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RESEARCH ARTICLE

Fungal synthesis of zinc oxide nanoparticles using *Aspergillus niger* for sustainable nanomaterial production and biological activity

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Abstract

This study investigates the production of zinc oxide nanoparticles (ZnO NPs) using *Aspergillus niger* culture filtrates as a sustainable and environmentally friendly approach, combining them with a zinc carbonate solution. The produced ZnO nanoparticles were examined using transmission electron microscopy (TEM), energy dispersive X-ray diffraction (EDX), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FT-IR). The characterization data validated the creation of highly crystalline ZnO NPs with an average size range from 27 to 40 nm. ZnO NPs effect on *A. ochraceus* and *A. niger* growth at ideal temperatures was investigated. At doses of 0.25%, 0.5%, and 1%, respectively, *Aspergillus niger* and A. ochraceus caused 56%, 81%, and 87% and 64%, 71%, and 86% of the inhibition of fungal growth, respectively. At the highest ZnO NPs concentration, the maximum inhibition rate was observed. This research highlights the potential of *Aspergillus niger* as a bio-factory to produce ZnO nanoparticles with promising applications in agriculture and other fields. The eco-friendly synthesis method, coupled with the antifungal properties of the synthesized ZnO nanoparticles, provides a sustainable and environmentally friendly alternative to conventional fungicides for plant disease management.

Keywords: *Aspergillus niger*; Zinc oxide NPs; Green synthesis; antifungal activity; sustainable agriculture.

1. Introduction

Microorganisms, such as bacteria, fungi, and algae, have been found to be useful bio-agents for the synthesis of nanoparticles in recent years. These bio-agents have advantages like being economical and highly efficient in reducing metal ions to form stable nanoparticles (Kato *et al.*,2020). Additionally, these bio-factories are interesting because they function as efficient and environmentally friendly metal ion- reducing agents (Pillai *et al.*,2020). Since its wide range of benefits, the field of nanotechnology has seen remarkable advancements, especially in the synthesizing nanoparticles with sizes below 100 nm. These nanoparticles, or NPs, differ from the particles from which they are composed in terms of their physical and chemical properties, which makes them valuable for a variety of applications and promising avenues for innovation across diverse fields, including medicine, biology, agriculture, environment, and industry (Sivasankarapillai *et al.*,2019; Sosna *et al.*,2020). There are several ways to generate nanoparticles, including chemical, biological, and physical processes. Among them, using inexpensive, non-toxic chemicals and being environmentally friendly are just a few benefits that biological

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synthesis has over chemical approaches (Sabir *et al.*,2014; Raliya and Tarafdar, 2012; Yedurkar *et al.,* 2016). These techniques are applied to the synthesizing various metal nanoparticles, including zinc, gold, and silver (Clarance *et al.*,2020; Feroze *et al.*,2020).

ZnO NPS have gained attention for their biocompatibility, their antibacterial qualities and photocatalytic capabilities render them advantageous in domains including chemical sensors, solar cells, and photocatalysis (Lee *et al.*, 2016; Xie *et al.*,2018; Grasland *et al.*,2019; Ahmoum *et al.*,2019). Fungi are a type of microorganism that has attracted a lot of attention due to their potential in NP synthesis. They are crucial in the intra- or extracellular reduction of metals (Pillai *et al.,* 2020). Compared to other microorganisms, fungi have several advantages, such as a high capacity for wall binding, an increased ability to absorb metals intracellularly, a high secretion of proteins, enzymes, and metabolites, a high growth rate, ease of handling in large-scale production, and low production procedure costs (Farrag *et al.,* 2020). being used, thus, to create stable, tiny ZnO-NPs (Pillai *et al.,* 2020). Alamdari *et al.,* (2020) claim that the extract's functional groups transfer electrons, turning Zn+2 into Zn+. The hydroxyl and phenolic compounds in the leaf extracts are responsible for the generation of NPs by changing Zn+2 into Zn+ ions (Khan *et al.,* 2019). Ezealisiji *et al.,* (2019) reported similar results, stating that zinc nitrate was ionized in an aqueous solution to generate Zn+2, which was subsequently reduced to Zn+ by a phytochemical present in the extract. The hydroxyl group found in polyphenols may change into zinc hydroxide during hydrolysis (Basnet *et al.,* 2018). Compared to bacterial cells, the fungus exhibits superior capability for secreting a higher concentration of metabolites into the media culture; so, it is preferable to green synthesis. Additionally, fungus cells appear more resilient to changes in process variables like pressure, flow rate, and stirring, which increases their potential application in large-scale synthesis (Li *et al.,* 2012). Consequently, it is more advantageous for fungus to synthesize high-yield, inexpensive, non-toxic, and environmentally benign nanoparticles. The fungi that are frequently found in a variety of situations include the *Aspergillus* genus. Meanwhile, only a small number of Aspergillus species have a substantial effect on animal or human health (Paulussen *et al.,* 2017; Zmeili and Soubani 2007). According to studies (Ansari *et al.*,2014; Ramesh *et al.*,2014), ZnO NPs have a strong antifungal effect on A. niger. The nanoparticles efficiently stop the fungal pathogen's growth and development. ZnO NPs' antifungal action against *A. niger* includes rupturing the fungal cell wall and membrane, producing reactive oxygen species (ROS), and interfering with cellular functions, all of which eventually cause the fungal cells to die (Parveen 2023; Tran and Webster 2011). Because of their larger surface area, ZnO NPs have better antifungal efficacy than bulk zinc oxide because interact more effectively with fungal cells (Parveen 2023; Ansari *et al.*,2014). ZnO NPs are a possible substitute for conventional fungicides in the context of sustainable plant disease management because of their special qualities, which include their antifungal action, targeted delivery, and decreased environmental effect (Sharma *et al.*,2023; Jamdagni *et al.*,2018).

