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The efficacy of some fungi in Bio-synthesis of silver nanoparticles and characterization through physical and microscopic approaches

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Abstract Green techniques for the production of metallic nanoparticles have emerged as a new and promising area of research in recent years. Several microbes produce metal nanoparticles that can be extracellular or intracellular and vary in efficiency, size, and form. In this investigation, five fungal mycelial filtrates (*Penicillium* spp., *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp. and *Trichoderma* spp.) were screened for the creation of silver nanoparticles (AgNPs). By examining the fungus filtrate optically only three fungal isolates (*Fusarium spp.*, *Aspergillus spp.* and *Trichoderma spp.*) were best able to reduce the silver salt ($AgNO_3$) into silver nanoparticles. The UV-visible spectra of the biosynthesized nanoparticles (AgNPs) by *Fusarium oxysporum* cell filtrate showed characteristic surface plasmon absorption at 432 nm. Fourier Transform Infrared (FTIR) analysis of AgNPs synthesized using *F. oxysporum* showed vibrational frequencies at 3449, 1630, 1025 and 535 cm^{-1}. A Transmission electron microscopy (TEM) image demonstrated the production of spherical AgNPs ranging in size from 5.36 to 20.11 nm in diameter. The green biosynthesis process used in this study is a non-toxic alternative to standard chemical and physical approaches, and it would be suitable for biological large-scale manufacturing and potential therapies.

keywords: Silver nanoparticles; Biological synthesis; *Fusarium oxysporum*; *Aspergillus spp.*; *Trichoderma spp.*

1.Introduction

Nano-biotechnology is an area of research that modifies the characteristics and behavior of materials at the nanoscale for a variety of human-beneficial uses **[1]**. A nanoparticle (NP) is a tiny particle that exhibits full-unit behaviour in terms of its attributes across a size range (1-100 nm). The specific surface area of a solid substance grows as its size decreases, which increases surface reactivity and quantum-related phenomena. The small size of nanoparticles causes their physical and chemical properties to differ from those of the same material in bulk form **[2]**.

A new synthesis method known as "green nanotechnology" incorporates biological systems as microorganisms, including bacteria, fungi, yeast, and plant extract **[3]**. Different transition metals can be used to synthesize nanoparticles, including silver (Ag), gold (Au), platinum, ferrous (Fe), cadmium sulfide (CdS) and zinc oxide (ZnO), each type differs from others in particular ways and in terms of its physical attributes **[4]**.

Silver nanoparticles (AgNPs) are being used more and more in a range of industries, including medicine, food, health care, and consumer products, as a result of their unique physical and chemical features **[5, 6].** They have been used in a variety of applications because of their peculiar properties, such as antibacterial agents, industrial, home, and healthcare-related products, consumer goods, medical device coatings, optical sensors, cosmetics, the food industry, drug delivery, the pharmaceutical sector, and as anticancer agents **[7]**.

Fungi are among the biological resources that exhibit increased metal bioaccumulation capabilities and tolerance, which are advantageous traits for the manufacturing of nanoparticles **[8]**. Fungi have the highest levels of intracellular metal uptake and wall binding due to their high levels of metal tolerance **[9,10]**. Comparatively to other plants and microorganisms, fungi have mycelia that are more successful at maintaining their position in the bioreactor during agitation and high flow pressure **[11]**. Additionally, the extracellular enzymes that fungi produce in large quantities lead to a tremendous quantity of enzyme production **[12]**. Metal nanoparticles (NP), nanostructure, and biomimetic mineralization can all be produced via internal and external ways of enzyme reduction **[10, 13]**.

