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PREPARATION OF SILVER NANOPARTICLES BY BIO-REDUCTION USING *NIGROSPORA ORYZAE* CULTURE FILTRATE AND ITS ANTIMICROBIAL ACTIVITY

SASWATI SAHA, DIPANKAR CHATTOPADHYAY[a], KRISHNENDU ACHARYA[*]
Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, Kolkata-700019
[2]Department of Polymer Science & Technology, University College of Science & Technology, University of Calcutta, Kolkata-700009

In the present article we describe a facile biosynthetic method for the silver nanoparticles highlighting myconanotechnology and its antimicrobial efficacy. The nanoparticles were characterized using UV-vis spectroscopy, dynamic light scattering (DLS), transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction (XRD) and fourior transform infrared (FTIR) spectroscopy. These methods allow validating the spherical nature of the produced nanoparticles ranging from 30 nm to ~90 nm in size. The crystallinity of the nanoparticles was confirmed by XRD. The probable mechanistic aspect for the mycogenesis of the silver nanoparticles are also evaluated which would facilitate for several industrial applications based on potential antimicrobials.

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1. Introduction

Nanotechnology is gaining incredible impetus in the present century due to its capability of modulating metals into their nanosize, which drastically changes the chemical, physical and optical properties of metals. The development of dependable, environmentally benign process for the synthesis of nanoscale materials is an important aspect of nanotechnology. Due to the outbreak of the infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and researchers in this field are searching for new antibacterial agents [1]. Metallic silver in the form of nanoparticles has made a remarkable response as a potential antimicrobial agent [2]. The current chemical methods for synthesizing nanoparticles are energy intensive, employ toxic chemicals which are becoming outdated, expensive and inefficient. These produce hazardous wastes which not only pose a sizeable risk to the environment but preclude them for any biomedical application. Conversely, the physical methods of nanoparticle synthesis such as sputter deposition, thin films, grown directly though a narrow size distribution of the particles or clusters were often difficult to achieve. Additionally, these methods were time consuming and still under development. Therefore, there is a growing need for the uses of bio-compatible, non-toxic, cost-effective and eco-friendly methods for production of silver nanoparticles [3].

Among the biological systems, the microorganisms are greatly desired for the syntheses of different nanoparticles [4, 5, 6] because of their diversity richness and high tolerance under ambient sconditions of temperature, pressure and acidity. Innumerable microorganisms including algae [7], bacteria [8, 9] and fungi have already been harnessed for the green synthesis of silver nanoparticles. Filamentous fungi are more advantageous over the bacteria and algae for having

[*]Corresponding author: krish_paper@yahoo.com

fungal mycelial mesh which can withstand flow pressure and agitation and other conditions in the bioreactors or other chambers. In addition, due to their fastidious growth, easy handling property and easy fabrication property, the process could be scaled-up. Moreover, they can accumulate metals by physico-chemical and biological mechanisms including extracellular binding by metabolites and polymers, binding to specific polypeptides and metabolism-dependent accumulation. However, an extracellular synthesis has obvious advantages over an intracellular process in case of downstream processing due to the very little handling of the fungal biomass and also presence of high levels of secreted proteins and/or enzymes that not only stabilize the particles but allows for an improved yield [10, 11]. Beside these, another advantage is that the fungus could grow on the surface of an inorganic vector during culture, which could distribute Ag nanoparticles in more efficient way as a catalyst [12].

The initial approach of mycosynthesis of silver nanoparticles was carried out by challenging an acidophilic pathogenic fungus of *Taxas* plant, *Verticillium* sp. with silver nitrate ($AgNO_3$) leading to the reduction and accretion of Ag-nanoparticles of about 25nm in diameter intracellularly within the biomass [13]. Beside this other silver tolerant fungi like *Fusarium oxysporum* [14], *F. solani* USM 3799 [15], *Aspergillus niger* [11], *Coriolus versicolor* [16], etc are capable of synthesizing silver nanoparticles of different sizes but with spherical shapes. Later on a number of fungi have been investigated by scientists and were found to be capable of biosynthesis of Ag-nanoparticles having different particle size and shape, both extra- and intra-cellularly [6, 17, 18].

