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Biosynthesis of Monodispersed Silver Nanoparticles and their Activity against *Mycobacterium tuberculosis*

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Abstract

Tuberculosis (TB) is a life threatening disease, caused by *Mycobacterium tuberculosis*. Emergence of high degree of drug-resistance and prevalence of *Mycobacterium* other than tuberculosis (MOTT) necessitates the search for new anti-tubercular agents. In that context, silver nanoparticles (AgNPs) which have been known for their antimicrobial activity were evaluated against clinical isolates of *M. tuberculosis* Multi-Drug Resistant (MDR), Extensive-Drug Resistant (XDR) and MOTT strains, through Microplate Alamar Blue Assay (MABA). All the clinical isolates got inhibited within the MIC range of 6.25 to 12.5 µg/ml of AgNPs. Silver nanoparticles were synthesized by bioreduction of silver nitrate ($AgNO_3$) solution with enzymes extracted from *R. stolonifer* and characterized using UV-Vis absorption spectrophotometry, FTIR, XRD, AFM. A spherical shaped AgNPs with the average particle size of 5 nm were synthesized and evaluated for their antimycobacterial activity.

Keywords: *Mycobacterium tuberculosis*; *Rhizopus Stolonifer*; Silver nanoparticles; Microplate alamar blue assay (MABA)

Introduction

The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology [1]. 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 [2]. Currently, the metallic nanoparticles are thoroughly being explored and extensively investigated as potential antimicrobials. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. The small size and the high surface to volume ratio i.e., large surface area of the nanoparticles enhances their interaction with the microbes to carry out a broad range of probable antimicrobial activities. Silver nanoparticles 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 [3].

Several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. It was also suggested that silver ions (particularly Ag+) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or can react with sulfur-containing proteins, leading to the inhibition of enzyme functions [4]. The general understanding is that Ag nanoparticle of typically less than 20 nm diameters get attached to sulfur-containing proteins of bacterial cell membranes leading to greater permeability of the membrane, which causes the death of the bacteria [5]. The dose dependent effect of silver nanoparticles (in the size range of 10-15 nm) on the Gram-negative and Grampositive microorganisms has been studied [6]. At micromolar levels of Ag+ ions have been reported to uncouple respiratory electron transport from oxidative phosphorylation, inhibit respiratory chain enzymes, or interfere with the membrane permeability to protons and phosphate [7]. In addition, higher concentrations of Ag+ ions have been shown to interact with cytoplasmic components and nucleic acids [8].

The emergence of multi-drug resistant 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 [9,10]. The Alamar blue oxidation-reduction dye is a general indicator of cellular growth and/or viability; the blue, nonfluorescent, oxidized form becomes pink and fluorescent upon reduction [11]. Growth can therefore be measured with a fluorometer or spectrophotometer or determined by a visual color change.

It is estimated that one-third of the world's population is infected with the tubercle bacillus [12]. While only a small percentage of infected individualswill develop clinical tuberculosis, 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 [13]. A number of efficacious anti-tubercular agents were discovered in the late 1940s and 1950s with the last, rifampin, introduced in the 1960s [14]. These agents had reasonable efficacy and, when used in combination, would preclude the development of drug resistance. The use or (in most cases) misuse

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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 [15].

Materials and Methods

Synthesis of silver nanoparticles

Fungi for the synthesis of AgNPs was 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) [16]. 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 intoErlenmeyer flaskcontaining 100 ml distilled water and were incubated at the above said condition. The biomass was filtered again, (Whatman filter paper No.1) 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 color change from yellow to brown were subjected to UV-Visible absorption spectrophotometric and SEM studies. Control (without silver ions) was also run along with the experimental flasks.

Characterization of silver nanoparticles

Formation of nanosilver was monitored using UV-Visible absorption Spectroscopy (T90+UV/vis spectrometer), which is one of the important technique to verify the formation of metal nanoparticles provided surface Plasmon resonance exists for the metal [17]. Absorption spectroscopy in the UV-Visible region has long been an important tool for the nanoparticle characterization. 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. Appearance of color arises from the property of the colored material to absorb selectively within the visible region of the electromagnetic spectrum. To detect silver nanoparticle the absorption range is 400 to 450 nm [18].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 biosynthesized AgNPs were studied by Atomic Force Microscopy (AFM).

Silver nanoparticles against *M. tuberculosis*

Drug preparation: Ten clinical isolates of *M. tuberculosis* obtained from Khwaja Bande Nawaz Hospital, Gulbarga and were sub culturedon Middlebrook 7H11 agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Suspensions were prepared in 0.04% (vol/vol) Tween 80-0.2% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) so that theirturbidities matched that of a McFarland no. 1 turbidity standard. Suspensionswere 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), rifampin (RMP), streptomycin (SM), and ethambutol (EMB) were obtained from Sigma. Stock solutions of INH, SM, and EMB were prepared in deionized 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.