Extracellular silver nanoparticles could be created by the fungus *Aspergillus favus* (Ag-NPs) **[14]**. It is known that the fungus *F. oxysporum* secretes the nicotinamide adenine dinucleotide (NADH) enzyme, specifically nitrate reductase, which may be in charge of the enzymatic metal reduction pathway that leads to the bioreduction of Ag+ to Ag^0 known as the electron shuttle **[13, 15]**. The mycosynthesis of silver NPs (SNPs) by *F. oxysporum* has been the subject of numerous reports **[16, 17]**. It has not yet been possible to fully understand the fundamental mechanics of nanoparticle production. The most effective biomolecules to be used in order to choose the best technical parameters to be employed in the biosynthesis, the reducing and stabilizing agents which are necessary to be determined, even though a number of factors may interact to decide the biological synthesis reaction **[18].**

The current work intends to utilize an effective, inexpensive and environmentally friendly method for green synthesis of silver nanoparticles (AgNPs) using five mycelial filtrates from *Penicillium* spp., *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp., and *Trichoderma* spp. Choosing the best fungal isolate for AgNP production and characterizing it with various physical and microscopic approaches.

2. Materials and methods

Fungal isolates

Penicillium spp., *Alternaria* spp., *Fusarium* spp., *Aspergillus* spp. and *Trichoderma* spp. isolates were obtained from Mycology laboratory, Botany Department, Faculty of Science, Mansoura University, Egypt.

Fungal biomass preparation for AgNPs biosynthesis

Potato Dextrose Agar (PDA) media (200 g of potato extract, 20 g of Dextrose, and 20 g of Agar) were dissolved in 1 L of distilled water (dist. H_2O) and autoclaved at 121°C for 20 minutes followed by cooling. The medium was mixed well before pouring. Five fungal isolates were cultured on PDA medium and then incubated at 25°C for five days till sufficient fungal growth occurred. For subsequent use in the biosynthetic process, the culture of these fungi was kept at 4°C [19].

Identificationoffungalisolates:Morphological identification on culture media

By examining the characteristics of the colony, the fungal morphology was examined macroscopically (color, shape, size and hyphae) **[20].**

Extracellular synthesis of silvernanoparticles

The purified fungal biomass was obtained by growing the fungus on agar discs (3-5 discs) in a liquid medium called PDA broth media, many discs of fresh culture were then inoculated in 500 ml Erlenmeyer flasks, each containing: 100 ml of PDA broth medium and incubated under continuous rotating conditions (180 rpm) for 72 hours at 25 ±1 °C. Following the incubation phase, the fungal biomass was filtered from the media, and the collected fungal biomass was thoroughly washed three times with sterile distilled water to remove any traces of media. To each 100 ml of sterile de-ionized water, 10 g wet fresh weight of fungal biomass were placed and incubated under the same circumstances as before for 24 hours. Carefully weigh $AgNO_3$ to make final concentration of 1mM. These flasks are rotated as previously mentioned condition and to avoid photooxidation of silver ions, keep the area dark, with the experimental flasks at the same conditions; control flasks (cell-free filtrate free from silver ions) were added.

Characterization of the biosynthesized nano-silver

Ultra Violet –Visible Spectrum

UV-Visible absorption spectroscopy is used to track the formation of nanosilver (Uni cam UV-VIS. Spectrometer UV2, U.S.A) according to the method of **[21].** A UV-VIS-NIR spectrophotometer was used to measure the absorption between the wavelengths of 200 and 800 nm (UV-1601, Shimadzu, Japan). The blank was distilled water.

To differentiate between five fungal isolates, optical density was measured and Plot was carried out using JMP®, Version 16 (SAS Institute Inc., Cary, NC, USA, 2020-2021).

FTIR (Fourier TransformInfrared)spectrum

The produced silver nanoparticles' surface chemistry was examined using FTIR spectroscopy. Depending on the infrared absorption frequency, the functional groups linked to the nanoparticles' surfaces were visible at 400–4000 cm^{-1}. AgNPs were dispersed in a dry matrix of KBr, which later became compressed to create a clear disc, as part of the sample preparation process. The JASCO FTIR (Japan) device employed a KBr pellet as a standard.

Transmission Electron Microscopic analysis (TEM)

The TEM (Transmission Electron Microscope JEOLJEM-2100, U.S.A.) used grid with carbon coating (Type G 200, 3.05 diameter, TAAP, U.S.A.) to visualize the size and shape of the nano-colloidal sample.

