Biosynthesis of monodispersed silver nanoparticles and their activity against *Mycobacterium tuberculosis*

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Abstract: In this study, we report the characterisation of biosynthesised silver nanoparticles and investigate the antibacterial effects of biologically synthesised silver nanoparticles (AgNPs) on *Mycobacterium tuberculosis*. There is an urgent need to develop new anti-tuberculosis drugs, highlighted by the ongoing rise in tuberculosis (TB) cases worldwide. One worrying factor in the current TB problem is the prevalence of multi-drug resistant (MDR) strains. Increased resistance of *Mycobacterium tuberculosis* (*M. tuberculosis*) to antibiotics has led the researchers to develop a good antibacterial agent to overcome this problem. A colorimetric, microplate-based Alamar Blue assay (MABA) method was used to determine the MICs of isoniazid (INH), rifampin, streptomycin (SM), and ethambutol (EMB) for *M. tuberculosis* clinical isolates and silver nanoparticles against multi-drug resistance strains of *M. tuberculosis* isolated from MABA method.

Keywords: *Rhizopus stolonifer*; AgNPs; *Mycobacterium tuberculosis*.

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1 Introduction

The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology (Raveendran et al., 2006). Today, nanometal particles, especially silver, have drawn the attention of scientists because of their

extensive application in the development of new technologies in the areas of electronics, material sciences and medicine at the nanoscale (Magudapathy et al., 2001). Silver nanoparticles (AgNPs) have many applications; for example, they might be used as spectrally selective coatings for solar energy absorption and intercalation material for electrical batteries, as optical receptors, as catalysts in chemical reactions, for biolabelling, and as antimicrobials (Joerger et al., 2000).

The emergence of multi-drug resistant (MDR) and extreme drug-resistant tuberculosis (TB) has emphasised the need for methods that will allow quick detection of the microbial agent, while simultaneously performing rapid antibiotic susceptibility tests. Due to the long generation time of *Mycobacterium tuberculosis*, antimicrobial susceptibility testing takes a few weeks, delaying treatment, which may negatively affect the patient's health, and lead to an increase in disease transmission (Akselband et al., 2005; Pina-Vaz et al., 2005). The Alamar Blue oxidation-reduction dye is a general indicator of cellular growth and/or viability; the blue, non-fluorescent, oxidised form becomes pink and fluorescent upon reduction (Ahmed et al., 1994). Growth can therefore be measured with a fluorometer or spectrophotometer or determined by a visual colour change.

It is estimated that one-third of the world's population is infected with the tubercle bacillus (Dye et al., 1999). While only a small percentage of infected individuals will develop clinical TB, each year there are approximately eight million new cases and two million deaths. *Mycobacterium tuberculosis* is thus responsible for more human mortality than any other single microbial species. Today, the HIV pandemic has exacerbated the problem by providing a large reservoir of highly susceptible individuals (Corbett et al., 2003). A number of efficacious anti-tubercular agents were discovered in the late 1940s and 1950s with the last, rifampin (RMP), introduced in the 1960s (Schraufnagel, 1999). These agents had reasonable efficacy and, when used in combination, would preclude the development of drug resistance. The use or (in most cases) misuse of these drugs over the years has led to an increasing prevalence of multiple-drug resistant (MDR) strains, establishing an urgent need to develop new effective agents (Cox et al., 2003).

2 Materials and methods

2.1 Synthesis of AgNPs

Fungi for the synthesis of AgNPs were inoculated in malt glucose yeast peptone (MGYP) broth containing yeast extract and malt extract-0.3% each, glucose-1%, peptone-0.5%, at 40°C, in shaking condition (180 rpm) (Banu et al., 2011). After the incubation period of about 72 h the biomass was filtered and then extensively washed with sterile distilled water to remove the medium components. This biomass was taken into Erlenmeyer flask containing 100 ml distilled water and incubated at the above said condition. The biomass was filtered again, (Whatman filter paper no. 1) and after 72 h the fungal filtrate was used further. Aqueous solution of $AgNO_3$ (1 mM $AgNO_3$ of final concentration) was mixed with fungal filtrate and the flasks were agitated at 40°C. Periodically, aliquots of only those isolates which showed colour change from yellow to brown were subjected to UV-

visible absorption spectrophotometric and scanning electron microscopy (SEM) studies. Control (without silver ions) was also run along with the experimental flasks.

