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BIOSYNTHESIS, CHARACTERISATION & ANTIFUNGAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED BY THE FUNGUS *ASPERGILLUS FOETIDUS* MTCC8876

SWARUP ROY, TRIPARNA MUKHERJEE, SHATARUPA CHAKRABORTY, TAPAN KUMAR DAS*
Department of Biochemistry and Biophysics, University of Kalyani, Kalyani – 741235, India

The silver nanoparticles synthesized biologically have been widely used in medicinal field. The synthesis of silver nanoparticles has been carried out by using the extracellular filtrate of the fungal strain, *Aspergillus foetidus* MTCC8876. The synthesis of silver nanoparticles was identified primarily by changing the color of the extracellular filtrate and confirmed with the help of the study of UV-Vis spectroscopy. The synthesized nano particles have further been characterized by the Biophysical techniques like Dynamic light scattering (DLS), Fourier transformation infrared (FTIR), Atomic force microscopy (AFM) and the Transmission electron microscopy (TEM) with studies of Energy dispersive X-ray (EDX) and it was confirmed that the silver nano particles were constituted extracellularly by an extracellular reductase like enzyme, available in the cell- filtrate separated from the biomass, mycelia of *Aspergillus foetidus*. The results obtained from the study of antifungal activities of the silver nanoparticles are very significant and indicate that the synthesized silver nanoparticles may have an important advantage over conventional antifungal antibiotics.

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Keywords: *Aspergillus foetidus*, Extracellular, Silver nanoparticles, Biophysical techniques, Antifungal activity

1. Introduction

In last few years, research in nanotechnology has been carried out extensively as nanoparticles possess increased structural integrity as well as unique chemical, mechanical, optical, electronic and magnetic properties compared to large particles of bulk materials [1]. In the synthesis of nano particles, the various conventional processes like a number of chemical and physical methods including chemical reduction in aqueous or non-aqueous solution [2], micro emulsion [3], sono-chemical [4] and microwave-assisted [5] methods have been applied . Since the chemicals like organic solvents, hydrazine, sodium borohydride and N, N-dimethyl formamide utilized in the synthesis of metal nano particles are found to be highly reactive and biological hazardous, the chemically synthesized metal nano particles could not accomplished with the biomedical field. On the other hand, the microbiologically synthesized metal nanoparticles are found to be eco-friendly, reliable, biocompatible and economic. It is reported from the ancient time that silver among the various metals has been considered as an effective antimicrobial agent, food preservative agent and water purifying agent [6]. Accordingly microorganisms such as fungi [7, 8], bacteria [9], actinomycetes [10] and plants [11, 12] have been used for the study of biosynthesis of silver nanoparticles. Synthesis of nanoparticles occurs intracellularly or extracellularly of which the extracellular process could be used preferably as it is less laborious and also is less costly. It is found that fungi are more suitable than bacteria for the use of the synthesis of nano particles

*Correspondent author: email: tapankumardas175@gmail.com

extracellularly as the fungi could form large biomass which may be handling easily. It is reported that the fungi such as *Aspergillus sp.* [7, 8, 13]. *Fusarium sp.*[10], *Trichoderma sp.*[14], and *Cladosporium sp*.[15] etc. could synthesize extracellularly the silver nano particles whereas Sastry et al. [16] reported that silver nanoparticles could be synthesized intracellularly by *Verticillium sp.* In spite of the cost effective process, report on the use of the fungal strains like Aspergillus *sp.* for the biosynthesis of silver nanoparticles extracellularly are found to be very scanty. As far as it is known that this is the first time attempt taken to investigate the biosynthesis of silver nanoparticles extracellularly by using the fungus, *Aspergillus foetidus*, and the synthesized silver nano particles has been characterized biophysically by various techniques. The present report is also concerned with the investigation of antifungal activity of the synthesized silver nano particles.

2. Experimental

2.1. Source of organism and composition of growth media

2.1.1. Organism

The identified strain, *Aspergillus foetidus* MTCC8876 isolated from Kalyani waste water treatment Centre, Kalyani, West Bengal, India was used for the study of biosynthesis of silver nanoparticles.

