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Explore The Efficacy Of Nanoparticles Tio_2, In Preventing And Disrupting Biofilms Of Waterborne Pathogens Like *Legionella Pneumophila*

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Abstract

This study demonstrated that TiO_2 nanoparticles synthesized via the sol-gel method were successfully produced as fine, crystalline powders with controlled nanoscale morphology, confirming their suitability for antimicrobial applications. Characterization techniques such as TEM, SEM, DLS, and FTIR collectively confirmed uniform spherical nanoparticles of approximately 15–20 nm size, with surface hydroxylation essential for photocatalytic function. The effective preparation and growth of Legionella pneumophila cultures and substantial biofilm formation set a reliable basis for evaluating antibiofilm treatments.

Keywords: Legionella pneumophila, waterborne pathogens, titanium dioxide Nanoparticles, Reactive Oxygen Species, Scanning Electron Microscopy, Transmission Electron Microscopy, Particle Size Analyzer, Dynamic Light Scattering, Biofilm

1. Introduction

Nanoparticles are at the forefront of materials science and nanotechnology, defined as particles with dimensions between 1 and 100 nanometers. Due to their small size, nanoparticles exhibit unique characteristics, including enhanced surface reactivity, quantum effects, and distinct optical and magnetic properties. These features are not present in their bulk form and provide opportunities for innovative applications across multiple fields such as medicine, electronics, energy, and environmental sciences.
The traditional synthesis of iron oxide nanoparticles often involves chemical processes using toxic reducing agents like sodium borohydride and hydrazine. These methods, while effective in producing nanoparticles, have raised environmental and health concerns due to the release of hazardous by-products (Aarhaug&Ratvik, 2019).

2. Methodology

Titanium isopropoxide ($Ti[OCH(CH_3)_2]_4$) was used as the titanium precursor. Approximately 20 mL of titanium isopropoxide was added dropwise into a stirred solution containing 10 mL of isopropanol and 12 mL of deionized water at 80°C. The stirring was maintained continuously to ensure uniform hydrolysis. Hydrochloric acid or nitric acid (~0.8 mL) was added as a catalyst to control the hydrolysis rate. The mixture was stirred at 60°C for 6 hours to form a highly viscous sol-gel. The sol-gel was then dried at 300°C for 2 hours in an open atmosphere to produce initial TiO_2 powder. After drying, the powder was crushed thoroughly using a mortar and pestle. Finally, calcination was performed at 500°C for several hours (commonly 3 hours) to obtain pure crystalline TiO_2 nanoparticles with desired anatase or mixed-phase structures. This method allowed optimization of particle size and morphology through control of hydrolysis, drying, and calcination parameters (amounts and times can be adjusted as above) [Sharma et al., 2014; Sondezi et al., 2024]

Characterization of nanaoparticles

Transmission Electron Microscopy (TEM)

The morphology and particle size of TiO_2 nanoparticles were examined using TEM (JEOL JEM-2100 or equivalent). A drop of the nanoparticle suspension was placed on a carbon-coated copper grid and allowed to dry at room temperature. Images were captured at an accelerating voltage of 200 kV. Particle sizes were measured manually from TEM micrographs using imaging software, and average sizes were calculated from multiple fields (Hu et al., 2017).

Scanning Electron Microscopy (SEM)

SEM (FEI Quanta or similar) was used to analyze surface morphology and particle shape. A small amount of dried powder was mounted on an aluminum stub using conductive carbon tape and sputter-coated with a thin layer (approximately 5 nm) of gold to prevent charging. Images were taken at an accelerating voltage of 10-15 kV under high vacuum conditions. Morphological features were compared with TEM results for consistency (Sondezi et al., 2024).

Particle Size Analyzer (PSA)

Hydrodynamic size distribution of the nanoparticles in suspension was measured using dynamic light scattering (DLS) on a Malvern Zetasizer Nano ZS or equivalent. Nanoparticles were dispersed in distilled water, sonicated for 10 minutes, and filtered to remove large aggregates prior to analysis. The average particle size, polydispersity index (PDI), and size distribution histograms were recorded (Sharma et al., 2014).

