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Extracellular biosynthesis of silver nanoparticles using the filamentous fungus *Penicillium* sp.

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ABSTRACT

The aim of the study was to synthesize silver nanoparticles by using filamentous fungus Penicillium sp. The fungal culture was isolated from the soil samples collected from agriculture fields in Vellore district, Tamil Nadu, India. The purified fungal isolates were inoculated in minimal medium and incubated at room temperature for three days. For the synthesis of silver nanoparticles, 50 ml of cell filtrate was mixed with equal volume of 1mM silver nitrate [AgNO3 (1 mM)] and agitated at room temperature in dark. The synthesis of silver nanoparticles was investigated by UV-Vis spectroscopy, Atomic force microscopy and Fourier Transform Infrared Spectroscopic analysis. Results indicate the synthesis of silver nanoparticles in the reaction mixture. Mechanism of silver nanoparticles synthesis was determined by nitrate reduction test.

Keywords: silver nanoparticles; Mucor sp.; silver nitrate; Atomic force microscopy.

INTRODUCTION

Nanotechnology is emerging field of science which involves synthesis and development of various nanomaterials. At present, different types of metal nanomaterials are being produced using copper, zinc, titanium, magnesium, gold, alginate and silver. These nanomaterials are used in various fields such as optical devices [1], catalytic [2], bactericidal [3], electronic [4], sensor technology [5], biological labelling [6] and treatment of some cancers [7]. In last decay, application of nano material has been extensively increase and the high demands leads to the bulk production of the nanomaterial. Classically the nanoparticles are produced by physical [8] and chemical methods [9], as these methods are costly, toxic and non eco friendly, scientists are looking forward to synthesize low cost, non toxic, eco friendly nanoparticles. Most recently, biosynthesis of nanoparticles using bacteria [10, 11], fungus and plants [12] have emerged as a simple and viable alternative to more complex physical and chemical synthetic procedures to obtain nanomaterials.

Silver nanoparticles are undoubtedly the most widely used nanomaterials among all. Silver nanoparticles are used in antimicrobial agents, textile industries, water treatment, sunscreen

lotions etc [3, 13]. Previous studies reported the biosynthesis of silver nanoparticles by plants such as *Azadirachta indica* [14], *Capsicum annuum* [15], *Carica papaya* [16], *Gliricidia sepium* [12], *Eucalyptus hybrida* [17] and microorganisms such as *Aspergillus fumigatus* [18], *Cladosporium cladosporioides* [19], *Fusarium oxysporum* [20], *Pseudomonas aeruginosa* [11] and *Rhodopseudomonas capsulate* [10]

The filamentous fungi possess some advantages over bacteria in nanoparticles synthesis, as most of the fungi are easy to handle, required simple nutrient, possess high wall-binding capacity, as well as intracellular metal uptake capabilities [21, 22]. This study involves the biological synthesis of silver nanoparticles using filamentous fungus *Penicillium* sp. and the characterization of the synthesized silver nanoparticles by UV - Visible spectroscopy, Atomic Force Microscopic (AFM) analysis and Fourier Transform Infrared Spectroscopy (FTIR) analysis. Future studies can be conducted to explore applications of the silver nanoparticles generated from the *Penicillium* sp.

MATERIALS AND METHODS

Chemicals

Potato Dextrose agar (PDA), silver nitrate, Lactophenol Cotton Blue Stain, Potassium bromide (FTIR grade), Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), magnesium Sulfate heptahydrate ($MgSO_4 . 7H_2O$), Ammonium Sulphate $(NH_4)_2SO_4$, yeast extract and glucose.

Sample collection

Soil sample were collected from different agriculture fields in Vellore district, Tamil Nadu, India. Soil samples were collected from 3 to 4 cm depth with help of sterile spatula. Samples were transferred in to sterile plastic bags and brought to the Molecular and Microbiology Research Laboratory and stored in a refrigerator at 4°C up to further processing.

Isolation of fungal cultures

Isolation of soil fungi was performed by serial dilution and spread plate method. One gram of soil sample was serially diluted in sterilized distilled water to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to PDA plates. The sample was uniformly distributed by using a sterile glass spreader. The plates were incubated at room temperature for 3 days.

The fungal isolates were further subcultured on the PDA plates in order to obtain pure culture. Pure isolates were maintained at 4ºC in refrigerator for further studies [23].

