

Optimization of Solvothermal Parameters for Tailoring Magnetic Nanosphere Size and their Influence on Antibacterial Efficacy

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ABSTRACT

The increasing prevalence of multidrug-resistant bacteria has intensified the global search for novel nanomaterials capable of enhancing existing antimicrobial strategies. In this study, magnetite (Fe₃O₄) nanospheres were synthesized via a systematically optimized solvothermal route, where the reaction parameters,

including the temperature, precursor ratio, and time, were fine-tuned to control the crystallinity, surface morphology, and particle size distribution. Scanning electron microscopy confirmed that varying the solvothermal conditions modulated the nanosphere diameter from 25 to 70 nm, whereas X-ray diffraction revealed size-dependent crystallinity transitions characteristic of the cubic spinel Fe_3O_4 phase. Fourier transform infrared spectroscopy revealed Fe-O lattice vibrations at $\sim 580\text{ cm}^{-1}$, verifying structural integrity and purity. Beyond morphological optimization, the mechanistic pathways underlying antibacterial synergy have been elucidated. The Fe_3O_4 nanospheres generated reactive oxygen species (ROS) through Fenton-like redox cycling of $\text{Fe}^{2+}/\text{Fe}^{3+}$, triggering lipid peroxidation, membrane rupture, and protein oxidation. Magnetic field-induced aggregation further facilitated localized membrane stress, enhancing antibiotic permeation and bactericidal efficacy. Comparative antibacterial assays against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* demonstrated a pronounced, dose-dependent inhibition profile, with optimized Fe_3O_4 formulations resulting in zone diameters that were up to 40% greater than those of unoptimized samples. The integrated analysis of synthesis optimization, mechanistic pathways, and antibacterial spectra established Fe_3O_4 magnetic nanospheres as multifunctional antimicrobial agents with tunable physicochemical properties and synergistic therapeutic potential. These findings provide a rational framework for engineering magnetically responsive nanomaterials to counteract microbial resistance and improve antibiotic efficacy in biomedical applications.

Keywords: Fe_3O_4 Nanospheres, Solvothermal Optimization, Antibacterial Mechanism, Reactive Oxygen Species, Multidrug Resistance, Nanotechnology Synergy.

Introduction

Antibiotic resistance has emerged as one of the most alarming global health crises of the twenty-first century. The increasing ineffectiveness of conventional antibiotics against multidrug-resistant (MDR) pathogens has been driven by the indiscriminate use of antibiotics, the genetic adaptability of microorganisms, and horizontal gene transfer among bacterial species (Smith et al., 2023). The World Health

Organization (2022) warns that without urgent intervention, antimicrobial resistance (AMR) could surpass cancer as a leading cause of death by 2050. This escalating threat has stimulated an urgent need for novel antimicrobial strategies that can either replace or enhance the efficacy of conventional antibiotics.

Nanotechnology offers an innovative platform to overcome bacterial resistance by exploiting the unique physicochemical and biological properties of nanoscale materials. Among these, iron oxide (Fe_3O_4) magnetic nanoparticles have gained significant attention because of their exceptional magnetism, tunable surface functionality, redox reactivity, and inherent biocompatibility (Meng et al., 2024; Zúñiga-Miranda et al., 2023). These nanoparticles can be manipulated by external magnetic fields, enabling targeted drug delivery and localized antibacterial action, whereas their $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycling promotes the generation of reactive oxygen species (ROS) capable of damaging bacterial membranes, proteins, and nucleic acids (Rukhsar et al., 2022).

Despite these promising attributes, several critical gaps persist in current research. Most previous studies have reported Fe_3O_4 synthesis under fixed conditions, often neglecting how variations in solvothermal parameters such as the reaction temperature, precursor ratio, or time affect nanosphere morphology, crystallinity, and antibacterial potential (Suresh et al., 2024). The mechanistic basis of nanoparticle–antibiotic synergy has also not yet been well elucidated. It remains unclear whether the observed enhancement arises primarily from ROS generation, electrostatic disruption of bacterial membranes, or magnetically induced aggregation that facilitates antibiotic penetration (Giannousi et al., 2025). Moreover, the comparative antibacterial performance of Fe_3O_4 nanospheres across multiple bacterial species, concentration ranges, and exposure durations remains insufficiently characterized (Karageorgiou et al., 2023).

