KAVAKA 49: 65-71 (2017)

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

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(Submitted in September, 2017; Accepted on December 25, 2017)

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* (Sanghi 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°18'35"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 27º ± 2ºC 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 sub cultured on a PDA medium by incubating at 28ºC for 5-7 days (Sharma *et al*., 2016). The pure culture of fungal isolate maintained at 4ºC 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 ± 2ºC 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 ± 2ºC 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).

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 37ºC, 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 32ºC 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.

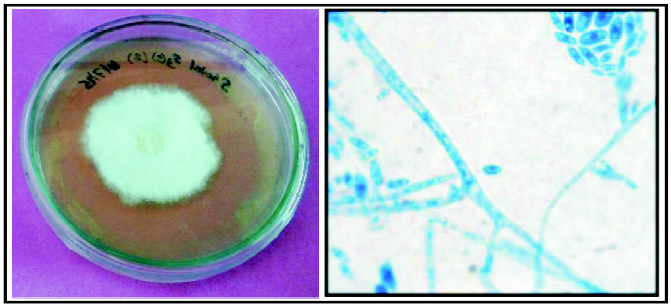

Fig. 1: a) Colony of *Pestalotiopsis versicolor* on the PDA media b) Reproductive structures of the endophytic fungal isolate under compound microscope

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.

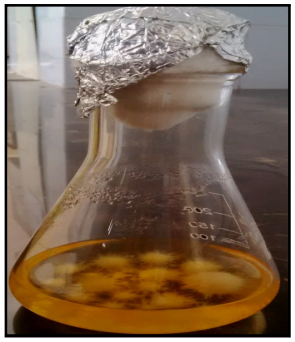

Fig 2 : Biomass of endophytic fungi in PDB after 7 days of incubation

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 SNPs (**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).

Fig 3: (a) Filtrate without $AgNO_3$ (b) Filtrate with synthesized SNP's

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).

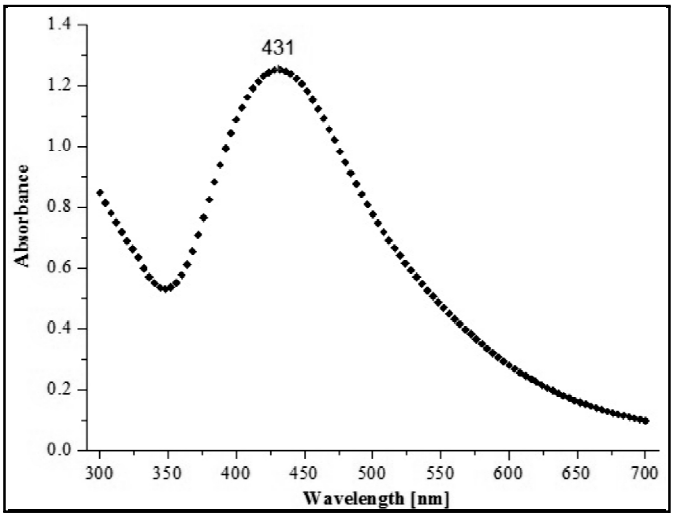

Figure 4: UV-visible absorption spectrum of synthesized SNPs from

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

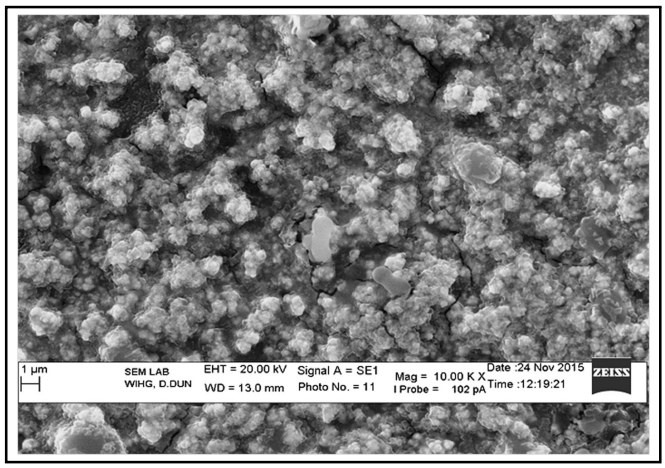

Fig 5: SEM image of Silver nanoparticles synthesized from *P. versicolor*

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 *Penicillium* (Sarsar *et al*., 2015), *Aspergillus* (Bhainsa and D'souza, 2006), *Pleurotus* (Devika *et al*., 2012), *Fusarium* (Duran *et al*., 2005), and *M. kobus* (Salunke *et al*., 2015).

X-Ray Diffraction analysis: The crystalline natures of the synthesized SNP's were characterized using XRD which showed four peaks of 2θ values at 38.45, 45.75, 66.45 and 77.95. XRD pattern clearly illustrates the crystalline nature of synthesised 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

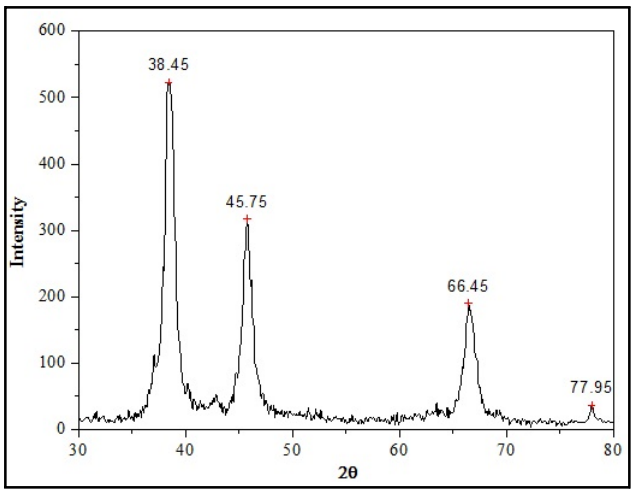

Fig 6: XRD analysis of silver nanoparticles synthesised from *P. versicolor*

Fusarium oxysporum.

