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Biosynthesis of highly stabilized silver nanoparticles by *Rhizopus stolonifer* and their Anti-fungal efficacy

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ABSTRACT

Highly stabilized monodispersed silver nanoparticles (AgNPs) were synthesized by *Rhizopus stolonifer* and the antifungal efficacy of silver nanoparticles (AgNPs) against *Candida sp.* were studied. Characterization of biosynthesized nanosilver was made by TEM-EDS and AFM. Minimum Inhibitory Concentration (MIC) of biosynthesized AgNPs, Amphotericin B, and Fluconazole have been studied on pathogenic fungi and the changes on membrane reactions have been elucidated by Scanning Electron Microscopy (SEM). The present study indicates AgNPs has considerable antifungal activity comparison with other antifungal drugs Nanosilver showed potent activity against pathogenic fungi. The results showed nano-Ag exerted activity on the mycelia. Thus, the present study indicates nano-Ag may have effective antifungal activity, deserves further investigation for clinical applications.

1. Introduction

Skin infections caused by fungi have become more common in recent years (Woodfolk et al., 2005). In particular, fungal infections are more frequent in patients who are immunocompromised because of cancer chemotherapy, or organ or human immunodeficiency virus infections (Mirmirani et al., 2001). This upward trend is concerning, considering the limited number of antifungal drugs available because prophylaxis with antifungals may lead to the emergence of resistant strains. Therefore, there is an inevitable and urgent medical need for novel antifungals. Since ancient times, it has been known that silver and its compounds are effective antimicrobial agents (Silver, 2003). In articular, because of the recent advances in research on metal nanoparticles, nano-Ag has received special attention as a possible antimicrobial agent (Sondi and Salopek-Sondi, 2004). Therefore, the preparation of uniform nanosized silver particles with specific requirements in terms of size, shape, and physical and chemical properties is of great interest in the formulation of new pharmaceutical products (Merisko-Liversidge et al., 2003) Many studies have shown their antimicrobial effects, but the effects of nano-Ag against fungal pathogens of the skin are mostly unknown. In this study,

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nanosilver was synthesized using *R.stolonifer* and its antifungal effects on *Candida sp* were investigated.

2. Materials and Methods

2.1. Synthesis of silver nanoparticles

Fungi isolated from soil samples were inoculated in Malt Glucose Yeast Peptone (MGYP) broth (Karbasian et al., 2008) 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 72h of incubation the biomass was filtered and then extensively washed with distilled water to remove the medium components. This biomass was taken into flasks containing 100 ml distilled water and were incubated at the above said condition. The biomass was filtered again, (Whatman filter paper No.1) after 72h the fungal filtrate was used further. Aqueous solution of AgNO3 (1mM AgNO3 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.

2.2. 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 (Basavaraja et al., 2008) Absorption spectroscopy in the UV-Visible region has long been an important tool for the nanoparticle characterization. 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 (Melissa et al., 2009) Sample for transmission electron microscopy (TEM) (Hitachi-H-7500) was prepared by drop-coating the AgNPs solution into the carbon-coated copper grid, which shows the size and morphology of the particles. The presence of elemental silver was confirmed through Energy Dispersed Spectroscope (EDS). Three dimensional picture of the biosynthesized AgNPs were studied by Atomic Force Microscopy (AFM). The interaction between protein and AgNPs was analysed by Fourier transform-infrared spectroscopy (FT-IR).

2.3. Microorganisms and culture conditions

Five pathogenic fungal strains were used in this study. *Candida glabrata* (ATCC 90030), *Candida parapsilosis* (ATCC 22019), and *Candida krusei* (ATCC 6258) were obtained from the American Type Culture Collection (ATCC). Clinical isolates of *Candida albicans* and *Trichophyton mentagrophytes* were obtained from the khwaja Banda Nawaz Hospital, Gulbarga, Karnataka India. *Candida* spp. and *Trichophyton mentagrophytes* were maintained in a Sabouraud dextrose agar (SDA) and a potato dextrose agar (PDA) at 35°C, respectively.

