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(Research Paper)

Eco-friendly synthesis of silver nanoparticles using cell-free extract from *Chaetomium olivaceum* B422: a novel approach for controlled nanoparticle production

Saydeh Fatemeh Hoseini-Nilaki
Department of Biological Science, Faculty of Science, University of Kurdistan, P.O. Box 416, Sanandaj, Kurdistan, Iran
bitahemedi229@gmail.com

Morahem Ashengroph[1]
Research Center for Nanotechnology, University of Kurdistan, P.O. Box 416, Sanandaj, Kurdistan, Iran
m.ashengroph@uok.ac.ir

Musa Moetasam Zorab
Department of Physics, College of Science, University of Halabja, Kurdistan region, Iraq
musa.zorab@uoh.edu.iq

Jafar Abdollahzadeh
Department of Plant Protection, Agriculture Faculty, University of Kurdistan, P.O. Box 416, Sanandaj, Kurdistan, Iran
j.abdollahzadeh@uok.ac.ir

Sodabeh Piri Kakihai
Department of Plant Protection, Agriculture Faculty, University of Kurdistan, P.O. Box 416, Sanandaj, Kurdistan, Iran
sodabehpiri2019@gmail.com

Abstract
The antibacterial and anticancer potential of silver nanoparticles (Ag-NPs) is promising for cancer treatment and fighting multidrug-resistant bacteria. This study introduces the first eco-friendly synthesis of Ag-NPs using a cell-free extract (CFE) of *Chaetomium olivaceum* strain B422 isolated from grapevine. In this study, ten fungal strains collected from grapevine trunks and twigs were purified and cultured on potato dextrose agar (PDA) at 25 °C (pH 5) under dark conditions for 7 to 14 days. Ag-NPs were synthesized using CFEs of the fungal strains and silver nitrate as precursor. The biosynthesis process was optimized by varying factors such as silver nitrate concentration, pH, temperature, agitation speed and incubation time using a one-factor-at-a-time approach. The synthesized Ag-NPs were characterized for various properties such as size, structure and stability using UV-Vis spectroscopy, field emission scanning electron microscopy (FESEM), dynamic light scattering (DLS), Raman spectroscopy and X-ray diffraction (XRD). Of the ten fungal strains studied, only strain B422, identified as *C. olivaceum* by phylogenetic analysis of DNA sequence data, successfully synthesized Ag-NPs by reduction of silver nitrate. This was confirmed by a color change to dark brown and a distinct UV-Vis absorption peak at 425 nm. Optimal conditions for biosynthesis were found to be 5 mM silver nitrate, pH 7, 35 °C, no shaking and an incubation period of 96-120 h. FE-SEM analysis revealed nanoparticles ranging in size from 2 to 92 nm, with an average size of 32 to 42 nm, while DLS analysis revealed a hydrodynamic size of 46.3 nm. Zeta potential measurements showed high stability (-23.5 mV). Raman spectroscopy identified functional groups associated with the Ag-NPs and XRD confirmed their crystalline structure. The sustainable synthesis of Ag-NPs using fungal CFE allows precise control of nanoparticle size and morphology, making it highly relevant for biomedical applications and a significant advancement in green nanotechnology for healthcare.
Keywords: Silver nanoparticles, Fungal cell-free extract, *Chaetomium olivaceum*, Nanoparticle synthesis, Green nanotechnology

[1] Corresponding Author

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Introduction

Nanotechnology is a cutting-edge field of research that focuses on the manipulation and control of materials at the nanoscale for various applications. Nanoparticles are highly effective agents in catalysis, healthcare and electronics due to their unique properties such as larger surface area and enhanced mechanical and optical properties (1). Ag-NPs are key factors in biomedicine due to their large surface area, potent antibacterial, anticancer and antioxidant properties, and precise nanoscale engineering potential. They are widely used in drug delivery, medical imaging and biosensor development, with significant potential for advancing new therapeutic strategies and improving public health (2). Ag-NP production methods, including spray pyrolysis, laser ablation, co-precipitation and hydrothermal techniques, have shown success but are limited in biological areas due to the use of harmful chemicals. Microbial synthesis, using organisms such as bacteria, yeasts and filamentous fungi, has emerged as an environmentally friendly alternative that reduces ecological damage while maintaining control over nanoparticle properties (3, 4). Fungal nanobiotechnology has attracted considerable attention for its eco-friendly production of nanoparticles. Fungi, which are considered to be natural nanofactories, can produce various metabolites and proteins that aid in the eco-friendly production of nanoparticles with specific size and shape (5). In comparison to other microorganisms, fungi present notable advantages such as simple cultivation, resistance to high levels of metal ions and the capacity to mass-produce nanoparticles inexpensively. Furthermore, fungi generate proteins and enzymes that convert metal ions into less toxic compounds, which is valuable for nanoparticle production (6). Several genera of filamentous fungi, including *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Sarocladium* and *Trichoderma*, have been used to produce silver, gold and zinc oxide nanoparticles. Among these, *Fusarium* and *Aspergillus* have been the focus of most studies related to silver nanoparticle synthesis (7-9). Recently, in a study of nanoparticle synthesis by some fungal isolates, *S. subulatum* AS4D (= IRAN 5131C), isolated from declined grapevines exhibited significant potential to produce Ag-NPs (9). In this study, we focused on the biosynthesis of Ag-NPs using *C. olivaceum* strain B422 isolated from grapevines showing symptoms of decline. We investigated the influence of the key factors precursor concentration, medium pH, temperature, agitation and incubation time on Ag-NPs synthesis using a cell-free extract (CFE) approach. This research presents a green, safe and eco-friendly method, highlighting the potential of using fungi as valuable biological resources in nanotechnology.

Materials and Methods

Fungal strains and culture conditions

Ten fungal strains collected from grapevine trunks and twigs across various regions of the country, specifically in Kouzaran, Kermanshah Province, were provided for study by the Mycology Laboratory of the Faculty of Agriculture, University of Kurdistan. Fungal strains were purified by single-spore or hyphal-tip methods on PDA. The cultures were then incubated on PDA in the dark at pH 5 for 7 to 14 days at 25 °C.

Biosynthesis of Ag-NPs

Fungal strains synthesized silver nanoparticles in a reaction medium containing silver nitrate (purity >99%) as precursor and fungal CFEs as biocatalyst. CFEs were prepared according to the method described by Mohammadjani *et al.* (9). Fungi were cultured in PDB at 25℃ and 200 rpm for 15 days. After centrifugation, the biomass was resuspended in sterile water and incubated for three days. The extract was then filtered and mixed with 50 ml of silver nitrate solution to a final concentration of 3 mM for nanoparticle synthesis. The mixture was incubated at 25 ℃ and 100 rpm for different time intervals. Controls were performed with silver nitrate or extract only.

Effect of different parameters on the synthesis of Ag-NPs

The optimal conditions for the biosynthesis of silver nanoparticles were determined using a single-factor optimization method as described by Bolbanabad *et al.* (10). In this approach, one synthesis parameter was varied while the others were kept constant. The initial conditions for Ag-NP biosynthesis were shaking speed 100 rpm, temperature 25 ℃ and incubation time 3 day. Surface plasmon resonance was monitored by UV-visible spectroscopy. The study systematically investigated the effects of precursor concentration (1–6 mM), pH (4–9), temperature (25–40 ℃), agitation speed (0–200 rpm), and incubation time (12–168 h) on Ag-NP synthesis. Statistical analysis was performed using ANOVA and Tukey's test with a significance level of 95%, using GraphPad software version 10.

