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Extracellular Synthesis of Silver Nanoparticles by *Aspergillus terreus*: Biosynthesis, Characterization and Biological Activity

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The development of fast, cost-effective, and "eco-friendly" method for the production of silver nanoparticles is an important aspect of nanotechnology today. In this paper, ten fungal strains isolated from marine sediment in Mediterranean Sea (Alexanderia) were screened for their abilities to synthesis silver nanoparticles. *Aspergillus terreus* MALEX was selected as the most active strain. The silver nanoparticles were characterized by UV–vis spectrophotometry, X-ray diffraction analysis, and Scanning electron microscopy (SEM). UV–visible spectrum of the aqueous medium containing silver ion showed a peak at 420 nm corresponding to the plasmon absorbance of silver nanoparticles. SEM studies showed formation of well-dispersed nanoparticles in the range of 15–29 nm and the shape of nanoparticles was spherical. The biosynthesized silver nanoparticles exhibited high activities against four pathogenic bacterial strains (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *E. coli*, *and Salmonella* sp.), four mycotoxigenic fungal strains (*Fusarium solani*, *Alternaria alternata*, *Aspergillus flavus*, *A. ochraceus*).

Key words: Marine fungi, *Aspergillus terreus*, silver nanoparticles, Antimicrobial**.**

Mycotoxins are a group of toxic chemical secondary metabolites produced by strains of some fungal species when they grow under favourable conditions on a wide range of foods and feeds[6]. Mycotoxins affect several agricultural products, including cereals, oilseeds, pulses, nuts, root crops, dried fruits, and coffee beans which form the agricultural economic backbone of most developing countries in Africa. Contamination of agricultural products occurs as a result of infection by toxigenic fungi under favourable environmental conditions. Mycotoxins generally are of concern in human health, food safety and trade because of their acute and chronic effects on humans and domesticated animals. Recently, the increase of resistance to commercially available antimicrobial agents by pathogenic bacteria and fungi has become a serious problem.[32,33]. The use of compounds that can reduce or inhibit the growth of mycotoxigenic fungi and pathogenic bacteria

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during storage of food is very valuable. Applying different chemicals, during storage of food and prior to feeding, were considered by many scientists in recent years for control of fungi and detoxification of mycotoxins [35]. The use of silver nanoparticles as inorganic antimicrobial agents, silver has been thoroughly investigated.

The biosynthesis of silver nanoparticles is rapidly gaining importance due to ease of formation of nanoparticles and environmental applications. Presently, both prokaryotic (bacteria) and eukaryotic organisms such as fungi have been found to produce silver nanoparticles. Because of their tolerance and metal bioaccumulation ability, fungi are taking the centre stage of studies on biological generation of metallic nanoparticles [26]. A distinct advantage of using fungi in nanoparticle synthesis is the ease in their scale-up (eg, using a thin solid substrate fermentation method). Given that fungi are extremely efficient secretors of extracellular enzymes, it is thus possible to easily obtain large-scale production of enzymes. Further advantages of using a fungal-mediated green approach for synthesis of metallic nanoparticles include economic viability and ease in handling biomass.

Silver nanoparticles are being extensively synthesized using various fungi such as *Fusarium oxysporum* [1], *Fusarium semitectum* [4], *Aspergillus fumigatus* [5], *Cladosporium cladosporioides* [3], *Aspergillus clavatus* [30], *Trichoderma reesei* [29], *Penicillium fellutanum* [14]. Vigneshwaran et al. employed *Aspergillus flavus* to produce silver nanoparticles. The applications of silver nanoparticles greatly depend on their size[27].

Recent research regarding the use of fungi has generally investigated potential redox systems using silver nitrate as the source of silver ions [18,25]. Several enzymes, α-NADPH-dependent reductases and nitrate-dependent reductases, were implicated in silver nanoparticles synthesis for *Fusarium oxysporum* [7]. Nitrate reductase was also suggested to initiate nanoparticles formation in a *Penicillium* species [18].

