ISSN : 0974 - 7494 Volume 7 Issue 5

Trade Science Inc.

Nano Science and Nano Technology
An Indian Journal

Full Paper

NSNTAIJ, 7(5), 2013 [163-171]

Biological synthesis of silver nanoparticles using filamentous fungi

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ABSTRACT

Background: The use of the highly activities of microbial cells for the synthesis of nanosized materials has recently emerged as a novel approach for the synthesis of metal nanoparticles.

Results: In this study, out of eleven fungal organisms were screened for their ability to produce nanosilver nanoparticles, three species, namely, *Aspergillus flavus, Penicillium citrinum* and *Fusarium oxysporum*, proved to be nanosilver producers of different sizes and shapes. These nanoparticles dislodged by ultrasonication showed an absorption peak at 425 nm in case of *A. flavus* as well as *F. oxysporum* while 450 nm for *P.citrinium* in UV–visible spectrum corresponding to the plasmon resonance of silver nanoparticles. Morphology and size details of the silver nanoparticles were proved by TEM and Zetasizer techniques. TEM results revealed that The sizes of the silver nanoparticles were found to be in the range of 7–38 nm and nano silver shape was different with different fungal species. Zetasizer data showed that the nano silver colloid of *A.flavus* showed more homogeneity than that other two colloids of *P. citrinum* and *F.oxysporium* respectively.

Conclusions: The use of fungus for silver nanoparticles synthesis offers the benefits of eco-friendliness and amenability for large-scale production.

KEYWORDS

Nanosilver;
Fungi;
Biosynthesis;
Nanoparticles;
Zetasizer.

BACKGROUND

One of the first and most natural questions asked when starting to deal with nanoparticles is "Why are nanoparticles so interesting? Why work with these extremely small structures that are challenging to handle and synthesize especially when compared with their macroscopic counterparts?" The answer lies in the unique properties possessed by these nanoparticles.

Nanoparticles have been synthesized by various physical and chemical processes; however, some chemical methods cannot avoid the use of toxic chemicals in the synthesis process[1,2]. Therefore, there is an urgent need to develop a green process of nanoparticle synthesis, biological methods of nanoparticle synthesis using either microorganisms or plant extracts have offered a reliable, eco-friendly alternative to chemical and physical methods. Some of these microorganisms can be used to remediate toxic metals. Metal ion reduction is recognized as one of the heavy metal resistance

mechanisms in microorganisms. In this biological process, the microorganisms reduce metals in the form of insoluble particles, and proteins secreted by these microorganisms act as capping agents to prevent agglomeration of the particles and to stabilize them in the medium. This capacity to produce metallic particles intra- or extracellularly through the reduction of metal ions has been implemented in the production of nanoparticls[3]. It can be categorized into intracellular and extracellular synthesis according to the place where nanoparticles are formed.

Many organisms including unicellular and multicellular microorganisms have been explored as a potential bio factory for synthesis of metallic nanoparticles (Cadmium sulfide, gold, silver) either intracellularly or extracellularly[4-6]. Recently many studies has been conducted to explore the synthesis of nanoparticles uses of microorganisms as a potential, bio sources; such as Au and Ag,[7] use edible mushroom extract for biosynthesis of Ag, Au and Ag-Au nanoparticles[8,9] has reported that tropical marine yeast *Yarrowia lipolytica* NCIM 3589 and immobilized fungus *Coriolus versicolor* when exposed to Au and Cd ions formed respective metallic bionanoparticles.