2.4. Determination of antifungal susceptibility

MIC of silver nanoparticles was determined against pathogenic fungi by broth micro dilution method based on the Clinical and Laboratory Standards Institute, CLSI, 2000 method outlined in documents M-27A (Mritunjai et al., 2008). An RPMI 1640 medium buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS) was used as the culture medium, and the inoculum size of *Candida sp*. Was 0.5×103 to 2.5×103 cells/ml, and that of *T. mentagrophytes* was 0.4×104 to 5×104 cells/ml. The microdilution plates inoculated with fungi were incubated at 35°C, and the turbidity of the growth control wells was observed every 24 h. The 90% inhibitory concentration (IC_{90}) was defined as the lowest concentration that inhibited 90% of the growth as determined by a comparison with the growth in the control wells. The growth was assayed with a microplate reader (Bio-Tek Instruments, Winooski, VT, U.S.A.) by monitoring absorption at 405 nm. In the current study, amphotericin B and fluconazole were used as a positive control toward fungi; amphotericin B is a fungicidal agent widely used in treating serious systemic infections (Sondi and Salopek-Sondi, 2004) and fluconazole is used in the

treatment of superficial skin infections caused by dermatophytes and *Candida sp* (Mirmirani et al., 2001)

3. Results

A detailed study on the extracellular biogenic synthesis of AgNPs using *R.stolonifer* and the anti-fungal effect of AgNPs against *Candida sp.* 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. The recorded UV-Visible absorption spectra of the reaction solution can be observed in Figure 1. The spectra exhibit an intense peak at 422 nm corresponding to the surface plasmon resonance frequency of silver nanoparticles (Mukherjee et al., 2008). The reduction of Ag^+ ions to elemental silver by *R.stolonifer* is characterized by EDS analysis, the spectrum shows the optical absorption peak approximately at 3kev as depicted in Figure 2. 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 30 nm as presented in Figure 3. All the particles were well dispersed and no agglomeration was noticed. Three dimensional images were studied by atomic force microscopy (AFM). FT-IR spectrum shows the presence of band at 1645(1), 1537(2) and 1454(3) cm^{-1}. The silver nanoparticles exhibited peaks of silver at 2θ=37°, 45°, 64° and 77° that can be indexed to the (111), (200), (220) and (311) facets of silver, respectively, biosynthesized nanoparticles were highly stabilized.

3.1. Determination of antifungal activity

Nanosilver with IC_{90} of 1-2 µg/ml, showed significant antifungal activity against *T.mentagrophytes* and *Candida sp*. While Amphotericin B and Fluconazole shows IC_{90} values of 6 and 8 µg/ml respectively. Toward all fungal strains, nano-Ag exhibited high activity compare to amphotericin B and fluconazole, showing IC_{90} values of 1-3 µg/ml. Amphotericin B shows more potent activity than Fluconazole which gives IC_{90} values of 2-6 µg/ml. While Fluconazole shows IC_{90} values of 8 to20 µg/ml. Amphotericin B gives the IC_{90} values against *C.glabrata* and *C.parasilosis* 2 and 5 µg/ml respectively. The IC_{90} values of nanosilver agaist *C.glabrata* and *C.parasilosis* 1 and 2 µg/ml respectively, 3 µg/ml for *C.krusei.* can be seen in Table 1

Table 1. Antifungal efficacy of nanosilver

Fungal strains	IC_{90} (µg/ml)		
	Nano-Ag	Amphotericin B	Fluconazole
C. albicans	1	6	8
C. glabrata	1	2	16
C. parapsilosis	2	5	13
C. krusei	3	4	13
T. mentagrophytes	2	5	20

3.2. Scanning Electron Microscopy (SEM)

The morphological changes of *Candida albicans*, influenced with AgNPs were observed in scanning electron microscope (SEM) (Figure 3).

4. Discussion

Micro-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 pathogenic fungi. Here we have reported a simple biological way for synthesizing the silver nanoparticles extracellularly using a cell free filtrate of *R. stolonifer.* The synthesis of nanoparticles were formed within 24 hours of silver ions coming in contact with cell filtrate, showed brown color solution after the incubation period of 24 hours. The appearance of brown color solution clearly indicates the formation of silver nanoparticles (Mukherjee et al., 2001). This event indicates that the reduction of the ions occur extracellularly through the enzymes secreted by the fungi in the solution. A representative TEM image recorded from drop coated film of silver nanoparticles. All the particles were well separated without any agglomeration. The size ranges between 5 to 50 nm was seen. It is suggested 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. EDS analysis gives the additional evidence for the reduction of silver nanoparticles to elemental silver. The optical absorption peak is observed approximately at 3kev, which is typical for the absorption of metallic silver nanocrystals due to surface Plasmon resonance, which confirms the

