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BIOSYNTHESIS OF SILVER NANOPARTICLES USING A PLANT PATHOGENIC FUNGUS, *FUSARIUM OXYSPORUM* F. SP. CUBENSE

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ABSTRACT

Development of reliable processes for the synthesis of silver nanomaterials is an important aspect of current bionanotechnology research. Many reports have been published on the extracellular as well as intracellular biosynthesis of silver nanoparticles using microorganisms. However, these methods of synthesis are rather slow. In present study, rapid and extracellular synthesis of silver nanoparticles using a plant pathogenic fungus *F. oxysporum* f.sp. *cubense* (Foc) is reported. Incubation of Foc mycelium with silver nitrate solution produce silver nanoparticles in 90 min. Silver nanoparticles were characterized by UV-Vis spectroscopy, FTIR and TEM. The particles synthesized were in range of 10-100 nm, capped by proteins and posses antimicrobial activity against *Pseudomonas* sp.

Key words: Silver nanoparticles; Extracellular synthesis; Fusarium oxysporum f. sp. cubense; TEM

INTRODUCTION

Nanotechnology is an emerging field in the area of interdisciplinary research, especially in biotechnology where efforts are being focused on integrating them with biology. The synthesis of silver nanomaterials/particles is extensively studied using chemical and physical methods [1]; however, development of reliable technology to produce nanoparticles is an important aspect of nanotechnology. Biological synthesis process provides wide range of environmentally acceptable methodology, low cost production, and minimum time required [2]. The synthesis and assembly of nanoparticle would benefit from the development of clean, nontoxic, and environmentally acceptable "green chemistry" procedures probably involving organisms ranging from bacteria to fungi. Synthesis of nanoparticles using biological entities has great interest due to their unusual optical [3] photoelectrochemical [4] and electronic properties [5]. Nanoparticles are also used as catalyst in chemical reaction [1], biolabeling [6], antimicrobial agent, anti HIV [7,8], electric batteries, and optical sensor [9].

Extracellular synthesis of nanoparticles using cell filtrate could be beneficial over intracellular synthesis. Hence, fungi could be extremely good candidates for extracellular and environmentally friendly process [2]. Extracellularly produced nanoparticles were stabilized by the proteins and reducing agents secreted by the fungus [10]. In addition to good nanodispensity, particles with well defined dimensions could be obtained of by using fungi. Rai et al., [11] coined the term 'Myconanotechnology' describing the use of various fungi in preparation of nanomaterials.

Silver nanoparticles have received considerable attention due to their attractive physical and chemical properties. Fungi are reported to produce array of proteins that reduces and convert silver in to silver nanoparticles [12]. Most of the fungi are opportunistic known plant pathogens. On invasion, plants thwart infection by producing oxidative burst as primary response [13]. For successful infection, pathogen must counter and survive the oxidative burst of plant usually by producing antioxidant enzymes, which reduce the reactive oxygen species and shift the redox potential. We hypothesized that plant pathogenic fungi, able to cause infection, can be the good candidate for silver nanoparticle synthesis. Fusarium oxysporum f.sp. cubense (Foc) used in this study was isolated from wilt infected banana plants. Therefore, in present study the synthesis of silver nanoparticles was carried out using a plant pathogenic fungus, Foc.

MATERIALS AND METHODS

Microorganism

Previously isolated Fusarium oxysporum f.sp. cubense (Foc) was maintained on potato dextrose agar (PDA) at 27°C. For liquid culture of fungus, 8-mm agar plug of 3-4 week old culture was inoculated in potato dextrose broth (PDB) and incubated at 27°C for 21 day.

Cultural Technique

Agar plug (8 mm dia) was cut using a sterile cork borer from three days old culture and inoculated in 250 ml Erlenmeyer flasks containing 100 ml of PDB. The growth obtained after three day at 27º C was used to inoculate three set of flasks viz., mycelial mat in distilled water, mycelial mat in medium and only medium, each augmented with 10 mM final concentration of AgNO3. Synthesis of silver nanoparticles was analyzed periodically by withdrawing samples by tracking plasmon resonance of silver nanoparticles using UV-Vis spectrophotometer.