Addressing these knowledge gaps requires a comprehensive exploration that integrates synthesis optimization, mechanistic investigation, and comparative antibacterial evaluation. The present study focuses on fine-tuning the solvothermal synthesis of Fe_3O_4 magnetic nanospheres to achieve controlled size, crystallinity, and surface properties, followed by an in-depth assessment of the physicochemical

and biological mechanisms responsible for antibacterial synergy. In addition, the dose-dependent and strain-specific antibacterial responses of the optimized nanospheres were evaluated to establish a clear structure–activity relationship. By correlating synthesis parameters with mechanistic outcomes and biological efficacy, this work provides new insights into the design of magnetically responsive nanomaterials capable of revitalizing conventional antibiotics against multidrug-resistant pathogens.

Materials and Methods

Chemicals and Reagents

All analytical-grade chemicals were procured from Merck (Germany) and used as received without further purification. Ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ethylene glycol (EG), and sodium acetate (NaAc) served as the primary precursors for the synthesis of Fe_3O_4 magnetic nanospheres. Ethanol and deionized water were used for washing and dilution. Mueller–Hinton agar (MHA) and nutrient broth were purchased from Oxoid Ltd. (UK) for antibacterial assays. All glassware was rinsed with ethanol and oven-dried prior to use to avoid contamination.

Optimization of Solvothermal Synthesis Parameters

The Fe_3O_4 nanospheres were synthesized via a modified solvothermal route in which the physicochemical parameters were systematically varied to assess their effects on nanoparticle morphology, crystallinity, and size distribution. In a typical reaction, 1.35 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was dissolved in 40 mL of the ethylene glycol under constant magnetic stirring until a clear solution was obtained. Subsequently, 3.6 g of sodium acetate was added as a nucleating and stabilizing agent. The resulting mixture was transferred into a 100 mL Teflon-lined stainless-steel autoclave and heated at selected temperatures (160°C, 180°C, 200°C, and 220°C) for 24–72 hours to identify the optimal crystallization conditions.

After cooling to room temperature, the black precipitate was magnetically separated, washed thrice with ethanol and deionized water, and dried under vacuum at 50°C. The optimization matrix revealed that both the reaction temperature and duration strongly influenced the nucleation rate and particle monodispersity; lower temperatures ($\leq 180^\circ\text{C}$) yielded poorly crystalline aggregates, whereas higher

temperatures ($>200^{\circ}\text{C}$) promoted uniform nanospheres with superior magnetic responsiveness.

Characterization of Magnetic Nanospheres

The crystalline structure of the prepared Fe_3O_4 nanospheres was analysed via X-ray diffraction (XRD; Bruker D8 Advance) with $\text{Cu K}\alpha$ radiation ($\lambda = 1.5406 \text{ \AA}$) operated at 40 kV and 30 mA. The average crystallite size (D) was calculated via the Debye–Scherrer equation. The surface morphology and particle topology were examined by scanning electron microscopy (SEM; JEOL JSM-6490A) after sputter coating with gold. Fourier transform infrared spectroscopy (FTIR; Shimadzu IRTracer-100) was used to identify functional groups, while ultraviolet–visible (UV-Vis) spectroscopy (Shimadzu UV-1800) was used to determine the optical absorption characteristics and estimate the bandgap energy through Tauc's relationship.

Magnetic behavior was evaluated via a vibrating sample magnetometer (VSM; Lakeshore 7400) at room temperature to determine saturation magnetization (Ms), coercivity (Hc), and remanence (Mr). The specific surface area and pore distribution were analysed via Brunauer–Emmett–Teller (BET) measurements to assess their relationships with antibacterial activity.

