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ORIGINAL ARTICLE

Mycosynthesis of silver nanoparticles bearing antibacterial activity

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Abstract Mycosynthesis of silver nanoparticles was achieved by endophytic *Colletotrichum* sp. ALF2-6 inhabiting *Andrographis paniculata*. Well dispersed nanoparticles were characterized using UV–Visible spectrometry with maximum absorption conferring at 420 nm. FTIR analysis revealed possible biomolecules reducing the metal salt and stabilization of nanoparticles. XRD analysis depicted the diffraction intensities exhibiting between 20 and 80 °C at 2theta angle thus conferring the crystalline nature of nanoparticles. Morphological characteristic using TEM revealed the polydispersity of nanoparticles with size ranging from 20 to 50 nm. Synthesized nanoparticles exhibited bactericidal activity against selected human pathogens. Nanoparticles mode of action was carried out to reveal DNA damage activity. Thus the present investigation reports facile fabrication of silver nanoparticles from endophytic fungi.

1. Introduction

Nanotechnology is emerging field of science which involves synthesis and development of materials at nanoscale (Naveen et al., 2010). It has opened new avenues by intersecting with interdisciplinary field of science for innumerable applications (Morones et al., 2005). These nanomaterials are used in various fields such as electronic devices, sensor technology, signal enhancers, optical sensors, biomarkers, magnetic, catalysis, optical polarizability, electrical conductivity, antimicrobialactivity and drug delivery to tumor cells (Nilsson et al., 2007; Duncan, 2011; Costa-Fernandez et al., 2006; Schrand et al., 2008; Naz et al., 2014; Shiraishi and Toshima, 2000; Ning et al., 2008; Sondi and Salopek-Sondi, 2004; Aliosmanoglu and Basaran, 2012; Syed et al., 2013). Hence nanoparticle research has gained tremendous interest especially use of silver nanoparticles has myriad applications in biomedical sector with large number of products already in market such as ointments, dressing materials and packaging materials (Sadowski et al., 2008). Silver nanoparticles are reported to bear antimicrobial property against array of pathogenic microorganisms. Mode of action of silver nanoparticles as per the scientific records suggests that silver nanoparticles have different mode of action for instance they are known to interact with the thiol groups of vital enzyme, cause pit on the cell wall and damage the DNA of the organism (Baker and Satish, 2012b).

In future decades much more applications of silver nanoparticles are expected to be reported but one of the major

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constraint is the synthesis protocols of nanoparticles. Most popular and widely used conventional methods for the synthesis of nanoparticles are bound with various implications such as use of toxic chemicals and generation of high energy resulting in environmental pollution (Baker et al., 2013a). Owing to which eco-friendly process for nanoparticle synthesis has gained impute importance in recent years with large number of biological entities are constantly being explored for reduction of metal salts and synthesize nanoparticles with desire size and shape (Baker et al., 2013b). Use of microorganisms is known to have better advantage compared to plant species as microorganisms can be cultured and preserved for constant usage whereas use of plant species may pose a risk and imbalance to plant diversity especially the harvesting of endangered species. Among the microbial diversity encompasses the plethora of microorganisms called endophytes which have reported to be one of the untapped and rich sources of bioactive compounds bearing biological activities. Endophytes are of great potentials to secrete structurally diversified metabolites (Baker and Satish, 2012a). But one of the least studied areas in the field of endophytes is their evaluation for nanoparticle synthesis (Baker and Satish, 2012c). The interference of endophytes with nanoparticles is relatively new and is expected to have significant impact. Fungal endophytes are reported to secrete diverse group of biomolecules extracellularly which are capable of reducing metal salts at rapid scale under optimized conditions. One such endophyte is isolated from healthy leaf of *Andrographis paniculata* and employed for rapid synthesis of silver nanoparticles. The synthesized nanoparticles were evaluated for bactericidal activity against significant human pathogens. Mode of action of nanoparticles was determined with treatment of DNA with silver nanoparticles. Thus the study highlights the mycosynthesis of silver nanoparticles bearing bactericidal activity using *Colletotrichum* sp. ALF2-6 and the results obtained are promising enough to envision the emerging role of endophytes for facile reduction of metal ions.