UV-Visible Spectroscopy

Samples were collected from broth surrounding mycelia mat at time intervals of 0 min, 30 min., 60 min., 90 min., 120 min., 22 h, and 24 h from three set of flasks viz., mycelial mat in distilled water, mycelial mat in medium and only medium, each augmented with 10 mM final concentration of AgNO3. Distil water and medium collected was filtered using 0.2µ filter unit. This filtered sample was used for further use. Synthesis of silver nanoparticles was analyzed using UV-Visible

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spectroscopy (Cyber lab spectrophotometer) operated at a resolution of 1 nm from 340 to 700nm range in a 1-cm path quartz cell.

Fourier Transform Infrared Spectrometry

FTIR analysis of silver nanoparticles was carried out at Sophisticated Instrumentation Centre for Applied Research & Testing (SICART), Vallabh Vidyanagar-388120, Gujarat, India. FTIR measurements carried out by preparing KBr pellet of the silver nanoparticle–fungus reaction solution.

Transmission Electron Microscopy

TEM was carried out at SICART using Holland Tecnai 20, Phillips with CCD Camera. TEM pictures were recorded from the silver nanoparticle deposited on a carbon-coated copper TEM grid.

Anti Bacterial Activity

Silver nanoparticles are extensively studied for preparation of smart surfaces which remain free of microbe. Hence in present study, antibacterial activity of silver nanoparticles was evaluated against Gram negative organism (*Pseudomonas* sp) after coating on cotton cloth. Cotton cloth (2×2 cm) was autoclaved and dried. In sterile condition, cloth was immersed in flask containing F. oxysporum and *F. oxysporum* augmented with $AgNO_3$ and kept for 1 hour. Then centrifuge at 3500 rpm for 15 minutes and dried. PDA Plates were spreaded with 0.1ml of overnight grown *Pseudomonas* sp. Impregnated cloth was kept in the centre of inoculated plate. After Incubation at 37ºC for 24 hours, plates were observed for zone of inhibition.

RESULTS AND DISCUSSION

Silver nanoparticles have applications in spectrally selective coating for solar energy absorption, optimal receptors in intercalation material for electrical batteries, polarizing filters, catalysts in chemical reaction, bio-labeling and as antimicrobial agents. These applications are dependent on synthesized silver particles and their chemical stability without undergoing degradation like partial oxidation. There are several physical and chemical methods for synthesis of metallic nanoparticles [1]. However, biological methods may be relatively simple, reliable, eco-friendly and promising. In this regard, microorganisms such as bacteria, fungi, and yeast are known for their ability to reduce metal ions to form metallic nanoparticles [14]. There are only few papers concerned with silver nanoparticle synthesis by fungi that too confined to terrestrial strains. The present study proved a rapid and extra-cellular biosynthesis of silver nanoparticles by a plant pathogenic fungus.

Silver Reduction

Present study was to investigate the ability of a plant pathogenic fungus *F. oxysporum* f.sp. *cubense*, isolated from wilt infected banana plants, for synthesizing silver nanoparticles. Changes in mycelial appearance and culture filtrate were depicted by incubating mycelial mat with silver solution. It was observed that the biomass has a pale yellow color before reaction with the silver ion (Fig. 1a), which changed to a brownish color on completion of the reaction (Fig. 1b). The appearance of a yellowish–brown color in solution containing the biomass was a clear indication of the formation of silver nanoparticles in the reaction mixture [15] and was due to the excitation of surface plasmon vibrations in the nanoparticles. Fungal culture filtrate when incubated with silver salt solution in medium as well as in distilled water and maintained under dark exhibited a gradual change in color towards brown on formation of silver particles. Change in the color of the culture filtrate was intense brown after 24 h of incubation. Control (without silver ion) did not exhibit any color change of the culture filtrate (Fig. 2a, b, c & d). This clearly depicted that the fungal mycelia is playing a role in synthesis of silver nanoparticles independent of the surrounding medium. Reports on several hydroquinones with excellent redox properties that could act as electron shuttle in metal reductions are given by many researchers [16, 17]. Thus, it was evident that electron shuttles or other reducing agents released by Foc are capable of reducing silver ions to silver nanoparticles.

