











Formulation, In Vitro Evaluation, and Antimicrobial Efficacy of Zinc Oxide Nanoparticles by *Lactobacillus*

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Abstract

Owing to their unique physical, chemical, and potential antibacterial characteristics, zinc oxide nanoparticles (ZnO NPs) are highly valuable for medical applications. In this study, we aimed to determine the antimicrobial activity of ZnO NPs. ZnO NPs extracted from *Lactobacillus* showed antifungal activity. The synthesis of ZnO NPs was characterized using Fourier-transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and UV-Vis diffuse reflectance spectroscopy (UV-Vis DRS). Using the well-diffusion method, the inhibition zones of ZnO NPs against *Malassezia globosa* and *Candida albicans* were approximately 28-29 mm in diameter, which compares favorably with those of fluconazole and ketoconazole. SEM analysis revealed a hexagonal morphology, with particle sizes ranging from 56.14-64.97 nm. UV-Vis analysis showed an absorption peak around 300 nm. This study revealed the dose-dependent antifungal effects of ZnO NPs and suggested that their interaction with fungal cells disrupts cell membrane integrity, potentially leading to cell death.

Keywords: ZnO Nanoparticles, Nanotechnology, *Candida albicans*, *Malassezia globosa*

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Citation: Ghyadh BA, Farhood AH, Shalal SH, et al. Formulation, In Vitro Evaluation, and Antimicrobial Efficacy of Zinc Oxide Nanoparticles by *Lactobacillus*. J Pure Appl Microbiol. 2026;20(2):1746-1753. doi: 10.22207/JPAM.20.2.65

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INTRODUCTION

Nanotechnology enables the creation of materials at the nanoscale (less than 100 nm) and has attracted scientific attention. Nanoparticles (NPs) are extremely small particles that have unique mechanical, optical, catalytic, and biological properties compared to their bulk counterparts, largely due to their high surface-area-to-volume-ratio. Metal oxide NPs have garnered particular interest because of their unique properties and broad range of potential applications.¹ The size and shape of NPs can be controlled during synthesis using various methods, including chemical, physical, and biological approaches.^{2,3} Biomedical nanomaterials have recently gained increasing attention due to their numerous biological properties and potential applications in medicine. Various nanomaterials, such as titanium dioxide (TiO₂ NPs), zinc oxide (ZnO NPs), gold (Au NPs), and silver (Ag NPs), have demonstrated the ability to inhibit microbial adherence and exhibit antibacterial activity.^{4,5} In particular, metal oxide NPs exhibit broad and promising potential in the biomedical field. ZnO NPs can generate reactive oxygen species (ROS) on their surface, which are associated with their strong antibacterial activity, while also being relatively nontoxic and biocompatible. These characteristics have attracted considerable research interest.^{6,7} ZnO NPs are more effective in various applications than their bulk counterpart (ZnO) due to their high surface-area-to-volume ratio. High production rates with controllable particle sizes can be achieved through physical and chemical synthesis methods. However, physical methods often require high pressure, temperature, and energy input, whereas chemical methods may involve hazardous substances that can negatively impact human and animal health and contribute to environmental degradation. Chemically produced ZnO NPs are often less biodegradable and may be toxic, as residual chemicals can adhere to or persist in the final NP products, potentially interfering with biological applications. This limits their use in biological and clinical settings. Therefore, there is a need to develop biocompatible, nontoxic, biosafe, affordable, and environmentally friendly ZnO NPs is required as alternatives. Environmentally friendly synthesis of ZnO-NPs

