RESEARCH PAPER

Biosynthesis of Iron Oxide Nanoparticles Using *Bacillus thermotolerans* and Their Antimicrobial Potential Against Multidrug-Resistant *Klebsiella Pneumoniae*

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ABSTRACT

Resistance to most antibiotics by pathogenic microbes has become a major international problem, making it necessary to find effective treatments. These resistant diseases must be stopped by effective microorganisms. A strong isolate (N11) was chosen from 30 probiotic strains screened for antibacterial activity. *Bacillus thermotolerans* was identified by appearance, microscopic inspection, and DNA sequencing of isolate N11. This study examined *B. thermotolerans*' iron oxide nanoparticle production. Their MDR action against *Klebsiella pnemoniae* from diabetic foot infections was examined. Morphological, biochemical, and VITEK2 systems revealed MDR *Klebsiella pnemoniae* isolates. UV-Vis spectrophotometry, FTIR, FSEM, AFM, and XRD confirmed IONP biosynthesis. IONPs from *B. thermotolerans* were effective against MDR *Klebsiella pnemoniae*. FSEM analysis showed particle sizes of 25.31–64.25 nm, averaging 42 nm. AFM measured 7.677 nm particle size, and XRD measured 20 nm. Antibacterial tests revealed a maximum inhibition zone of 31 mm at 500 µg/mL against *Klebsiella pneumoniae*. Additionally, the IONPs exhibited antibiofilm activity with the highest recorded biofilm inhibition of 97.50% at 1000 µg/mL, while no haemolysis activities in all concentrations. These findings confirm the effectiveness of biosynthesized IONPs as a potential therapeutic agent for treating antimicrobial resistance in MDR pathogens.

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INTRODUCTION

Diabetic Foot Ulcer (DFU) is one of the most significant diabetic complications with serious consequences. Improper management of DFU can lead to osteoporosis, gangrene, and amputation. For amputees, the risk of mortality increases, and survivors are more susceptible to microbial resistance. There is an increasing struggle over selecting the most effective antibiotic(s) for DFU. Factors associated with mortality in patients with DFU, as well as antimicrobial resistance in affected individuals, remain critical research priorities [1,2]. Antimicrobial resistance (AMR) has existed since the discovery of the first antibiotic, "penicillin," in 1940, as part of bacteria's natural evolutionary process. Genes for resistance existed in healthy bacterial species millions of years ago. However, AMR has now become a significant global health concern, largely due to the irrational overuse and abuse of antibiotics, which leads to prolonged hospital stays, economic strain, and even fatal outcomes [3-6].

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Antibiotic resistance is one of the most critical public health threats worldwide. It is estimated to cause 700,000 deaths annually and may rise to 10 million deaths per year by 2050 [7,8]. In the United States alone, at least 2 million people annually become infected with antibiotic-resistant microorganisms, resulting in at least 23,000 deaths [9]. The impact of AMR is disproportionately higher in poorer countries with underdeveloped healthcare systems [10-12]. In recent years, antibiotic consumption has continued to rise in many low- and middle-income countries [13,14]. Even when antibiotics are used correctly, their overuse can still lead to resistance. This creates an urgent need to develop innovative strategies and technologies to combat microbial resistance [15-22].

Nanotechnology offers promising solutions to global challenges, including AMR. Its advanced techniques can support many biomedical applications including drug and vaccine delivery [23,24], antibacterial activity [25-33], cancer treatment [34-37], development of nanoparticles with antioxidant properties [38-41], and even modifying nanostructures for specific applications [43-46]. Nanoparticles exhibit unique electrical, catalytic, magnetic, and optical properties that differ from bulk materials [47-49]. For example, metallic nanoparticles like gold, silver, platinum, and palladium, as well as inorganic oxides like zinc oxide and titanium oxide, are valued for their exceptional mechanical, chemical, and magnetic properties [50-53]. Recently, Artificial Intelligence (AI) has emerged as a transformative tool in advancing nanotechnology, enabling more precise design, synthesis, and analysis of nanomaterials [54]. Biological methods for synthesizing nanoparticles are increasingly favored due to their environmentally friendly, non-toxic nature and, in some cases, superior efficacy against microorganisms. Unlike physical and chemical methods, which are energy-intensive, costly, and potentially hazardous to the environment, biological synthesis offers a sustainable alternative [55-57].

