CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Media

Potato Dextrose Agar (PDA) was used for the isolation of fungi. Malt Extract Glucose Yeast Extract Peptone (MGYP) broth was used for screening of fungi for AgNPs production. Nutrient Agar was used for preservation of bacterial strains. Tryptic Soy broth, Luria-Bertani broth and Congo red agar were used to study antibiofilm activity of AgNPs. Muller Hinton Agar (MHA) was used for antibacterial activity of AgNPs. Silver nitrate and MHA were procured from Hi-Media (Mumbai, India).

2.2 Isolation and Screening of Fungi from Different Soil Samples

Different soil samples from Hatti gold mines, Raichur district Karnataka and Yadgir district (Yadgir, Surpur and Shahapur) were collected in polythene bags and aseptically brought to the laboratory. For the isolation of fungi potato dextrose agar medium (Potato 200 g, Dextrose 20 g , Agar 20 g and pH 6 at 29ºC for 24-48 hrs) was prepared and inoculated with soil samples by serial dilution technique. The isolated fungi were subcultured on same medium to obtain pure cultures. Further, these pure cultures were stored under refrigeration for future use. The isolated fungi were subjected for the synthesis of AgNPs.

2.3 Silver Nanoparticles Biosynthesis

The fungus isolated from soil samples were maintained on PDA slants. The MGYP was prepared by mixing malt extract (0.5%), glucose (1.0%), yeast extract (0.3%) and peptone (0.5%) in distilled water for biomass production. Of the 15 fungi isolated
from the soil samples only one fungus, identified as *Aspergillus niger* depending on its efficiency was only used for further work. The Erlenmeyer flask containing 100 ml MGYP broth was inoculated with the test fungus, *A. niger* under sterile aseptic conditions, and allowed for biomass production at 29°C for 48-72 h. After 72 hrs of incubation the biomass was filtered and washed with distilled water to remove any traces of media content. Experiment was repeated thrice.

The biomass taken into fresh Erlenmeyer flask containing 100 ml distilled water and further kept at afore mentioned conditions for 48 hrs. The biomass was filtered again using Whatman’s filter paper No.1, the extracellular filtrate was used for the synthesis of AgNPs. Synthesis of AgNPs is extracellular, so aqueous solution of AgNO$_3$ (1mM AgNO$_3$ concentration) was challenged with fungal filtrate and the flasks were agitated. Periodically, aliquots of those isolates which showed colour change from yellow to brown were subjected to UV-Vis spectrometer analysis along with control. The biosynthesis of AgNPs is depicted as flow chart (Fig. 2.1),

2.4 Characterization Studies of Silver Nanoparticles

The fundamental of nanotechnology lies in the fact that properties of materials change dramatically when their size is reduced to the nanometer range. But measuring this nano dimension is not a very easy task. Although research is going on to synthesize nanostructure and nanophasic materials, characterizing these nano sized materials is also an emerging field posing lot of challenges to scientists and technonologists. Thus, nanotechnology has motivated the upsurge in research activities on the discovery and
invention of sophisticated nano characterization techniques to allow a better control of morphology, size and dimensions of materials in nano range.

**Fresh culture of fungus, *A. niger***

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Erlenmeyer flask of 100 ml MGYP broth

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Incubated at 29°C for 48-72 h

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Mycelia harvested & washed with Dist. Water

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Mycelia + Dist. Water incubation at aforesaid condition

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Filtrate collected using Whatman’s filter paper No.1

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Extracellular filtrate + AgNO₃

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Biosynthesis of AgNPs  (Murali et al., 2003)

**Fig. 2.1: Biosynthesis of AgNPs using *A. niger***
Optical microscopes are generally used for observing micron level materials with reasonable resolution. Magnification cannot be achieved through optical microscopes due to aberrations and limit in wavelength of light. Hence, the imaging techniques such as, Transmission Electron Microscopy (TEM/HRTEM), Scanning Electron Microscopy (SEM), Atomic Force Microscopy (AFM), etc. have been developed to observe the sub micron size nanoparticles. Though the principles of all the techniques are different but one common thing is that they produce a highly magnified image of the surface or the bulk of the sample. Nanoparticles can only be observed through these imaging techniques as human eye as well as optical microscope cannot be used to see dimensions at nano level.

