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STRUCTURAL CHARACTERIZATION AND EVALUATION OF MYCOGENIC ZINC OXIDE NANOPARTICLES FROM THE CELL-FREE CULTURE-EXTRACT OF *Aspergillus niger*

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ABSTRACT

Advances in the biological synthesis of nanoparticles have attracted decisive research attention in recent years. This is due to their eco-friendliness, nontoxicity and large spectra of applications. In this work, the structural characteristics and the purity of biogenic zinc oxide (ZnO) nanoparticles were assessed. Zinc oxide (ZnO) nanoparticles were synthesized extracellularly using the culture filtrate of *Aspergillus niger*, in the presence of zinc acetate dihydrate, as a precursor. The structural characteristics and purity of the nanoparticles were examined using standard characterization methods viz *UV-visible spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), Fourier-transmission infrared (FT-IR) spectroscopy and X-ray diffraction (XRD).* Results revealed a peak at 311nm and whitish and spherical particles with particulate sizes between 30 and 40 nm for the *UV-visible* spectroscopy and SEM respectively. On the FT-IR scale, absorption peaked at 548 cm^{-1} in the spectra region known for the functional groups of ZnO nanoparticles; while the average crystalline size was 21 nm based on XRD analysis. Findings in this study revealed that the ecofriendly biogenic nanoparticles synthesized by common fungi, such as *Aspergillus niger,* possess desirable qualities comparable to those from non-ecofriendly and costly chemical processes, which are currently employed for an array of applications.

Keywords: Zinc-oxide Nanoparticles, Microbial Synthesis, Mycogenic Nanoparticles, *Aspergillus niger; Cell-free culture-extract.*

INTRODUCTION

The emergence of nanotechnology has piqued the interest of researchers in creating nanoparticles with sizes as small as 100 nm due to its numerous benefits and applications (Kalpana *et al.,* 2018). This technology transformed metal salts into metal oxide particles of specific sizes and shapes. Zinc oxide (ZnO) nanoparticles have, among these metal oxides, attracted special attention worldwide, due to their wide range of applications and uses including industrial coatings, sunscreen products, textiles, paints, and as antibacterial agents (Raghupathi *et al.,* 2011). According to the US Food and Drug Administration (FDA), zinc oxide is listed as generally recognized as safe (GRAS) among other four zinc compounds (acetate, carbonate, chloride, and sulfate) (Espitia *et al.,* 2016). It is the second most abundant metal oxide after iron, and it is inexpensive, safe, and can be easily prepared (Lakshmipriya and Gopinath, 2021). More importantly, the ZnO nanoparticles have smaller sizes so they have a large surface area and have been synthesized by different methods.

Several physical and chemical methods have been reported for the production of ZnO nanomaterials. Methods such as arc discharge, chemical vapour condensation, hydrogen plasma-metal reaction, micro-emulsion, laser pyrolysis in the vapour phase, hydrothermal, sol-gel, sonochemical, and ball milling (Low *et al.*, 2020), and recently, the use of laser-ablation technique (El-Gendy *et al.*, 2022). Although the physical and chemical methods are generally considered the best to get uniform-sized nanoparticles with long-term stability (Augustine and Hasan, 2020) these techniques are complicated, costly, inefficient, and outdated (Patra and Baek, 2014) and involve the use of toxic and hazardous chemicals which may result to pollution in the environment (Mohd Yusof *et al.,* 2019).

On the other hand, the biological process has, in recent times, been gaining attention from researchers due to its Eco-friendliness and nontoxicity. The biological process involves the use of plants or the biomass (cell or cell-free supernatant) of bacteria, fungi, yeast, and algae with the extracts being biocompatible and which help as capping agents for stabilizing the NPs (Droepenu *et al.,* 2022). A large number of plants (stems, roots, seeds, and leaves) have been reported to produce zinc oxide nanoparticles, but a few microorganisms have been earlier reported to synthesize nanoparticles (Mohd Yusof *et al.,* 2020). Generally, organisms (unicellular and multicellular) can synthesize nanoparticles intracellularly or extracellularly based on their general propensities to produce both extracellular or intracellular inorganic materials (Thakkar *et al.,* 2010). The intracellular method is laborious and involves transporting ions into the microbial cell biomass to form the nanoparticles based on the activities of certain enzymes. On the other hand, extracellular synthesis is more advantageous and involves trapping the metal ions on the surface of the cells and reducing ions in the presence of certain enzymes (Zhang *et al.,* 2011).

