BIOMIMETIC SYNTHESIS OF SILVER NANOPARTICLES FROM AN ENDOPHYTIC FUNGUS AND THEIR ANTIMICROBIAL EFFICACY

Nameirakpam Nirjanta Devi[1]*, Dheeban Shankar. P[2], Sutha.S[3]

[1]Department of Biotechnology, K.S.Rangasamy College of arts and science, Tiruchengode -- 637 215, Tamil Nadu, India
[2]Department of Biotechnology, Nandha Arts and Science College, Erode, Tamil Nadu, India.
[3]Centre for Nano Science and Technology, K.S.Rangasamy College of Technology, Tiruchengode - 637 215, Tamil Nadu, India

E-mail of Corresponding Author: nnirjan@gmail.com

Abstract
In this work, an endophytic fungus, *Penicillium sps.* was isolated from the medicinal plant, *Centella asiatica*. The extracellular biosynthesis of silver nanoparticles using the filtrate of cell mass of an isolated *Penicillium sps* was monitored and the UV-Vis absorption spectrum recorded for the solution shows the characteristic surface plasmon resonance band for silver nanoparticles in the range of 390-440 nm. The SEM studies confirmed the formation of silver particles in the size of 100 nm, a clear indication of the formation of silver nanoparticles. The silver nano particle synthesised using filtrate of endophytic fungal biomass shows the antimicrobial effect on *Proteus mirabilis*, *Shigella dysentriae, Klebsiella pneumoniae,* and *Staphylococcus aureus* , and fungal pathogens such as *Candida albicans.* Finally it is revealed that nowadays the disease causing microbes have become resistant to drug therapy. So this environmental friendly method was analyzed to be faster when compared to other chemical methods and was evaluated as the very good choice of antimicrobial agent.

Keywords: Silver nanoparticles; Green Synthesis; Endophytic fungi; Antimicrobial activity

1. Introduction
Generally the extracts of medicinal plants, metabolites of endophytic fungi and silver ions are having antimicrobial activity. Despite, the extracellular synthesis of silver nanoparticles by exploiting the biomass of endophytic fungus with 1mM silver nitrate was found to have an additional antimicrobial activity[1]. Endophytes are microbes that inhabit plant tissues in their life cycle without causing any apparent harm to their host[2]. Their presence implied a symbiotic interaction, in all the plants investigated until now[3]. Metal nanoparticles show promising applications in the fields of medicine, electronics, agriculture, etc. In the present scenario pharmaceutical and biomedical sector was facing the challenge of continuous increase in the emerging pathogens, with their antibiotic resistance profiles, with fear about the emergence and re-emergence of multi-drug resistant pathogens and parasites[4]. Nanoparticles present a higher surface-to-volume ratio with decreasing size and the specific surface area was relevant for catalytic reactivity and other related properties such as antimicrobial activity. Because of the pathogenic organism resistance to the already available drugs, there was always a need for an alternative approach to search for new bioactive compounds. Plants which were natural products and present abundantly all over world can be use to search for new bioactive compounds; especially the plants are searched to isolate an endophytic fungus, which found to have a very good bioactive compound. The term endophyte was applied to fungi (or bacteria) which live within plant tissues, for all or part of their life cycle and cause no apparent infections[5-7]. This definition excludes the mycorrhizal fungi but doesn't imply that endophytic fungi were not cultivable on artificial media. Some species of endophytic fungi were identified as sources of anticancer, antidiabetic, insecticidal and immunosuppressive compounds. The use of microorganisms such as bacteria, yeast, fungi and *Actinomycetes* were described for the formation of nanoparticles and their applications. Silver (Ag) was used to control

bacterial growth in a variety of application including dental work, catheters and burn wound, because Ag ions and Ag-based compounds were highly toxic to microorganism[8]. The reduction of the Ag^+ ions occurs due to reductases released by the fungus into the solution, thus opening up a novel fungal/enzyme-based approach of synthesis of nanomaterials[9,10]. The long-term stability of the nanoparticles in the solution may be due to the stability of proteins like cystine[11]. With these backgrounds the present investigation is aimed at screening endophytic fungus for the ability to bioreduce aqueous silver nitrate to silver nanoparticles, characterizing the silver nanoparticles and checking its antimicrobial activity.

