ISSN Online: 2349-7785

Synthesis and Comparative Characterization of Fungal AgNPs from Isolated *Colletotrichum ti* and *Paecilomyces sinensis*: Endophytes of *Oroxylum indicum* (L.) Vent.

Mamta Gokhale*, Shivani Choudhary, Isha Deshpande
Department of Botany and Microbiology, St. Aloysius' College, Autonomous, Sadar, Jabalpur, India

Received 10 August 2019; accepted in revised form 19 December 2019

Abstract: The study emphasizes on the use of a biological approach for the synthesis of silver nanoparticles (AgNPs). The fungal endophytes have been of great importance for such an approach. An experiment was designed to employ *Colletotrichum ti* & *Paecilomyces sinensis: fungal* endophytes of *Oroxylum indicum* (L.) Vent. (an important medicinal tree) for green synthesis of AgNPs which are well characterized by the implementation of fluorescence microscopy, UV-visible spectroscopy, fourier transform infrared spectroscopy and X-ray diffraction techniques. The fluorescence microscopy helps to define cell physiology more precisely whereas UV-visible spectroscopy shows a characteristic peak of silver nanoparticles at 420 nm. FTIR graph implies that with increasing concentrations of silver nitrate in fungal samples, the association is stronger which is reflected by the strong vibrations in the similar regions of wavelength. The size of the synthesized nanoparticles was determined by XRD analysis. The calculated crystalline size of the intracellular AgNPs is 14.69 nm. A comparative study was developed by synthesizing extracellular and intracellular nanoparticles with *Paecilomyces* and *Colletotrichum* respectively.

Key words: Endophytic fungi; *Oroxylum indicum*; Green Synthesis; *Colletotrichum*; *Paecilomyces sinensis*; Fourier Transform Infrared Spectroscopy.

Introduction

The term endophyte and endophytic fungi have been used often frequently since the past 30 years in the mycological literature to report the internal mycota of living plants. In a broad sense, the fungi that colonize living plant tissue without causing any effect are endophytic fungi. Recently endophytes are viewed as an outstanding source of biologically active secondary metabolites natural antimicrobial products [1]. These endophytic fungi have been employed for the green synthesis of nanoparticles.

Nanotechnology

The nanoparticle is a base of nanotechnology i.e., clusters of atoms ranging from 1 to 100 nm. Nanoparticles are viewed as the fundamental building block of nanotechnology i.e., they are the initial points for preparing many devices and nanostructured materials [2].

Nanoparticles can be synthesized by various methods such as physical, chemical or biological protocols, therefore it covers a diverse area of research and technology [3]. Nanoparticles synthesized by these process are known as engineered nanoparticles. These penetrate inside the cell and induces the alteration of the cell membrane, cell structure, molecules and to other protective mechanisms, possibly by generating free radicals [4]. Nanoparticles production has traditionally been

*Corresponding author (Mamta Gokhale)
E-mail: < mamtashrirang@gmail.com >

through chemical and physical methods. However, these routes for the synthesis of particles/ crystallites require tedious and environmentally challenging techniques [5]. Therefore scientists are approaching towards "green" approaches i.e. biological methods for the synthesis of nanoparticles.

Biological synthesis of nanoparticles

Nanoparticles synthesis by biological methods is increasing prominently as they are cost-effective, rapid synthesizing, having mild reaction conditions in a variety of hosts and produce stable nanoparticles with non-toxic and eco-friendly approaches [6]. This biological synthesis of nanoparticles is effectuated by using plants [7], bacteria [8,9] and fungi [10,11]. The implication and exploration of fungi in Nanobiotechnology are considered important. Due to their toleration and metal bioaccumulation capability, fungi have attracted more attention regarding the research on biological production of metallic nanoparticles. The additional prerogatives: of utilizing them in nanoparticle synthesis is the easiness of fungi scale-up i.e., utilizing a thin solid substrate fermentation technique [12]. As fungi are very effective secretors of the extracellular enzyme, therefore achieving vast production of enzyme is feasible [13]. Another merit for the utilization of green approach mediated by fungi to synthesize metallic nanoparticles is economic livability and facility of employing biomass. Moreover, numbers of species of fungus grow very fast therefore culturing and keeping them in the laboratory are very simple. Also, the fungus is metabolic diverse microorganisms, they have become one of the major biological entity for the production of nanoparticles [14]. High wall-binding and intracellular metal uptake are the capacities of most fungus. There are many fungal species which have been studied for their ability to synthesize silver nanoparticles.