2. Materials and methods

2.1. Sample collection and isolation of endophytes

Healthy leaves of *A. paniculata* were collected from southern part of India. The samples were thoroughly washed in running tap water followed by sterile distilled water to remove adhered soil particles. Samples were excised into small segments (0.4–0.5 cm) using sterile scalpel and segments were subjected to surface sterilization by sequential steps as followed by protocol of Rakshith et al. (2013). Segments were placed on to the surface of water agar media amended with chloramphenicol (150 mg/L) and incubated at 26 °C in an alternate cycle of 12 h dark and 12 h light for 3 weeks. Colonies emerging from surface sterilized plant segments were subcultured until further use.

2.2. Optimization and mycosynthesis ofsilver nanoparticles

Fermented cell free extract of *Colletotrichum* sp. ALF2-6 was treated with 1 mM of silver nitrate and incubated at different temperatures ranging from 30 to 80 °C and pH of the reaction mixture was varied from acidic to alkaline. Samples were monitored periodically for the synthesis of nanoparticles with the aid of UV–Visible spectrophotometer operating at a resolution of 1 nm (Baker et al., 2014).

2.3. Characterization of mycosynthesized silver nanoparticles

The X-ray Diffraction (XRD) patterns were obtained on desktop X-ray diffractometer operating at 30 kV and at a current of 15 mA with Cu radiation (k = 1.5404 Å). The diffracted intensities were recorded from 0° to 80° of 2θ angles. X-ray photoelectron spectra were recorded Rigaku miniflex 2 instrument. FTIR spectra of silver nanoparticle solution were recorded on Perkin Elmer spectrum one B in diffuse reflectance (DRS) mode at a resolution of 2 cm^{-1}. Transmission electron microscopy (TEM) analysis of silver nanoparticles was prepared on carbon-coated copper TEM grids. TEM scan was performed using a TECHNAI-T12 JEOL JEM-2100 Transmission electron microscope operated at a voltage of 120 kV with Bioten objective lens. Subsequently, the particle size was ascertained using a Gatan ccd Camera (Baker et al., 2014).

2.4. Phenotypic and genotypic characterization of the fungal endophyte

Phenotypic characterization was carried out by mounting part of the viable culture and observed under microscope to determine the morphological characteristics (Naveen et al., 2010). Genotypic characterization of fungus was carried out using DNA isolation kit (Hi pura, HiMedia, Mumbai, India) according to manufacturer's instruction. In brief, isolation of fungal genomic DNA and 18S rDNA region was amplified, the PCR product was bi-directionally sequenced using forward (ITS1) and reverse (ITS4) primers which produced an expected amplicon size of ~500–600 base pairs. Sequencing results were processed using Bio Edit software (Hall, 1999). Processed sequences were subjected to BLAST tool at NCBI to assign putative identity, designation of operational taxonomic units based on sequence similarity measures and phylogenetic inference. Partial nucleotide sequences were deposited in NCBI GenBank to procure accession number. Neighbor joining analysis of endophyte mediating silver nanoparticle synthesis and close relatives retrieved from Genbank using Clustal W and Bio Edit softwares (Hall, 1999; Thompson et al., 1997). Alignments were manually edited where necessary and phylogenetic analyses were performed to assess phylogenetic affiliation using Molecular Evolutionary Genetics Analysis software MEGA6 (Tamura et al., 2011).

2.5. Bactericidal activity of mycosynthesized silver nanoparticles

Bactericidal activity of mycosynthesized silver nanoparticles was evaluated against *Escherichia coli* (MTCC 7410), *S*almonella *typhi* (MTCC 733), *B*acillus *subtilis* (MTCC 121) and *Staphylococcus aureus* (MTCC 7443) and all test pathogens were procured from Microbial Type Culture Collection, Chandigarh, India. Inoculum of test pathogens was prepared to obtain 5×10^5 CFU (Colony forming unit) and bactericidal activity was determined via CFU assay. In brief, Mueller–Hinton agar plates were supplemented with silver nanoparticles with different concentrations (25, 50, 75 and 100 μg/mL). Test inoculum was smeared onto the plates and incubated for 24 h at 37 °C and one control was maintained without addition of silver nanoparticles. The colonies were counted and validated with the control plate to determine the effect of nanoparticles (Sondi and Salopek-Sondi, 2004). Minimal Inhibitory

Concentration was determined by broth micro-dilution technique based on the protocol described by Sarker et al. (2007). Resazurin dye was used as a growth indicator to check the efficacy of nanoparticles against the test organisms. Gentamicin was used as positive control and bacterial growth in the plate was inspected visually as well as ELISA microtitre plate reader.