Colony characterization

The fungal isolates were observed using hand lens and the colony morphology was recorded with respect to color, shape, size and nature of colony.

Microscopic characterization

Fungal isolates were microscopically characterized by Lactophenol Cotton Blue mounting. The cell morphology was recorded with respect to spore chain morphology, hyphae and mycelium structure.

Biosynthesis of silver nanoparticles

The *Penicillium* sp. was selected for the further studies for the production of silver nanoparticles. The *Penicillium* sp. 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 25°C. After the incubation, the cell filtrate was obtained by passing it through Whatman filter paper no. 1. Filtrate was collected and used further for nanoparticles synthesis.

For the synthesis of silver nanoparticles, 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. Control (without the silver ion, only biomass) was also run along with the experimental flask [24].

Characterization of synthesized silver nanoparticles

UV–visible spectroscopic analysis

The reduction of silver ions was confirmed by qualitative testing of supernatant by UV–visible spectrophotometer. 1 ml of sample supernatant was withdrawn after 24 hr and absorbance was measured by using UV–visible spectrophotometer between 400-600 nm.

Fourier Transform Infrared (FTIR) Spectroscopy analysis

The lyophilized sample was subjected to FTIR Spectroscopy analysis. Two milligrams of the sample was mixed with 200 mg KBr (FT-IR grade) and pressed into a pellet. The sample pellet was placed into the sample holder and FT-IR spectra were recorded in the range 4000-450 cm^{-1} in FT-IR spectroscopy at a resolution of 4 cm^{-1}.

Atomic force microscopy

A thin film of the sample was prepared on a glass slide by dropping 100 µl of the sample on the slide, and was allowed to dry for 5 min. The slides were then scanned with the AFM.

Nitrate reductase assay

Nitrate reductase is an enzyme that converts nitrate to nitrite. The enzyme activity was measured using the method described by Harley, 1993 and Saifuddin et al., 2009 [25, 26]. The activity was measured by putting in the substrate for the enzyme (nitrate) and then measuring the amount of nitrite after 1 h. The net increase in nitrite at 1 h is the amount of nitrate reductase activity.

RESULTS AND DISCUSSION

Isolation and identification of *Penicillium* sp.

Fungal cultures were isolated from the soil samples collected from various agricultural lands in Vellore district, Tamil Nadu, India. The fungal isolates were characterized on the basis of colony characteristics and microscopic appearance [27]. *Penicillium* sp. was further selected for the biosynthesis of silver nanoparticles because of very few reports only reported the synthesis of silver nanoparticles using *Penicillium* sp. Earlier Shaligram et al, 2009 have reported the biosynthesis of silver nanoparticles using aqueous extract of *Penicillium brevicompactum* WA 2315. The size of synthesized nanoparticles was found to be 58.35±17.88 nm by SEM and TEM analysis [28]. Kathiresan et al, 2009 have reported the biosynthesis of silver nanoparticles by a marine fungus, *Penicillium fellutanum.* The fungal culture was isolated from the rhizosphere soil of *Rhizophora annamalayana* Kathir. The synthesis of silver nanoparticles was controlled with respect of pH, temperature, silver ion concentration and exposure time to silver nitrate [29].

Penicillium sp. colonies appeared as velvety and sulcate with green color on PDA medium plates. Reverse side of the colony was yellow in color. Results are displayed in Figure 1A. Microscopic identification of the fungal isolates was performed by LPCB mounting. *Penicillium* sp. appeared as highly branched mycelium, septate hyphae. Conidiphores sprouted on the mycelium and conidiospores were visualized. Results are displayed in Figure 1B.