Types of biological nanoparticles

There are two major ways for the fungal mediated nanoparticle synthesis: (a) extracellular, (b) intracellular using dried or wet mycelia. Homogeneous distribution of nanoparticles in the cell wall of *Verticillium* spp during its biosynthesis have been reported [22]. In the fungus *Aspergillus flavus* [23] accumulation of silver nanoparticles on the surface of its cell wall was observed when placed in silver nitrate solution are some example of a fungal mediated intracellular synthesis of silver nanoparticles.

Materials and methods

Strain collection

Both fungal strains of *Paecilomyces sinensis* and *Colletotrichum ti* were collected from the Department of Botany and Microbiology Laboratory (identified by National Centre for Cell Science [NCCS], Pune, India). The fungal strains were isolated from a medicinally important endangered tree *Oroxylum indicum*. The tree is located in the reserve forest area of Jabalpur district Madhya Pradesh, India.

Biosynthesis of AgNPs

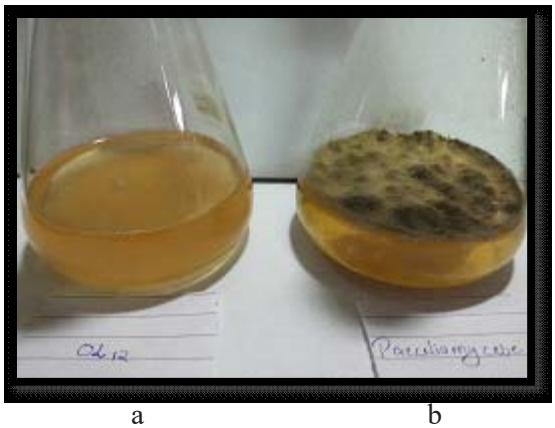
The two fungal strains i.e. *Paecilomyces* and *Colletotrichum* were grown in 250 ml conical flask in PDB separately (Fig. 1). Each flask was incubated at 28°C at 140 rpm for 7 days. After incubation, fungal biomass of each strain was separated by filtration, washed with sterile distilled water to remove the traces of culture media components respectively. After washing, fungal biomasses were re-suspended in 100 ml autoclaved distilled water separately for one day.

Preparation and optimization for extracellular AgNP synthesis

Fungal biomass of every strain (Fig. 2 and 3) was filtered with Whatman's filter paper no. 1 in sterile conditions, separately. Solutions of silver nitrate of two concentrations (1 and 1.5 mM), were prepared and 10 ml of filtrate (CFCF) of *P. sinensis* and *Colletotrichum* was added (in the ratio 1:9 v/v) separately to initiate the formation of AgNPs. The reaction mixtures were incubated at 28°C for 48 hours. Fungal filtrate (CFCF) without the addition of AgNO3 was incubated under the same condition was presumed as control.

Preparation and optimization for intracellular AgNP synthesis

Fungal biomass was filtered and added to the two concentrations of silver nitrate (1 mM, 1.5 mM) to promote the formation of AgNPs. The



a b

Fig. 1. Fungal growth in potato dextrose broth (PDB)a. *Colletotrichum ti*, b. *Paecilomyces sinensis*

Fig. 2. Dried Mycelia of *P. sinensis*

Fig. 3. Mycelia of *Colletotrichum ti*

reaction mixture was incubated at 28ºC for 48 hours. Fungal biomass in distilled water without the addition of AgNO3 was incubated under the same condition presumed as control.

Characterization of AgNPs

Fluorescent microscopy

Colour change was observed in the reaction mixtures after synthesis of fungal AgNPs, is the initial indication of nanoparticle synthesis. Slides were prepared for both intracellular (inside fungal hypha) and extracellular (in suspension) synthesis of NPs and were observed under a fluorescent microscope to observe fluorescence (life technologies EVOS). To monitor cell physiology more precisely, fungal sample (OL12) was subjected to fluorescence microscopy.