2.6. DNA damage activity of mycosynthesized silver nanoparticles

DNA damage study was demonstrated according to the protocol of Vahdati and Sadeghi, 2013. Silver nanoparticles were treated with DNA isolated from *Escherichia coli* and incubated for 30 min. Control DNA without treatment of nanoparticles was served as positive control and silver nanoparticles without bacterial DNA as negative control. Electrophoration was carried out using 1% agarose gel at 75 V for 30 min.

3. Results and discussion

The results obtained in the present investigation attributes toward the emerging role of endophytes for synthesis of nanoparticles. Reduction of silver nitrate to silver nanoparticles was observed visually with change in color of the reaction mixture to brown (Fig. 1). This is due to the surface plasmon resonance of the silver nanoparticles (Krishnaraj et al., 2010). The synthesis was rapid and completed within 20 min under optimized condition. It was observed that the synthesis was maximum at elevated temperature above 50 °C as the temperature increased the synthesis was efficient and rapid which confirmed that elevated temperature influenced the synthesis. Similarly alkaline pH favored the synthesis compared to acidic pH, these results are in accordance with the previous scientific reports (Rashidipour and Heydari 2014). When cited for reports on *Colletotrichum* sp., it is reported that *Colletotrichum gloeosporioides* could efficiently synthesize silver nanoparticles this finding highlights the possible metabolic process and secretion of reducing agent as their extracellular metabolite which is mediating the synthesis (Ravindra and Rajasab, 2014).

3.1. Biosynthesis and characterization of mycosynthesized silver nanoparticles

Colletotrichum sp. ALF2-6 culture filtrate reduced silver nitrate and synthesized well dispersed silver nanoparticles which was confirmed using UV visible spectrum with peak conferring between 300 nm and 600 nm and maximum absorbance at 420 nm as shown in (Fig. 2). Further possible interaction of biomolecules present in the culture filtrate of *Colletotrichum* sp. ALF2-6 which mediates the nanoparticle synthesis and stabilized nanoparticles was evaluated using FTIR spectra which displayed different vibrational stretches between 400 and 4000 cm^{-1}. These vibrational stretches were predicted based on their earlier reports showed at 3325.22 cm^{-1} which corresponds to primary amines (Hussein, 2010), 1635.2 cm^{-1} corresponds to carbonyl group (Kunwong et al., 2011), 664.69 cm^{-1} and 601.63 cm^{-1} corresponds to C–H bend alkynes (Baseri and Baker, 2011) stretch as shown in (Fig. 4). These results obtained correlate the findings of previous scientific literatures which clearly suggest the metabolic diversity of the fungal communities responsible for

Figure 1 Color change in synthesized SNP.

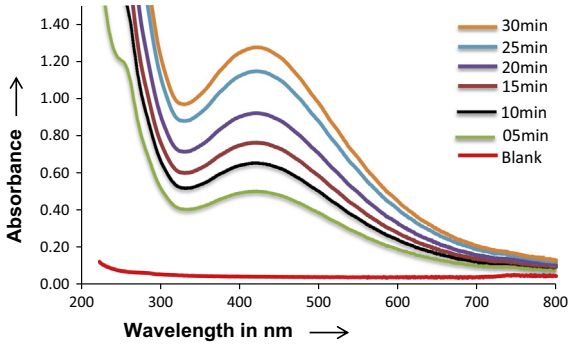


Figure 2 UV-Visible spectra of mycosynthesized silver nanoparticles.

