Mycosynthesis of silver nanoparticles using endophytic fungus *Pestalotiopsis versicolor* and investigation of its antibacterial and azo dye degradation efficacy

Kavish Rajput¹, Shruti Agrawal², Jyoti Sharma¹, and Pavan Kr. Agrawal³

¹Department of Biotechnology, Govind Ballabh Pant Engineering College, Pauri Garhwal, Uttarakhand, India-246194
²Department of Microbiology, Sai Institute of Paramedical and Allied Sciences, Dehradun, Uttarakhand, India-248001
³Corresponding Author Email: p_k_agarwal@rediffmail.com

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**ABSTRACT**

The aim of this study was to synthesize safe, novel and cost-effective silver nanoparticles (SNPs) without using any synthetic reducing and capping agents. Endophytic fungal extract of *Pestalotiopsis versicolor* (Speg.) Steyaert was used for the synthesis of SNPs. The synthesized SNPs were tested for their antibacterial and azo dye degradation efficacy. The synthesized SNPs showed signature surface plasmon resonance at 425 nm. The size and morphology of SNPs were confirmed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Crystallite nature of the SNPs was confirmed by using X-ray diffraction (XRD). The SNPs exhibited strong antibacterial activity against both Gram positive and Gram negative bacteria and also showed good azo dye-degrading potential against Congo red, Rhodamine B and Orange G.

Keywords: Green synthesis, silver nanoparticles (SNPs), TEM; antibacterial, dye decolourization

**INTRODUCTION**

Nanotechnology is an emerging field of science that is making its way into a large assortment of marketed products including in health care, chemical sensing, biomedical sciences, drug-gene delivery and catalysis (Bindhani and Panigrahi, 2014). Plethoras of studies have shown that metal (eg. gold, silver, selenium, tellurium, platinum, palladium, silica, titanium, zirconium, quantum dots and iron ore) nanoparticles can be biosynthesized by various microorganism groups like actinomycetes, fungi and viruses. In recent years, silver nanoparticles have duly attracted special attention in different fields of sciences and being hailed as one of the superior metallic nanomaterials due to its successful applications in various products, such as medical devices, antimicrobial coatings, biosensing, food storage, paints, sunscreens, wound dressings and cosmetics (Ahamed et al., 2010).

Silver nanoparticles for the above mentioned purposes, can be synthesized using either physical, chemical or biological processes (Rajput et al., 2017). But both physical and chemical methods employ undesirable and hazardous chemicals in the synthetic process, which generate hazardous by-products and often leave unwanted residues in the final product (EL-Moslamy et al., 2017). Consequently, research on the use of biological systems like microbes, plants and enzymes for the synthesis of various nanoparticles is gaining importance (Rajput et al., 2016).

Studies on the microbial synthesis of SNPs demonstrated the use of certain fungi and bacteria in the successful synthesis of nanoparticles of varied sizes and shapes with potential medical applications. Fungi can produce larger amounts of nanoparticles as they secrete larger amounts of proteins which proportionately translate to higher productivity of nanoparticles (Mohanpuria et al., 2008). The mechanism of silver nanoparticle synthesis using fungi involve the following steps: 1. trapping of Ag⁺ ions at the surface of the fungal cells 2. reduction of the adsorbed silver ions by the enzymes present with the fungal system (Mukherjee et al., 2001). Examples of fungi used for the synthesis of silver nanoparticles so far are *Verticillium* sp. (Mukherjee et al., 2001), *Phoma* sp. 3.2883 (Chen et al., 2003), *Fusarium oxysporum* (Duran et al., 2005), *Phanerochaete chrysosporium* (Vigneshwaran et al., 2006), *Aspergillus fumigatus* (Bhainsa and D’ Souza, 2006), *Aspergillus flavus* (Vigneshwaran et al., 2007), *Fusarium semitectum* (Basavaraja et al., 2008), *Coriolus versicolor* (Singhi and Verma, 2009), *Fusarium solani* (Gade et al., 2009), *Aspergillus clavatus* (Verma et al., 2010) (*Trametes versicolor* (Duran et al., 2014).