In the present work, we explore the potentiality of *Nigrospora oryzae*, a saprophytic fungus causing minute leaf and grain spot disease in rice, for the first time to synthesize Ag-nanoparticles extracellularly when its mycelia free medium was seeded with 1 mM silver nitrate ($AgNO_3$) solution and the possible involvement of secreted fungal enzymes or biomolecules for its synthesis. Moreover, we also investigated the antimicrobial effects of the synthesized silver nanoparticles against representative microorganisms of public concern.

2. Materials and Methods

2.1. Preparation of mycelia free culture filtrate

The fungus *Nigrospora oryzae* isolated previously, was grown aerobically in liquid medium containing malt (0.3%), glucose (1%), yeast extract (0.3%), peptone (0.5%) and distilled water. The Erlenmeyer flasks of capacity 250 ml were inoculated with fungal mycelia and incubated at 25-30°C with shaking at 150 rpm.

2.2. Synthesis of silver nanoparticles from mycelia free culture filtrate

The fungal culture filtrate was separated from the mycelial mat aseptically only after 8-9 days of its incubation by filtration through Whatman filter paper No.1. To 100 ml of the mycelia free culture filtrate (MFCF), appropriate amount of silver nitrate ($AgNO_3$) salt was added to make the final concentration of 1 mM solution. Simultaneously, a positive control (only the MFCF without $AgNO_3$) and negative control (only 100 ml of 1mM $AgNO_3$ in de-ionized water) was also checked for comparison. All the 3 sets were kept under agitation at room temperature in the dark. The formation and time dependant production of silver nanoparticles were observed by their color change and further validated spectrophotometrically. The silver nanoparticles were separated out by centrifugation (at 12000 g for 10 min) and the settled nanoparticles were washed with deionized water (three times). The purified silver nanoparticles were re-dispersed in water by ultrasonication (Piezo-u-sonic ultrasonic cleaner, Pus-60w).

2.3. Characterization of the synthesized silver nanoparticles

To measure the time dependent synthesis of silver nanoparticles, periodically aliquots of 2 ml of the sample (showing a change in color) were scanned in a UV/Vis Spectrophotometer (HITACHI-1130 Spectrophotometer) at 1nm resolution, after 24 hrs, 48 hrs and 72 hrs. The particle sizes were measured by laser diffractometer using a Nano Size Particle Analyzer (Zen 1600 Malvern USA) in the range between 0.6 nm to 6.0 μm. The morphology and sizes were also detected by transmission electron microscopy (Model: FP 5018/40, Tecnol G^2 Spirit Bio TWIN). Atomic Force Microscopy (AFM) and Fourier Transform Infrared Spectroscopy (FTIR) were also

carried out using Nanoscope® 111a Veeco multimode, USA and Shimadzu FTIR 8400S spectroscopy respectively. The XRD spectra were recorded from 5° to 90° 2θ angles using X-ray diffractometer (model: Seifert XDAL 3000) using Cu Kα radiation operated at 45 kV and 30 mA. EDAX was also performed using EDAX$_{®}^{TSL™}$ to confirm the presence of silver in the nanoparticles.

2.4. Determination of the presence nitrate reductase

The reduction of nitrate was revealed by a fluorometric method [19] using fluorescence spectrophotometer, Hitachi F-7000, model no. 5J0-0139. Nitrate reductase assay was performed with the same culture filtrate (MFCF) of *Nigrospora* oryzae in the same way as done for the silver nanoparticle production. Here, the silver nitrate was replaced by 0.1% potassium nitrate (KNO_3), considered as the treated sample. The culture filtrate (without KNO_3) was kept for control. The assay for nitrate reductase was executed after 48 hrs of incubation. To 400 µl of MFCF (both control and treated), 800 µl deionized water was added. To these 40 µl of freshly prepared 2,3-diaminonapthalene (DAN) (0.05 mg/ml in 1N HCl) was added and mixed well. After 10-15 min of incubation at 20°C, the reaction was stopped by the addition of 20 µl of 0.1N NaOH.

2.5. Assay for the antibacterial property of the silver nanoparticles

The silver nanoparticles suspended in deionized water were examined for their antibacterial activity by the method of agar diffusion. Six bacterial strains, *Bacillus subtilis* [MTCC 736], *Bacillus cereus* [MTCC 306], *Escherichia coli* [MTCC 68], *Proteus vulgarius* [MTCC 426], *Pseudomonas aeruginosa* [MTCC 8158] and *Micrococcus luteus* [MTCC 1538] were used for this analysis. These bacterial strains were grown on nutrient broth (NB) media (purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India.) for 24 hrs prior to the experiment, seeded in agar plates by the pour plate technique. Cavities were made using a cork borer (5 mm diameter) at an equal distance and were loaded with the silver nanoparticle solution and then incubated at 37°C for 24 hrs.