the mediating the synthesis of the nanoparticles. Earlier FTIR analysis reveals the role of biomolecules which reduce the silver nitrate and bind onto the nanoparticles and stabilize them which prevents the aggregation. The crystalline nature of the synthesized nanoparticles depicted with Bragg's peak 38.2°, 44.42°, 64.5°, 77.4° (Fig. 3) corresponding to the cubic facets of the particles which justifies the standard diffraction pattern of silver nanoparticles and the result obtain is in agreement with the standard diffraction of earlier scientific reports (Qian et al., 2013). TEM micrograph revealed the polydispersity of nanoparticles with size ranging from 5 to 60 nm with myriad shapes such as spherical, near to spherical, triangular and hexagonal (Fig. 5a). Histogram was plotted by counting the number of silver nanoparticles and grouped according to size as in (Fig. 5b). The extracellular synthesis of silver nanoparticles is more advantageous owing to the fact that it forms easy to downstream the nanoparticles compared to the intracellular synthesis. The obtained nanoparticles will be free from any bio-mass, toxic material and any solvent residues.

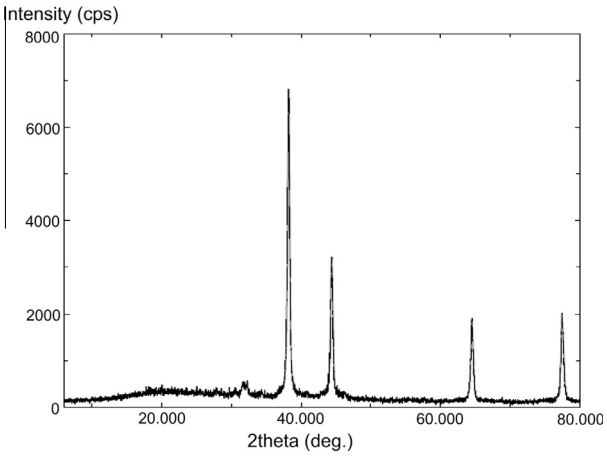


Figure 3 X-ray diffraction pattern of mycosynthesized silver nanoparticles.

3.2. Fungal Identification and phylogenetic affiliation

Phenotypic characteristic served conidial masses with light salmon color. The size of the conidia ranged from 10 to 25 µm long with curved tapered ends, truncated base, aseptate and hyaline. Chlamydospores were dark brown, clustered and sometimes chained together (Fig. 6a and b). Genotypic characterization was successful with PCR amplification and production of amplicons ranging 600–700 bps. ITS sequence was deposited in Genbank Database and avail the accession number KM113381. Phylogenetic analysis by neighbor-joining method indicates the closest proximity and exhibited 100% homology with *Colletotrichum chlorophyti* (Fig. 7).

3.3. Evaluation of antibacterial activity of mycosynthesized silver nanoparticles.

Antibacterial activity of silver nanoparticles synthesized from *Colletotrichum* sp. via colony forming unit assay showed increased number of colonies in control plate compared to silver nanoparticles treated plates and colonies number reduced gradually as the concentration of nanoparticles increased from 0 to 100 µg/mL as shown in the Fig. 8. Among the test pathogens *S. aureus* was more sensitive to silver nanoparticles at 50 µg/mL concentration with no growth and rest of the test organisms were susceptible at 100 µg/mL with no colonies observed onto the plate. This result was in accordance with