is gaining increasing attention as a potential alternative physical and chemical methods. In this approach, numerous bioactive substances derived from microorganisms and plants act as reducing and stabilizing agents.^{8,9} In addition to plants, bacteria, actinomycetes, fungi, and yeast, both multicellular and unicellular organisms, can be used for the biosynthesis of ZnO-NPs.¹⁰ The green synthesis method is considered safe for biological systems because it is energy-efficient, nonhazardous, and ecologically conscious.¹¹ The production of anion superoxide (O₂⁻), hydroxyl radicals (•OH), and perhydroxyl radicals (HO₂•) on the surface of ZnO NPs is considered a key factor in their cytotoxicity, although the exact mechanisms remain unclear. When the cells are exposed to NPs, defense mechanisms are triggered to prevent damage. However, if the production of highly reactive free radicals surpasses the antioxidative protective capacity of the cell, oxidative damage to biomolecules can occur, potentially leading to cell death.¹² The intracellular synthesis of NPs is significantly influenced by the ionic charge and structure of the microbial cell wall. In this process, zinc ions are taken up by microbial cells and interact with biomolecules such as enzymes and coenzymes to form ZnO NPs. Various polysaccharides and proteins present in microbial cell walls provide binding sites for metal ions. Bacteria are extremely vulnerable to heavy metal ions because, in response to metal stress, electrostatic interactions facilitate the adsorption and uptake of these ions into the cell wall structure.¹³ This is attributed to the presence of polypeptides, enzymes, and amino acids such as cysteine in microbial membranes, which contain negatively charged carboxylate groups that attract metal ions. Similarly, electrons are transferred from NADH via membrane-bound electron carriers, such as NADH-dependent reductases, which reduce metal ions into their elemental form.¹⁴ These atoms subsequently nucleate to form NPs, which accumulate in the periplasmic or cytoplasmic regions near the cell wall. Proteins, peptides, and amino acids, including cysteine, tryptophan, and tyrosine play an important role in stabilizing NPs within the cell.¹⁵ Probiotic lactic acid bacteria (LAB), commonly used for the biosynthesis of ZnO NPs, have attracted considerable attention due to their safety and beneficial properties. The

thick cell walls of LAB contain several functional groups and biostructures.¹⁶ Studies have shown that ZnO NPs biosynthesized using *Lactobacillus* spp. extracts exhibit strong antimicrobial activity against *Salmonella typhi*, *Clostridium difficile*, *Clostridium perfringens*, and *Escherichia coli*, as well as fungal, antiviral, and antialgal effects.¹⁷ These characteristics make LAB effective biological systems for NP production. The thick cell walls of Gram-positive bacteria, including LAB, are composed of polysaccharides, lipoteichoic acids, proteins, and peptidoglycan.¹⁸ These components provide negatively charged sites that attract metal cations, facilitating their adsorption and contributing to the biogenesis of NPs.¹⁹ Recent studies have focused on the synthesis of ZnO NPs using *Lactobacillus* spp., particularly employing cell-free supernatant (CFS) as a reducing agent. The biosynthesized ZnO NPs are typically characterized using techniques such as scanning electron microscopy (SEM), UV-Vis spectroscopy, and Fourier-transform infrared spectroscopy (FTIR). The aim of this study was to evaluate the antifungal activity of these NPs against dandruff-associated *Malassezia globosa* and *Candida albicans* isolates from the vulvovaginal region.

MATERIALS AND METHODS

Due to their high biosynthesis efficiency, *Lactobacillus* spp. were selected as the biological model for ZnO NP synthesis. The isolate was obtained from dairy products maintained at the University of Thi-Qar Advanced Microbiology Laboratory. The isolate was cultured for 24 hrs at

38 °C on De Man, Rogosa, and Sharpe (MRS) broth. Identification was performed using the VITEK 2 system.

Lactobacillus-mediated synthesis of ZnO NPs

ZnO NPs were biosynthesized using a slightly modified method described by Durazzo et al.²⁰ Briefly, *Lactobacillus* spp. were cultured in MRS broth and incubated for 24 hrs at 37 °C. The bacterial cells were then harvested by centrifugation at 5,000 rpm for 15 min at 20 °C. The supernatant was collected and subjected to continuous stirring at 1,500 rpm. The bacterial filtrate was added to sterilized deionized water containing zinc (5,000 mM), followed by incubation for 24 hrs at 37 °C. The resulting ZnO NPs were recovered by high-speed centrifugation at 13,000 rpm for 15 min, and any excess material was removed by washing with 80% ethanol. The ZnO NPs were collected and air-dried at 60 °C.

Evaluation of the manufactured zinc oxide nanoparticles

Physical characterization was performed to determine the optical and structural properties of the synthesized ZnO NPs.

UV-visible spectroscopy

A UV-Vis spectrophotometer (T80+ UV-Vis) was used to obtain the optical absorption spectra of the NPs. One milligram of the ZnO NP sample was dispersed in 1.5 mL of toluene solution and sonicated for 30 min. The absorption spectra were recorded over a wavelength range of 200-600 nm.²¹