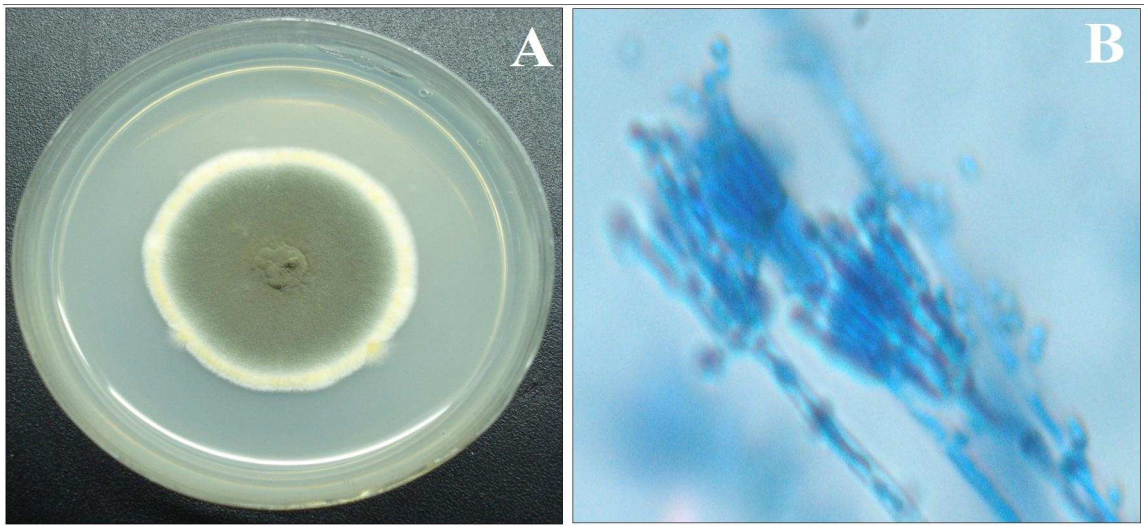

Figure 1: Colony morphology of the *Penicillium* sp. isolate (A) and microscopic view of the isolate (B)

Characterization of silver nanoparticles

Color change

Cell free filtrate of *Penicillium* sp. was mixed with silver nitrate solution and incubated in dark in rotary shaker. Samples showed changed in colour from almost colourless to brown, this is a clear indication of the formation of silver nanoparticles in the reaction mixture. The intensity of the colour was increased during the period of incubation. The appearance of brown colour was due to the excitation of surface plasmon vibrations [20]. Control showed no change in colour of the mixture when incubated in the same conditions. Results are reported in Figure 2.

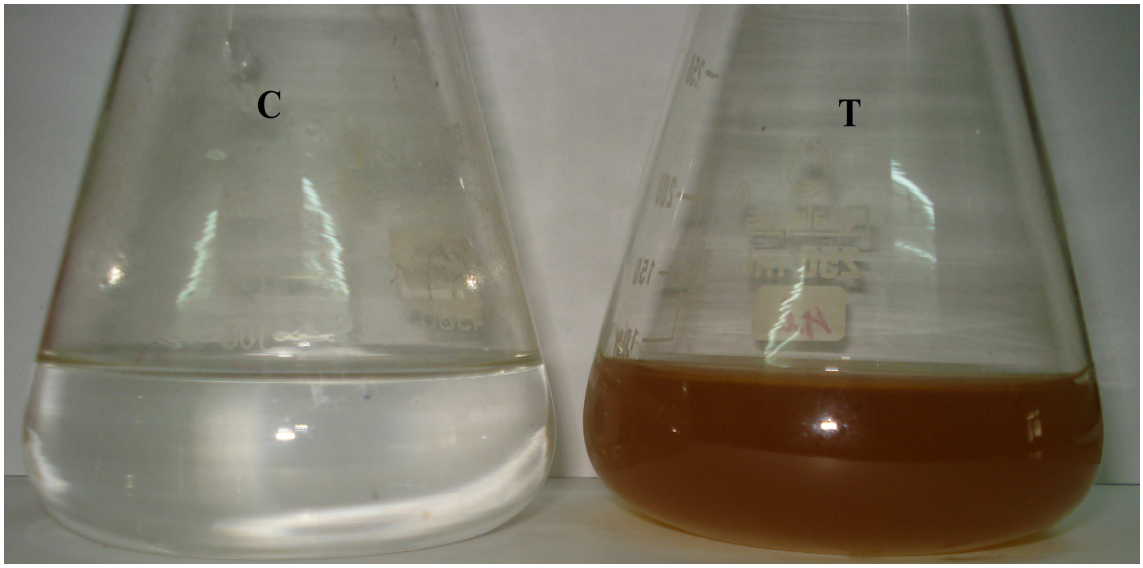

Figure 2: Biosynthesis of silver nanoparticles- colour change reaction: conical flasks containing the extracellular filtrate of the *Penicillium* sp. biomass (C) and conical flasks containing the extracellular filtrate of the *Penicillium* sp. biomass after exposure to $AgNO_3$ solution for 24 h (T)

UV–visible spectroscopic analysis

Synthesis of colloidal silver nanoparticles was initially performed by UV - Visible spectroscopic analysis. In UV – Visible spectrum, a strong peak was observed between 400-420 nm, indica5te the presence of silver nanoparticles [30]. UV – visible spectra is reported in Figure 3.