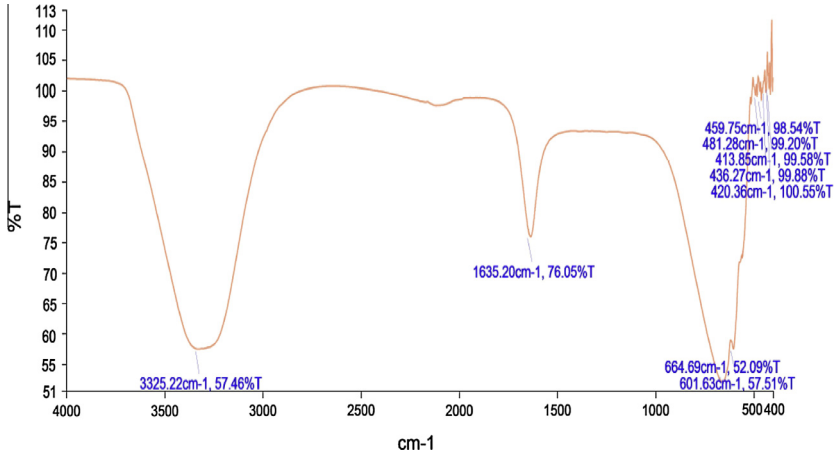
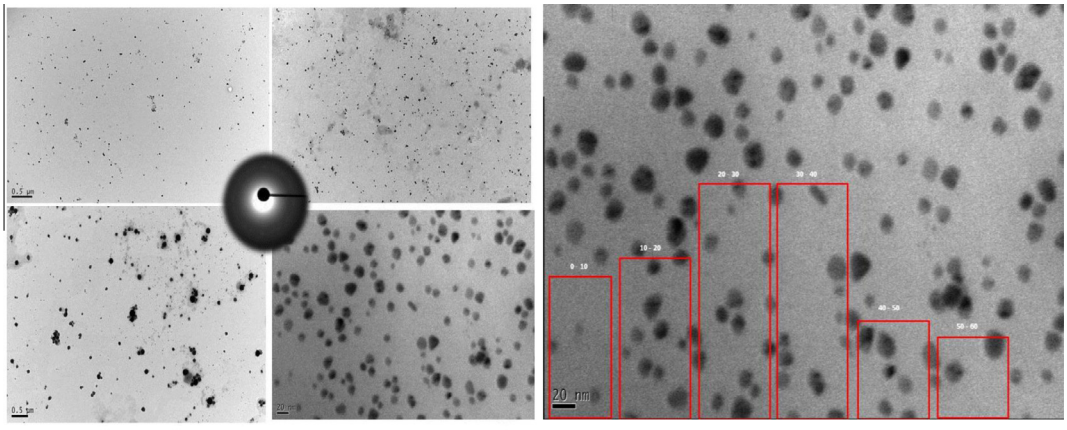


Figure 4 FTIR spectra of mycosynthesized silver nanoparticles.



(a) **(b)**

Figure 5 TEM micrograph of mycosynthesized silver nanoparticles.

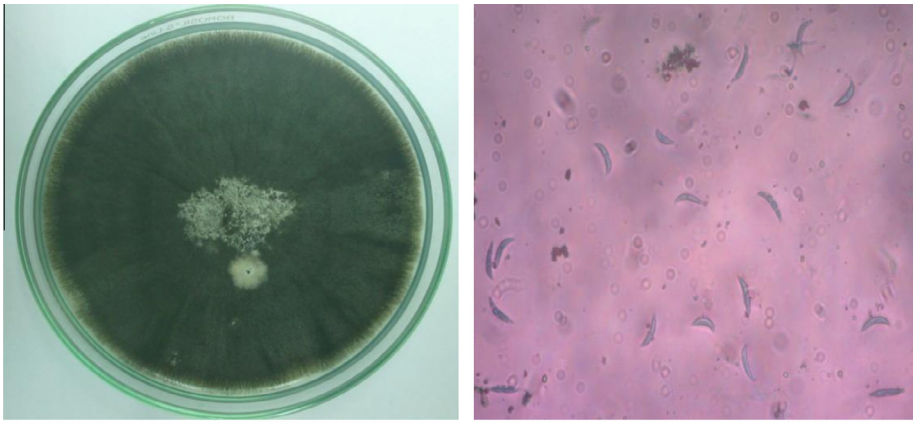
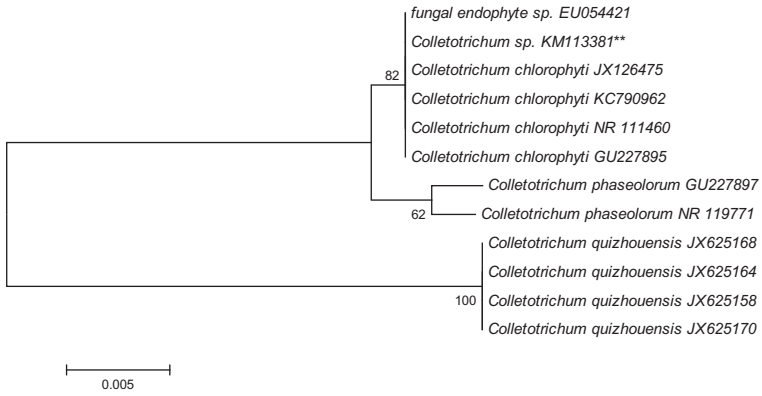


Figure 6 Morphology of *Colletotrichum* sp. ALF2–6.