Ahmad et al. [15] suggested that NADPH dependent nitrate reductase is specific to Fusarium oxysporum prolonged reaction of Ag+ ions with another fungus, Fusarium moniliforme, did not result in the formation of silver nanoparticles, neither intracellularly nor extracellular. The long-term stability of the nanoparticle solution mentioned earlier may be due to the stabilization of the silver particles by the proteins. Silver nanoparticles have been reported to interact strongly with enzymes such, as cytochrome x and a similar binding mechanism may be operative in this study.

Upon filtration, biomass was still pale yellow and aqueous solution contained the silver nanoparticles, characterized by an intense brown color. This indicated that the reduction of the Ag+ ions took place extracellularly. The results corroborate with the earlier reports that the silver nanoparticles are extracellular synthesis by microbial candidates [15, 18].

UV Visible Spectroscopy

Silver nanoparticles exhibit new optical properties, which are observed neither in molecules nor in the bulk metals [1]. One example is presence of absorption band in visible region. This band appears due to the surface plasmon-oscillation modes of conduction electrons, which coupled through the surface to external electromagnetic fields [19]. The surface plasmon resonance and large effective scattering cross section of individual metal nanoparticles make them ideal candidate for molecular labeling [20].

Therefore, synthesis of silver nanoparticles was assayed using spectral scan by UV–visible spectroscopy in the spectral region 340-700 nm. This technique outlined above has proved to be very useful for the analysis of nanoparticles [21]. As illustrated in Fig. 3a, the UV-Visible spectra recorded as a function of time of reaction of control having only fungal biomass in media (Control) whereas Fig. 3b shows the UV-Vis spectra of an aqueous solution of 10^{-2} M $AgNO_3$ with the fungal biomass and media. A broad peak located between 420 and 500 nm was found to increase with time representing the silver nanoparticles synthesis. Our observations are in corroboration with Kuber and D'Souza [18] who also reported blunt peak of silver nanoparticles. However, on the contrary Ahmad et al. [15] reported development of strong and sharp peak between 420 and 450 nm representing the silver nanoparticles instead of blunt peaks. The peak was developed in 90 min of reaction, indicating rapid synthesis of silver nanoparticles using FOC. In earlier studies on the synthesis of silver and gold nanoparticles using bacteria [22] and fungi [23], the time required for completion of the reaction (i.e., complete

reduction of the metal ions) ranges from 24 to 120 h, while the 22 h incubation exhibited maximum synthesis of silver nanoparticles (Fig. 3b).

The increase in color intensity of culture filtrate was due to increasing number of nanoparticles formed because of reduction of silver ions present in the aqueous solution. Silver nanoparticles synthesized by using Fusarium oxysporum are reported to have very good monodispersity as well stability for even up to 4 months of incubation at 25°C and these are favorable characters for potential application of nanoparticles [15].

FTIR Spectra of Silver Nanoparticles

FTIR measurement carried out shows the amide bands, which were due to –N-H stretch and carbonyl stretch vibration in the amide linkages of the protein. The bands at 1634 and 1560 cm –1 were identified as the amide I and II bands [24] and arise due to carbonyl stretch and –N –H stretch vibrations in the amide linkages of the proteins, respectively [25]. The positions of these bands are close to that reported for native proteins. The FTIR results thus indicate that secondary structure of the proteins is not affected as a consequence of reaction with the Ag+ ions or binding with the silver nanoparticles. The band at ca. 1458 cm –1 is assigned to methylene scissoring vibrations from the proteins in the solution (Fig. 4(a)&(b)).

The absorption spectra of individual silver nanoparticles were correlated with their size and shape determined by transmission electron microscopy (TEM) [3]. Extracellular reduction of the metal ions by Foc resulted in the rapid formation of the highly stable silver nanoparticles of 10-100 nm dimensions.

TEM Analysis

The silver nanoparticles are highly variable in shape with spherical and occasionally triangular spherical with size ranging from 10-100 nm with highest proportion of 70 nm size particles, as evident by TEM studies (Fig. 5). The nanoparticles were not in direct contact even within the aggregates, indicating stabilization of the nanoparticles by capping agents. The solution of silver nanoparticle, synthesized by the reaction of Ag+ ions with Foc is exceptionally stable – the stability is likely to be due to capping with proteins secreted by the fungus. The separation between the silver nanoparticles seen in the TEM image could be due to capping by proteins and would explain the UV–Visible spectroscopy measurements, which is characteristic of well dispersed silver nanoparticles [15].

