

Anti-biofilm Activity of CuO/ZnO Nanoparticles Biosynthesized by Psychrotrophic Bacteria

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ABSTRACT

Bacterial biofilms are a major contributor to persistent infections because biofilm-associated cells exhibit increased tolerance to antimicrobial agents. This study aimed to investigate the potential of cold-tolerant bacteria isolated from the Zagros highlands for the extracellular biosynthesis of copper oxide (CuO) and zinc oxide (ZnO) nanoparticles and to evaluate their antibacterial and antibiofilm activities against *Staphylococcus epidermidis*. CuO and ZnO nanoparticles were synthesized using cell-free supernatants obtained from psychrotrophic bacteria and subsequently characterized using UV-Vis spectroscopy, dynamic light scattering (DLS) and X-ray diffraction (XRD). DLS analysis revealed mean hydrodynamic diameters of 63.21 nm for CuO and 59.16 nm for ZnO, with corresponding polydispersity index (PDI) values of 0.271 and 0.190, respectively. XRD analysis confirmed the crystalline nature of both nanoparticles, with estimated crystallite sizes of 63.21 and 59.19 nm for CuO and ZnO, respectively. Their antimicrobial and biofilm-inhibitory activities were evaluated using crystal violet staining and colony-forming unit assays. ZnO exhibited stronger concentration-dependent activity and completely inhibited *S. epidermidis* biofilm formation at 250 µg/mL, whereas CuO required a concentration of 500 µg/mL. Both nanoparticles also reduced the number of viable biofilm-associated cells as their concentrations increased. Overall, psychrotrophic bacteria-mediated biosynthesis yielded CuO and ZnO nanoparticles with promising antimicrobial and antibiofilm properties, with ZnO demonstrating greater activity against *S. epidermidis* biofilms. These findings support their potential application as eco-friendly agents for biofilm control.

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INTRODUCTION

The present study was designed to investigate the potential of cold-tolerant bacteria isolated from the Zagros highlands for the extracellular biosynthesis of CuO and ZnO nanoparticles and to evaluate their antibacterial and antibiofilm activities against *Staphylococcus epidermidis*. Specifically, the study aimed to screen cold-tolerant bacterial isolates for their

ability to mediate the extracellular synthesis of CuO and ZnO nanoparticles, select the strain exhibiting the highest synthesis yield, identify the selected nanoparticle-producing strain based on 16S rRNA gene sequencing, and purify and characterize the resulting nanoparticles using UV–Vis spectroscopy, XRD and DLS. In addition, the study aimed to evaluate the antibacterial and anti-biofilm activities of the synthesized nanoparticles against *Staphylococcus epidermidis*, including their effects on biofilm formation and the disruption of established biofilms.

Conventional physical and chemical routes for nanoparticle synthesis offer considerable control over nucleation, particle size, morphology and crystallinity, but commonly rely on energy-intensive processing, elevated temperatures, specialized equipment and synthetic reducing or stabilizing agents, which may increase production costs and generate environmentally undesirable residues. In contrast, biological synthesis exploits naturally occurring biomolecules from microorganisms, plants, or other biological systems to mediate nanoparticle formation and stabilization under relatively mild and aqueous conditions, thereby offering a potentially lower chemical and environmental burden (Ghosh et al., 2024; Tanwar, 2024). Nevertheless, biological approaches remain constrained by biological variability, slower reaction kinetics, difficulties in achieving precise control over nanoparticle characteristics and challenges associated with reproducibility and scale-up (Liu et al., 2024; Ghosh et al., 2024). Within this framework, microbial synthesis is particularly attractive because extracellular enzymes, proteins, metabolites and other biomolecules can participate in nanoparticle nucleation and stabilization, providing a rational and potentially sustainable platform for the biosynthesis of CuO and ZnO nanoparticles (Liu et al., 2024; Amin & Aziz, 2025).

Biofilms are structured microbial communities embedded in a self-produced extracellular polymeric substance (EPS) matrix that promotes surface attachment, persistence and protection from antimicrobial agents and host defenses. This biofilm lifestyle contributes substantially to persistent and recurrent infections and represents an important challenge in antimicrobial resistance (Algadi et al., 2024; Generalova & Dushina, 2025). Among clinically important biofilm-forming bacteria, *Staphylococcus epidermidis* is a major opportunistic pathogen associated with indwelling medical devices. Although a normal component of human skin microbiota, its ability to adhere to abiotic surfaces and form mature biofilms facilitates persistent device-associated infections. Biofilm formation is therefore a key determinant of the clinical persistence and pathogenicity of *S. epidermidis* (Cheung & Otto, 2025; Fernández-Calderón et al., 2025).