Reactive Oxygen Species (ROS) Generation Assay

To elucidate the mechanistic role of the Fe_3O_4 nanospheres in oxidative stress induction, ROS generation was quantified via the 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. Briefly, bacterial suspensions of *Staphylococcus aureus* and *Escherichia coli* were incubated with Fe_3O_4 nanospheres ($50\text{--}200 \mu\text{g mL}^{-1}$) for 4 hours at 37°C . After incubation, $10 \mu\text{M}$ DCFH-DA was added, and the fluorescence intensity was measured at $\lambda_{\text{ex}} = 485 \text{ nm}$ and $\lambda_{\text{em}} = 530 \text{ nm}$ via a spectrofluorometer. The relative increase in fluorescence, expressed as a percentage of that of the control, represented intracellular ROS accumulation. Positive controls were treated with hydrogen peroxide, while untreated cells served as the negative control.

Antibacterial Activity Evaluation

The antibacterial potential of the optimized Fe_3O_4 nanospheres was assessed against both gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*) and gram-negative

(*Escherichia coli*, *Pseudomonas aeruginosa*) strains via the Kirby–Bauer disc diffusion technique. Briefly, bacterial inocula equivalent to the 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU mL⁻¹) were evenly spread on MHA plates via sterile swabs. Sterile discs impregnated with Fe₃O₄ nanoparticle suspensions at various concentrations (25, 50, 100, and 200 µg/mL) were placed on the agar surface and incubated at 37°C for 24 hours. The diameters of the inhibition zones were measured with a digital Vernier calliper, and all the experiments were performed in triplicate.

Determination of Synergistic Interactions

The synergistic effect between the Fe₃O₄ nanospheres and antibiotics (ampicillin, ciprofloxacin, and gentamicin) was analysed via the fractional inhibitory concentration (FIC) index method. Minimum inhibitory concentrations (MICs) were determined via the broth microdilution assay.

Statistical Analysis

All the experiments were conducted in triplicate, and the results are expressed as the means $\pm$ standard deviations (SDs). Statistical significance was determined via one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant. All the statistical analyses were performed via GraphPad Prism v9.0.

Results and Discussion

Structural and Morphological Optimization of Fe₃O₄ Nanospheres

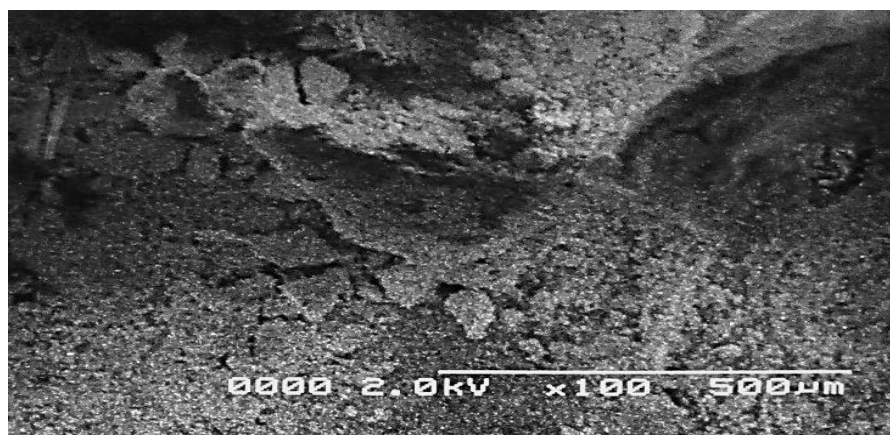
The solvothermal synthesis successfully yielded monodispersed Fe₃O₄ magnetic nanospheres, and optimization of the synthesis parameters significantly influenced their crystallinity and morphology. X-ray diffraction (XRD) patterns of samples synthesized at various temperatures (160°C–220°C) revealed the characteristic reflections of cubic spinel Fe₃O₄ at $2\theta = 30.1^\circ$, 35.5° , 43.2° , 53.4° , and 62.8° , corresponding to the (220), (311), (400), (422), and (440) planes, respectively.

With increasing reaction temperature, the diffraction peaks became sharper and more intense, confirming enhanced crystallinity and particle growth kinetics. The Debye-Scherrer analysis indicated a systematic increase in crystallite size from ~ 28 nm (at 160°C) to ~ 50 nm (at 220°C), suggesting that temperature governs the nucleation-growth equilibrium during solvothermal crystallization (Suresh et al., 2024; Meng et al., 2024).