Environment friendly microorganisms may minimize the involvement of hazardous chemicals in the production of SNPs, while bimetallic nanoparticle production may also find new avenues through reduction of the metal ions together (Iravani, 2014). Biotechnology approaches towards the synthesis of nanoparticles enjoy several advantages including easy scale up possibilities and economical feasibility (Cauerhff and Castro, 2013).

In this study, SNPs production using environment friendly processing with live microorganism *Pestalotiopsis versicolor* was tried successfully. The physical characteristics of the SNPs were evaluated using UV-visible Spectrophotometer, Scanning Electron microscopy (SEM), X-Ray Diffraction (XRD) and Transmission Electron Microscopy (TEM). SNPs were also subjected to biological assays to evaluate their potential antibacterial activity against Gram positive *Bacillus subtilis* and Gram negative *Pseudomonas aeruginosa* and *Salmonella enterica*. Experiments were also carried out to see the efficacy of the produced SNPs to decolourize azo dyes (eg. Congo red, Rhodamine B and Orange G).

**MATERIALS AND METHODS**

**Chemicals and reagents:** All chemicals and reagents used in the present study were of analytical and reagent grade. Congo red, Orange G and Rhodamine B were procured from Sigma and Fluka, USA. Silver Nitrate (Sigma Aldrich), Muller Hinton Agar and Malt Extract Agar were procured from Himedia.
Collection of leaves: Mature leaves and twigs of *Cupressus torulosa* D. Don, a gymnospermous tree (Accession number 115744, Botanical Survey of India, Dehradun) were collected from different locations of Ghurdauri, Pauri, Uttarakhand, India (latitude - 30° 18'55"N and longitude -78° 69'30"E). The collected material was transported to work place aseptically by keeping in sterile polyethylene bags stored at 4°C till further processing.

Isolation, sub-culturing and identification of endophytic fungus: For isolation of endophytic fungi, healthy plant tissues (leaves and twigs) of *Cupressus torulosa* were washed under running tap water and cut into inch sized pieces followed by surface sterilization with alcohol (70 %, 3 min), sodium hypochlorite (0.5 %, 1 min) and finally with sterile water before drying on a sterile filter paper. The dried samples were plated on Water Agar (WA) media amended with streptomycin (200 mg/L) and sealed with parafilm and incubated at 27°± 2°C for 2-4 weeks in an incubator. Fungal growth was observed in the form of cottony outgrowth from the incubated water media plates with sterilized plant tissue. From germinating fungi, hyphal tips were isolated and subcultured on a PDA medium by incubating at 28°C for 5-7 days (Sharma et al., 2016). The pure culture of fungal isolate maintained at 4°C and was sent to the National Fungal Culture Collection of India (NFCCI), ARI, Pune, India which was identified as *Pestalotiopsis versicolor* (Speg.) Steyaert (NFCCI 3978) belonging to the family *Amphisphaeriaceae*. This fungus was subsequently used for the biological synthesis of SNP's during the present investigations.

Biological synthesis of SNP's: For the synthesis of silver nanoparticles, the biomass of endophytic fungal isolate *P. versicolor* was grown aerobically in MGYP broth containing 0.3% Malt extract, 1% Glucose, 0.3% Yeast extract and 0.5% Peptone per 100 ml of distilled water. The inoculated flasks were incubated on orbital shaker at 25 ± 2°C and agitated at 120 rpm for 96 h (Selvi and Sivakumar, 2012). After incubation, biomass was harvested by filtering through Whatman filter paper followed by repeated washing with double distilled water to remove any medium component from the biomass. For this purpose 10 g (wet weight) was brought in contact with 100 mL of sterilized double distilled water for 48 h at 25 ± 2°C in a 250 mL Erlenmeyer flask and agitated again at 120 rpm. After the incubation, the cell filtrate was obtained by filtering it through Whatman filter paper No. 1. The filtrates were treated with 1 mM silver nitrate (Sigma Aldrich) solution in an Erlenmeyer flask and incubated for 24 hour at room temperature in the dark (Nabikhan et al., 2010). Control containing cell-free filtrate without silver nitrate solution was also run as standard.