Fungi are becoming the primary organism used in biological production of nanoparticles due to their tolerance and metal bioaccumulation ability[10]. The possibility of large-scale production of extracellular enzymes, economic viability and simplicity in handling biomass are distinctive advantages of using fungi in nanoparticles synthesis. However, genetic manipulation of eukaryotic organisms as a technique for over-expressing specific enzymes involved in nanopartcles synthesis is more difficult than in prokaryotes[11]. The extra cellular production of metal nanoparticles by several strains of the fungus *Fusarium oxysporum* has been described by[12]. The presence of hydrogenase in the *F. oxysporium* broth was demonstrated. This extra cellular enzyme shows excellent redox properties and it can act as an electron shuttle in metal reduction. It was evident that electron shuttles or other reducing agents (e.g., hydroquinones) released by microorganisms are capable of reducing ions to nanoparticles. *Aspergillus fumigatus* and *Phanerochaete chrysoporium* produced stable silver nanoparticles when challenged with silver nitrate in aqueous medium. The extra cellular synthesis of stable silver nanoparticles using the fungus *Aspergillus flavus* has also been reported[13]. Recently[9] has reported the synthesis of silver nanoparticles using white rot fungus *C.versicolar.* Working towards the goal to enlarge the scope of bio-organisms in the biosynthesis of nanomaterials, we explore the potential of *Penicillium citrinum, Fusarium oxosporum, Cephalosporium maydis, Sclerotium rolfsii, Fusarium rosum, Fusarium monoloforum, Asperigillus niger, Macrophmina sp., Acremonium strticum* and *Asperigillus flavous* for extracellular biosynthesis of silver nanoparticles.

RESULT

The detailed study on extracellular biosynthesis of silver nanoparticles by the eleven fungi eleven fungi (*Penicillium sp., Fusarium oxosporum, Cephalosporium maydis Sclerotium rolfsii, Fusarium rosum, Fusarium monoloforum, Asperigillus niger, Macrophmina sp., Acremonium strticum* and *Asperigillus flavous*) were screened for synthesis of silver nanoparticles in from Ag^{+}. The fungal biomass of each strain, after incubation for 72 h. with deionized water, was separated by filtration and to the cell- free filtrate silver nitrate was added. The formation of silver nanoparticles by the cell – free filtrate of the fungi studied was investigated by visual observation of the change in the color of the solution. The appearance of a pale to intense brown color in the reaction vessels and the solution remained as hydrosol and no precipitation was observed even after 72 h of incubation suggested the formation of silver nanoparticles. Control (without silver ion) and negative fungal results showed no change in color of the cell filtrate when incubated in the same environmental condition (data not shown). Only three of eleven tested fungi showed changes in color at the end of incubation. Figure 1(3,5,6) shows two conical flasks with the cell-free filtrate of *Asperigillus flavus, Fusarium oxysporun and Penicillium citrinum* in aqueous solution of 10^{-3} M $AgNo_3$ at the beginning of the reaction (left flask) and after 72h of reaction (right flask). It is observed that the biomass has a pale yellow color before reaction with the silver ions (left flask), which changes to a brownish color on completion of the reaction (right flask).

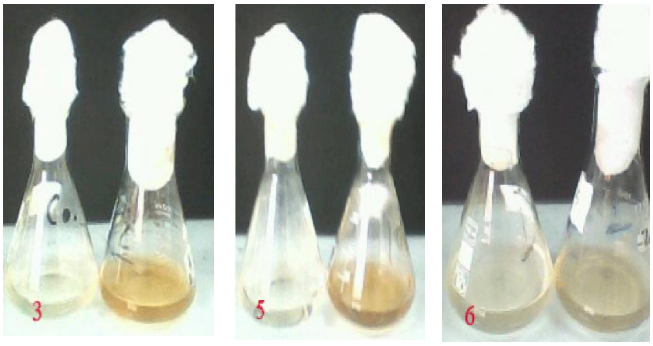

Figure 1 : Pictures of conical flask containing cell-free extract in aqueous solution of 10^{-3} M of $AgNO_3$ for : 3, *A. flavus* ; 5, *F. oxysporum* and 6, *P. citrinum* at the beginning of the reaction (left flask) and after 72 h of reaction (flask 2).

The light absorption pattern of the cell filtrate was monitored in the range of 200–800 nm using a UV–visible spectrophotometer. Figure 2 shows the UV–visible spectra of the silver nitrate solutions challenged with the fungal filtrates. While no absorption band was observed in control, a characteristic surface Plasmon absorption band at 425 nm was observed at 72 h for *Asperigillus. flavus and Fusarium. oxysporium* (Figure 2 a, b &c). On the other hand, the absorption band was observed at 450 nm in case of *Penicllium. citrinum*.