The study demonstrates how *Aspergillus niger* can produce zinc oxide nanoparticles (ZnO NPs), which have exciting potential for use in agriculture. For the treatment of plant diseases, the environmentally friendly synthesis process and the antifungal qualities of ZnO NPs offer a viable and sustainable substitute to traditional fungicides.

2. Materials and methods

The lab for this experiment was located in the Faculty of Agriculture, South Valley University, Qena, at the Agricultural Botany Department.

2.1. Isolation and preparations Aspergillus niger

There were multiple processes involved in producing pure cultures of the fungi linked to sick onion bulbs. Initially, the onion sections that were impacted were sliced into little pieces. After that, these parts were disinfected by submerging them for a minute in a 2% mercuric chloride solution. After sterilization, the pieces were dried between two filter sheets and carefully cleaned with distilled water. Next, the sterilized pieces were put straight onto Petri dishes that were filled with Potato Dextrose Agar (PDA) medium, which was made by mixing one liter of water with 200 grams of potato extract, 15 grams of agar, and 20 grams of dextrose(Chrapačienė *et al.,* 2022)**.** For seven days, the Petri plates were incubated at 30 °C. Fungal hyphal tips and individual spores were extracted from the developing colonies at this time and placed in PDA medium slant tubes. Before being kept in a refrigerator at 5 °C for additional research, these tubes were incubated at 30 °C for an additional seven days.

2.2. Isolation and preservation of Aspergillus ochraceous

Isolates of *A. ochraceous* were obtained from the Aswan University Faculty of Science's Botany Department. Initially, these isolates were cultivated for five days at 27 °C on PDA in slant tubes. They were then kept at 4 °C in a refrigerator. After 30 minutes of boiling 200g of potatoes in 1 liter of distilled water, the PDA medium was prepared by filtering the mixture through cheesecloth. After that, this solution was mixed with 20g of dextrose, 20g of agar, and water to create a 1-liter medium. To guarantee disintegration, the medium was then heated. The medium was autoclaved for 20 minutes at 121°C' as the last stage.

2.3. Biosynthesis of zinc oxide nanoparticles by Aspergillus niger

Every technique was adjusted to fit the *Aspergillus niger* biosynthetic process. The fungus was cultured in potato dextrose broth (PDB) at 28 °C using a rotary shaker running at 150 rpm for ninety-six hours. Using Whatman filter paper No. 1, the biomass was collected, and any leftover medium components were removed by washing with distilled water. According to (Sagar and Ashok 2012), a wet biomass of 25 grams was added to each flask holding 100 milliliters of Milli-Q water. Again, the flasks were shaken for twenty-four hours at 150 rpm and 28 °C. Whatman No. 1 filter paper separated each fungus's biomass following the incubation period (Raliya and Tarafdar 2014). ZnO nanoparticles were subsequently biosynthesized using the obtained cell-free filtrate. 50 ML of distilled water were used to dissolve one gram of zinc carbonate to create zinc oxide nanoparticles. After adding 50 ML of 0.2 M acetic acid, the mixture was agitated for one and a half hours. After this, after adding the extract from *Aspergillus niger*, the mixture was agitated for an hour at 70 °C. The mixture was then mixed with ammonia solution to maintain a pH of 10–12 until all zinc particles precipitated as zinc hydroxide. Following the completion of precipitation, the precipitation was digested in the mother liquor for 12 hours at 90 °C in a water bath. After centrifuging the solution, the precipitate was dried overnight at 120 °C. Ultimately, zinc oxide nanoparticles were obtained by calcining the dry precipitate for three hours at 450 °C.

2.4. Antifungal activity of ZnO NPs on Aspergillus niger and A. ochraceous growth at optimal temperatures

Three distinct zinc oxide nanoparticle concentrations (0.25, 0.5, and 1 g/100 ml of PDA medium) were used to examine the impact of these particles on the development of *Aspergillus niger* and A. ochraceous. The control group was infected with fungal isolates following the combination of each concentration with the medium and the pouring of the mixture into 9 cm diameter plates . Following the inoculation process, all plates were incubated at 27 °C for seven days . The inhibition % was calculated

using the following formula. (Al-Wahab and Hussain, 2020):

Inhibition (%) = ("Control sample diameter - treated colony diameter" / "Control sample diameter") × 100

