RESEARCH PAPER

Molecular and Antibacterial Evaluation of Biosynthesized TiO_2 Nanoparticles Against Multidrug-Resistant Pseudomonas Aeruginosa and Acinetobacter Baumannii

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ARTICLE INFO

Article History:
Received 16 May 2026
Accepted 27 August 2026
Published 01 October 2026

Keywords:
Antibiofilm
Biosynthesis
Nanoparticles
Resistance Gene
TiO_2

ABSTRACT

Green synthesis of metal nanoparticles has emerged as a sustainable approach for developing alternative antimicrobial agents against multidrug-resistant pathogens. This study aimed to biosynthesize titanium dioxide nanoparticles (TiO_2 NPs) using *Enterococcus faecalis* isolated from soil and to evaluate their antibacterial, antibiofilm, and molecular effects against clinical multidrug-resistant isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* obtained from burn and wound infections. The synthesized TiO_2 NPs were characterized using UV–Visible spectroscopy, FESEM, AFM, XRD, and FTIR analyses. The nanoparticles exhibited an anatase crystalline structure with an average particle size of approximately 39.6 nm.Antibacterial activity was evaluated using agar well diffusion, showing maximum inhibition zones of 30 mm for *P. aeruginosa* and 28 mm for *A. baumannii* at 1000 µg/mL. A concentration-dependent antibiofilm effect was observed, with inhibition rates reaching 92.6% for *P. aeruginosa* and 89.5% for *A. baumannii*.Gene expression analysis was performed exclusively in *P. aeruginosa*, where TiO_2 NPs significantly down regulated*Intl2* expression (fold change = 0.42) compared to untreated controls.These findings suggest that biosynthesized TiO_2 nanoparticles possess strong antibacterial and antibiofilm properties and may interfere with resistance-associated genetic elements in *P. aeruginosa*, highlighting their potential application in burn wound infection management.

How to cite this article
Wali H. Aldujaili N., Kadium S. Molecular and Antibacterial Evaluation of Biosynthesized TiO_2 Nanoparticles Against Multidrug-Resistant Pseudomonas Aeruginosa and Acinetobacter Baumannii. J Nanostruct, 2026; 16(4):5173-5184. DOI: 10.22052/JNS.2026.04.056

INTRODUCTION

Burns destroy the skin, the natural barrier to the external environment. As a result, the burned area is prone to infection and microbial colonization. The most common pathogenic colonizing bacteria are *Staphylococcus aureus (S. aureus)* and *Pseudomonas aeruginosa (P. aeruginosa)*. *S.aureus* is a human commensal bacterium, as many individuals carry it, and a potentialinfectious pathogen. Other prevalent bacteria in burn wounds include *Acinetobacterbaumannii* (*A. baumannii*) and *Klebsiellapneumoniae* [1]; these pathogens can contribute to the inflammatory immune responses induced by thermal injuries. The intact human skin surface is essential for protecting

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against infection, preserving homeostasis of body fluids, and regulating body temperature.Burn injury compromises the skin barrier and facilitates bacterial infection, thereby delaying burn wound healing [2]. *Enterococcus faecalis* (*E. faecalis*) is a gram-positive bacterium that can cause a variety of nosocomial infections,with urinary tract infections being the most common. These infections can be difficult to treat because of drug resistance in many *E. faecalis* isolates. Despite their troublesome nature, little is known about the host or bacterial factors required for *E. faecalis* to cause urinary tract disease [3].