Iron oxide nanoparticles (IONPs) have gained widespread recognition for their exceptional magnetic, chemical, and biocompatible characteristics, making them highly valuable in both biomedical and environmental applications. Their utility spans various fields, including targeted drug delivery, magnetic resonance imaging, and antimicrobial therapies [58-61]. The development of IONPs through biologically derived and environmentally sustainable methods presents a promising alternative to traditional chemical synthesis, offering enhanced safety and efficiency. This study aims to explore the biosynthesis of iron oxide nanoparticles using *Bacillus thermotolerans* and assess their potential as inhibitors of multidrug-resistant *Klebsiella pneumoniae* isolated from diabetic foot infections. Through advanced characterization methods, the research seeks to uncover the therapeutic potential of IONPs in addressing the global challenge of antimicrobial resistance.

MATERIALS AND METHODS

Isolated and Identification of bacteria from a diabetic foot infection

A total of 75 swab samples were collected from patients with multidrug-resistant (MDR) diabetic foot disease from three hospitals in Hilla province (Marjan Hospital and the diabetic foot center in Alsadeq Hospital) between October 2023 and January 2024. Among these, *Klebsiella pneumoniae* was the predominant and most common Gram-negative bacilli, by 40 isolates (53%), followed by *Staphylococcus aureus* (15 isolates, 20%), *Pseudomonas aeruginosa* (9 isolates, 13%), *Escherichia coli* (5 isolates, 7%), *Proteus mirabilis* (3 isolates, 4%), and other species (3 isolates, 4%). Swabs from patients with diabetic foot infections were transported to the laboratory, where they were cultured by streaking on blood agar and MacConkey agar. In this study, the MDR isolates were identified based on their morphological properties, biochemical tests, and results obtained from the VITEK2 system [62].

Bacterial Isolates Used to Synthesize Nanoparticles

Different bacterial isolates from soil were tested (N1-N30); bacterial isolate no. N11 was selected based on color change and biological activity by growing on brain heart infusion agar at 37°C for 24-48 h. Colony morphology of differentiated-on medium and basic biochemical tests, molecular examination through gene expression through the extraction of genomic DNA, DNA sequencing assay, and agarose gel electrophoresis [63].

Synthesis of Iron Oxide Nanoparticles by N11 (Bacillus thermotolerans*)*

Isolate no. N11 were cultured in brain heart infusion broth at 37°C for 24 hours. After incubation, iron (ferric chloride, $FeCl_3$, 0.0081 mg) was added, followed by incubation for another 24 hours at 37°C in a shaking incubator at 150 rpm as shown in Fig. 1. The resulting colloidal suspension was centrifuged at 10,000 rpm for 15 minutes, and the supernatant was precipitated and used as a mechanical iron oxide nanoparticle (IONPs) suspension for separation [64,65].

Detection of Biosynthesis Iron Oxide Nanoparticles

The synthesis of IONPS of nanosize with a brown shade is proven in Fig. 1. The structural properties of the prepared nanoparticles were characterized by UV, FESEM, AFM, XRD, and FTIR evaluations [66], and analyses of these NPs were conducted at the

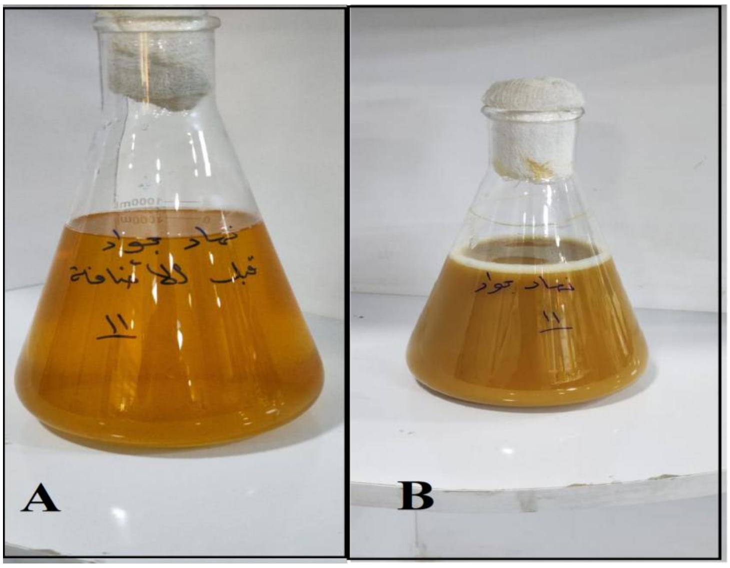

Fig. 1. Iron Oxide Nanoparticle Biosynthesis. A) Supernatant of *Bacillus thermotolerans* **before** incubation with iron salt. B) Reaction mixture after 24-hour incubation with iron salt showing a color change.