Results

Identification of studied Fungal isolates based on the cultural morphological characteristics

The tested fungi for producing nano-silver were identified morphologically as *Penicillium spp.* in which its aerial hyphal growth has characteristic green color (Figure 1A), *Alternaria spp.* in which its aerial hyphal growth has characteristic dark color (Figure 1B), *Fusarium oxysporum* in which its aerial hyphal growth has characteristic pinkish white color (Figure 1C), *Aspergillus flavus* in which its aerial hyphal growth has characteristic green yellowish color (Figure 1D) and *Trichoderma asperellum* in which its aerial hyphal growth has characteristic green color (Figure 1E). These fungi were cultured and maintained on Potato Dextrose Agar (PDA) at 25°C for 7 days and then stored at 4°C until used.

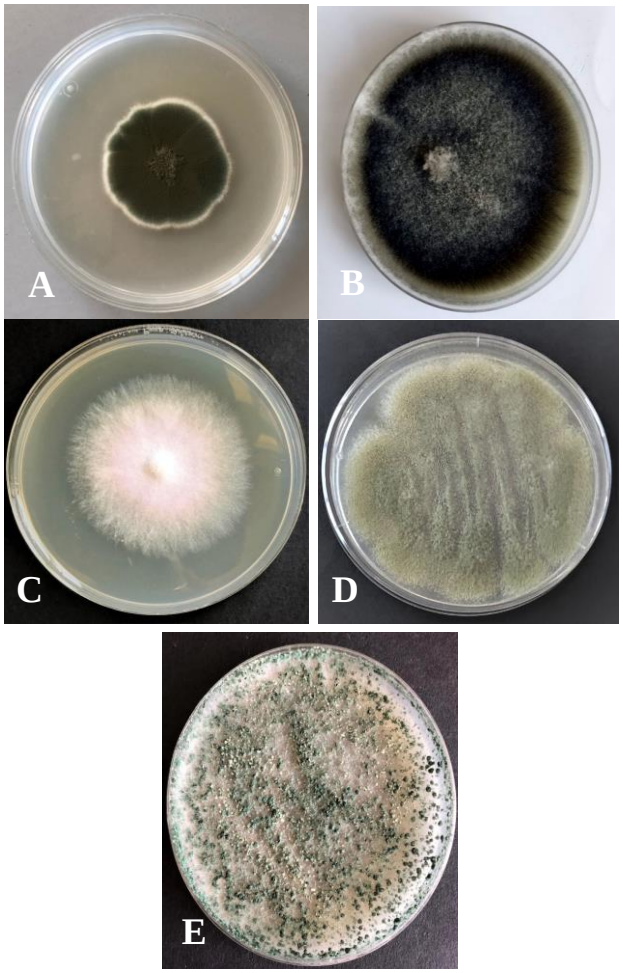

Figure 1. Characteristic growth of the studied five fungal isolates on PDA medium after 7 days incubation at 25°C. A) *Penicillium spp.*; B) *Alternaria spp.*; C) *Fusarium oxysporum*; D) *Aspergillus flavus*; E) *Trichoderma asperellum*

Screening of fungal isolates for AgNPs synthesis

The tested fungi were cultured on Potato Dextrose broth medium for fungal biomass production, followed by addition of 1mM $AgNO_3$. After the cell-free water extract has been incubated and $AgNO_3$, silver nanoparticles have been synthesized. The noticeable colour change was identified as a sign of the development of nano-silver. This might occur as a result of gradual reduction of silver ions to zero state silver atoms. The significant optical change in color which indicated to synthesis of AgNPs followed by measuring optical density (OD) using UV-VIS. spectrometer as shown in **(Figure 2).**

