INTERNATIONAL JOURNAL OF ENVIRONMENTAL SCIENCES Volume 1, No 7, 2011

Research article ISSN 0976 – 4402

Synthesis of monodispersed silver nanoparticles by Rhizopus Stolonifer and its antibacterial activity against MDR strains of Pseudomonas Aeruginosa from burnt patients

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ABSTRACT

The extracellular synthesis of silver nanoparticles by *Rhizopus stolonifer* and its efficacy against multidrug resistant **(**MDR) strains isolated from burnt cases from hospitals at Gulbarga region, Karnataka, India is reported here. Synthesis of silver nanoparticles was carried out using fungal filtrate and an aqueous solution of silver nitrate ($AgNO_3$). Characterization of silver nanoparticles was done by UV-Visible absorption spectroscopy shows maximum absorption at 422 nm, Transmission Electron Microscope (TEM) revealed the formation of spherical nanoparticles with size ranging between 5 to 50 nm. Energy Dispersive Spectroscope (EDS) shows the optical absorption peak at 3kev, Fourier Transform Infrared (FT-IR) shows the bands at 1633, 1554 and 1423 cm-1 which confirms the presence of protein in the sample which coat covering the silver nanoparticles known as capping proteins, and Atomic Force Microscope (AFM) revealed three dimensional structures of the nanoparticles. Two MDR-strains of *Pseudomonas aeruginosa* (P1and P2) from burnt patients were selected for the antibacterial study with AgNPs.

Keywords: *Rhizopus stolonifer*, silver nanoparticles, AFM, MDR-strains

1. Introduction

The field of nanoscience has blossomed over the last twenty years and the need for nanotechnology will only increase as miniaturization becomes more important in areas such as computing, sensors, and biomedical applications (David D, Evanoff Jr, Chumanov G. 2005). Silver's antibiotic properties have made the metal a popular treatment for wounds and burns. Special dressings for burns provide antimicrobial barrier protection using concentrations of nanosilver particles. The medical bandages help skin to heal by preventing infections during treatment. The silver impregnated dressings require fewer painful changings of dressings than previous silver treatments. Historically, silver has been known to have a disinfecting effect and has been found in applications ranging from traditional medicines to culinary items. Moreover, several salts of silver and their derivatives are commercially manufactured as antimicrobial agents (Krutyakov Y.A, Kudrynskiy A.A, Olenin A.Y, Lisichkin G.V. 2008). Nanotechnology is expected to open some new aspects to fight and prevent diseases using atomic scale tailoring of materials. The ability to uncover the structure and function of biosystems at the nanoscale, stimulates research leading to improvement in biology, biotechnology, medicine and healthcare. Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging, sensing, targeted drug delivery and gene delivery systems and artificial implants. The new age drugs are nanoparticles of polymers, metals or ceramics, which can combat conditions like cancer and fight human pathogens like bacteria (Singh M, Singh S, Prasad S, Gambhir I.S. 2008).

Received on April 2011 Published on June 2011

The development of new resistant strains of bacteria to current antibiotics has become a serious problem in public health; therefore, there is a strong incentive to develop new bactericides. Bacteria have different membrane structures which allow a general classification of them as Gram-negative or Gram positive. The structural differences lie in the organization of a key component of the membrane, peptidoglycan. Gram negative bacteria exhibit only a thin peptidoglycan layer (~2–3 nm) between the cytoplasmic membrane and the outer membrane; in contrast, Gram-positive bacteria lack the outer membrane but have a peptidoglycan layer of about 30 nm thick. Silver has long been known to exhibit a strong toxicity to a wide range of bactericidal applications (Gupta A, Maynes M, and Silver S. 1998). Silver compounds have also been used in the medical field to treat burns and a variety of infections (Feng Q.L, Wu J, Chen G.Q, Cui F.Z, Kim T.N, Kim J.O. 2000). However, the bactericidal property of these nanoparticles depends on their stability in the growth medium, since this imparts greater retention time for bacterium-nanoparticle interaction. There lies a strong challenge in preparing nanoparticles of silver stable enough to significantly restrict bacterial growth.