UV-visible spectroscopy analysis: Change in colour of the mycelium free filtrate incubated with 1 mM silver nitrate solution visually observed over a period of time indicates the reduction of silver ions to silver nanoparticles. The silver nanoparticles formed in the mycelium free fungal filtrate were monitored by sampling of aliquots (1 mL) at different time intervals. Filtrate without aqueous solution of 1mM silver nitrate was maintained as control (Selvi and Sivakumar, 2012). Colour change was monitored under UV-visible spectrophotometer. Absorption measurements were carried out on UV-visible spectrophotometer (PerkinElmer Lambda-35 UV spectrometer) at a resolution of 1 nm between 300 and 700 nm ranges. The synthesized silver nanoparticles were stored at ambient temperature for six months and their stability was checked by measuring absorbance at 431 nm (Musarrat et al., 2010).

Scanning Electron Microscopy analysis: Scanning Electron Microscopy was used for observing the distribution and morphology of the silver nanoparticles (Sunkar and Nachiyar, 2012) and ZEISS machine was used for SEM analysis. Sample was filtered followed by loading of about 25 µl of the sample on to the stub provided for SEM analysis. Then the images of the synthesized silver nanoparticles were taken at different resolutions.

X-Ray Diffraction analysis: XRD analysis of the sample was carried out in the dry form of the sample, which was loaded on to a clean slide provided for XRD analysis so as to know the presence of the silver nanoparticles (Raja et al., 2017).

Transmission Electron Microscopic analysis: Sample was loaded on the carbon coated copper grid for the TEM analysis. Electronic beam was transmitted through an ultra-thin specimen (Swamy et al., 2014). The size of the silver nanoparticles was measured from image obtained by TEM (Ishida et al., 2014). The synthesized silver nanoparticles were stored at 4°C and was sent to the National Fungal Culture Collection of India (NFCCI), ARI, Pune, India which was identified as *Pestalotiopsis versicolor* (Speg.) Steyaert (NFCCI 3978) belonging to the family *Amphisphaeriaceae*. This fungus was subsequently used for the biological synthesis of SNP's during the present investigations.

Study of antibacterial activity: Antibacterial activity of the synthesized silver nanoparticles from fungus *P. versicolor* was investigated by standard agar-well diffusion method (Sondi et al., 2003). Bacterial pathogens used are Gram positive *Bacillus subtilis* and Gram negative *Salmonella enterica* and *Pseudomonas aeruginosa*. Muller Hinton Agar media was prepared, autoclaved and poured into Petri plates. After media solidification each bacterial strain was swabbed uniformly. Wells of equal diameter were made by the aid of cork borer. Different concentrations of synthesized silver nanoparticles were loaded into the wells having concentration 50, 100, 150 and 200 µl using sterile micropipette. The plates were then incubated for 24 h at 37°C, and the antibacterial activity was determined by measuring the diameter of the inhibition zone which is expressed in mm.

Dye decolourization using SNP's synthesized by *P. versicolor*: Silver nanoparticles synthesized from fungus *P. versicolor* were tested against Congo red, Rhodamine B and Orange G dyes. For decolourization study, 1 ppm concentration of Congo red, Rhodamine B and Orange G were prepared in the media (Deb, 2014). Dye in the media with fungal culture was maintained as control. Samples were incubated at 32°C for 24 h. After incubation, absorbance of the samples was taken using UV-visible spectrophotometer. For Congo red decolourization was tested at 498 nm, for Rhodamine B at 550 nm and for Orange G at 476 nm.

Statistical analysis: All experiments were carried out in triplicates and the collected data was statistically analysed.
RESULTS AND DISCUSSION

Morphotypic characterization of endophytic fungus:
Endophytic fungus isolated from C. torulosa was identified as Pestalotiopsis versicolor (Speg.) Steyaert (NFCCI 3978) which is an appendage-bearing anamorphic form and belongs to the family Amphisphaeriaceae. On the PDA medium, the isolated fungal culture appeared olive green in colour with thread like mycelia having colony with wavy margins, which turns white in colour after a week of incubation (Fig. 1a). The slide cultures prepared from this fungus showed septate hyphae with pigmented crystals along with. (Fig. 1b). Pestalotiopsis species have a worldwide distribution, particularly in tropical and temperate ecosystems, and are pathogenic to a wide range of hosts. Molecular studies have shown Pestalotiopsis to be a monophyletic genus. It is often isolated as endophyte and has been shown to produce a variety of bioactive secondary metabolites with potential medicinal use (Sharma et al., 2016). Many endophytic and pathogenic Pestalotiopsis species are also reported to inhabit on dead leaves, bark and twigs as saprophytes.