Several bacterial (Mohd Yusof *et al.,* 2020; Al-Kordy *et al.,* 2021 and Faisal *et al.,* 2022) and fungal (Guilger-Casagrande and Lima., 2019; Dias *et al.*, 2022 and Qanash *et al.,* 2023) species have been used for the synthesis of various nanoparticles. However, few of the studies reported on the desirable properties such as structural characteristics and purity of the biogenically synthesized nanoparticles. In this work, ZnO nanoparticles were synthesized using an indigenous fungus, *Aspergillus niger,* characterized using standard procedures and compared with those reported in previous studies to determine its comparative advantage over, most especially, the non-ecofriendly and expensive chemically-synthesized nanoparticles.

MATERIALS AND METHODS

Microorganism

Aspergillus niger was collected aseptically from the Culture Collection Centre, Department of Microbiology, University of Ilorin. Fungal identity was verified based on colonial and cultural features as well as the morphological characteristics of the sporangia and conidia using standard methods as described in the Pictorial Atlas of Soil and Seed Fungi by Watanabe (2010). For colonial identification, direct observation of colony features on PDA was used as a yardstick. Morphological features were observed under a high-powered imaging microscope on a slide preparation after staining with lactophenol cotton blue. Pure culture was maintained at 4°C on potato dextrose agar (PDA) slant until needed.

Preparation of fungal extract

Spores from a 48-72 h-old culture of *Aspergillus niger* were cultivated aerobically in a 250mL Erlenmeyer flask containing a 100 mL potato dextrose broth (PDB; Sigma-Aldrich). The flask was incubated at 30°C and 120 rpm for 72 h. Fungal biomass was harvested after cultivation. Thereafter, ten grams of the fungal biomass was suspended in 100 mL of sterile distilled water in an Erlenmeyer flask (250 mL) and stirred at 150 rpm and 30°C for 72 h. The cell was discarded after filtration (using Whatman No.1 filter paper) to obtain a filtrate useful for nanoparticle synthesis according to the method described by Mekky *et al.* (2021).

Biogenic Synthesis of Zinc Oxide (ZnO) nanoparticles

Zinc Oxide NPs were synthesized by using the filtrate of the fungus *Aspergillus niger* obtained as described in the Section above. During biological synthesis, 2.5 grams of zinc acetate dihydrate was dissolved in 250 mL of deionized distilled water and mixed with the biomass filtrate at 1:1 ratio of the aqueous solution of zinc acetate. Sodium hydroxide NaOH (0.1 M) was prepared and added in drops with constant stirring using a magnetic stirrer for an hour until the pH increased to 11. The solution was placed in a dark shaking incubator for 3 days and then filtered. A pale white filtrate of ZnO nanoparticles was obtained as described by Shamim *et al.* (2019).

Characterisation of the Zinc oxide nanoparticles

The structural characteristics of the synthesised nanoparticles were determined following standard procedures. Spectroscopy was performed using UV–Vis (SPECORD®200 PLUS) for presumptive confirmation of the synthesis. Surface morphology was analysed using scanning electron microscopy (SEM; JEOL-JSM-7600F). Transmission electron microscopy (TEM) (TEM-ARM200F-G Verios 460L) was used for the determination of the sizes of NPs. Fourier transform infrared spectroscopy (FTIR) (Nicolet iS10 FT-IR Spectrometer) was used to identify the various functional groups present in synthesized nanoparticles. Finally, the crystallinity of the nanoparticles was determined using X-ray diffraction (XRD) (Rigaku D/MaxIIIC, PW1800).