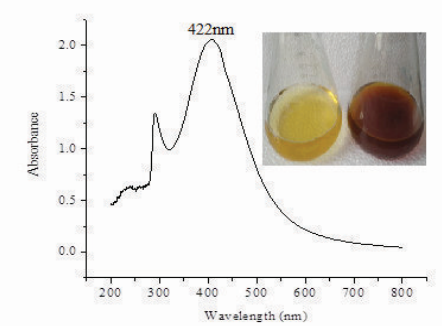

Figure 1. UV-Visible absorption spectra of AgNPs produced by *R.stolonifer*

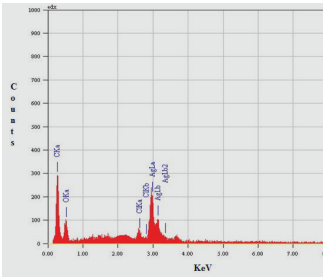

Figure 2. EDS of the nanosilver synthesized by *R.stolonifer*

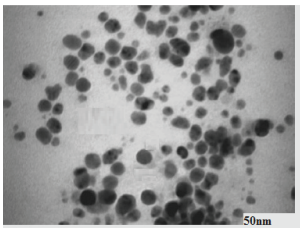

Figure 3. TEM images of silver nanoparticles Synthesized by *R.stolonifer*

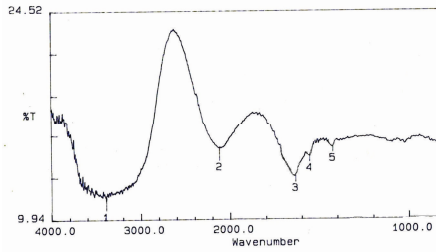

Figure 4. FT-IR spectra recorded from a drop-coated film of silver nanoparticles Synthesized by *R.stolonifer*

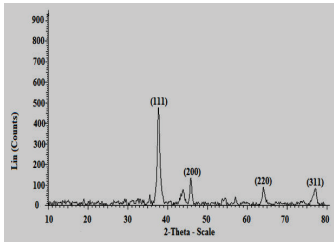

Figure 5. XRD patterns recorded showing 4 sharp peaks of silver with fcc lattice

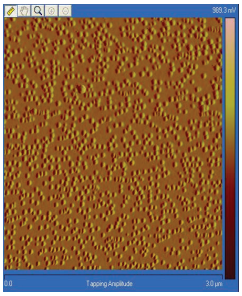

Figure 6a. AFM picture of the sample

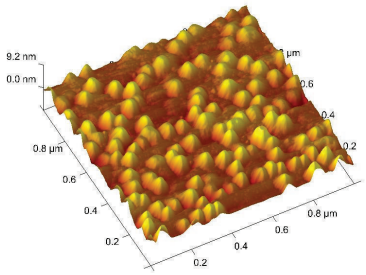

Figure 6b. AFM shows the three dimensional image of the silver nanoparticles

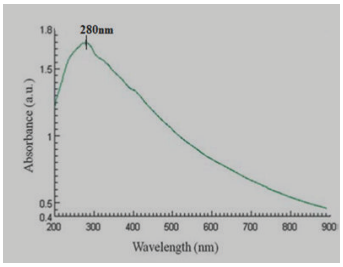

Figure 7. UV-Vis. spectra of cell-free filtrate showing presence of proteins

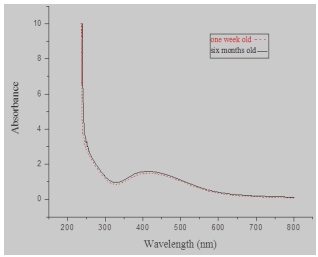

Figure 8. Absorption spectra of AgNPs recorded one week after the synthesis and after six months

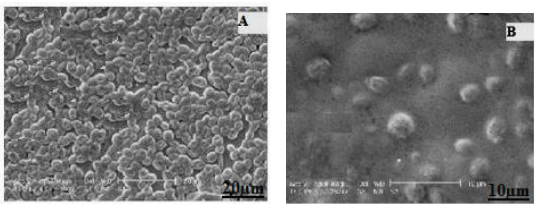

Figure 9. SEM images of *Candida albicans*. (A)Normal cells and (B) cells influenced by AgNPs

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.