Nanobiotechnology is a modern nanoscience field that utilizes nanoscale systems across various biomedical fields and applications. Metal nanoparticles (NPs) have a large surface area-to-volume ratio, and their high percentage of surface atoms has made them widely studied for their unique physicochemical characteristics, including catalytic, electronic, optical, magnetic, and antibacterial properties [4-17]. Artificial intelligence (AI) has become a transformative tool for advancing nanotechnology, enabling the design, synthesis, and optimization of nanoscale materials with unprecedented precision. AI-driven algorithms enable fast analysis of complex datasets, prediction of nanoparticle properties, and identification of optimal conditions for targeted applications. The synergy between AI and nanotechnology hastens novelty in drug delivery, antimicrobial materials, and energy systems [18]. Recent developments highlight the integration of nanotechnology with antioxidants and chemotaxis, enabling the development of nanoparticle-based delivery systems that improve stability and bioavailability [19,20]. Functionalized nanoparticles such as iron oxide, zinc oxide, and silver oxide nanoparticles have emerged as effective vehicles for drugs, proteins, and antigens due to their biodegradability, biocompatibility, ease of surface modification, and efficient delivery properties [20-27]. In addition, these nanoparticles showed great antimicrobial and antibiofilm activities [28-32]. In addition, advances in nanotechnology have enabled techniques to precisely modify the physical and chemical properties of biomaterials. One such method is focused ion irradiation, which has been effectively utilized to tune the surface properties of nanomaterials at the nanoscale. This approach enables precise control over surface energy, adhesion, and roughness, thereby enhancing the functionality of nanomaterials in biomedical applications [33]. Nanoparticles have been synthesized using numerous physical and chemical approaches [34]. Green synthesis of biogenic nanoparticles (NPs) from plant or microbial sources has emerged as an area of interest due to its eco-friendly, fast, simple, safe, energy-efficient, cost-effective, and less toxic nature [35,36]. Green synthesis of nanoparticles can be achieved using various microorganisms, especially bacteria and fungi, which are preferred for synthesizingmany metal nanoparticles, including titanium dioxide (TiO_2) nanoparticles. This is due to their low growth time and eco-friendly nature [37]. TiO_2 occurs naturally and has many properties, such as high refractive index, light absorption, low toxicity, stability, and low production cost [38]. Although green-synthesized TiO_2 nanoparticles have demonstrated antimicrobial properties, few studies have investigated their effects on resistance-associated genetic elements, such as

Fig. 1. Biosynthesis TiO_2 nanoparticles steps.

integrons, in multidrug-resistant burn isolates. Furthermore, few reports have evaluated their simultaneous antibacterial, antibiofilm, and molecular regulatory effects. Therefore, this study addresses this gap by assessing the impact of biosynthesized TiO_2 nanoparticles on *Intl2* gene expression in clinical *Pseudomonas aeruginosa* isolates.

MATERIALS AND METHODS

Bacterial Isolates Used for Nanoparticle Synthesis

Twelve bacterial isolates (H1 to H12) from soil were screened for nanoparticle synthesis. Isolate H6 was selected based on color changes and biological activity in brain-heart infusion broth incubated at 37 °C for 24 hours. Identification was confirmed via morphological, biochemical, and molecular methods. After screening various bacterial isolates (H1 to H12) naturally isolated from soil (Fig. 1), isolate H6 was identified by microscopy, morphology, and basic biochemical tests.

Molecular Identification ofEnterococcus faecalis

Genomic DNA from *E. faecalis* was extracted using the Favorgen kit following the manufacturer's protocol. The Purity and the concentration of the extracted DNA were assessed using a NanoDrop-1000 spectrophotometer. PCR amplification of the 16S rRNA gene was carried out using specific primers listed in Table 1. The PCR reaction mixture (50 µL) contained 25 µL of Taq Master Mix, which included DNA polymerase, dNTPs, PCR buffer, and an optimized concentration of 1.5 mMMgCl₂. Additionally, 200 nM of each primer and the DNA template were added, and the final volume was adjusted to 50 µL with PCR-grade water. Following PCR confirmation, the amplified products were purified and subjected to Sanger sequencing using both forward and reverse primers. Sequencing reactions were performed in a 10 µL reaction volume according to the manufacturer's guidelines. The sequences were analyzed using a 3730xl DNA Analyzer, and the obtained results were submitted to the GenBank database.

Biosynthesis of TiO_2 Nanoparticles

Titanium dioxide salt was added to a pure culture of H6 in brain-heart broth, and the mixture was incubated at 37 °C for 24 hours in a shaking incubator. The product was centrifuged at 5000 rpm for 5 minutes; this step was repeated several times until a pure solution was obtained and then dried at 50 °C to obtain TiO_2 NPs in powder form.

Characterization of TiO_2NPs

Absorption spectra using UV-visible spectroscopy were used to confirm nanoparticle formation between 200 nm and 800 nm. FESEM was used to measure nanoparticles, which indicated an average size of approximately 39.61 nm, with some agglomeration. Atomic Force Microscopy (AFM) was used to examine the surface morphology and revealed the presence of nanoparticle clusters. X-ray diffraction (XRD) was employed to analyze the crystal phase, and the results were confirmed. FTIR peaks specific to Ti-O bonds were identified, indicating high purity. All the previously mentioned analyses were conducted at the University of Tehran in the Islamic Republic of Iran.

Antibacterial Activity

Fifty *P. aeruginosa* and *A.baumannii* isolates were collected from patients visiting AL-Najaf Hospital and were grown on blood agar, MacConkey agar, and chromium agar at a temperature of 37 C° for 24 hours. Bacteria were diagnosed by microscopic examination using Gram stain of the colony, Colony Morphology on different media, basic biochemical tests, and finally the Vitek 2 system. The antibacterial activity of TiO_2 NPs was tested using Mueller-Hinton agar at concentrations of 1000, 500, 250, and

Table 1. Primer Sequences for 16SrRNAgene amplification.