Figure 1. Growth of *Lactobacillus* isolate on MRS agar

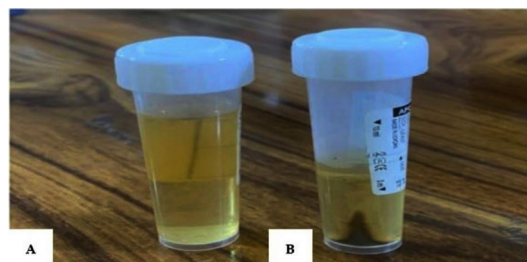


Figure 2. Biomanufacture of ZnO NPs using *Lactobacillus* spp. (A) Within 1 hr of adding zinc salts to the *Lactobacillus* cell-free supernatant. (B) After 72 hrs of incubation with zinc salts in the *Lactobacillus* cell-free supernatant

Field-Emission Scanning Electron Microscopy (FE-SEM)

The morphology of the synthesized ZnO NPs was examined using a field-emission scanning electron microscope (SEM) (model S-1640, HITACHI, Japan). The sample was imaged by scanning with a focused electron beam at various magnifications to analyze surface topography and particle size. Prior to analysis, the samples were cleaned and dried. The instrument was operated under high vacuum conditions (10^{-5} mbar), which required approximately 3-4 min to stabilize. The extra high tension (EHT) voltage was set to the required value, which is typically 5 kV. To prevent surface charging, gold was applied to the sample's surface.²²

Antimicrobial effect of ZnO NPs

The antimicrobial activity of ZnO NPs was evaluated using the disc diffusion method. The antimicrobial effects of the cell-free supernatant and ZnO NPs were assessed against *M. globosa* and *C. albicans* on Mueller-Hinton (MH) agar plates. Briefly, the turbidity of each microbial suspension was adjusted to match the 0.5 McFarland standard. Next, 100 μ L of the ZnO NP suspension was transferred to wells created using a sterile 6 mm cork borer. The plates were then incubated at 37 $^{\circ}$ C for 24 hrs, after which the diameter of the inhibition zone was measured as the clear area surrounding the wells where microbial growth was inhibited. Each experiment was performed at least twice.²³

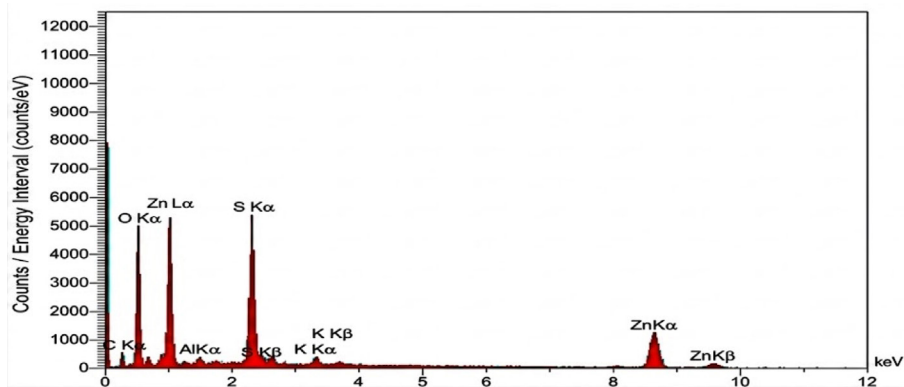


Figure 3. FE-SEM for ZnO nanoparticles

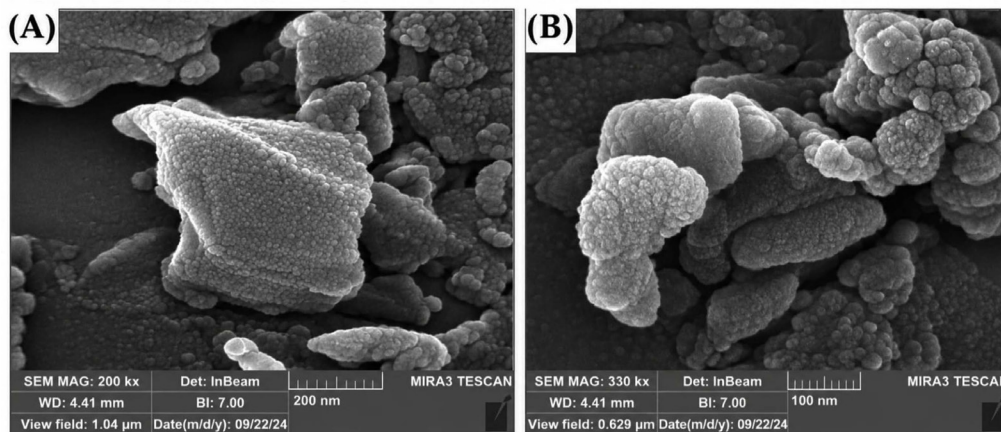


Figure 4. SEM micrographs of the prepared sample at different magnifications: (A) SEM image recorded at 200 k \times magnification (scale bar = 200 nm), showing the platelet-like morphology of the particles; (B) SEM image recorded at 330 k \times magnification (scale bar = 100 nm), showing the surface morphology and particle aggregation in greater detail

