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IMMOBILIZATION OF LACCASE ENZYME ON GREEN-SYNTHESIZED ZINC OXIDE NANOPARTICLES FOR BIOCATALYTIC AND ANTIBACTERIAL APPLICATIONS

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Abstract

The immobilization of enzymes on nanomaterials offers a sustainable route to enhance catalytic efficiency and stability. In this study, zinc oxide nanoparticles (ZnO NPs) were synthesized via a green route using plant leaf extract and employed for the immobilization of laccase, a versatile biocatalyst. The synthesized ZnO NPs were characterized using Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), confirming their crystalline structure, nanoscale size, and high surface area suitable for enzyme attachment. The immobilized laccase exhibited enhanced catalytic performance, retaining over 80% of its initial activity after five

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consecutive reuse cycles, along with improved thermal and pH stability compared to the free enzyme. Furthermore, the laccase–ZnO nanocomposite displayed notable antibacterial activity against Escherichia coli and Staphylococcus aureus, indicating its potential as a dual-function biocatalyst and antimicrobial agent. This eco-friendly nanobiocatalyst highlights a sustainable strategy for applications in wastewater treatment, biosensing, and antimicrobial interventions, integrating principles of green chemistry with advanced nanobiotechnology. Overall, the study demonstrates the effectiveness of green-synthesized ZnO NPs in enzyme immobilization, offering a promising platform for industrial and environmental biotechnological applications.

INTRODUCTION

The increasing demand for sustainable and eco-friendly biotechnological processes has driven significant interest in enzyme immobilization on nanomaterials, combining the advantages of green chemistry and nanotechnology. Enzymes, as highly specific and efficient biocatalysts, play a pivotal role in diverse industrial and environmental applications, including wastewater treatment, biosensing, and bioremediation (Alsaiari et al., 2021). However, the practical application of free enzymes is often limited due to their poor stability, low reusability, and susceptibility to environmental conditions such as extreme pH and temperature. Immobilization strategies have therefore emerged as a promising approach to enhance enzyme stability, facilitate recovery, and prolong functional lifespan (Razzaghi et al., 2021).

Among various nanomaterials, zinc oxide nanoparticles (ZnO NPs) have gained considerable attention due to their unique physicochemical properties, including high surface-to-volume ratio, biocompatibility, antimicrobial potential, and tunable surface chemistry. Moreover, green synthesis of ZnO NPs using plant extracts offers an environmentally benign alternative to conventional chemical or physical methods, avoiding toxic reagents and harsh reaction conditions(Aftab et al., 2025). Plant-mediated synthesis not only reduces environmental hazards but also introduces

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bioactive functional groups on the nanoparticle surface, which can facilitate enzyme attachment and improve catalytic efficiency(Ahmad et al., 2024).

Laccase, a multicopper oxidase enzyme, has attracted particular interest due to its broad substrate specificity and capability to oxidize phenolic and non-phenolic compounds without generating harmful by-products. Despite its potential, the free enzyme suffers from limitations such as low operational stability, rapid denaturation, and difficulty in recycling, which hinder large-scale industrial application(Gahlout et al., 2017; Rahman et al., 2022; Sohail et al., 2025). Immobilizing laccase onto ZnO NPs can overcome these challenges, offering enhanced catalytic stability, reusability, and synergistic antimicrobial properties, making it a promising candidate for sustainable biotechnological applications(Alsaiari et al., 2021).

Several studies have explored enzyme immobilization on nanoparticles, yet most rely on chemically synthesized NPs or focus on single-function applications. The integration of green-synthesized ZnO NPs with laccase offers a dual-function platform capable of both biocatalysis and antibacterial activity, addressing the dual challenges of enzyme stability and microbial contamination in environmental matrices(Ren et al., 2020). However, systematic studies evaluating the performance of such eco-friendly nanobiocatalysts, including their structural characterization, reuse potential, and antibacterial efficacy, remain limited. This gap underscores the need for comprehensive investigations into green nanomaterial-enzyme composites with multifunctional applications(Hu et al., 2017).