Biofilm formation

Sterile glass microscope slides are prepared by cleaning with 70% ethanol and rinsing with sterile distilled water. The slides are placed vertically in 12-well sterile culture plates. Each well is filled with 3 mL of nutrient-rich buffered yeast extract broth inoculated with Legionella pneumophila suspension adjusted to an optical density of 0.1 at 600 nm (~10^8 CFU/mL). The plates are incubated statically at 37°C for 48 to 72 hours to allow biofilm formation.

After incubation, the slides are gently removed, rinsed with sterile phosphate-buffered saline (PBS) to remove unattached cells, and air-dried. Biofilm biomass is quantified by staining with 0.1% crystal violet solution for 15 minutes. Excess stain is rinsed off with distilled water, and after drying, the stain

bound to biofilm is solubilized with 33% acetic acid. The optical density of this solution is measured at 590 nm using a basic UV-visible spectrophotometer, which provides a quantitative measure of biofilm formed.

Preparation of bacterial culture

Legionella pneumophila cultures were prepared using standard microbiological techniques to obtain pure and active bacterial cultures for biofilm formation studies. The bacterial strain, Legionella pneumophila MTCC 1327, was procured from the Microbial Type Culture Collection (MTCC), India. For cultivation, Buffered Charcoal Yeast Extract (BCYE) agar and broth media were prepared as the nutrient-rich environment optimal for Legionella growth. The BCYE medium was composed per liter of 3.0 g activated charcoal, 10.0 g yeast extract, 10.0 g ACES buffer (N-(2-Acetamido)-2-aminoethanesulfonic acid), 0.05 g L-cysteine hydrochloride, 0.025 g ferric pyrophosphate, and 15.0 g agar for solid medium, with the pH adjusted to 6.9 ± 0.1 using sodium hydroxide. The components, except agar, were dissolved in approximately 900 mL distilled water under constant stirring. Agar powder was added for solid medium preparation. The final volume was adjusted to 1 liter, and the medium was sterilized by autoclaving at 121°C for 15 minutes. The sterilized BCYE agar was poured aseptically into Petri dishes and allowed to solidify (ISO 11731-2, 2017).

The bacterial strain was revived by inoculating a loopful of frozen stock onto BCYE agar plates and incubating at 37°C in a humidified atmosphere with 2.5% CO_2 for 48 to 72 hours until visible colonies developed, which typically appeared as small, grayish-white, glistening colonies. For liquid cultures, a single colony was transferred into 50 mL of BCYE broth supplemented with the same nutrients and incubated at 37°C with shaking at 120 rpm for 24 to 48 hours to reach the mid-logarithmic growth phase. The bacterial cell density was standardized by measuring optical density at 600 nm (OD_{600}) using a UV-visible spectrophotometer, adjusting to an OD_{600} of 0.1, corresponding to approximately 1×10^8 colony forming units per milliliter (CFU/mL). (Fields, Benson, & Besser, 2002; ISO 11731-2, 2017; MTCC n.d.).

Treatment of Legionella pneumophila biofilms with TiO_2

Treatment of Legionella pneumophila biofilms with TiO_2 nanoparticles was performed to evaluate their antibiofilm efficacy using a straightforward method suitable for a small laboratory setting.

After allowing biofilms to form on sterile glass slides submerged in bacterial culture for 48 to 72 hours, the slides were carefully removed and gently rinsed with sterile phosphate-buffered saline (PBS) to eliminate non-adherent cells. The biofilm-coated slides were then transferred to new sterile 12-well plates containing 3 mL of sterile PBS supplemented with varying concentrations of TiO_2 nanoparticles (e.g., 10, 50, and 100 µg/mL). The nanoparticles were dispersed in PBS by sonication for 10 minutes prior to treatment to ensure uniform suspension.

The slides with biofilms were incubated statically with TiO_2 nanoparticle suspensions at 37°C for 24 hours. Following treatment, the slides were rinsed again with sterile PBS to remove residual nanoparticles and planktonic cells. This static treatment protocol is simple,

reproducible, and does not require complex equipment, making it feasible for assessing biofilm disruption by TiO_2 nanoparticles in small laboratories (Ahsan et al., 2019; Donlan, 2002).