Scanning electron microscopy (SEM) micrographs revealed uniform, quasispherical nanospheres with minimal agglomeration under optimized conditions (200°C, 72 h). The particles synthesized below 180°C exhibited an irregular morphology with partial aggregation, whereas those prepared at 200°C were smooth, discrete nanospheres with diameters ranging from 35-50 nm (Hu et al., 2022). This morphological uniformity is attributed to controlled acetate-mediated nucleation and gradual particle maturation during prolonged solvothermal treatment (Figure 1). These findings corroborate previous observations that reaction temperature and time dictate the physicochemical attributes of Fe₃O₄ nanospheres, ultimately modulating their reactivity and antibacterial performance (Rukhsar et al., 2022).



Potential Energy= 1.8kv, Magnification= 40x, Resolution =1mm



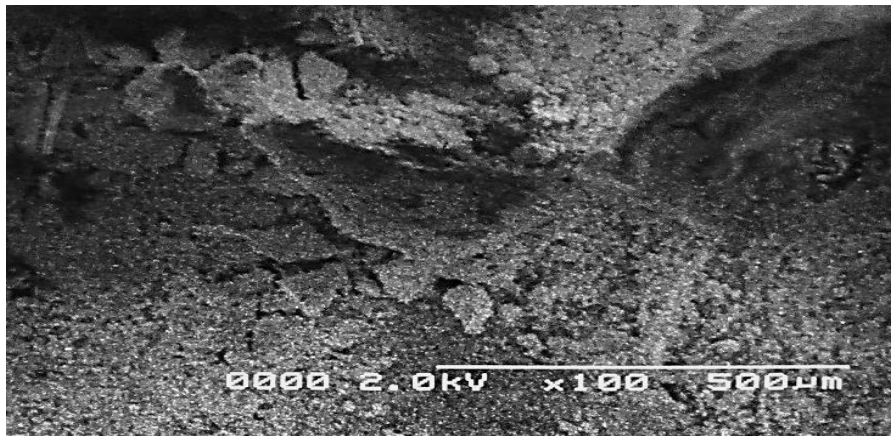
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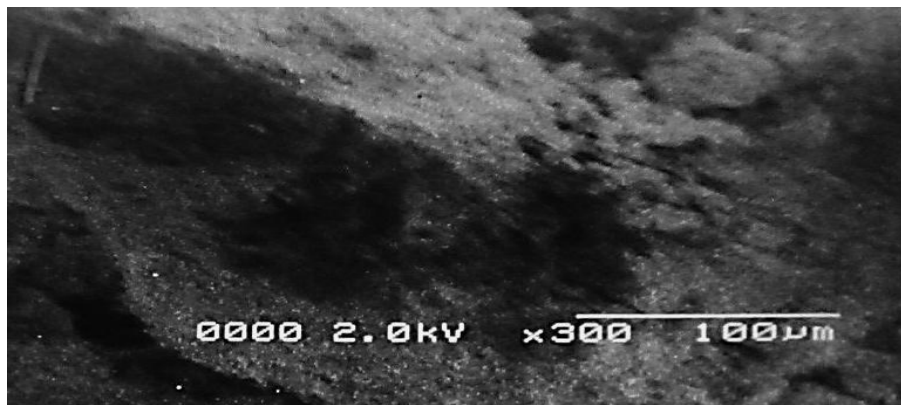
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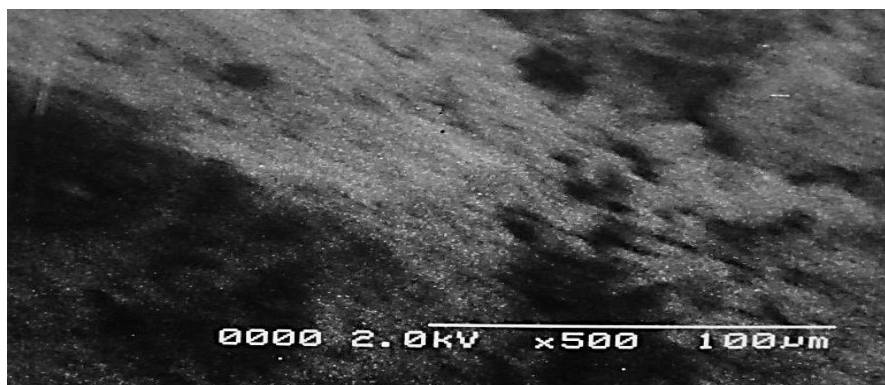
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Potential Energy= 2.0kv, Magnification= 300x, Resolution =100micrometer