1. Materials and methods

1.1 Isolation, cultivation and identification of endophytic fungi: The collected plant sample was thoroughly washed with sterile distilled water and air dried before they are processed. The materials were then surface sterilized by immersing them sequentially in 70% ethanol for 3min, 0.5% sodium hypochloride for 1min and again in 70% ethanol for 30 s. Finally, the leaves were rinsed three times in sterile distilled water. The excess water was dried under laminar airflow chamber. Then, with a sterile scalpel, the leaves of 0.5cm size were carefully dissected and placed on petri-plates containing Potato dextrose agar, Rose Bengal agar and Sabouraud dextrose agar. The media were supplemented with streptomycin sulphate (100mg/L) to suppress bacterial growth. The plates were then incubated at 25 ± 2°C until fungal growth**.** The plant segments were observed once a day for the growth of endophytic fungi. Hyphal tips growing out the plated segments were immediately transferred into PDA slant and maintained at 4°C. The fungal isolates were identified based on their morphological and reproductive characters using standard identification manuals[12, 13] (Barnett and Hunter, 1972; Subramanian, 1971). Isolated fungus was inoculated in 250ml conical flask containing 100 ml potato dextrose broth, kept at 120 rpm over orbital shaker for about 3 days. Fungal spores were found to be well grown within 3 days.

1.2 Synthesis of silver nanoparticles: 10 gm of fresh and clean fungal biomass were taken in 100 ml Deionised sterile distilled water and Kept over shaker at 120 rpm for about 3 days incubation. After the proper growth of fungal spores, the spore containing water was filtered and 1mM Concentration of silver nitrate was added to the Filtrate of biomass and kept in shaker at 120 rpm for about 3 days to have complete synthesis of silver nanoparticles. For characterization of silver nanoparticles formed in the filtrate of fungal biomass, the bioreduced solution consisting of hydrosols of silver nanoparticles and biomolecules from the filtrate of fungal biomass was subjected to centrifugation at 5000 rpm for 10minutes, washed twice and the pellet was discarded. Later the supernatant was subjected to centrifuge at 25900 rpm (75000 x g), for 30minutes. The pellet formed was dissolved in 1.0ml of deionized water and air dried.

1.3 Characterization of green synthesized silver nanoparticles: The wide distribution range of silver nanoparticles in the bioreduced solution was analysed through dynamic light scattering technique. The dried Ag nanoparticles were subjected to FTIR analysis for analyzing the capping ligand of silver nanoparticles which act as a stabilizing agent. The phase evaluation of the dried Ag nanoparticles were analysed through X-Ray Diffractometer with Cu-Kα as a radiation source. The diffracted intensities were recorded from 10˚ to 80˚ of 2θ angles. The size and surface morphology of the biosynthesized silver nanoparticles using the filtrate of fungal biomass was characterized using scanning electron microscopy analysis.

1.4 Antimicrobial activity: Silver nanoparticles synthesized using the filtrate of fungal biomass were tested for its potential antibacterial activity against a few gram negative bacterial pathogens such as *Proteus mirabilis*, *Shigella dysentriae, Klebsiella pneumoniae.* Among gram positive microorganism *Staphylococcus aureus* were assayed. *Candida albicans* was used as a fungal strain. Agar Well diffusion assay method was followed, which involves swabbing the cultures in pre-sterilized nutrient agar plates and Sabouraud dextrose agar plates and four wells were cut in the same using sterile cork borer. Each well was loaded with different concentration of Ag nano particle like 20, 40 and 60 µl of the solutions in the following order: water as negative control, filtrate of fungal biomass, solution of silver nanoparticles, and silver nitrate. Then the sample loaded in nutrient agar plates and

sabouraud dextrose agar plates were incubated at 37^0C for 24 hrs and 27ºC for 3 days respectively.

2. Results and discussion

Totally 4 different fungal strains were isolated from *Centella asiatica*. In that only 1 strain *Penicillium sps* is used for the synthesis of silver nano particles. The microscopic image of the isolated *Penicillium sps* (endophytic fungi) from *Centella asiatica* was shown in Fig.1 (a). The size of the nanoparticles depends upon the nature of the medium used. Normally by chemical method, the corresponding chemical may act as a stabilizing agent. From *Centella asiatica*, the endopytic fungal such as *Penicillium sps* culture was isolated and it was confirmed through the morphological study. But in case of biomimetic method, instead of chemicals, the filtrate of fungal biomass is used as the medium to the synthesis silver nanoparticles. For silver nano particles synthesis, addition of culture supernatant of the isolated *Pencillium* strain to 1mM solution of silver nitrate. The appearance of colour changes from pale yellow to brown colour after 24h of reaction indicates the formation of silver nanoparticles in the solution, which was depicted in the fig 1 (b). Fig.2 shows the UV-Vis absorption spectrum recorded for the solution of the synthesised silver nano particles. The image shows the characteristic surface plasmon resonance band for silver nanoparticles in the range of 390-440 nm. The increase in UV value shows the ion formation. From this observation, it was predicted that the rate of reduction of the Ag^+ ions took place extracellularly. The rate formation is literally rapid, comparable to the chemical method of synthesis. When *Vipul Bansal et al*, are using fungus like *Fusarium* species and *Actinomycetes* and bacteria like *Rhodopseudomonas* the synthesis of nanoparticles[14], it took approximately 72-96 hours, When compared with them the *Pencillium sps,* an endophytic fungus isolated from *Centella asiatica* synthesized the silver nanoparticles within 24 hours. This made the investigation highly significant for rapid synthesis of silver nanoparticles.