2.5. Characterization techniques of zinc oxide nanoparticles

ZnO was subjected to X-ray diffraction (XRD) investigation in the 2θ range of 5 to 80° using a German Brucker D8 Advance. FTIR spectra were obtained with a Shimadzu FTIR from Kyoto, Japan, covering the 400–4000 cm–1 range. FT-IR and XRD characterizations were carried out at South Valley University's Central Laboratory. An FEI Quanta 250 FEG MKII scanning electron microscopy (SEM) was utilized for morphological characterization. Using an electron dispersive X-ray (EDX) detector and XT Microscope Control software, the samples were SEM imaged on the same FEI Quanta 250 FEG MKII equipment. Aztec® EDX analysis software was utilized with a 10 mm2 SDD Detector-x-act from Oxford Instruments as the EDX system. The produced nanoparticles' shape and surface structure were observed using transmission electron microscopy (TEM, JEOL JEM-100CX II). Characterizations (SEM, EDX, and TEM) were carried out at Minia University's Central Laboratory for Microanalysis and Nanotechnology.

3. Results and discussion

3.1. Identification and characterization of Aspergillus niger

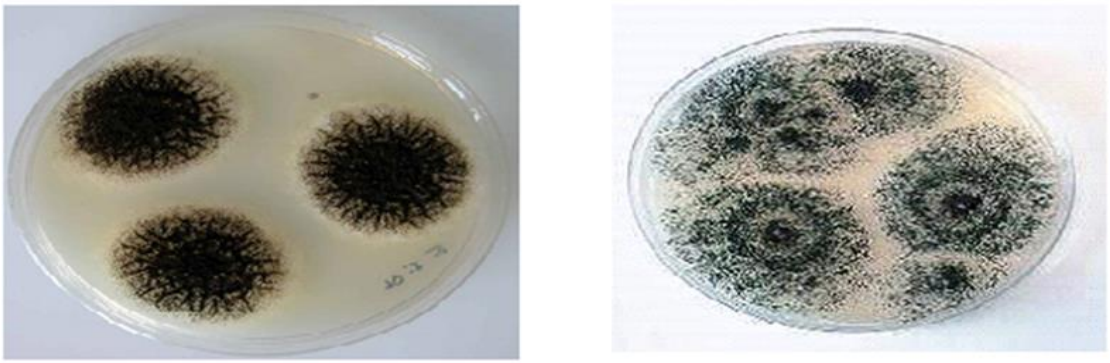


Figure 1. Morphological of *Aspergillus niger* on Potato Dextrose Agar PDA medium.

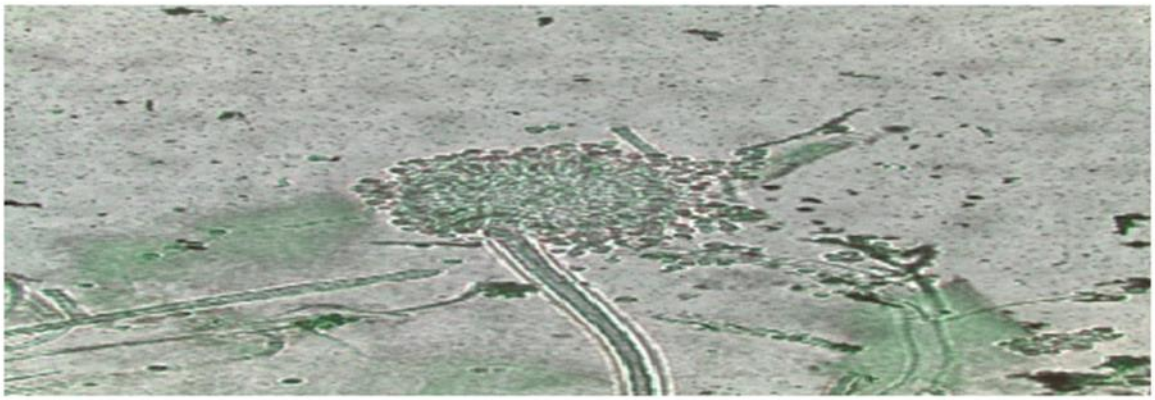


Figure 2. Hypha and holders of Aspergillus niger.

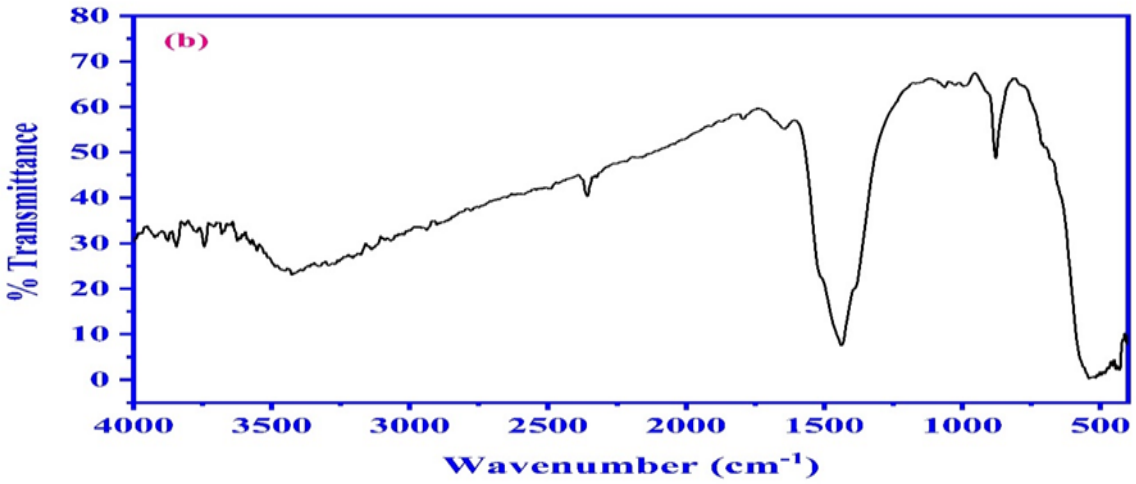
3.2. Characterization of ZnO NPs synthesized by Aspergillus niger