RESULTS AND DISCUSSION

Biogenic synthesis of Zinc Oxide Nanoparticles by *Aspergillus niger*

In the present study, *Aspergillus niger* was used to synthesize zinc oxide nanoparticles. This is based on the general ability of Fungal species including those of *Aspergillus* to secrete a large number of extracellular enzymes and redox proteins (Ajijolakewu *et al.,* 2017). Previous work by Lahiri *et al.,* (2021) has shown that fungal species can synthesize nanoparticles because they possess intrinsic mechanisms which enhance the synthesis from metallic salts. Generally, the presence of a large number of extracellular enzymes and redox proteins contributes to the conversion of metal ions in growth media into nanoparticles through the reduction process. Gomaa (2020) however observed that these secreted proteins conferred stability to the synthesized NPs by acting as capping agents which encapsulated and bound their surfaces. Table 1 shows the colonial and morphological properties of *Aspergillus niger.*

Table 1: Colonial and Morphological Characteristics of *Aspergillus niger*

Colony Size (cm) 3days	**Colony Color**	**Reverse Color**	**Colony edge**	**Mycelia form**	**Mycelia colour**	**Conidia Structure**	**Conidia wall**	**Conidia color**	**Phialides & Metulae**	**Condiophore Branch**
7-8	Black	Cream dull yellow	Rough	Clavate non-septate hypha	Hyalin	Globose	Rough	Black	Present & dense	Regular

Characterization of Biogenic Nanoparticles from *Aspergillus niger*

UV-vis absorbance spectra analysis

The UV-visible spectrum of the biogenic (ZnO) nanoparticles was recorded in the liquid phase at the range between 200 and 800 nm. Figure 1 shows the UV-spectrum analysis of ZnO nanoparticles noted at absorbance at 309 nm. This is similar to the findings of Al-Kordy *et al.* (2021) who observed a peak at 310 nm. Gurgur *et al.* (2020) mentioned that UV peak which lies between 300-400 nm are characteristic of ZnO nanoparticles which may be due to the electron transition from the valence band to the conduction band.

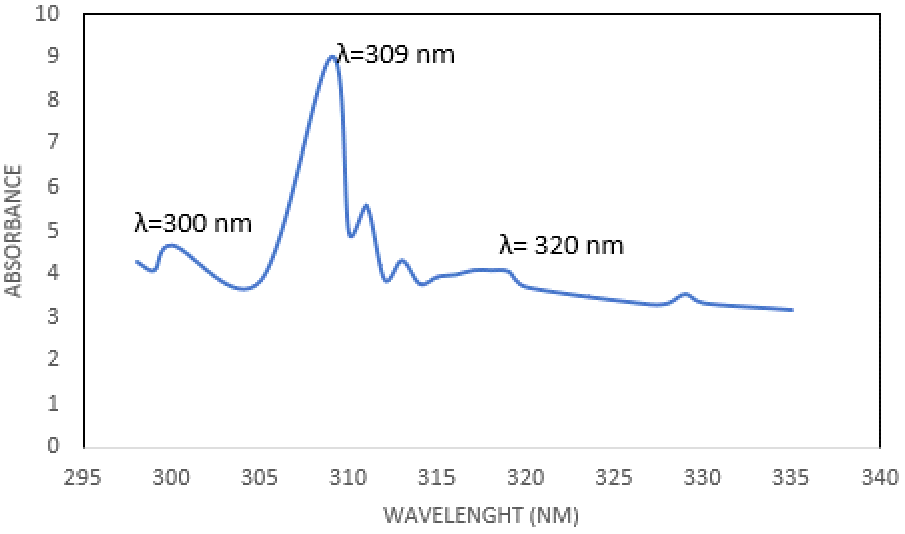

Figure 1. UV-vis spectrum of Biogenic Zinc Oxide Nanoparticles from *A. niger culture extract*