3. Results and discussion

3.1. UV-Vis spectroscopic analysis

The reduction of silver ions from Ag^{+1} to Ag^0 state and formation of silver nanoparticles was preliminarily determined by the change in color (set containing MFCF and $AgNO_3$) from straw yellow to golden brown to light brown (Fig. 1) within 24 hrs. No color change was observed in other two sets (kept as a positive and negative control). As the color of silver colloid is attributed to specific surface plasmon resonance (SPR) arising due to the collective oscillation of free conduction electrons induced by an interacting electromagnetic field, the formation and stability of the nanoparticles were ascertained by UV-Vis spectroscopy. The intensity of peak at 425 nm increases with time and becomes sharper indicating formation of spherical silver nanoparticles (Fig. 2).

Fig. 1 Mycelia free culture filtrate showing color change (c) on addition of 1 mM $AgNO_3$. No change in color observed in (a) negative and (b) positive controlled sets.

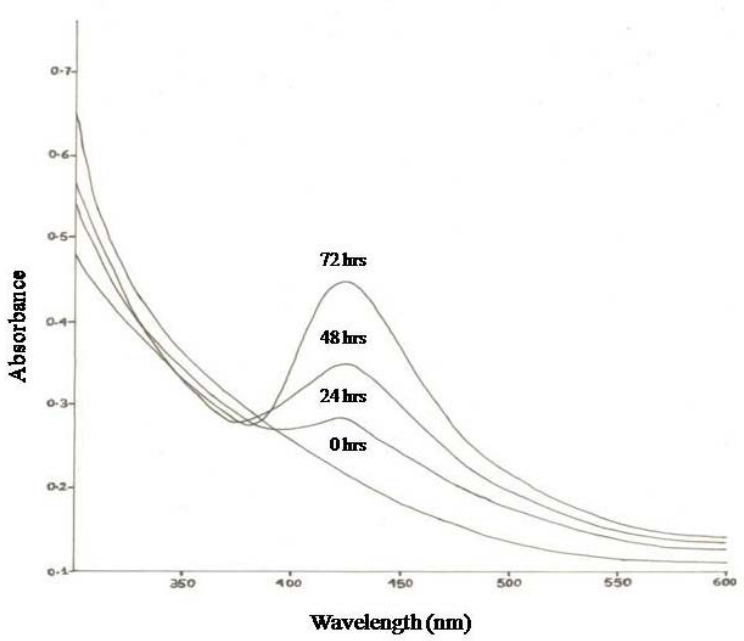

Fig. 2 UV-Vis spectra of the synthesized Ag-nanoparticles showing a sharp rise of peak with time.

Morphological studies of silver nanoparticles

Dynamic light scattering measurements showed the monodispersed spherical nature of produced silver nanoparticles ranging from 30 to ~90 nm size (Fig. 3) whose morphology was further confirmed by TEM (Fig. 4) and AFM (Fig. 5) images.

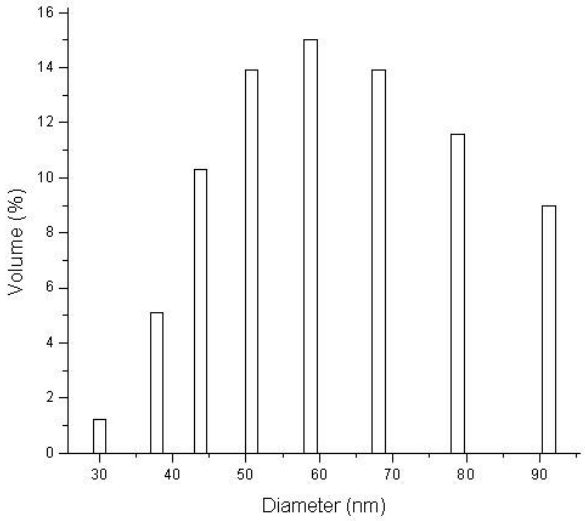

Fig. 3 DLS data plotted graphically showing the range of Ag-nanoparticle size.

