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Biosynthesis of silver nanoparticles from *Trichoderma* species

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A total of 75 isolates belonging to five different species of *Trichoderma viz., T. asperellum, T. harzianum, T. longibrachiatum, T. pseudokoningii* and *T. virens* were screened for the production of silver nanoparticles. Although all the isolates produced nanoparticles, *T. virens* VN-11 could produce maximum nanoparticles as evident from the UV-Vis study. The highest Plasmon band was observed at 420 nm at every 24 h that attained maximum intensity at 120 h (0.543). The high resolution transmission electron microscopy (HRTEM) further provided the morphology of the nanoparticles. These nanoparticles were found single or aggregated with round and uniform in shape and 8-60 nm in size. The nitrate reductase activity of VN-11 was found to be 150 nmol/h/mL which confirmed the production of silver nanoparticles through reduction of Ag^{+} to Ag^{0}.

Keywords: Plasmon band, Silver nanoparticles, Transmission electron microscopy, *Trichoderma*

With the development of different physiological races of pathogen due to indiscriminate use of chemical pesticide, alternatively the researchers are looking for an alternative strategy to combat the pathogens without polluting the environment and avoiding the threat for the development of super race of pathogen. Thus there was a shift to begin synthesis process, which happens to be mostly of biological nature depending upon the theme of nano biotechnology.

In recent years, the green approach of nanoparticles synthesis by biological entities has been gaining great interest over various other physico-chemical methods, which are laden with many disadvantages. Biological systems offer unique promising features to tailor nanomaterials with predefined properties. It is known that certain microorganisms play an important role in remediation of toxic metals through reduction of the metal ions so long as they are not toxic in other ways. Fungi become the favourite choice for the nanotechnologist due to the wide variety of advantages they offer over bacteria, yeast, actinomycetes, plants, and other physico-chemical properties[1,2]. They are easy to handle, required simple nutrient, possess high wall-binding capacity, as well as intracellular metal uptake capabilities. Some of the fungi, which have been widely used for synthesis of nanoparticles include, *Trichoderma reseei*[3], *T. viride*[4], *Phytophthora infestans*[5], *Aspergillus niger*[6], *A. flavus*[7], *A. clavatus*[8], *Fusarium oxysporum*[9], *Verticillum* sp.[10,11], *Penicillium* sp.[12], *Pleurotus sajor-caju*[13].

However, the important challenging issues in current nanotechnology include the development of reliable experimental techniques for the synthesis of nanoparticles of different compositions and sizes along with high monodispersity. The use of microorganisms for the deliberate synthesis of nanoparticles is a fairly new and exciting area of research with considerable potential for further development. This study involves screening for the biological synthesis of silver nanoparticles using different species of the fungus *Trichoderma* and characterization of the synthesized silver nanoparticles by UV - Visible spectroscopy and transmission electron microscopy (TEM).

Materials and Methods

The present study was undertaken with a view to study the biosynthesis of silver nanoparticles in different *Trichoderma* species. The *Trichoderma*

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species were collected from Indian Type of Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi. Five different species were used in the study were *T. asperellum* (15 isolates), *T. harzianum* (14 isolates),. *T. longibrachiatum* (17 isolates), *T. pseudokoningii* (17 isolates), and *T. virens* (12 isolates)

All these isolates were cultured in PDB and incubated at 25 ºC in the BOD incubator for 5 days and used for the extracellular synthesis of silver nanoparticles.

Extracellular biosynthesis of Ag^+ nanoparticle using culture supernatant of Trichoderma species—For the synthesis of silver nanoparticles extracellularly, 50 mL aqueous solution of 1 mm silver nitrate ($AgNO_3$) was treated with 50 mL of *Trichoderma* supernatant solution in a 250 mL conical flask (*p*H adjusted to 8.5). The whole mixture was treated at 40 °C (200 rpm) for 5 days and maintained in the dark. Control experiments were conducted with uninoculated set.

UV visible studies—The reduction of silver ions was monitored by measuring the UV-VIS spectrum of the reaction medium at 24 h with time interval upto 120 h and their absorbance was recorded at 380, 400 and 420 nm using spectrophotometer.