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

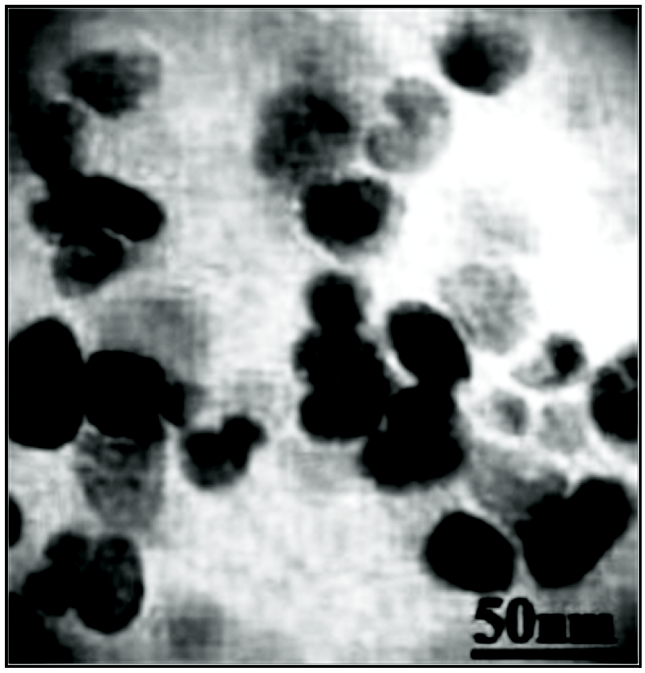

Fig 7: TEM image of synthesized SNP's from fungal

different sizes (Eppler *et al*., 2000; Mittal *et al*., 2013; Shankar *et al*., 2004).

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 *B. subtilis, S. enterica* and *P. aeruginosa.* Antibacterial activity of SNP's synthesized using leaf extract of containing endophytic *P. versicolor* was evaluated against human bacterial pathogens such as *Bacillus subtilis* (441), *Salmonella enterica* (733) and *Pseudomonas aeruginosa* (424) (Sondi *et al*., 2003) (**Fig. 8**). The zone of inhibition (**Table 1**) of Ag NPs against *Bacillus subtilis* was found to be

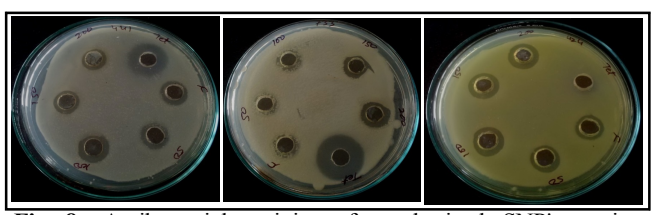

Fig 8: Antibacterial activity of synthesized SNP's against (a) *Bacillus subtilis (441*), (b) *Salmonella enterica (733*) and (c) *Pseudomonas aeruginosa* (424)

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

S. No.	Human Pathogen	Antibiotic (Tetracycline)	Fungi extract	Ag NP's(50µl)	100µl	150µl	200µl
1.	*Bacillus subtilis*	12	5	6	6	6	7
2.	*Salmonella enterica*	15	5	6	6	6	7
3.	*Pseudomonas aeruginosa*	1	4	5	6	7	8

6

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

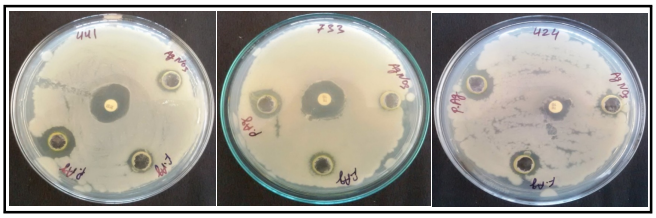

Fig 9: Antibacterial test of $AgNO_3$ against (a) *Bacillus subtilis (441*) (b) *Salmonella enterica (733*) and (c) *Pseudomonas aeruginosa* (424)

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

Table 2: Zone of inhibition (mm) of $AgNO_3$ and silver nanoparticles synthesized from *P. versicolor* of *C. torulosa*

S. No.	Bacterial pathogen	$AgNO_3$	Fungi SNPs
1	*Bacillus subtilis*	4	6
2	*Salmonella enterica*	1	3
3	*Pseudomonas aeruginosa*	2	4

fungal isolates of *C. torulosa* (**Fig. 9**), the zone of inhibition of $AgNO_3$ 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)**.

Dye decolourization 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

Table 3: % Decolourization of dyes by silver nanoparticles synthesized from *P. versicolor.*

Time duration (in days)	% reduction of dye Congo red	% reduction of dye Orange G	% reduction of dye Rhodamine B
1	9.70	16.90	12.24
2	14.76	26.47	22.49
3	22.63	35.64	34.68
4	41.35	43.99	48.40
5	57.38	52.54	62.83
6	70.46	60.08	75.73
7	89.45	69.65	82.62
8	91.56	83.50	89.10

decolourization absorbance has been depicted in the table. The percentage (%) of dye decolourization was calculated by using the formula:

$$\% \text{ Decolourization} = (O.D_{control} - O.D_{test} / O.D_{control}) \times 100$$

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.

ACKNOWLEDGMENT

We gratefully acknowledge TEQIP-II and G. B. Pant Institute of Engineering and Technology, Pauri, Garhwal for providing financial support for conducting this research.

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