Primer name	Sequence 5′-3′	Reference
27F	AGAGTTTGATCCTGGCTCA	39
1492R	GGTTACCTTGTTACGACTT	

125 µg/mL. Zones of inhibition were measured in millimeters. The antibacterial activity was measured by preparing plates of Muller-Hinton Agar medium and cultivating the *P. aeruginosa* and *A.baumannii* bacteria on the culture medium, then drilling using a cork drill and, filled with 100 µl of TiO_2 NPs at concentration of 1000 µg/ml, 500 µg /ml, 250 µg/ml, 125 µg/ml, each MHA plate was incubated at 37 °C for 24 hours, after incubation, the determination of the inhibition zone was measured on the dishes in millimeters with ruler. *In vitro*, antibiofilm activity was measured using the 96-well microtiter plate technique. The first well of the microtiter plate was filled with 100 µL BHIB broth medium containing 1% glucose and 100 µL of biogenic Titanium oxide NPs, then prepared at several dilutions (1000, 500, 250, and 125 µg/ml). After that, we added 100 µL of the diluted concentration till the last one. This was used as a control to confirm bacterial biofilm development and the inhibition of biofilm formation by TiO_2 NPs. Then we added 10 µL of overnight-cultured isolated bacteria to each well of the microtiter plate. So, each well of the microtiter plate will contain 110 µL of mixed suspensions [40].

Effect of TiO_2 NPs on gene expression of Intl2in Pseudomonas aeruginosa

To extract the RNA of two *P. aeruginosa* samples, one untreated (controlled) and the other treated with nanoparticles, the supernatant was initially discarded. The samples were frozen at -80 C and allowed to thaw at room temperature. Once the RNA was completely thawed, the RNA extraction workflow was initiated, and the resulting pellet was stored at −80°C until further processing. RNA was extracted using 1ml of TRIzol reagent, with incubation at room temperature for 5 minutes. Subsequently, 100 µl of chloroform was added, and the samples were mixed vigorously for 30 seconds, then incubated at room temperature for 3 minutes. Centrifugation

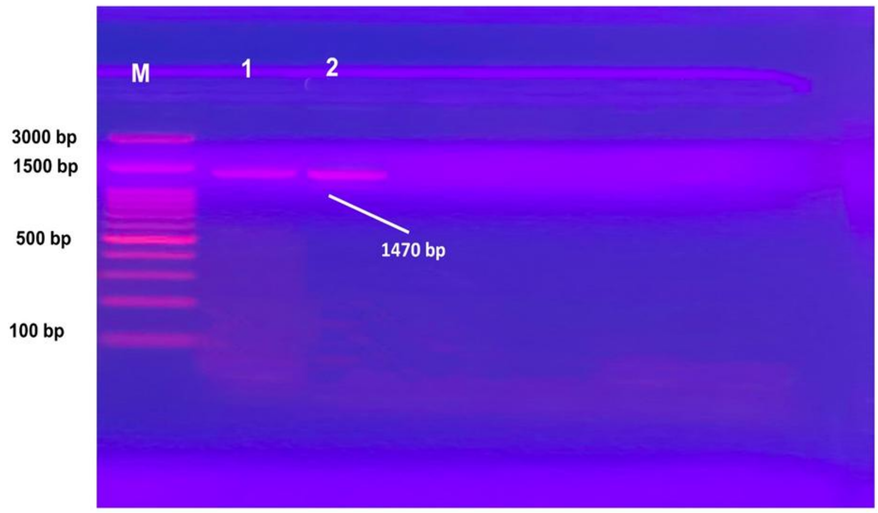

Fig. 2. PCR assay for E. faecalis using genus-specific16S rRNA gene primers.

Table 2. Primer Sequences for *intl2* Gene Amplification.

Targetgene	Primer name	Oligonucleotideprimer Sequence (5'-3')	Ampliconsize (bp)	References
IntlI (RT)	F	CCTCCCGCACGATGATC	280	41
	R	TCCACGCATCGTCAGGC		

was performed at 10000 × g for 15 minutes at 2-8 °C. Following centrifugation, the upper colorless layer was carefully transferred to a new, fresh tube, and 250 µl of isopropanol was added. The mixture was gently inverted and incubated at room temperature for 10 minutes, followed by centrifugation at 10000 × g for 10 minutes at 2-8 °C. After centrifugation, the supernatant was discarded, and the RNA precipitate was visible on the walls and bottom of the tube. Then, 1 ml of 75% ethanol was added to the pellet, and the solution was vortexed before centrifugation at 7500 × g for 5 minutes at 2-8 °C. The supernatant was discarded, and the RNA pellet was air-dried for 5 minutes. The pellet was then resuspended in 50 µl of RNA dissolving solution and incubated at 55-60̊ C for 10 minutes to facilitate complete dissolution. For gene expression analysis, RT-PCR was performed using specific primers for the *intl2* gene Table 2.