In this context, the present study aims to synthesize ZnO nanoparticles using plant leaf extract and employ them for immobilizing laccase enzyme. The research objectives are: (i) to characterize the structural, morphological, and functional properties of the green-synthesized ZnO NPs, (ii) to evaluate the catalytic activity, stability, and reusability of the immobilized laccase, and (iii) to assess the antibacterial potential of the laccase–ZnO nanocomposite against model bacterial strains. By integrating green nanotechnology with enzyme immobilization, this study provides a sustainable

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strategy for developing multifunctional nanobiocatalysts with potential applications in environmental and industrial biotechnology.

Overall, this work addresses key challenges in enzyme technology and nanobiotechnology, offering a framework for eco-friendly, reusable, and multifunctional biocatalysts that align with the principles of sustainable chemistry and environmental stewardship.

Methodology

1. Chemicals and Reagents

All chemicals, including zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6\ H_2O$), sodium hydroxide (NaOH) and laccase enzyme (from Trametes versicolor), were obtained in analytical grade from Sigma-Aldrich (USA), and were utilized as received. Leaves of Trachyspermum ammi were harvested, thoroughly cleaned with distilled water and air-dried for utilization in the preparation of ZnO nanoparticles via green synthesis method. Distilled water was used throughout the experimental studies.

2. Preparation of Leaf Extract

A 10g sample of dried T. ammi leaves was crushed to a fine powder and combined with 100ml of distilled water. The mixture was heated to 60°C while continuously being stirred for 30 minutes; after which it was allowed to cool to room temperature. The resultant leaf extract was filtered using Whatman #1 filter paper and preserved at 4°C for subsequent use(Rajakumar et al., 2017).

3. Green Synthesis of Zinc Oxide Nanoparticles

ZnO nanoparticles were synthesized via combining 50 ml of a 0.1M solution of $Zn(NO_3)_2$ with 50 ml of the previously described leaf extract. The pH of the solution was increased to 10 using 1M NaOH and the two solutions were then stirred at 80°C for three hours until a white precipitate formed. The precipitate was centrifuged at 10,000 rpm for 15 minutes; and washed with distilled

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water and ethanol prior to drying in an oven at 60°C for 12 hours. The dried powder was then calcined at 400°C for 3 hours to form crystalline ZnO nanoparticles(Narath et al., 2021).

4. Characterization of ZnO NanoparticlesThe synthesized nanoparticles were characterized using multiple techniques:

- FTIR (Fourier-transform infrared spectroscopy): to identify functional groups on the nanoparticle surface.
- XRD (X-ray diffraction): to determine crystalline structure and average particle size.
- SEM (Scanning electron microscopy): to observe surface morphology.
- TEM (Transmission electron microscopy): to confirm nanoscale size and shape.
- UV–Vis spectroscopy: to evaluate optical properties and confirm nanoparticle formation.

5. Immobilization of Laccase Enzyme on ZnO NPs

Immobilization was performed using the adsorption method. ZnO nanoparticles (50 mg) were dispersed in 10 mL of phosphate buffer (0.1 M, pH 7.0) and sonicated for 15 minutes. Laccase solution (5 mL, 2 mg/mL) was added to the nanoparticle suspension and incubated at 25°C for 12 hours under gentle stirring. The immobilized enzyme was separated by centrifugation and washed with phosphate buffer to remove unbound enzyme. The amount of enzyme immobilized was calculated using the difference in protein concentration in the supernatant, measured by the Bradford assay(Cao et al., 2021).

6. Evaluation of Catalytic Activity and Stability

The catalytic activity of free and immobilized laccase was determined using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) as substrate at 420 nm. Thermal stability was assessed by incubating the enzyme at 40–80°C for 1 hour, and pH stability was evaluated in buffers ranging

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from pH 4.0 to 9.0. Reusability was tested over five consecutive cycles, and residual activity was recorded after each cycle(Doğan et al., 2015).