Studies were carried out on MDR-strains of *P.aeruginosa.* The effect of the nanoparticles was found to be significantly more pronounced on MDR-strains, irrespective of whether the strains were resistant or not. We attribute this enhanced antibacterial effect of the nanoparticles to their stability in the medium as a colloid, which modulates the phosphotyrosine profile of the bacterial proteins and arrests bacterial growth.

Experimental evidence suggests that DNA loses its replication ability once the bacteria have been treated with silver ions (Feng Q.L, Wu J, Chen G.Q, Cui F.Z, Kim T.N, Kim J.O. 2000). Other studies have shown evidence of structural changes in the cell membrane as well as the formation of small electron-dense granules formed by silver and sulfur (Feng Q.L, Wu J, Chen G.Q, Cui F.Z, Kim T.N, Kim J.O. 2000). The bactericidal effect of silver ions on micro-organisms is very well known; however, the bactericidal mechanism is only partially understood. It has been proposed that ionic silver strongly interacts with thiol groups of vital enzymes and inactivates them (Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T. 2003). Silver ions have been demonstrated to be useful and effective in bactericidal applications, but due to the unique properties of nanoparticles nanotechnology presents a reasonable alternative for development of new bactericides. Metal particles in the nanometer size range exhibit physical properties that are different from both the ion and the bulk material.

For the synthesis of AgNPs biological method have an advancement over physical and chemical methods as it doesn't require high energy, pressure and toxic chemicals, we report the synthesis of silver nanoparticles biologically using fungi *R. stolonifer* which is cost effective, eco-friendly, acceptable technology. Filamentous fungi are easy to handle and capable of synthesizing AgNPs extracellularly, and hence are preferred over bacteria and unicellular organisms (Mukherjee P, Roy M, Mandal B.P. 2008). The nanoparticles synthesized using fungi present good monodispersity and stability (Govindaraju K, Tamilselvan S, kiruthiga V, Singaravelu G. 2010).

Silver nanoparticles have shown bactericidal activity against various strains of bacteria including *salmonella, staphylococcus and pseudomonas* (Patil S.S, Dhumal R.S. 2009). Antimicrobial silver is now used extensively to combat organisms in wounds and burns. It works because pathogens cannot mutate to avoid its antimicrobial effect. In the process of developing burn and wound silver technologies, researchers have studied the antimicrobial properties of silver nanoparticles to remain effective in the face of virulent pathogens. The positively charged ionic form is highly toxic for microorganisms but has relatively low

toxicity for human tissue cells (Patil S.S, Dhumal R.S. 2009). Nanosilver works in a number of ways to disrupt critical functions in microorganisms, for example it has high affinity for negatively charged side groups on biological molecules. These include groups such as sulfhydryl, carboxyl, phosphate and other charged groups distributed throughout microbial cells. This binding reaction alters the molecular structure of the macromolecules rendering it worthless to the cell. Silver simultaneously attacks multiple sites within the cell to inactivate critical physiological functions such as cell wall synthesis, membrane transport, nucleic acid (such as RNA and DNA) synthesis and translation, protein folding and function, and electron transport, which is important in generating energy for the cell. Without these functions, the bacterium is either inhibited from growth or, more commonly, the microorganism is killed (Gibbins B, Warner L. 2005).

Antibiotic resistant bacteria increasing in frequency and numbers can cause an environmental health problem in the community. The emerging antimicrobial resistance trends in burn wound represents a serious therapeutic challenge for clinicians. Currently the investigation of this phenomenon has regained importance due to the increase of bacterial resistance to antibiotics, caused by their overuse.