SEM and TEM analysis of Zinc Oxide Nanoparticles

The SEM analysis of the studied nanoparticles shows white spherical particles that are highly compacted (Fig. 2a). A similar observation was reported by Kalpana *et al.* (2018) who observed particles of ZnO NPs that were compacted and almost spherical in size. During the synthesis of nanoparticles, a white precipitate was formed. This may likely be a result of the reduction of Zn^{2+} ions in the zinc acetate to ZnO NP in the aqueous medium (Mekky *et al.,* 2021). Meanwhile, the TEM analysis (Fig. 2b) revealed an average particle size between 20-40 nm. An earlier report by Raj *et al.* (2015) showed that the average sizes of zinc oxide nanoparticles were between 30 and 40 nm. On the other hand, Fig. 2c shows the Selected Area Electron Diffraction (SAED) patterns of ZnO nanopowder. The bright distinct bright rings confirm the preferential orientation of the nanocrystals

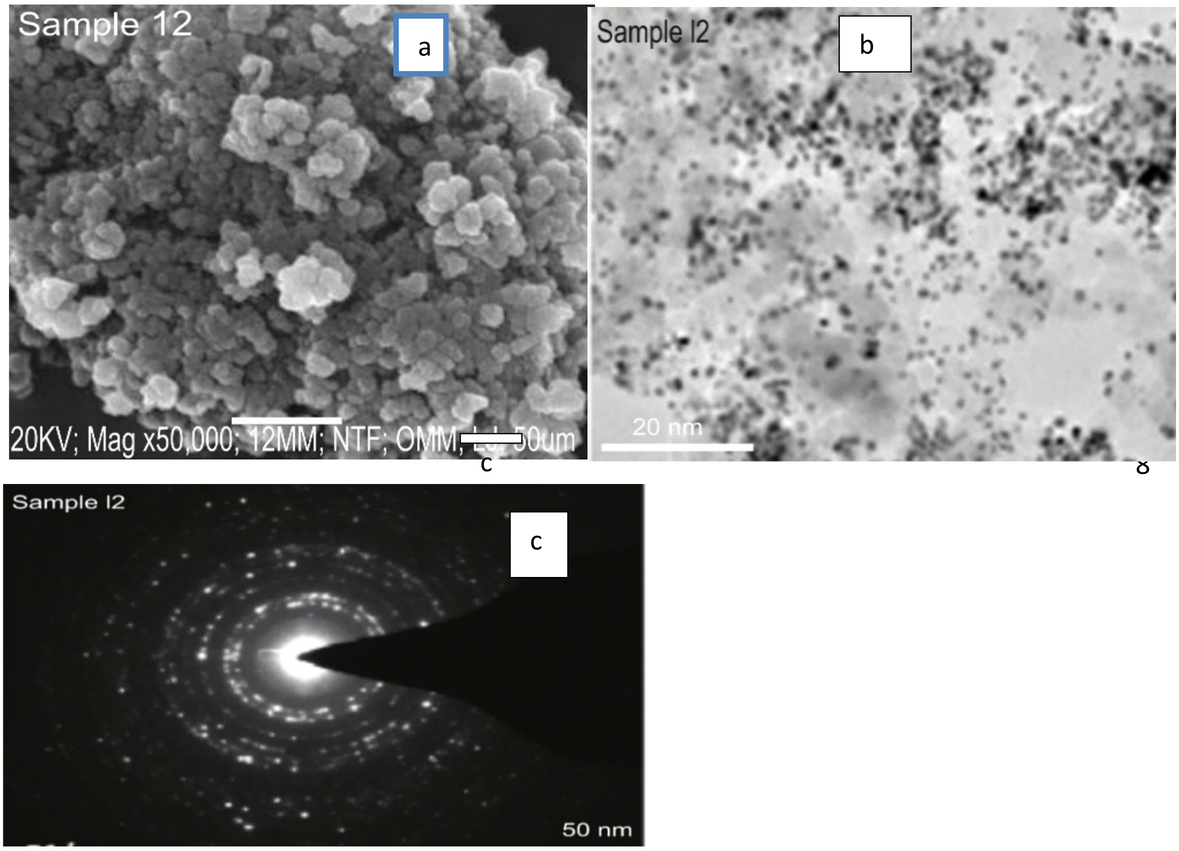

Figure 2: Electron Microscopy Analysis of the Mycogenic ZnONPs (a) SEM analysis; (b) TEM analysis; (c) Selected Area Electron Diffraction (SAED patterns