The particle size of the bioreduced silver nano particles using *Pencillium sps* was analysed through dynamic light scattering techniques shown in Figure 4. The particle size graph indicates the average particle size of 119 nm with the wide distribution of 20 to 200 nm. This wide distribution of silver nano particles was due to the slow reduction of silver nitrate to silver nano particles. Figure 5 shows the XRD pattern of the synthesised silver nano particles. From the XRD pattern, it was revealed that, the silver nano particle synthesised was not well crystallized. The crystalline peak appeared in the XRD pattern corresponds to the cubic shapes of the silver with the average grain size of the 32 nm. An interesting observation was made that, the biomimetic synthesis affects the formation of crystalline nano particles and which tends to semi crystalline nano particles formation. The morphology of the prepared nano particle was confirmed by scanning electron microscopy which was shown in Figure 6. The oval shape of the formed nano particles was confirmed through the SEM image.

FTIR analysis was used to characterize the nature of capping ligands that stabilizes the silver nanoparticles formed by the bioreduction process. The FTIR spectrum of the synthesised silver nano particles are shown in Figure 6. The broad absorption band in the range of 3265 cm^{-1} was due to the presence of surface hydroxyl group. The sharp band at 1380 cm^{-1} was due to the C-N stretching vibration of aliphatic and aromatic amines. The IR band at 1316 cm^{-1} assigned as carbonate bond. The absorption band at 1076 cm^{-1} was attributes to the presence of typical phosponic acid group. The vibration band at 2921 cm^{-1} attributes to the side chain consisting of C-H stretching symmetric and asymmetric mode. These data suggests that the stabilizing agents may be present in the culture supernatant of *Pencillium sps*.

In further experiments, the antimicrobial activity was checked aganist bacterial (gram positive and gram negative) and fungal strain with silver nano particles having 20, 40 and 60 µl. The silver nanoparticles synthesized in our study effectively inhibited the growth of the pathogens like *Proteus mirabilis*, *Shigella dysentriae, Klebsiella pneumoniae,* and *Staphylococcus aureus* are shown in Figure 7. Silver nano particles shows promising results as an antimicrobial agent against gram positive and gram negative bacteria. The toxicity of the silver nano particle against the microorganism is mainly due to the free silver ions. However, in the case of bacterial strains *Shigella dysentriae* shows minimum bacterial activity at the lowest silver concentration. But

the rate of bacterial inhibition was high for *Staphylococcus aureus* against silver nano particle. From this observation it was concluded that, at the lowest concentration *Staphylococcus aureus* exhibit better antibacterial activity against silver nano particle. In the case of fungal analysis, silver nano particle shows a promising growth inhibition. While increasing the concentration of the silver nano particles exhibit significant growth inhibition towards *Candida albicans* are shown in Figure 8. The higher inhibition zone was observed for the higher concentration. From this result it was observed that, nano particles shows better growth inhibition against the tested microorganisms.

Conclusion

The biomimetic synthesis of silver nano particle using *Penicillium sps* have successfully prepared with the particle size of 100 nm. At the minimum concentration of silver nano particle the antibacterial activity was analysed and *Staphylococcus aureus* shows higher antibacterial activity. Similarly, the anti fungal activity of the nano particle also beneficial while testing with the nano particle. Further investigations in the field can lead to the improvement of the medical methods for the treatment of microbial infections.

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Figure 1(a). Optical microscopic view of the *Pencillium sps* extracted from *Centella asiatica* leaf and (b). Colour change of the silver nitrate during reaction.