3.2.1. X-ray diffraction (XRD) analyses

At the micro- and nanoscales, one of the most potent techniques for analyzing material structures is X-ray diffraction (XRD). To dissect the phase composition and crystallite size of a sample, XRD analysis has been performed. The XRD pattern, as depicted in Figure 3a, exhibited distinct peaks at 2θ angles of 31.8°, 34.5°, 36.2°, 47.5°, 56.5°, 62.8°, 66.5°, 68.5°, 69.1°, 72.6°, and 77.1°, corresponding to crystallographic planes such as (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202). Using the Scherrer equation (Fakhari *et al.*, 2019), the average crystallite size was determined to be 39.74 nm, affirming the nanoparticles' nanocrystalline nature, as evidenced by their sharp and intense diffraction peak (101). Notably, the absence of impurity peaks in the XRD spectra underscores the sample's purity and excellent crystalline quality. These results were matched with the literature (Selvarajan and Mohanasrinivasan, 2013; Shamim *et al.*, 2019).

3.2.2. Fourier transform infrared (FT-IR) analyses

The functional groups present in the synthesized zinc oxide nanoparticles were identified through FT-IR characterization. In Figure 3b, peaks at 3422.24, 2356.17, 1644.96, 1440.40, 880.24, 527.57, and 439.59 cm^{-1} were observed, highlighting specific functional groups. The broad peak at 3422.24 cm^{-1} in spectra corresponds to the stretching vibration of the OH group in water (Gao *et al.*, 2019). The peak at 2356.17 cm^{-1} indicates O=C=O stretching (Abdelbaky *et al.*, 2022), while the C=C stretching vibrations were noted at 1644.96 cm^{-1} (Gao *et al.*, 2019). Additionally, peak at 1446 cm^{-1} signify C=C stretching (Bashir *et al.*, 2022), and those at 880.24, 527.57, and 439.59 cm^{-1} confirm the presence of Zn-O bonds in the sample (Es-haghi *et al.*, 2019). These findings suggest that a swift, uncomplicated, and environmentally friendly synthesis method could yield highly crystalline nanomaterials. Consequently, this study demonstrates superior energy efficiency, establishing it as a more sustainable and effective approach for synthesizing zinc oxide nanoparticles.