2.1.2. Growth Media

Czapek-Dox (CD) as broth medium as described by Raper and Thom (1949), that contained (per liter): KH_2PO_4 (1g), $NaNO_3$ (2g), $MgSO_4$ (0.5g), KCl (0.5g), $FeSO_4$ (0.01g), $ZnSO_4$ (0.01g), glucose(40g) and as solidified media(CDA) were used for the growth of the strain, *Aspergillus foetidus*. The p^H of the medium was adjusted to 8 before autoclaving . The medium was solidified with 2 % agar as solid CD medium (CDA).

2.2. Biologically Extracellular Synthesis Of Silver Nanoparticles

2.2.1. Cell Filtrate preparation

Spores suspension of *Aspergillus foetidus* (10^{11}conidia in one liter) was inoculated to liquid CD medium and incubated at 28±2 °C for 96 h in a orbital shaker (120 rpm). The harvested biomass was filtered through Whatman filter paper no.1 and washed thrice with sterile distilled water to remove any medium component. 20 g of biomass was taken in a conical flask and 200 mL of Milli-Q deionized water (18.2 MΩ at 25 °C) was added in order to contact with the biomass. The mixer was agitated at 150 rpm & incubated at 28±2 °C for 72 h and the Live cell filtrate (LCF) was obtained by passing it through Whatman filter paper no. 1. The LCF was centrifuged at 5000 rpm for 10 min. to sediment the cell debris from this filtrate and the supernatant was used to reduce the silver nitrate solution.

2.2.2. Biosynthesis of Silver nanoparticles

The fungal extract LCF was used as a reducing agent when challenged with 1mM final concentration of $AgNO_3$. For synthesis of silver nanoparticles, $AgNO_3$, 1mM final concentration was mixed with 20 ml of cell filtrate in a 250 ml flask and incubated at 28±2 °C & agitated at 150 rpm in dark. Positive Control (without the silver nitrate solution, only biomass) & only silver nirate solution (1mM) as a negative control was also run along with the experimental flask.

2.3. Characterisation of Synthesized Silver nanoparticles

2.3.1. UV-Visible spectroscopy analysis

The bio-reduction of precursor silver ions was monitored by sampling of aliquots (1 mL) at different time intervals. Absorption measurements were carried out in schimadzu UV-Visible dual beam Spectrophotometer at a resolution of 1 nm. The color change found visually may be considered as first observation for the preliminary synthesis of silver nanoparticles. UV-Vis. Spectroscopic analysis of several weeks old samples was also carried out to check the stability of the synthesized silver nanoparticles.

2.3.2. FTIR spectroscopy analysis

The prepared cell-free filtrate was freeze- dried in lyophilizer and the potassium bromide was added to the lyophilized sample (100:1).The FT-IR spectrum of the prepared sample was recorded in a PerkinElmer FT-IR in the range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}.

2.3.3. Particle size (DLS) analysis

The aqueous suspension of the synthesized silver nanoparticles was filtered through a 0.22 μm syringe driven filter unit and the size of the distributed silver nanoparticles were measured by using the principle of dynamic light scattering (DLS) technique made in a Malvern Zetasizer Nano series compact scattering spectrometer.

2.3.4.The Zeta potential measurement

The suspension of the synthesized silver nano particles was diluted 10 times and the diluted sample was allowed to filter through a 0.22 μm syringe driven filter unit. The filtered sample of silver nano particles was considered to measure The Zeta potential was measured in Zetasizer Nano ZS (Malvern).

2.3.5. AFM analysis

For atomic force microscopy(AFM) analysis, 100μL of 10 times diluted solution of the synthesized silver nanoparticles is used to filter through 0.22 μm syringe driven filter unit .The filtered sample was placed on clean cover slip and kept under vacuum desiccation for overnight before loading them onto a specimen holder. The slides were then scanned in the AFM.

2.3.6. TEM & EDX analysis

For transmission electron microscope (TEM) measurements, a drop of 10 times diluted sample of the synthesized silver nanoparticles was placed on the carbon coated copper grids and kept under vacuum desiccation for overnight before loading them onto a specimen holder. The elemental analysis was carried out in spectrum of energy dispersive X-ray.