Ultraviolet-visible (UV-Vis) spectroscopy

The formation of silver nanoparticles was monitored by visual observation of colour change from pale white to dark brown. On addition of AgNO3 colour change was the initial indicator of the formation of AgNPs. The colour is due to the formation of silver nanoparticles and the excitation of surface plasmons [15]. To observe the intensity of colour change in both intra & extracellular AgNP synthesizing solutions, absorbance was read and were further confirmed by sharp peaks given by silver nanoparticles in the visible region from UV-spectrum of the reaction solution. The samples were scanned in UV-vis spectrophotometer 1800 (Shimadzu A116352) at 300 to 600 nm. Scanning was performed after the reaction time of 24 hrs.

Fourier transform infrared (FTIR) spectroscopy

With the help of Fourier transform infrared spectroscopy, the biotransformed product present in synthesized silver nanoparticles were analysed. The AgNPs was centrifuged at 10,000 rpm for 10 min. The pellets were collected, dried and ground with potassium bromide (KBr) in the ratio of (1:100 w/w) and analysed by detecting it with the formation of peaks and then compared with control (fungal suspension without the addition of AgNO3) and powder of silver nitrate (Blank). The instrument used for FTIR analysis was IRAffinity -1S (Shimadzu A221353).

X-ray diffraction (XRD)

XRD analysis was carried out using X-ray powder diffractometer Bruker D2 PHASER 2nd Gen. The air-dried nanoparticles were coated onto XRD grid and analysed at a voltage of 30 kV and a current of 10 mA with monochromatic radiation and on 2u wavelength 1.54060.

Result and discussion

Biosynthesis of AgNPs

Extracellular synthesis

Different concentrations of silver nitrate (AgNO3) (1 mM, 1.5 mM) upon incubation with the filtrate of both fungi i.e. *Paecilomyces sinensis* and *Colletotrichum ti*, turned dark brown colour only in *P. sinensis* in all concentrations of silver nitrate (Fig. 4).. Darkest colour intensity was observed in the AgNO3 solution (1.5 mM). The other fungal filtrate and control flasks remained as such (no colour change) after 72 h incubation period (Fig. 5).

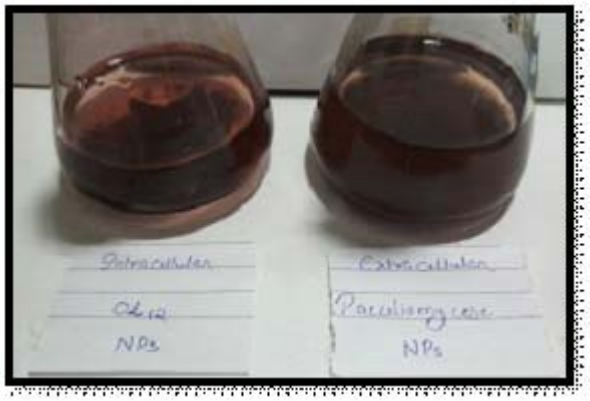
Intracellular synthesis

For examination of intracellular synthesized nanoparticle, silver nitrate in different concentrations were added with the biomass of both fungi i.e. *Paecilomyces sinensis* and *Colletotrichum ti*. Fungal biomass of *Colletotrichum* turned from white to the dark brown colour in both concentrations of AgNO3. High intensity of colour change was observed in AgNO3 solution of 1.5 mM concentration. The fungal biomass in control flasks and of *P. sinensis* remained as such after the incubation period of 72 hrs (Fig. 6).

The generation of dark brown colour is due to the surface plasmons resonance (SPR) exhibited by nanoparticles and is typical of the silver nanoparticles. Structure and size of AgNPs synthesized by both fungi were further observed under the fluorescent microscope.

Fluorescent microscopy

Synthesized AgNPs were observed under a fluorescent microscope in both fungi. It was observed that fungal biomass of *Paecilomyces sinensis* in AgNO3 solution was not fluorescent, while it's cell filtrate mixture with AgNO3 solution showed the fluorescence (extracellular AgNPs). On the



a b

Fig. 4. Synthesized fungal AgNPs. a. *Paecilomyces* AgNPs (extracellular), b. *Colletotrichum* AgNPs (intracellular)

Fig. 5. Paecilomyces AgNPs (extracellular)

Fig. 6. Colletotrichum AgNPs (intracellular)

other
hand fungal mycelium of *Colletotrichum ti* was showing fluorescence in fungal biomass when incubated with AgNO3 solution (intracellular AgNPs).