Here we report the synthesis of stabilized silver nanoparticles using a cell-free aqueous filtrate from *R. stolonifer*, its characterization by UV-Visible absorption spectra, SEM-EDS, TEM, AFM and FT-IR. Further studies were also done on antibacterial activity of biosynthesized AgNPs against multidrug resistant (MDR) strains of *P.aeruginosa* isolated from burnt patients.

2. Materials and Method

2.1 Synthesis of silver nanoparticles

Silver nitrate was purchased from High media, Mumbai. *R. stolonifer* biomass was grown in Malt Glucose Yeast Peptone (MGYP) broth, containing yeast extract and malt extract- 0.3% each, glucose-1%, peptone-0.5%, the inoculated broth was maintained at 40^{o}C, under constant agitation at 180 rpm in an orbital shaker for three days (Karbasian M, Atyabi SM, Siyadat SD, Momen SB and Norouzian D. 2008). After 72 h of incubation the fungal biomass was filtered and then extensively washed with distilled water to remove any adhering media components. The wet fungal mycelia was suspended in 100 ml distilled water and incubated at the above said conditions. The mycelial suspension so obtained was filtered again, (Whatman filter paper No.1) and after 72 h, the fungal filtrate was used further. Aqueous solution of AgNO3 (1mM AgNO3 of final concentration) was mixed with fungal filtrate and the reaction was allowed to proceed at 40^{o}C under constant agitation at 180 rpm. Completion of the reaction was adjudged by UV-Visible absorption spectroscopy. Further characterization was done by SEM-EDS, TEM, FT-IR, and AFM.

2.2 Characterization of silver nanoparticles

Absorption spectroscopy in the UV-visible region has long been an important tool for the nanoparticle characterization. UV-Visible absorption spectrophotometer (T90/T90+ doublebeam) with a resolution of 1 nm was used for recording the UV-Visible absorption spectrum, which is one of the important technique to verify the formation of metal nanoparticles provided surface plasmon resonance exists for the metal (Basavaraja S, Balaji S.D, Arunkumar L, Rajasab A.H, Venkataraman A. 2008). The presence of elemental silver was confirmed through energy dispersed spectroscope. Transmission electron micrograph

pattern were recorded on a carbon coated copper grid on a Hitachi-H-7500 machine. The interaction between protein and AgNPs was analysed by fourier transform infrared spectroscope (JASCO FT/IR-3500). Three dimensional structures of biosynthesized silver nanoparticles were observed by atomic force microscopy.

2.3 Source of MDR-strains

Two MDR strains of *Pseudomonas aeruginosa* (P1 and P2) from burnt cases from hospitals at Gulbarga region, Karnataka, India were subjected to study the antibacterial efficacy of silver nanoparticles. These strains which were completely resistant to ampicillin, ciprofloxacine, cefuroxime, gentamicin and tobramicin were considered as multidrug resistant and selected for the study. AgNPs (silver nanoparticles) produced by *R.stolonifer* gave good antibacterial efficacy against these multidrug resistant strains of *P. aeruginosa* isolated from burnt patients.

2.4 The antibacterial efficacy of nanosilver

Antibacterial activity of nanosilver was studied against multidrug resistant clinical isolates by agar diffusion method. The bacterial suspension was made in Muller-Hinton liquid medium at 37^{o}C. The bacterial inoculum was prepared by diluting the overnight culture with 0.9% NaCl to a 0.5 McFarland standard and inoculated on Muller-Hinton Agar, cavities were made with the help of gel puncture. 20µL (0.001mg) of freshly prepared silver nanoparticles were used to check the antibacterial activity. The zone of inhibition was measured.

2.5 Synergistic effect of silver nanoparticles with antibiotics

A disc diffusion method was used to assay the synergistic effect of antibiotics with biosynthesized silver nanoparticles for antibacterial activity against clinically isolated strains of *P.aeruginosa* on Muller-Hinton agar plates. The standard antibiotic discs were purchased from Himedia (Mumbai,India). To determine the synergistic effect, standard antibiotic disc was further impregnated with 20µL of freshly prepared silver nanoparticles (final content 0.001mg of AgNPs per disc), further these discs were subjected for antibacterial activity.

