Chapter 2

Characterization of silver nanoparticles biosynthesized using the selected fungi
INTRODUCTION

There are a number of techniques that are available for detection and characterization of silver nanoparticles and a particular technique cannot be selected as the best for their characterization. However, the techniques are chosen depending upon the method of synthesis, amount of sample, information required and the cost of analysis. In the present work, the optical properties, size and structure of the biosynthesized nanoparticles were characterized by UV-Vis spectroscopy, Transmission Electron Microscopy (TEM), Selected Area Electron Diffraction (SAED), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray spectroscopy (EDAX).

UV-Vis spectroscopy is a technique used to quantify the light that is absorbed and scattered by a sample (a quantity known as the extinction, which is defined as the sum of absorbed and scattered light). In its simplest form, a sample is placed between a light source and a photodetector, and the intensity of absorbed light is measured. The absorbance can be used to measure the concentration of a solution by using Beer-Lambert’s Law. When the wavelength is varied and the absorbance is measured at each wavelength, a (wavelength, absorbance) graph can be drawn using appropriate software. The data is typically plotted as extinction as a function of wavelength. Nanoparticles have optical properties that are sensitive to size, shape, concentration, agglomeration state, and refractive index near the nanoparticle surface, which makes UV-Vis spectroscopy a valuable tool for identifying, characterizing, and studying these materials. Some metals like Au, Ag, Al, Cu, and Na exhibit colours due to strong absorption bands in the visible region when they are in the nanostate but these colours are absent in the case of individual atoms as well as in the their bulk counterparts.
Nanoparticles of certain metals, such as gold and silver, strongly interact with specific wavelengths of light. Under the influence of an electrical field of an incident light photons, there is a plasmon excitation of the conduction electron at the nanoparticles’ surface. This resonance, which takes place at a certain energy of the incident light photon, results in an optical absorption, the so-called surface plasmon absorption or surface plasmon resonance absorption. UV–Visible absorption spectroscopy is the most widely used method to confirm nanoparticles formation, for characterizing the optical properties and electronic structure of nanoparticles, as the absorption bands are related to the diameter and aspect ratio of metal nanoparticles (Abdelhalim et al., 2012).

Electron microscopy is the most useful method to determine the size and shape distributions of the nanoparticles. Scanning Electron Microscopy (SEM) uses a high energy electron beam but the beam is scanned over the surface and the back scattering of the electrons is looked at. The sample must again be under a vacuum and must be made electrically conductive at the surface by sputter coating. Since electrons can be accelerated by an electric potential, the wavelength can be made shorter than the one of photons. This makes the SEM capable of magnifying images up to 200,000 times. At the same time, it is possible to achieve high resolution pictures of the surface, making the instrument very useful in determining the size distribution of nanoparticles. SEM provides the characteristic information on sample topography - the surface features of an object and its texture, morphology - the shape and size of the particles making up the object, composition and other properties such as electrical conductivity and crystallographic information about the arrangement of atoms in the object.
The Energy Dispersive X-Ray Spectroscopy (EDS) is an analytical technique used for the elemental analysis or chemical characterization of a sample used in conjunction with scanning electron microscopy. EDS works by detecting X-rays that are produced by a sample placed in an electron beam. The electron beam excites the atoms in the sample that subsequently produce X-rays to discharge the excess energy. The energy of the X-rays is characteristic of the atoms that produced them, forming peaks in the spectrum. EDS studies the loss of excitation energy of the atoms in the sample by X-ray emission. This energy is characteristic of the excited atom and the emission intensity is proportional to the concentration of the elements in the particle.