Anti-TB activity using Alamar Blue Method: The anti-mycobacterial activity of antibiotics and nanosilver were assessed against *M. tuberculosis* using Microplate Alamar Blue Assay (MABA) [19]. This methodology is non-toxic, uses a thermally stable reagent. Briefly, 200 µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized 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 were made directly on plate. The drug concentrations ranges between 8 and 64 µg/ml. Plates were covered and sealed with parafilm and incubated at 37°C 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 color in the well was interpreted as no bacterial growth, and pink color was scored as growth.The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

The strains which is having 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 were taken from 0.2, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml.

Results and Discussion

Silver nanoparticles were synthesized biologically from *R. stolonifer* using $AgNO_3$**.** 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 color after the incubation. The appearance of brown color solution (Figure 1) clearly indicates the formation of silver nanoparticles [20,21]. The color change was caused by the surface plasmon resonance of silver nanoparticles in the visible region [22]. Silver nanoparticles are known to exhibit size and shape dependent surface plasmon resonance bands which are characterized by UV-Visible absorption spectroscopy [23]. This event clearly indicates that the reduction of the ions occur extracellularly through reducing agents released in to the solution by fungi**.** 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 oxidizes it to NAD+. The enzyme is then oxidized by the simultaneous reduction of metal ions [24]. The mechanistic aspect was explained by Duran et al. [25] that apart from enzymes, quinine derivates of napthoquinones and anthraquinones also act as redox centres in the reduction of silver nanoparticles [25].

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 characterization of silver nanoparticles. Silver nanoparticles 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

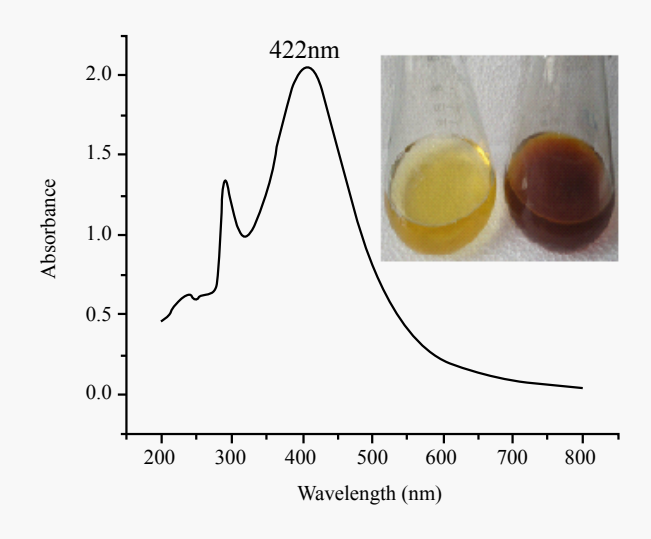

Figure 1: UV-visible absorption spectroscopy of silver nanoparticles of *R. stolonifer*.

silver nanoparticles presents a maximum between 420 nm and 450 nm (Maliszewska, 2008).

Transmission electron microscopy

Transmission electron microscopic study shows the morphology and size details of synthesized silver nanoparticles (Figure 2). In general the particles are nano sized and well dispersed. The silver nanoparticles 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 [26]. It is suggest that the biological molecules could possibly perform the function for the stabilization of the AgNPs.

Fourier transforms 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 amide I and II bands in the FTIR spectra of proteins is a sensitive indicator of conformational changes in the protein-secondary structure [27]. The FTIR spectrum of the SNPs produced by *R. stolonifer* is shown in (Figure 3). This spectrum shows the presence of band at 1645(4), 1537(5) and 1454(6) cm^{-1}, the bands at 1645 cm^{-1} corresponds to primary amine NH band [28]. The band at ca.1454 cm^{-1} due to methylene scissoring vibrations present in the proteins. Overall the observation confirms the presence of protein in the samples of silver nanoparticles. It is reported earlier that proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins.

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 stabilizing in the medium. This evidence suggests that the biological molecules could possibly perform the function for the formation and stabilization of the AgNPs in the aqueous medium.

Atomic force microscopy

The Atomic Force Microscopic (AFM) picture of the sample was analysed. Figure 4a shows the particles which are spherical in shape and monodispersed in nature under optimized condition for the production of silver nanoparticles. The topography of the picture shows the particles from three different places seen in figure 4b. The height and width of the particle is measured (5 nm) using the software.

M. tuberculosis against silver nanoparticles

MIC test results for all of the clinical isolates of *M. tuberculosis* were available by the 8th day of incubation. After 5 days of incubation, the AlamarBlue reagent was added to the control wells. Following incubationBlue reagent was added to the control wells. Following incubationat 37°C for 24 h, most control wells became pink can be seen in figure 5. For those that remained blue, Alamar Blue was added to the nextcontrol well and the plates were reincubated for another 24 huntil all control wells were pink (indicating sufficient growth todetermine drug susceptibility). Alamar Blue was then added toall remaining wells, and the results were determined on thefollowing day (day 7 or 8).