2.6 MIC of silver nanoparticles against *P.aeruginosa*

To examine the MIC of AgNPs and the growth curve of *P.aeruginosa* to AgNPs, different concentration of nanosilver 5, 10, 20, 25, 30µL/ml was added in LB medium. Bacterium culture (*P.aeruginosa*) was controlled at 105-106 CFU/ml and incubated at 37^{o}C. To establish the antibacterial activity of nanosilver on bacterial growth, the MIC of AgNPs was determined by optical density of the bacterial culture solution containing different concentration of nanosilver particles.

3. Results and Discussion

Study on the extracellular biosynthesis of AgNPs using *R.stolonifer* and the antibacterial effect of biosynthesized AgNPs against *P.aeruginosa* (MDR strain) were reported. The color change of the fungal filtrate was noted by visual observation. The characterization of AgNPs was done by UV-Visible absorption spectroscopy, which shows an intense peak at 422 nm can be seen in Figure 1. EDS analysis, gives the optical absorption peak approximately at 3kev depicted in Figure 2.

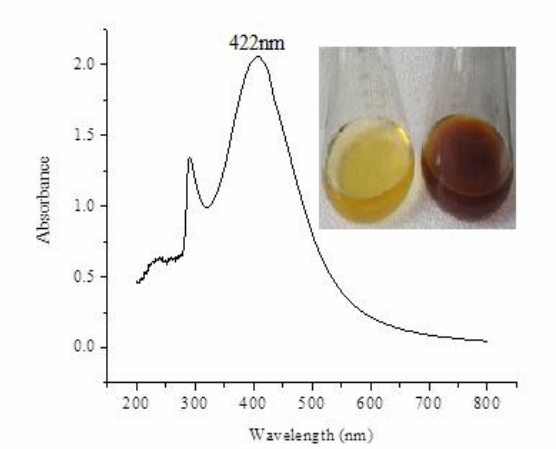

Figure 1: UV-Visible absorption spectra of AgNPs produced by *R.stolonifer*

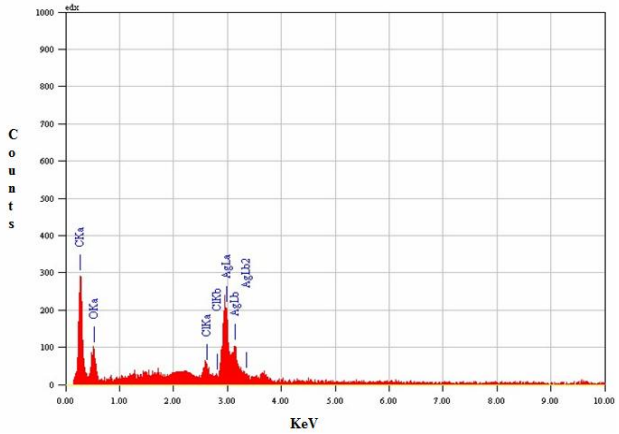

Figure 2: EDS of the nanosilver synthesized by *R.stolonifer*

The TEM technique used to visualize size and shape of the biosynthesized silver nanoparticles have shown spherical shaped structures with size ranging between 5 to 50 nm presented in Figure 3. All the particles were well separated and no agglomeration was noticed. The FT-IR spectrum of extracellular biosynthesized AgNPs has shown the presence of bands at 1645, 1537 and 1454cm^{-1} can be observed in Figure 4. Three dimensional images of silver nanoparticles produced by *R.stolonifer* are presented in Figure 5.