Fig. 1: a) Colony of Pestalotiopsis versicolor on the PDA media b) Reproductive structures of the endophytic fungal isolate under compound microscope

Production of biomass of endophytic fungus: Fungal biomass obtained after an incubation period (Fig. 2) in the MGYP broth media appears like feathered balls. The biomass is then separated and further used for the synthesis of silver nanoparticles.

Synthesis of silver nanoparticles from fungus P. versicolor: Silver ions were reduced when exposed to endophytic fungal extract of P. versicolor. The reaction mixture depicted a gradual change in colour at room temperature from pale yellow to yellowish brown after 24 hours of incubation, which indicates the formation of the SNP's (Fig. 3). This colour shift seems to be a result of the property of quantum confinement that could be a size dependent property of nanoparticles which affects their optical property. Synthesis of SNPs has been investigated by number of investigators utilizing many isolated fungal species including Penicillium (Sarsar et al., 2015), Aspergillus (Bhainsa and D'souza, 2006), Pleurotus (Devika et al., 2012), Fusarium (Duran et al., 2005), Trichoderma (Vahabi et al., 2011; Basavaraja et al., 2008).

Characterization of SNP’s

UV-visible Spectroscopy: The preliminary confirmation of SNPs synthesis can be done by UV-visible spectroscopy (Baker et al., 2015). The sharp absorption peak at 431 nm showed the synthesis of SNPs. The absorption peak of silver nanoparticles is reported to range from 400-450 nm (Ramteke et al., 2013). Absorption of silver nanoparticles was observed between 300-700 nm with peak at 450 nm which confirmed the formation of silver nanoparticles in the present case (Fig. 4). As has been already reported in the previous study SNPs showed peak in between the range of 400-450 nm in case of Pleurotus (Devika et al., 2012), Fusarium (Duran et al., 2005), Trichoderma (Vahabi et al., 2011 and Basavaraja et al., 2008).

Scanning Electron Microscopy analysis: Scanning electron microscopy (SEM) is employed for morphological characterization of nanoparticles at the nanometer to micrometer scale (Schaffer et al., 2009). SEM images were taken at the magnification of 10,000x for SNPs synthesized from fungus P. versicolor. To look at under SEM the SNPs were of variable sizes...
and shapes including spherical (Fig. 5). The results obtained by the SEM analysis of SNPs synthesized by the endophytic fungus are in conformity to the previous studies, undertaken on number of fungal genera including \textit{Penicillium} (Sarsar et al., 2015), \textit{Aspergillus} (Bhainsa and D’souza, 2006), \textit{Pleurotus} (Devika et al., 2012), \textit{Fusarium} (Duran et al., 2005), and \textit{M. kobus} (Salunke et al., 2015).

\textbf{X-Ray Diffraction analysis:} The crystalline natures of the synthesized SNPs were characterized using XRD which showed four peaks of 20 values at 38.45, 45.75, 66.45 and 77.95. XRD pattern clearly illustrates the crystalline nature of synthesized silver nanoparticles (Bar et al., 2009). The diffraction peaks obtained by the XRD analysis corresponds to the (1 1 1), (2 0 0) (2 2 0) and (3 1 1) planes of face centred cubic structure of metallic silver nanoparticles (Fig. 6) The reported size of these particles ranges between 5-50 nm (Theivasanthi and Alagar, 2012; Arokiyaraj et al., 2014). Selvi and Sivakumar (2012) also reported peaks at 38.28, 44.38, 64.54 and 77.54 of the SNP’s synthesized from \textit{Fusarium oxysporum}.

\textbf{Transmission Electron Microscopy analysis:} Size and morphology of the synthesized silver nanoparticles was determined by Transmission Electron Microscopy (TEM) analysis (Sadeghi and Gholamhoseinpoor, 2015). Silver nanoparticles showed highly variable morphology. Separation of silver nanoparticles could be due to capping by proteins (Vahabi et al., 2011). TEM image confirmed crystalline nature of silver nanoparticles (Fig. 7). The particles were monodisperse, with only a few particles of different sizes (Eppler et al., 2000; Mittal et al., 2013; Shankar et al., 2004).