Specific primers for the Intl2 gene used in RT-PCR reaction, when quantifying mRNA, real-time PCR can be performed as either a one-step reaction, where the entire reaction from cDNA synthesis to PCR amplification is performed in a single tube, or as a two-step reaction, where reverse transcription and PCR amplification occur in separate tubes. There are several pros and cons associated with each method.

RESULTS AND DISCUSSION

In the present study, genomic DNA was successfully extracted from *E. faecalis* strains isolated from soil. The purity and integrity of the extracted DNA were assessed using agarose gel electrophoresis. The purified bacterial DNA served as a template for the conventional PCR to analyze the clonal diversity of *E. faecalis*. The PCR amplification products generated by the selected primers for the isolated *E. faecalis* strain were high, with patterns showing two well-separated bands, as shown in Fig. 2. However, restriction PCR on the isolated *E. faecalis* was performed in two steps, including amplification and purification of the 16S rDNA. The 16S rDNA genes of the isolated *E. faecalis* were successfully amplified, yielding the expected gene length of 1470 bp. The obtained sequence was submitted to the GenBank database under the accession number PP825415.1.

UV-visible spectroscopy characterized the production of TiO_2 nanoparticles by *E. faecalis*. Fig. 3 shows the absorption spectra at 200 nm and 800 nm, which are due to the surface Plasmon resonance /vibration in the reaction mixture. The absorption peak provides evidence of nanoparticle formation in the bacterial culture [42]. These results show that the prepared surfaces exhibit good stability and higher crystal organization, but also high agglomeration. As illustrated in Fig. 4 of TiO_2 NPs, it is clear that the surface attained lower crystallinity, and the size of the synthesized TiO_2 NPs from isolated (H6) was about 39.61 nm.

Fig. 5 shows the surface morphology of the TiO_2 NPs; the images were obtained using an atomic force microscope (AFM; XE100, Park Systems) over a scanning range of 10 × 10 µm. Fig. 6 shows the

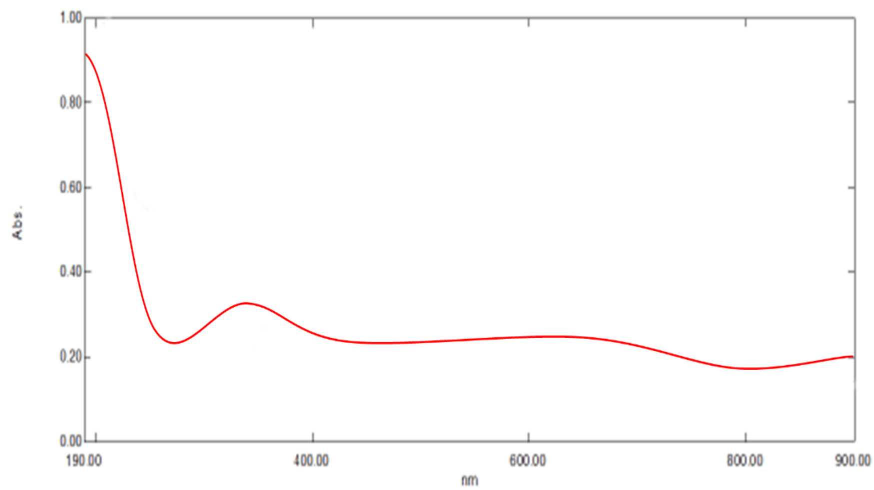

Fig. 3. UV assay of TiO_2 nanoparticles.