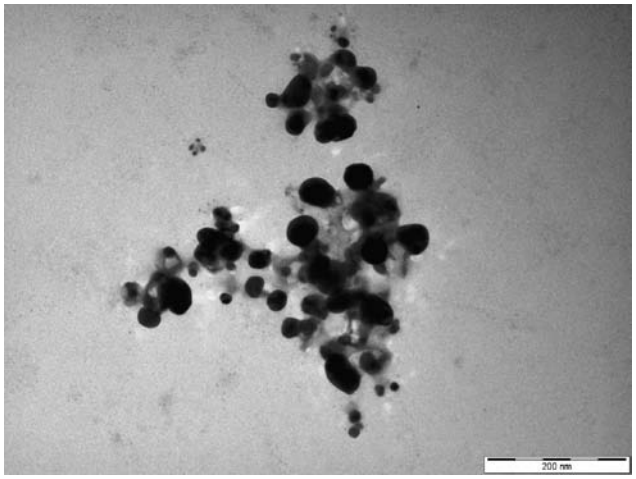

Fig. 4 TEM image of the Ag-nanoparticles showing its spherical nature.

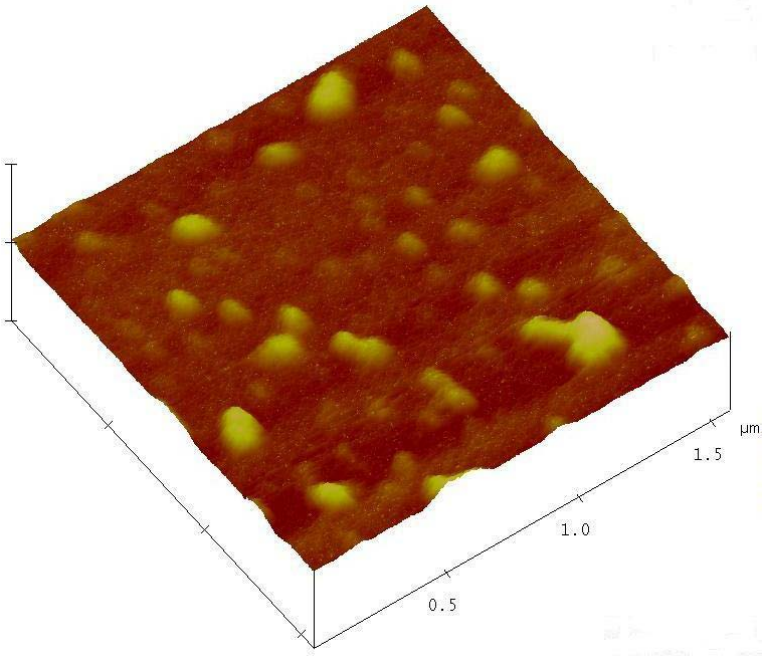

Fig. 5 AFM image showing 3-D view of Ag-nanoparticles.

XRD studies

The crystallographic analysis of powdered samples by XRD (Fig. 6) showed intense peaks of silver at 2θ =33°, 47°, 64° and 78° that can be indexed at (111), (200), (220) and (311) facets which agree with the values reported for face centered cubic (fcc) silver nanocrystals (JCPDS card file NO. 4-783).