Characterization of fungal-synthesized Ag-NPs

Biosynthesized Ag-NPs were separated by centrifugation at 13000 × g for 30 min. They were then freeze-dried (Alpha 1-2 LDplus, Martin Christ, Germany) and analyzed by a variety of microscopic and spectroscopic techniques. Field emission

scanning electron microscopy (FESEM, TSCAN, MIRA3, Czech Republic) was used to analyze the dimensions and shape of the freeze-dried nanoparticles at magnifications of 20,000 to 30,000. Energy dispersive X-ray (EDX) analysis was performed to determine the elements present by examining images at 500 and 200 nm. The particle size distribution was evaluated using ANIX Emica software. The XRD analysis of the crystalline properties of the Ag-NPs was carried out using a Philips PW1730 system, with angles ranging from 10° to 80°. Raman spectroscopy (UniDRON, Taiwan) was used to detect functional groups, while dynamic light scattering (DLS) using a Malvern instrument was used to assess the average size and distribution of the particles in solution. Zeta potential (Malvern, UK) analysis was used to assess the stability of the nanoparticles.

Molecular characterization of the selected fungi

Genomic DNA was extracted from 7-day-old pure cultures growing on PDA at 25℃ according to the modified method of Raeder and Broda (11) as described by Abdollahzadeh *et al.* (12). The partial gene sequence of β-tubulin (*tub2*) was amplified using the primer pairs Bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC-3′) and Bt2b (5′- ACCCTCAGTGTAGTGACCCTTGGC-3′) (13). The PCR reaction was performed in a final volume of 25 µl consisting of 12.5 µl master mix, 1 µl of each primer (10 µM), 1 µl template DNA (50-100 ng/µl) and 9.5 µl PCR grade water. The PCR reaction was as follows: an initial denaturation step of 3 min at 95 ℃, followed by 35 cycles of 60 s at 94 ℃, 60 s at 52 ℃ and 90 s at 72 ℃, with a final extension of 5 min at 72℃. PCR products were purified and sequenced by BGI (China) via BMG (Bio Magic Gene) Co. (Karaj, Iran). Consensus sequence extraction was performed using BioEdit Sequence Alignment Editor v.7.0.9.0. (14) and submitted to GenBank. The generated sequence was subjected to BLAST analysis and aligned with the sequences of the type or authentic specimens of existing related species using online MAFFT v. 7 (15). The phylogenetic analysis of the aligned sequences was performed by Maximum Parsimony method using PAUP v. 4.0b10 (16) according to Abdollahzadeh *et al.* (17). Phylogenetic trees were plotted using FigTree v. 1.4.3 and edited in Adobe Illustrator CS2 v. 12.0.0.

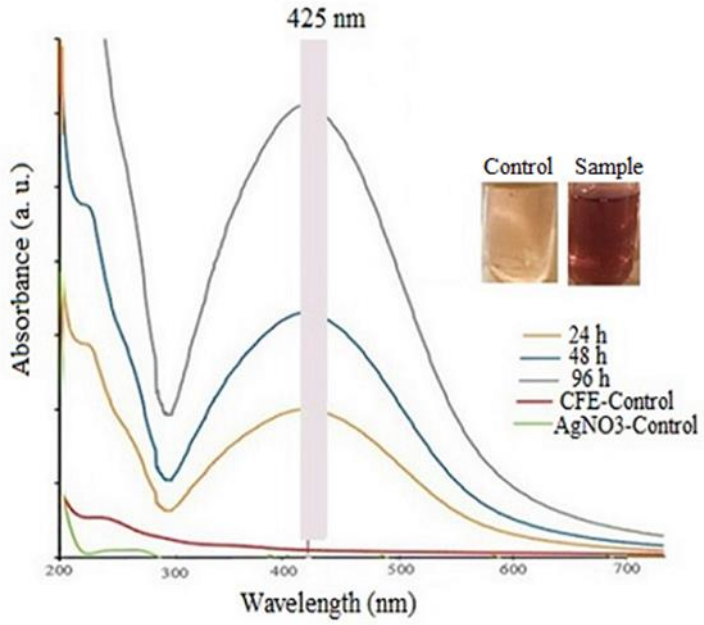

Fig. 1. Visual observations (transition from light to brown) and absorption spectra from spectrophotometric analysis of a 3 mM silver nitrate solution at pH 6.0. Changes were monitored at 24, 48 and 96 h of reaction at 25°C on a rotary shaker (100 rpm) using CFE from fungal strain B422.

Results

Characterization of fungal strains of synthesizing Ag-NPs

Ten selected fungal strains were screened for their ability to synthesize Ag-NPs using a CFE strategy. Among the strains tested, only strain B422 reduced silver nitrate to Ag-NPs at 3 mM (Fig. 1). The untreated silver nitrate solution (control) remained bright yellow in color, whereas the sample treated with fungal CFE turned dark brown, indicating the

formation of nanoparticles. UV-Vis spectrophotometry revealed a distinct absorption band at 425 nm, in the range characteristic of Ag-NPs (400 to 450 nm), attributed to surface plasmon resonance, which is related to the oscillation of free electrons on the nanoparticle surface (18).

A BLAST search of the NCBI database using the generated sequence (PQ202824) showed that our strain was close to members of *Chaetomium*. Based on phylogenetic analysis, strain B422 was identified as *Chaetomium olivaceum* (Fig. 2).

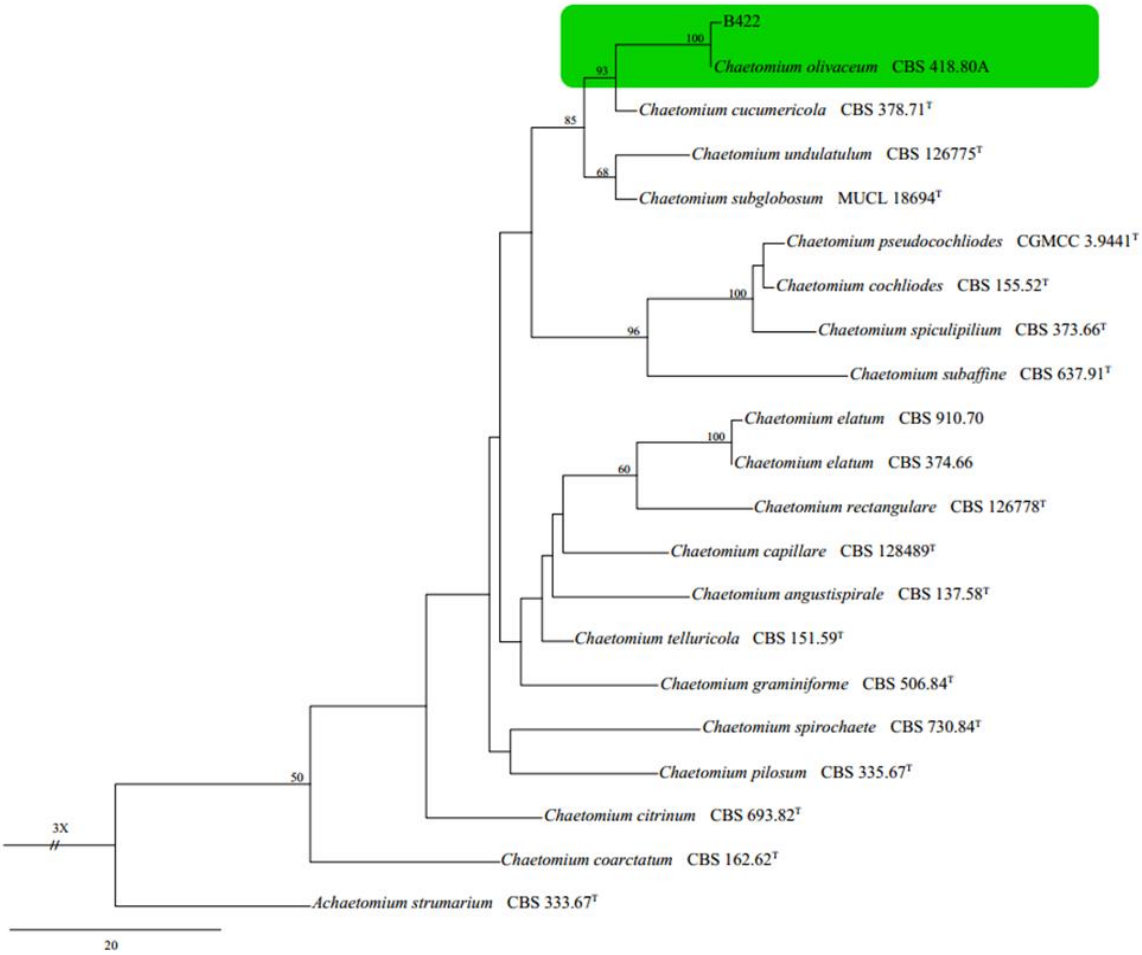

Fig. 2. One of the six most parsimonious trees of the genus *Chaetomium* based on phylogenetic analysis of *tub2* sequence data. Bootstrap values from MP analysis are shown at the nodes. *Achaetomium strumarium* was used as outgroup.