High resolution transmission electron microscopy analysis—The high resolution transmission electron microscopy (HRTEM) analysis of extracellular by synthesized silver nanoparticle were prepared by drop-coating biosynthesized silver nanoparticles solution on carbon coated TEM grids (40 × 40 µm mesh size). Sample were dried and kept under vacuum in desiccators before loading them onto a specimen holder. HRTEM measurements were performed on a JEOL model 1200EX electron microscope operated at an accelerating voltage at 120 kV.

Nitrate reductase assay—The enzyme-nitrate reductase in culture filtrate of *Trichoderma* species with $AgNO_3$ was assayed according to the procedure followed by Harley[14] and Saifuddin *et al*[15]. An aliquot (5 mL) of 5-day fungal filtrate was mixed with 10 mL of assay medium (30 mM KNO_3 and 5% propanol in 0.1*M* phosphate buffer of *p*H 7.5) and incubated in the dark for 60 min. After incubation, nitrites formed in the assay mixture were estimated by adding 5 mL of sulphanilamide and NEED (N-(1-naphthyl) ethylene diamine dihydrochloride) solutions in to it. The developed pink color was measured in an UV–vis spectrophotometer. The enzyme activity was finally expressed in terms of nmoles of nitrite/mL/h.

Results

Extracellular synthesis of silver nanoparticles—In this study, five different species of *Trichoderma viz., T. asperellum, T. harzianum, T. longibrachiatum, T. pseudokoningii* and *T. virens* were screened for the synthesis of stable silver nanoparticles.

It was observed that the fungal supernatant (positive control) retained its original colour but the silver nitrate treated fungal supernatant turned dark brown at 10 h due to the deposition of silver nanoparticles.

The brown color of fungal cells can clearly be observed in Fig. 1. The picture of the conical flask containing the fungal cells after immersion in 1mM $AgNO_3$ solution after 120 h is shown in Fig. 1. It can be observed that the previous pale yellow colour of the reaction mixture is changed to the brownish colour after 120 h reaction.

Ultra violet-visible (UV-Vis) spectroscopy—Figure 2 shows the UV-Visible spectra of the silver nitrate solution challenged with the fungus. While no absorption band was observed in control a characteristic surface Plasmon absorption band at 420 nm was observed at every 24 h that attained maximum intensity at 120 h. It was found that the highest absorption band was found for *T. virens* isolate, VN-11 (0.543) followed by *T. longibrachiatum* isolate TL-3 (0.448); *T. asperellum* isolate TV-3 (0.411); *T. pseudokoningii* isolate TP-7 (0.387) and *T. harzianum* isolate TH-13 (0.232). The spectrum clearly shows the increase in intensity of silver nitrate solution with time, indicating the formation of increased number of silver nanoparticles in the solution. The solution was extremely stable even after a month of reaction, with no evidence of aggregation of particles.

Fig. 1—Conical flask containing *Trichoderma* biomass before (C) and after (A) exposure of Ag^+ ions for 120 h. B - $AgNO_3$ solution alone, D–PDB alone

Transmission electron microscopy—Transmission electron microscopy has provided further insight into the morphology and size details of the silver nanoparticles. The representative HRTEM picture recorded from the silver nano particle film deposited on a carbon coated copper TEM grid is shown in Fig. 3. In general, particles are isotropic (i.e., low aspect ratio) in shape and reasonably monodisperse. The sizes of silver nanoparticles were found 8-60 mm from the HRTEM images. The separation between the silver nanoparticles seen in the HRTEM image could be due to capping of proteins and would explain the UV-Vis spectroscopy measurements, which is characteristic of well- dispersed silver nanoparticles.