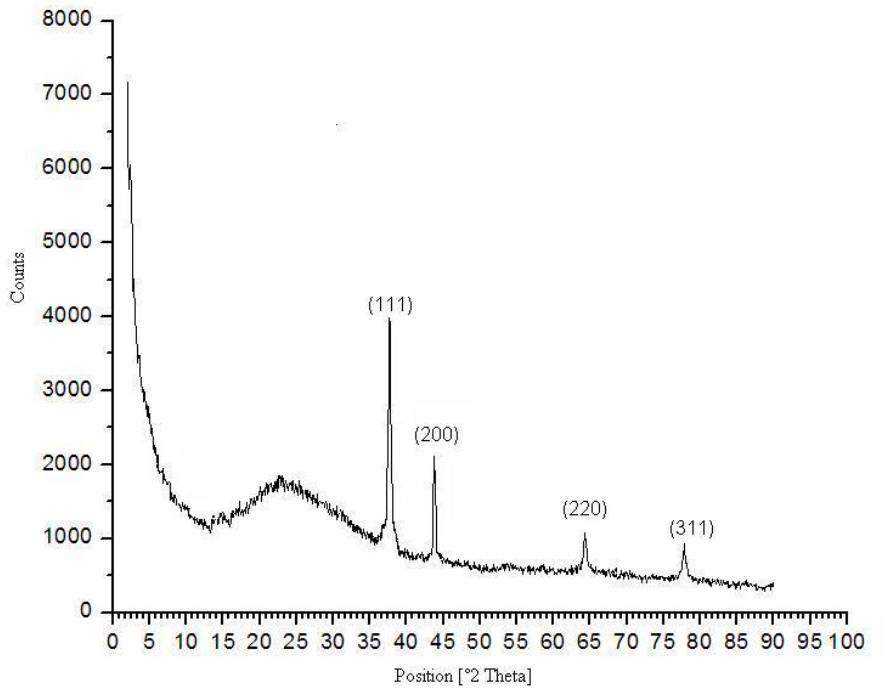

Fig. 6 XRD patterns showing 4 peaks confirming fcc nature of Ag- nanocrystallites.

EDAX analysis

The presence of silver is further confirmed by EDAX by the presence of sharp optical absorption peak (Fig. 7) within the range of 3 to 4 keV which is typical for the absorption of metallic silver nanocrystallites [20].

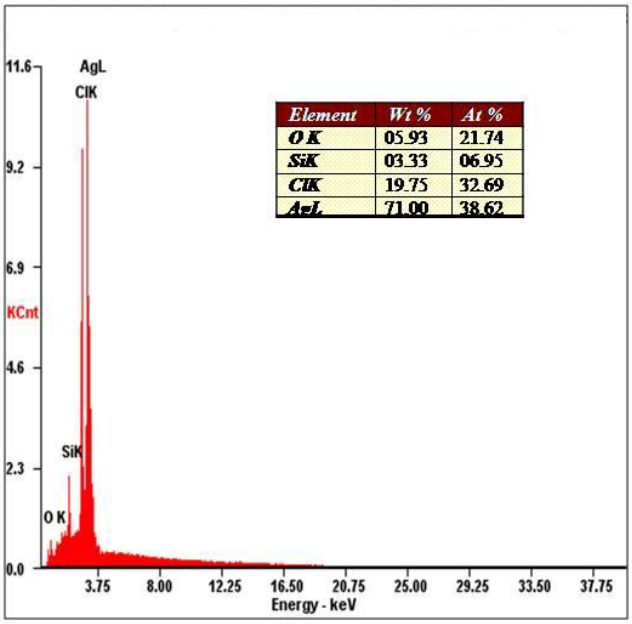

Fig. 7 EDAX recorded shows sharp peak confirming presence of silver.

FTIR spectroscopic studies

FTIR measurements (Fig. 8) were carried out for the verification of possible interactions between silver salt and MFCF for the formation and stabilization of synthesized silver nanoparticles. The strong band peak at 3300-3500 cm^{-1} which is the characteristic of N-H stretching vibrations [21] indicates strong hydrogen bonding. Compared to the FTIR bands of fungal mycelia free broth (kept as a control), the peak of representing N-H stretching vibration in the nanoparticles is much narrower. This change may be due to the breakage of H-bonds between the amide groups present in the media, which were adsorbed onto silver nanoparticle surface and intend to form stronger bonds with Ag atoms thus leading to the narrowing of N-H peaks. The bands at 2925 cm^{-1} and 2848 cm^{-1} are attributed to the side chain vibrations of symmetric and antisymmetric modes corresponding to aliphatic and aromatic respectively. The band at 1652 cm^{-1} represents the presence of amide I [22]. The presence of band at about 1743 cm^{-1} corresponds to carbonyl stretch vibrations in ketones, aldehydes and carboxylic acids is noteworthy and infer that the reduction of silver ions may be coupled to the oxidation of the hydroxyl groups in fungal hydrolysates released in Ag/fungal mycelia free medium.