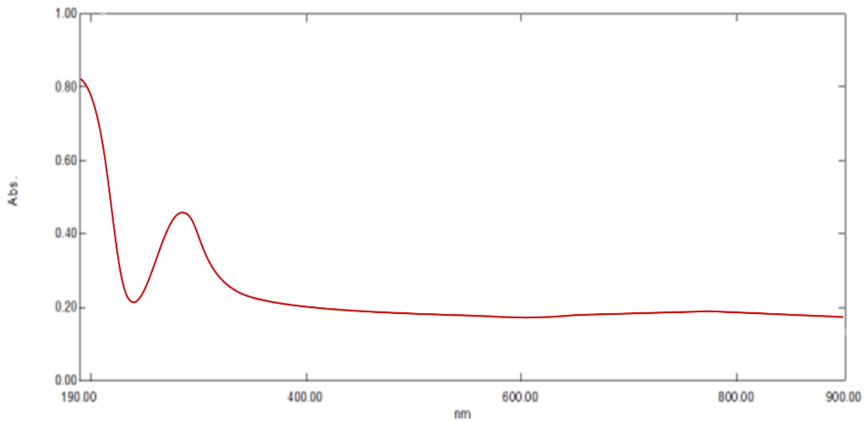

Fig. 2. UV Assay of biosynthesized IONPS.

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Antibacterial Activity of Iron Oxide NPs

The antibacterial activity of IONPs was examined using an agar diffusion methods [67,68] against multi-drug-resistant (MDR) *Klebsiella pneumoniae* bacteria isolated from diabetic foot infections. Four pure isolated colonies of fresh culture were suspended in five milliliters of brain heart infusion broth and incubated at 37°C for four to eight hours. The turbidity produced by the growth culture was calibrated with sterile broth to achieve an optical density comparable to the 0.5 McFarland requirements (equivalent to 1.5 x 10^8 cells/mL). A sterile cotton swab was dipped into the suspension, and used to streak the entire surface of a Mueller Hinton agar (MHA) dishes. The wells in MHA are prepared using a sterile cork borer (7 mm diameter of pores), and filled with 100 µl of IONPs of 1000 µg/ml, 500 µg/ml, 250 µg/ml, and 125 µg/ml compared with well filled by Distilled

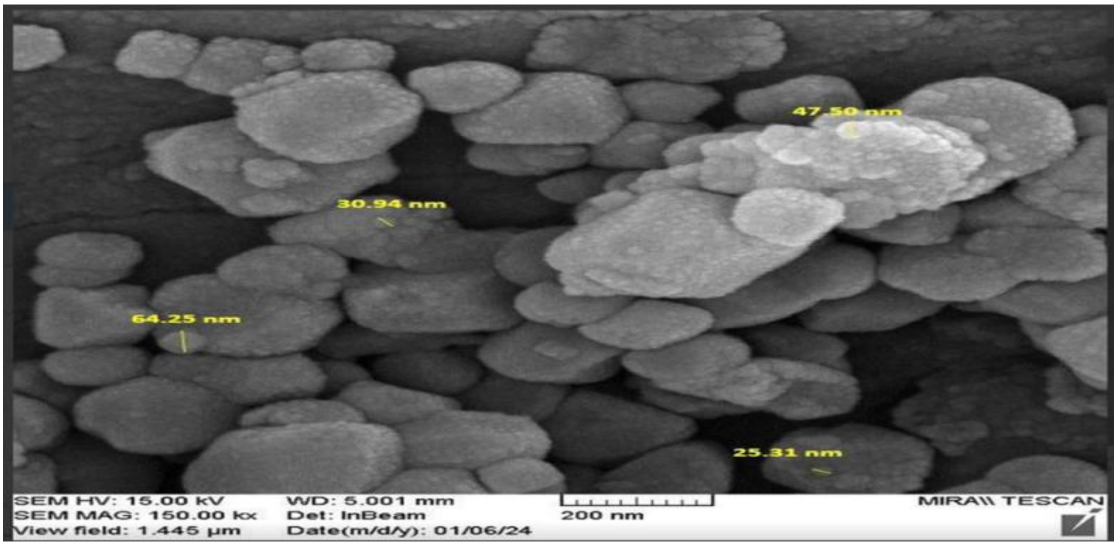

Fig. 3. FESEM Assay of biosynthesized IONPS.