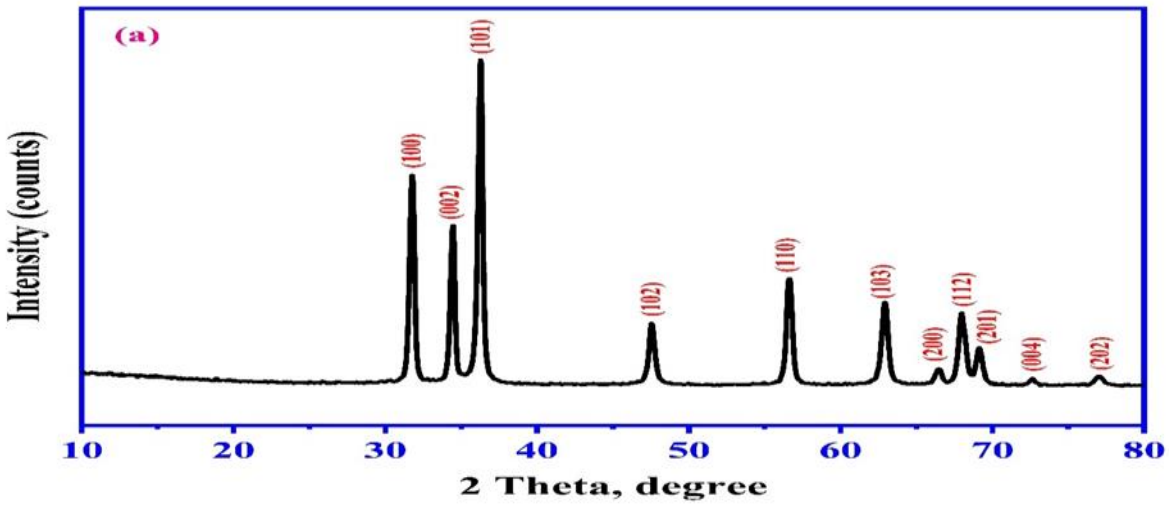


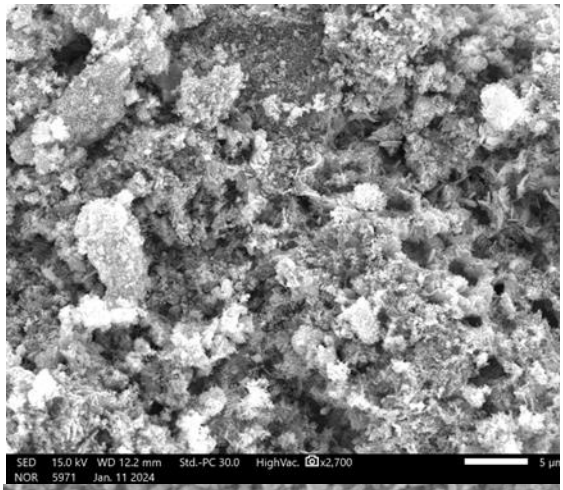
Figure 3. (a) XRD diffraction patterns of ZnO nanoparticles synthesized by *Aspergillus niger* calcined at 450 °C and (b) FT-IR spectra of ZnO nanoparticles synthesized by *Aspergillus niger* calcined at 450 °C.

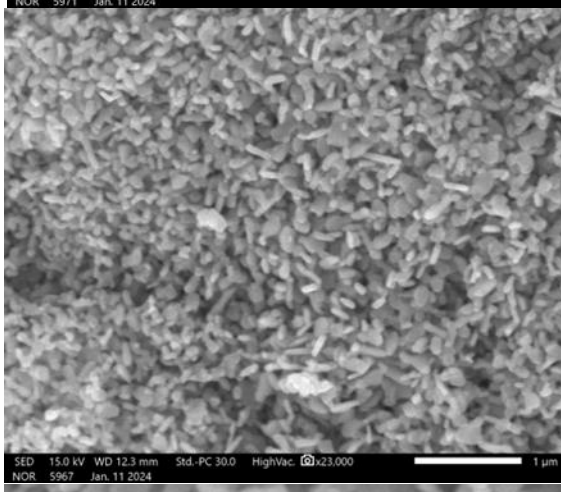
3.2.3. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray (EDX) analyses.

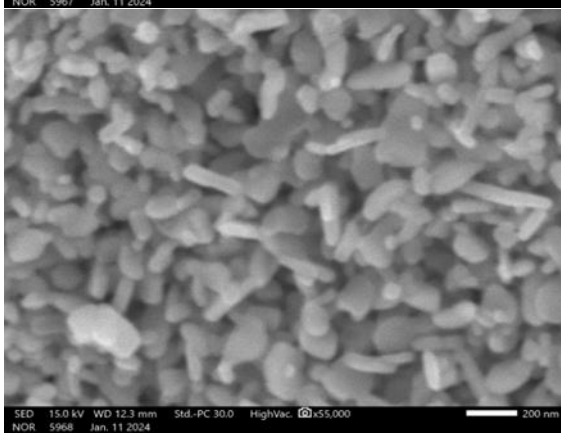
These scanning electron microscope (SEM) images show ZnO nanoparticles synthesized using *Aspergillus niger* at magnifications of 2700x, 23000x, and 55000x. The scale bar represents x 5 nm, 1 nm, and 200 nm, respectively. The nanoparticles exhibit a mostly root-like morphology. The size distribution is relatively uniform, with little variation in particle size. The surface of the nanoparticles appears rough with a series of grooves and pits that increase the adsorption capacity of the material. The nanoparticles are mostly individually dispersed, with minimal agglomeration observed. The images indicate that the fabrication process resulted in well-defined and uniformly sized ZnO nanoparticles.

EDX technique is used to determine the chemical composition of a nano sample of zinc oxide prepared from *Aspergillus niger*. The higher the peak, the higher the concentration of the element in the tested sample. The EDX spectrum plot identifies the element corresponding to each peak, and the type of X-rays it corresponds to. Figure 4 shows the EDX analysis of the prepared zinc oxide sample. It was found that the weight percentage of O and Zn were 17.49 and 69.90 weight percent, respectively. The atomic ratios were found to be 34.04 and 33.29 for O compared to Zn. This indicates the purity of the prepared sample and its freedom from any impurities. The spectrum reveals a very small concentration of carbon. The presence of carbon in the sample can be attributed to the carbon adhesive tape.

Element	Mass (%)	Atom (%)
C	12.61±0.13	32.67±0.33
O	17.49±0.15	34.04±0.30
Zn	69.90±1.00	33.29±0.48