Antimicrobial Assay

Silver nanoperticles are used to create antimicrobial surfaces like surgical instruments, smart cloths etc. Hence, piece of cotton cloth was impregnated with silver nanoparticles synthesized by Foc strain. Silver nanoparticles incorporated in cotton cloth exhibited antibacterial activity against *Pseudomonas* sp. Control plate without nanoparticles showed lawn growth of pseudomonas but cloth with silver nanoparticles inhibited the growth of Pseudomonas and diameter of the inhibition zone was 50.33mm± 1.52. The mechanism of the inhibitory effects of Ag ions on microorganisms is partially known. Some studies have reported that the positive charge on the Ag ion is crucial for its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged nanoparticles [26]. In contrast, Sondi and Salopek-Sondi [27] reported that the antimicrobial activity of silver nanoparticles on Gram-negative bacteria was dependent on the concentration of Ag nanoparticle, and was closely associated with the formation of pits in the cell wall of bacteria. Then, Ag nanoparticles accumulated in the bacterial membrane can cause the increase in membrane permeability, resulting in cell death. However, because those studies included both positively charged Ag ions and negatively charged Ag nanoparticles, it is insufficient to explain the antimicrobial mechanism of positively charged Ag nanoparticles. Therefore, there is another possible mechanism. Amro et al. [28] suggested that metal depletion might cause the formation of irregularly shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins. In addition, Sondi and Salopek-Sondi [27] speculate that a similar mechanism may cause the degradation of the membrane structure of *E. coli* during treatment with Ag nanoparticles. Although their inference involved some sort of binding mechanism, still the mechanism of the interaction between Ag nanoparticles and component(s) of the bacterial cell's outer membrane is unclear. Recently, Kim et al. [29] reported that, free radicals derived from the surface of Ag nanoparticles induced membrane damage and be responsible for the antimicrobial activity.

The silver nanoparticles are capable of purifying drinking water, degrading pesticides and killing human pathogenic bacteria [18]. Recent studies of microorganisms in the synthesis of nanoparticles are a new and exciting area of research with considerable potential for development [30]. The use of fungi in the synthesis of nanoparticles is potentially exciting since they secrete large amounts of enzymes and are simpler to deal within the laboratory [23].

CONCLUSION

Present study focused on environment friendly, rapid and extracellular biosynthesis of silver nanoparticles by a fungus, Foc, isolated from wilt infected banana plants. Upon addition of the silver ion (10mM) into the flask containing the mycelial mat, the color of the medium changed very rapidly to brown, which could be due to the excitation of surface plasmon vibrations, typical of the silver nanoparticles. This indicated the presence of silver nanoparticles. The FTIR spectra suggested the capping of silver nanoparticles by protein. However, further studies must be conducted to verify if bacteria develop resistance towards the nanoparticles and to examine cytotoxicity of nanoparticles towards human cells before proposing their use.

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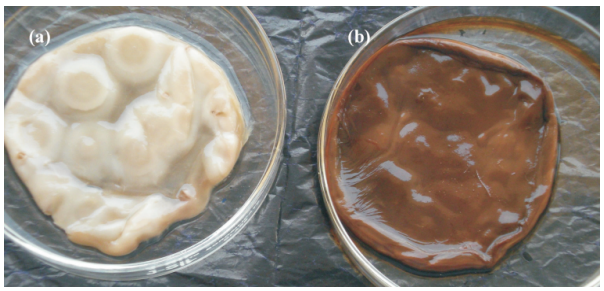


Figure 1: Appearance of Mycelial mat grown in (a) absence and (b) presence of silver ions