A previous investigation has shown that many terrestrial fungi do not produce nitrate reductase (Na-R). Recently, an examination of marine fungi revealed that all strains tested produce Na-R. Other authors, however, showed absence of this enzyme in a few marine strains [28]. Hence, the present study was undertaken to explore a new fungal strains isolated from marine environment have ability to synthesize extracellular silver nanoparticles and to investigate the antimicrobial activity of biosynthesized silver nanoparticles against some fungal and bacterial strains.

MATERIALS AND METHODS

Screening of fungal isolates for synthesis silver nanoparticles

Ten fungal strains isolated from marine sediments in the Mediterranean Sea (Alexanderia) were screened for their ability to synthesis silver nanoparticles (table1). To obtain biomass for biosynthesis studies 100µL of fungal conidia will be inoculated in flasks containing 100mL of potato dextrose broth (PDB).The cultures were incubated in an orbital shaker at 28 °C at 150rpm for 72 h. After incubation, the biomasses were harvested by filtration, followed by extensive washing with sterile distilled water to remove any residual growing media. For the biosynthesis experiments, approximately, 10 g of fungal biomass was transferred into 250mL Erlenmeyer flask containing 100mL double distilled water and incubated for 24 h in an orbital shaker at speed of 150rpm and 28 °C. After incubation, the fungal filtrate was obtained by passing through Whatmann No.1 filter paper. $AgNO_3$ was added to 100mL of fungal filtrate at a concentration of 1mM and incubated at 28 °C and speed of 150rpm in an orbital shaker. Conical flasks with either fungal filtrate or $AgNO_3$ served as positive and negative control. The change in the color was indication of biosynthesis of nanoparticles.

Aspergillus terreus MALEX (M is encoded for marine and ALEX for Alexandria) was selected as the most active strain to synthesis of silver nanoparticles and was used for further studies. Different concentrations of $AgNO_3$ (1mM, 2mM, 5mM, and 10mM) were added to 100mL of fungal filtrate in separate conical flasks and incubated at 28 °C and speed of 150rpm in an orbital shaker.

Characterization of silver nanoparticles

The characterization of the biosynthesized silver nanoparticles was achieved at the central labs of the new material institute in the city of scientific research and technological

applications using the different characterization equipments such as UV-Vis spectrophotometer, transmission electron microscope (TEM), and X-ray diffraction (XRD) according to Zaki, et al method [36]. In order to study the formation of nano-materials, sample of 1ml was withdrawn and the absorbance for sample was scanned over a wide wavelength range 200-700nm using UV–visible spectrophotometer (Labomed. model UV–Vis Double beam spectrophotometer). The silver nanoparticle solution thus obtained was centrifuged at 12,000 rpm for 15 min, after which the pellet was redispersed in deionized water to get rid of any uncoordinated biological molecules. The purified pellets were then freeze-dried, powdered, and used for XRD, SEM.

Scanning electron microscope (JEOL JSM 6360LA, Japan) was utilized to confirm and proof the previously detected morphology of nano-materials; samples were prepared by placing a drop of hydrophobic nano-material colloid or its aqueous coordinate on carbon-coated copper grids and dried at room temperature. The dried silver nanoparticles sample was placed into a flat aluminum sample holder, where the X-ray source (Schimadzu-7000, USA) was a rotating anode operating at 30 kV and 30 mA with a copper target. Data was collected between 10º and 90º in 2¸.

Antimicrobial activity

Screening for antibacterial activity

The antibacterial activity of synthesized silver NPs was evaluated against four bacterial strains (*Staphylococus aureus*, *Klebsiella pneumonia*, *E. coli*, and *Salmonella* sp.). The tested bacterial strains were cultivated on Nutrient agar medium; three wells were made having a diameter 7mm. Different concentrations of silver nanoparticles colloidal solution were prepared (1 mM, 2mM, 5mM, 10 mM and 20 mM). Water and aqueous fungal extract were used as negative controls for antibacterial activity. All plates were incubated at 37 °C for 24 h., after incubation period was finished; all plates were observed for determination of the inhibition zone around the wells. The minimum inhibitory concen-tration (MIC) was the lowest concentration of silver NPs that resulted in visual inhibition of bacterial growth [11].