Fourier Transmission Infrared (FTIR) Spectroscopy of zinc oxide nanoparticles

Fourier Transmission Infrared (FTIR) spectroscopy in Fig 3 shows the different functional groups associated with synthesized zinc oxide nanoparticles with absorption peaks in the range of 3687.00cm^{-1}, 3620.55 cm^{-1}, 3433.00 cm^{-1} indicating O-H stretching, 2935.66 cm^{-1} and 2373.66 cm^{-1} indicates C-H stretching vibration of alkenes.1633.25 cm^{-1} indicates C=C stretching of the alkene functional group, and 1027.45 cm^{-1} indicates the C-OH group of the phenols. The absorption peaks at 548.62cm^{-1} indicates the region of ZnO nanoparticles which corresponds to metal-oxygen similar to a study by Handore *et al.* (2014) with an absorption peak at 545 cm^{-1}. The spectra peak between 400 and 600 cm^{-1} is known for Zn-O (Dallatu *et al.,* 2020).

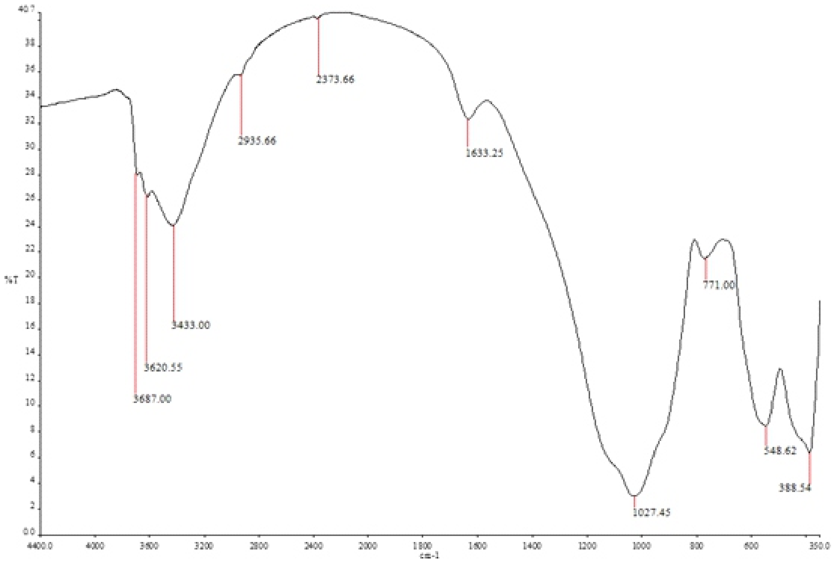

Figure 3: FT-IR spectrum of biosynthesized ZnO NPs.

X-ray diffraction (XRD) analysis of zinc oxide nanoparticles

The X-ray diffraction pattern of synthesized ZnO NPs exhibits well-defined diffraction peaks and all peaks are indexed based on the standards of the joint committee on powder diffraction standards (JCPDS-36-1451). These peaks correspond to (005), (100), (002), (101), (102), (110), (103), (112) and (201) narrow intense peaks indicating nanoparticles are pure and crystalline (Fig 4). Meanwhile, the average crystalline size (D) was estimated using the Debye Sheerer equation (Equation 1) as described by Kadhim *et al.* (2017) to be 21 nm.

$D = K\lambda / \beta\cos\theta$------------------------ Equation 1

where K is the Scherrer constant crystallite shape factor which depends on the shape of the particle and its value is 0.90, λ is the wavelength of CuKa radiation (0.1541 nm), β is the full width at half maximum of the selected diffraction peak corresponding to (101) plane and θ is the Bragg angle obtained from 2θ of the same plane.