A representative TEM micrograph of silver nanoparticles obtained after 72 h of incubation is presented in Figure 3. The micrograph showed nano-particles with variable shape, most of them present in spherical in nature with some others having occasionally triangular shape. The size of the particle ranged from 7 to 38 nm. Majority of the silver nanoparticles were scattered with only a few of them showing aggregates of varying sizes as observed under TEM. Under observation of such images. In the (Figure 3a) of *Asperigillus flavous* it could be observed that the nanopartecles were well separated. The most dominating morphology shape was spherical and the size in the range 7nm to 37 nm. The image in the Figure 3b of *Pencillium citrinum* suggested that the particles are polydiperse and are mostly spherical in shape. Its size ranged from 8 nm to 38 nm. *Fusarium oxysporum* shown in Figure 3c were formed in several different sizes, ranged from 9 nm to 27 nm mostly have triangular shape.

Zeta average diameter was used for measurement of average hydrodynamic diameters and particle size distribution (polydispersity indexes). Figure 4(a, b and c) show size distributions of nanoparticles in three colloids. While most of them were around 10-100 nm, the silver nanoparticles in colloidal solution had a diameter of approximately 50.75 nm, 32.07 nm and 37.84nm which produced by *Asperigillus flavous, Fusarium oxysporum* and *Pencillium citrinum* respectively.

On the other hand, polydispersity index (Pdi) (data not shown) were recorded as 0.414, 0.7 and 0.528 for *Asperigillus flavous, Fusarium oxysporum* and *Pencillium citrinum* respectively.

DISCUSSION

Today, nano metal particles, such as silver and gold, have drawn the attention of scientists because of their extensive application to new technologies in chemistry, electronics, medicine, and biotechnology. Beside many physical and chemical methods which have been developed for preparing metal nanoparticles, nanobiotechnology also serve as an important method in the development of clean, nontoxic, and environmentally friendly procedures for the synthesis and assembly of metal nanoparticles. It is known that the physical properties of biologically produced nanoparticles may vary among different types of organisms[14]. In this study a variety of fungal species were screened for their ability to produce silver nanoparticles of uniform size and shape. Out of Eleven fungal species only three species identified as; *Asperigillus flavous, Pencillium citrinum* and *Fusarium oxysporum;* were showed the ability to produce exteracellular silver nanoparticles. In this context several works[15-18] pointed out that, extracellular biosynthesis of silver nanoparticles achieved by different fungal species. In this regard,[11] reported that, Because of their tolerance and metal bioaccumulation ability, fungi are taking the center stage of studies on biological generation of metallic nanoparticles.

In our results, the color of the medium changed very rapidly to brown upon addition of the silver ion (1 mM) into the flask containing the cell filtrate of *Asperigillus flavous, Pencillium citrinum* and *Fusarium oxysporum*. The appearance of the brown color was an indication of formation of colloidal silver particles in the medium. The brown color of the medium could be due to the excitation of surface plasmon vibrations, typical of the silver nanoparticles[10]. As shown in this study, the silvernanoparticles were synthesized in the extracellular cell filtrate of the filamentous fungus. This offers

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a great advantage over an intracellular process of synthesis from the application point of view[13]. Since the nanoparticles formed inside the biomass would have required additional step of processing for release of the nanoparticles from the biomass by ultrasound treatment or by reaction with suitable detergents.