The synthesized silver nanoparticles were characterized by various techniques such as- Visual observation, UV-Vis spectrophotometer (T90+ UV-Vis spectrophotometer), Transmission Electron Microscopy (TEM- Hitachi0 H 7500 ID, Japan), ZETA-sizer (Malvern Instruments Ltd.) Fourier Transform-Infrared Spectroscopy (FTIR- Perkin Elmer model 783 spectrophotometer), Scanning Electron Microscopy (SEM- JEOL Model JSM- 6390 LV), Energy Dispersive Spectroscopy (EDS- JEOL Model JED - 2300), Atomic Force Microscopy (AFM) and X-ray Diffraction (XRD- Philips PAN analytical, The Netherland) which provide physicochemical characteristics of the produced nanoparticles, from *A.niger*.

### 2.4.1 Visual Observation

The preliminary synthesis of AgNPs was indicated by visual observation. After production the mycelia was taken and washed with sterile distilled water, repeated to
remove any traces of previous media content then was re-suspended in Erlenmeyer flask containing distilled water. After incubation the fungal filtrate was filtered using Whaman filter paper No.1 then collected filtrate was challenged with AgNO₃. Before addition of silver nitrate the fungal filtrate was pale yellow in colour. Visual observation was done after adding 1mM AgNO₃. Color change was inspected.

2.4.2 UV-Vis Spectroscopy

The UV-Vis spectrum (T90+ UV-Vis spectrophotometer) for AgNPs is obtained by exposing the sample to UV-light from a light source. The specific surface plasmon resonance is responsible for their unique remarkable optical phenomenon. Color transitions arise due to molecular and structural changes in the substances being examined, leading to corresponding changes in the ability to absorb light in the visible region of the electromagnetic spectrum.

Principle

The reference beam in the spectrophotometer travels from the light source to the detector without interacting with the sample. The sample beam interacts with the sample exposing it to ultraviolet light of continuously changing wavelength. When the emitted wavelength corresponds to the energy level which promotes an electron to a higher molecular orbital, energy is absorbed. The detector records the ratio between reference and sample beam intensities ($I_o/I$). The computer determines at what wavelength the sample absorbed a large amount of ultraviolet light by scanning for the largest gap between the two beams. When a large gap between intensities is found, where the sample beam intensity is significantly weaker than the reference beam, the computer plots this
wavelength as having the highest ultraviolet light orientation of AgNPs (Avinash et al., 2005 and Joshia et al., 2008).

2.4.3 Transmission Electron Microscopy (TEM)

TEM is unique technique because it can provide a real micrograph image on the atom distribution in nanoparticles and on its surface. The topographic information obtained by TEM in the vicinity of atomic resolution can be utilized for structural characterization and identification of various phases of nanoparticles, such as spherical, cubic, irregular or hexagonal. TEM is a microscopic technique whereby beam of electrons is transmitted through an ultra thin specimen and interacts as it passes through the sample. An image is formed from the electrons transmitted through the specimen, magnified and focused by an objective lens which appears on an imaging screen. A drop of AgNPs solution was placed on the carbon coated copper grids and kept under vacuum before loading them onto a specimen holder. Then TEM micrographs were taken by prepared grids to determine the size and shape of the produced AgNPs.

Principle

In TEM, the crystalline sample interacts with the electron beam mostly by diffraction rather than by absorption. The intensity of the diffraction depends on the orientation of the planes of atoms in a crystal relative to the electron beam; at certain angles the electron beam is diffracted strongly from the axis of the incoming beam, while at other angles the beam is largely transmitted. Specimen holder which was provided in TEM allows the specimen to tilt a range of angles in order to obtain specific diffraction conditions. Therefore, a high contrast image can be formed by blocking electrons
deflected away from the optical axis of the microscope by placing the aperture to allow only unscattered electrons through. This produces a variation in the electron intensity that reveals information on the crystal structure. This technique, particularly sensitive to extended crystal lattice defects, is known as ‘bright field’ or ‘light field’. It is also possible to produce an image from electrons deflected by a particular crystal plane which is known as a dark field image (Joshia et al., 2008). The specimens should be very thin and able to withstand the high vacuum present inside the instrument so that the electron beam can penetrate.

2.4.4 ZETA Sizer or Particle Size Analyzer

There are different techniques for the measurement of particle size and its distribution (PSD) such as sieve analysis, optical counting methods, electro resistance counting methods, sedimentation techniques, laser diffraction methods, dynamic light scattering method, acoustic spectroscopy, etc. Among them dynamic light scattering is mostly used for obtaining size distribution of AgNPs. We have analyzed the size distribution and zeta potential of the nanoparticles using ZETA sizer (Malvern Zetasizer Ltd. Bangalore).