\textbf{Antibacterial activity of synthesized silver nanoparticles:} In this study, the application of silver nanoparticles as an antibacterial agent was evaluated. The result of this study clearly demonstrated that the colloidal silver nanoparticles inhibited the growth and multiplication of the tested bacterial strains such as \textit{B. subtilis}, \textit{S. enterica} and \textit{P. aeruginosa}. Antibacterial activity of SNP’s synthesized using leaf extract of containing endophytic \textit{P. versicolor} was evaluated against human bacterial pathogens such as \textit{Bacillus subtilis} (441), \textit{Salmonella enterica} (733) and \textit{Pseudomonas aeruginosa} (424) (Sondi et al., 2003) (Fig. 8). The zone of inhibition (Table I) of Ag NPs against \textit{Bacillus subtilis} was found to be
mm at 50 µl, 100 µl, and 150 µl and 7 mm at 200 µl concentrations. Exactly similar pattern was observed against Salmonella enterica. As compared zone of inhibition observed against Pseudomonas aeruginosa was 5 mm at 50 µl, 6 mm at 100 µl, 7 mm at 150 µl and 8 mm at 200 µl of the sample. The SNPs synthesized from various endophytic fungi including Penicillium (Sarsar et al., 2015), Aspergillus (Bhainsa and D'Souza, 2006), Pleurotus (Devika et al., 2012) and Fusarium (Duran et al., 2005) are also reported to show antibacterial activity.

Antibacterial test of 1mM aqueous solution of silver nitrate was tested against Bacillus subtilis (441), Salmonella enterica (733) and Pseudomonas aeruginosa (424). When compared with the silver nanoparticles synthesized from the fungal isolates of C. torulosa (Fig. 9), the zone of inhibition of AgNO₃ against Bacillus subtilis was found to be 4 mm while it was 1mm against Salmonella enterica and 2 mm against Pseudomonas aeruginosa when 50 µl of the sample was used in each case (Table 2).

Table 1- Zone of inhibition (mm) of SNPs synthesized from P. versicolor

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Human Pathogen</th>
<th>Antibiotic (Tetracycline)</th>
<th>Fungi extract</th>
<th>Ag NPs(50µl)</th>
<th>100µl</th>
<th>150µl</th>
<th>200µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus subtilis</td>
<td>12</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Salmonella enterica</td>
<td>15</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas aeruginosa</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
</tbody>
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Fig 9: Antibacterial test of AgNO₃ against (a) Bacillus subtilis (441) (b) Salmonella enterica (733) and (c) Pseudomonas aeruginosa (424)

Dye decolourization activity by activity of synthesized silver nanoparticles: Decolourization of the dyes Congo red, Orange G and Rhodamine B using silver nanoparticles synthesized from endophytic fungal extract of P. versicolor (Table 3) was investigated. After 24 hour samples were withdrawn and analysed by UV-visible spectrophotometer at 498 nm for Congo red, 476 nm for Orange G and 550 nm for Rhodamine B. Significant decolourization of Congo red, Orange G and Rhodamine B was observed. The decolourization absorbance has been depicted in the table. The percentage (% of dye decolourization was calculated by using the formula:

% Decolourization = (O.D_control - O.D_sn) / O.D_control x 100

Silver nanoparticles synthesized from endophytic fungal extract of P. versicolor decolourized Congo red quite fast as compared with Orange G and Rhodamine B. These results suggested that SNP's can be used for the treatment of textile effluents also which is in conformity with the observations of Jalandoni-Buan et al. (2015).

CONCLUSION

In this study, SNPs were synthesized by biological means using endophytic fungal extract. The SNPs synthesis process at laboratory scale is quite inexpensive and non-toxic, eco-friendly as compared to the chemical methods. This bio-process yielded stable, spherical SNPs displaying considerable antibacterial activity. The synthesized SNPs also showed efficient degradation of azo dyes like Congo red dye and thus have potential in industrial application other than possible pharmaceutical application as antibacterial agents.

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