Effect of different parameters on fungal-synthesized Ag-NPs: Fungi are widely used for the preparation of metal nanoparticles due to their high metal tolerance and ease of cultivation. Optimization of parameters such as precursor concentration, temperature, pH and reaction time is crucial for the synthesis of nanoparticles with desirable stability and biocompatibility (19). The concentration of both the precursor and the fungal extract determines the properties of the nanoparticles. Lower precursor concentrations slow down the synthesis, while higher concentrations accelerate it. Therefore, the balance between the fungal extract and the metal salt is crucial for optimal nanoparticle production (Fig. 3) (20). Fig. 3a shows that increasing the silver nitrate concentration from 1 to 5 mM resulted in a significant increase in nanoparticle uptake. This was followed by 72 h of incubation at 25 °C, pH 6 and a shaking speed of 100 rpm. After analyzing the results, 5 mM silver nitrate was selected as the most effective concentration for subsequent tests. The pH of the reaction medium is another key parameter influencing nanoparticle properties. Higher pH levels favour the synthesis of metal nanoparticles by affecting the competition between protons and metal ions for binding to negatively charged groups. As shown in Fig. 3b, the effect of various pH levels (4 to 9) on Ag-NPs synthesis under optimal conditions (5 mM silver nitrate, 72 h at 25 °C and 100 rpm). The highest absorption peak corresponding to nanoparticle biosynthesis was observed at pH 7.

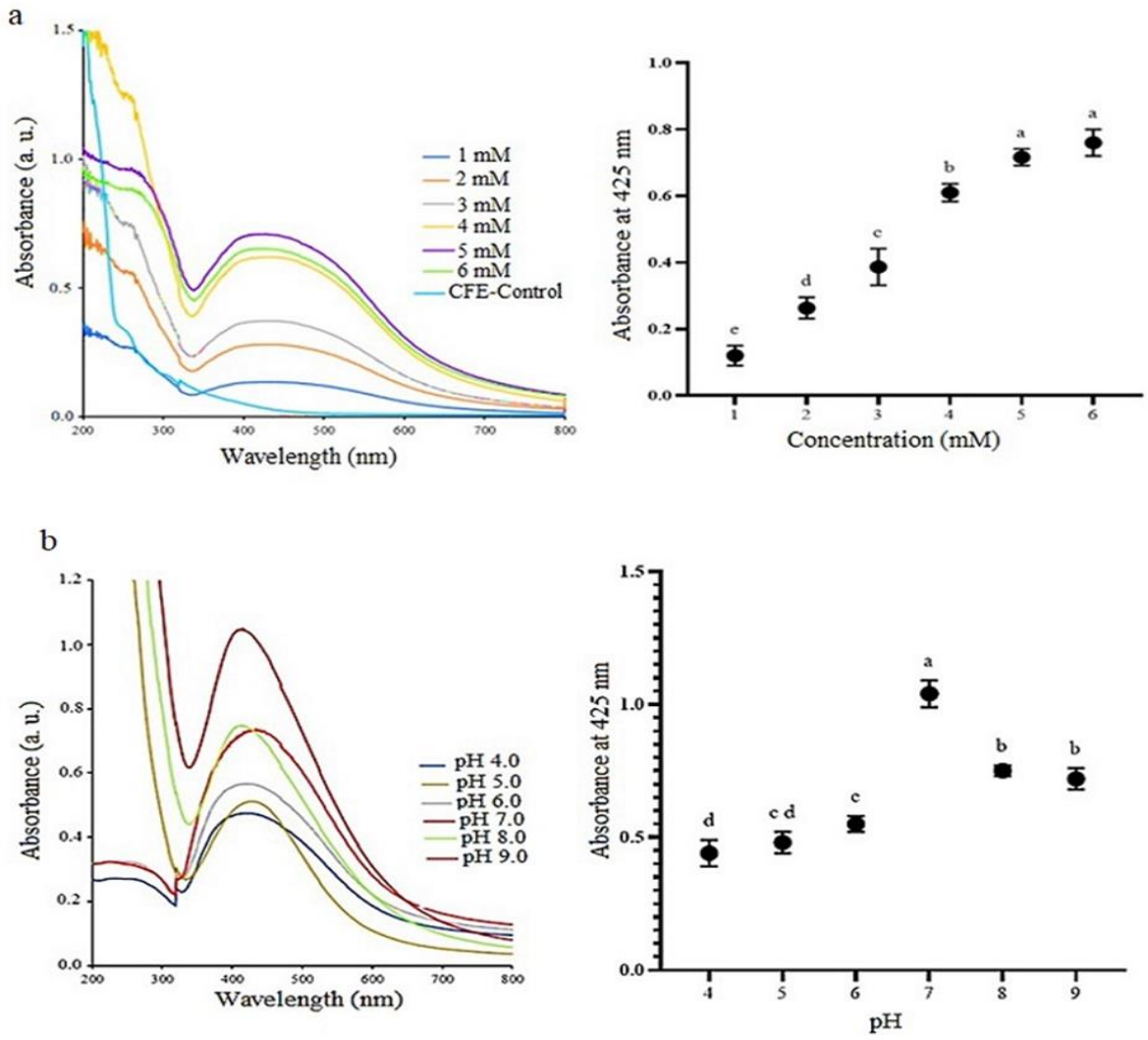

Fig. 3. Effect of (**a**) silver nitrate ($AgNO_3$) precursor concentration and (**b**) pH on the biosynthesis of Ag-NPs using the CFE of fungal strain B422. Statistical analysis was performed using the Tukey method, with different lowercase letters indicating significant differences at the 5% level ($P < 0.05$).

Temperature is crucial for the green production of nanoparticles by fungal strains. It affects the time taken to produce them, as well as their size and stability (Fig. 4). Fig. 4 as hows the effect of different temperatures on the synthesis rate of silver nanoparticles under optimal conditions (5 mM silver nitrate, pH 7) using the fungal strain B422. The maximum absorption occurred at 35 °C, but further increases in temperature reduced the efficiency of silver nitrate bioreduction to nanoparticles. The effect of agitation on the synthesis of Ag-NPs was also examined (Fig. 4b). Maximum absorption was achieved under static conditions without agitation. As the shaker speed increased, the bioreduction efficiency decreased.