2.2 Characterisation of AgNPs

Formation of nanosilver was monitored using UV-visible absorption spectroscopy (T90+UV/vis spectrometer), which is one of the important techniques to verify the formation of metal nanoparticles provided surface plasmon resonance exists for the metal (Basavaraja et al., 2008). Absorption spectroscopy in the UV-visible region has long been an important tool for the nanoparticle characterisation. Colour 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. Appearance of colour arises from the property of the coloured material to absorb selectively within the visible region of the electromagnetic spectrum. To detect AgNP the absorption range is 400 to 450 nm (Kemp et al., 2009). This surface plasmon resonance is caused by the coherent oscillation of the free conduction electrons induced by light. Samples for transmission electron microscopy (TEM) (Hitachi-H-7500) were prepared by drop-coating the AgNPs solution into the carbon-coated copper grid, which shows the size and morphology of the particles. The interaction between protein and AgNPs was analysed by Fourier transform-infrared spectroscopy (FT-IR). Three-dimensional picture of the biosynthesised AgNPs were studied by atomic force microscopy (AFM).

2.3 AgNPs against M. tuberculosis

2.3.1 Drug preparation

Ten clinical isolates of *M. tuberculosis* obtained from Khwaja Bande Nawaz Hospital Gulbarga and were sub cultured on Middlebrook 7H11 agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Suspensions were prepared in 0.04% (vol/vol) between 80–0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) so that their turbidities matched that of a McFarland no. 1 turbidity standard. Suspensions were further diluted 1:25 in 7H9GC broth (4.7 g of Middlebrook 7H9 broth base [Difco, Detroit, Mich.], 20 ml of 10% [vol/vol] glycerol, 1 g of Bacto Casitone [Difco], 880 ml of distilled water, and 100 ml of oleic acid, albumin, dextrose, and catalase.

Isoniazid (INH), RMP, streptomycin (SM), and ethambutol (EMB) were obtained from Sigma. Stock solutions of INH, SM, and EMB were prepared in deionised water, and RMP was prepared in dimethyl sulfoxide. Stock solutions were diluted in 7H9GC broth to two times the maximum desired final testing concentrations prior to their addition to microplates.

2.3.2 Anti-TB activity using Alamar Blue method

The anti-mycobacterial activities of antibiotics and nanosilver were assessed against *M. tuberculosis* using microplate Alamar Blue assay (MABA) (Lourenço et al., 2007).This methodology is non-toxic, uses a thermally stable reagent. Briefly, 200 µl of

sterile deionised water was added to all outer perimeter wells of sterile 96 wells plate to minimised evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds was made directly on plate. The drug concentrations ranged between 8 and 64 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25 µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink.