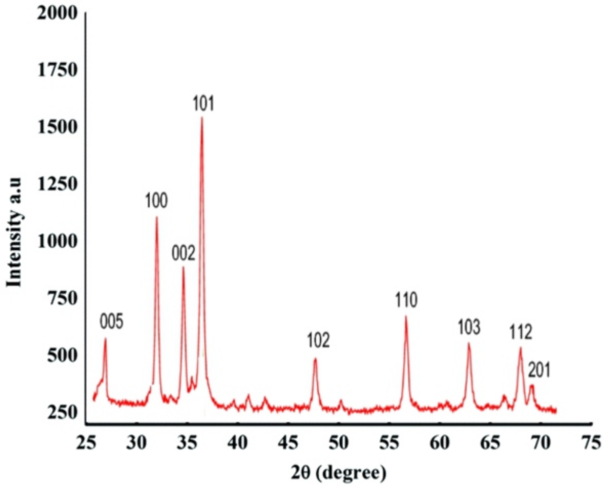

Figure 4: XRD pattern of Zinc Oxide Nanoparticles

Comparative evaluation of Biogenic Zinc Oxide Nanoparticles and Chemically synthesized Nanoparticles.

Unlike the common chemical cost-intensive approach, the Zinc oxide nanoparticles produced in this study were synthesized through an eco-friendly biogenic technique using *Aspergillus niger.* Table 2 below provides a holistic comparison between the structural characteristics of a list of chemically synthesised and those biogenically synthesized as produced in this work and other studies.

Table 2: Comparative evaluation of Biogenic Zinc Oxide Nanoparticles and Chemically synthesized Nanoparticles.

Nanoparticles	**Method of Synthesis**	**Organisms / Method**	**Pre Cursors**	**Structural Characteristics**					**Reference**
				Uv-Vis	**X-Ray D**	**SEM**	**FTIR**	**TEM**	
Zinc Oxide	Biogenic	*Aspergillus niger*	Zinc Acetate	Spectrum peak at 310 nm	21 nm	Spherical and highly compacted particles	Absorption peaks at 548.62cm^{-1}	30-40 nm	*This work*
Zinc Oxide	Chemical	*Solgel*	Zinc Acetate	-	50 nm	-	-	Size distribution 50-70nm	Kumar *et al* (2020)
Zinc Oxide	Chemical	*One-point synthesis via chemical precipitation*	Zinc Acetate	357nm	47.2nm	Irregular surface structure particle aggregation. -Average size of 65.3nm	887 cm^{-1} and 550 cm^{-1}	-	Akpomie *et al* (2021)
Zinc Oxide	Chemical	*sol-gel*	Zinc Acetate	370nm 374nm 376nm 377nm	-	Particles have rod shapes	436 cm^{-1}	Average diameter 37 cm^{-1} and 395 cm^{-1}	Ismail *et al* (2019)
Zinc Oxide	Biogenic	*Daedalea sp.*	Zinc Acetate	350 – 380nm	Average crystalline size of 14.53nm	Irregular in shape	550.05 cm^{-1}		Kamal *et al* (2023)
Zinc Oxide	Biogenic	*Aspergillus niger*	Zinc nitrate	320nm	41nm	Compactly arranged and almost spherical particles; diameter 53.69nm; average size 61±0.65nm	487cm^{-1}	-	(Kalpana *et al*., 2018)
Zinc Oxide	Biogenic	*Haloalaliphic Alkalibacillus sp W7*	Zinc Sulphate	spectrum peak at 310nm	19.5nm	Nanosize range; irregular to nearly spherical; average size ranging from 5-45nm	578.95cm^{-1}	1-30nm and average size of 17±1nm	(Al-kordy *et al.,* 2021)

Table 2: Continued Comparative evaluation of Biogenic Zinc Oxide Nanoparticles and Chemically synthesized