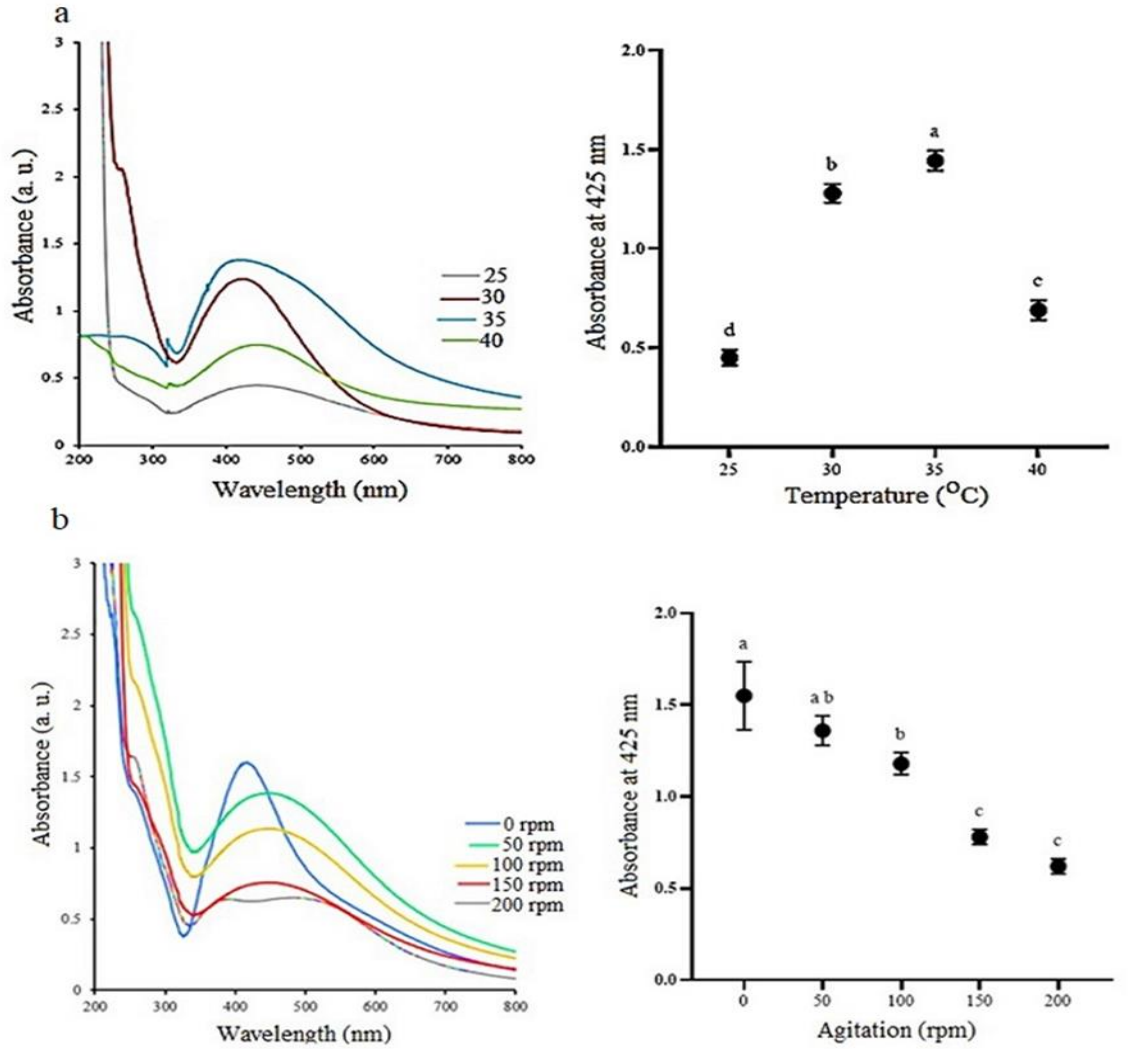

Fig. **4.** Effect of temperature (**a**) and agitation (**b**) on the biosynthesis of Ag-NPs using CFE from fungal strain B422. Statistical analysis was performed using the Tukey method, with different lowercase letters indicating significant differences at the 5% level ($P < 0.05$).

To further improve the silver nitrate reduction efficiency, the effect of incubation time was investigated under optimized conditions (5 mM silver nitrate, pH 7, 35 ℃ and static conditions) (Fig. 5). The absorption peak associated with silver nanoparticles increased consistently from 12 to 96 h, indicating continuous nanoparticle formation. Therefore, the optimal incubation time for Ag-NP biogenesis by the strain was 96 h. Overall, the optimal conditions for the biosynthesis of Ag-NPs by the fungal strain *C. olivaceum* B422 using the CFE strategy were determined to be a 5 mM concentration of $AgNO_3$, a temperature of 35 ℃, pH 7, no agitation (0 rpm) and an incubation time of 96 h.

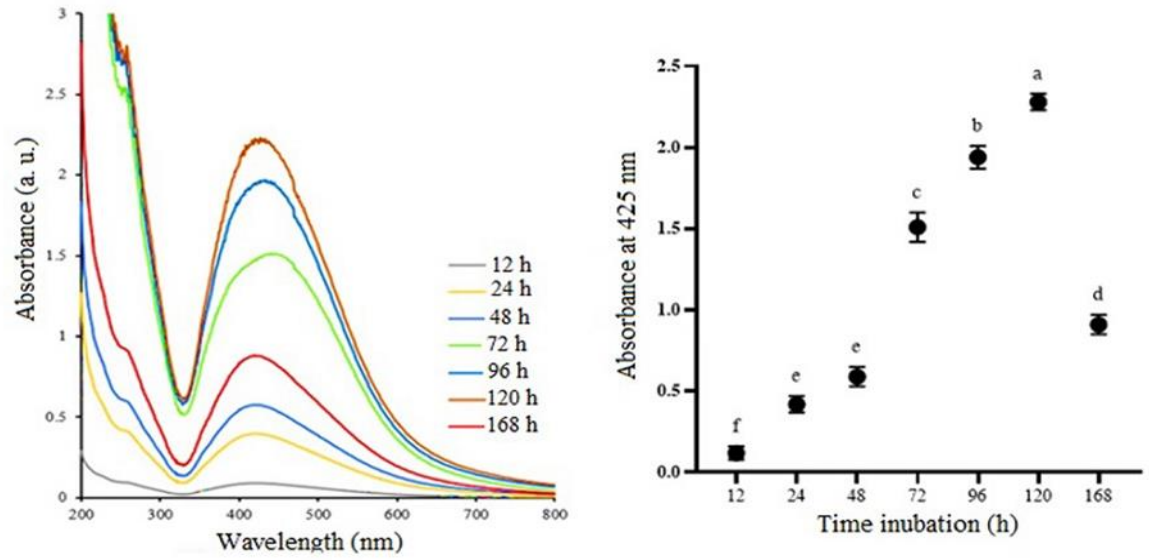

Fig. **5.** Effect of incubation time on the biosynthesis of Ag-NPs using CFE from fungal strain B422. Statistical analysis was performed using the Tukey method, with different lowercase letters indicating significant differences at the 5% level ($P < 0.05$).