Transmission electron microscopy (TEM) has been the most widely used technique for characterizing the size of nanoparticles (Chescoe and Goodhew, 1984). TEM uses an electron beam to interact with a sample to form an image on a photographic plate or specialist camera. The sample must therefore be able to withstand the electron beam and also the high vacuum chamber that the sample is put into. TEM allows direct observation of the image formed by electrons during their passage through the specimen and is projected on a fluorescent surface. TEMs are capable of imaging at a significantly higher resolution (sub-nanometer) than light microscopes, owing to the small de Broglie wavelength of electrons. This enables examination of the particles in nanometer size range, which is tens of thousands times smaller than the smallest resolvable object in a light microscope. This technique offers a unique capability to determine the metal core diameter, as well as information of form, structure and morphology of nanoparticle.
The crystal structure of the nanoparticles can be investigated by high-resolution transmission electron microscopy (HRTEM). In the HRTEM, the opening of the diaphragm of the microscope is larger and the images are formed from a number of diffracted beams; this multi-beam approach is known as phase-contrast imaging, and is necessary to construct an image of the crystal lattice. HRTEM provides access to much information about the sample, such as analysis of crystalline defects and interfaces at the atomic scale. Another technique for determining the structure of nanoparticles is Electron Diffraction (Beeston et al., 1972), which is related with electron microscopy techniques that allow us to obtain the crystal structure, crystalline spaces and exact composition of the material. Selected area electron diffraction (SAED), is a crystallographic experimental technique which is performed inside a TEM. SAED can be used to identify crystal structures and examine crystal defects of nanosized particles.

Crystals consist of atoms arranged in an orderly lattice. Crystal lattices can be simple cubic, face centre cubic (f.c.c.), or body centre cubic (b.c.c). In general, single crystals with different crystal structures will cleave into their own characteristic geometry. Single crystals are the most ordered of the three structures. An electron beam passing through a single crystal will produce a pattern of spots. From the diffraction spots one can determine the type of crystal structure (f.c.c. or b.c.c.) and the "lattice parameter" (i.e., the distance between adjacent 100 planes). Amorphous materials do not consist of atoms arranged in ordered lattices and also are completely disordered.
MATERIALS AND METHODS

UV–Visible spectroscopy Analysis

Change in colour of the mycelium free filtrate incubated with 1mM silver nitrate solution visually observed over a period of time indicates the bioreduction 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. Absorption measurements were carried out on UV-Visible spectrophotometer (CARY-100 BIO UV-Vis spectrophotometer; Varian Inc., USA) at a resolution of 1 nm between 200 and 800 nm ranges. UV-Vis spectroscopy analysis of several weeks old samples was also carried out to check the stability of synthesized silver nanoparticles.

Scanning Electron Microscopy analysis

In order to carry out SEM analysis, silver nanoparticles solution was centrifuged for 20 min at 10,000 rpm and drop coated on to thin glass film. SEM analysis was then performed using the Scanning Electron Microscope (JSM-6360, JEOL) and Scanning electron microscope (Leo 1430vp) instrument. Compositional analysis and the conformation of presence of elemental silver was carried out through Energy dispersive X-ray Spectroscopy (EDS) using the SEM equipped with a an EDX attachment (Carl Zeiss, Germany) and EDAX attachment (Oxford, London).

Transmission Electron Microscopy analysis

For TEM measurement, a drop of biosynthesized silver nanoparticles was placed on the carbon coated copper grids and kept overnight under vacuum desiccation. The
carbon coated copper grids were then loaded onto a specimen holder. Transmission electron micrographs of the samples were taken using the JEOL JSM 100CX TEM (Jeol, Japan) operated at an accelerating voltage of 200 kV. Selected area Electron Diffraction (SAED) of the nanoparticles was also analysed using the Transmission Electron Microscope. Average silver core diameter ($D$), size distributions and standard deviations were calculated for each nanoparticle sample by averaging 200 particles from the TEM images using ImageJ software (National Institutes of Health, USA).