Antifungal activity assay

The antifungal activity of synthesized silver nanoparticles was evaluated against four fungal strains (*Fusarium solani*, *Alternaria alternata*, *Aspergillus flavus*, *A. ochraceus*). The in vitro antifungal activity of the silver NPs was evaluated using the well method. The petri plates containing Czapkes medium were inoculated with the fungal strains; three wells were made having a diameter 7mm. Different concentrations of silver nanoparticles colloidal solution were prepared (1 mM, 2mM, 5mM, 10 mM and 20 mM). Water and aqueous fungal extract were used as negative controls for antifungal activity. All plates were incubated at 28°C for 3-5 days. After incubation period was finished; all plates were observed for determination of the inhibition zone around the wells. The minimum inhibitory concentration (MIC) was the lowest concentration of silver nanoparticles that resulted in visual inhibition of fungal growth.

RESULTS

The ability of fungal isolates to synthesis silver nanoparticles

The screening of fungal strains isolated from marine sediment for their potentiality to synthesize silver nanoparticles revealed that 7 fungal strains does not have ability to synthesize silver nanoparticles, while 3 fungal strains (*Aspergillus terreus*, *Aspergillus versicolor*, and *Talaromyces* sp.) had the ability to synthesize silver nanoparticles depending on color change (Table1). Figure 1 shows the photographs of *Aspergillus terreus* filterate (left flask) and silver nitrate in the presence of *Aspergillus terreus* filterate after completion of the reaction (right flask). The appearance of a yellowish-brown color confirms the existence of silver nanoparticles in the filterate (right flask). Our study reported for the first time that *Aspergillus terreus* have ability to synthesize extracellular silver nanoparticles.

The precipitation of silver nanoparticles was observed after 24 h of incubation. Control (without silver ion) showed no change in color of the cell filtrate when incubated in the same environmental condition. The flasks being incubated in the dark with different concentrations of silver ions showed gradual change in color of the medium to brown, with intensity increasing with the increase silver ions concentration until 5mM and then decreased (Figure2).

Biosynthesized silver nanoparticles characterization

The absorption spectrum (Figure 3) of the yellowish-brown silver nanoparticle solution prepared with the proposed method showed a surface Plasmon absorption band with a maximum of 425 nm, indicating the presence of spherical Ag nanoparticles. This structure was confirmed by SEM images. A representative SEM micrograph of silver nanoparticles obtained after 72 h of incubation is presented in Figure 4. The micrograph showed nanoparticles with spherical shape. The size of the particle ranged from 15 - 29 nm. Majority of the silver nanoparticles were scattered with only a few of them showing aggregates of varying sizes as observed under SEM. Further studies were carried out using X-ray diffraction to confirm the crystalline nature of the particle and the XRD pattern obtained has been represented in Figure 5. The XRD pattern showed four intense peaks in the whole spectrum of 2θ value ranging from 20 to 90 at 2 theta angles. The diffraction pattern corresponds to pure silver metal powder. The XRD pattern indicates that the nanoparticles had a spherical structure. No peaks of the XRD pattern of Ag_2O and other substances appear in Figure 5, and it can be stated that the obtained silver nanoparticles had a high purity.

Biological activity of biosynthesized silver nanoparticles

The results from the agar diffusion method revealed that the biosynthesized silver nanoparticles by *A. terreus* has broad spectrum antibacterial activities against the bacterial strains were tested. Among bacterial strains *E. coli* was the most susceptible to silver nanoparticles, as indicated by a growth inhibition zone of 7 mm when incubated with silver nanoparticles produced from 2 mM AgNO3 (MIC ~0.33 µg). By contrast, the other bacterial strains were less susceptible to silver nanoparticles (Figure6 and Table 2). In our work, we employed agar diffusion method to determine the susceptibility of four fungal strains. The agar diffusion method resulted in the formation of zones of growth inhibition ranging from 3-13 mm and MICs of 0.84-1.68 µg/mL. *Fusarium solani* and *Alternaria alternate* were the most susceptible to silver nanoparticles, as indicated by a growth inhibition zone of 3 mm when incubated with silver nanoparticles produced from 5 mM AgNO3 (MIC