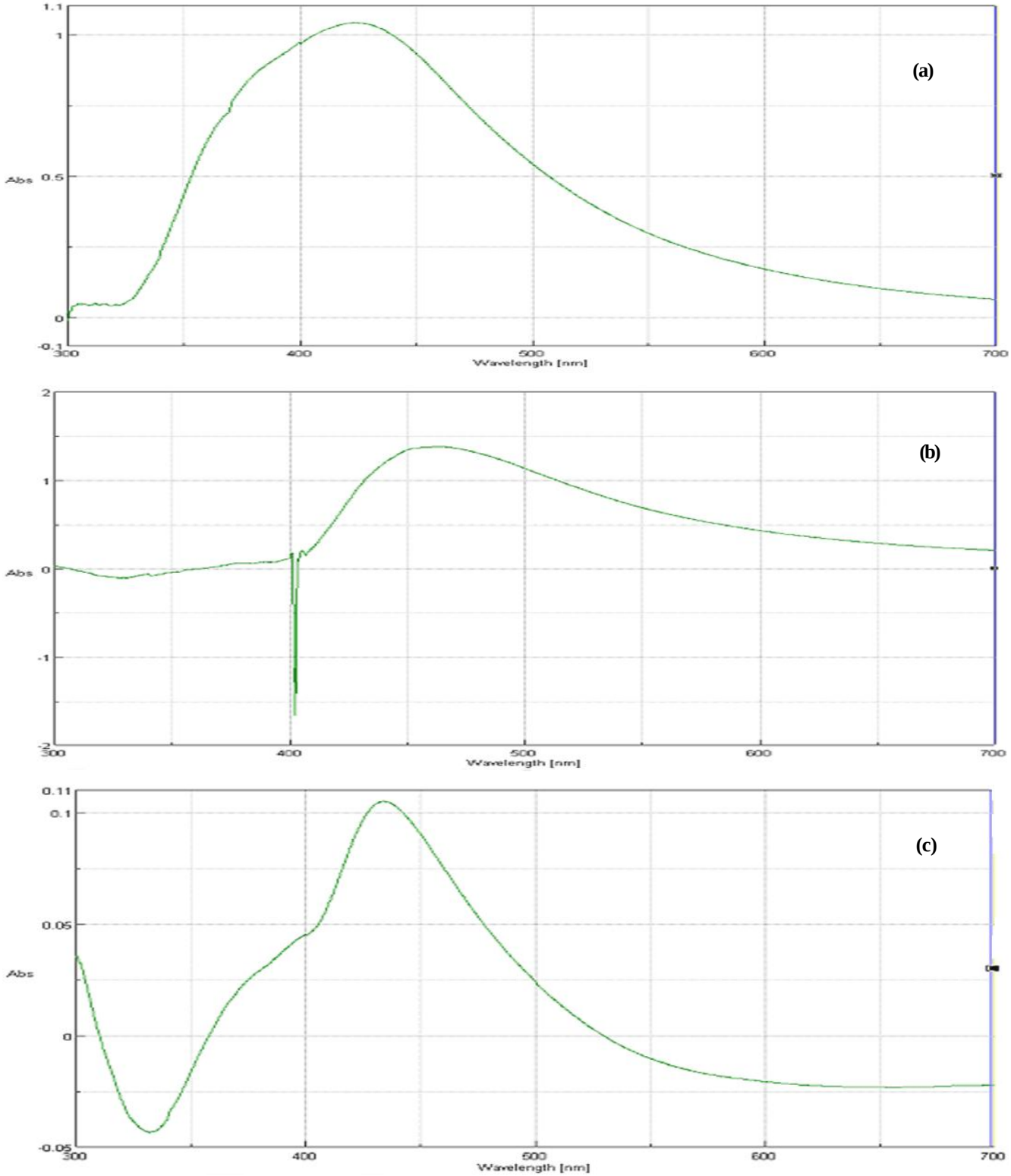

Figure 2 : UV – Vis spectra recorded for a : *A. flavor* ; b : *P. citrinum* and c: *F. oxysporum* using cell-free extract of after 72 h of incubation period.