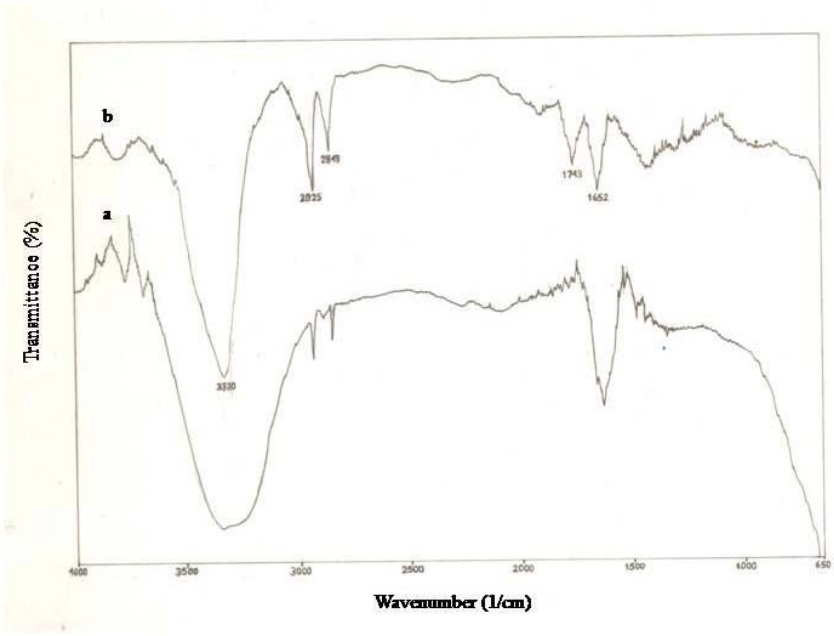

Fig. 8 FTIR data recorded from a drop coated film of (a) mycelia free culture filtrate and (b) the synthesized Ag-nanoparticles.

UV-Vis analysis for the presence of aromatic amino acids

Previously, Willner and co-workers used enzymes as catalysts to mediate the growth of metallic nanoparticles, which were useful as optical sensors for substrates such as glucose, L-DOPA, alcohols, lactate and nerve gas analogues [23]. Active biomolecules secreted by the fungal cells, including proteins and enzymes, organic acids, lipids and sugars, could be present in the mycelia free medium. So, the enzyme mediated reaction proposed by Willner et al., might have worked in the present case, with small molecules as the substrates and enzymes as the catalysts. Interestingly, the presence of strong absorption in the UV region at ~270 nm (Fig. 9) infers the existence of aromatic side groups in the amino acid residues (tyrosine, tryptophan and phenylalanine).

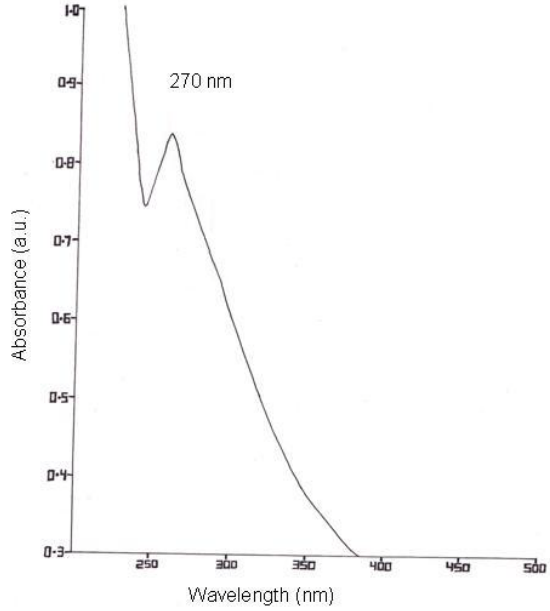

Fig. 9 Confirmation of the presence of aromatic amino acids in the MFCF by UV-Vis spectroscopy.

Fluorometric assay for nitrate reductase

The hypothesis that the reduction of silver ions in the solution by cell free supernatant is most likely to occur perhaps due to the presence of nitrate reductase was previously evaluated in *Fusarium* sp. The (Fig. 10) confirms the presence of nitrate reductase showing the presence of 2,3-diaminonapthotriazole emission peak at around 408 nm when excited at maximum wavelength of 375 nm by spectrofluorometer. Here the nitrite reacts with 2,3-diaminonapthalene to reduce it to 2,3-diaminonapthotriazole. The intensity of the band was increased on addition of 0.1% KNO_3 to the MFCF, confirming the presence of nitrate reductase.

