* sp. and *Fusarium oxysporum*, when exposed to gold and silver ions, reduced the metal ion fairly rapidly and formed respective metallic nanoparticles. Considering all these factors, an extensive screening study was carried out involving several filamentous, fungal biomasses to identify a biological system for the extracellular biosynthesis of silver nanoparticles. Preliminary results indicated *Alternaria alternata* (MSU-351) as a potential candidate for this purpose. Hence, in the present work we have investigated extracellular biosynthesis of silver nanoparticles using the endophytic fungus, *A. alternata*. Further these biologically synthesized nanoparticles were found highly toxic against different pathogenic bacteria and fungi.

MATERIALS AND METHODS

Collection of plant samples

Karanja Plants samples (*Pongamia pinnata*L.) from unique environmental niches of selangor region, especially those with an unusual biology and possessing novel strategies for survival were selected for study.^[11-13] The samples were collected in sterilized polythene bags from the different locations of shah alam region of Selangor state, during the months of July-November.

Isolation of fungal endophytes

The leaves and nodes were used as explants for isolation of fungal endophytes.^[10-13] All explants were surface-sterilized by dipping in 75% ethanol for 1 minute, 4 % sodium hypochlorite for 5 minutes followed by rinsing three times in sterilized distill water. In each petri dish (9 cm diameter), a total of four-five processed explants were evenly spaced onto the surface of potato dextrose agar (PDA) media supplemented with 200 µg /ml tetracycline incubated at 28°C and daily observation was recorded (Fig.1a). The sporulating mycelia of fungi appeared on the plates were carefully isolated, sub-cultured and maintained the pure culture on PDA slants (Fig. 1 b & c).

Identification of fungal endophytes

The isolated endophytic fungi have been described and identified on the basis of morphological features like colony characterization, growth of fungi on different media, colour of colony (front and reserve), conidial development, size, shape, conidia, attachment of conidia and shape of conidial head. Then the fungus is grown in a slide culture, sporulation characteristics and the spores of the fungus remain undisturbed and attached to the sporophores thus facilitating in identification. This technique was performed for various stages of conidia formation and proper identification of the sporulating fungi.^[8, 11-13]

Synthesis of silver nanoparticles (SNPs)

The fungal endophyte *A. alternata* (MSU-351) isolated from the karanja plants were further investigated for nanoparticles synthesis and to obtain cell biomass the fungus was grown aerobically in a liquid media containing (g/l) KH₂PO₄, 7.0; K₂HPO₄, 2.0; MgSO₄·7H₂O, 0.1; (NH₄)₂SO₄, 1.0; yeast extract, 0.6; and glucose, 10.0. The flasks were inoculated, incubated on orbital shaker at 25°C and agitated at 150 rpm.^[6-7] The biomass was harvested after 72 h of growth by sieving through a plastic sieve, followed by extensive washing with distilled water to remove any medium component from the biomass. Typically 20 g of biomass (fresh weight) was brought in contact with 200 ml of Milli-Q deionized water for 72 h at 25°C in an Erlenmeyer flask and agitated in the same conditions described earlier. After the incubation, the cell filtrate was obtained by passing it through Whatman filter paper no. 1. For synthesis of silver nanoparticles, AgNO₃, 1mM final concentration was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 25°C in dark. Control (without the silver ion, only biomass) was also run along with the experimental flask. Sample of 1ml was withdrawn at different time intervals and the absorbance was measured at a resolution of 1 nm using UV-visible spectrophotometer. After 72 h of incubation the cell filtrates containing nanoparticles were used for transmission electron microscopy (TEM). The silver nanoparticles film was formed on carbon coated copper TEM grids and analysed by transmission electron microscopy.

Antimicrobial activity of SNPs

Antibacterial assays

The antibacterial assays were done on *Escherichia coli* and *Pseudomonas aeruginosa* by standard disc diffusion method. Briefly Luria Bertani (LB) agar medium was used to cultivate bacteria. Fresh overnight cultures of inoculum (100 µl) of each culture were spread on to LB agar plates. Sterile paper discs of 3mm diameter (containing 50mg/litre silver nanoparticles) along with four standard antibiotic containing discs were placed in each plate.^[6-8]

Antifungal Assays

The antifungal assays were done on *Aspergillus flavus* by food poisoning method. Potato dextrose agar (PDA) medium was used in the study. The medium of each Petri dish contains 30 ppm silver nanoparticles were inoculated each alone at the centre with 5mm Inoculum disc of each pathogenic fungus and incubated at 25 °C for 7 days. The medium with Inoculum disc of each fungus but without silver nanoparticles served as control.^[6-8]

RESULTS AND DISCUSSION

The detailed study on extracellular biosynthesis of silver nanoparticles by the *Alternaria alternata* biomass was carried out in this work. The fungal biomass after incubation for 72 h with Milli-Q water was separated by

filtration (Fig. 1d). The cell filtrate of the filamentous fungus incubated with aqueous silver nitrate solution and incubated in the dark in an environmental shaker which showed gradual change in colour of the medium to brown, with intensity increasing during the period of incubation. The change in colour of the medium was noted by visual observation. In comparison control showed no change in colour of the cell filtrate when incubated in the same environmental conditions. The light absorption pattern of the cell filtrate was monitored in the range of 200–800 nm using a UV–visible spectrophotometer. Short term incubation carried out within few minutes of introducing silver ions into the flask containing the cell filtrate resulted in the spectrum of increasing intensity in the range of 350–600 nm. UV–visible spectrum of the medium was also recorded to study the change in light absorption profile of the medium and change in intensity of the brown colour during long term incubation. The UV–visible spectra