The persistent nature of biofilm-associated infections and the limitations of existing approaches for controlling biofilm formation highlight the need for alternative antimicrobial strategies with effective anti-biofilm activity. Conventional nanoparticle production may involve energy-intensive processes and chemical reagents, while biological synthesis can be affected by biological variability, slower reaction kinetics, limited control over nanoparticle characteristics, and difficulties in reproducibility and scale-up. Furthermore, despite

increasing interest in biologically synthesized nanoparticles, the potential of psychrotrophic bacteria as biological platforms for the extracellular production of CuO and ZnO nanoparticles and the comparative evaluation of their activity against *S. epidermidis* biofilms remains insufficiently investigated. Exploring cold-tolerant bacterial isolates as nanoparticle-producing systems may therefore provide an additional biological resource for the development of metal oxide nanoparticles with antibacterial and anti-biofilm properties.

Metal oxide nanoparticles, particularly CuO and ZnO, have emerged as promising anti-biofilm agents because of their multiple antimicrobial actions, including membrane disruption, reactive oxygen species (ROS) generation, metal-ion release and damage to cellular components. Their biological activity, however, is influenced by physicochemical properties such as particle size, crystallinity, surface characteristics, aggregation and concentration (Algadi et al., 2024; Generalova & Dushina, 2025). Recent studies have demonstrated concentration-dependent antibacterial and anti-biofilm activities of green-synthesized CuO and ZnO nanoparticles against bacterial pathogens (Khairy et al., 2024; Atapakala et al., 2024; Shakerdarabad et al., 2024).

Biological synthesis offers an environmentally compatible alternative to conventional nanoparticle production by utilizing microbial biomolecules involved in nanoparticle formation, stabilization and capping. In particular, psychrotrophic microorganisms represent an underexplored resource for nanobiotechnology because their adaptation to low temperatures may support nanoparticle production under relatively mild conditions. Cold-tolerant *Streptomyces* strains have previously been shown to mediate extracellular synthesis of metal nanoparticles, demonstrating the potential of cold-adapted bacteria as biological platforms for nanoparticle production (Bakhtiari-Sardari et al., 2020; Ramasamy et al., 2023).

Although interest in biologically synthesized metal oxide nanoparticles has increased, the use of psychrotrophic bacteria for the extracellular production of CuO and ZnO nanoparticles, together with a comparative assessment of their anti-biofilm effects against *Staphylococcus epidermidis*, has not yet been adequately investigated.

METHODS AND MATERIALS

Investigated Strains

During the initial stage of the investigation, 44 cold-resistant bacterial strains isolated from the Zagros Highlands of Lorestan Province, Iran, together with selected *Streptomyces* strains isolated from the Oman Sea, were recovered, purified and subjected to preliminary phenotypic characterization. All procedures related to isolation, purification and initial characterization were conducted at Ferdowsi University of Mashhad, Iran. The resulting pure cultures were then assessed under controlled laboratory conditions for their capacity to mediate the biosynthesis of CuO and ZnO nanoparticles. The synthesized nanoparticles were subsequently screened for their antibiofilm activity against the selected bacterial test strains to evaluate their potential to inhibit biofilm formation. This preliminary screening was conducted at the same research centre to identify promising bacterial isolates and their

corresponding nanoparticle preparations exhibiting superior nanoparticle-producing capacity and antibiofilm potential. The most promising candidates were then selected for further optimization of synthesis, physicochemical characterization and assessment of their antibiofilm efficacy.

Maximum Tolerable Concentration

The Maximum Tolerable Concentration (MTC) of the bacterial isolates was determined using a standard metal-tolerance assay. Briefly, 200 mL of solid Tryptic Soy Broth (TSB) medium was prepared in separate flasks and supplemented with copper and zinc salts at concentrations of 2.5, 5, 10, 25 and 50 mM/L. The supplemented media were subsequently dispensed into sterile Petri plates and incubated at 30°C for 24 h to ensure uniform distribution and complete solidification. The solidified plates were then divided into equal sections. Plates without copper or zinc supplementation were used as controls to assess normal bacterial growth.