Fluorescent microscopic images showed that extracellular silver nanoparticles in *P. sinensis* were in clusters of an irregular shape with variable sizes**.** Hyphal cells of *Colletotrichum ti* were fluorescent, following the pattern of normal *Colletotri-chum* hypha (Fig. 7). There is no any type of distortion or breakage in a hypha. Fluorescent cells formation is a clear indication of intracellular AgNPs synthesis as observed in the images taken with a fluorescent microscope for *Colletotrichum t*i (Fig. 8).

Fig. 7. *F*luorescence microscopy of *Colletotrichum ti*

Fig. 8. Fluorescence microscopy of *P. sinensis*

UV-visible spectrophotometer

Extracellular AgNP (P. sinensis)

The synthesis of AgNPs was detected (Fig. 9) and monitored by UV-visible absorption spectrum scanning in the range of 300-600 nm of both the NPs. The UV-vis spectra of fungal cell filtrate of *P. sinensis* treated with the 1.5 mM AgNO3 solution shows a characteristic surface plasmons peak at 420 nm, and the maximum colour intensity obtained after 3 days. A specific absorption peak was detected at 420 nm, indicating the formation of maximum AgNPs in 1.5 mM AgNO3.

This spectroscopic pattern results from interactions of free electron limited to tiny metallic spherical objects with episode electromagnetic wave, we also found that with the increase in incubation time the absorbance also increases and AgNPs were synthesized by 24 h and there was almost no increase in absorbance after 48 h of incubations. The electrons of silver nanoparticles are excited in the presence of UV light toward the surface and form a characteristic peak of silver nanoparticles [16].

Intracellular AgNP (Colletotrichum ti)

The UV-vis spectra of fungal mycelium of *Colletotrichum ti* (Fig. 10) induced with AgNO3 (1.5 mM) solution was showing a characteristic surface plasmons peak at 420 nm. The maximum colour intensity was obtained after 2 days. A specific absorption peak was detected at 420 nm, indicating the formation of maximum AgNPs in 1.5 mM AgNO3 in *Colletotrichum ti*.

During synthesis, change in the colour of biomass from white to brown was the indication of conversion of Ag++ to Ag0. This colour change could be due to the shift in the surface plasmons resonance were silver as Ag++ is reduced to silver nanoparticles.

Ag++ +e2->Ag0(s)

The particles were scanned at 200-600 nm where the classic UV-Vis peak of silver nanoparticles shifted from 420 to 400 nm may be due to various mechanism involved during their synthesis. A characteristic peak for silver nanoparticle has been reported at 420 nm by Sivakumar and Vidyasagar [20].

Entrapment of silver nanoparticles on the cell wall of microbes is suggested in the study. The presence of the carboxyl group of amino acid residue and the amine of peptide chain along with reducing groups like aldehyde and ketone gov-