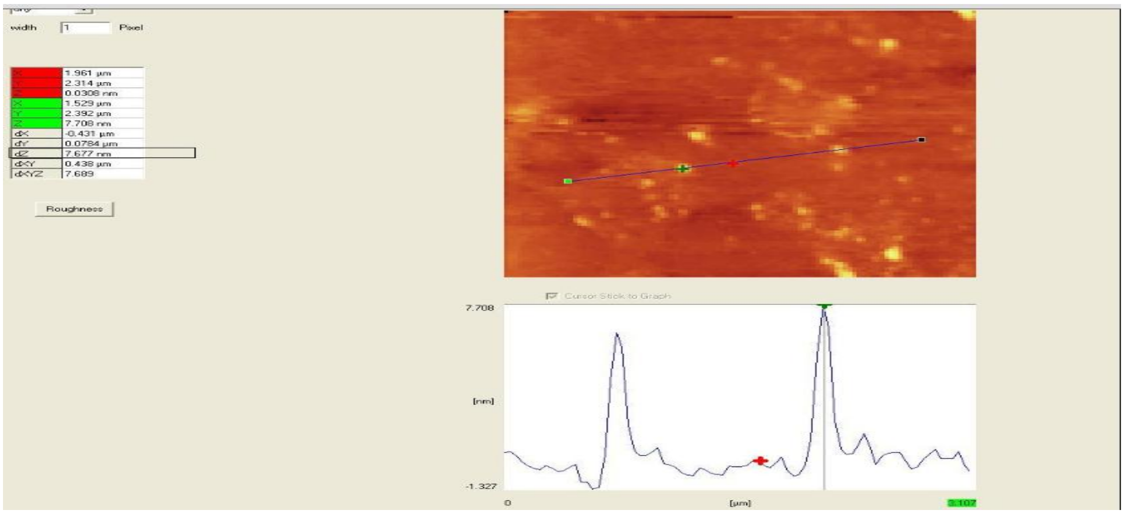

Fig. 4. AFM Assay of biosynthesized IONPS.

water as control. The dishes are incubated at 37°C for twenty- four hours after the incubation period, the diameter of the inhibition zones in millimetres was measured to determine antimicrobial activity.

RESULTS AND DISCUSSION

Formation of iron oxide nanoparticles by color change as shown in Fig. 1, and their size estimation within the composite suspension that was confirmed by UV-visible spectroscopy as shown in Fig. 2. The absorption spectrum of nanoparticles produced in the reaction mixture peaks at 280 nm, possibly due to the oxidation of zero-valent iron to iron oxide nanoparticles. The spectra clearly show maximum absorption peaks, indicating the formation of an increased number of iron oxide nanoparticles in the solution. The absorption peaks at wavelengths of 204 nm and 320 nm further indicate the formation of iron oxide nanoparticles [69,70].

The field emission scanning electron microscopy (FESEM) was used to determine the surface morphology and scale of nanoparticles in composite films [71]. The specimen was prepared by grinding iron oxide nanoparticles, preparing a colloidal suspension of the nanoparticles, and attaching a droplet of the suspension to the fixing matrix. Before FESEM characterization and after FESEM characterization, the samples were again air-dried and stored in a drying