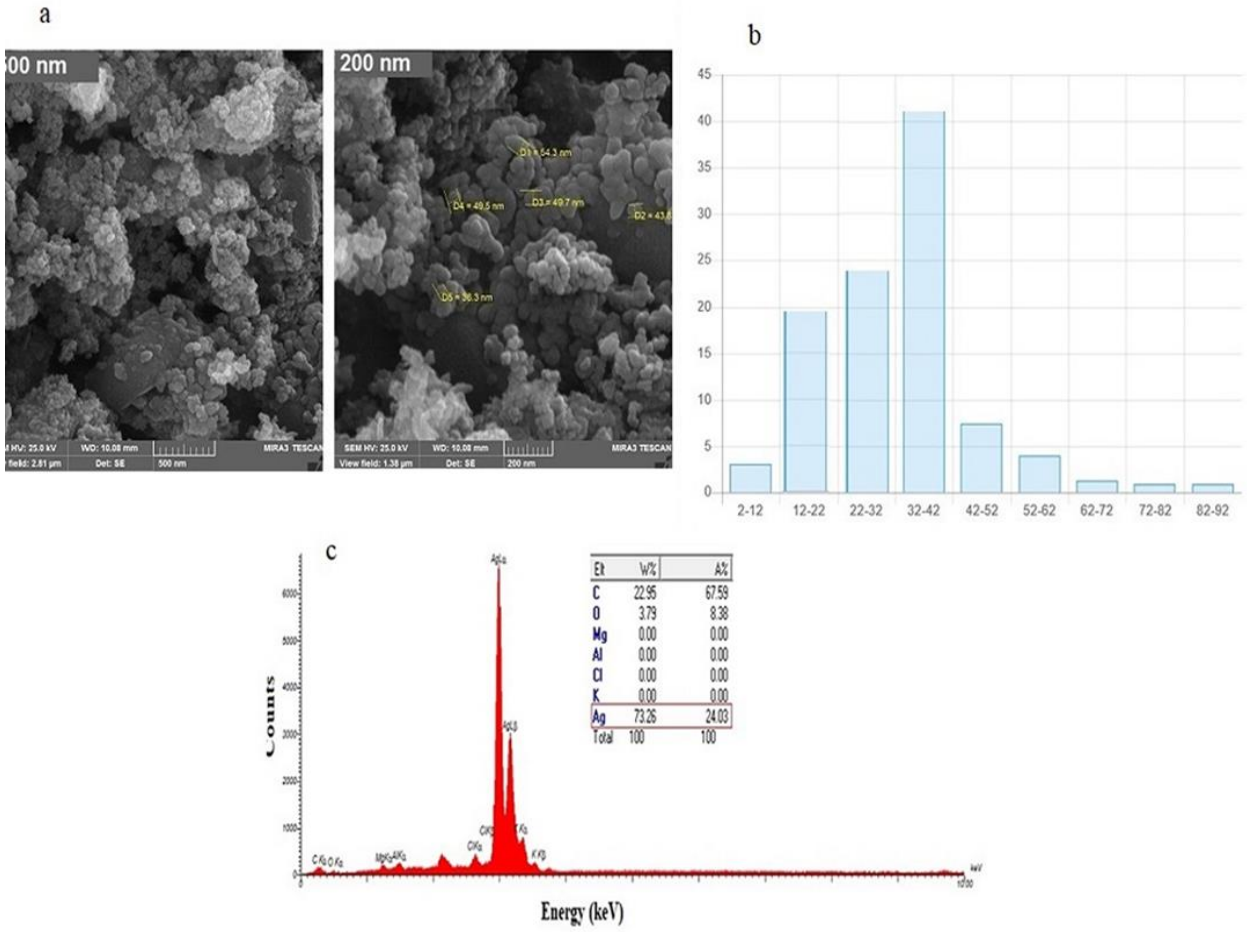

Fig. **6.** FESEM images (**a**), particle size distribution histogram (**b**) and EDX analysis (**c**) of Ag-NPs synthesized under optimized conditions using CFE from fungal strain B422.

Characterization of Fungal-Synthesized Ag-NPs

The SEM images (Fig. 6a and b) showed that the Ag-NPs were predominantly spherical or irregular in shape, with most sizes in the range of 32-42 nm. The nanoparticles often formed agglomerates, probably due to factors such as temperature, incubation time or the composition of the reaction solution. These clusters may be beneficial for applications requiring a large surface area or increased reactivity. The EDX spectrum reveals the chemical composition of silver nanoparticles synthesized by a fungus (Fig. 6c). Prominent silver peaks (Ag Lα at 2.98 keV and Ag Lβ at 3.15 keV) confirm the successful synthesis of the nanoparticles. The presence of carbon and oxygen peaks is probably due to capping or reducing agents used during synthesis. Silver accounts for 73.26% by weight and 24.03% by atomic percentage. Carbon and oxygen are indicative of biological coatings or surface oxidation, potentially enhancing the stability of the nanoparticles and their suitability for biotechnological and industrial applications.

Fig. 7 shows the DLS analysis and Zeta potential measurements. The average hydrodynamic diameter of the synthesized silver nanoparticles was 46.3 nm, in agreement with the size estimates obtained by electron microscopy (Fig. 7a). The zeta potential was measured to be -23.5 mV, indicating the high physical stability of the nanoparticle colloids (Fig. 7b). This stability suggests that the capping or reducing agents were effective in preventing nanoparticle aggregation and maintaining their integrity, which is critical for biological and industrial applications (21).

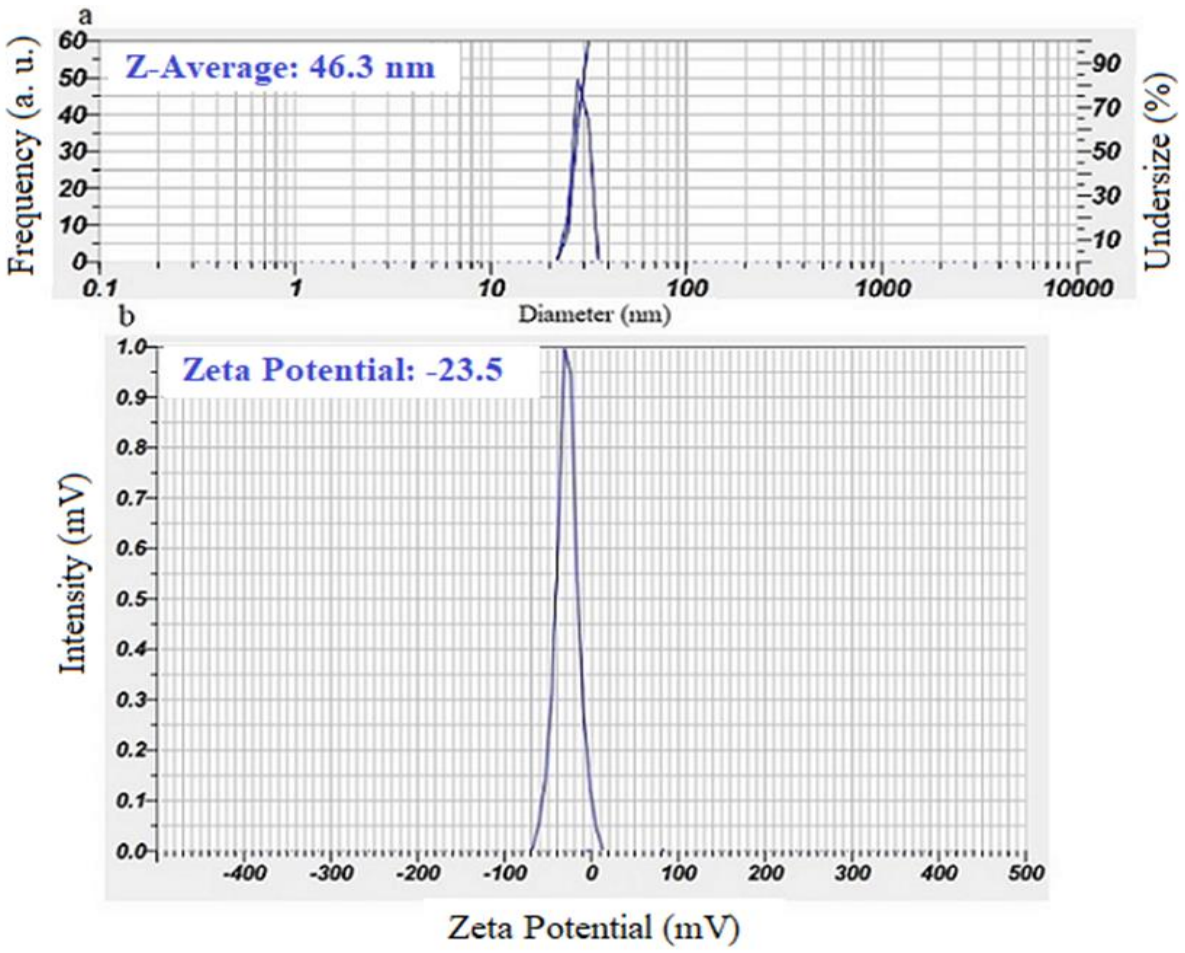

Fig. 7. DLS analysis (**a**) and zeta potential analysis (**b**) of Ag-NPs synthesized under optimal conditions using CFE from fungal strain B422.