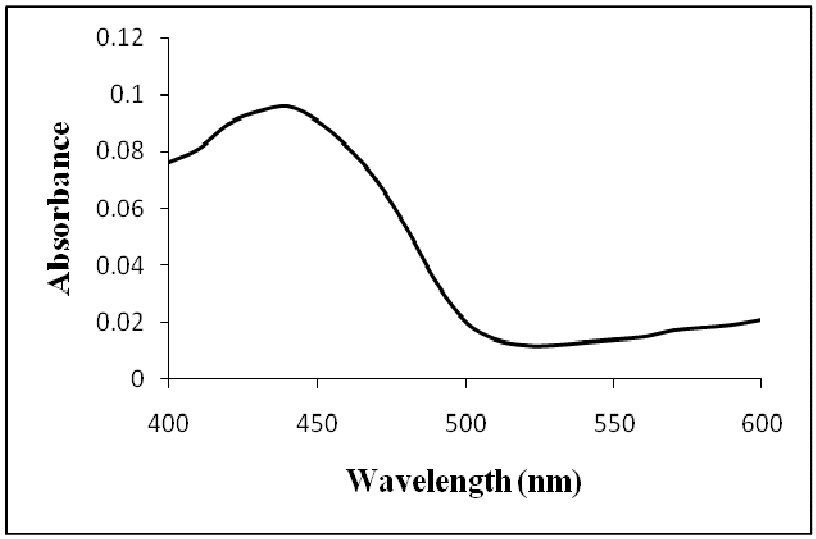

Figure 3: UV–visible spectrum of silver nanoparticles synthesized using *Penicillium* sp. after 24 h

FTIR analysis

The lyophilized nanoparticle samples were analyzed in FTIR to identify the possible bio molecules responsible for the reduction of the Ag+ ions by the cell filtrate. The FTIR spectrum is presented in Figure 4. The representative spectra of nanoparticles obtained manifests absorption peaks located at about 3843.68 cm^{-1} (–NH group of amines), 3597.73 cm^{-1} (-OH group of phenols), 2080.65 cm^{-1} (aromatic –CH stretching), 1631.66 cm^{-1} (-NHCO of amide) and 767.16 cm^{-1} (C-Cl).