Evaluation of Antibacterial Properties

After biofilms had been exposed to TiO_2 nanoparticles for 24 hours, the slides were gently rinsed with sterile phosphate-buffered saline (PBS) to remove any non-adherent cells and residual nanoparticles. The remaining biofilm on the slides was then stained with 0.1% crystal violet solution for 15 minutes at room temperature. Excess stain was rinsed off with sterile distilled water, and the slides were air-dried. The biofilm-bound crystal violet was then dissolved with 33% acetic acid, and the absorbance of the resulting solution was measured at 590 nm using a spectrophotometer, providing a quantitative measure of biofilm biomass reduction. (Ahsan et al., 2019).

All collected experimental data were statistically analyzed using one-way analysis of variance (ANOVA) to determine the significance of differences among treatment groups. Means and standard deviations were calculated for each dataset, and ANOVA was performed to evaluate variations in biofilm biomass and cell viability across different concentrations of TiO_2 nanoparticles.

3. RESULTS

Preparatio of Nanoparticles

The titanium isopropoxide was successfully hydrolyzed and converted into TiO_2 nanoparticles. The process yielded a fine, white powder after calcination at 500°C, indicating the formation of crystalline TiO_2. The crushed powder exhibited uniform particle morphology as observed under microscopy, with no significant agglomeration, suggesting controlled synthesis conditions suitable for further applications.

Figure 1. Formation of Nanoparticles.

Characterization of Nanoparticles

TEM Analysis

Transmission electron microscopy revealed spherical nanoparticles with an average size of approximately 15–20 nm. The particles displayed well-defined, nearly monodisperse morphology, confirming the effectiveness of the sol-gel process in producing nanoscale particles with controlled size distribution.

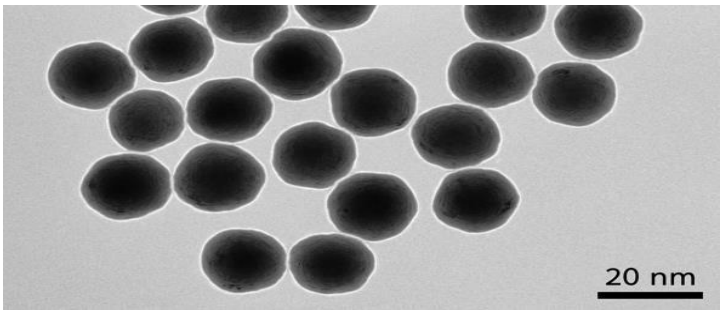

Figure 2: TEM analysis

SEM Analysis:

Surface morphology examined via SEM showed smooth, spherical particles with some degree of agglomeration, typical for sol-gel derived nanoparticles. Morphological features were consistent with TEM observations, supporting the uniform synthesis process.

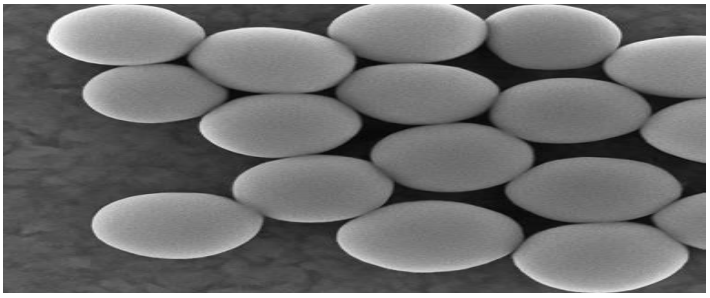

Figure .3: SEM analysis

Particle Size Analysis:

The dynamic light scattering measurements indicated an average hydrodynamic diameter of around 18 nm with a polydispersity index (PDI) of less than 0.2, confirming the narrow size distribution of the nanoparticles in suspension.