Table 1. Source and name of isolates screened for their abilities for silver nanoparticle biosynthesis

Strains	Source	Silver NPs biosynthesis
Alternaria alternate	Sea water	-ve
Alternaria chlamydospora	Sea water	-ve
Aspergillus tamarii	Sea water	-ve
Aspergillus terreus	Sea water	+ve
Aspergillus versicolor	Sea water	+ve
Penicillium corylophulum	Sea water	-ve
Scopulariopsis halophilica	Sea water	-ve
Talaromyces sp.	Sea water	+ve
Trichoderma viride	Sea water	-ve
Verticillium sp.	Sea water	-ve

Table 2. Minimum inhibitory concentration (MIC) of silver nanoparticles (SNPs) for bacterial strains and fungal strains concentrations

Bacterial strains	MIC (µg/ml)	Fungal strains	MIC (µg/ml)
Staphylococcus aureus	0.84	*Fusarium solani*	0.84
Klebsiella pneumoniae	0.84	*Alternaria alternata*	0.84
E. coli	0.33	*Aspergillus flavus*	1.68
Salmonella sp.	0.84	*Aspergillus ochraceus*	1.68

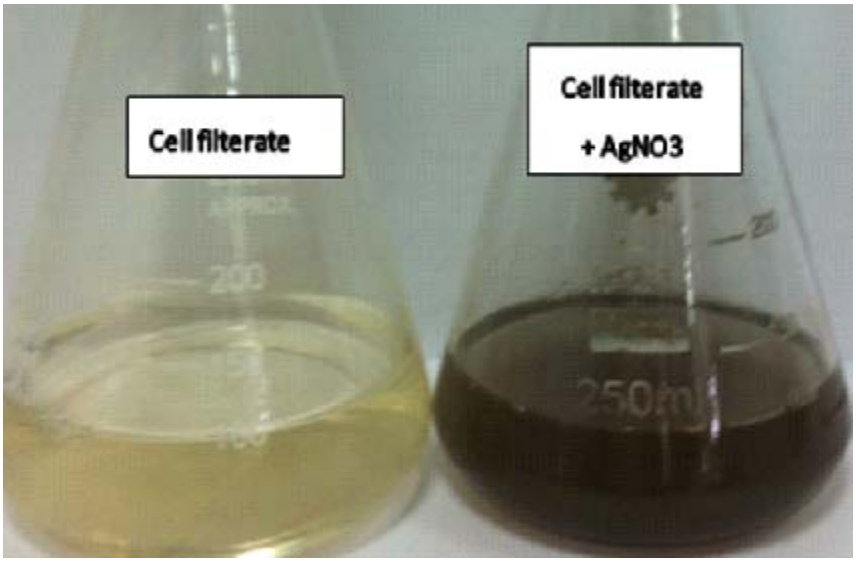

Fig. 1. Cell filtrate (72 h) of Aspergillus terreus with silver ion (5 mM): (left) at the beginning of the reaction and (righ) after 24 h of reaction

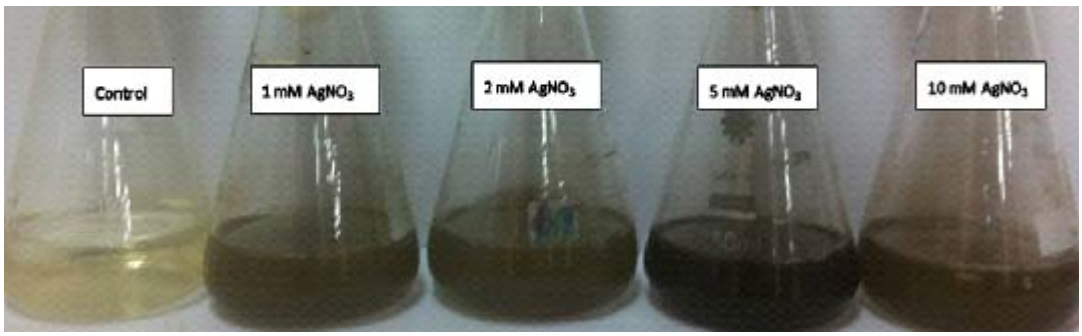

Fig. 2. Incubation of cell filtrates of *Aspergillus terreus* with different concentrations of silver ion