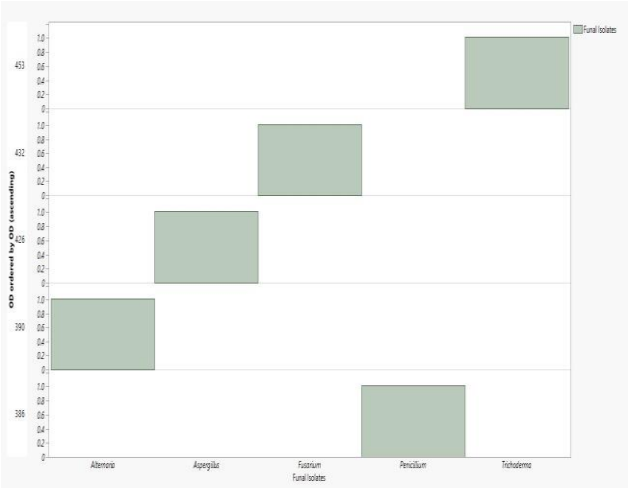

Figure 2. Histogram showing the optical density of AgNPs synthesized via the five studied fungal isolates.

Characterization of synthesized silver nanoparticles by *F. oxysporum*Optical observation and Ultra Violet –Visible Spectrum

The optical observation showed in color change was recorded **(Figure 3).** Color changed from pink to brown after incubation period. Also, The U.V-VIS spectra of Nano-silver particles produced by *F. oxysporum* ranged from 200 to 600 nm, a distinctive peak at 432 nm was recorded. This peak is taken into consideration as a sign of the bio-synthesized nano-silver **(Figure 4)**.

A

B

Figure3. Thebiosynthesis of silver nanoparticles. Color changed from pink to brown after incubation period, flask (A) control (cell free filtrate without silver ions), and flask (B) is a test flask (cell free filtrate with silver ions) after 72 h. incubation.

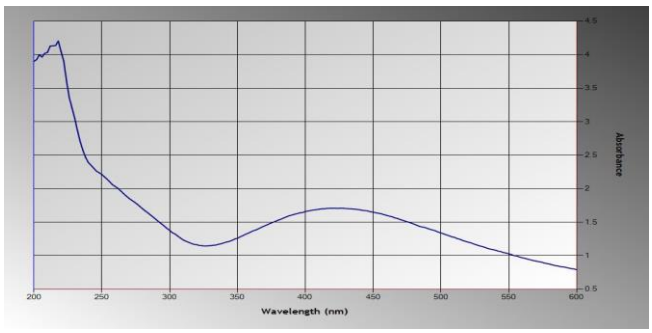

Figure 4. (UV-VIS) spectrum of the biosynthesis of silver nanoparticles.

FTIR (Fourier Transform Infrared) spectrum

FTIR analysis was used to look into the various functional groups responsible for the stabilization and reduction of silver nanoparticles. FTIR analysis of AgNPs synthesized using *F. oxysporum* showed vibrational frequencies at (3449, 2925, 2856, 1747, 1630, 1025 and 535 cm^{-1}), as seen in **(Figure 5)**. The peak at (3449 and 2925 cm^{-1}) refers to (N–H) stretch vibration of primary and secondary amides of protein and (O–H) stretch of alcohols and phenols. The peak at (3449 and 2925 cm^{-1}) refers to (N–H) stretch vibration of primary and secondary amides of protein and (O–H) stretch carboxylic acids. The peak at (2856 cm^{-1}) refers to (C–H) symmetrical stretch vibration of alkanes. The peak at (1747 cm^{-1}) refers to (C=O) carboxylic acid. The peak at (1630 cm^{-1}) refers to carbonyl stretch, which is assigned to the amide I bond of protein. The peaks at (1025 cm^{-1}) refer to

aliphatic amines. The peaks at (535 cm^{-1}) refer to (C–Cl) stretch alkyl halides.