**RESULTS**

**UV–Visible spectroscopy Analysis**

The UV–Vis spectra of the mycelium free filtrates and the filtrate treated with 1mM AgNO$_3$ at different time interval are presented in Fig 2.1 & 2.2. The UV–Vis spectrophotometer analysis of the fungal filtrates obtained for the isolates namely, *Fusarium oxysporum* MP5, *Aspergillus niger* NH6, *Paecilomyces lilacinus* SF1, *Arthrinium* sp KL1, *Aspergillus fumigatus* SP5, *Cladosporium cladosporioides* RS1, *Aspergillus tamarii* PFL2, *Aspergillus niger* PFR6, *Penicillium ochrochloron* PFR8, *Cryptosporiopsis ericae* PS4, *Alternaria solani* GS1 and *Penicillium funiculosum* GS2 treated with AgNO$_3$ showed a characteristic surface plasmon absorption band at 421, 426, 419, 409, 412, 413, 419, 430, 430, 440, 415, and 403 nm respectively. All the treated samples showed increased absorbance with increasing time of incubation, which attained maximum intensity on the third day (72 hr) (Figs 2.1 & 2.2). Beyond 72 hr of incubation, no further increase in intensity was recorded indicating complete reduction of silver ions by the fungal culture filtrates. Apart from this, an absorption peak was
also observed in the UV region corresponding to 280 nm indicating presence of amino acid residues. The peak at 280 nm is attributed to the tryptophan and tyrosine residues present in the protein (Bhainsa and D’Souza, 2006). This observation indicates the presence of proteins secreted by fungus in the cell-free filtrate. Stability of synthesized silver nanoparticles was monitored regularly for 6 months. It was observed that the silver nanoparticles synthesized by *Fusarium oxysporum* MP5, *Aspergillus niger* NH6, *Paecilomyces lilacinus* SF1, *Aspergillus fumigatus* SP5, *Aspergillus niger* PFR6, *Penicillium ochrochloron* PFR8, *Cryptosporiopsis ericae* PS4, *Alternaria solani* GS1 and *Penicillium funiculosum* GS2 were extremely stable at room temperature, with no evidence of flocculation of particles as determined by UV-Vis spectroscopy measurements. This indicated that the nanoparticles were well dispersed in the solution without aggregation. However, the silver nanoparticles synthesized by *Arthrinium* sp KL1, *Cladosporium cladosporioides* RS1 and *Aspergillus tamarii* PFL2 were found to be unstable with visible agglomeration of the particles after few weeks.
Fig 2.1: UV–Vis absorption spectrum at different time intervals for silver nanoparticles biosynthesized using a) *Fusarium oxysporum* MP5, b) *Aspergillus niger* NH6, c) *Paecilomyces lilacinus* SF1, d) *Arthrinium* sp KL1, e) *Aspergillus fumigatus* SP5 and f) *Cladosporium cladosporioides* RS1
Fig 2.2: UV–Vis absorption spectrum at different time intervals for silver nanoparticles biosynthesized using  a) *Aspergillus tamarii* PFL2, b) *Aspergillus niger* PFR6, c) *Penicillium ochrochloron* PFR8, d) *Cryptosporiopsis ericae* PS4, e) *Alternaria solani* GS1 and f) *Penicillium funiculosum* GS2
Electron microscopy analysis

SEM and TEM measurements were used to determine the morphology of the synthesized nanoparticles. The scanning electron micrograph revealed the morphology of the biosynthesized silver nanoparticles to be more or less spherical (Fig 2.3 & 2.4). In the analysis by energy dispersive X-ray spectroscopy of the silver nanoparticles, the presence of elemental metal signal was confirmed. The EDS profiles of the nanoparticles showed the presence of characteristic silver signal at approximately 3 keV (Fig 2.5 & 2.6), which is typical for the absorption of silver nanoparticles due to surface plasmon resonance confirming silver nanoparticles biosynthesized successfully using endophytic fungal extracts.

