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**Biogenic silver nanoparticles by Aspergillus terreus MP1 and its promising antimicrobial activity**

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**ABSTRACT**

Developments of nanotechnology are leading to a rapid proliferation of nanomaterials that are likely to become a source of many different engineered nanoparticles in the environment. The unique properties, such as high specific surface area and mobility, could potentially lead to effective nanomaterial that can be applied in diverse sectors. Nano materials are at the leading edge of the rapidly developing field of nanotechnology. In the present study Aspergillus terreus MP1 genbank Accession Number: HQ49678 derived from marine sponges were tested for its efficacy to synthesise silver nanoparticles. Extracellularly produced metal nanoparticles were characterised using analytical instruments to reveal their size and shape. The potency of silver nanoparticles was tested against antagonistic human pathogens and found to inhibit them effectively.

**Key words:** Silver Nanoparticle, Marine sponge, Aspergillus terreus , antibacterial analysis.

**INTRODUCTION**

Nature has devised various processes for the synthesis of nano- and micro-length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials (Sastry et al., 2004). The synthesis of nanomaterials of specific composition and size is a burgeoning area of materials science research. The properties of these materials in applications as diverse as catalysis, sensors and medicine depend critically on the size and composition of the nanomaterial(Nalenthiran, 2009). Thus, researchers have used biological synthesis, since this technique provides particles with good control over the size distribution. The main reason for this may be that the processes devised by nature for the synthesis of inorganic materials on nano- and micro- scales have contributed to the development of relatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials (Mandal et al., 2006; Sastry et al., 2004). The ability of some microorganisms such as bacteria and fungi to control the synthesis of metallic nanoparticles should be employed in the search for new materials (Mandal et al., 2006). The metabolic activity of microorganisms can lead to precipitation of nanoparticles in external environment of a cell, the fungi being extremely good candidates for such processes. Although it is known that microorganisms such as bacteria, yeast and now fungi play an important role in remediation of toxic metals through reduction of the metal ions, this was considered interesting as nanofactories very recently (Fortin, 2000). Using these dissimilatory properties of fungi, the biosynthesis of inorganic nanomaterials using eukaryotic organisms such as fungi may be used to grow nanoparticles of gold and silver (Mukherjee, 2001) intracellularly. Both unicellular and multicellular organism are known to produce inorganic materials either intra or extracellularly (Mann, 1996). The use of microorganism such as bacteria, yeast, fungi, and actinomycetes has been described for the formation of nanoparticles and their applications (Sastry et al., 2004; Mandal et al., 2006; Gerick and Pinches, 2006).

The rate of intracellular particle formation and therefore the size of the nanoparticles could, to an extent, be manipulated by controlling parameters such as pH, temperature, substrate concentration and exposure time to substrate (Gerick and Pinches, 2006). In addition to good monodispersity, nanoparticles with well defined dimensions can be obtained by using fungi. It can be concluded from their study that compared to bacteria... fungi could be a source for large amount production of nanoparticles. Since fungi are known to secrete much higher amounts of proteins, thus might have significantly higher productivity of nanoparticles in biosynthetic approach.

**MATERIALS AND METHODS:**

**Isolation of Fungi:**
The sponge sample was washed with sterile water (distilled water: sea water: 1:1) and ground in a mortar and pestle under aseptic conditions. Serial dilution was performed and from each dilution, plating was done in Sabourauds agar by spread plate technique. The plates were then incubated at 27°C for 5 days. After 5 days, the plates were examined and the pure culture was isolated on pure agar plate.

**Molecular characterization and Identification of elite fungi by ITS sequencing:**
The fungi were grown in culture in potato dextrose broth at room temperature in the dark for 48 to 72 hours. The genomic DNA was isolated and the ITS region of 5.8S rRNA was amplified using primer ITS1 TO 5' TCCGTAAGTTGAACCTGCGG 3' and primer ITS5 5' TCTCCGCTTATTGATATGC 3' and sequenced using automated sequencer.

**Synthesis Of Silver Nanoparticles:**
7 ml of the fungal mycelial extract was added to 60 ml of 1 mM AGNO₃ solution and kept below 60°C. Bioreduction of silver ions in the solution was monitored by measuring UV-Vis spectra of the solution at periodic intervals. The nanoparticle synthesized was confirmed by UV-Vis spectra plasma curve. The solution was centrifuged and the particles are characterized.

**UV-VIS Spectra Analysis:**
The bioreduction of Ag⁺ in aqueous solution was monitored by periodic sampling of aliquots (0.2ml) of the suspension, then diluting the samples with 2ml deionized water and subsequently measuring UV-Vis spectra, at the wave length of 200 to 700 nm. UV-Vis spectra were recorded at initial, 5min, 10min, 15min, 30min, 1hr, 2hr, 3hr, 4hr, 7hr, and 8hr.

**Scanning Electron Microscopy (SEM)**
After synthesis of nanoparticles, the sample was filtered through Millipore filters of 0.2 μm pore size, to remove any contaminants interfering with the SEM images. About 25 μl of the sample was pipette out and loaded on a ‘stubs’ provided for SEM analysis. The stub is made of copper, in the shape of a small cylinder about the size of 1 cm dia. One side of the stub was stuck with double sided carbon material. After loading the sample on the carbon material, the stub was fixed to a holder. The holder accommodates about 4 samples at a time.

**Transmission Electron Microscopy (TEM)**
TEM is a method of producing images of a sample by illuminating the sample with electronic radiation (under vacuum), and detecting the electrons that are transmitted through the sample.

**XRD Measurement:**
The air dried nanoparticles were coated onto XRD grid and analysed for the formation of Ag nanoparticle by Philips X-Ray Diffractionometer with Philips PW 1830 X-Ray Generator operated at a voltage of 40kV and a current of 30mA with Cu Kα radiation. The diffracted intensities were recorded from 10° to 80° of 20 angles.