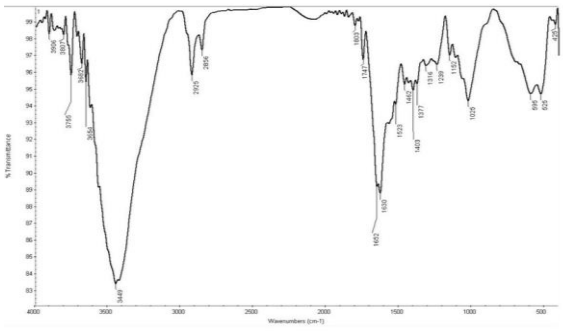

Figure 5. Fourier transform infrared (FTIR) spectrum of AgNPs synthesized by *Fusarium oxysporum* showing different functional groups responsible to stabilize or cap AgNPs.

Transmission Electron Microscopic analysis

Morphology and size distribution are easily discernible from TEM images. This micrograph shows the presence of individual and aggregated particles in the size range (5-20 nm); Due to the capping agent around protein-made nanoparticles, the nanoparticles are segregated from one another even within aggregates. The repulsive force that exists between particles is caused by this capping agent. The nano-silver that was visible in the micrograph had a primarily spherical shape **(Figure 6**).

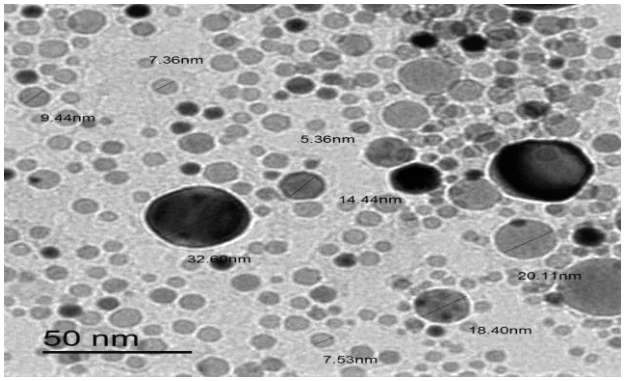

Figure 6. TEM micrograph of bio-synthesized nano-silver by *Fusarium oxysporum*, This image displayed the size distribution range of (5.36- 20.11 nm) and spherical shape of nanocolloids.

Discussion

Using bio-prospection in various and less frequently studied settings, we can analyze the microbial diversity and encounter microbes specialized in certain bio-products, such as metal nanoparticles. Microorganisms can produce nanoparticles faster, more cheaply, more effectively, and without the use of dangerous chemicals when compared to physical-chemical processes **[22]**. In this investigation, we substitute a biological technique for the conventional chemical and physical approaches. The use of fungi in biosynthesis is intriguing since they are available in bulk and the release of extracellular enzymes makes the downstream processing simpler. Extracellular methods are favored over intracellular methods because the latter require an additional purification step to achieve pure nano-silver **[23]**. Since fungi produce more proteins than bacteria do, employing them for biosynthesis is preferable since more NPs can be produced **[24]**.

In this investigation, out of five fungal species screened; *Penicillium* spp., *Alternaria spp., Fusarium* spp., *Aspergillus* spp. and *Trichoderma* spp.*;* only three fungal species; namely *Fusarium oxysporum, Aspergillus flavus* and *Trichoderma asperellum* were found to be the best for synthesis of AgNPs. These five fungal filtrates gradually changed to brown, which demonstrated the formation of AgNPs. After 24 h of incubation, the color of the culture filtrate containing silver nitrate solution turned intensely brown, whereas, the control (without silver nitrate salt) did not exhibit any color change. *Fusarium oxysporum, Aspergillus flavus* and *Trichoderma asperellum* exhibited the most intense brown color compared to the other fungal species which is consistent with the outcomes listed by **[25].**

The surface plasmon resonance characteristic of silver nanoparticles is responsible for the formation of dark brown **[26-28].** In this investigation, AgNPs produced by *F. oxysporum* were characterized using a variety of analytical methods, including as UV-visible spectroscopy, TEM and FTIR, to precisely describe their morphological and physical characteristics in accordance with **[6, 29, 30].**