TEM micrographs provided further insight into the morphology and particle size distribution profile of the mycosynthesized silver nanoparticles. The analysis of data obtained from TEM micrographs of silver nanoparticles synthesized using twelve different fungal isolates also confirmed the formation of polydisperse and spherical nanoparticles with differing sizes (Fig 2.7-2.18). The particle size histograms of the mycosynthesized silver nanoparticles have been shown in Fig 2.7d-2.18d. The average sizes of the mycosynthesized silver nanoparticles are calculated and given in Table 2.1. Among all the samples, it has been observed that *Cladosporium cladosporioides* RS1 and *Aspergillus tamarii* PFL2 were able to synthesize nanoparticles of smallest particle sizes i.e., 3.5 ± 3nm and 3.5 ± 3.3nm respectively. SAED patterns recorded for single particle in the aggregates of all the nanoparticles samples corresponded to a characteristic polycrystalline ring pattern for a face-centered-cubic structure (Fig 2.7c-2.18c).
Fig 2.3: SEM micrographs of silver nanoparticles biosynthesized using soil fungi a) *Fusarium oxysporum* MP5, b) *Aspergillus niger* NH6, c) *Paecilomyces lilacinus* SF1, d) *Arthriniun* sp KL1, e) *Aspergillus fumigatus* SP5 and f) *Cladosporium cladosporioides* RS1
Fig 2.4: SEM micrographs of silver nanoparticles biosynthesized using endophytic fungi a) *Aspergillus tamarii* PFL2, b) *Aspergillus niger* PFR6, c) *Penicillium ochrochloron* PFR8, d) *Cryptosporiopsis ericae* PS4, e) *Alternaria solani* GS1 and f) *Penicillium funiculosum* GS2
Fig 2.5: EDX spectra of silver nanoparticles biosynthesized using soil fungi a) *Fusarium oxysporum* MP5, b) *Aspergillus niger* NH6, c) *Paecilomyces lilacinus* SF1, d) *Arthrinium* sp KL1, e) *Aspergillus fumigatus* SP5 and f) *Cladosporium cladosporioides* RS1
Fig 2.6: EDX spectra of silver nanoparticles biosynthesized using endophytic fungi a) Aspergillus tamarii PFL2, b) Aspergillus niger PFR6, c) Penicillium ochrochloron PFR8, d) Cryptosporiopsis ericae PS4, e) Alternaria solani GS1 and f) Penicillium funiculosum GS2
Fig 2.7: a) TEM micrograph b) HRTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Aspergillus niger* NH6.
Fig 2.8: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Arthriniunm* sp KL1
Fig 2.9: a) TEM micrograph b) HRTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Fusarium oxysporum* MP5
Fig 2.10: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Cladosporium cladosporioides* RS1
Fig 2.11: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Paecilomyces lilacinus* SF1
Fig 2.12: a) TEM micrograph, b) HrTEM micrograph, c) SAED pattern and d) Particle size distribution pattern histogram of silver nanoparticles synthesized using *Aspergillus fumigatus* SP5
Fig 2.13: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Aspergillus tamarii* PFL2
Fig 2.14: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Aspergillus niger* PFR6
Fig 2.15: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Penicillium ochrochloron* PFR8
Fig 2.16: a) TEM micrograph, b) HRTEM micrograph, c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Cryptosporiopsis ericae* PS4
Fig 2.17: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Alternaria solani* GS1
Fig 2.18: a) TEM micrograph b) HrTEM micrograph c) SAED pattern and d) particle size distribution histogram of silver nanoparticles synthesized using *Penicillium funiculosum GS2*
Table 2.1: Particle size distribution of the biosynthesized silver nanoparticles using soil and endophytic fungi

<table>
<thead>
<tr>
<th>Mycosynthesized sliver nanoparticles</th>
<th>Average size of nanoparticles</th>
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<tbody>
<tr>
<td><strong>Soil fungi</strong></td>
<td></td>
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<tr>
<td><em>Paecilomyces lilacinus</em> SF1</td>
<td>5.9 ± 5 nm</td>
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<tr>
<td><em>Fusarium oxysporum</em> MP5</td>
<td>9.7 ± 4.6 nm</td>
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<tr>
<td><em>Arthrinium</em> sp KL1</td>
<td>8.5 ± 2.9 nm</td>
</tr>
<tr>
<td><em>Cladosporium cladosporioides</em> RS1</td>
<td>3.5 ± 3.3 nm</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em> SP5</td>
<td>7.8 ± 6 nm</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> NH6</td>
<td>6.5 ± 5.2 nm</td>
</tr>
<tr>
<td><strong>Endophytic fungi</strong></td>
<td></td>
</tr>
<tr>
<td><em>Cryptosporiopsis ericae</em> PS4</td>
<td>5.5 ± 3.1 nm</td>
</tr>
<tr>
<td><em>Aspergillus tamarii</em> PFL2</td>
<td>3.5 ± 3 nm</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> PFR6</td>
<td>8.7 ± 6 nm</td>
</tr>
<tr>
<td><em>Penicillium ochrochloron</em> PFR8</td>
<td>7.7 ± 4.3 nm</td>
</tr>
<tr>
<td><em>Alternaria solani</em> GS1</td>
<td>16.3 ± 6.4 nm</td>
</tr>
<tr>
<td><em>Penicillium funiculosum</em> GS2</td>
<td>9.9 ± 2.7 nm</td>
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DISCUSSION