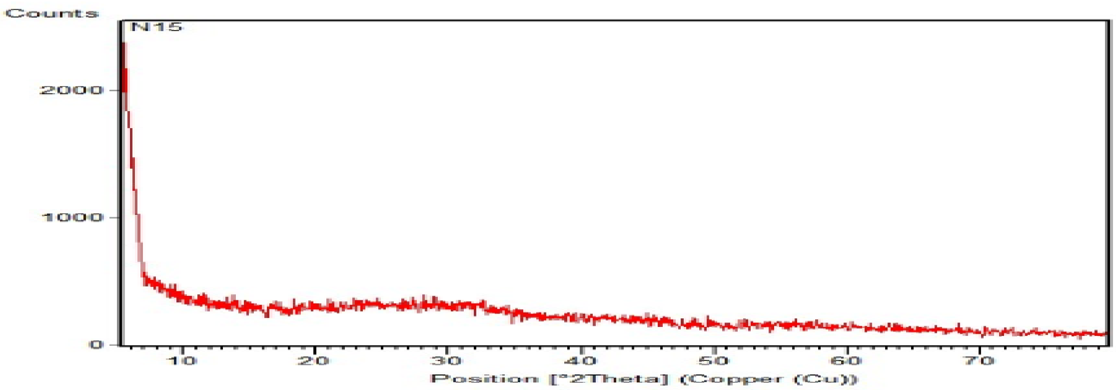

Fig. 5. XRD Assay of biosynthesized IONPS.

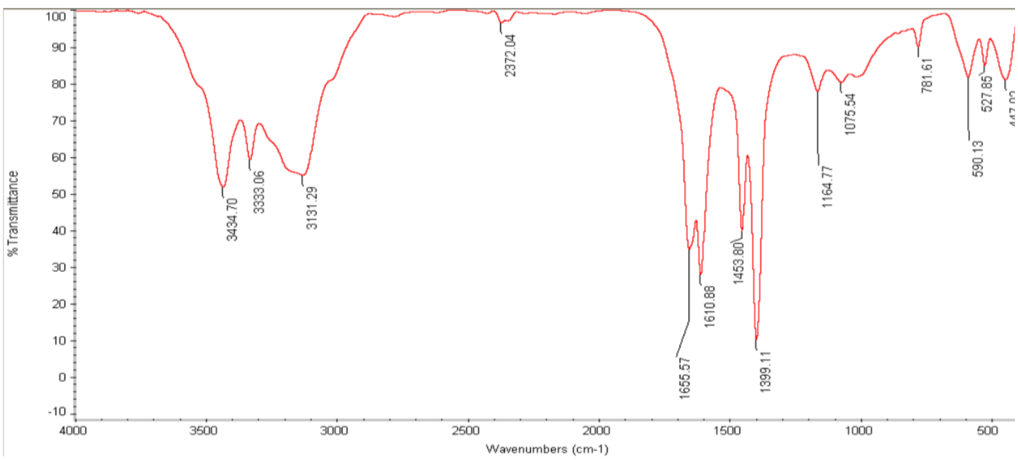

Fig. 6. FTIR Assay of biosynthesized IONPS.

chamber. Images were carried out in low vacuum mode at an accelerating voltage of 10-12 kV, at different magnification forces. *Bacillus thermotolerans* particles with sizes in the range of 25.31–64.25 nm, with an average size of 42 nm, were observed, as shown in Fig. 3. FESEM analysis showed the average particle size and detected their structure. However, large nanoparticles were seen due to aggregation, which may be due to the presence of cell components on the surface of nanoparticles acting as a capping agent [72]. The nanoparticles were not in direct contact, even within the aggregates, indicating the stabilization of the nanoparticles by a capping agent [73].

Fig. 4 shows that the atomic force microscope was used to investigate the dispersion and aggregation of nanomaterials, as well as their shape and size. (AFM; XE100 Park systems) at a scanning range of 10 x 10 µm finally formed an agglomeration with a large size of IONPS particle about 7.677 nm from *B. thermotolerans*. Atomic Force Microscopy's extraordinary resolution allows for precise three-dimensional visualization of molecular structures, as well as atomic-scale strategies. The procedure for preparing samples for AFM is straightforward. Because samples can be viewed under near-physiological conditions, AFM can record the critical procedures of molecules, organelles, and other structures in living cells in real-time [74,75].