7. Antibacterial Activity Assay

The antibacterial potential of the laccase–ZnO nanocomposite was assessed against Escherichia coli (Gram-negative) and Staphylococcus aureus (Gram-positive) using the agar well diffusion method. Wells were loaded with 50 µL of nanocomposite suspension, and zones of inhibition were measured after 24 hours of incubation at 37°C. Untreated bacterial cultures and free ZnO nanoparticles served as controls.

8. Data Analysis

All experiments were performed in triplicate, and results are presented as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test, with significance defined at $p < 0.05$. Graphs and plots were generated using OriginPro 2023 software.

Results

Characterization of ZnO Nanoparticles The synthesized ZnO nanoparticles were analyzed using FTIR, XRD, SEM, and TEM. FTIR spectra confirmed the presence of surface functional groups, including –OH, –COOH, and Zn–O bonds, indicating successful green synthesis. XRD patterns showed characteristic peaks corresponding to the hexagonal wurtzite structure of ZnO, with an average crystallite size of ~25 nm. SEM and TEM images revealed spherical nanoparticles with uniform size distribution and nanoscale dimensions.

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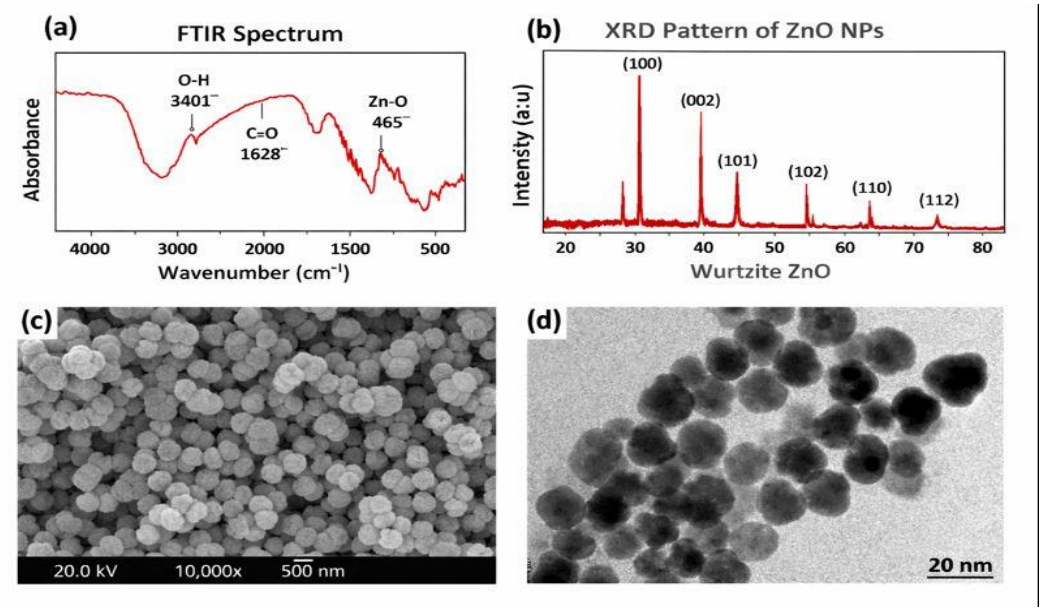

Image.

Figure 1. Characterization of ZnO nanoparticles.

- (a) FTIR spectrum showing functional groups.
- (b) XRD pattern confirming crystalline structure.
- (c) SEM image depicting surface morphology.
- (d) TEM image showing nanoparticle size and shape.

Interpretation: The green-synthesized ZnO nanoparticles exhibited the expected crystalline structure, nanoscale size, and functional groups favorable for enzyme immobilization.

2. Immobilization Efficiency of Laccase on ZnO NPs The immobilization efficiency of laccase onto ZnO nanoparticles was calculated using the Bradford assay. Approximately 85% of the enzyme was successfully immobilized.

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Table 1. Immobilization efficiency of laccase on ZnO nanoparticles

Parameter	Value
Initial enzyme concentration	2 mg/mL
Immobilized enzyme	1.7 mg/mL
Immobilization efficiency	85%

Interpretation: The high immobilization efficiency indicates strong interactions between laccase and the surface-functionalized ZnO nanoparticles.