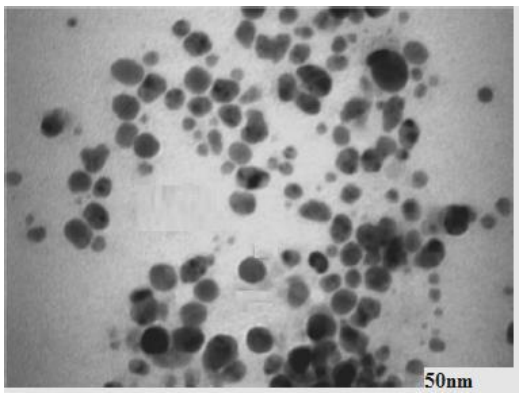

Figure 3: TEM images of AgNPs Synthesized by *R. stolonifer*

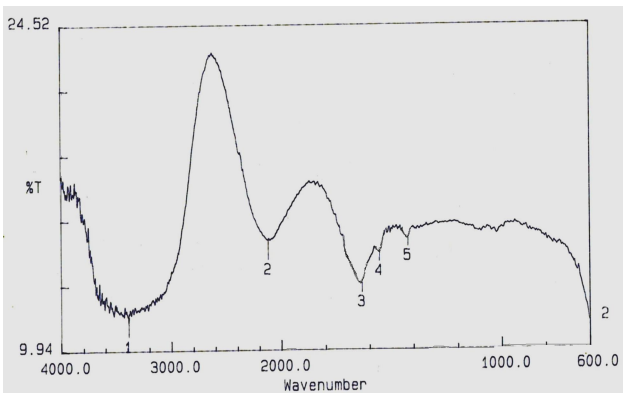

Figure 4: FT-IR spectra of silver nanoparticles

In our experiment the biosynthesized nanosilver showed excellent antibacterial activity against multidrug resistant *P aeruginosa* isolated from burnt infections can be seen in Figure 8. Two strains of *P. aeruginosa* (P1 and P2) were selected for the study. Agar diffusion method was employed to study the antibacterial effect. Biologically synthesized nanosilver showed zone of inhibition (mm) of about 33mm and 30.5mm in diameter for P1 and P2 strains of *p.aeruginosa* respectively. The combination of these biosynthesized silver nanoparticles with antibiotics was also investigated against MDR-strains using disc diffusion method can be seen in Figure 9. The diameter of the zone of inhibition around the antibiotic discs with and without silver nanoparticles against the test strains is shown in Table 1.

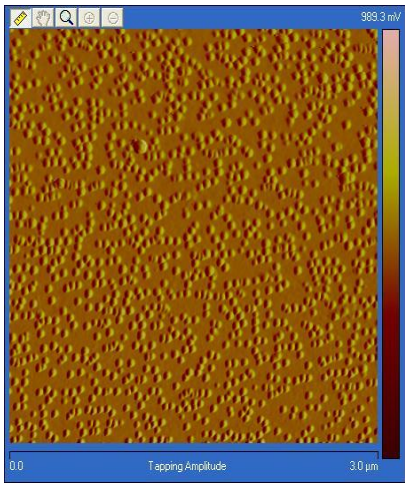

Figure 5a: AFM picture of the sample

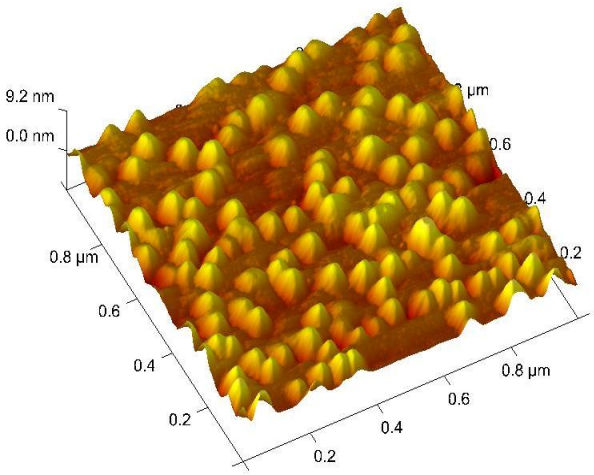

Figure 5b: AFM shows the three dimensional images of AgNPs.