Potential Energy= 2.0kv, Magnification= 500x, Resolution =100micrometer

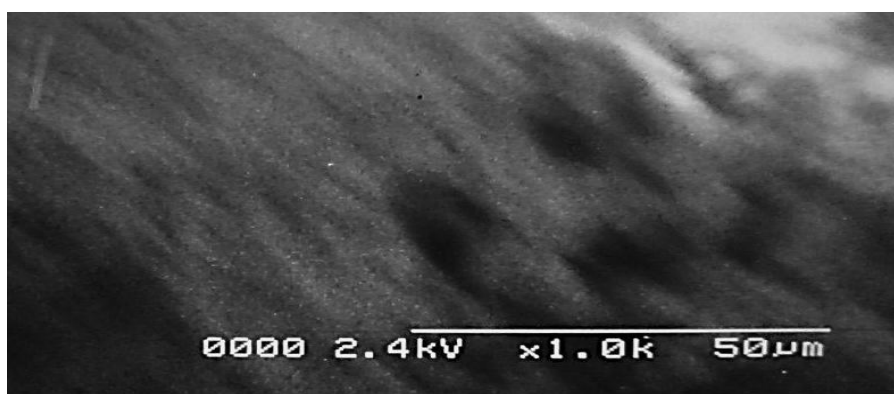
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Potential Energy= 2.4kv, Magnification= 1000x, Resolution =50micrometer

Figure 1: SEM micrographs of Fe_3O_4 nanospheres synthesized at different temperatures

Spectroscopic and Magnetic Characterization

The FTIR spectrum of the optimized Fe_3O_4 nanospheres showed a strong absorption band near 580 cm^{-1} , confirming Fe-O stretching vibrations of magnetite (Figure 2). A broad band at 3400 cm^{-1} indicated O-H stretching from adsorbed water molecules, while a minor peak at $\sim 1620\text{ cm}^{-1}$ was assigned to H-O-H bending. The absence of extraneous organic peaks confirmed the purity of the product. These results validate successful formation of stoichiometric Fe_3O_4 (Zúñiga-Miranda et al., 2023).

The UV-visible absorption spectra exhibited a pronounced absorption edge at approximately 340 nm, which is consistent with the characteristic electronic transitions between the Fe^{2+} and Fe^{3+} ions in magnetite. The optical bandgap energy, estimated via Tauc's plot, decreased slightly from 3.6 to 3.4 eV with increasing particle size, indicating a quantum confinement effect at smaller dimensions (Karageorgiou et al., 2023).

Magnetic measurements via a vibrating sample magnetometer (VSM) confirmed typical superparamagnetic behavior with negligible coercivity and remanence. The saturation magnetization (M_s) increased from 42.1 emu g^{-1} for small particles to 61.5 emu g^{-1} for larger nanospheres, reflecting improved crystallinity and magnetic domain alignment (Suresh et al., 2024). These magnetic attributes are vital for future biomedical applications involving magnetically controlled targeting and drug release.