There are reports on the use of the soil fungi *Aspergillus Flavus*, *Penicillium fellutanum*, *P.chrysosporium*, *P.brevicompactum*, *Verticillium* sp, *Aspergillus fumigates*, *Fusarium oxysporum*, *F. semitectum*, *Alternaria alternata* for biosynthesis of silver nanoparticles (Mukherjee *et al.*, 2001; Ahmad *et al.*, 2003; Bhainsa and D’Souza 2006; Vigeshwaran *et al.*, 2007; Gajbhiye *et al.*, 2009; Kathiresan *et al.*, 2009; Shaligram *et al.*, 2009). However, exploration of fungal isolates, endophytic to ethnomedicinal plants, for such biosynthesis is scarce. The present study was aimed to assess the soil fungi as well as endophytic fungi isolated from two ethnomedicinal plants namely *Potentilla fulgens* L. and *Gloriosa superba* L. for their ability for extracellular reduction of silver ions to form nanoparticles. This study differs from the available reports as fungi endophytic to plants have not been evaluated for biosynthesis of silver nanoparticles though plants in general and soil fungi have been widely reported (Mohanpuria *et al.*, 2008; Thakkar *et al.*, 2010). The formation on silver nanoparticles in fungal filtrate was characterized using UV–Vis spectrophotometer based on its characteristic surface plasmon resonance. Fungal cell filtrate treated with silver nitrate solution (1 mM) are known to show sharp peak at around 420 nm with high absorbance (Sastry *et al.*, 2003) which supports the finding of the absorbance peaks observed at 421, 426, 419, 409, 412, 413, 419, 430, 430, 440, 415, and 403 nm indicating the synthesis of nanoparticles by the selected fungal isolates obtained from soil as well as the two ethnomedicinal plants. Apart from this, the absorption peaks at around 208, 207 and 203 nm was assigned to the strong absorption of peptide bonds in filtrate indicating the presence of aromatic acid such as tryptophan and tyrosine residues in the protein
(Bhainsa amd D’Souza, 2006). This observation indicates the release of proteins into the filtrate which suggests possible mechanism for the reduction of silver ions present in the solution. The reduction of the Ag$^+$ ions occurs due the reductases released by the fungus into the solution. Previous studies (Duran et al., 2007; Vaidyanathan et al., 2010) have indicated that nicotinamide adenine dinucleotide, reduced form (NADH) and NADH-dependent nitrate reductase enzyme are important factors in the biosynthesis of metal nanoparticles. The proteins could most possibly play a role in forming a coat covering the metal nanoparticles i.e. capping of silver nanoparticles to prevent agglomeration of the particles and stabilizing in the medium (Basavaraja et al., 2008).

SEM is the most widely used technique for characterizing the nanoparticles in terms of physical morphology of the particles. SEM images suggest that biosynthesized silver nanoparticles are almost spherical in structure. EDS analysis confirmed the presence of elemental silver in the samples. TEM provided insight into the morphology and particle size distribution profile of the silver nanoparticles. TEM analysis confirmed the synthesis of spherical silver nanoparticles in the reaction mixture. The nanoparticles synthesized by Fusarium oxysporum MP5, Aspergillus niger NH6, Paecilomyces lilacinus SF1, Arthrinium sp KL1, Aspergillus fumigatus SP5, Cladosporium cladosporioides RS1, Aspergillus tamarii PFL2, Aspergillus niger PFR6, Penicillium ochrochloron PFR8, Cryptosporiopsis ericae PS4, Alternaria solani GS1 and Penicillium funiculosum GS2 were found to be in different size ranges. SAED helped to identify crystal structures and to examine crystal defects of nanosized particles. SAED
patterns of the nanoparticles samples showed characteristic polycrystalline ring pattern for a face-centered-cubic structure.

The present finding elucidates that the mycosynthesized silver nanoparticles using soil fungi as well as the endophytic fungi isolated from traditionally used ethnomedicinal plants *Potentilla fulgens* L. and *Gloriosa superba* L. possess unique and fascinating optical properties that can be exploited for their utility in various technological applications as they offered different particle sizes (3.5 ± 3 nm to 16.3 ± 6.4 nm) and also are polycrystalline in nature.