Nanoparticles	**Method of Synthesis**	**Organisms / Method**	**Pre Cursors**	**Structural Characteristics**					**Reference**
				Uv-Vis	**X-Ray D**	**SEM**	**FTIR**	**TEM**	
Zinc Oxide	Green	Gum tragacanth	Zinc acetate	308nm	32nm	Hexagonal structure; uniform in size	absorption peak at 457-545cm^{-1}	- Average size of 20nm	(Handore *et al*., 2014)
Zinc Oxide	Green	*Root extract of zingiber officinale*	Zinc Acetate			Sizes between 30-50nm and spherical shapes	1566 cm^{-1}	-	(Raj *et al*., 2015)
Zinc Oxide	Biological	*Bacillus foraminis*	Zinc Sulphate	380nm	-	-	519cm^{-1}	16-25nm	EL-GHWAS (2022)
Zinc Oxide	Biogenic	*Actinomycetes*	Zinc Sulphate	310nm	11.76nm 14.58nm 11.57nm	Needle shape structure of average size 321.3nm	403-418cm^{-1}	12-35nm	(Rajivgandhi, *et al*., 2021)
Zinc Oxide	Biogenic	*Pseudomonas aeruginosa*	Zinc Acetate	380nm	21nm	-	520-630cm^{-1}	Between 6-21nm	(Abdo *et al*., 2021)
Zinc Oxide	Biogenic	*Bacillus cereus strain RNT6*	Zinc Sulphate	382nm	-	Spherical shape 21 to 35	621cm^{-1}	Spherical 21 to 35 nm	(Ahmed *et al* 2021)
Zinc Oxide	Biogenic	*Phanerochaete chrysosporium*	Zinc Sulphate	349nm	-	Hexagonal form with varied sizes	531cm^{-1}	5-200nm	Sharma *et al.* (2021)
Zinc Oxide	Biogenic	*Pseudomonas putida*	Zinc Nitrate	360nm	50nm	Clusters of spherical agglomerated in nano size	460cm^{-1} and 505cm^{-1}	Average diameter 44.5nm	Jayabalan *et al* (2019)
Zinc Oxide	Biogenic	*Xylaria acuta*	Zinc Nitrate	370nm	35-45nm	Hexagonal nanoparticles diameter of 40-55nm	386.07 cm^{-1} 385.11 cm^{-1} 401.40 cm^{-1} 389.90 cm^{-1}	Particle size ranges from 30-50nm	Sumanth *et al* (2020)
Zinc Oxide	Biogenic	*Lactobacillus plantarum TA4 (supernatant)*	Zinc Nitrate	349nm	-	Nano flowerlike shape with diverse size	455.29 cm^{-1}	average size of 291 ± 98.1 nm	Mohd Yusof *et al* (2020)
Zinc Oxide	Biogenic	*Lactobacillus plantarum TA4 (Cell Biomass)*	Zinc Nitrate	351nm	-	Irregular shape as agglomerated spherical, hexagonal and oval	545.80 cm^{-1} and 513.18 cm^{-1}	ranging from 49.2 to 369.5 nm,	Mohd Yusof *et al* (2020)

Comparatively, the biogenic ZnO generally presented structural characteristics with obvious advantages over the non-ecofriendly and expensive chemically synthesised ones. For instance, the structural properties of the ZnO nanoparticles based on chemical synthesis are characterized by irregular surfaces, larger particulate sizes and inordinate absorption peaks and diameters far from the usual (Table 2). In contrast, the biogenic ZnO nanoparticles are characterized by spherical surfaces, smaller particulate sizes and moderate absorption peaks in the spectra range common with standard ZnO nanoparticles. Furthermore, it is worth noting that based on the evaluation of the various structural characteristics of the biogenic ZnO nanoparticles (Table 2), the results in this study are comparatively better (Table 2) than most other biogenically-sourced or, at least, in the range of standard referenced biogenic ZnO nanoparticles presented in Table 2.

CONCLUSION

Zinc oxide nanoparticles were synthesized through an eco-friendly myco-synthetic technique using the cell-free filtrate of *Aspergillus niger* isolated from a soil sample. This biosynthesized nano powder was characterized by UV-visible spectroscopy, SEM, TEM, FT-IR, and XRD analysis. The study has carefully compared the synthesized ZnONPs with other studies. The study carefully compared the synthesized ZnONPs with other studies involving most especially, the chemical synthesis. Findings in this study have justified that biogenic nanoparticles synthesized by common fungi could present desirable qualities and prospects better than those from non-ecofriendly and costly chemical synthesis, which are currently employed for an array of applications.

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CONFLICT OF INTEREST

No conflict of interest.

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