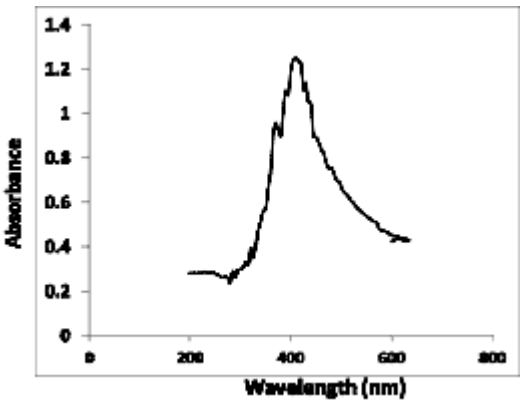

Fig. 3. UV-Vis absorption spectrum of obtained silver nanoparticles

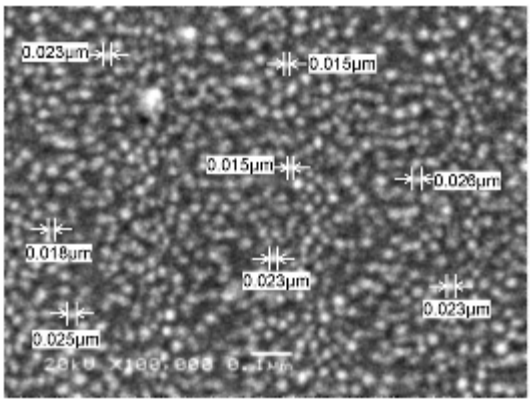

Fig. 4. SEM micrograph of silver particles synthesized by *Aspergillus terreus*

~0.84 µg). By contrast, the other fungal strains were less susceptible to silver nanoparticles (Figure7 and Table 2).

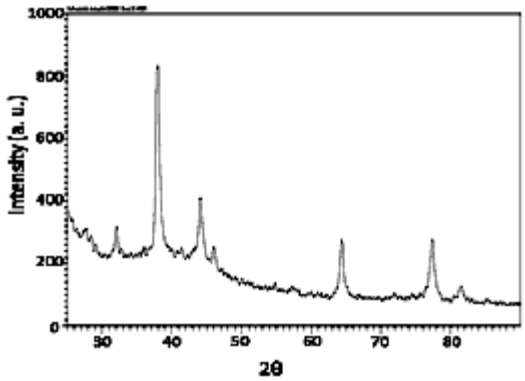

Fig. 5. X-ray diffraction pattern of silver nanoparticle film obtained from cell filtrate of *A. terreus*

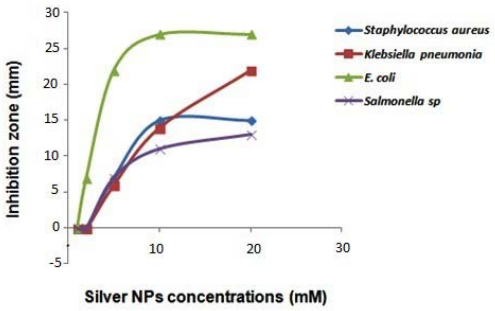

Fig. 6. Inhibition zones (mm) of bacterial strains after 24 h of incubation in contact with different concentrations of silver nanoparticle colloidal solutions biosynthesized by *A. terreus*