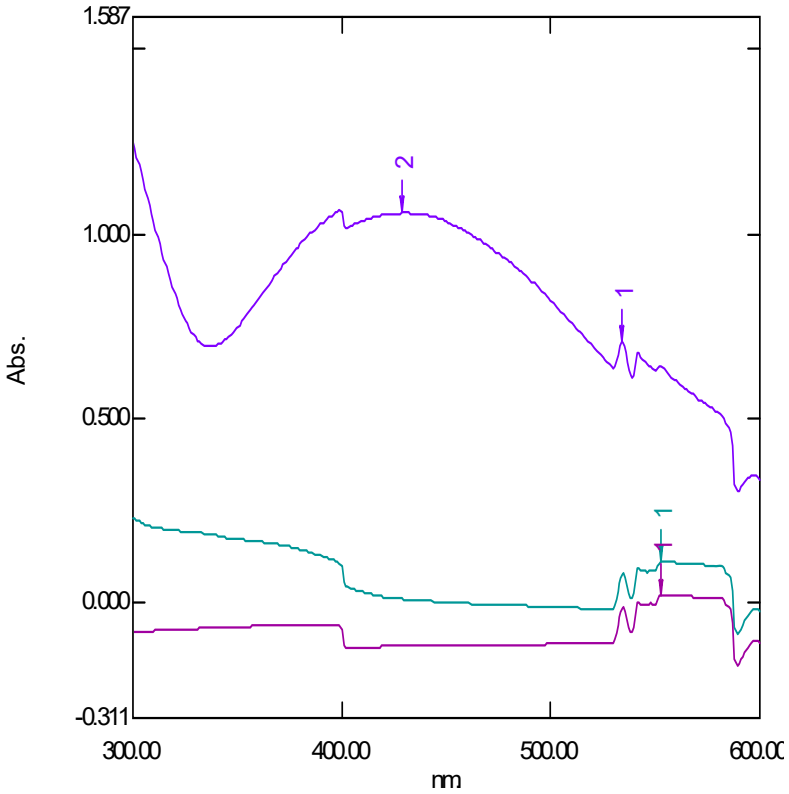


Fig. 9. UV vis spectroscopic analysis of *P. sinensis*

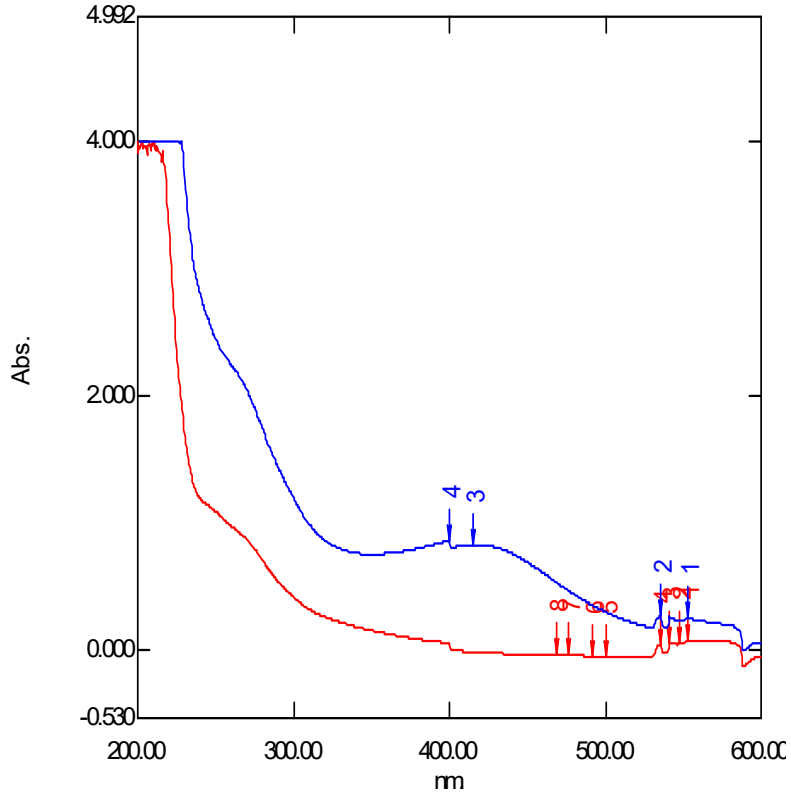


Fig. 10. UV vis spectroscopic analysis of *Colletotrichum ti*

ern the bioreduction of silver ions to [Ag(NH3)2] group [17]. It has also been reported that the presence of dehydrogenase and nitrate reductase play an important role in the synthesis of silver nanoparticles [18,19]. Hence fungal cell wall mediated silver nanoparticle synthesis could be possible while the presence of carboxyl group of amino acid enhances the entrapment of nanoparticles intra-cellularly.

FTIR spectroscopy

Extracellular AgNPs (P. sinensis)

Fourier Transform Infrared Spectroscopy (FTIR), provides evidence for the presence of proteins as a capping agent, which helps in increasing the stability of the synthesized silver nano-particles [21]. The recorded FTIR spectra (Fig. 11) was analysed and the regions of vibrations were found to be 1000 cm^{-1}, 1050 cm^{-1}, 1100 cm^{-1}, and 1380 cm-1 in *P. sinensis*. The band at 1000 cm^{-1} corresponds to C-N stretching of amines. The band at 1050 cm^{-1} and 1100 cm^{-1} suggests presents of alkoxy group (C-O). The bands at 1380 cm^{-1} exemplify the N=O symmetry stretching typical of the nitro compound.

The recorded graph also implies that with increasing concentrations of silver nitrate in the fungal sample, the association is stronger which is reflected by the strong vibrations in the similar regions of wavelength.