Susceptibility of the tested isolates to antibiotics

The test was conducted according to the 2015 CLSI guidelines.²⁴ *M. globosa* and *C. albicans* were cultured on MH agar plates supplemented with ketoconazole and fluconazole.

antifungal effects. Hydroxyl radicals generated in the aqueous NP suspension can disrupt fungal cell membranes, inhibiting growth and potentially causing cell death.²⁴ The growth of the *Lactobacillus* isolate from dairy products on MRS medium is shown in Figure 1.

RESULTS AND DISCUSSION

Microbe-mediated ZnO NPs have antifungal properties similar to their antibacterial activity. The presence of ZnO NPs promotes the generation of ROS, which strengthens their

ZnO NP biosynthesis by *Lactobacillus*

Several studies have explored the synthesis of ZnO NPs using various biological components. After synthesis, the reaction mixture appeared whitish and turbid, with NPs eventually

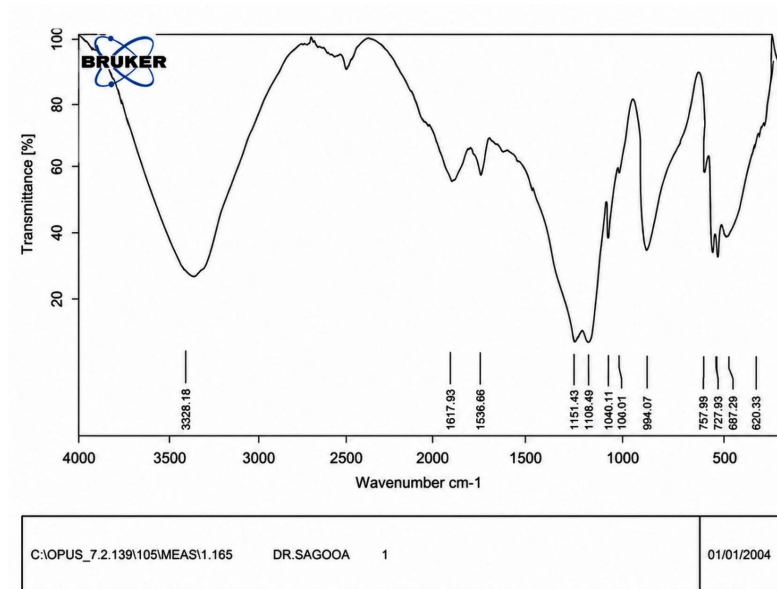


Figure 5. FTIR measurements for ZnO NPs

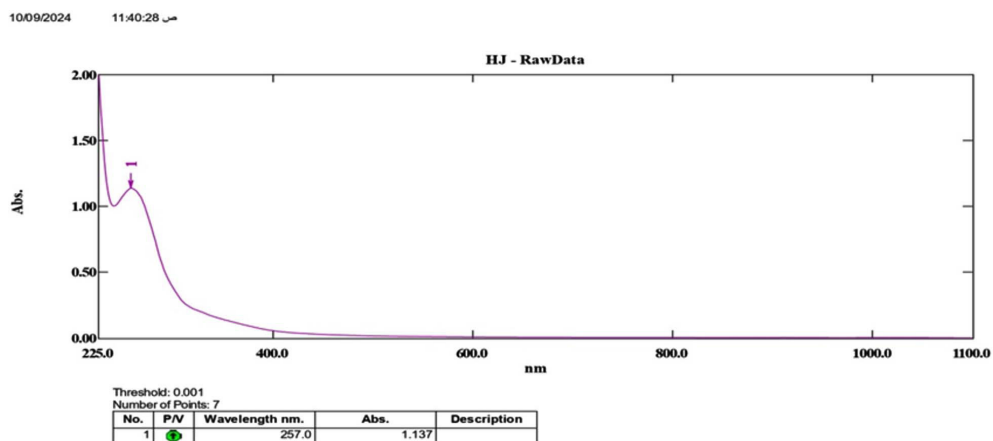


Figure 6. UV-Vis spectroscopic analysis of ZnO NPs

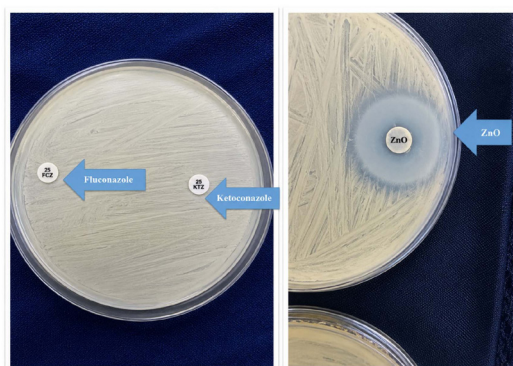


Figure 7. Comparison of antifungal activity between ZnO NPs and antibiotics against *Malassezia globosa*