MIC of AgNPs against *p.aeruginosa* was studied in LB liquid media supplemented with different concentrations of AgNPs. Figure 7 shows the increasing concentration of nanoparticles progressively inhibited the growth test strain. The concentration of 20µL/ml was found to be strongly inhibitory for *P.aeruginosa*.

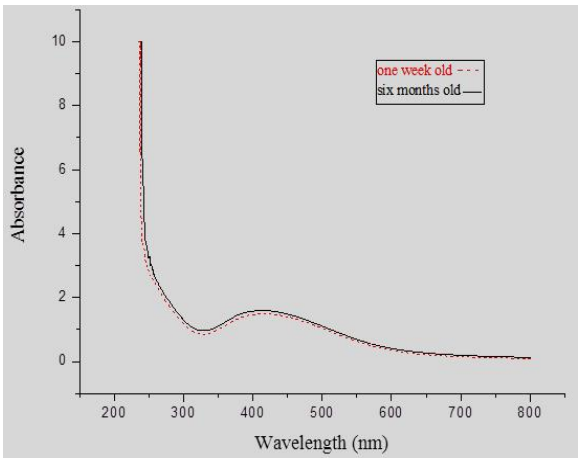

Figure 6: Absorption spectra of AgNPs recorded one week after the synthesis and after six months.

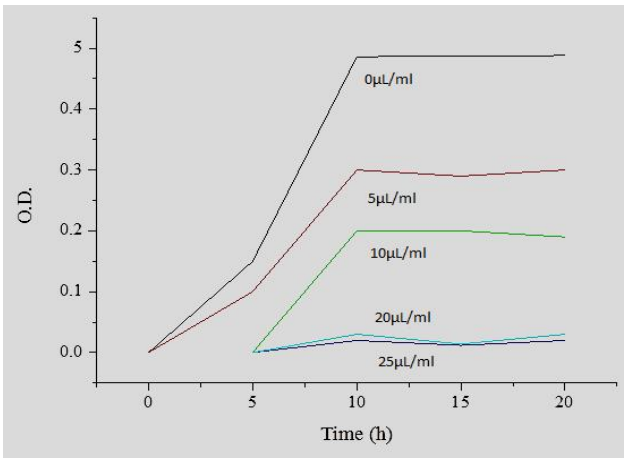

Figure 7: Bacterial *(P.aeruginosa)* curve in LB media at different concentration of AgNPs.

Living organisms have huge potential for the production of silver nanoparticles of wide applications. This study demonstrated the green synthesis of silver nanoparticles and their activity against MDR strains of *P.aeruginosa*. Here we have reported a simple biological way for synthesizing the silver nanoparticles extracellularly using a cell free filtrate of *R.stolonifer*. The silver nanoparticles were formed within 24 h of silver ions coming in contact with cell filtrate, showed brown color. The appearance of brown color solution clearly indicates the formation of silver nanoparticles (Mukherjee P, Ahmed A, Mandal D, Senapati S, Sainkar SR, Khan M.I. 2001a, 2001b). This event indicates that the reduction of the ions occur extracellularly through the enzymes secreted by the fungi in the solution. The AgNPs produced by *R. stolonifer* were characterized by UV-Visible absorption spectroscopy and the maximum absorption was observed at 422 nm. Our results are correlating with the reports of

Sadowski et al, 2008 and Maliszwaska et al 2009 with the fungus *penicillium*. Mukherjee et al 2007 reported an intense peak at 410 nm. Surface Plasmon peaks were also located at 420 nm using *klebsiella pneumonia* (Minalian et al 2008). It is reported that the absorption spectrum of spherical silver nanoparticles presents a maximum between 420 nm and 450 nm (Maliszewska 2008). Silvercnanocrystals are known to exhibit size and shape-dependent SPR bands which is characterized by UV-Visible absorption spectroscopy (Xie J, Lee J.Y, Ting Y P. 2007).