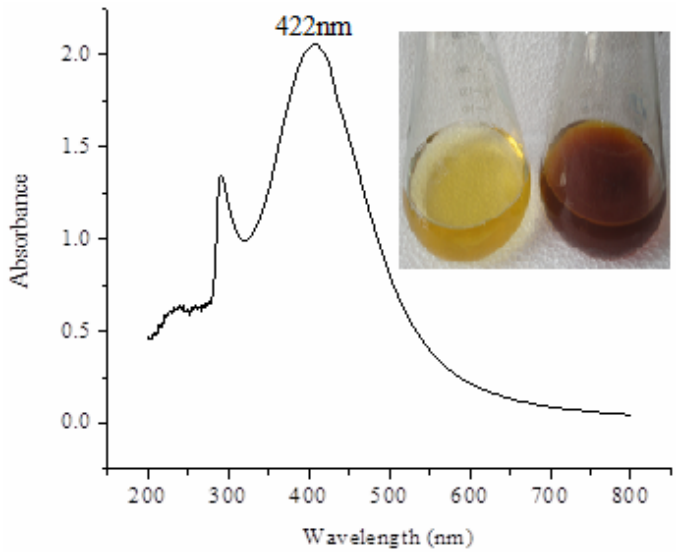
The strains with IC_{90} values of > 16 µg/ml, were considered as resistance strains, these resistance strains were tested against nanosilver particles by Alamar Blue method as mentioned above, the concentration of nanosilver was taken from 0.2, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50, 100 µg/ml.

3 Results and discussion

AgNPs were synthesised biologically from *R. stolonifer* using$AgNO_3$. The use of specific enzymes such as reductase secreted by fungi opens up exciting possibilities of designing a rational biosynthesis strategy for metal nanoparticles of different chemical composition. A number of different genera of fungi have been investigated for biotechnological process research and it has been shown that fungi are extremely good candidates in the synthesis of nanoparticles. The fungi were grown in MGYP broth, after 72 h of incubation period the fungal biomass was separated by filtration. The biomass was suspended in distilled water after several washings again for 72 h, and then was subjected to filtration for the separation of fungal biomass. The fungal filtrate was used for the synthesis of nanosilver. This fungal filtrate was mixed with aqueous solution of $AgNO_3$, which shows brown colour after the incubation. The appearance of brown colour solution (Figure 1) clearly indicates the formation of AgNPs (Mukherjee et al., 2001a, 2001b). The colour change was caused by the surface plasmon resonance of AgNPs in the visible region (Varshney et al., 2009). AgNPs are known to exhibit size and shape dependent surface plasmon resonance bands which are characterised by UV-visible absorption spectroscopy (Xie et al., 2007). This event clearly indicates that the reduction of the ions occur extracellularly through reducing agents released in to the solution by fungi. The nanoparticles were formed extracellular when the cell wall reduction enzymes were responsible for metal ions reduction as well as when the reduction enzymes secreted extracellular. The extracellular synthesis by comparison is more adaptable to the synthesis of a wider range of nanoparticles. Recent studies reveal that fungal sources are extremely efficient secretors of extracellular enzymes; it is thus possible to easily obtain large-scale production of enzymes (Kannan and Subbalaxmi, 2011).

The use of fungi in the synthesis of nanoparticles is relatively recent addition to the list of microorganisms possessing nanoparticle biosynthesis ‘ability’. Application of fungi to produce nanoparticles is potentially exciting because of their ability to secrete large amounts of enzymes. Some of the microorganisms, which have been widely used for synthesising AgNPs include: *Verticillium spp*., *Fusarium oxysporum*, *Pseudomonas stutzeri* AG259 (Sastry et al., 2003) and *Aspergillus fumigatus* (Bhainsa and D’Souza, 2006).

Figure 1 UV-visible absorption spectroscopy of AgNPs of *R. stolonifer* (see online version for colours)



Towards elucidating mechanism of nanoparticles formation, protein assays indicate that an NADH-dependent reductase is the main responsible factor of biosynthesis processes. This reductase gains electrons from NADH and oxidises it to NAD+. The enzyme is then oxidised by the simultaneous reduction of metal ions (Senapati and Ahmad, 2005). The mechanistic aspect was explained by Duran et al. that apart from enzymes, quinine derivates of napthoquinones and anthraquinones also act as redox centres in the reduction of AgNPs (Narayanan and Sakthivel, 2010). The demonstration of green synthesis of nanoparticles includes two steps: first bioreduction of silver ions to produce AgNPs, secondly, stabilisation and encapsulation of the same by using a suitable capping agent.