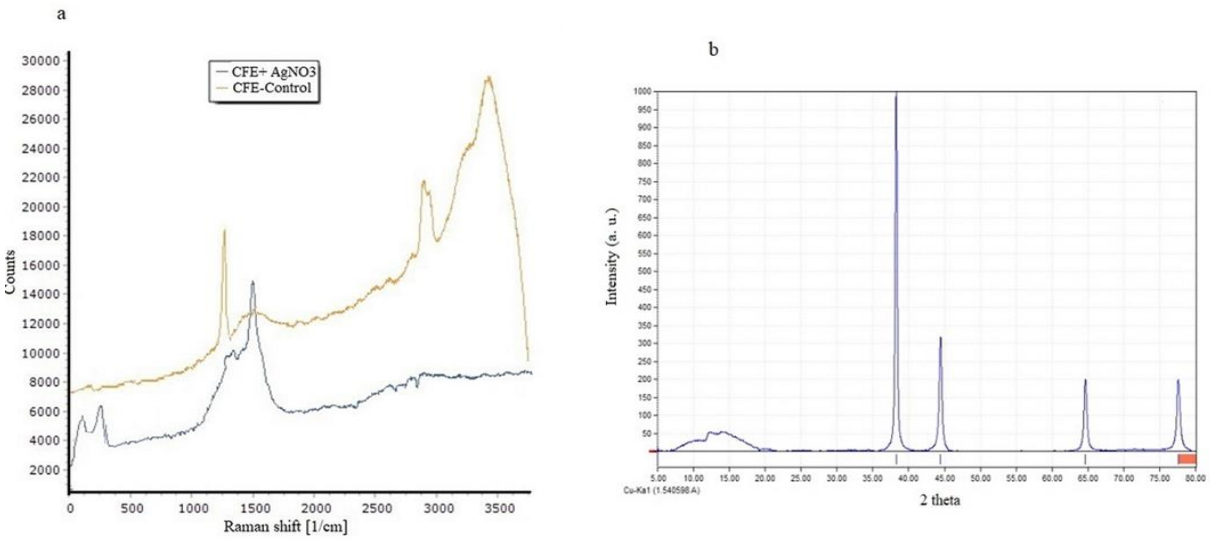

Figure 8. Raman analysis (**a**) and XRD analysis (**b**) of Ag-NPs synthesized under optimal conditions using CFE from fungal strain B422.

As shown in Fig. 8a, Raman spectroscopy analysis of Ag-NPs synthesized with fungal extract compared to a control medium (CFE not treated with silver nitrate). The Raman spectrum of Ag-NPs shows significant changes in peak intensity and position compared to the control sample. A prominent peak at around 1500 cm^{-1} is attributed to vibrational modes of functional groups associated with Ag-NPs. Additional Raman peaks in the 100 to 200 cm^{-1} region are associated with metal-metal (Ag-Ag) or metal-oxygen (Ag-O) vibrations, indicating lattice vibrations of metal nanoparticles. The overall reduction in peak intensity in the Ag-NPs spectrum is probably due to Raman signal quenching caused by nanoparticle formation. In contrast, the control spectrum shows a wide variety of peaks. This indicates the presence of different organic compounds or biomolecules in the fungal extract. The intense peaks found in the 2900-3500 cm^{-1} range are associated with C-H stretching vibrations, a common feature of organic molecules. The 1200 cm^{-1} peak could be related to C-H bending or C-N stretching vibrations, which are common in biomolecules such as proteins and amino acids in the fungal extract. The changes in the Raman peaks confirm the effective formation of Ag-NPs and their association with biomolecules in the extract (22). X-ray diffraction (XRD) analysis further confirmed the crystalline nature of the synthesized Ag-NPs. As shown in the diffraction pattern in **Error! Reference source not found.**b, distinct peaks were observed at angles of 38.26°, 44.44°, 64.57° and 77.52°, corresponding to the (111), (200), (220) and (311) planes of the Ag-NPs crystals, respectively. These peaks are characteristic of the crystalline structure of Ag-NPs and correspond to standard reference patterns for Ag-NPs (23).

Discussion and Conclusion

The biological synthesis of Ag-NPs using fungi has attracted considerable attention due to the ability of fungi to degrade organic matter and use various enzymes and molecules to reduce silver ions. Enzymes such as protease, cellulase, chitinase and glucosidase actively accelerate and enhance Ag-NP synthesis. Additionally, enzymes like NADH and NADH-dependent nitrate reductase effectively reduce Ag^{+} ions to Ag^{0} in fungal systems, facilitating nanoparticle formation (24). Ag-NPs can be synthesized intracellularly or extracellularly, the latter being preferred due to its practicality and efficiency. In our study, we used the extracellular method with CFE, which uses compounds secreted by microorganisms as reducing agents, eliminating the need for direct involvement of microbial cells. After separating the culture medium from the biomass, the supernatant, which is rich in biological molecules, is used for nanoparticle synthesis. This approach simplifies the process by avoiding the complexities of maintaining microbial cells, saving time and resources. The use of CFE also improves the scalability of nanoparticle production and enables rapid synthesis initiation, as nanoparticle formation begins immediately upon the addition of silver salts. This leads to faster production, which is critical for applications in medicine and biotechnology. In addition, CFE provides better control over synthesis conditions, optimizing nanoparticle characteristics for consistent and desirable products (10, 25). Filamentous fungi are