The synthesis of nanosilver by *R.stolonifer* is further characterized by EDS analysis, which gives the additional evidence for the reduction of silver nanoparticles to elemental silver (Amanulla M.F. 2010). The optical absorption peak is seen approximately at 3kev, which is typical for the absorption of metallic silver nanocrystals due to surface plasmon resonance, which confirms the presence of nanocrystalline elemental silver. Spectrum shows strong silver signal along with weak oxygen and carbon peak, which may be originate from the biomolecules that are bound to the surface of nanosilver particles can be observed in Figure 2.

A representative TEM image recorded from drop coated film of a silver nanoparticles sample preserved for over 6 months. This has been deliberately done to observe the effect of ageing on the size of the particles. The silver nanoparticles are spherical in structure. All the particles are well separated and no agglomeration was noticed. The size ranges between 5 nm to50 nm was seen. The process of growing silver nanoparticles comprises of two key steps: (a) bioreduction of AgNO3 to produce silver nanoparticles and (b) stabilization and/or encapsulation of the same by suitable capping agents (Mukherjee P, Roy M, Mandal B.P. 2008). It is suggest that the biological molecules could possibly perform the function for the stabilization of the AgNPs. Silver nanoparticles synthesized by this route are fairly stable even after prolonged storage. This may be concluded that there is not much agglomeration of the AgNPs even after preserving the colloidal solution for extended periods. Fig. 6 clearly represents the stability of AgNPs even after 6 months of storage.

The aim of IR spectroscopic analysis is to determine chemical functional groups in the sample. The amide linkages between amino acid residues in polypeptides and proteins give rise to well-known signatures in the infrared region of the electromagnetic spectrum. Different functional groups absorb characteristic frequencies of IR radiation. Thus, IR spectroscope is an important and popular tool for structural elucidation and compound identification. IR spectra shows the bands at 1633(3) and 1554(4) cm^{-1} are identified as the amide I and amide II and arises due to carbonyl stretch and –N-H stretch vibrations in the amide linkages of the proteins, respectively. The band at 1423(5) cm^{-1} is assigned to methylene scissoring vibration from the protein in the solution. Overall the observation confirms the presence of protein in the sample which coat covering the silver nanoparticles known as capping proteins. Capping protein stabilizes the metallic nanoparticle and prevents agglomeration in the medium. This study gives the evidence of formation and stabilization of silver nanoparticles in the aqueous medium by using biological molecules.

For more information about the biosynthesized silver nanoparticles the sample was subjected to atomic force microscopic study. Figure 5a shows the particles which are spherical in shape, smooth surface 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 5b. The height and width of the particle is measured (5 nm) using the software.

3.1 Antibacterial efficacy of AgNPs

The biosynthesized nanosilver showed excellent antibacterial activity against multidrug resistant *Pseudomonas aeruginosa* isolated from burnt infections from hospitals at Gulbarga region, Karnataka, India. Two strains of *P. aeruginosa* (P1 and P2) were selected for the study. Agar diffusion method was employed to study the antibacterial effect. Biologically synthesized nanosilver showed zone of inhibition (mm) of about 33mm and 30.5mm in diameter for P1 and P2 strains of *p.aeruginosa* respectively. Here we report the efficacy of mycogenic metal nanosilver to kill MDR strains which is difficult through the conventional chemotherapy. It is reasonable to state that the binding of particles to the bacteria depends on the surface area available for interaction. Smaller particles having the larger surface area available for interaction will give more bactericidal effect than the larger particles. The gram negative bacteria have a layer of lipopolysaccharide at the exterior, followed by a thin layer of peptidoglycan negative charges on the lipopolysaccharides are attracted towards positive charges available on silver nanoparticles and disturb its power function such as permeability and respiration (Raffi M, Hussain F, Bhatti3 T.M, Akhter J.I, Hameed A, HasanM.M. 2008). Nanosilver may also penetrate inside the bacteria and cause damage by interacting with phosphorus and sulfur-containing compounds such as DNA (Morones J.R, Elechiguerra J.L. 2005). The results of this study clearly demonstrated that the colloidal silver nanoparticles inhibited the growth and multiplication of the test organisms. Such high antibacterial activity was observed at very low concentration of nanosilver 20µL (0.001mg).