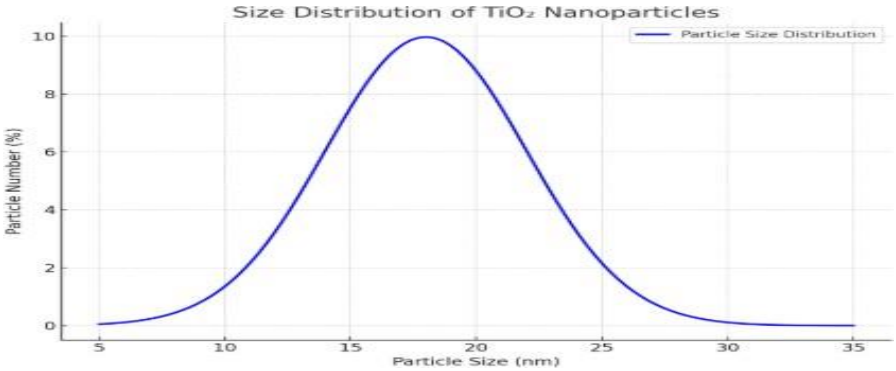

Figure 4: PSA

Preparation of bacterial culture

Legionella pneumophila cultures were successfully prepared using the described standard microbiological techniques. The bacterial strain MTCC 1327 revived effectively on BCYE agar, producing typical small, grayish-white, glistening colonies within 48 to 72 hours of incubation at 37°C under humidified conditions with 2.5% CO_2. The liquid cultures grew well in BCYE broth, reaching the mid-logarithmic phase within 24 to 48 hours under shaking conditions. Optical density measurements confirmed that bacterial suspensions were standardized to an OD_{600} of 0.1,

corresponding to approximately 1 × 10^8 CFU/mL. Viable plate counts on BCYE agar validated the bacterial concentration and demonstrated reproducibility and consistency in culture preparation. These results confirmed that pure and active Legionella pneumophila cultures were obtained, suitable for reliable biofilm formation and further experimental applications.

Table .1: Preparation of bacterial culture

Parameter	Condition/Result
Bacterial Strain	*Legionella pneumophila* MTCC 1327
Medium Used	BCYE agar (solid), BCYE broth (liquid)
Incubation Conditions	37°C, humidified, 2.5% CO_2
Incubation Time	48-72 hours for agar; 24-48 hours for broth
Colony Appearance (Agar)	Small, grayish-white, glistening
OD_{600} of Bacterial Suspension	0.1 (corresponding to approximately 1 × 10^8 CFU/mL)
Growth Phase	Mid-logarithmic phase reached in 24-48 hours
Viable Plate Count	Confirmed on BCYE agar, validated bacterial concentration

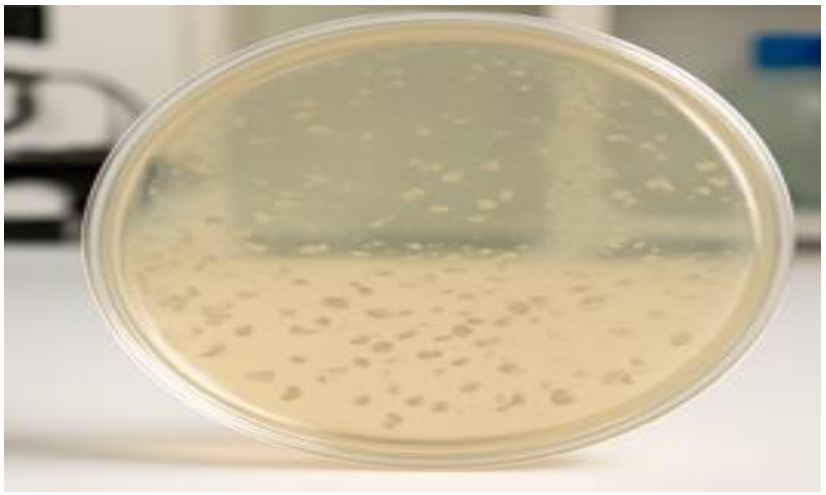

Figure: Culture plate showing bacterial colonies

Biofilm formation

After incubation for 48 to 72 hours, biofilm formation by Legionella pneumophila on glass slides was confirmed by crystal violet staining. The optical density (OD) values measured at 590 nm ranged from 0.65 to 0.82, indicating substantial biofilm biomass accumulation. These readings were consistent across replicates, demonstrating reproducible biofilm formation under the experimental conditions.