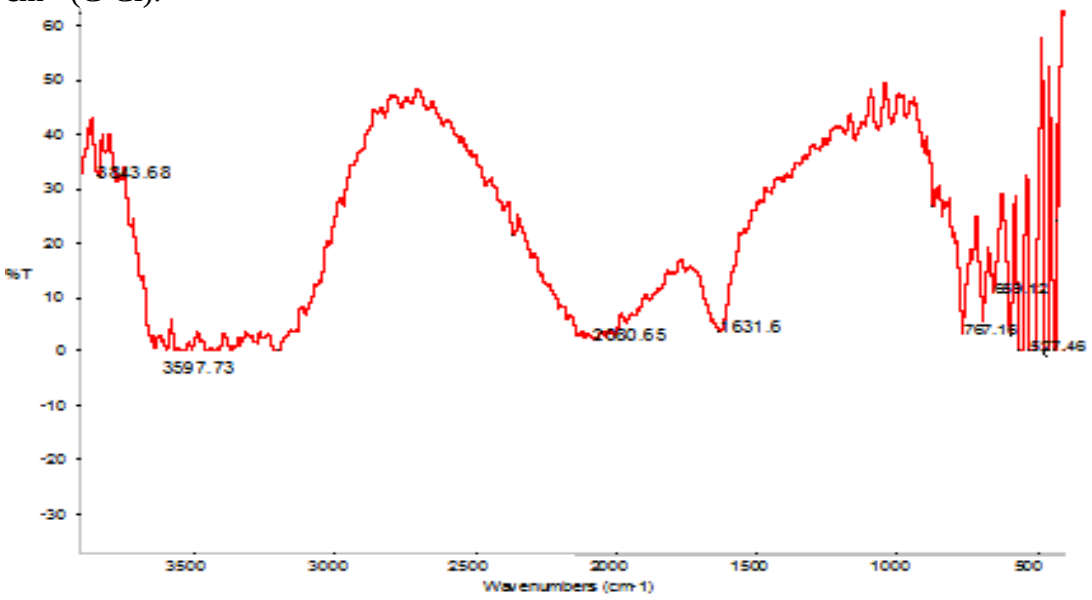

Figure 4: FTIR analysis of silver nanoparticles biosynthesis by using *Penicillium* sp.

Atomic force microscopic analysis

The silver nanoparticles were characterized by AFM for its detail size, morphology and agglomeration of silver. AFM images were taken with silicon cantilevers with force constant 0.02 – 0.77 N/m, tip height 10–15 nm, contact mode. It was observed that the silver

nanoparticles agglomerated and formed distinct nanostructures (nanoparticles). The topographical image of irregular silver nanoparticles is reported in Figure 5. Formation of silver nanoparticles and its agglomeration was clearly observed in figure. The particle size of the silver nanoparticles ranges in size from 52-104nm.

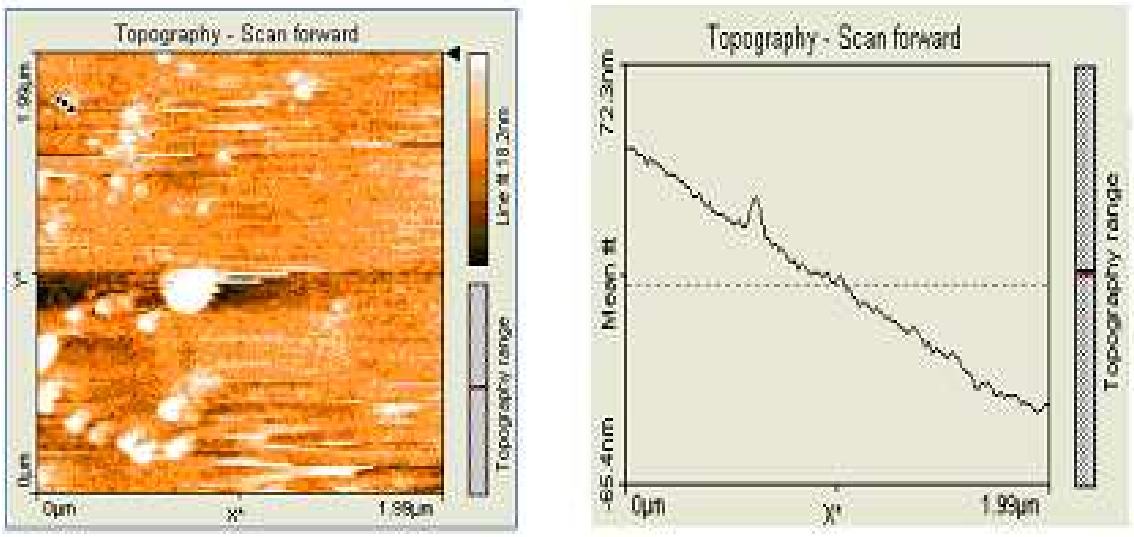

Figure 5: Atomic force microscopy image shows formation of nanoparticles by *Penicillium* sp.

Nitrate reductase assay

In this study nitrate reductase activity of the culture supernatant of *Penicillium* sp. was detected by nitrate reductase assay. The nitrate reductase activity of the culture supernatant was found to be 270 nmol/ h/ml. Nitrate reductase activity of the isolate indicates the possible resion of the reduction of silver nitrate in to silver nanoparticles [25, 26].

CONCLUSION

In this study silver nanoparticles were biologically synthesized using filamantus fungi *Penicillium* sp. isolated from the soil samples. The cell filtrate of *Penicillium* sp. was challenged with 1 mM of silver nitrate, change of the mixture from colorless to orange brown indicate the synthesis of silver nanoparticles in the reaction mixture, size of the synthesized nanoparticle was measured 52-104 nm by AFM analysis. Results conclude that isolated *Penicillium* sp. is a prominent producer of silver nanoparticle.

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