especially valued for their ability to produce silver nanoparticles with desirable morphology, high stability and broad applicability. The use of fungi as reducing and stabilizing agents in the biogenic synthesis of Ag-NPs offers several advantages, including high protein production, ease of handling and low-toxicity residues. The nanoparticles are coated with biomolecules from the fungi, which increases their stability and potentially provides additional biological activity (26, 27). Recent studies have highlighted the significant potential of fungal-mediated biogenic synthesis of Ag-NPs for healthcare and agriculture, offering numerous advantages. By selecting specific fungal species and adjusting synthesis conditions such as precursor concentration, temperature, pH and incubation time, nanoparticles with diverse physicochemical properties can be produced. This method surpasses plant and bacterial synthesis in achieving optimal culture conditions and scalability, resulting in nanoparticles of desired shape and size. Unlike chemical methods, it is non-toxic, cost-effective and sustainable. However, challenges remain in selecting suitable fungi, maintaining sterile conditions and optimizing synthesis parameters to ensure monodispersity, stability and biocompatibility (28, 29). In the current study, the first step was to screen for a competent fungal strain capable of producing Ag-NPs. The selected strain B422 was obtained from *Vitis vinifera* trunks and twigs and identified as *C. olivaceum* based on phylogenetic analysis of a partial sequence of the *β-tubulin* gene (*tub2*). The *β-tubulin* gene is one of the main DNA barcodes used to identify fungal species in phylogenetic studies. Our study is the first to report the green, extracellular synthesis of Ag-NPs by *Chaetomium* members, which are known to produce industrial enzymes and valuable metabolites with antifungal and antibacterial properties (30, 31). The research aimed to optimize the synthesis of silver nanoparticles by investigating various parameters to maximize production while ensuring proper dispersion and morphology. A concentration of 5 mM $AgNO_3$ was found to be optimal, providing a balance between nanoparticle yield, size, morphology and stability. While previous studies have often used 1 mM $AgNO_3$ for fungal synthesis (32, 33), our results are consistent with research showing that different concentrations produce different results (9, 10, 34, 35). This highlights the importance of selecting an optimal $AgNO_3$ concentration to balance synthesis efficiency and desired nanoparticle characteristics, while avoiding problems associated with excess silver (5, 24). The pH of the synthesis medium is critical for optimizing nanoparticle morphology, size and stability. Research on fungi like *Epicoccum nigrum* (36) and *Fusarium oxysporum* (35) indicates that alkaline pH levels optimize synthesis by reducing competition between protons and metal ions. In contrast, excessive pH can inhibit enzyme activity. We determined pH 7 to be optimal, confirming findings that a neutral to slightly alkaline pH range is most effective (9, 37). Temperature is crucial for determining nanoparticle size and stability. Higher temperatures accelerate reactions, and produce larger nanoparticles, but excessively high temperatures can denature proteins, leading to aggregation and reduced stability. For example, *T. harzianum* thrives at around 40 °C, while *F. oxysporum* performs best between 60 °C and 80 °C (5). In this study, 35 °C was found to be optimal for Ag-NP synthesis using a cell-free extract, highlighting the need for precise temperature control to ensure high quality nanoparticle production. Agitation plays a crucial role in nanoparticle formation. For example, in a study using *Isaria fumosorosea*, an agitation speed of 150 rpm was found to enhance nanoparticle synthesis (38). However, in the present study, static conditions with minimal agitation resulted in maximum absorption and more efficient bioreduction of silver nitrate, indicating that limited agitation may be more favorable for controlled nanoparticle formation. Excessive agitation can hinder nanoparticle formation by disrupting the biological and physical processes required to reduce silver ions into nanoparticles. In contrast, static conditions promote stable formation and uniform distribution of nanoparticles, a key factor in biological synthesis methods. This finding aligns with previous studies, such as in *T. longibrachiatum*, where the absence of agitation improved the bioreduction process (39). The incubation period for Ag-NP synthesis was optimized using 5 mM $AgNO_3$, pH 7 and a temperature of 35 ℃ under static conditions. The absorption peak increased steadily between 12 and 96 h, stabilizing afterward, indicating that nanoparticle formation was most active during this period. Prolonged incubation led to larger nanoparticles, as indicated by a red shift in the absorption peak. The synthesized Ag-NPs were predominantly spherical or irregular in shape, with sizes ranging from 36.5 to 64.3 nm, the majority between 32 and 42 nm. This size uniformity is typical of biologically synthesized nanoparticles, where capping agents from the fungal extract stabilize the particles and prevent aggregation. This stabilization is critical to maintaining the dispersion of the nanoparticles, thereby enhancing their functionality for biomedical and environmental applications. The controlled size and shape of the nanoparticles are essential in determining their reactivity and stability, underlining the success of the optimization process in producing stable

nanoparticles with desirable properties for practical use. The EDX spectrum confirmed the successful synthesis of Ag-NPs, showing strong silver signals alongside carbon (C) and oxygen (O), probably from organic capping agents. The absence of significant impurities highlights the purity and efficiency of the synthesis process. DLS measurements indicated a hydrodynamic diameter of 46.3 nm with a zeta potential of -23.5 mV, suggesting high physical stability. Raman spectroscopy and XRD further validated the crystalline structure and interaction of the Ag-NPs with biomolecules from the fungal extract. Taken together, the SEM, EDX, DLS, Raman and XRD results show that the Ag-NPs produced have controlled size, morphology and stability, making them well suited for biomedical applications.

This study highlights the potential of *C. olivaceum* strain B422 for the green synthesis of biocompatible silver nanoparticles (Ag-NPs) suitable for medical and biotechnological applications. By optimizing key factors such as $AgNO_3$ concentration, pH, temperature and incubation time, we successfully produced Ag-NPs with controlled characteristics. This sustainable synthesis method offers a scalable approach to the production of nanoparticles for drug delivery, antimicrobial coatings and diagnostic tools. The research confirms the biocompatibility of the synthesized Ag-NPs and highlights the importance of exploring fungal strains for nanoparticle production. Future investigations should aim to elucidate the mechanisms of extracellular nanoparticle synthesis in order to expand their applications.

Acknowledgments
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Declaration of interest statement
Authors declare no conflict of interest.

References

(1) Ramezani Farani M., Farsadrooh M., Zare I., Gholami A., Akhavan O. Green synthesis of magnesium oxide nanoparticles and nanocomposites for photocatalytic antimicrobial, antibiofilm and antifungal applications. *Catalysts* 2023; 13(4): 642. https://doi.org/10.3390/catal13040642

(2) Meher A., Tandi A., Moharana S., Chakroborty S., Sovalin S., Mondal A., Dey S., Chandra P. Silver nanoparticle for biomedical applications: A review. *Hybrid Advances* 2024; 6: 100184. https://doi.org/10.1016/j.hybadv.2024.100184

(3) Zhang XF., Liu ZG., Shen W., Gurunathan S. Silver nanoparticles: synthesis, characterization, properties, applications, and therapeutic approaches. *Int J Mol Sci* 2016; 17(9): 1534. https://doi.org/10.3390/ijms17091534

(4) Alavi M., Ashengroph M. Mycosynthesis of AgNPs: Mechanisms of nanoparticle formation and antimicrobial activities. *Expert Rev Anti Infect Ther* 2023; 21(4): 355-363. https://doi.org/10.1080/14787210.2023.2179988

(5) Guilger-Casagrande M., Lima R. Synthesis of silver nanoparticles mediated by fungi: A Review. Front Bioeng Biotechnol 2019; 7: 287. https://doi.org/10.3389/fbioe.2019.00287

(6) Zambonino MC., Quizhpe EM., Jaramillo FE., Rahman A., Santiago Vispo N., Jeffryes C., Dahoumane SA. Green synthesis of selenium and tellurium nanoparticles: current trends, biological properties and biomedical applications. *Int J Mol Sci* 2021; 22: 989. https://doi.org/10.3390/ijms22030989

(7) Lateef Agbaje., Evariste Bosco GK., Nandita D., Shivendu R. *Microbial nanobiotechnology: principles and applications*. Springer Nature. 2021.

(8) Srivastava S., Bhargava A. *Green nanoparticles: the future of nanobiotechnology*. Springer. 2022.

(9) Mohammadjani N., Ashengroph M., Abdollahzadeh J. Untargeted metabolomics and molecular docking studies on green silver nanoparticles synthesized by *Sarocladium subulatum*: Exploring antibacterial and antioxidant properties. *Chemosphere* 2024; 355: 141836. https://doi.org/10.1016/j.chemosphere.2024.141836

(10) Bolbanabad EM., Ashengroph M., Darvishi F. Development and evaluation of different strategies for the clean synthesis of silver nanoparticles using *Yarrowia lipolytica* and their antibacterial activity. *Process Biochem* 2020; 94: 319-328. https://doi.org/10.1016/j.procbio.2020.03.024

(11) Raeder U., Broda P. Rapid preparation of DNA from filamentous fungi. *Appl Microbiol* 1985; 1: 17–20. https://doi.org/10.1111/j.1472-765X.1985.tb01479.x

(12) Abdollahzadeh J., Mohammadi Goltapeh E., Javadi A., Shams-bakhsh M., Zare R., Phillips AJL. *Barriopsis iraniana* and *Phaeobotryon cupressi*: two new species of the *Botryosphaeriaceae* from trees in Iran. *Persoonia* 2009; 23: 1-8. https://doi.org/10.3767/003158509X467552

(13) Glass NL., Donaldson GC. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes.