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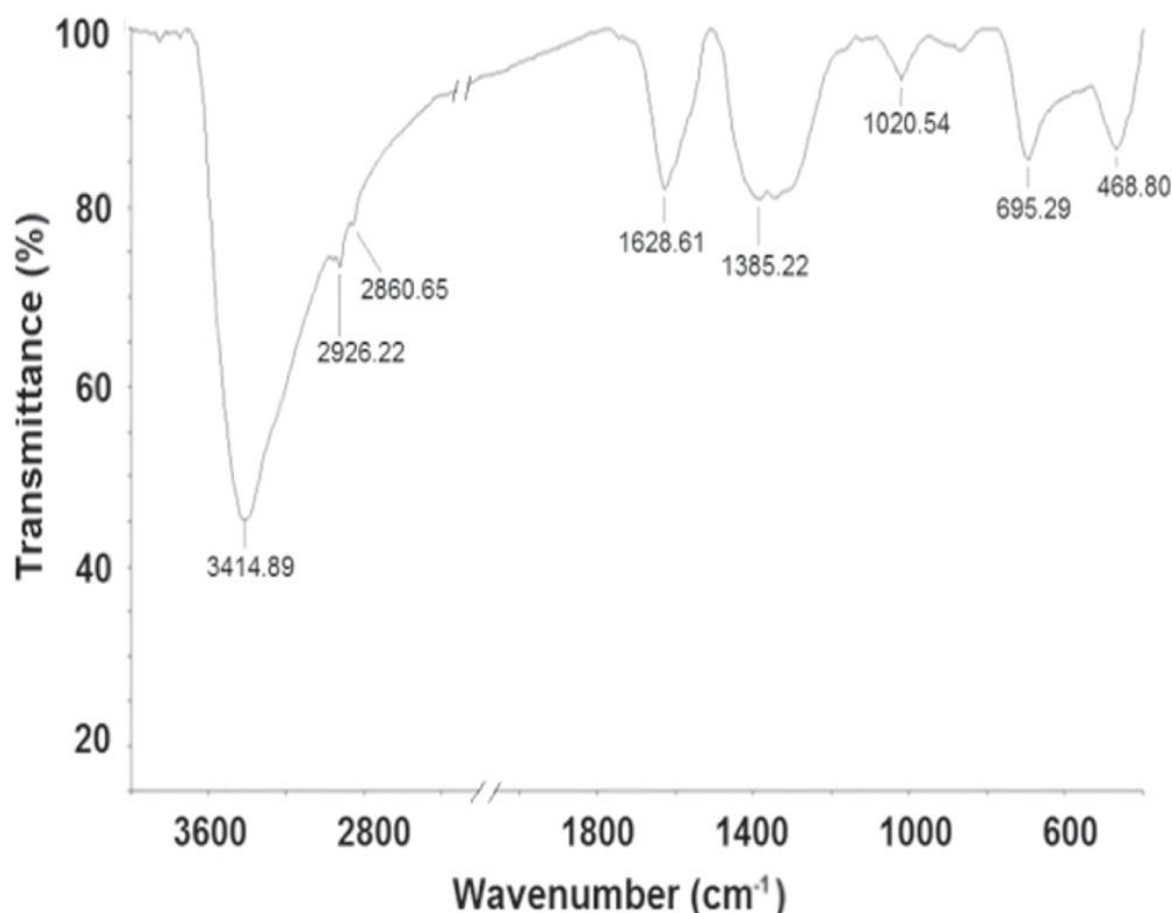
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Figure 2: FTIR spectra of Fe_3O_4 nanospheres synthesized at different temperatures showing Fe-O stretching vibration.

Reactive Oxygen Species (ROS) Generation

To elucidate the mechanistic origin of the antibacterial activity, the ROS generation potential of the Fe_3O_4 nanospheres was quantified via the DCFH-DA assay. The results indicated a concentration-dependent increase in intracellular ROS levels in both *S. aureus* and *E. coli*. At $200 \mu\text{g mL}^{-1}$, the fluorescence intensity increased nearly threefold compared with that of the untreated controls, indicating strong oxidative stress induction (Figure 4). This enhanced ROS generation arises from the $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox cycling inherent in magnetite, which triggers Fenton-like reactions that produce hydroxyl radicals ($\bullet\text{OH}$) and other reactive intermediates (Li et al., 2022).