Intracellular AgNPs (*Colletotrichum ti*)

In *Colletotrichum ti*, dry sample of mycelia, with AgNO3 and silver nitrate (control) was showing similar bending vibrations at 730 cm^{-1} and 880 cm-1 which are associated with C-H bond or C=C

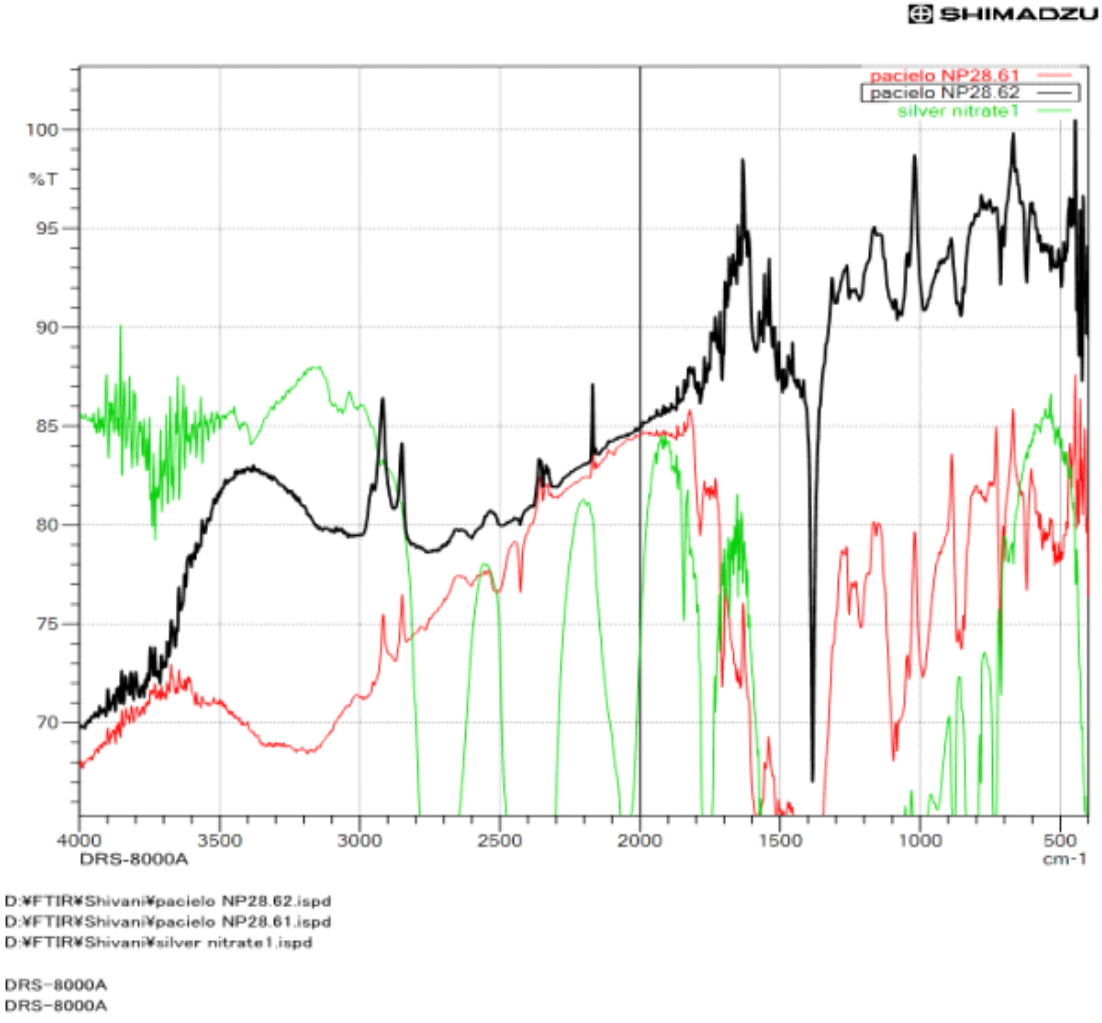


Fig. 11. FTIR analysis of *P. sinensis*

(alkenes), whereas the association causes a shift of vibrations from 1400 cm-1 to 1460 cm-1 which suggest the bending vibrations of C-H bonds (Fig. 12).