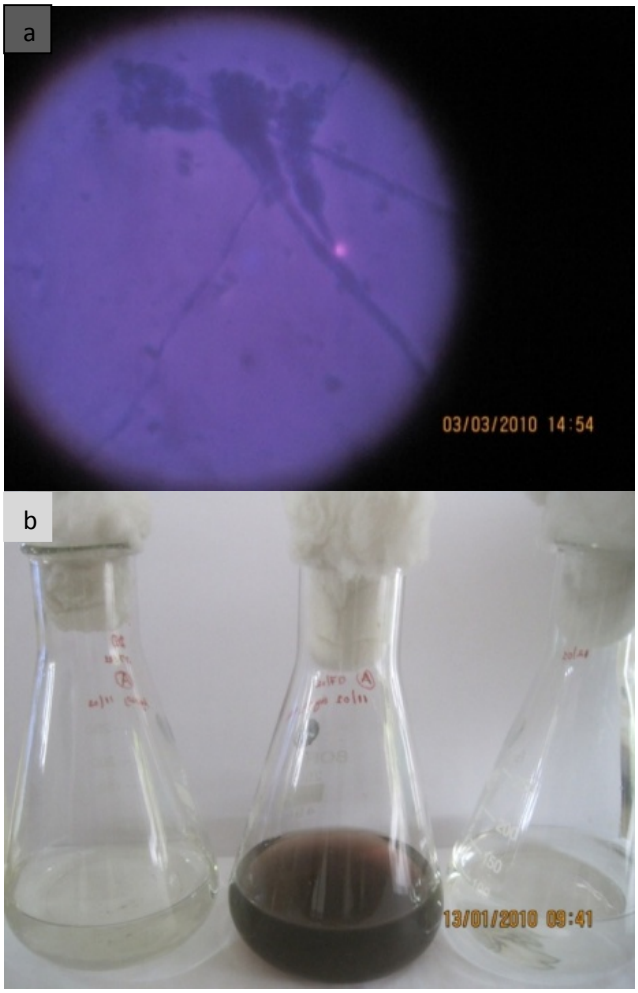

Figure 2. UV absorption spectrum of the silver nano particles prepared using *Pencillium sps.*

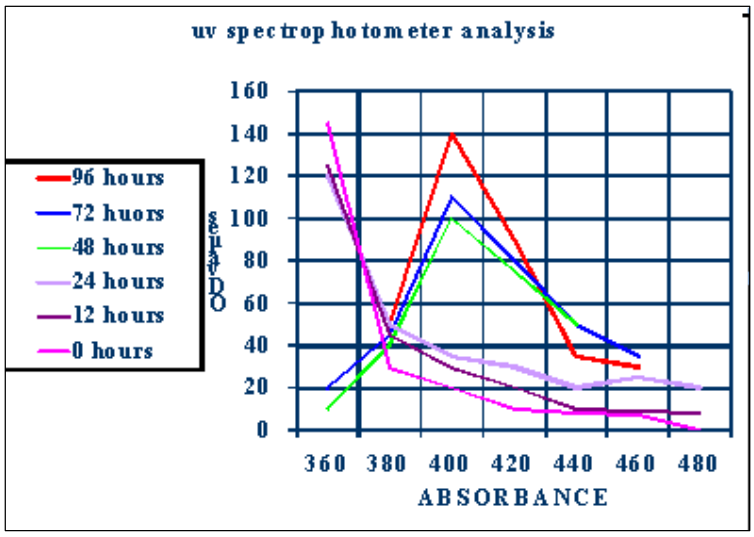

Figure 3. Particle size of the silver nano particle through dynamic light scattering technique.