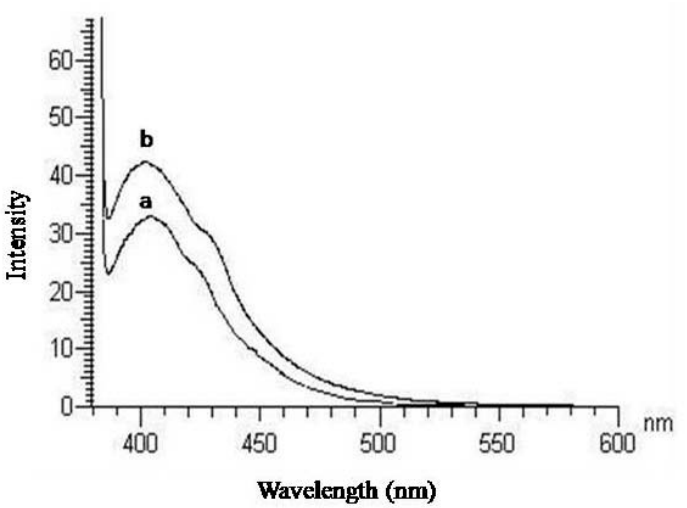

Fig. 10 Fluorescence emission spectra for the confirmation of nitrate reductase. In emission spectra curves (a) and (b) represent respectively for MFCF and MFCF along with 0.1% KNO_3.

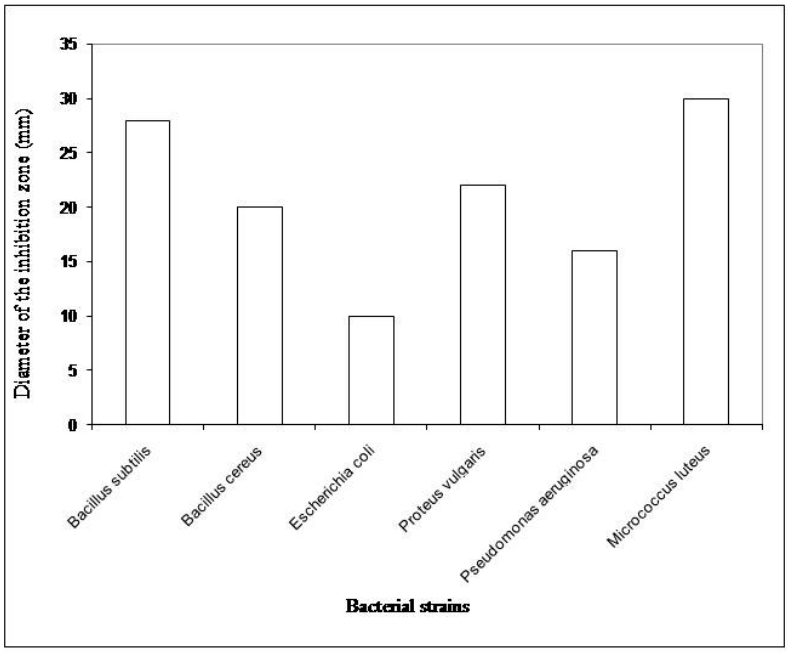

Fig. 11 The antibacterial efficacy of the synthesized Ag-nanoparticles plotted graphically comparing their diameters of the inhibition zone.

Assay for antibacterial property of the silver nanoparticles

On evaluation of the antimicrobial property of the produced silver nanoparticles against *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Micrococcus luteus* by cup plate method, showed its specificity at a concentration of 100 µg/ml against those pathogenic bacteria. The diameter of the inhibition zones for all the tested bacteria were measured and plotted graphically (Fig. 11).

4. Conclusions

The present study revealed the extracellular mycosynthesis of isotropic spherical silver nanoparticles ranging from 30-80 nm in diameter when the mycelia free culture filtrate of the pathogen was seeded with 1 mM silver nitrate. The silver nanoparticles thus formed were characterized by UV-Vis spectroscopy, DLS, AFM and TEM. XRD and EDAX analysis confirmed the presence of silver nanocrystallites. FTIR spectroscopic analysis indicates that the coordination behaviors between amino groups of the secreted fungal proteins/biomolecules and the chemiadsorbtion of the aldehyde and keto groups present in the MFCF may be liable for the reduction of silver ions to form stabilized protein-capped silver nanoparticles. The presence of aromatic amino acids was confirmed by UV-Vis spectroscopy. Furthermore, the presence of nitrate reductase in the MFCF may give an idea of the possible mechanism for the reduction of silver salt. These produced silver nanoparticles also showed excellent antibacterial property. Thus, it could be concluded that these protein-conjugated silver nanoparticles could be used in developing novel antimicrobial agents which may find potential applications in antimicrobial packaging materials and wound dressing/wound burns.

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