The main goal of IR spectroscopic analysis is to determine chemical functional groups in the sample. 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 (Mritunjai 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 1645(1), 1537(2) and 1454(3) cm^{-1}, the bands at 1645 cm^{-1} corresponds to primary amine NH band (Mritunjai et al., 2008). 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.

The XRD pattern obtained for the extracellular AgNPs showed four intense peaks in the spectrum of 2th values ranging from 20 to 80. X-ray diffraction (XRD) further confirmed the generation of AgO. Inspection of the XRD patterns of dried silver nanoparticles reveal the existence of sharp diffraction lines at low angles (2° to 99°). The silver nanoparticles exhibited peaks of silver at 2θ=37°, 45°, 64° and 77° that can be indexed to the (111), (200), (220) and (311) facets of silver, respectively (Figure 5) which agree with the values reported for face centered cubic (fcc) silver nanocrystals (JCPDS card file no. 4-783). Earlier studies have also reported the crystalline nature of biosynthesized AgNPs using different fungi (Mohammed Fayaz, 2010). The lattice parameters calculated by the PowderX software revealed that the maximum deviation between the observed and calculated values of interplanar spacing (d) was below 0.002 Å. The full-width-at-half-maximum (FWHM) value for (111) plane of reflection was used to calculate the size of the nanoparticles, and the average particle size of the AgNPs has been determined to be 9 nm.

For more information about the biosynthesized silver nanoparticles the sample was subjected to atomic force microscopic study. Figure 6a 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 6b. The height and width of the particle is measured (5nm) using the software.

Figure 7 shows the UV-Visible absorption spectrum recorded from the reaction vessel after 72 h of reaction. An absorption peak at ca. 280 nm was observed which corresponds to aromatic amino acids of proteins. It is well known that the absorbance peak at 280 nm arises due to electronic excitations in tyrosine and tryptophan residues of the protein (Xie et al., 2007). This observation indicates the presence of proteins secreted by fungus in the cell-free filtrate. The particles in the solution are thus stabilized by the capping agent that is likely to be proteins present in the cell-free filtrate.

Stability of bio-synthesized silver nanoparticles was monitored regularly for more than six months. It was observed that the nanoparticle solution was extremely stable at room temperature; with no evidence of flocculation of particles as determined by UV-Visible absorption spectra can be seen in Figure 8. This indicates that the nanoparticles were well dispersed in the solution without aggregation. Monodispersity and stability are highly desirable characteristics of the nanoparticles (Bhainsa and D'Souza, 2006). This is an important aspect of synthesis of nanoparticles, since the lack of sufficient stability of many nanoparticles preparation has to some extent impeded the development of the real world applications of nanomaterials (Shankar et al., 2003).

In our experiment the biosynthesized nanosilver showed excellent anti-fungal activity against fungal species. Here we report the efficacy of mycogenic metal nanosilver to kill pathogenic fungi which is difficult through the conventional chemotherapy. It is reasonable to state that the binding of particles to the mycelia depends on the surface area available for interaction. Smaller particles having the larger surface area available for interaction will give more fungicidal effect than the larger particles. AgNPs attach to cell membrane and penetrate in the fungi then produce a site with little molecular weight in center of fungi, and then AgNPs attach to respiratory sequence and finally cell division stop lead to cell death, can be seen in the Figure 9 which shows the SEM images of the activity of AgNPs on pathogenic *Candida sp.* AgNPs release silver ions in fungal cell which increase antifungal activity (Alt et al., 2004).

These results suggested that nanosilver is a potential compound in the treatment of fungal infectious diseases. Many studies have shown the antimicrobial effect of nanosilver (Klasen, 2000), but the effect of nanosilver against the fungal pathogens of the skin which includes *Candida sp* are mostly unknown. The primary significance of this study is to observe that, the nanosilver could inhibit the growth of dermatophytes. Secondly the fact that this process of the production of silver nanoparticels is environmental friendly as it is cost-effective and free from any solvent and toxic chemicals is also of importance. The filamentous fungi are easily in handling and also easily amenable on large scale production. The nanoparticles synthesized using fungi present good monodispersity and stability. The potential applications of nanosilver partilces in different fields have revolutionized the health care textile and agricultural industry.

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