The biogenic iron oxide nanoparticles were analyzed using X-ray diffraction (XRD) [76]. XRD is a common analytical technique. The 2θ peak position was correctly marked using JCPDS NO: 01-076-1363 to analyze the molecular crystal structure and identify qualitatively different molecules. Quantitative chemical analysis was used to calculate crystallinity, symmetrical substitution, and particle size. The crystal size was estimated using the Debye-Scherrer formula: $D = 0.94\gamma/\beta \cos\theta$. The diameter of the nanoparticles was determined to be 20 nm for *Bacillus*

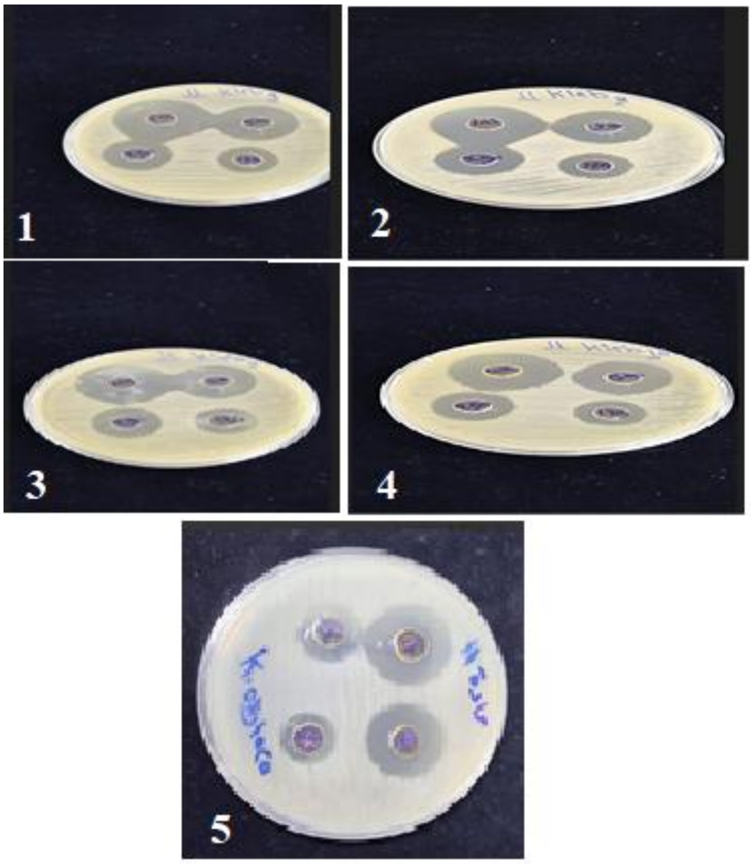

Fig. 7. Effect of IONPS on *Klebsiella pnemoniae* (1-4) and *Klebsiella oxytoca* (5).

thermotolerans, as shown in Figure 5.

Fourier Transform Infrared Spectroscopy (FTIR) was used to evaluate the iron oxide powder supplied from Sigma Aldrich (USA) for the sample in the *Bacillus thermotolerans* group, as shown in Fig. 6. The FTIR spectrum of the oxide chain ranged between 500 and 4000 cm^{-1}, and the absorption peak was observed at 165557 cm^{-1} in *Bacillus thermotolerans*. This was defined by group frequencies, the alcoholic (alcohol) ligand of the carbonyl C=O in the asymmetric stretching of the carbonate ion (CO_3^{2-} species). OH group stretching and bending vibrations were assigned to the stages of the (CO_3^{2-})-water interaction. The FT-IR spectrum of IONPS synthesized by bacteria showed a band between 500 and 800 cm^{-1}, associated with the chain oxide. The peak at 165557 cm^{-1} was assigned to the O-H stretch [77, 78].

Genomic DNA of *Bacillus thermotolerans* was isolated and then electrophoresed on an agarose gel, and documented visually. The DNA samples were used as templates for the PCR reaction aimed at amplifying the 16S rDNA gene (universal primers), 27F: AGAGTTTGATCCTGGCTCA and 1492R: GGTTACCTTGTTACGACTT [79]. Electrophoresis was performed with the PCR product 1470bp in agarose gel, which was then visualized.