Application of biological systems for synthesis of AgNPs has already been reported by Sastry et al. (2003). However, the exact mechanism leading to the formation of AgNPs by organisms is yet to be elucidated. Ahmad et al. (2003) have reported that certain NADH dependent reductases of *F. oxysporum* were involved in the reduction of silver ions. Nonetheless, it would be far more practical if the metal ion exposed to the fungus could be reduced outside the fungal biomass, leading to the formation of metal nanoparticles in solution (Mandal et al., 2006). Therefore, in this study *R. stolonifer* was used to reduce silver ions extracellularly.

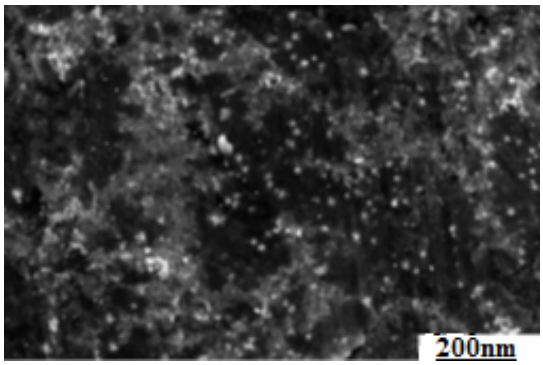
3.1 UV-visible absorption spectroscopy

The synthesis of nanosilver was confirmed by UV-visible absorption spectroscopy which is one of the most widely used technique for structural characterisation of AgNPs. AgNPs from *R. stolonifer* showed maximum absorbance at 422 nm, implying that the bioreduction of the silver nitrate has taken place following incubation of the $AgNO_3$ solution in the presence of cell-free fungal filtrate. It is reported that the absorption spectrum of spherical AgNPs presents a maximum between 420 nm and 450 nm (Maliszewska and Sadowski, 2009).

3.2 Scanning electron microscopy

SEM is a method for high resolution surface imaging. The SEM uses electrons for imaging, much as light microscopy uses visible light. The advantages of SEM over light microscopy include greater magnification (up to 100,000×) and much greater depth of field. Different elements and surface topography emit different amounts of electrons, due to this the contrast in the electron micrograph (picture) is representative of surface topography and composition. Figure 2 shows SEM picture of the fungal filtrate after exposure to 1.0 mM aqueous $AgNO_3$ solution for 72 h. The overall morphology of AgNPs is more clearly seen, with well dispersed particles.

Figure 2 SEM – well dispersed AgNPs



3.3 Transmission electron microscopy

TEM study shows the morphology and size details of synthesised AgNPs (Figure 3). In general, the particles are nano sized and well dispersed. The AgNPs formed were predominantly spherical in shape, with the size ranging between 3 to 20 nm. Nanosilver was produced by the reaction of silver ions with the enzymes released by *R. stolonifer*, is exceptionally stable and the stability is due to the capping proteins secreted by the fungus (Mohanpuria, 2008). It is suggest that the biological molecules could possibly perform the function for the stabilisation of the AgNPs.

3.4 Fourier transform-infrared spectroscopy

The chemical functional groups in the sample were determined by IR spectroscopic analysis. Different functional groups absorb characteristic frequencies of IR radiation. Thus, IR spectroscopy is an important and popular tool for structural elucidation and compound identification.

The position of the amides I and II bands in the FTIR spectra of proteins is a sensitive indicator of conformational changes in the protein-secondary structure (Singh et al., 2008). The FTIR spectrum of the SNPs produced by *R. stolonifer* is shown in Figure 4. This spectrum shows the presence of band at 1,645(4), 1,537(5) and 1,454(6) cm^{-1}, the bands at 1,645 cm^{-1} corresponds to primary amine NH band (Fayaz, 2010). The band at ca. 1,454 cm^{-1} is due to methylene scissoring vibrations present in the proteins. Overall, the observation confirms the presence of protein in the samples of AgNPs. It is reported

earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins.