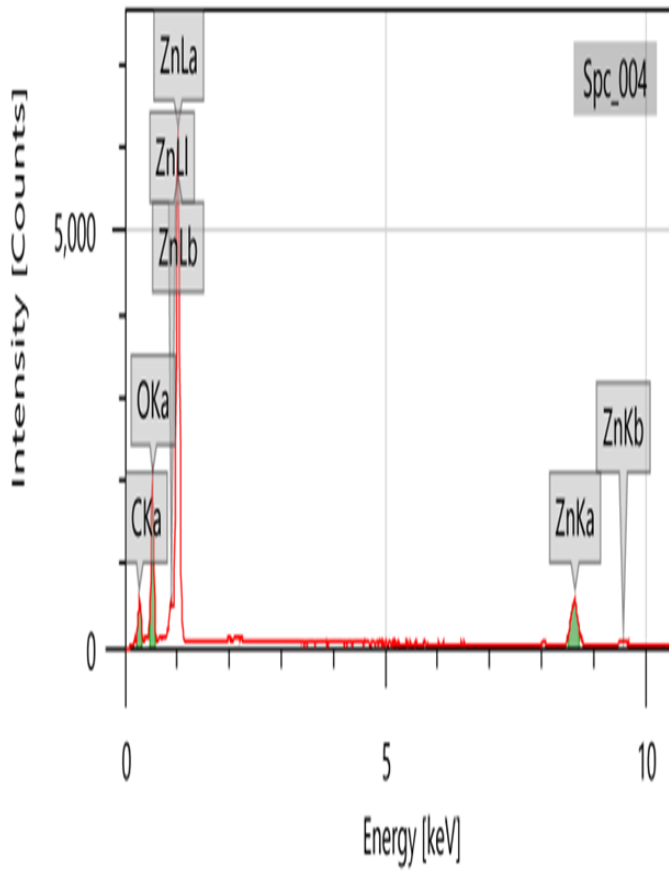


Figure 4. *Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray (EDX) analyses* of ZnO nanoparticles synthesized by *Aspergillus niger* calcined at 450 °C.

3.3. Transmission electron microscopy analysis (TEM)

The structure and morphology of ZnO NPs were evaluated using TEM. Figure (5) shows the transmission electron microscope (TEM) image of ZnO nanoparticles obtained from green-colored ZnO nanoparticles synthesized by *A. niger*. After calcining the precipitates at 450 °C for 3 h, the ZnO nanoparticles were found to be in the average range of 27 nm which is consistent

with the XRD results obtained for the same sample. The particles were found to be well-aggregate in rod-like shape. These aggregations may have occurred during the calcination process. This may be due to the large specific surface area and high surface energy of the nanoparticles.

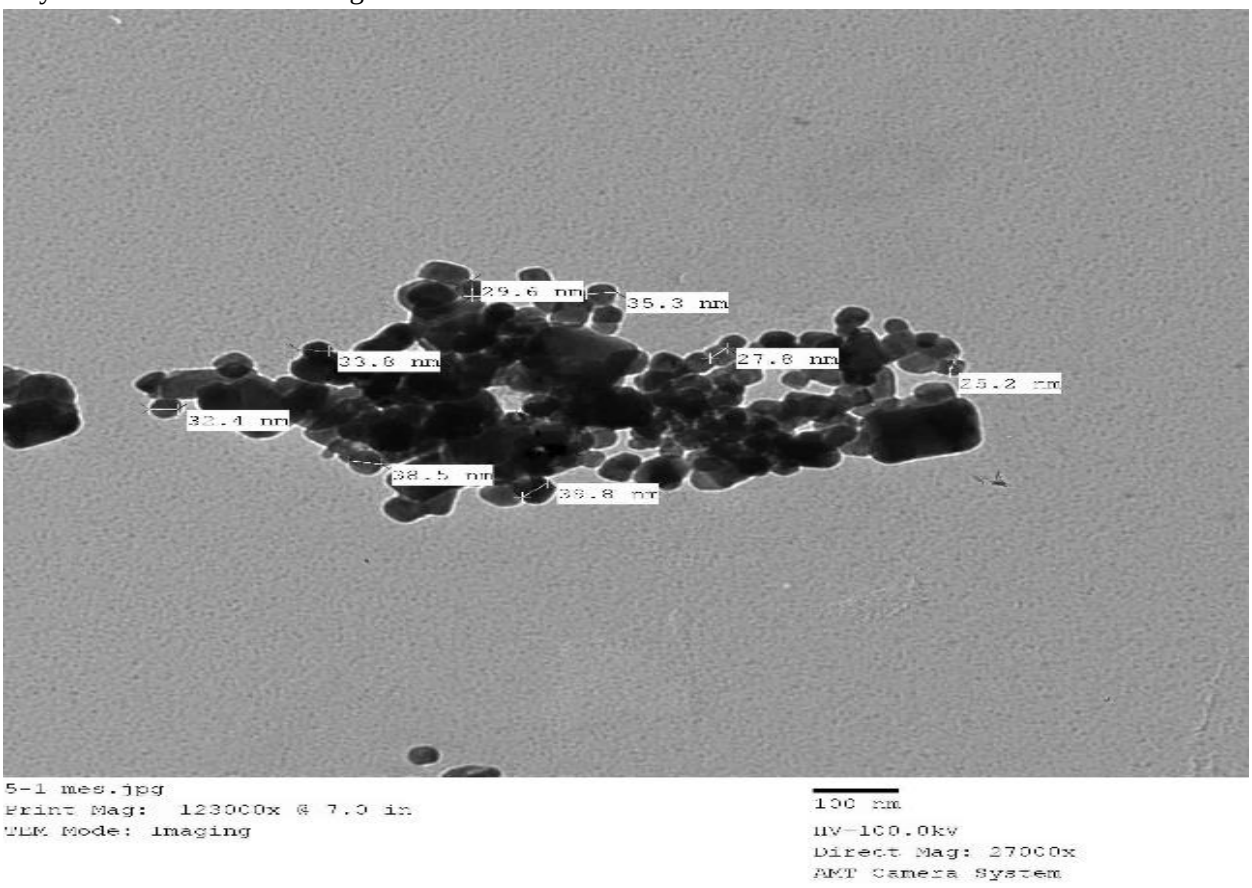


Figure 5. Transmission electron microscopy analysis (TEM) of ZnO nanoparticles synthesized by *Aspergillus niger* calcined at 450 °C.