2.4. Nitrate reductase assay

The prepared fungal filtrate was considered for the assay of nitrate reductase according to the method of Harley [17]. 2 mL of fungal LCF was mixed with 2 mL of the assay medium (30mM KNO_3 and 5% iso-propanol in 0.1M phosphate buffer of pH 7.5) and incubated at 25 ^{0}C in the dark for 1 hours and then 1mL of 50 mM sulphanilamide and 1mL of 10 mM NEED (N-(1-naphthyl) ethylene diamine dihydrochloride) solutions were added .The intensity of the developed color was estimated in an UV–Vis spectrophotometer at 540 nm.

2.5. Effect of silver nanoparticles on the morphology of *Aspergillus foetidus*

Any variation of the morphology of the fungal strain was characterized microscopically by Lacto phenol Cotton Blue mounting method in presence of silver nano particle.

2.6. Antifungal activity

The antifungal activity of the synthesized silver nanoparticles was examined considering *Aspergillus niger*, *Aspergillus foetidus*, *Aspergillus flavus*, *Fusarium oxysporum*, *Aspergillus oryzae*, *Aspergillus parasiticus* as testing fungal strain. 0.1 mL of spore's suspension of the testing strains was aseptically spread onto CD agar plates. The cavities of 3-4 mm were made in the middle of the agar plates and were filled up with the solution of the synthesized silver nanoparticles. The plates were incubated at 28±2 $^{\circ}$C for 2-3 days. The two cavities were considered as testing for control by filling up one cavity with the double distilled water and another with $AgNO_3$ solution only.

3. Result and discussion

3.1. Characterization of the synthesized silver nanoparticles

The flask containing silver nitrate solution showed change in colour of the reaction mixture from light yellow to light brown after 24h of incubation as well as of agitation with increasing intensity of the colour during the incubation period as shown in the figure (Fig. 1). The positive control (without the silver nitrate solution, only biomass) and the negative control (pure silver nitrate solution without cell-free filtrate) did not show any change of colour of the reaction mixture .The appearance of the brown colour was an indication of the formation of silver nano particles in the medium .The UV-visible spectroscopy studies could be considered as the most useful technique for structural characterization of silver nanoparticles. The absorption spectra of nanoparticles showed highly symmetric single-band absorption with peak maximum (Surface Plasmon Resonance, SPR) at the wavelength, 425 nm with steadily increased in intensity as a function of time of reaction without any shift in the peak (Fig. 2). The increase in intensity of colour occurred due to gradually increasing the number of silver nanoparticles synthesized from

the reduction of silver ions available in the aqueous solution. There is no further increase of intensity as recorded after 96 h of incubation due to complete reduction of silver ions in the reaction mixture. The silver nanoparticles peak remained same at 425 nm even after 96 h of incubation indicated that the particles were well dispersed in the solution and there was not very much aggregation. The size and shape of the synthesized silver nanoparticles reflects the absorbance peak as reported earlier [18, 19]. The shifting of the SPR peak to longer wavelengths with increase in the size of particle was found to be similar as reported by Brause et. al. [20]. The synthesized silver nanoparticles were found to remain stable for several weeks.

Fig.-1 the change of color from light yellow (b) to light brown (a) of live cell filtrate (LCF) of Aspergillus foetidus after addition of AgNO3 solution (1 mM).

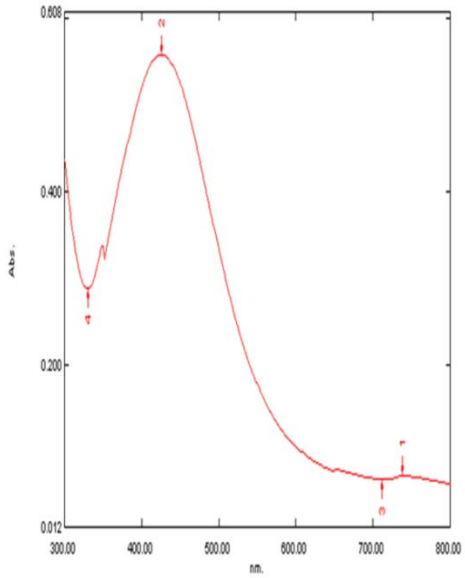

Fig. 2. UV-VIS spectrum of LCF and $AgNO_3$ solution (1 mM) indicates the presence of highly Symmetric peak at wavelength 425 nm.