settling at the bottom of the flask after several hours.²⁵⁻²⁷ The formation of a white pellet, as shown in Figure 2, served as visual confirmation of ZnO NP production. The collected material was placed on a small plate and dried in an oven at 40 °C for 8 hrs, yielding powdered ZnO NPs. These results are consistent with previous studies using *Lactobacillus* for ZnO NP synthesis^{28,29} and align with reported effects observed in similar research.³⁰

Analysis of ZnO NPs Using FE-SEM

FE-SEM was used to examine the morphology and size of ZnO NPs biosynthesized by *Lactobacillus* spp. After synthesis, the NPs were calcined at 500 °C for 1 hrs. Figures 3 and 4 show the FE-SEM images of the ZnO NPs. The images reveal that the NPs are hexagonal in shape, with diameters ranging from 56.14-64.97 nm, consistent with their classification as ZnO NPs.³¹

FTIR analysis

The functional groups involved in the synthesis of ZnO NPs were analyzed using FTIR spectroscopy. It is suggested that interactions between bacterial biomolecules and functional groups on bacterial cells contribute to the formation of ZnO NPs.²⁷ Similarly, Hu et al. reported that functional groups derived from proteins, including -NH₂, -OH, and -COOH, act as binding sites that facilitate zinc reduction (Figure 5).^{32,33}

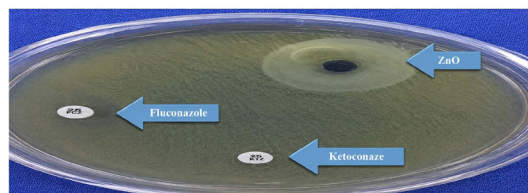


Figure 8. Comparison of antifungal activity between ZnO NPs and antibiotics against *Candida albicans*

UV-Vis diffuse reflectance

The interference that affected the UV/Vis absorbance of the colloidal ZnO NPs at 257 nm was eliminated when the biomass was extracted from the samples. At first, centrifugation of the samples was considered a useful step. Nevertheless, the solution turned yellow at 257 nm, and the absorbance of the colloidal NP suspension dropped dramatically to nearly zero. This could be explained by the NPs adhering to each other or becoming entangled in the biomass. Therefore, centrifugation should be avoided, as it can destroy the NPs. The absorbance of the NPs was measured at 257 nm was measured and corrected before starting further operations. UV spectroscopy was used to track the colloidal ZnO NP solution in the 300-700 nm range (Figure 6).

Antifungal Activity

The ZnO NPs demonstrated remarkable antifungal activity against the tested fungi, resulting in inhibitory zones ranging from 28-29 mm in diameter (Figures 7 and 8). In comparison, the antibiotic controls showed much smaller inhibitory effects, with fluconazole and ketoconazole generating zones of only 6 mm.³⁴⁻³⁶

CONCLUSION

Biosynthesized ZnO-NPs are considered to have significant growth-stimulating potential, in addition to their antimicrobial properties. In this study, a strain of *Lactobacillus* was isolated and identified using several characterization

techniques for its ability to produce ZnO NPs. The results of multiple characterization procedures confirmed the successful synthesis of ZnO NPs with a spherical morphology and average diameters ranging from 56.14-64.97 nm. Furthermore, ZnO-NPs demonstrated strong antifungal activity against *C. albicans* and *M. globosa*, the latter being associated with dandruff on the scalp. These findings suggest that biosynthesized ZnO NPs could serve as a promising alternative for the treatment of various fungal infections. However, additional investigation is required to validate this hypothesis.

ACKNOWLEDGMENTS

The authors express their gratitude to the Department of Pharmacognosy, University of Thi-Qar, Thi-Qar, Iraq, for their valuable support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

SHS and AHF conceptualized the study. HJM and HSY collected resources. MHM, JHA and AJH performed the experiments. BAG supervised the study. HKA wrote the manuscript. HKA and AHK reviewed and revised the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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