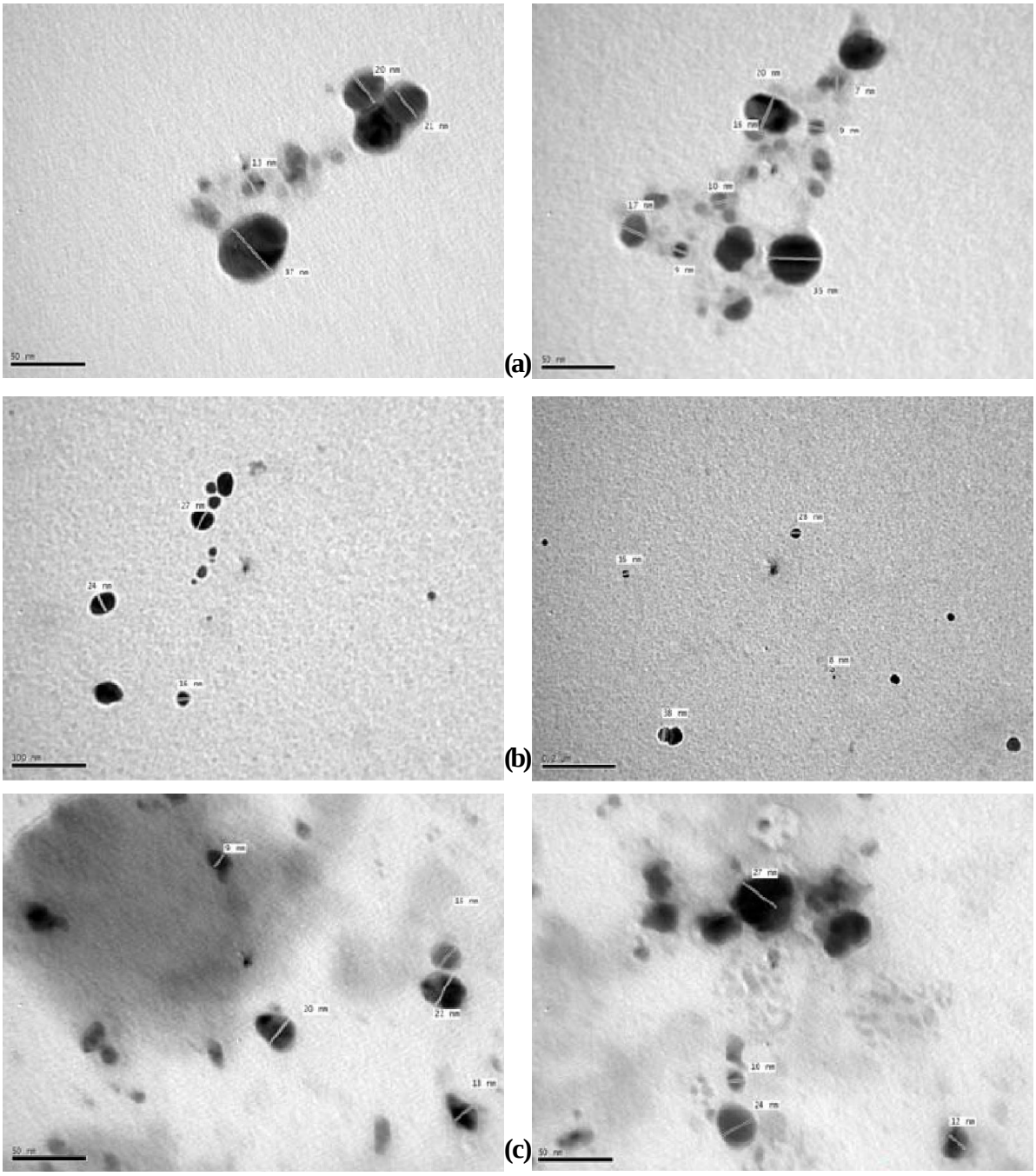

Figure 3 : TEM image of silver nanoparticles synthesized using cell-free extract of a : *A. flavus,* b : *P. citrinum* and c : *F oxosporum*.

In the present investigation, the absorption spectrum of the medium containing the silver ions showed increased intensity at 425 nm in case of *Asperigillus flavous*, and *Fusarium oxysporum* after 72 h of incubation, while at 450 nm in case of *Pencillium citrinum*. The increase in intensity could be due to increasing number of nanoparticles formed as a result of reduction of silver ions present in the aqueous solution[16,19]. The peaks in case of *Pencillium citrinum* (450 nm) showed higher than that in case of *Asperigillus flavous*, and *Fusarium oxysporum* (425nm) clearly indicates increase in silver nanoparticles size[10,17]. The fact that silver nanoparticles peak remained close to 425 nm after 72 h of incubation indicates that the particles were well dispersed in the solution and there was not much aggregation. Monodispersity is an important characteristic of the nanoparticles[3,12,20,21].