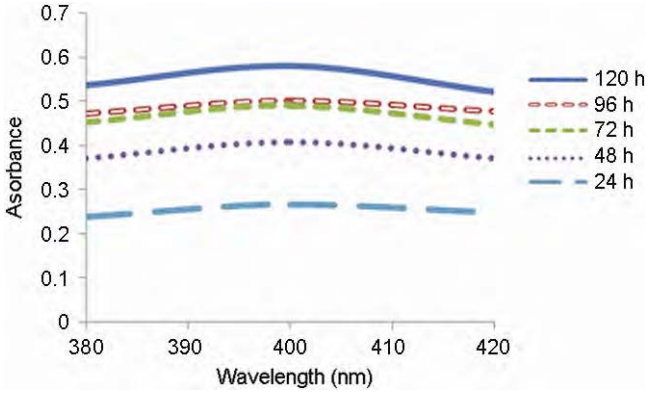

Fig. 2—UV–visible spctra of *Trichoderma virens* isolate VN-11 filtrate as function of time. The peak 420 nm corresponds to the surface Plasmon resonance of silver nanoparticles

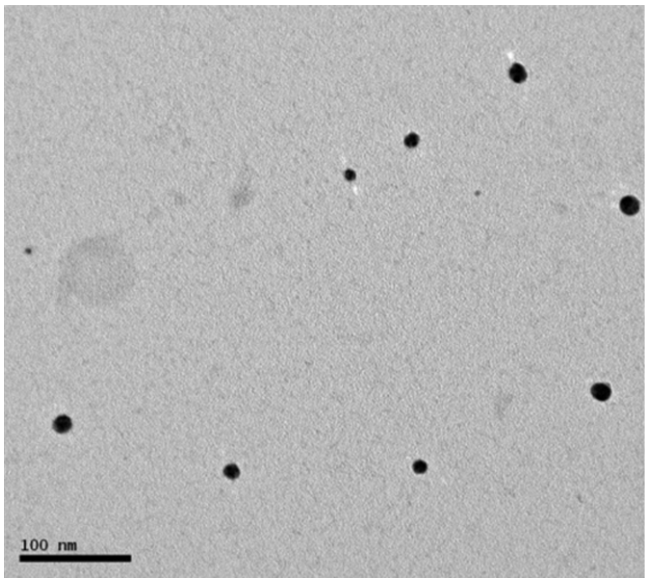

Fig. 3—HRTEM micrograph recorded from a drop-coated film of an aqueous solution incubated with *Trichoderma virens* isolate, VN-11 reacted with Ag^+ ions for 24 h. Bar represents 100 nm.

Nitrate reductase assay—The extracellular proteins secreted by the fungus are responsible for the reduction of Ag^+ to Ag^0. Hence, the role of reductases in the fungal filtrate was investigated by nitrate reductase assay. The nitrate reductase activity of the culture supernatant of of *T. virens, T. asperellum, T. harzianum, T. pseusokoningii* and *T. longibrachiatum* were found as 150, 250, 200, 50, 150 nmol/h/mL respectively (Fig. 4). Nitrate reductase activity of the isolate indicates the possible reason of the reduction of silver nitrate in to silver nanoparticles.

Discussion

Due to incredible properties, nanoparticles have become significant in many fields in the recent years, such as energy, health care, environment, agriculture, *etc*. The silver nanoparticles are prepared by using physical, chemical and biological methods[16]. However biological methods of nanoparticles synthesis would help to remove harsh processing conditions by enabling the synthesis at physiological *p*H, temperature, pressure, and at the same time at lower cost. Large number of micro organisms have been found capable of synthesizing inorganic nanoparticles composite either intra or extracellularly[17]. Among all, the fungi taking the centre stage of studies on biological generation of nanopartilces because of the tolerance and bioaccumulation[18]. Fungi are

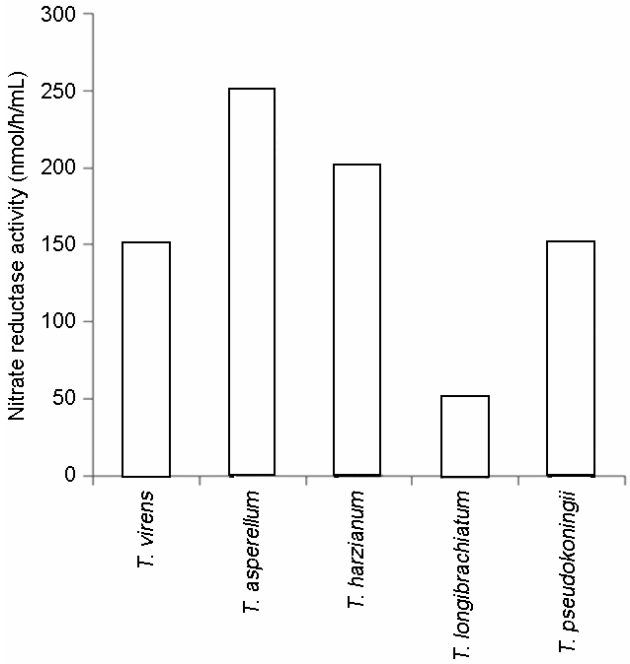

Fig. 4—Nitrate reductase activity (nmol/h/mL) of different *Trichoderma* species at 5th day of incubation with $AgNO_3$ solution.