Four strains were susceptible to INH and six were resistant. RMP gives five 50% activity against *M. tubersulosis*, SM shows seven strains resistant and 3 susceptible, EMB against test strains gives six resistant

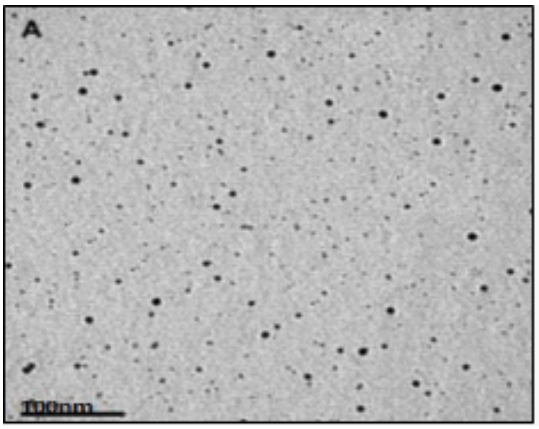

Figure 2: TEM image show silver nanoparticles, synthesized by *R. stolonifer*.

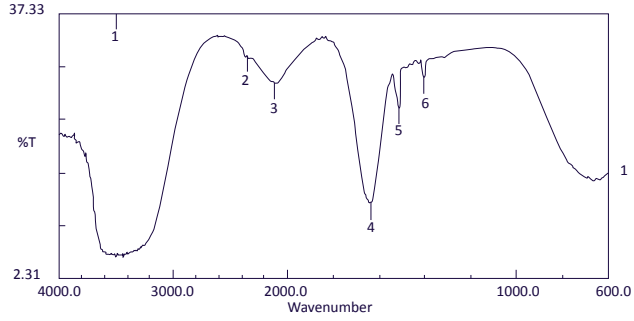

Figure 3: FT-IR spectra recorded from a drop-coated film of silver nanoparticles synthesized by *R. stolonifer*.

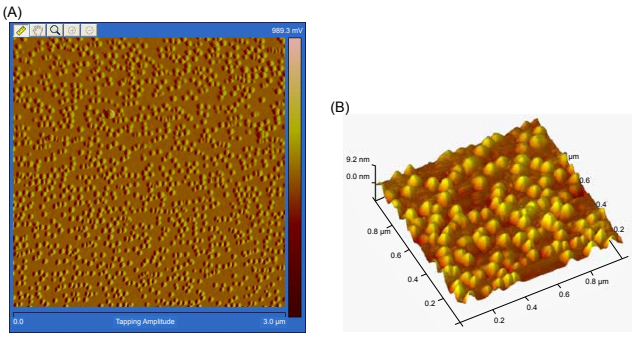

Figure 4: (a) AFM picture of the sample and (b) AFM shows the three dimensional image of the silver nanoparticles.

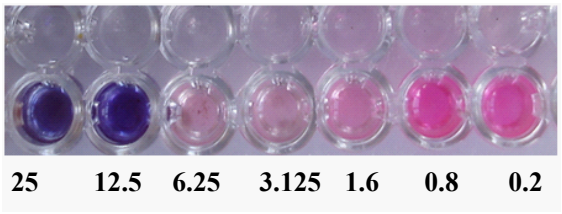

Figure 5: Alamar Blue assay (MABA) method was used to determine the MICs of biosynthesized AgNPs against *M. tuberculosis*.

and four susceptible with MABM. IC_{90} value of 0.25 µg/ml for RMP, INH, EMB and 4 µg/ml with EMB have been observed. Strains showing IC_{90} values of >16 µg/ml were considered as resistant strains, these resistant strains of *M. tuberculosis* were tested against biologically synthesized silver nanoparticles, which has given minimum inhibition concentration of 12.5 µg/ml against *M. tubersulosis.*

Conclusion

Living organisms have huge potential for the production of silver nanoparticles and nanodevices of wide applications. This study demonstrated the green synthesis of silver nanoparticles and their activity against ESBL strains. In conclusion, we have reported a simple biological way for synthesizing the silver nanoparticles. The kinetics of extracellular synthesis of silver nanoparticles 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 image suggests that the particles are 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 stabilization of silver nanoparticles through coating of protein moiety on the nanosilver particles. Three dimensional structures of silver nanoparticles have been studies by Atomic Force Microscopy (AFM) clearly show the monodispersed AgNPs the average size of 5 nm. The synergistic effect of biosynthesized silver nanoparticles with antibiotics has shown the excellent antibacterial activity against clinically isolated ESBL-strain. This process of the production of silver nanoparticles 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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