Due to the simultaneous vibration of the metal nanoparticles' free electrons in resonance with a light wave, metal nanoparticles exhibit the Surface Plasmon Resonance (SPR) absorption band in UV-visible spectroscopy. In the present study a characteristic, SPR band of absorption was seen in the supernatant of *F. oxysporum* treated with 1mM $AgNO_3$ at 432 nm and this is in agreement with earlier works which declared that the SPR band for silver nanoparticles occurs between 300 and 500 nm **[21, 31].**

To describe and distinguish biomolecules that were bound specifically to the bio-synthesized AgNPs, FTIR spectroscopy was performed. In this investigation, the spectra of bio-synthesized nanoparticles revealed a clear peak in the region of (3449, 2925, 2856, 1747, 1630, 1025 and 535 cm^{-1}). The spectra indicate that amide functional groups, carbonyl and amines (both aromatic and aliphatic), phenols, and alcohol were used in the synthesis of silver nanoparticles **[32, 33].** In the current study, the

capping of synthesized AgNPs maybe as a result of the various extracellular proteins produced. Proteins can screen the surface charges that keep the particles from repelling one another or interact with the particles through bridging-type interactions, among other things, to affect the dispersion **[17].** Furthermore, our findings, which are consistent with the findings of Roy et al. [34], who biosynthesized AgNps from Aspergillus foetidus, show that the presence of different functional groups such as amide linkages and -COO- carbonyl groups confirm protein in the sample forms a coating known as a capping protein over the silver nanoparticles, which stabilizes the metallic nanoparticles and inhibits agglomeration in the medium. Likewise, **Gupta *et al*. [35]** stated that, to identify the functional groups responsible for stabilizing and capping mycosynthesized silver nanoparticles as well as reducing silver ions, FTIR readings were acquired. The spectra of *F. oxysporum* revealed numerous peaks at (3305.99, 2927.94, 1693, 1650, 1553, 1375, 1147, 1076, 1043 cm^{1}).

The shape of the produced silver nanoparticles was visualized using the TEM technique, which revealed both single particle and groups of aggregated, spherical particles, The particles size ranges from 5.36 - 20.11 nm. Even within the aggregates, the nanoparticles were not in direct touch with one another and were covered by a thin layer of organic material, which suggested that a capping agent had stabilised the nano-particles **[33, 36].** In this investigation, the AgNPs produced by *F. oxysporum*, were shown to be the most efficient possibly due to their small particle size. In reality, numerous investigations have demonstrated that AgNPs activity is strongly dependent on the NP size **[22, 37].** Also, **Roy *et al*. [34]** stated the spherical-shape of the Ag-NPs, with the size ranging between (3 and 20 nm) in *A. foetidus MTCC8876*.

Although the precise mechanism by which fungi produce AgNPs is not yet fully understood, earlier research has shown that NADH and NADH-dependent nitrate reductases are crucial components in the manufacture of metal nano-particles **[38, 39]**. In the present study, the chemical environment, pressure, temperature, and time all affect the crystal structure; the last three of these variables were constant for all fungi, We may thus infer from the nitrate reductase activity patterns in the extracellular extracts that the fungi's chemical environment played a role in the differentiation of the structures that were formed.

The potential for creating a logical and environmentally friendly method for the bio-synthesis of nanomaterials from a variety of chemical compounds, including oxides, nitrides, and other compounds, is what we think is the main benefit of our protocol based on fungal enzymes.

Conclusion

In the process of producing metal nanoparticles, fungus expose their mycelium to a metal salt solution. For its own life, the fungus is prompted to create enzymes and metabolites. The extracellular enzyme and fungal metabolites act as catalysts in this process to convert harmful metal ions to non-toxic metallic solid nanoparticles. More research is needed to understand the specific chemical mechanism causing the formation of AgNPs via biological techniques in order to have better control over their size and polydispersity. In addition, it's crucial to monitor the environmental effects of silver when it's used in the real world to determine how it affects both the environment and human health. This biosynthetic approach has the potential to be a successful technology for producing metal nanoparticles and useful in biotechnological and environmental applications.

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