X-ray diffraction pattern of the synthesized Titanium nanoparticles. The following peak signals in the planes confirm that the formation of the anatase crystal phase largely coincides with the JCPD 89-4921 standard. The intensity of the sample's XRD peaks reflects that the formed nanoparticles are crystalline, and broad diffraction peaks indicate very small crystallites [43]. FTIR studies of the TiO_2 NPs showed characteristics consistent with the formation of high-purity products. The FTIR spectra (Fig. 7) of these nanoparticles showed peaks only corresponding to TiO_2. The peak observed at 590 cm−1 is due to the vibration of the TiO- O bond. The FTIR spectrum firmly suggests the presence of TiO bonds and the absence of peroxo and OH groups in the final product. The TiO_2 NPs prepared

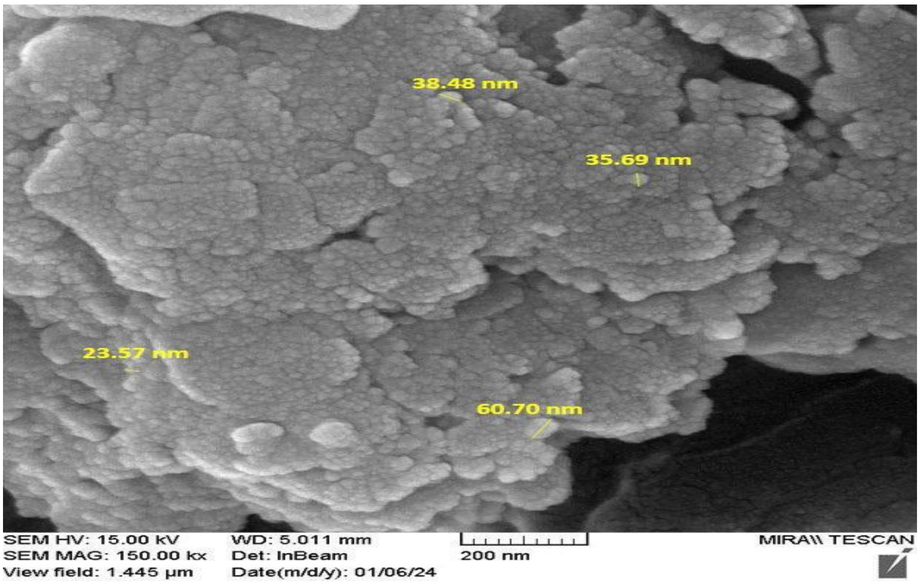

Fig. 4. FESEM assay of TiO_2 nanoparticles.

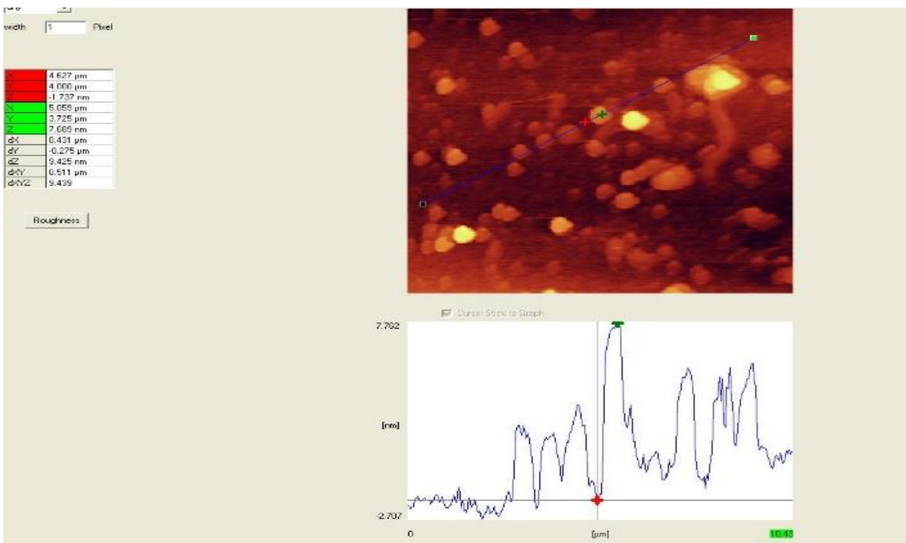

Fig. 5. AFM assay of TiO_2 nanoparticles.

by this method are high-quality and suitable for further applications. The carboxylic groups are known to coordinate with metal ions, which may act as a nucleation site for nanoparticle formation.

Tables 3 and 4 show the antibacterial activity of TiO_2 NPs (1000,500,250,125 µg/ml) concentrations against *P. aeruginosa* and *A. baumannii* isolated from burns and wound samples, showing high measurement diameter inhibition zones on Muller-Hinton agar of *P. aeruginosa* about 30mm while *A. baumannii* about 28mm in concentration 1000 µg/ml and a low measurement diameter inhibition zone of P. aeruginosa about 16mm while *A.baumannii* about 15mm in concentration 125 µg/ml by nanoparticles in all concentrations. The antibacterial test results showed that TiO_2 NPs had a greater inhibitory effect on P. aeruginosa than the others, as shown in Fig. 8. The synthesized TiO_2 NPs were determined against *P.aeruginosa* and *A. baumannii* by antibiofilm activity. The antibiofilm activity corresponded to the nanoparticle dose, and a dose effect was observed with isolated nanoparticles. The maximum anti-biofilm action recorded was 96.70% at 1000 µg/ml TiO_2 NPs treatment against *A. baumannii*, while the minimum was 28.40% at 125 µg/ml. In the case