****Colletotrichum** sp. ALF2-6- Isolate studied under present study.

Figure 7 Neighbor-joining phylogenetic tree showing the relationship among *Colletotrichum* sp.

the findings of Sondi and Salopek-Sondi, 2004, which reports that *E. coli* was susceptible at concentration 50–60 µg/mL. Similarly minimal inhibitory concentration of silver nanoparticles showed silver nanoparticles at concentration 12.5 µg/mL were minimal to inhibit the growth of *S. aureus*, *S. typhi*, *B. subtilis* and 25 µg/mL concentration of silver nanoparticles showed inhibition against *E. coli* (Table 1). The result was validated with standard gentamicin.

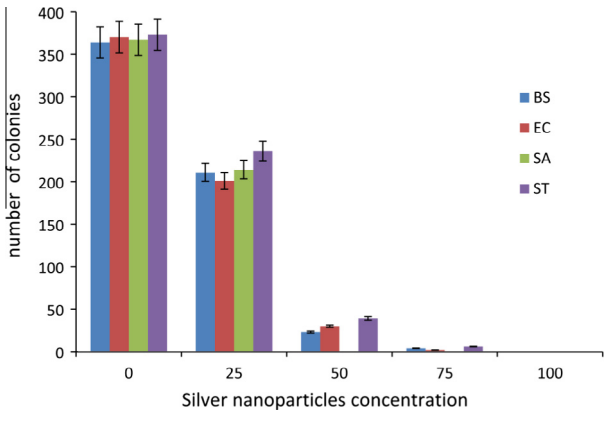


Figure 8 Graphical representation for antibacterial activity of mycosynthesizedsilver nanoparticles.

3.4. DNA damage activity

The electrophoresis gel showed intact band with the control DNA without silver nanoparticles. Whereas DNA treated with silver nanoparticles showed deformed and damage of DNA indicating the action of silver nanoparticles by forming a smear in lane3 as shown in (Fig. 9). These results were in accordance with the findings of the Vahdati and Sadeghi

Table 1 MIC of SNP against test pathogens.

Sl no.	Test pathogens	Standard gentamicin	MIC of SNP *Colletotrichum* sp.
1	*B. subtilis*	1.56 µg/mL	12.5 µg/mL
2	*E. coli*	1.56 µg/mL	25 µg/mL
3	*S. aureus*	1.56 µg/mL	12.5 µg/mL
4	*S. typhimurium*	1.56 µg/mL	12.5 µg/mL

Note: + control(Gentamicin), SNP-Silver nanoparticle.

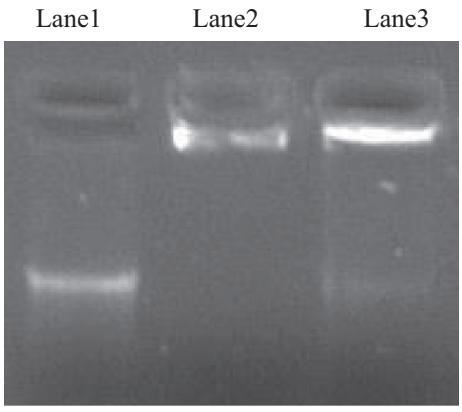


Figure 9 DNA damage study of mycosynthesized silver nanoparticles. Note: Lane1-DNA, Lane2-SNP, Lane3-SNP + DNA.

(2013), showing action of nanoparticles on plasmid DNA of *E. coli*.

4. Conclusion

Till date majority of the reports on biological synthesis of nanoparticles have just reported the synthesis protocol and very limited number of studies demonstrate the application part keeping this lacuna the present study was designed and executed to report the facile mycosynthesis of silver nanoparticles using potent endophyte *Colletotrichum* sp. ALF2-6 The study not only highlights the emerging role of endophytes for the synthesis of nanoparticles but also envisions the mode of action of these biologically synthesized nanoparticles on DNA damage of the *E. coli*. Thus the present study forms first report of endophytic *Colletotrichum* sp. ALF2-6 from plant *A. paniculata* mediating silver nanoparticle synthesis.

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