XRD analysis

Extracellular AgNPs (*P. sinensis*)

***Intracellular AgNPs* (*Colletotrichum ti*)**

The diffracted intensities were recorded from 10o to 90ofor dry sample of *Colletotrichum ti* (Fig. 13). Three strong braggs reflections at 27.16o, 31.52o and 45.50o were observed. The interplanar spacing (dcalculated) values are 3.14, 2.28and 1.98 for 27.16o, 31.52o and 45.50o reflections respectively. The average crystalline size is calculated using the Debye-Scherrer formula:

$D = k\lambda / \beta\cos\theta$

Where D is the average crystalline size of the nanoparticles, k is a geometric factor (0.9), λ is the wavelength of X-ray radiations source and β is angular FWHM (full-width at half maximum) of the XRD peak and the diffraction angle θ. The calculated crystalline size of the AgNPs is 14.69 nm.

Conclusion

In recent years a lot of work has been carried out for synthesis of fungal nanoparticles, showing their importance in different fields of science. *Oroxylum indicum* (L) vent. is an important medicinal tree, every part of this tree possesses medicinal properties. The endophytic fungi (*Colletotrichum ti* and *Paecilomyces sinensis*) isolated from this plant are employed for the biological synthesis of silver nanoparticles. More intense silver nanoparticles were obtained on the concen-