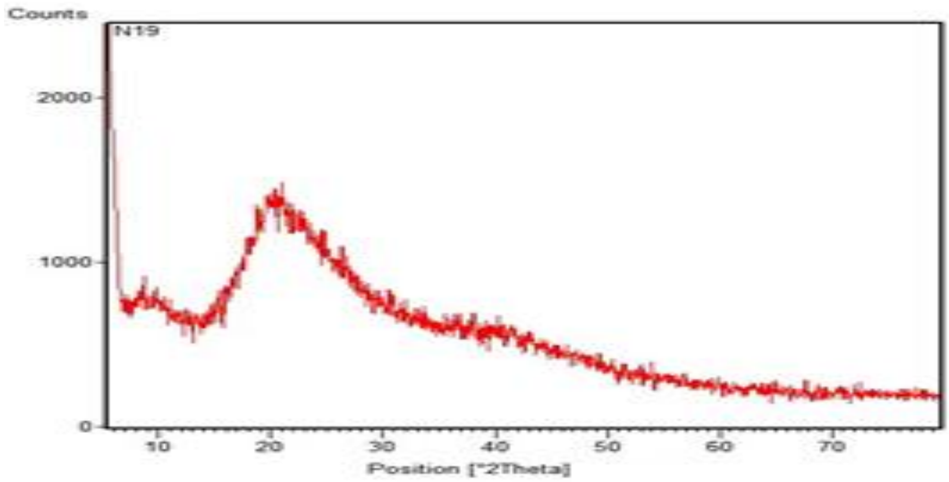

Fig. 6. XRD assay of TiO_2 nanoparticles.

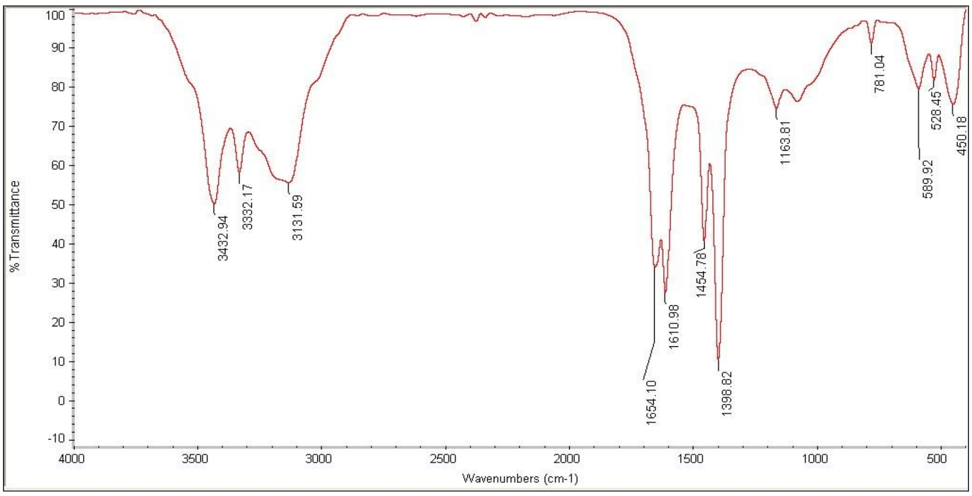

Fig. 7. FTIR assay of TiO_2 nanoparticles.

of *P. aeruginosa*, the highest biofilm inhibition rate observed was 92.60% at 1000 µg/ml TiO_2 NP exposure, while the lowest was 53.40% at 125 µg/ml. Many studies confirm the antimicrobial activity of TiO_2 nanoparticles [44,46]. The bacteria were treated with TiO_2 NPs and incubated for 24 hours. The results, shown in Table 5 and Fig. 9, indicate that TiO_2 NP treatment decreased Intel gene expression by 0.42 compared to the control. This agrees with studies [45].