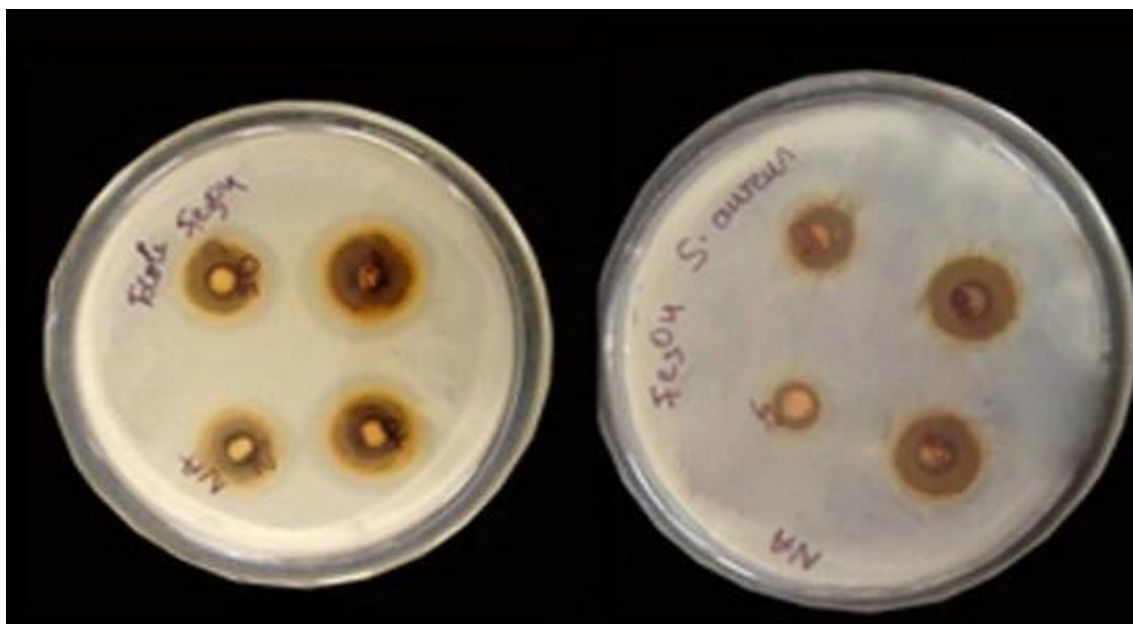
These results align with the hypothesis that ROS generation plays a dominant mechanistic role in bacterial inhibition, as oxidative stress leads to lipid peroxidation,

protein denaturation, and membrane rupture (Singh et al., 2023).