Principle

Dynamic light scattering is a non-invasive, well-established technique for measuring the size of molecules and particles typically in the submicron region, and with the latest technology lower than one nanometer. Particles, emulsions and molecules in suspension undergo Brownian motion. This is the motion induced by the bombardment of solvent molecules that themselves are moving due to their thermal energy. If the particles
or molecules are illuminated with a laser, the intensity of the scattered light fluctuates at a rate that is dependent upon the size of the particles as smaller particles are “kicked” further by the solvent molecules and move more rapidly (Joshia et al., 2008). Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size (radius $r_k$) using the Stokes-Einstein relationship, as shown below:

$$ r = \frac{kT}{\pi 6 \eta D} $$

Where, $k$ is the Boltzmann's constant; $T$, the temperature in K; $\eta$, the solvent viscosity; and $D$, the diffusion coefficient. Therefore the size and size distribution of AgNPs was studied using ZETA sizer.

**2.4.5 Fourier Transform-InfraRed Spectroscopy (FT-IR)**

The interaction between protein and AgNPs was analyzed by Fourier Transform-InfraRed Spectroscopy analysis. The main aim of the FT-IR analysis is to determine the different functional groups present in the sample. The AgNPs suspension was centrifuged at 10,000 rpm/10 min and dried sample analysis was recorded on Perkin Elmer one FT-IR spectrophotometer in the range 450 to 3000 cm$^{-1}$. Different functional groups absorb the characteristic frequencies of FT-IR radiation. The amide linkages between amino acid residues in polypeptides and proteins gives rise to well known signatures in the infrared region of the electromagnetic spectrum. The positions of the amide I and II bands in the FT-IR spectra of proteins are sensitive indicator of conformational changes in the protein secondary structure.
Principle

The spectrometer consists of a source of infrared light, emitting radiation throughout the whole frequency range of the instrument. Light from the source is split into two beams of equal intensity. One beam is made to pass through the sample while the other is allowed to behave as the reference beam. The function of such a double beam operation is to measure the difference in intensities between the two beams at each wavelength.

Now the two beams are reflected to a chopper which is rotating at a speed of 10 rotations per second. The chopper makes the reference and sample beam to fall on the monochromator grating alternately. The grating also rotates, albeit slowly. This rotation sends individual frequencies to the detector. It is the function of the detector to convert infrared thermal energy to electrical energy. At the wavelength where the sample has absorbed, the detector will receive a weak beam from the sample while the reference beam will retain full intensity. This will lead to a pulsating or alternating current to flow from the detector to the amplifier (Avinash et al., 2005 and Joshia et al., 2008).

2.4.6 Scanning Electron Microscopy (SEM)

SEM is a most widely used characterization technique to determine the shape and surface morphology of AgNPs. SEM was used to record the photomicrograph images of synthesized AgNPs. The scanning electron microscope is an electron microscope that images the sample surface by scanning it with a high energy beam of electrons. Conventional light microscopes use a series of glass lenses to bend light waves and create a magnified image while the scanning electron microscope creates the magnified images
by using electrons instead of light waves. A small volume of AgNPs suspension was taken for SEM analysis on electromicroscope stub. The stubs were placed briefly in a drier and then coated with gold in an ion sputter. Pictures were taken by random scanning of the stub. Shape and surface morphology of AgNPs were studied by SEM.

**Principle**

When the beam of electrons strikes the surface of the specimen and interacts with the atoms of the sample, signals in the form of secondary electrons, back scattered electrons and characteristic X-rays are generated that contain information about the sample's surface topography, composition, etc. The SEM can produce very high-resolution images of a sample surface, revealing details about 1-5 nm in size in its primary detection mode i.e. secondary electron imaging. Characteristic X-rays are the second most common imaging mode for SEM. These characteristic X-rays are used to identify the elemental composition of the sample by a technique known as energy dispersive X-ray (EDX) (Joshia et al., 2008).

**2.4.7 Electron Dispersive Spectroscopy (EDS)**

Energy dispersive X-ray analysis is a technique to analyze near surface elements and estimate their proportion at different position, thus giving an overall mapping of the sample. The reduction of Ag+ to elemental silver was confirmed by using EDS analysis. Samples were prepared on a copper substrate by drop coating of AgNPs. The elemental analysis was examined by EDS.
Principle

This technique is used in conjunction with SEM. An electron beam strikes the surface of a conducting sample. The energy of the beam is typically in the range 10-20keV. This causes X-rays to be emitted from the material. The energy of the X-rays emitted depends on the material under examination. The X-rays are generated in a region about 2 microns in depth, and thus EDX is not truly a surface science technique. By moving the electron beam across the material an image of each element in the sample can be obtained. Due to the low X-ray intensity, images usually take a number of hours to acquire. The composition or the amount of AgNPs near and at the surface can be estimated using the EDX, provided they contain some heavy metal ions (Avinash et al., 2005 and Joshia et al., 2008).