In addition to the present findings, several recent regional investigations further support the growing integration of molecular diagnostics and green nanotechnology in combating antimicrobial resistance. Molecular surveillance studies have detected extended-spectrum β-lactamase genes,

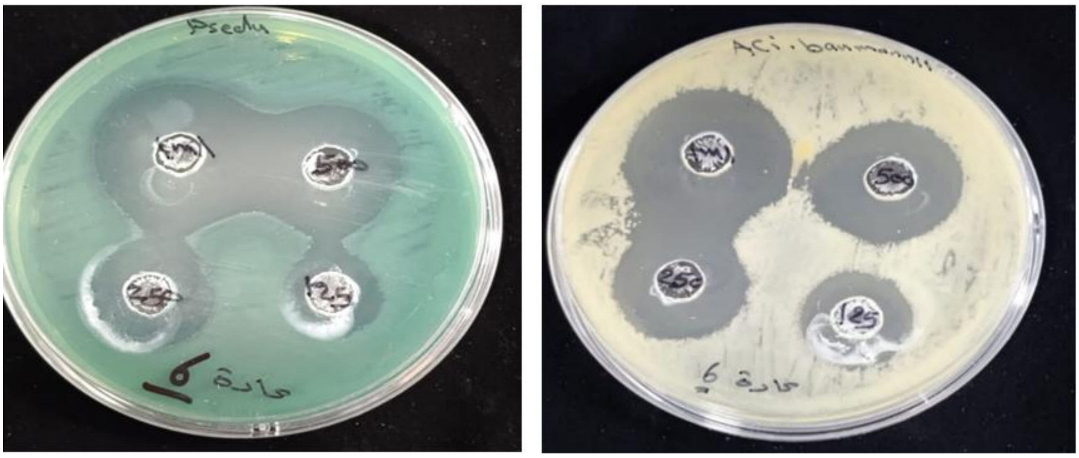

Fig. 8. Effect of TiO_2 NPs on *P. aeruginosa* and *A.baumannii* bacteria.

Table 3. Antibacterial efficacy of TiO_2 nanoparticles against *P. aeruginosa* and *A.baumannii.*

Bacteria	*P.aeruginosa*	*A. baumannii*
TiO_2nanoµg/ml.		
Con.1000	30	28
Con.500	26	25
Con.250	20	18
Con.125	16	15

Table 4. Antibiofilm efficacy of TiO_2Nps against *A.baumannii* and *P. aeruginosa.*

Pathogenic Bacteria	Concentration µg/ml	Inhibition rate (%)
A.baumannii	1000µg/ml	89.50%
	500 µg/ml	82.40%
	250µg/ml	80.10%
	125 µg/ml	28.40%
P. aeruginosa	1000µg/ml	92.60%
	500 µg/ml	90.70%
	250µg/ml	86.70%
	125 µg/ml	53.40%

such as *blaTEM* and *blaCTX-M*, in *Klebsiellaoxytoca* isolated from tonsillitis cases, highlighting the increasing dissemination of resistance determinants in community-associated infections [47]. Similarly, the identification of class 1 integron genes in Proteus mirabilis isolates from diabetic foot infections underscores the critical role of mobile genetic elements in the propagation of resistance [48]. Recent advances in nanobiotechnology have demonstrated the promising antimicrobial activity of biosynthesized reduced graphene oxide against multidrug-resistant uropathogenic bacteria [49], while environmental isolation of metallic *Klebsiellapneumoniae*strains capable of AgNPsnanobiosynthesis from iron-rich soils illustrates the ecological diversity of nanoparticle-producing microorganisms [50]. Moreover, engineered nanocomposites such as Ag/F–TiO_2 nanoparticles have shown targeted inhibitory activity against virulence-associated genes, including algD and plcH, in *Pseudomonas aeruginosa* isolated from cystic fibrosis patients