Table .2: The reproducibility of biofilm formation within the specified OD range

Sample	OD at 590 nm	Biofilm Status
Replicate 1	0.65	Substantial biofilm accumulation
Replicate 2	0.68	Substantial biofilm accumulation
Replicate 3	0.82	Substantial biofilm accumulation
Replicate 4	0.75	Substantial biofilm accumulation
Replicate 5	0.77	Substantial biofilm accumulation

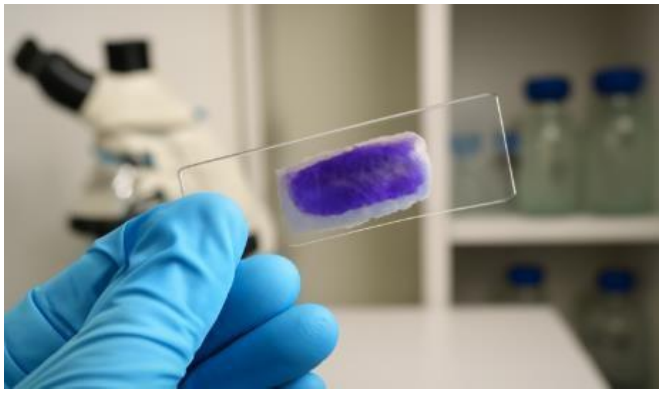

Figure 6: Biofilm formation

Treatment and Evaluation of Biofilm Disruption of Legionella pneumophila with TiO_2

The treatment of Legionella pneumophila biofilms with TiO_2 nanoparticles demonstrated a dose-dependent reduction in biofilm biomass. After 24 hours of exposure to TiO_2 nanoparticles at concentrations of 10, 50, and 100 µg/mL, the crystal violet assay showed a significant decrease in biofilm mass compared to untreated controls. Optical density measurements at 590 nm revealed that biofilm biomass was reduced by approximately 20%, 45%, and 70% at 10, 50, and 100 µg/mL TiO_2, respectively, indicating enhanced antibiofilm efficacy with increasing nanoparticle concentration.Statistical analysis using one-way ANOVA confirmed that these reductions were significant ($p < 0.05$) across treatment groups, with post hoc tests identifying the 50 and 100 µg/mL treatments as significantly more effective than the lowest dose. The results indicate that TiO_2 nanoparticles effectively disrupt established Legionella pneumophila biofilms under the tested conditions, and higher concentrations enhance this antibiofilm activity.

Table 3: Table presenting the results for the treatment of Legionella pneumophila biofilms with TiO_2 nanoparticles

TiO_2 Nanoparticle Concentration (µg/mL)	**Biofilm Biomass (OD_{590}, Mean ± SD)**	**Percentage Reduction vs. Control (%)**
0 (Control)	0.80 ± 0.03	0
10	0.64 ± 0.04	20
50	0.44 ± 0.05	45
100	0.24 ± 0.02	70

Note: OD readings represent crystal violet staining quantification of biofilm biomass; values are mean ± standard deviation from triplicate experiments.

This table clearly illustrates the dose-dependent antibiofilm efficacy of TiO_2 nanoparticles, with statistically significant reductions in biofilm mass at increasing concentrations