Sequences of *B. thermotolerans* were obtained online and aligned to the NCBI database using BLAST software. Matching numbers were identified using BioEdit software, and the sequences were submitted to NCBI in FASTA format using Sequin software. Pairwise alignment and distance phylogeny were investigated for the *Bacillus thermotolerans* 16S rRNA gene sequences. The online NCBI BLAST software

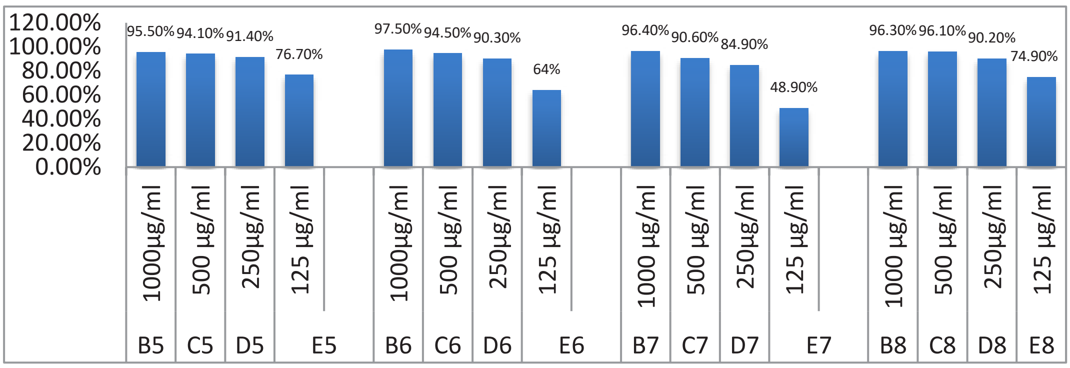

Fig. 8. Anti-biofilm effect of iron oxide NPs synthesized by *Bacillus. thermotolerans* against pathogenic bacteria (MDR *Klebsiella pneumonia*).

Table 1. Antibacterial activity of iron oxide nanoparticles synthesis by *B. thermotolerans* against MDR *Klebsiella pnemoniae* and *Klebsiella Oxytoca.*

Pathogenic bacteria	Zone inhibition (mm)			
	1000 µg /ml	500 µg /ml	250 µg /ml	125 µg /ml
Klebsiella pneumonia 1	29.5	31	29	24.5
Klebsiella pneumonia 2	26	25	24	20.5
Klebsiella pneumonia 3	22	21	20.5	17.6
Klebsiella pneumonia 4	18	18	17.5	17.7
Klebsiella oxytoca	22	20	16	14

compared the resulting sequences.

The antibacterial activities of iron oxide NPs as shown in Fig. 7 and Table 1 against multi-drug-resistant (MDR) *Klebsiella* bacteria isolated from diabetic foot infections. The effect of the nanoparticles on *Klebsiella pnemoniae* (1-4) and *Klebsiella oxytoca* (5) appears great effect in all concentrations.

Klebsiella pnemoniae exposed to nanoparticles at a concentration of 1000 µg/mL. Gram-negative bacteria were more sensitive to biogenic iron NPs and this align with [70]. The antibacterial action against many bacteria, including Gram-negative, aerobic, and anaerobic organisms, has been demonstrated. Because these materials have no harmful impact on humans, they are considered excellent antibiotic substitutes [80-82]. Many studies showed antibacterial activity of different nanoparticles against microorganisms such as titanium nanoparticles[83] and silver