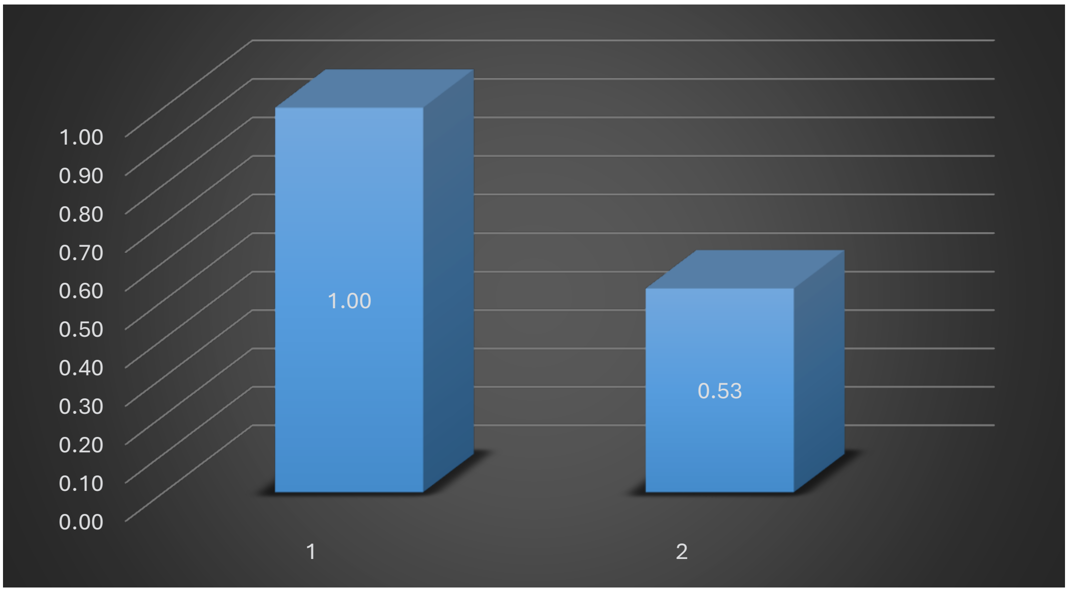

Fig. 9. TiO_2 NPs Effected on Gene expression of *P. aeruginosa* bacteria genome .

Table 5. TiO_2 NPs Effected on Gene expression of *P. aeruginosa* genome.

	16srRNA	IntI				
Control	average Ct	average Ct	dCT	ddCT	fold change	averag F change
1	18.49	26.45	7.96	0.620	0.651	
2	19.45	25.75	6.3	-1.040	2.056	
3	16.84	24.6	7.76	0.420	0.747	1.00
			7.34			
NANO						
1	19.35	27.53	8.18	0.840	0.559	
2	16.42	24.83	8.41	1.070	0.476	
3	16.61	24.78	8.17	0.830	0.563	0.53

[51], and ZnO nanoparticles have demonstrated suppressive effects on virulence genes in burn-associated *P. aeruginosa* isolates from the Al-Muthanna population [52]. Epidemiological assessments of antibiotic resistance profiles in thermophilic*Bacillus licheniformis* isolated from soil, along with clinical identification of pathogenic bacteria causing otitis media, further reinforce the importance of local microbial surveillance in guiding antimicrobial strategies [53,54]. Complementary investigations into the antibacterial and anticancer activities of indolebutanoic acid, as well as the synthesis and biological evaluation of novel tetrazole derivatives incorporating oxazine and thiazine compounds, reflect the expanding intersection between medicinal chemistry and nanotechnology-based therapeutic innovation [55,56]. Collectively, these studies align with the current work in emphasizing molecular targeting and eco-friendly nanomaterial development as promising approaches for addressing multidrug-resistant bacterial infections.

Previous investigations have consistently demonstrated the antimicrobial and biomedical relevance of metal-based nanomaterials and infection control strategies. Silver-ion technologies have demonstrated efficacy in reducing device-associated infections [57], while studies using silver-containing bioactive glass and surface-enhanced Raman spectroscopy have highlighted the physicochemical mechanisms underlying antibacterial interactions with metals [58,59]. Molecular insights into nosocomial infections further underscore the genetic basis of antibiotic resistance dissemination [60]. Foundational studies on the photochemical and catalytic properties of metal nanoparticles have established their mechanistic role in antimicrobial activity [61], alongside broader investigations into microbiota modulation and probiotic-host interactions [62]. Green microbial synthesis approaches, including silver nanoparticles produced by *Enterococcus faecalis* and Spirulina-mediated TiO_2 nanoparticles, have demonstrated promising activity against multidrug-resistant pathogens [63,64]. Additionally, advances in cellulose-templatedTiO_2 nanomaterials and nanoscale surface engineering have improved therapeutic delivery and photocatalytic efficiency [65,66]. Emerging applications of gold nanoparticles in biomedical and food systems, as well as titanium dioxide nanoparticles and selenium nanoparticles biosynthesized via microbial routes, further confirm the expanding role of nanotechnology as a sustainable antimicrobial strategy [67–69].

CONCLUSION

This study successfully demonstrated the green biosynthesis of anatase-phase TiO_2 nanoparticles using *Enterococcus faecalis*. The nanoparticles exhibited strong concentration-dependent antibacterial and antibiofilm activities against multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Importantly, TiO_2 nanoparticles significantly down-regulated the *Intl2* gene in *P. aeruginosa*, indicating potential interference with resistance-associated genetic mechanisms. These findings support the potential application of biosynthesized TiO_2 nanoparticles as alternative antimicrobial agents in burn wound infection management. Further in vivo and cytotoxicity studies are recommended to validate clinical applicability.

CONFLICT OF INTEREST

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

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