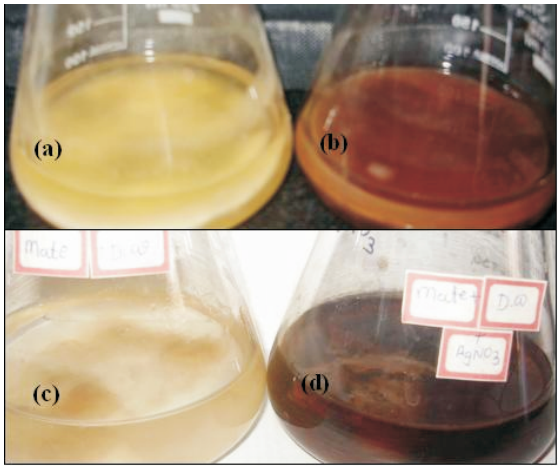
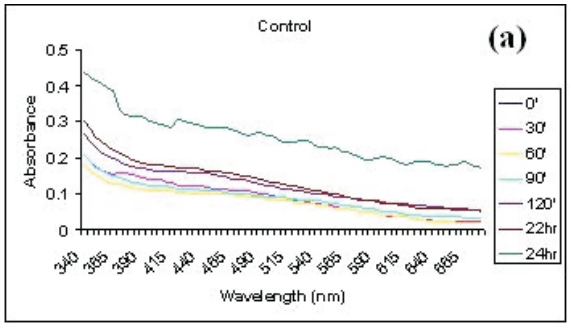


Figure 2: Changes in appearance of cultural filtrate on addition of Mycellial mat in (a) medium control, (b) medium augmented with silver ion, (c) distilled water control and (d) distilled water augmented with silver ion



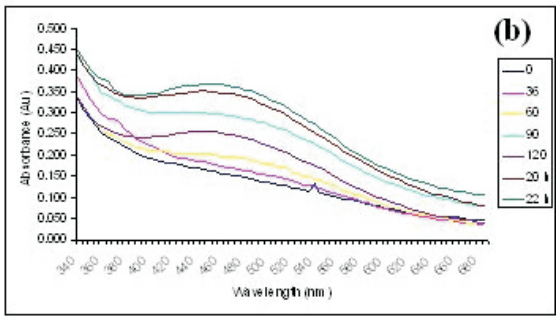


Figure 3: UV Vis spectral scan as function of time for tracking synthesis of silver nanoparticles by Foc (a) Control- without silver ion (b) experimental- with silver ion

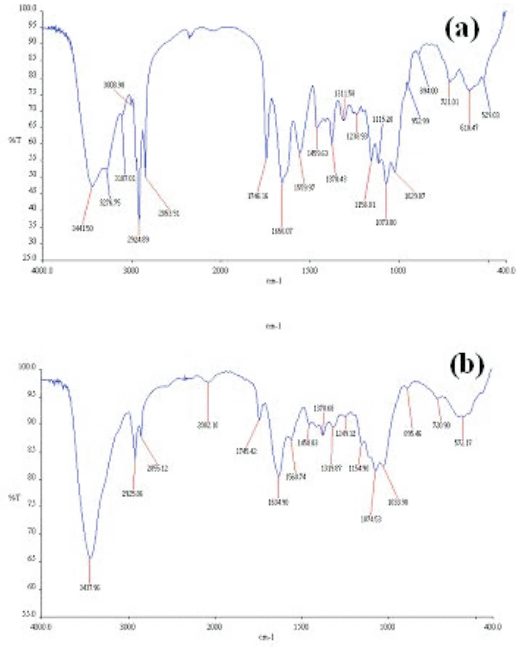


Figure 4: FTIR spectra of the mycelial mat (a) control- mycelial mat without silver ions (b) experimental- mycelial mat with silver nanoparticles

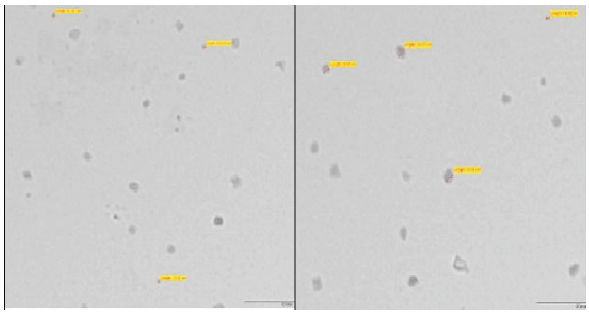


Figure 5: TEM micrograph of silver particles synthesized by Foc (Scale bar: 500 nm)