2.4.8 Atomic Force Microscopy (AFM)

AFM is ideal for quantitatively measuring the nanometer scale surface roughness and for visualizing the surface nano-texture of AgNPs. Advantages of the AFM for such applications are derived from the fact that the AFM is non-destructive technique and it has a very high three dimensional spatial resolution. The AFM analysis provides the size and shape of the nanoparticles and also it provides surface topography and three dimensional structures of AgNPs. The analysis of size and surface topography of the drop coated film of synthesized AgNPs was done using AFM. A small volume of sample was spread on a well cleaned glass cover slip surface mounted on the AFM stub, and was dried with nitrogen flow at room temperature. Micrographs were obtained in tapping mode using a silicon probe cantilever.
Principle

AFM is based on the existence of a separation-dependency force between any two bodies. It is the force between the tip and the substrate that is present at close separations. Typically, pyramidal silicon nitride tips are used, which have a radius of curvature on the order of 100Å. These are made by etching process that removes silicon from the substrate, leaving an etched or sharpened tip behind. The force is detected by placing the top on a flexible cantilever that deflects proportionally to the exerted force. The deflection is then measured by some convenient procedure, such as laser deflection or some other device. Actually, the main innovation may be seen as being a copy of the principle behind the record player.

Tapping mode AFM, the most commonly used of all AFM modes, that maps topography by lightly tapping the surface with an oscillating probe tip. The cantilever’s oscillation amplitude changes with sample surface topography, and the topography image is obtained by monitoring these changes and closing the feedback loop to minimize them. Tapping mode has become an important AFM technique, as it overcomes some of the limitations of both contact and non-contact AFM. By eliminating lateral forces that can damage soft samples and reduce image resolution, Tapping mode allows routine imaging of samples once considered impossible to image with AFM, especially in contact mode (Joshia et al., 2008).

2.4.9 X-ray Diffraction (XRD)

X-ray method of characterization is a powerful approach to study the crystalline nature of silver nanoparticles. X-rays are electromagnetic radiation similar to light, but
with a much shorter wavelength (few Angstrom) and produced when electrically charged particles of sufficient energy are decelerated. In an X-ray tube, the high voltage maintained across the electrodes draws electrons toward a metal target (the anode). X-rays are produced at the point of impact, and radiate in all directions. The advantage of this technique is to provide meaningful information about both medium ranges, local, atomic structure in AgNPs.

The AgNPs suspension was centrifuged at 10,000 rpm/10 min and the phase evolution of dried AgNPs powder samples was studied by X-ray diffraction technique using Cu Kα radiation. The generator voltage and current was set at 35 KV and 25 mA respectively. The AgNPs samples were scanned in the 2θ ranges 0 to 80˚ ranges in continuous scan mode.

**Principle**

If an incident X-ray beam encounters a crystal lattice, general scattering occurs. Although most scattering interferes with itself and is eliminated (destructive interference), diffraction occurs when scattering in a certain direction is in phase with scattered rays from other atomic planes. Under this condition the reflections combine to form new enhanced wave fronts that mutually reinforce each other (constructive interference). The relation by which diffraction occurs is known as the Bragg’s law or Bragg’s equation. As each crystalline material including metal and metal nanoparticles have a characteristic atomic structure, it will diffract X-rays in a unique characteristic diffraction order or pattern. X-ray diffraction data from AgNPs generally provide information about crystallinity, crystallite size, orientation of the crystallites and phase composition of AgNPs (Avinash et al., 2005 and Joshia et al., 2008).
2.4.10 Determination of Tryptophan/Tyrosine Residues

Presence of tryptophan/tyrosine residues in proteins release in the fungal filtrate was analyzed using spectrometer. The measurements were carried out on a UV-Vis spectrum. The excitation wavelength band close to maximal optical transitions of the tyrosine/tryptophan was detected. Ultraviolet spectrophotometers consist of a light source, reference and sample beams, a monochromator and a detector.

The ultraviolet spectrum for a compound is obtained by exposing a sample of the compound to ultraviolet light from a light source, such as a Xenon lamp. The surface Plasmon’s propagate around the interface between the surface of a metal nanoparticle and the medium. Therefore they are sensitive to changes at this interface and can be used as a characteristic tool to observe the interaction of surface adsorbed species.