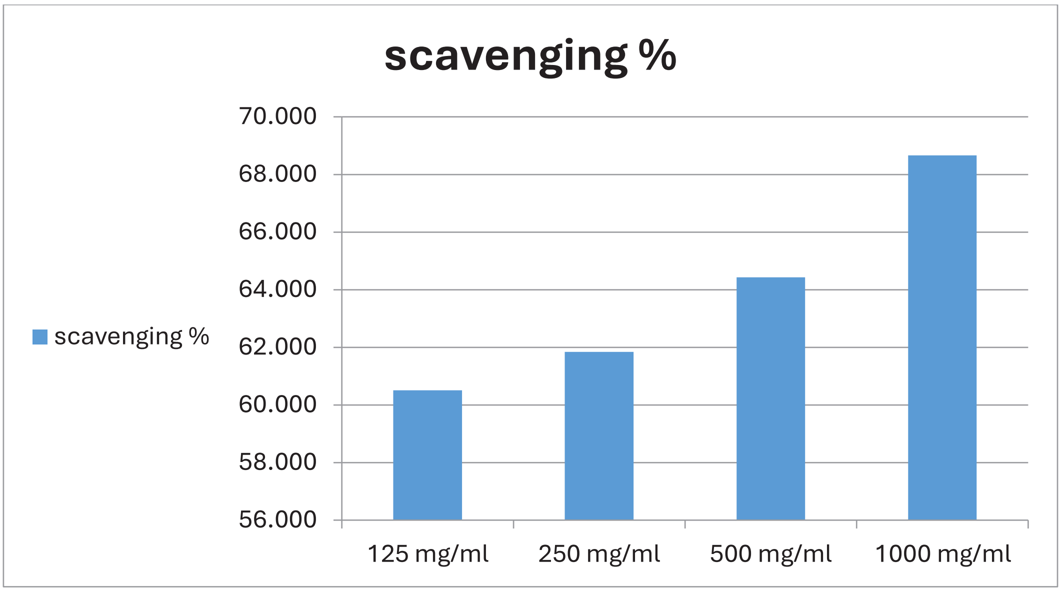

Fig. 9. Antioxidant effect of Iron oxide NPs synthesized by (*B. thermotolerans*) in the DPPH test.

Table 2. Iron oxide NPs synthesized by *Bacillus thermotolerans* effect on hemolysis.

concentration (mg/ml)	test result	hemolysis %
1	0.7759	0
0.5	0.7344	0
0.25	0.4998	0
0.12	0.4573	0
POSITIVE	2.6052	100
NEGATIVE	0.4354	0

nanoparticles [84].

Fig. 8 illustrates the development of synthetic IONPS with ant biofilm activity against *Klebsiella pnemoniae*. The antibiofilm effect is proportional to nanoparticle size [85]. The highest recorded anti-biofilm activity was observed with 97.50%. at 1000 µg/mL of IONPS against *Klebsiella pnemoniae*, although the lowest activity observed with 48.90% at 125 µg/mL of IONPS. These finding align with those studies demonstrating that the toxicity and bactericidal effects depends on concentration, species, and particle size [86]. These results match those of research showing that particle size, species, and concentration define the toxicity and bactericidal properties [86]. These can be explained by the size of the nanoparticles since they can reach into the biofilm matrix really deeply. Furthermore, these nano-agents have a high surface area to volume ratio, which facilitates efficient interaction with bacteria [87].

The radical scavenging assay was modified to determine the antioxidant effect of iron oxide NPs metabolites by reducing DPPH free radicals as shown in Fig. 9. The absorbance at 517 nm was measured 30 minutes after incubation of the nanoparticles in the DPPH solution at concentrations of 1000, 500, 250, and 125 µg/mL. The ability of nanoparticles to scavenge DPPH free radicals was validated by the color change measure-up [88,89].

The NPs synthesized by *Bacillus thermotolerans* showed scavenging percentages of 86.665% at 1000 µg/mL, 64.428% at 500 µg/mL, 61.840% at 250 µg/mL, and 60.505% at 125 µg/mL. Traditionally, the antioxidant radical scavenging potential of 1-diphenyl-2-picrylhydrazyl in vitro (DPPH) was considered to be a stable free radical, acting as a donor of hydrogen or an agent of electron absorption reduction. The antioxidant radical scavenging capacities of 1-diphenyl-2-picrylhydrazyl were also calculated in vitro, as it is a strong and well-known free radical, reliant on decreasing donor hydrogen or electron absorption [90]. Moreover, the antioxidant qualities of these nanoparticles help to explain their efficiency since oxidative stress is fundamental in immune resistance systems and bacterial pathogenicity [91].

Table 2 presents information on hemolysis, a condition in which red blood cells (RBCs) rupture and release their components, causing anemia, jaundice, and renal disease [92]. Because all substances entering the bloodstream interact with RBCs upon contact, it is important to evaluate the hemolytic properties of the materials. These finding highlights the biological applicability of nontoxic iron oxide NPs and these align with [93].

CONCLUSION

This study successfully demonstrated the biosynthesis of iron oxide nanoparticles (IONPs) using *Bacillus thermotolerans* and evaluated their antimicrobial potential against multidrug-resistant (MDR) *Klebsiella pneumonia*. Characterization techniques confirmed the formation of IONPs with nanoscale morphology, exhibiting significant antibacterial activity and biofilm inhibition. Unlike conventional antibiotics, these nanoparticles employ a dual-action mechanism by disrupting both planktonic bacterial growth and biofilm integrity, making them a promising alternative for combating antibiotic resistance. IONPs' non-hemolytic character suggests their possible safety for biological uses. Future research should explore their in vivo applications, long-term stability, and interactions with biological systems to advance their clinical and biomedical use.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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