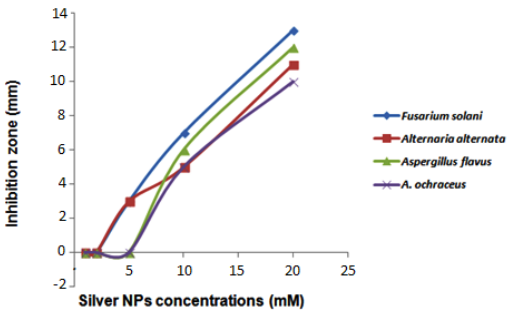

Fig. 7. Inhibition zones (mm) of fungal strains after 5days of incubation in contact with different concentrations of silver nanoparticle colloidal solutions biosynthesized by *A. terreus*

DISCUSSION

A variety of chemical and physical procedures could be used for synthesis of silver nanoparticles. However, these methods are fraught with many problems including use of toxic solvents, generation of hazardous by-products, and high-energy consumption. Accordingly, there is an essential need to develop environmentally benign procedures for synthesis of silver nanoparticles. A promising approach to achieve this objective is using fungi for production of silver nanoparticles. Biologically synthesized silver nanoparticles could be of immense use in medical textiles for their efficient antimicrobial properties[23]. In the last decade, the development of biological systems as an environmentally friendly method for metal nanoparticle formation has emerged as an interesting and important scientific field. A wide number of microorganisms, including bacteria, yeast, filamentous fungi, algae and plants, have been shown to be capable of fabricating various types of metal nanoparticles like silver, gold, palladium and others[20].

It is well known that silver nanoparticles exhibit a yellowish-brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles [13]. Reduction of silver ions to silver nanoparticles could be followed by a color change and UV-Vis spectroscopy. Therefore, the progress in conversion reaction of silver ions to silver nanoparticles was followed by a color change can be detected by visual observation.

This is the first time to use *A. terreus* in extracellular silver nanoparticles biosynthesis. The use of this organism will offer several advantages since it is considered as a non-pathogenic organism, has a fast growth rate, rapid capacity of metallic ions reduction, nanoparticles stabilization and facile and economical biomass handling. Previous studies have demonstrated that filamentous fungi, such as *F. oxysporum* [7], *Fusarium accuminatum* [12], *Aspergillus niger* [10], *Amylomyces rouxii* [19] and the endophytic fungus *Epicoccum nigrum* [23] are most efficient at producing silver nanoparticles. The reducing agent, reaction medium and silver nanoparticles stabilisation are three key factors in the synthesis of metallic nanoparticles[17]. According to Durán *et*

al. [7], reductases in the aqueous extracts of *F. oxysporum* are responsible for the reduction of Ag cations and subsequent silver nanoparticles production.

The silver nanoparticles were characterized by UV-Vis spectroscopy, one of the most widely used techniques for structural characterization of silver nanoparticles [24]. This result was in agreement with those results that reported the absorption spectrum of spherical silver nanoparticles present a maximum between 420nm and 450nm [2,16].

In our study, the biological activity of biosynthesized silver nanoparticles was applied on four mycotoxigenic strains and four pathogenic bacteria, which cause food quality problems. Our results revealed that the biosynthesized silver NPs by *A. terreus* has broad-spectrum antimicrobial activities against the fungal and bacterial strains were tested. This could be used as feed additives for their higher antimicrobial effect and are more resistant to deactivation by gastric acids and have a low absorption rate through the intestinal mucosa, thus minimising its potential risk of toxicity. Besides, it has been shown that the doses that promote animal physiological and productive effects are very low (20 to 40 ppm) thus precluding a harmful environmental effect [8,9,21]. Recently, silver nanoparticles have been used to cover the wheat grain to protect it from development of pathogenic microorganisms and accumulation of their toxic metabolites[22]. Previous studies have shown that silver NPs exhibit antimicrobial activity against different bacterial species, such as *Shigella dysenteriae* type I, *Staphylococcus aureus*, *Citrobacter* sp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* [17] and some fungal species, such as *Trichophyton mentagrophytes* and *Candida* spp [15,19]. Recently, Qian *et al.* [23] and Xu *et al.* [34] observed silver nanoparticles antifungal activity against several fungi as *Candida* spp, *Aspergillus* spp, *Fusarium* spp, *C. neoformans* and *Sporothrix schenckii* presenting MIC values of 0.12-1 µg/mL.

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