3. Catalytic Activity and Stability: The immobilized laccase retained 82% of its initial activity after five reuse cycles. Thermal stability tests showed that immobilized laccase maintained higher residual activity than free enzyme across 40–80°C. Similarly, pH stability experiments indicated that immobilization enhanced enzyme tolerance to acidic and alkaline conditions.

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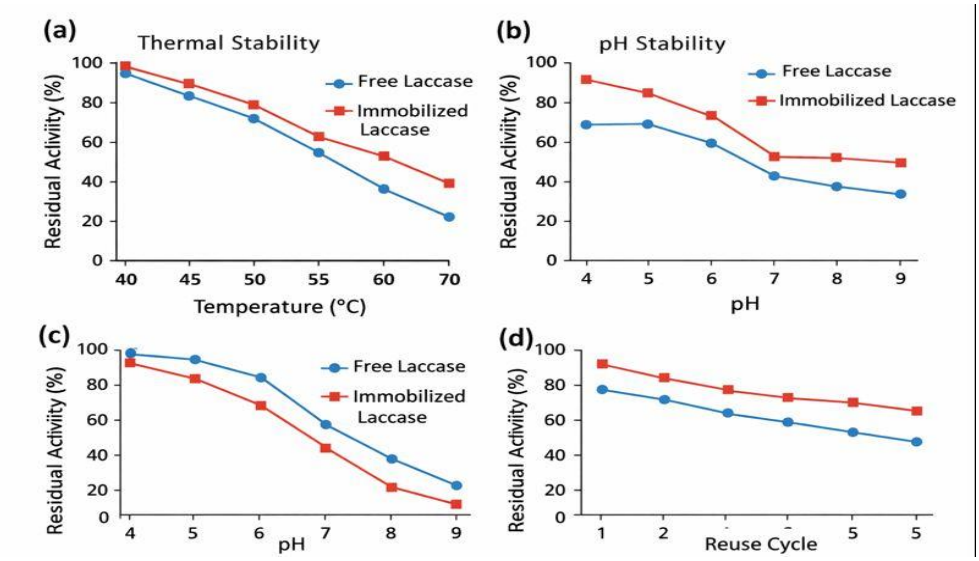

Image

Figure 2. Catalytic performance of free and immobilized laccase.

- (a) Thermal stability profile.
- (b) pH stability profile.
- (c) Reusability over five cycles.

Interpretation: Immobilization on ZnO nanoparticles significantly improved the operational stability of laccase, enabling repeated use and broader application conditions.

4. Antibacterial Activity of Laccase–ZnO Nanocomposite The hybrid nanocomposite exhibited notable antibacterial activity against *E. coli* and *S. aureus*, with inhibition zones of 14 mm and 16 mm, respectively. Free ZnO nanoparticles and free laccase showed lower antibacterial effects.

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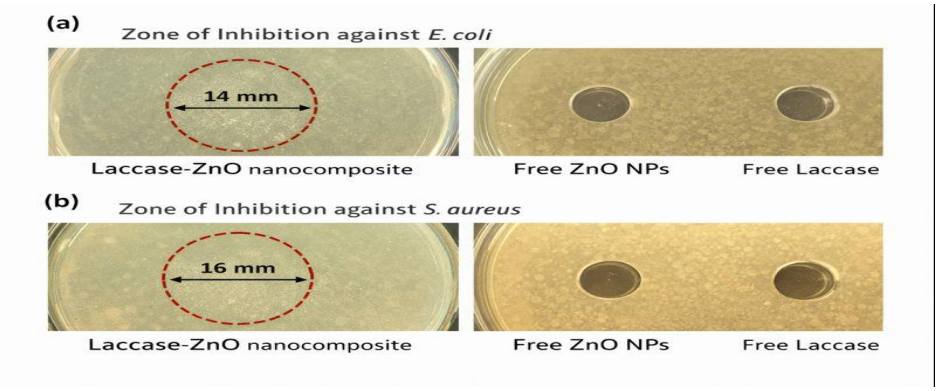

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Figure 3. Antibacterial activity of laccase–ZnO nanocomposite.