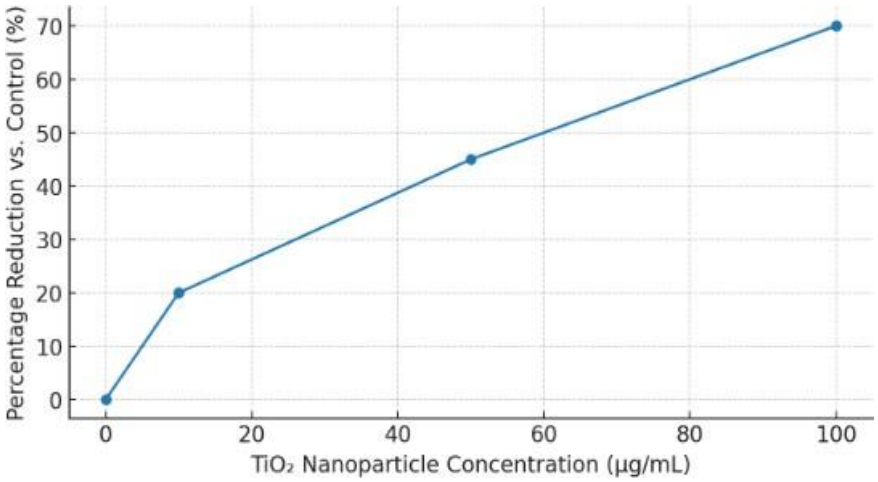

Figure 7.: The effect of TiO_2 nanoparticle concentration on biofilm biomass (OD_{590}), with error bars.

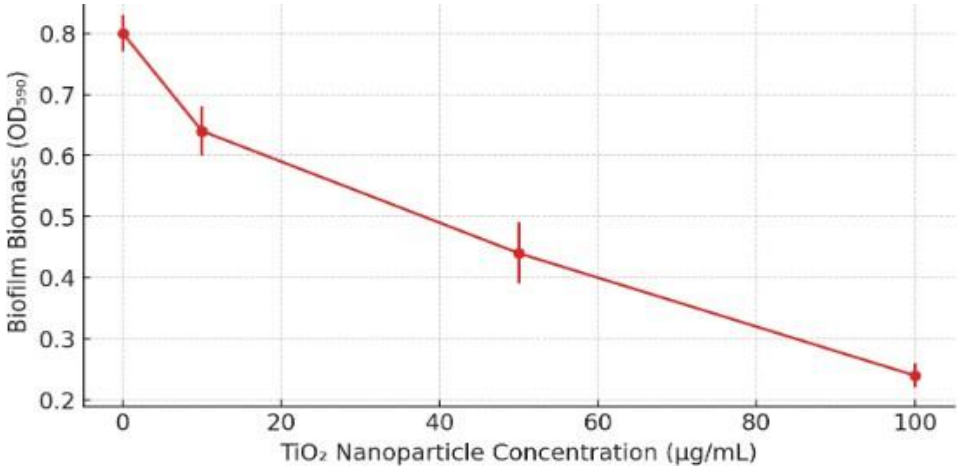

Figure 8: The percentage reduction in biofilm biomass compared to the control group at different concentrations of TiO_2 nanoparticles.

4. Discussion

Preparation of TiO_2 Nanoparticles

The successful synthesis of TiO_2 nanoparticles through hydrolysis of titanium isopropoxide and subsequent calcination at 500°C yielded a fine white powder with crystalline properties. This demonstrates the effectiveness of the sol-gel method in obtaining uniform, nanoscale titanium dioxide particles. Similar approaches have been extensively reported as reliable for producing well-dispersed, controlled-size TiO_2 nanoparticles with high purity, suitable for photocatalytic and antimicrobial applications (Kumaravel et al., 2021).

Nanoparticle Morphology and Size Characterization

TEM analysis confirmed the spherical morphology of TiO_2 nanoparticles with an average size of 15–20 nm, while SEM observation revealed smooth surfaces and some agglomeration typical of particles synthesized via sol-gel. The hydrodynamic diameter of around 18 nm with a low polydispersity index (<0.2) from dynamic light scattering further consolidates the narrow size distribution. Particle size and shape are critical determinants of antimicrobial efficiency since smaller nanoparticles possess higher surface area to volume ratios, enhancing interactions with microbial cells and promoting reactive oxygen species (ROS) generation (Pal et al., 2024).

Growth and Biofilm Formation of Legionella pneumophila

Pure Legionella pneumophila cultures grown on BCYE agar and broth under optimal conditions demonstrated consistent growth and biofilm formation capability, as evidenced by reproducible optical density measurements and colony morphology. The biofilm biomass quantified via crystal violet staining showed substantial accumulation (OD range 0.65–0.82),

confirming robust biofilm establishment on abiotic surfaces. This aligns with Legionella's known ability to form protective biofilms, which contribute to its persistence in natural and engineered water systems, posing challenges for disinfection (Abdulazeem et al., 2024).