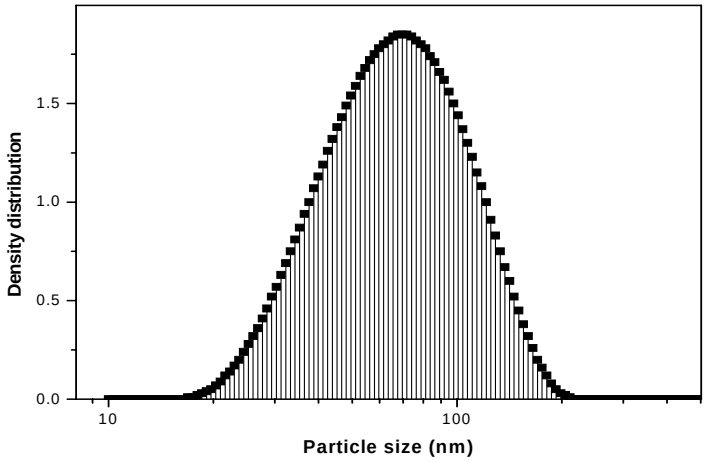

Figure 4. XRD pattern of the silver nano particle

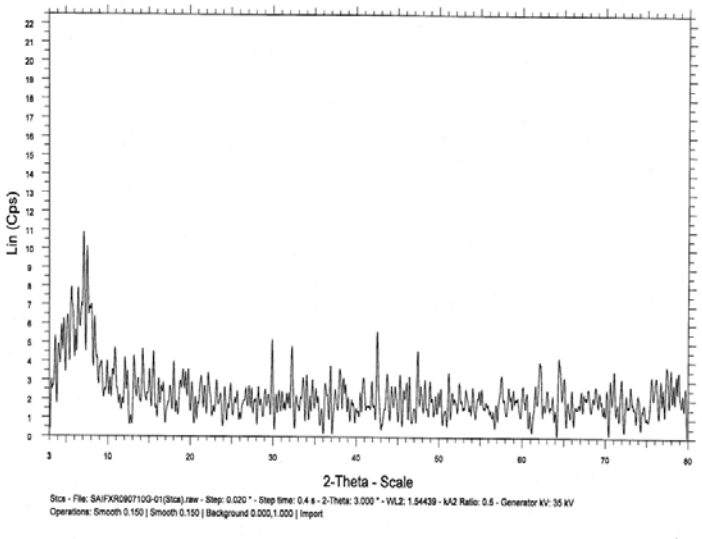

Figure 5. SEM image of the silver nano particle

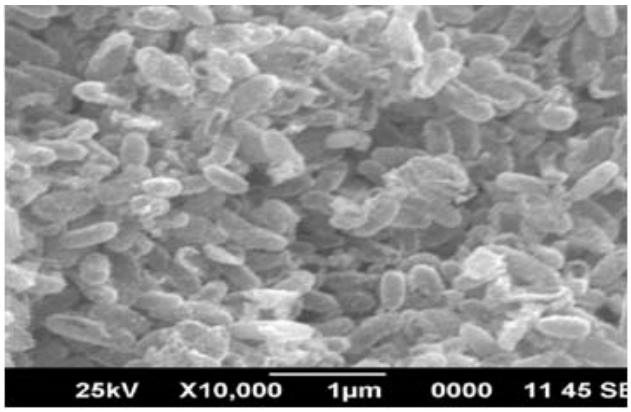

Figure 6. FTIR spectra of the silver nano particle

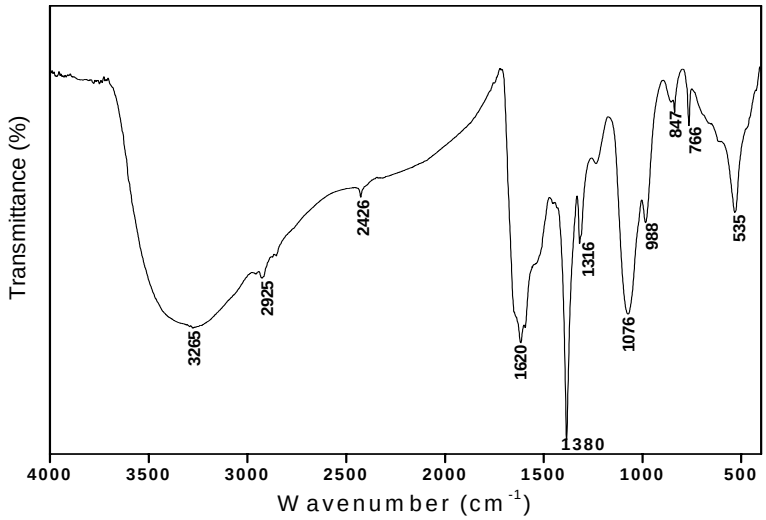

Figure 7. Antimicrobial activity of *Shigella dysentriae*, *Klebsiella pneumonia* *Staphylococcus aureus*, *Proteus mirabilis, and Candida albicans* using Silver nanoparticles.

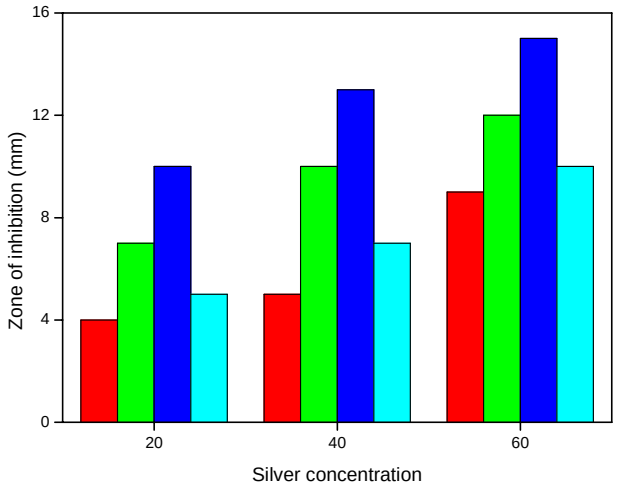

Figure 8 Growth inhibition zones against *Candida albicans*