Appl Environ Microbiol 1995; 61: 1323-1330. https://doi.org/10.1128/aem.61.4.1323-1330.1995

(14) Hall TA. *BioEdit. Version 7.0.0*. Department of Microbiology (United States: North Carolina State University). 2004. Available online: http://www.mbio.ncsu.edu/bioedit/bioedit.html

(15) Katoh K., Rozewicki J., Yamada KD. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief. Bioinform* 2019; 20: 1160–1166. https://doi.org/10.1093/bib/bbx108

(16) Swofford DL. *PAUP*: phylogenetic analysis using parsimony* (*and other methods) (Sunderland, MA, USA: Sinauer Associates). 2003. http://dx.doi.org/10.1111/j.0014-3820.2002.tb00191.x

(17) Abdollahzadeh J., Zare R., Phillips AJL. Phylogeny and taxonomy of *Botryosphaeria* and *Neofusicoccum* species in Iran, with description of *Botryosphaeria scharifii* sp. nov. *Mycologia* 2013; 105: 210–220. https://doi.org/10.3852/12-107

(18) Majumdar R., Kar PK. Biosynthesis, characterization and anthelmintic activity of silver nanoparticles of *Clerodendrum infortunatum* isolate. *Sci Rep* 2023; 13(1): 7415. https://doi.org/10.1038/s41598-023-34221-9

(19) Rai M., Golinska P. *Mycosynthesis of nanomaterials: Perspectives and challenges*. 2023. http://dx.doi.org/10.1201/9781003327387

(20) Santos TS., Eliana MDP., Matheus GDJS., Eliana BS., Patrícia S., Marcelo DCM. Entomopathogenic fungi biomass production and extracellular biosynthesis of silver nanoparticles for bioinsecticide action. *Appl Sci* 2021; 11(6): 2465. https://doi.org/10.3390/app11062465

(21) Gharari Z., Hanachi P., Sadeghinia H., Walker TR. Eco-friendly green synthesis and characterization of silver nanoparticles by *Scutellaria multicaulis* leaf extract and its biological activities. *Pharmaceuticals* 2023; 16(7): 992. https://doi.org/10.3390/ph16070992

(22) Franceschinis G., Beverina M., Corleto M., Sosa AM., Lillo C., Casará LA., Alonso SV., Maffia P., Montanari J., Tuttolomondo ME., Calienni MN. Green-synthesized silver nanoparticles using *Aloe maculata* extract as antibacterial agent for potential topical application. *OpenNano* 2023; 12: 100148. https://doi.org/10.1016/j.onano.2023.100148

(23) Lotfy WA., Alkersh BM., Sabry SA., Ghozlan HA. Biosynthesis of silver nanoparticles by *Aspergillus terreus*: characterization, optimization, and biological activities. *Front Bioeng Biotechnol* 2021; 9: 633468. https://doi.org/10.3389/fbioe.2021.633468

(24) Nguyen NPU., Dang NT., Doan L., Nguyen TTH. Synthesis of silver nanoparticles: From conventional to 'modern' methods—A Review. *Processes* 2023: 11:2617. https://doi.org/10.3390/pr11092617

(25) Ashengroph M., Khaledi A., Bolbanabad EM. Extracellular biosynthesis of cadmium sulphide quantum dot using cell-free extract of *Pseudomonas chlororaphis* CHR05 and its antibacterial activity. *Process Biochemistry* 2020; 89 :63-70. https://doi.org/10.1016/j.procbio.2019.10.028

(26) Adebayo EA., Azeez MA., Alao MB., Oke AM., Aina DA. Fungi as veritable tool in current advances in nanobiotechnology. *Heliyon* 2021; 7: e08480. https://doi.org/10.1016/j.heliyon.2021.e08480

(27) Osman AI., Zhang Y., Farghali M. *et al.* Synthesis of green nanoparticles for energy, biomedical, environmental, agricultural, and food applications: A review. *Environ Chem Lett* 2024; 22: 841–887. https://doi.org/10.1007/s10311-023-01682-3

(28) Balakumaran MD., Ramachandran R., Kalaicheilvan PT. Exploitation of endophytic fungus, *Guignardia mangiferae* for extracellular synthesis of silver nanoparticles and their in vitro biological activities. *Microbiol Res* 2015; 178: 9–17. https://doi.org/10.1016/j.micres.2015.05.009

(29) Rami MR., Meskini M., Sharafabad BE. Fungal-mediated nanoparticles for industrial applications: synthesis and mechanism of action. *Journal of Infection and Public Health* 2024; 102536. https://doi.org/10.1016/j.jiph.2024.102536

(30) Abdel-Azeem AM. Biodiversity and Industrial Applications of Genus *Chaetomium*. In: Abdel-Azeem AM, Yadav AN, Yadav N, Usmani Z. (eds) *Industrially Important Fungi for Sustainable Development. Fungal Biology*. Springer, Cham. 2021. http://dx.doi.org/10.1007/978-3-030-67561-5_5

(31) Kedves O., Kocsubé S., Bata T., Andersson MA., Salo JM., Mikkola R., Salonen H., Szűcs A., Kedves A., Kónya Z. et al. (2021) *Chaetomium* and *Chaetomium*-like Species from European Indoor Environments Include *Dichotomopilus finlandicus* sp. nov. *Pathogens* 2021; 10(9): 1133. https://doi.org/10.3390/pathogens10091133

(32) Du L., Xu Q., Huang M., Xian L., Feng JX. Synthesis of small silver nanoparticles under light radiation by fungus *Penicillium oxalicum* and its application for the catalytic reduction of methylene blue. *Mater Chem Phys* 2015; 160: 40–47. https://doi.org/10.1016/j.matchemphys.2015.04.003

(33) Phanjom P., Ahmed G. Effect of different physicochemical conditions on the synthesis of silver nanoparticles using fungal cell filtrate of *Aspergillus oryzae* (MTCC No. 1846) and their antibacterial effects. *Adv Nat Sci Nanosci Nanotechnol* 2017; 8: 1–13. http://dx.doi.org/10.1088/2043-6254/aa92bc

(34) Korbekandi H., Ashari Z., Iravani S., Abbasi S. Optimization of biological synthesis of silver nanoparticles using *Fusarium oxysporum*. *Iran J Pharm Res* 2013; 12: 289–298. https://doi.org/10.22037/ijpr.2013.1329

(35) Saxena J., Sharma PK., Sharma MM., Singh A. Process optimization for green synthesis of silver nanoparticles by *Sclerotinia sclerotiorum* MTCC 8785 and evaluation of its antibacterial properties. *Springerplus* 2016; 5: 861. https://doi.org/10.1186/s40064-016-2558-x

(36) Qian Y., Yu H., He D., Yang H., Wang W., Wan X., et al. Biosynthesis of silver nanoparticles by the endophytic fungus *Epicoccum nigrum* and their activity against pathogenic fungi. *Bioprocess Biosyst Eng* 2013; 36: 1613–1619. https://doi.org/10.1007/s00449-013-0937-z

(37) Liaqat N., Jahan N., Khalil-Ur-Rahman, Anwar T., Qureshi H. Green synthesized silver nanoparticles: Optimization, characterization, antimicrobial activity, and cytotoxicity study by hemolysis assay. *Front Chem* 2022; 10: 952006. https://doi.org/10.3389/fchem.2022.952006

(38) Banu AN., Balasubramanian C. Mycosynthesis of silver nanoparticles using *Beauveria bassiana* against dengue vector, *Aedes aegypti* (Diptera: Culicidae). *Parasitol Res* 2014; 113: 2869–77. https://doi.org/10.1007/s00436-014-3948-z

(39) Elamawi RM., Al-Harbi RE., Hendi AA. Biosynthesis and characterization of silver nanoparticles using *Trichoderma longibrachiatum* and their effect on phytopathogenic fungi. *Egypt J Biol Pest Control* 2018; 28: 28. https://doi.org/10.1186/s41938-018-0028-1