Figure 3 TEM image show AgNPs, synthesised by *R. stolonifer*

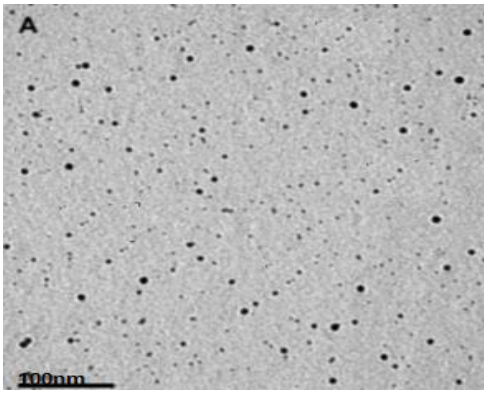
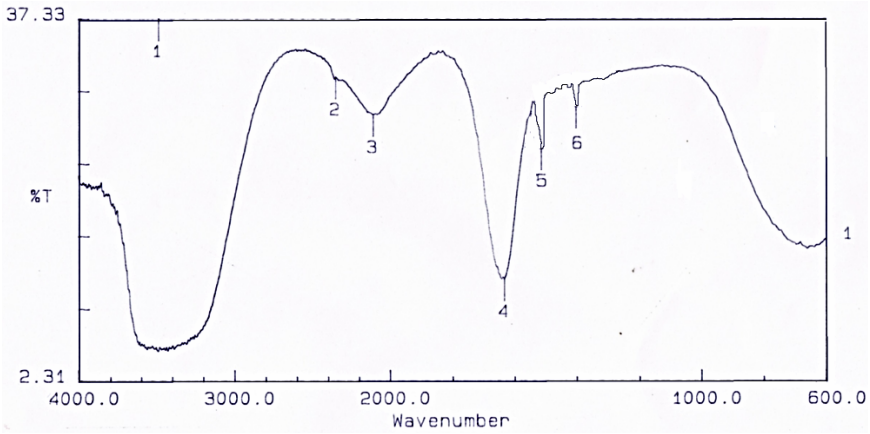


Figure 4 FT-IR spectra recorded from a drop-coated film of AgNPs synthesised by *R. stolonifer*

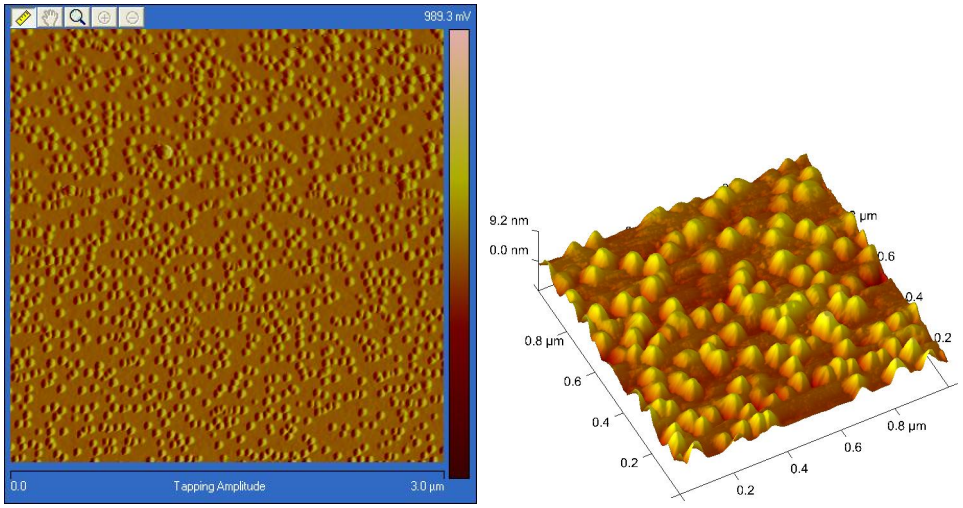


IR spectroscopic study has confirmed that the carbonyl group from amino acid residues and peptides of proteins has the stronger ability to bind metal, so that the proteins could most possibly form a coat covering the metal nanoparticles to prevent agglomeration of the particles and stabilising in the medium. This evidence suggests that the biological molecules could possibly perform the function for the formation and stabilisation of the AgNPs in the aqueous medium.