3.4. Impact of zinc oxide NPs on Aspergillus ochraceous and Aspergillus niger growth

Zinc oxide nanoparticles (ZnO) showed an inhibitory effect against *Aspergillus* species in PDA media. The results in Table 1 and Figure 6 showed the inhibitory action of ZnO NPs at varying doses against A. ochraceous and A. niger. The addition of ZnO nanoparticles at concentrations of 0.25%, 0.5%, and 1% was found to limit fungal growth in *Aspergillus niger* by 56%, 81%, and 86%, and in *Aspergillus ochraceous* by 64%, 71%, and 86%, respectively. ZnO NPs showed this effect when each isolate's ideal temperature was used to cultivate the fungus. The effectiveness of different ZnO nanoparticle concentrations on the growth of fungi such as *A. niger* and A. ochraceous was also investigated in this study.

The results showed that different concentrations of ZnO nanoparticles significantly inhibited the growth of fungus as compared to the controls. The highest concentration of nanoparticles produced the largest inhibition, which was often followed by a lower concentration of nanoparticles. The growth of the fungal species was inhibited by 56%, 81%, and 87% for *A. niger*

and 64%, 71%, and 86% for A. ochraceous at dosages of 0.25%, 0.5%, and 1%, respectively. Studies by Erazo *et al.,* (2019) and Kamal *et al.,* (2023) reinforce the conclusion that higher concentrations were more advantageous than lower values. ZnO nanoparticles have an inhibitory effect because they can break through the cell walls of fungi and stop the growth of new fungi through direct interaction with the fungal cell membrane (Ashwini *et al.*,2021).

The antifungal activity of ZnO NPs has been associated with the intracellular production of various free radicals, including as hydroxyl and superoxide radicals. According to Ipovsky *et al.,* (2011), DNA damage and irreversible chromosomal cell death are caused by the lack of smoothness in the cell membrane. As shown in the case of *Trichoderma reesei* and other fungi, the inhibitory effect of nanoparticles may be due to the inhibition of the production of extracellular enzymes and metabolites, which function as factors for their survival when exposed to stress from toxic substances and temperature changes (Vahabi *et al.*,2011). Fungal cellular processes like transport systems, signalling pathways, and enzyme activity may be hampered by zinc oxide nanoparticle-induced Zn^{2+} ion release, which could ultimately lead to fungal cell death. (Sirelkhatim *et al.*,2015; Jayaseelan *et al.*,2013). According to research by Jiang *et al.,* (2009) and Akhtar *et al.,* (2012), zinc oxide nanoparticles' tiny size and high surface area-to-volume ratio enable them to interact with the fungal cell wall and membrane more efficiently, disrupting it and ultimately causing fungal cells to die.

Additionally, fungal spore germination and the development of fungal biofilms—which are necessary for fungal survival and dissemination—can be impeded by zinc oxide nanoparticles (Saba *et al.*,2022; Behera *et al.*,2022). In a different investigation, Baskar *et al.,* (2013) discovered that the production of extremely reactive species such OH^{-}, H_2O_2, and O^{22-} is the primary mechanism by which zinc oxide nanoparticles exhibit antibacterial activity. While OH and O^{22-} harm the cell wall and membrane from the outside, H_2O_2 enters the cell.

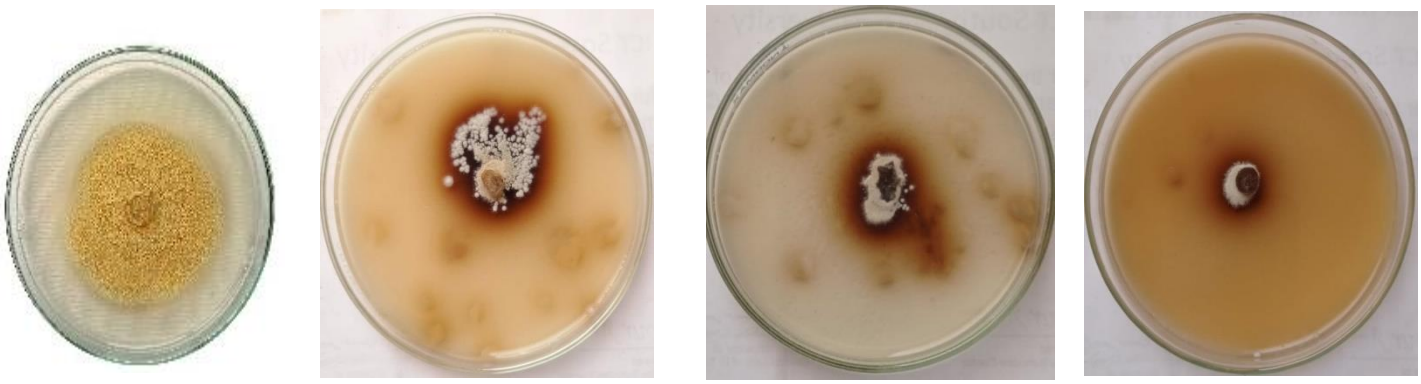
Table 1. lists the inhibitory effects of various doses of zinc oxide NPs on the development of *A. ochraceous* and *A. niger* at optimal temperatures.