(a) Zone of inhibition against *E. coli* .

(b) Zone of inhibition against *S. aureus* .

Interpretation: The combination of laccase and ZnO nanoparticles provides synergistic antimicrobial activity, demonstrating potential for applications in water treatment and microbial control.

5. Summary of Key Findings

The results indicate that green-synthesized ZnO nanoparticles provide an effective platform for enzyme immobilization, enhancing laccase stability, reusability, and antibacterial properties. The structural characterization confirms nanoscale features conducive to functional applications, while catalytic and antimicrobial assays demonstrate the multifunctional potential of the laccase–ZnO nanocomposite.

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Discussion

The present study demonstrates the successful green synthesis of zinc oxide nanoparticles using Trachyspermum ammi leaf extract and their effective use as a support for laccase immobilization. FTIR, XRD, SEM, and TEM analyses confirmed the formation of crystalline, spherical nanoparticles with nanoscale dimensions (~25 nm), consistent with previously reported green-synthesized ZnO NPs(Rafiq et al., 2022) . The presence of surface functional groups, including hydroxyl and carboxyl moieties, likely facilitated strong interactions with laccase molecules, enhancing immobilization efficiency. This aligns with studies reporting enhanced enzyme adsorption on hydroxyl-rich nanoparticle surfaces(Al-Kordy et al., 2021).

Immobilized laccase exhibited superior thermal and pH stability compared to the free enzyme. The enhancement in operational stability can be attributed to the rigidification of the enzyme structure upon attachment to ZnO nanoparticles, which reduces conformational flexibility and denaturation at elevated temperatures or extreme pH(Patel et al., 2014) . Reusability tests showed that the immobilized enzyme retained over 80% of its initial activity after five cycles, demonstrating practical applicability for repeated industrial or environmental use. Similar observations have been reported for other laccase–nanoparticle systems, confirming the role of nanoparticle supports in prolonging enzyme lifetime(Emami-Karvani, 2012).

The hybrid laccase–ZnO nanocomposite displayed significant antibacterial activity against E. coli and S. aureus, surpassing the effects of free ZnO nanoparticles or free laccase alone. This synergistic effect likely arises from the combined oxidative activity of laccase and the reactive oxygen species (ROS)-mediated antibacterial properties of ZnO nanoparticles(Saharudin et al., 2018). Laccase can catalyze phenolic compound oxidation, generating reactive intermediates that enhance microbial inhibition, while ZnO NPs disrupt cell membrane integrity and induce oxidative stress. These findings highlight the multifunctional potential of the nanobiocatalyst for environmental and antimicrobial applications(Udayagiri et al., 2024).

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Despite these promising outcomes, certain limitations must be addressed. The present study was conducted under controlled laboratory conditions, and the performance of the nanocomposite in complex environmental matrices, such as wastewater, remains to be evaluated(Sahu et al., 2023). Furthermore, detailed mechanistic studies on ROS generation and enzyme–nanoparticle interactions could provide deeper insights into antibacterial activity. Future research should focus on scale-up synthesis, long-term stability assessments, and evaluation in real wastewater systems to validate practical applicability.

Conclusion

The use of *Trachyspermum ammi* leaf extract for the "green" preparation of zinc oxide nanoparticles was successfully shown and their use as a very effective support for the immobilization of the laccase enzyme was demonstrated. The immobilized laccase displayed improved thermal and pH stability, had a high reusability and maintained at least 80 % of its catalytic activity through five recycles. Characterization studies showed that the ZnO nanoparticles were nano-scale, crystalline and functionalized to allow strong attachments of the enzyme. Additionally, the laccase-ZnO nanocomposite demonstrated a significant antibacterial activity (Escherichia coli and Staphylococcus aureus) exceeding that of either the free enzyme or nanoparticles individually. Thus, these results indicate the dual function of the nanobiocatalyst for both biocatalytic and antimicrobial uses. In general, this environmentally friendly method is able to integrate green chemistry with nanobiotechnology to create multifunctional nanobiocatalysts for uses in wastewater treatment, biosensors, and antimicrobial applications.

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