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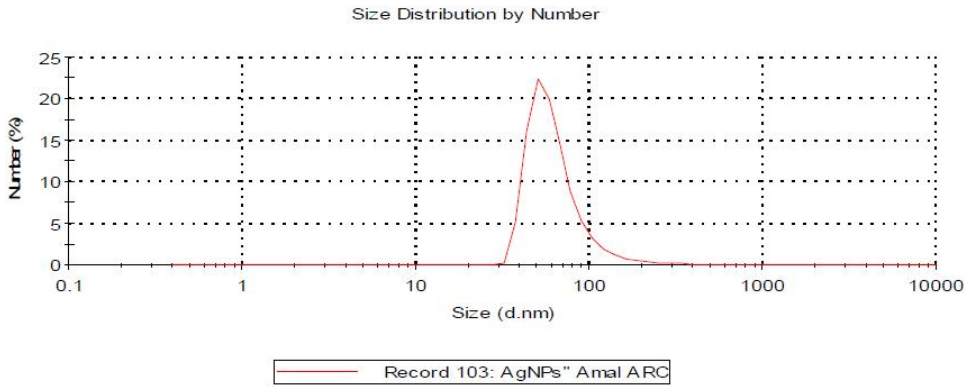
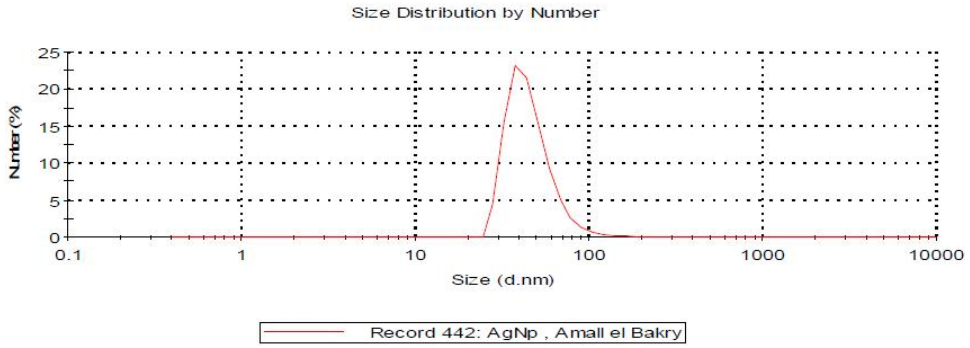
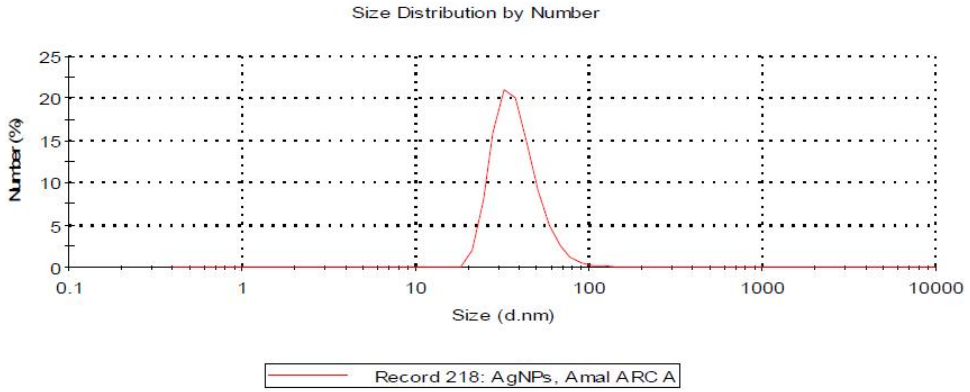


(a) *Asperigillus flavus*



(b) *Penicillium citrinum*



(c) *Fusarium oxysporum*

Figure 4 : Zetasizer average diameter a, for *A.flavus* with pdi 0.414, b for *P.citrinum* with pdi 0.528 and c for *F.oxysporum* with pdi 0.7.

Transmission electron microscopy provided further insight into the morphology and size details of the silver nanoparticles. The biosynthesized silver nanoparticles were previously characterized using TEM by several investigators[22,23]. As shown in our results, majority of the nanoparticles were spherical in shape produced by both *Asperigillus flavus* and *Pencillium citrinum* while it was appeared triangular by *Fusarium oxysporum*. The sizes of the silver particles were found to be in the range of 7–38 nm. These results indicate on the potentiality of fungi to nanosilver productivity were variable according to type species of the fungi. This may be attributed to potentiality of the species to produce oxidoreductase enzymes as well as reducing agents. Similar results were obtained from previous work[12,15,24,25] and[11], who pointed out that the mechanism of formation of silver nanoparticles from *Trichoderma asperellum* comprises two key steps : bioreduction of AgNO3 to produce silver nanoparticles followed by stabilization and/or encapsulation of the same by a suitable capping agent.