No.	Identification morphologically	Concentration of ZnO NPS (%)	Temperature (°C)	Inhibition percentage (%)
1	*Aspergillus niger*	0.25		56%
		0.5		81%
		1	28	87%
2	*Aspergillus ochraceous*	0.25		64%
		0.5		71%
		1		86%

Effect of zinc oxide nanoparticles on the growth of *Aspergillus niger.*

Control **0.25%** **0.5%** **1%**

Effect of zinc oxide nanoparticles on the growth of *Aspergillus ochraceous.*



Control **0.25%** **0.5%** **1%**

Figure 6. ZnO NPs action inhibition of *Aspergillus niger* and *Aspergillus ochraceous* under optimal temperatures on PDA medium.

4. Conclusion

This study successfully demonstrated the green manufacturing of zinc oxide nanoparticles (ZnO NPs) using the fungus *Aspergillus niger*, which is a sustainable and environmentally beneficial technology. The characterization techniques confirmed the generation of highly crystalline ZnO NPs with an average size range of 27–40 nm. To investigate the antifungal activity of the synthetic ZnO NPs, two fungus species were used: *Aspergillus niger* and *Aspergillus* ochraceous. The data showed a significant inhibition of fungal growth, with the greatest inhibition seen at the highest concentration of ZnO NPs tested. ZnO NPs were found to have a potent antifungal effect because of their ability to disrupt fungal cell membranes, generate reactive oxygen species, and hinder essential cellular processes.

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All authors are contributed in this research
Funding
There is no funding for this research.
Institutional Review Board Statement
All Institutional Review Board Statements are

confirmed and approved.

Data Availability Statement

Data presented in this study are available on fair request from the respective author.

Ethics Approval and Consent to Participate

Not applicable

Consent for Publication

Not applicable.

Conflicts of Interest

The authors disclosed no conflict of interest.

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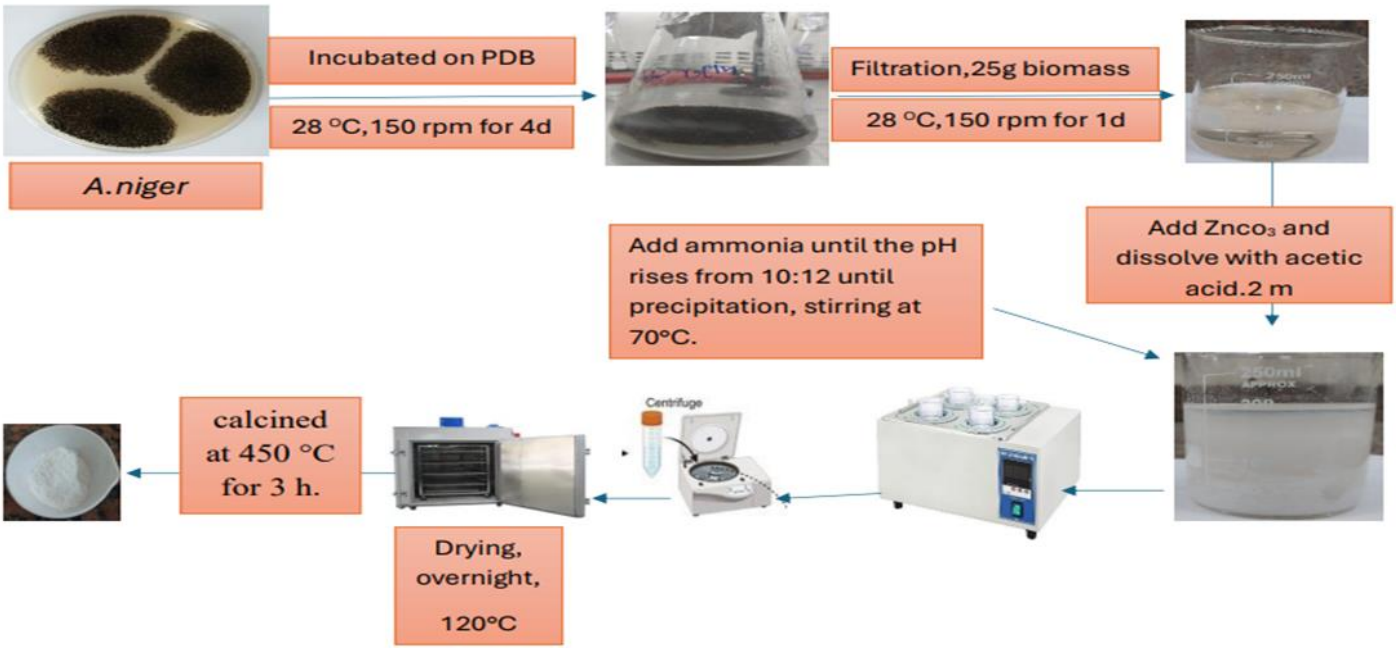

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