The FTIR measurements of the freeze-dried lyophilized samples were carried out to identify the possible interactions between silver and bioactive molecules, which may be responsible for the synthesis and stabilization of silver nanoparticles with the capping agent available in the fungal filtrate. The amide linkages between amino acid residues in proteins give rise to well known signatures in the infrared region of the electromagnetic spectrum. The FTIR spectrum reveals two bands at 1625 and 1516 cm^{-1} that correspond to the bending vibrations of the amide I and amide II bands of the proteins respectively (Fig. 3).

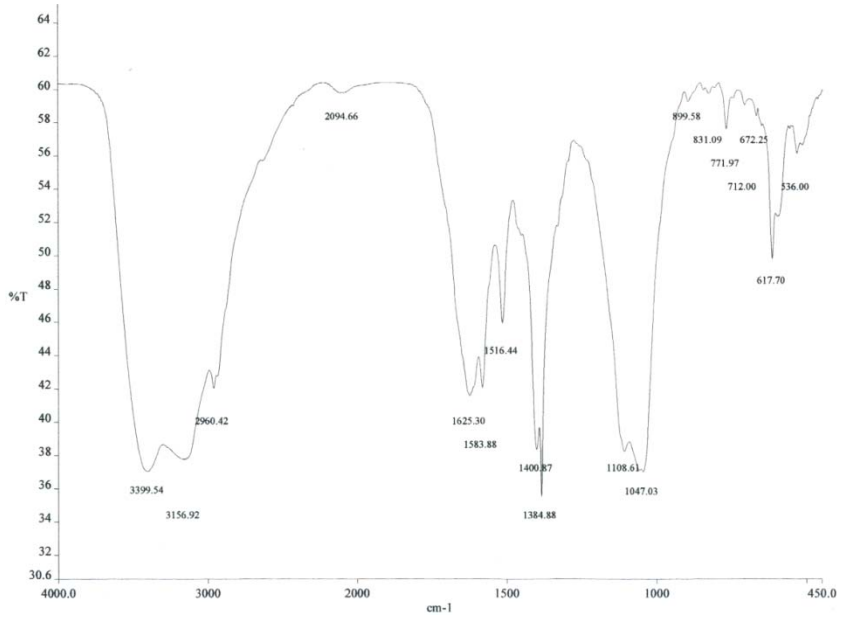

Fig. 3. FTIR spectrum of silver nanoparticles showing characteristics peak at 1625 and 1516 1384 and 1047 cm^{-1}.

The presence of the peaks of amino acids supports the presence of proteins in cell-free filtrate as observed in UV-Vis. spectra. It is well known that protein-nanoparticles interactions can occur either through free amino groups or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes. The two bands observed at 1384 and 1047 cm^{-1} can be assigned to the C–N stretching vibrations of the aromatic and aliphatic amines, respectively [21].These observations indicate the presence and binding of proteins with silver nanoparticles which may be the possible reason of their stabilization.

The data of Dynamic light scattering (DLS) supported that the average size of the synthesized nanoparticles are 104.9 nm and 0.24 PDI value and the obtained single peak indicated that the quality of the synthesized silver nano particles is good (Fig. 4) [22].

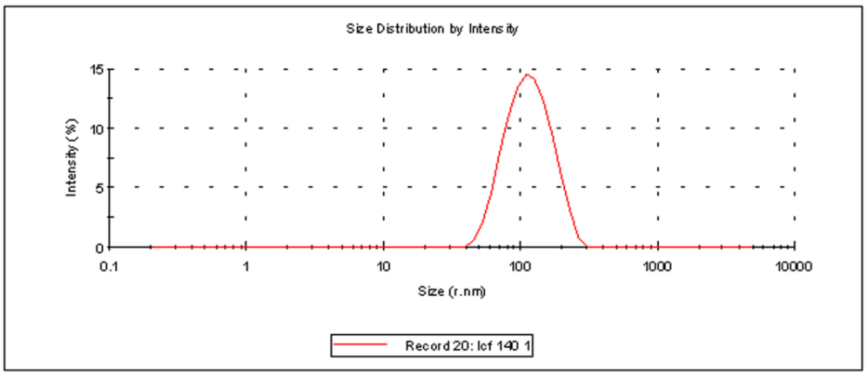

Fig. 4. Dynamic light scattering (DLS) of the synthesized silver nanoparticles.