Z average diameter was used as a second tech-

nique in the present investigation to confirm the nanoparticle sizes and nanoparticle size distribution (pdi). The results showed that the lowest particle size of the three fungi was observed for *F. oxysporum* nanoparticles and it was in agreement with that obtained from TEM results. On the other hand, the larger nano size was recorded from *A. flavus* nanoparticles measured as 50.75 nm in case of zetasizer instead of 38.8 nm from *P. citrinum* nanoparticles in case of TEM. The difference between the results of both techniques may be attributed to the quantity of samples and method of preparation were actually differs, however, the two techniques confirmed the production of silver nanoparticles from the three tested fungi in the present investigation. The polydispersity results suggested that the nano silver colloid of *A.flavus* showed more homogeneity than that othor two colloids of *P. citrinum* and *F.oxysporium* respectively. Zetasizer was previously used for characterization on nanoparticles produced either by green biosynthesis or chemical and physical synthesis[26,27].

According to the results of this research and previous research, we can say that To be utilized nanometal particles in different scientific fields, biological synthesis still requires the optimization of reaction conditions, and an understanding of the biochemical and molecular mechanisms of the reaction for obtaining better chemical composition, shape, size and monodispersity.

METHODS

Biological synthesis of silver nanoparticles

Eleven filamentous fungi strains isolated from environment (*Penicillium sp., Fusarium oxosporum, Cephalosporium maydis Sclerotium rolfsii, Fusarium rosum, Fusarium monoloforum, Asperigillus niger, Macrophmina sp., Acremonium strticum* and *Asperigillus flavas*). Identified by Dis. Dept., Plant Path. Res. Inst., Agric. Res. Center, Giza, Egypt have been used in the study. Inoculated fungi were prepared in Petri dishes at room temperature using 2% malt extract with 0.5% yeast extract or Czapek Dox Agar (Difco). Fungal biomass used for biosynthetic experiments was grown aerobically in liquid medium containind (g/l): KH2PO4 7.0; K2HPO4 2.0; MgSO4-7H2O 0.1; yeast extract 0.6 ; glucose 20.0). The Erlenmeyer flasks were inoculated with spores and incubated at 25°C with shaking (150 rpm) for 72h- 96h. After the incubation, the biomass was filtered (Whatman filter paper No.1) and then extensively washed with distilled water to remove any medium component. Fresh and clean biomass (10g) was taken into the Erlenmeyer flasks, containing 100 ml. of Milli-Q deionized water. The flasks were agitated at the same conditions as described above, then the biomass was filtered again (Whatman filter paper No.1) and cell-free filtrate was used in next experiments. A carefully weighed quantity of $AgNO_3$ was added to the conical flask to yield an overall Ag^+ ion concentration of 10^{-3} M in the aqueous solution. $AgNO_3$ (1mM of final concentration) was mixed with cell-free filtrate in an Erlenmeyer flask and agitated at 25c in dark. The control (without the silver ions) was also run along with the experimental flasks. To verify reduction of silver ions, the solution was scanned in the range of 200-800 nm in a UV- visible spectrophotometer (Jasco V630 made in Europe). The size and morphology of the nanoparticles were analysis with the transmission electron microscope (TEM) (Joel 1230 operated at 100KV connected with CD camera, Japan). The sample was prepared by placing a drop of silver nanoparticles on carbon-coated copper grid and subsequently drying in air before transferring it to the microscope[28]. ZetaSizer was used for measurement of average hydrodynamic diameters and polydispersity indexes (Pdis) [(Nano ZS) Malvern, UK. Zeta potential rang (mV): (-200:200mV)]. Each sample was analyzed in triplicate at 25æ%$_C$ at a scattering angle of 173æ%. Size distribution of the particles was estimated using Laser particle analyzer (LPA) images by measurement of diameters[15].

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