efficient secretor of extra cellular enzymes and it can easily obtain large scale production of enzymes. Further advantages of using fungal mediated green approach for synthesis of metallic nanoparticles include economic viability and ease in handling biomass. Many fungi like *Verticilium* sp[10], *Fusarium oxysporum*[8], *Aspergillus fumigatus*[19], *Trichoderma asperellum*[20], *Phoma glomerata*[21] have been extensively used in the production of nanoparticles.

In the present investigation, *Trichoderma* species were evaluated for the production of nanoparticles. All these species invariably produced nanoparticles which was evident from the change of colour from pale yellow to dark brown. However the intensity of the colour produced was highest for *T. virens* isolate VN-11. The change of colour is primarily due to the surface of Plasmon resonance of deposited silver nano particles ie., the colour of the nanoparticles was due to coherent and collective oscillations of the surface electrons[22]. This was further confirmed by UV-vis spectra and HRTEM analysis. It is generally recognized that UV-Vis spectroscopy could be used to examine the size and shape controlled nanoparticles in aqueous suspensions[23]. The spectra clearly shows the increase in intensity of silver nitrate solution with time, indicating the formation of increased number of silver nanoparticles in the solution. It was observed that the peak was at 420 nm region is a characteristic of proteins and enzymes that have been found responsible for the reduction of metal ions by the fungal mediated synthesis of nanoparticles. The HRTEM analysis showed that the particles were almost uniform in shape and dimension. They were present as individual nanoparticle or as aggregates with size ranging from 8-60 nm. The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by a capping agent. As mentioned earlier, the silver nanoparticle solution, synthesized by the reaction of Ag-ions with *Trichoderma* spp., is exceptionally stable. This stability is likely to be due to capping with proteins secreted by the fungus and would explain the UV-Vis spectroscopy measurements, which is characteristic of well dispersed silver nanoparticles. Although the nitrate reductase activity of *T. asperellum* was found to be the highest, the isolate VN11 of *T. virens* could also reduce the Ag^+ to Ag^0 thus forming the silver nanoparticles. Various studies have indicated that NADH and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles[24-28] The fungus *Trichoderma* is also known to secrete the cofactor NADH and NADH-dependent enzymes, especially nitrate reductase, which may be acting as a scaffold or nucleating agent and might be responsible for the bioreduction of Ag^+ to Ag^- and the subsequent formation of silver nanoparticles[3]. The same enzyme later then acts as a capping agent, thus ensuring complete formation of stable nanoparticles[29].

Although in the present study *T. virens* was found to produce maximum nanoparticles, all of the *Trichoderma* species studied were efficient in production of nanoparticles. Thus these species could also be used in future to explore applications of the silver nanoparticles generated from the *Trichoderma* spp.

In conclusion, the synthesis of silver nanoparticles using different species of *T. asperellum, T. harzianum, T. longibrachiatum, T. pseudokoningii* and *T. virens* is reported. The nanoparticles were characterized by UV–vis and HRTEM. UV–vis results showed maximum silver nanoparticles production by culture filtrate of *T. virens* which was incubated with $AgNO_3$ solution. Crystalline nature of the nanoparticles is evident from bright circular spots and clear lattice fringes in HRTEM images. HRTEM analysis confirmed the uniform distribution of nanoparticles in all the above five species with an average size of 8-60 nm.

In the recent era, the main problem with the crop protection application is residue effect. Therefore, application of nano based formulation as compared to standard pesticides will be a better alternative to avoid excess chemicals in soil. Silver nanoparticles based formulations which are produced by strong biocontrol fungi can be evaluated against different plant pathogens in further studies.

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