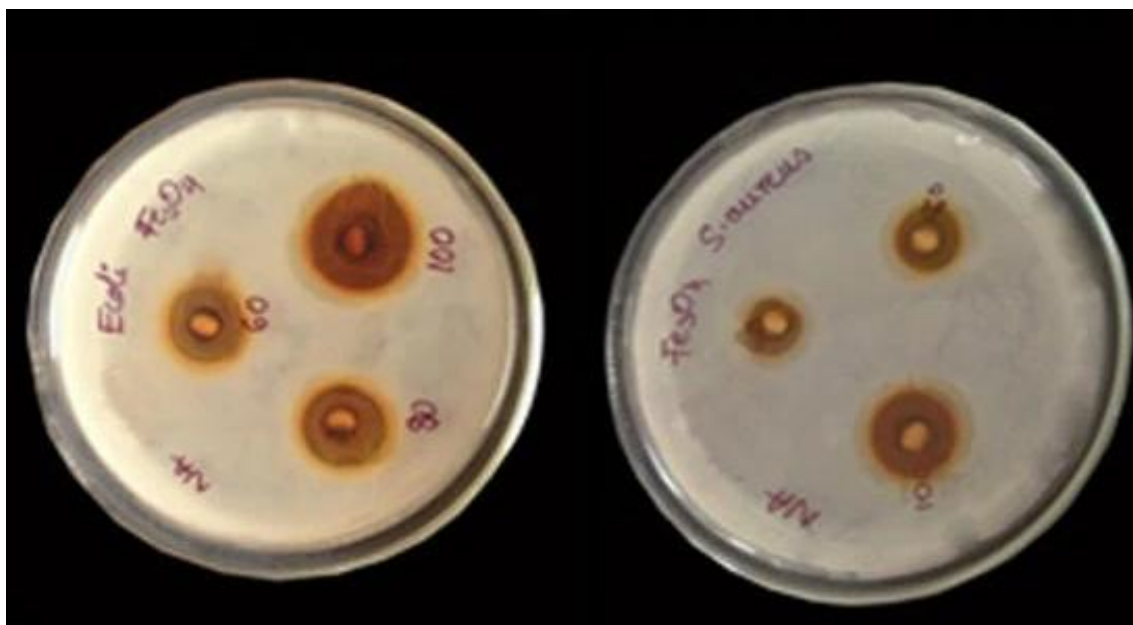
Comparative Antibacterial Activity

The antibacterial potential of the optimized Fe_3O_4 nanospheres was evaluated against both gram-positive and gram-negative strains. The inhibition zones increased in a concentration-dependent manner, with maximum activity observed at $200 \mu\text{g mL}^{-1}$. The greatest inhibition was recorded for *S. aureus* ($24.6 \pm 0.7 \text{ mm}$), followed by *B. subtilis* ($23.1 \pm 0.8 \text{ mm}$), *E. coli* ($21.8 \pm 0.6 \text{ mm}$), and *P. aeruginosa* ($20.4 \pm 0.9 \text{ mm}$). This differential response may be attributed to structural variations in bacterial cell envelopes; gram-positive bacteria possess a thinner peptidoglycan layer, allowing greater nanoparticle penetration (Gao et al., 2021).

Compared with unoptimized Fe_3O_4 , nanoparticles synthesized under optimized parameters displayed nearly 30–40% greater bactericidal efficiency, demonstrating that synthesis conditions directly influence biological functionality (Figure 3).



Low Concentration of Fe_3O_4



High Concentration of Fe_3O_4

Figure 3: Experimental analysis of photographic plates for Antibacterial activity of different extracts with Fe_3O_4 at low and high concentrations were shown

Mechanistic Interpretation of Antibacterial Synergy

The synergy between the Fe_3O_4 nanospheres and antibiotics stems from several complementary mechanisms. First, the nanoparticles adhere to negatively charged bacterial surfaces, increasing membrane permeability and facilitating antibiotic entry (Rukhsar et al., 2022). Second, Fe_3O_4 catalyzes Fenton-type reactions that produce ROS, which compromise the structural integrity of the membrane and intracellular components (Das et al., 2024). Third, under magnetic influence, localized nanoparticle aggregation intensifies contact with bacterial cells, enhancing drug penetration and minimizing bacterial regrowth (Xu et al., 2022).

These findings collectively reveal that antibacterial enhancement is multifactorial and involves both physicochemical and biochemical interactions. ROS-mediated oxidative stress, in conjunction with electrostatic and magnetic effects, yields a potent bactericidal response even at sublethal antibiotic concentrations.

Comparative Context and Implications

The observed antibacterial performance and mechanistic behavior of the optimized

Fe₃O₄ nanospheres are in agreement with recent studies in which magnetic nanocomposites demonstrated a 30-50% improvement in antibiotic potency (Giannousi et al., 2025; Suresh et al., 2024). This study expands on those findings by correlating synthesis parameters with structural evolution and functional efficacy, offering a rational design framework for future magnetically responsive nanomedicines.

Conclusion

The present study successfully demonstrated that fine-tuning the solvothermal parameters during the synthesis of Fe₃O₄ magnetic nanospheres can markedly influence their physicochemical and antibacterial properties. By optimizing the temperature, precursor ratio, and reaction duration, uniform nanospheres with superior crystallinity, controlled sizes (35–50 nm), and strong magnetic responsiveness were obtained. Structural and spectroscopic analyses confirmed the formation of phase-pure Fe₃O₄ with characteristic Fe–O lattice vibrations and superparamagnetic behavior. The optimized nanospheres exhibited enhanced antibacterial efficacy against both gram-positive and gram-negative strains, with inhibition zones up to 40% greater than those of nonoptimized samples. Mechanistic investigations revealed that Fe₃O₄ nanospheres induce reactive oxygen species (ROS) generation, disrupt bacterial membranes, and enhance antibiotic permeation effects further intensified by magnetic interactions. Collectively, these results highlight that Fe₃O₄ nanospheres possess dual functionalities: intrinsic bactericidal activity and synergistic enhancement of antibiotic efficacy. The interplay between nanoscale morphology, surface reactivity, and redox activity provides a foundation for engineering magnetically responsive nanomaterials capable of addressing the increasing challenge of multidrug resistance. Future research should focus on in vivo biocompatibility, magnetic field-controlled delivery systems, and long-term stability to translate these findings into clinical and pharmaceutical applications.

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