**FTIR Analysis:**
The dried Ag nanoparticles were subjected to FTIR analysis by KBr pellet (FTIR grade) method in 1: 100 ratios and spectrum was recorded in Nicolet Impact 400 FT-IR Spectrophotometer using diffuse reflectance mode operating at a resolution 4.

**Antibacterial Activity:**
Agar diffusion assay is used widely to determine the antibacterial activity of crude extract. The technique works well with defined inhibitors. Nutrient agar prepared and was poured in the petridish and allowed for solidification, 24 hours growing bacterial culture where swabbed on it. The wells(8 mm diameter) were made by using cork borers. The difference concentration of the crude extract were loaded in the well. The plate was then incubated at 37°C for 24 hours.

**RESULTS:**
In the present study, the 10⁻⁵ dilution of the sponge sample yielded three different isolates. The characterization and analysis was performed for Isolate1. Pure culture of fungal isolate

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Molecular characterization and Identification of elite fungi:
The ITS region is now perhaps the most widely sequenced DNA region in fungi. It is most useful for molecular systematics at the species level, and even within species. In the present study, the DNA was isolated from the Isolate1 and the ITS region of 5.8s rRNA was amplified using specific primers ITS 1 and ITS4 and sequence was determined using automated sequencers. Blast search sequence similarity was found against the existing non-redundant nucleotide sequence database thus, identifying the fungi as \textit{Aspergillus terreus}. The percentage of similarity between the fungi and database suggests it as novel strain. Thus, the novel strain was named as \textit{Aspergillus terreus} MP1 and made publically available in GenBank with an assigned accession number : HQ449678 .

Biosynthesis of Silver Nanoparticles
Biosynthesis of silver nanoparticles by the fungal filtrate was confirmed by change in the colour of the filtrate to brown after addition of silver nitrate. (Fig.2). This arises due to excitation of surface plasmon vibrations in the metal nanoparticles.

UV-Vis Spectral Analysis
The bioreduction of Ag+ in the filtrate reaction solution was monitored by periodic sampling of the reaction mixture at regular intervals by using UV-Vis spectroscopy. Control flasks maintained with silver nitrate solution (without culture filtrates) did not show any change of colour and its absorbance maximum was found to be at 340 nm, which is specific for silver nitrate solution. Whereas reaction mixture consisted of culture filtrate with silver nitrate (Fig. 3) showed a strong characteristic absorbance peak at around 420 nm. Analysis by spectrophotometer was made up to 8hrs.

SEM Analysis:
Scanning electron microscopic analysis of the silver nitrate solution (Control) and reduced form of silver nitrate solution are clearly distinguishable owing to their size difference. It is clearly seen that control silver nitrate particles are more than 1000nm size, whereas silver particles in the bio reduced colloidal suspensions measured 15-20nm in size.

TEM Analysis
TEM analysis revealed that the synthesized nanoparticles are stable in solution at room temperature. The size of nanoparticle range from 15-20nm. The decrease in anisotropy and particle size is evident from the images. The TEM images revealed equal spherical shape.

Fig.1a. Pure culture of isolate Fig.1b. SEM micrograph of isolate.

Fig.2. Synthesis of nanoparticle (a) control (b) positive silver nanoparticle synthesis

Fig.3: UV-Vis spectroscopy of silver nanoparticle

Fig.4a. SEM image of Silver nitrate in control sample

Fig.4b. SEM analysis of silver nanoparticle - 1µm and Fig.4c. silver nanoparticle 5µm

Fig.5a. TEM analysis of silver nanoparticle in 100nm Fig 5b. silver nanoparticle 150nm

Fig.5c: TEM analysis of silver nanoparticle - 200nm

Fig. 5c: TEM analysis of silver nanoparticle - 200nm

Fig. 5c: TEM analysis of silver nanoparticle - 200nm
XRD Analysis
XRD analysis showed three distinct diffraction peaks at 38.18°, 44.18°, 64.1° and can be indexed the angle vales of (111), (200), (220) crystalline planes of cubic Ag. This analysis revealed that nanoparticles are in orthorhombic crystals. The high peaks in the analysis indicate the active silver composition with the indexing.

FTIR Analysis
FTIR spectral analysis showed array of absorbance bands in 500 cm⁻¹ – 2000 cm⁻¹. The spectral band peaks are along the range of between 1500cm⁻¹ – 1750cm⁻¹ with prominent peaks at 1504.23cm⁻¹, 1514.74cm⁻¹, 1556.31cm⁻¹, 1574.22cm⁻¹, 1592.04cm⁻¹, 1650.63cm⁻¹ and 1657.64cm⁻¹ which were interpreted for the identification of the functional moieties in the air dried silver nanoparticles.

CONCLUSION:
Many nanotechnology applications are still at the concept level, requiring much more basic research before they can be incorporated into a viable product. Although this technology often conjures images of tiny robots traveling around inside the body repairing damaged tissue, we are not at that stage just yet. The current reality is much more subtle but the future looks set for some exciting developments that could potentially be very significant to the field of medicine. Much has already been studied in synthesis of nanomaterials through physical and chemical processes. But synthesis of nanomaterials by biological agents offer several advantages like establishment of ‘green chemistry’ leading to a pollution free production of nanomaterials in contrast to energy dependent, pollution based physical and chemical methods. Against this back ground, importance is being given more on the lines of producing nanomaterials or nanoparticles using bacteria, fungi, actinomycetes and plants. Evidences start accumulating on the synthesis of nanoparticles by these biological agents and quality of these biosynthesized nanoparticles are proved to be on par with that of produced physical and chemical process.

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