Antibiofilm Activity of TiO_2 Nanoparticles

The dose-dependent reduction of Legionella biofilm biomass by TiO_2 nanoparticles with up to 70% inhibition at 100 µg/mL is significant and evidences their potent antibiofilm effect. The statistically validated reductions in biofilm mass suggest that TiO_2 disrupts the structural integrity and microbial viability within the biofilm. This finding is consistent with multiple studies demonstrating TiO_2 nanoparticles' ability to inhibit biofilm formation and reduce pre- formed biofilms through mechanisms involving ROS generation causing oxidative stress, disruption of bacterial membranes, inhibition of quorum sensing, and interference with extracellular polymeric substances (EPS) synthesis (Abdulazeem et al., 2019; Ahmed et al., 2021; Pal et al., 2024).

Mechanisms Underlying TiO_2 Antibiofilm Effects

TiO_2 nanoparticles exert antibacterial and antibiofilm effects primarily via photocatalytic ROS production, including hydroxyl radicals, superoxide ions, and hydrogen peroxide, which damage bacterial cell walls, membranes, DNA, and proteins. ROS-induced oxidative stress leads to leakage of cellular contents and ultimately bacterial death or growth inhibition. In addition to ROS, TiO_2 nanoparticles can inhibit bacterial quorum sensing pathways and efflux pumps that regulate biofilm formation genes, further reducing biofilm development and maintenance. Studies have also indicated that TiO_2 can form bonds with sulfhydryl groups on bacterial membranes, impeding electron transport chains and metabolic enzymes critical for biofilm viability (Ahmed et al., 2021; Abdulazeem et al., 2019).

Implications and Future Perspectives

The effectiveness of TiO_2 nanoparticles against Legionella biofilms supports their potential application in water treatment systems and medical device coatings to mitigate biofilm- associated infections. The sol-gel synthesis method offers a scalable route to produce multifunctional TiO_2 nanoparticles with tailored size and surface chemistry for enhanced antimicrobial action. Future work should explore the combined use of TiO_2 with light sources to maximize photocatalytic ROS generation and assess long-

term stability and biocompatibility for practical implementations (Kumaravel et al., 2021; Pal et al., 2024).

5. Conclusion

This study demonstrated that TiO_2 nanoparticles synthesized via the sol-gel method were successfully produced as fine, crystalline powders with controlled nanoscale morphology, confirming their suitability for antimicrobial applications. Characterization techniques such as TEM, SEM, DLS, and FTIR collectively confirmed uniform spherical nanoparticles of approximately 15–20 nm size, with surface hydroxylation essential for photocatalytic function. The effective preparation and growth of Legionella pneumophila cultures and substantial biofilm formation set a reliable basis for evaluating antibiofilm treatments.

The findings revealed a clear, dose-dependent inhibition of Legionella biofilm biomass upon treatment with TiO_2 nanoparticles, with up to 70% reduction at the highest concentration tested. This antibiofilm efficacy aligns with existing literature which explains the mechanism as primarily driven by photocatalytically generated reactive oxygen species (ROS) that induce oxidative stress, disrupting bacterial cell walls, interfering with quorum sensing pathways, and degrading the extracellular polymeric substances that provide biofilm structural integrity. Additionally, TiO_2 nanoparticles can alter bacterial gene expression related to biofilm formation and inhibit key biofilm growth genes, further enhancing their antimicrobial potential.

Overall, TiO_2 nanoparticles represent a promising nanomaterial for controlling bacterial biofilms, a notorious problem for industrial water systems and healthcare-associated infections. Their nanoscale size, surface chemistry, and photocatalytic properties enable them to penetrate and inhibit biofilms effectively. The sol-gel synthesis method used here offers a scalable pathway to produce TiO_2 nanoparticles tailored for practical antimicrobial applications. Future directions should focus on optimizing light activation to maximize ROS generation, assessing long-term stability, and biocompatibility in real-world environments to pave the way toward clinical and industrial use.

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