Figure 8: Nanosilver shows the zone of inhibition against *P. aeruginosa*

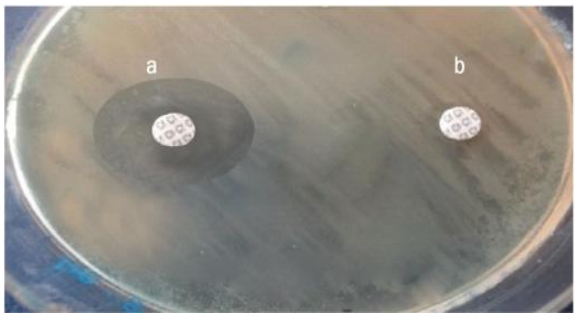

Figure 9: AgNPs shows the Zone of inhibition against *P. aeruginosa*
a) Antibiotic with AgNPs b) only antibiotic

The synergistic effect of biosynthesized silver nanoparticles with antibiotics was also investigated against MDR-strains using disc diffusion method. The diameter of the zone of inhibition around the antibiotic discs with and without silver nanoparticles against the test strains is shown in Table 1.

Table.1: Zone of inhibition (mm) of AgNPs (with and without antibiotic) against MDR-strains

Microorganisms	Antibiotics Zone(mm)	AgNPs (a) Zone(mm)	Antibiotic+AgNPs (b)	Fold increase %
P.aeruginosa (p1)	-	33	34.5	4.54%
P.aeruginosa (p2)	-	30.5	31	1.63%

The increase in synergistic effect may be caused by the bonding reaction between antibiotic and nanosilver. The antibiotic molecules contain many active groups such as hydroxyl and amido groups, which react easily with nanosilver by chelation (Mukherjee P, Roy M, Mandal B.P. 2008). Here we present a possible explanation for the enhancement of the synergistic antibacterial mechanism. This research provides helpful insight into the development of new antimicrobial agents.

In further experiment, *p.aeruginosa* was inoculated in LB liquid media supplemented with different concentrations of AgNPs. Fig.7 shows the increasing concentration of nanoparticles progressively inhibited the growth test strain. The concentration of 20µL/ml was found to be strongly inhibitory for *P.aeruginosa*. Biosynthesized SNPs were found to have a good antibacterial activity on the growth of bacteria.

4. Conclusion

Extracellular biosynthesis of highly stabilized AgNPs using *R.stolonifer* and the antibacterial effect of biosynthesized AgNPs against *P.aeruginosa* (MDR strain) were reported. The characterization of AgNPs was made by UV-Visible absorption spectroscopy, which shows an intense peak at 422 nm. EDS analysis, gives the optical absorption peak approximately at 3kev. The TEM micrograph shown spherical shaped structures with size ranging between 5 to 50 nm, with no agglomeration. The FT-IR spectrum of AgNPs shows the bands at 1645, 1537 and 1454cm^{-1}. AFM has shown the three dimensional images of silver nanoparticles. The biosynthesized nanosilver showed excellent antibacterial activity against multidrug resistant *Pseudomonas aeruginosa* isolated from burnt infections. Biologically synthesized nanosilver showed zone of inhibition (mm) of about 33mm and 30.5mm in diameter for P1 and P2 strains of *p.aeruginosa* respectively. 20µL/ml is the minimum inhibitory concentration of AgNPs which was found to be strongly inhibitory for *P.aeruginosa*.

The process for 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. The nanoparticles synthesized using fungi present good monodispersity and stability. The potential application of nanoparticles in different fields has revolutionized the health care textile and agricultural industry.

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