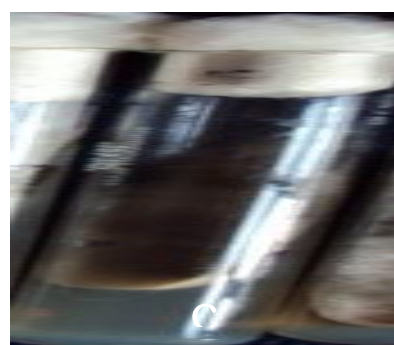
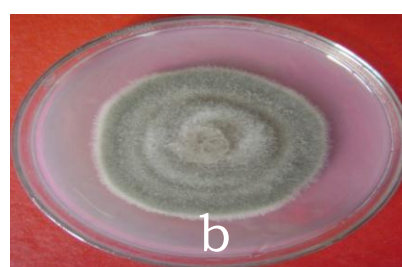
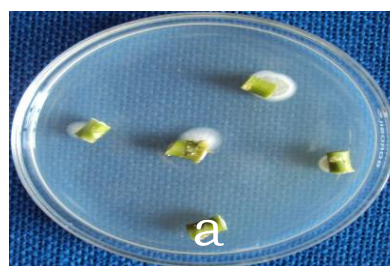
recorded at different time intervals showed increased absorbance with increasing time of incubation at around 420 nm. A representative TEM micrograph of silver nanoparticles obtained after 72 h of incubation is presented in (Fig. 1e). The micrograph showed nanoparticles with variable shape, most of them present in spherical nature with some others having occasionally triangular shape. The size of the particle ranged from 10–30 nm.^[9] Majority of the silver nanoparticles were scattered with only a few of them showing aggregates of varying sizes as observed under TEM.^[6-7] Further the nanoparticles synthesized by green route are found highly toxic against pathogenic bacteria and fungi at a concentration of 30 ppm (Table 1). Antibacterial effects of Ag nanoparticles obeyed a dual action mechanism of antibacterial activity, *i.e.*, the bactericidal effect of Ag⁺ and membrane-disrupting effect of the polymer subunits.

Table: 1 Antimicrobial activity of SNPs on human pathogens

Name of the Pathogen	Disease	Zone of Inhibition (mm)	
		Food poisoning method, 50 ppm SNPs	
		Treated	Control
<i>Escherichia coli</i> (Bacteria)	Urinary tract infection, Bacteremia, Diarrhea, meningitis	18 ± 0.5	48 ± 0.5
<i>Salmonella typhi</i> (Bacteria)	Typhoid, ulcer formation, organ failure	15 ± 0.5	45 ± 0.5
<i>Aspergillus flavus</i> (Fungi)	Mycotoxicosis	12 ± 0.3	40 ± 0.3
<i>Trichophyton mentagrophytes</i> (Fungi)	Respiratory tract mycotoxicosis	10 ± 0.3	32 ± 0.3

CONCLUSION

In conclusion, the bio-reduction of aqueous Ag⁺ ions by the *A. alternata* aqueous extract has been demonstrated. The reduction of the metal ions through the extract leading to the formation of silver nanoparticles of fairly well-defined dimensions. But the capabilities of fungi as a capping and reducing agent is not tested and not well defined. In the present study we found that this fungi can be also good source for synthesis of silver nanoparticles. This green chemistry approach toward the synthesis of silver nanoparticles has many advantages such as, ease with which the process can be scaled up, economic viability, etc. Applications of such eco-friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic materials (nano materials). Toxicity studies of silver nanoparticles on human pathogen opens a door for a new range of antibacterial agents. The reduction of silver ions and stabilization of the silver NPs was thought to occur through the participation of Leaf proteins and metabolites. Most importantly, the reaction was simple and convenient to handle and it is believed that it has advantages over other biological syntheses.



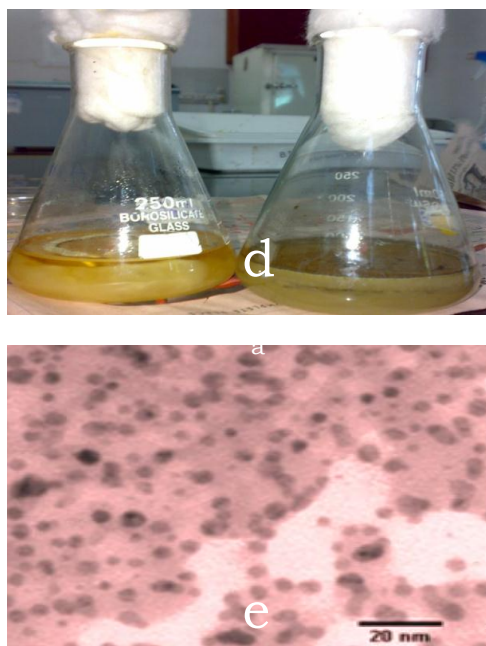


Figure: 1

- (a) Isolation of fungal endophyte *A. alternata* from Karanja nodal explants
 (b) & (c) Pure culture of *A. alternata* on PDA media in plate and slant
 (d) Culture filtrate of *A. alternata* with silver nitrate solution
 (e) TEM micrograph of novel silver nanoparticles (SNPs)

ACKNOWLEDGEMENT

The author would like to express their gratitude to the Management & Science University, Shah Alam, Selangor, Malaysia for providing instruments and reagents for this research.

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