The value of zeta potential of the nano particles as -19.3mV with a single peak signified that the presence of repulsion among the synthesized nano particles is present. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel with each other and there will be no tendency of the particles to assemble together. However, if the particles have low zeta potential values then there will be no force to prevent the particles coming together and flocculating.

In the study of Atomic force microscopy (AFM), the shape of the synthesized nanoparticles are found to be roughly spherical with the size in the range of nm (Fig. 5) and some

silver nanoparticles are also found to be present as cluster form as images formed in AFM .

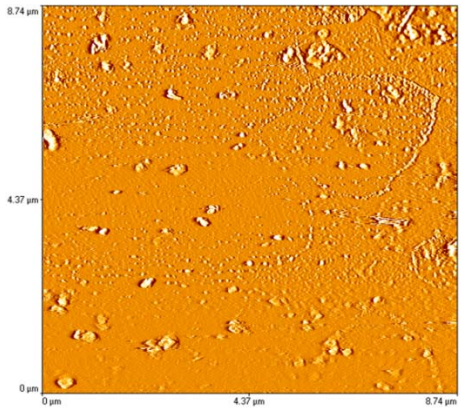

Fig. 5. Atomic force microscopy (AFM) of Silver nanoparticles formed by the reduction of silver ions using Aspergillus foetidus.

The transmission electron microscopy (TEM) studies characterized the shape and size of the synthesized silver nano particles as shown in Fig. 6. The results obtained from the study of TEM indicated that the same nanoparticles are poly-dispersed roughly spherical shaped with the size of 20-40 nm and some particles are found to be very small in size which are nearer to 5 nm . The same type of the nano particles having variable shape and size was observed in the common biological systems [13].The spectrum of the Energy Dispersive X-ray (EDX) studies showed a peak at 3 eV identified the presence of silver in the sample and it was further confirmed from the data of the elemental analysis obtained from EDX which reflected the confirm formation of silver nanoparticles (Fig. 6).

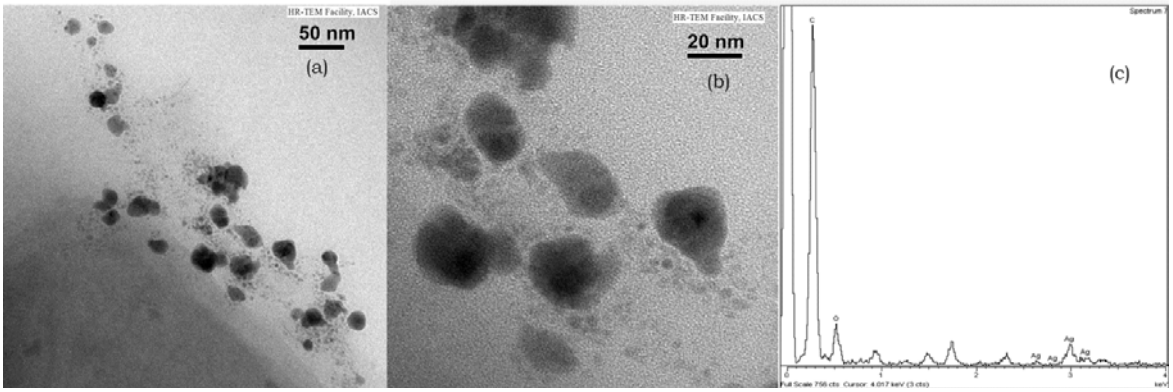

Fig. 6. TEM picture of silver nanoparticles synthesized by Aspergillus foetidus showing nano size particles of size ranges 20-40 nm (a & b respectively), EDS spectra of silver nanoparticles showing presence of Ag atom(c).