3.5 Atomic force microscopy

The AFM picture of the sample was analysed. Figure 5(a) shows the particles which are spherical in shape and monodispersed in nature under optimised condition for the production of AgNPs. The topography of the picture shows the particles from three different places seen in Figure 5(b). The height and width of the particle is measured (5 nm) using the software.

Figure 5 (a) AFM picture of the sample and (b) AFM shows the three-dimensional image of the AgNPs (see online version for colours)



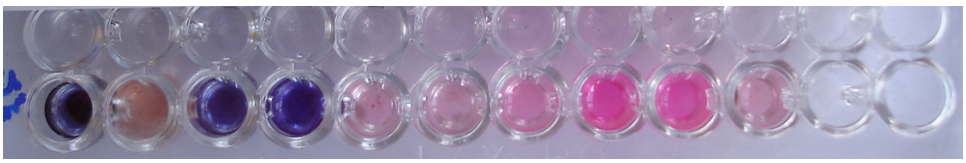
(a) (b)

3.6 M. tuberculosis against AgNPs

MIC test results for all of the clinical isolates of *M. tuberculosis* were available by the eighth day of incubation. After five days of incubation, the Alamar Blue reagent was added to the control wells. Following incubation blue reagent was added to the control wells. Following incubation at 37°C for 24 h, most control wells became pink as can be seen in Figure 6. For those that remained blue, Alamar Blue was added to the next control well and the plates were reincubated for another 24 h until all control wells were pink (indicating sufficient growth to determine drug susceptibility). Alamar Blue was then added to all remaining wells, and the results were determined on the following day (days 7 or 8).

Four strains were susceptible to INH and six were resistant. RMP gives five 50% activity against *M. tuberculosis*, SM shows seven strains resistant and three susceptible, EMB against test strains gives six resistant and four susceptible with MABM. IC90 values of 0.25 µg/ml for RMP, INH, EMB and 4 µg/ml with EMB have been observed. Strains showing IC90 values of > 16 µg/ml were considered as resistant strains, these resistant strains of *M. tuberculosis* were tested against biologically synthesised AgNPs, which has given minimum inhibition concentration of 12.5 µg/ml against *M. tuberculosis*.

Figure 6 Alamar Blue assay (MABA) method was used to determine the MICs of biosynthesised AgNPs against *M. Tuberculosis* (see online version for colours)



4 Conclusions

Living organisms have huge potential for the production of AgNPs and nanodevices of wide applications. This study demonstrated the green synthesis of AgNPs and their activity against ESBL strains. In conclusion, we have reported a simple biological way for synthesising the AgNPs. The kinetics of extracellular synthesis of AgNPs using a cell free filtrate of *R. stolonifer* is presented. The synthesis process was quite fast and nanoparticles were formed within 24 hrs of silver ions coming in contact with cell filtrate. The maximum absorbance was observed at 422 nm. The SEM and TEM images suggest that the particles are well separated, monodispersed and spherical in shape. The size ranges from 3 to 20 nm. The FTIR study suggests that the protein might have played an important role in the stabilisation of AgNPs through coating of protein moiety on the nanosilver particles. Three-dimensional structures of AgNPs have been studied by AFM and clearly show the monodispersed AgNPs with an average size of 5 nm. The synergistic effect of biosynthesised AgNPs with antibiotics has shown excellent antibacterial activity against clinically isolated ESBL-strain. This process of the production of AgNPs is environmental friendly as it is cost effective and free from any solvents and toxic chemicals. The filamentous fungi are easy in handling and also easily amenable on a large scale production.

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