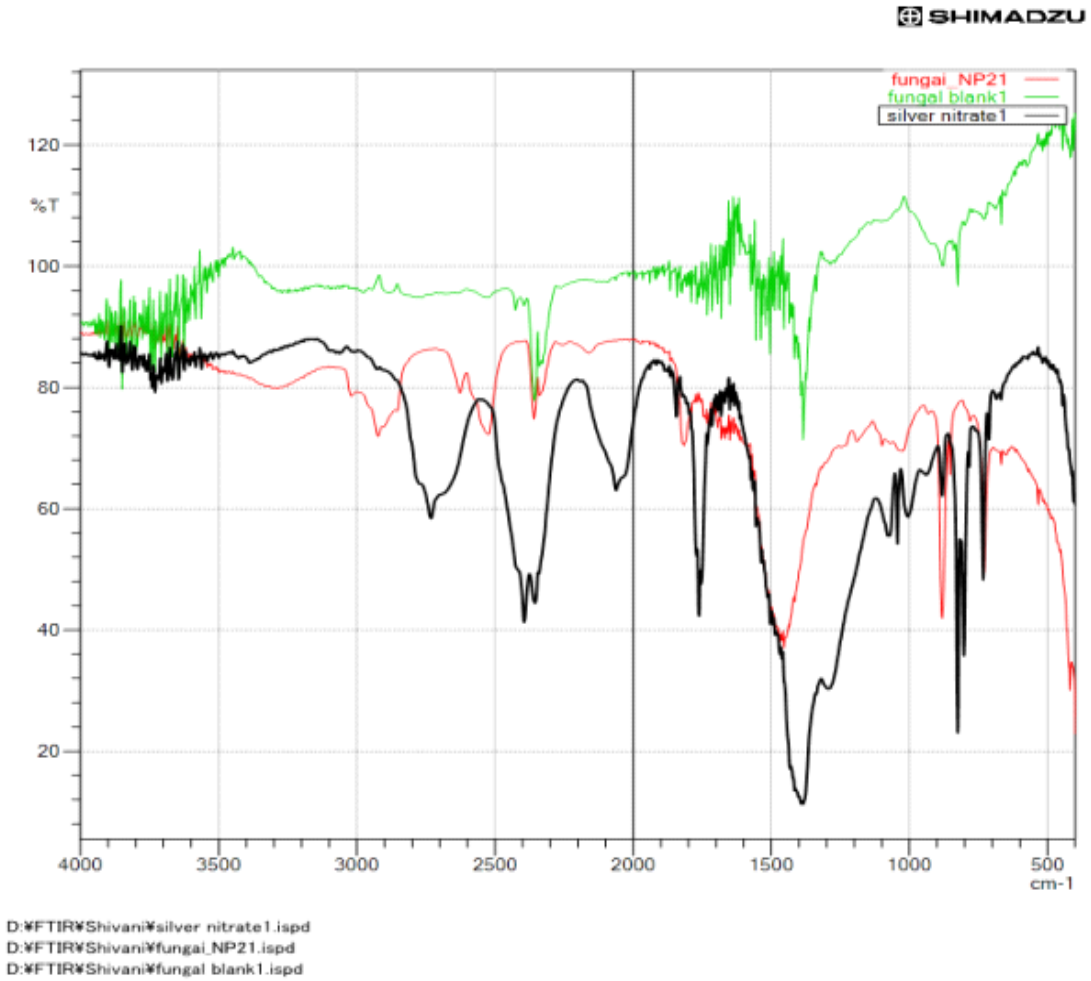


Fig. 12. FTIR analysis of *Colletotrichum ti*

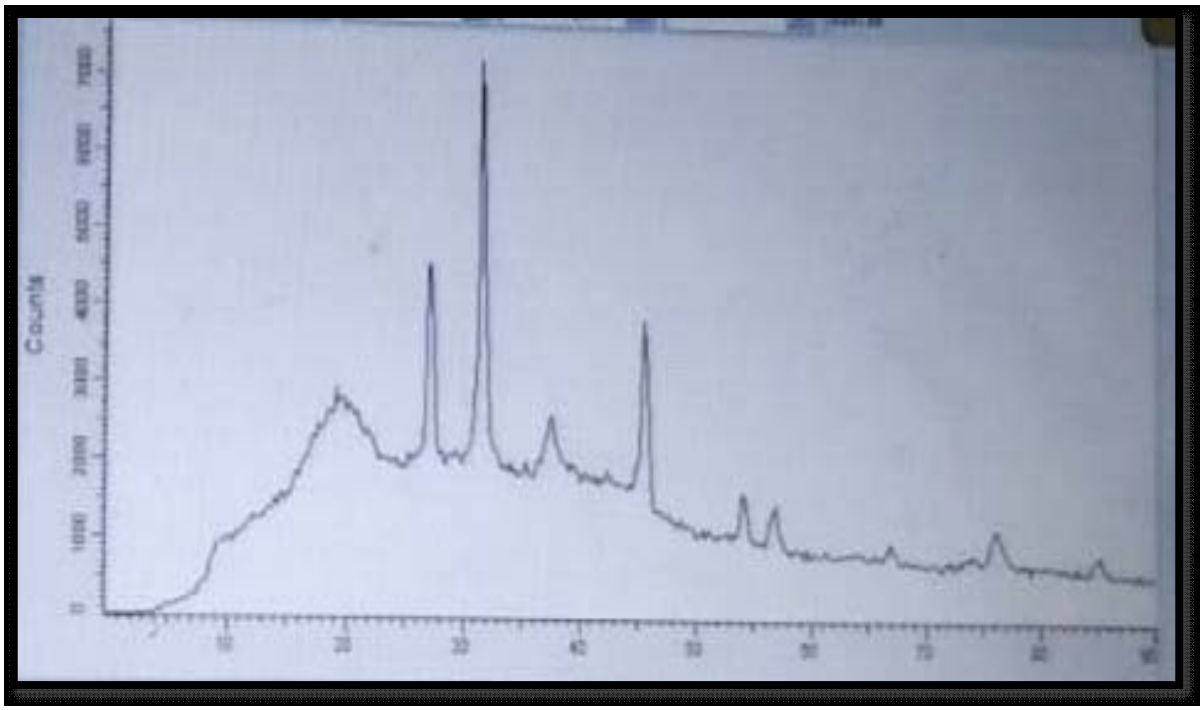


Fig. 13. XRD analysis of *Colletotrichum ti*

tration of 1.5 mM of silver nitrate. Intracellular AgNPs were synthesized from *Colletotrichum ti,* while in *Paecilomyces sinensis* created extracellular AgNPs. Synthesis of AgNPs from both fungi was confirmed with UV- Vis Spectrophotometer, as both of them showing highest absorbance on approx. 420 nm. The recorded FTIR spectra were analysed and the regions of vibrations were found to be 1000 cm^{-1}, 1050 cm-1, 1100 cm^{-1} and 1380 cm^{-1} in *P. sinensis*. In *Colletotrichum ti*., dry sample of mycelia, with AgNO3 and silver nitrate (control) was showing similar bending vibrations at 730 cm^{-1} and 880 cm^{-1} which are associated with C-H bond or C=C (alkenes), whereas the association causes a shift of vibrations from 1400 cm-1 to 1460 cm-1 which suggest the bending vibrations of C-H bonds. Size of intracellular AgNP in *Colletotrichum ti* and extracellular AgNP in *P. sinensis* was determined by XRD.

Acknowledgements

We acknowledge our sincere thanks to MHRD for providing financial assistance under the project of Bio- design Innovation Centre. The authors are grateful to Principal and Management of St. Aloysius College (Autonomous), Jabalpur (M.P) India for providing lab facility for the work.

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