In the present work, the extracellular filtrate of the fungal strain has been used to reduce Ag^{+} to Ag^{0} as reported as the nitrate reductase, an extracellular enzyme leading to formation of silver nano particle [23]. Duran et al. [24] reported that there are two possible mechanisms involved in the formation of silver nanoparticles by *Fusarium oxysporum*; one is following the reductive process carried out by the extracellular enzyme, nitrate reductase and the other by shuttle quinine process. It is confirmed that NADH-dependant nitrate reductase is the main enzyme available extracellularly involved in the reduction of silver ions to silver in *Fusarium oxysporum*[25] and *Bacillus licheniformis* [26]. Similarly, the presence of the extracellular enzyme, nitrate reductase in *Aspergillus foetidus* is confirmed and predicted the involvement of the same enzyme in the formation of silver nanoparticles by *Aspergillus foetidus.*

It was found that the formation of normal conidiophores was affected when the fungal

strain of *Aspergillus foetidus* was treated with silver nanoparticles (Fig. 7).

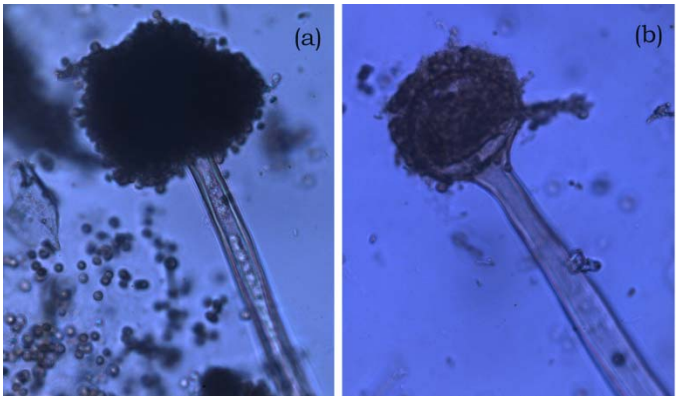

Fig. 7. Morphology of the fungi Aspergillus foetidus, (a) Normal conidiophores, (b) conidiophores in presence of silver nanoparticles.

The colloidal suspension of silver nanoparticles was used to study the antifungal activities against the fungal strains of *Aspergillus species* like *Aspergillus niger, Aspergillus flavus, Aspergillus foetidus , Aspergillus oryzae & Aspergillus parasiticus* and *Fusarium oxysporum* by agar well diffusion method and it was observed that a clear zone of inhibition was formed around the cavities at which suspension of silver nanoparticles was added. The diameter of growth inhibitory zone around the cavities at which silver nanoparticles were added was found to be higher compared to the diameter of zone inhibition formed around the cavities at which $AgNO_3$ solution was added as the respective. On the other hand no zone of inhibition was observed when distilled was considered as a control (Table 1). Dorau et al. [27] reported that silver nanoparticles exhibit the antimicrobial activity due to the formation of insoluble compounds by inactivation of sulfhydryl groups in the fungal cell wall and disruption of membrane bound enzymes and lipids resulting lysis of cell.

Table-1. The diameter of growth inhibitory zones in presence of synthesized silver nanoparticles against the fungal strains.

Name of the Test fungi	**Diameter of growth inhibitory zones in presence of**	
	Silver nitrate as control(cm)	**Silver nanoparticles as experimental (cm)**
A) *Aspergillus niger*	1.5	2.0
B) *Fusarium oxysporum*	1.3	1.7
C) *Aspergillus Oryzae*	1.5	1.8
D) *Aspergillus parasiticus*	1.4	1.9
E) *Aspergillus foetidus*	1.1	1.7
F) *Aspergillus flavus*	1.2	1.5

4. Conclusion

The extracellular filtrate of the fungal strain, *Aspergillus foetidus* could synthesize silver nano particles from the aqueous solution of silver nitrate. Such type of the synthesis of silver nano particles may be considered as eco-friendly as it is free from any toxic chemicals or organic solvent. The synthesized silver nano particles are found to be relatively stable by the synthesis

system itself. So it can be considered as low cost process compared to the other process, like chemical process as there is no requirement of any sort of capping agent for making the synthesized silver nano particles stable. In the present study it is found that the dimension of the synthesized silver nano particles is in the range of 20-40 nm. Investigation of antifungal activities of silver nano particles against some fungal strains of *Aspergillus sp.* indicated that it could be considered as a potential antifungal agent implicating its biomedical application.

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