For inoculation, a 5 µL aliquot of each bacterial suspension, prepared from a 24-h culture and adjusted to a turbidity equivalent to the 0.5 McFarland standard, was spot-inoculated onto each designated section of the plates. The inoculated plates were incubated at 20°C and 30°C for 48 h. The highest metal concentration at which no visible bacterial growth was observed was recorded as the MTC. Strains capable of growing at higher metal concentrations were classified as metal-resistant isolates and considered to have greater potential for the reduction of Cu²⁺ and Zn²⁺ ions (Shakya et al., 2012).

Biosynthesis and Characterization of Copper Oxide and Zinc Oxide Nanoparticles and Molecular Identification of the Producing Strain

The selected bacterial isolates were cultured in 400 mL of Tryptic Soy Broth (TSB) and incubated under controlled laboratory conditions at 20 °C with continuous agitation at 150 rpm for 48 h. Following sufficient cellular growth, the culture broth was centrifuged at 1,000 rpm for 15 min to obtain the cell-free supernatant. Equimolar (0.01 M) solutions of zinc nitrate and copper sulphate were subsequently added to the supernatant at a 1:1 volumetric ratio to initiate the biosynthesis of nanoparticles. The resulting reaction mixtures were then autoclaved at 121 °C for 15 min to promote the formation of metal oxide nanoparticles.

After incubation for 24 h at ambient temperature, the synthesized nanoparticles were recovered by centrifugation, thoroughly washed three times with deionized water and ethanol and dried at 80 °C. The dried products were subsequently calcined at 470 °C for 4 h to remove residual organic and biological moieties adsorbed onto the nanoparticle surfaces. Among the tested isolates, the strain exhibiting the most pronounced color transition in the supernatant and the highest degree of sediment formation was selected for further purification and physicochemical characterization of the synthesized zinc oxide nanoparticles.

The optical absorption spectra of the purified ZnO and CuO nanoparticles were recorded using a UV–Vis spectrophotometer over wavelength ranges of 200–400 nm and 250–350 nm,

respectively, to assess their characteristic optical absorption profiles and provide preliminary evidence of nanoparticle formation. The hydrodynamic diameter, size distribution and dispersity of the synthesized nanoparticles were determined by DLS to characterize their colloidal size and dispersion behavior. In addition, the crystalline structure and phase composition of the nanoparticles were analyzed using XRD to confirm the formation of the respective metal oxide phases and evaluate their crystallinity. For molecular identification, genomic DNA was extracted from the selected strain and the 16S rRNA gene was amplified and sequenced to confirm the taxonomic affiliation of the nanoparticle-producing microorganism.

Investigating the Anti-biofilm Activity of Nanoparticles

Staphylococcus epidermidis and other coagulase-negative staphylococci (CoNS) are opportunistic pathogens that constitute part of the normal human skin microbiota. *S. epidermidis* is frequently identified as a contaminant in blood cultures and represents a major cause of nosocomial infections, including bacteremia, catheter-associated infections and device-related infections. Its capacity to produce biofilms is a major determinant of pathogenicity, particularly among immunocompromised individuals and patients with indwelling medical devices. Biofilms are robust, multilayered microbial communities that adhere strongly to surfaces and exhibit substantial resistance to antimicrobial therapies and host immune defenses (Sani et al., 2014; Namvar et al., 2014). The relationship between virulence factors and biofilm formation in clinical *S. epidermidis* isolates highlights the bacterium's ability to colonize host tissues, establish persistent biofilms and contribute to chronic or device-associated infections in susceptible individuals.

Determination of MIC and MBC of Nanoparticles Against *S. Epidermidis*

The broth microdilution method was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CuO and ZnO nanoparticles against *Staphylococcus epidermidis*. A 96-well micro liter plate was used for the assay. Single colonies of *S. epidermidis* were inoculated into TSB supplemented with glucose and incubated at 37 °C for 18 h. Stock suspensions of CuO and ZnO nanoparticles (16,000 mg/L) were prepared in 40% glycerol and stirred with a sterile magnetic stirrer for 24 h. Subsequently, 100 µl of Mueller–Hinton Broth (MHB) was added to each well and serial two-fold dilutions of the nanoparticle suspensions were prepared across ten wells to obtain concentrations ranging from 8000 to 15.6 mg/L. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard and further diluted 1:20 in sterile physiological saline to achieve approximately 7.5×10^5 CFU/ml. Ten microliters of this bacterial suspension were added to each well, resulting in a final volume of 110 µl per well. Negative control wells contained 100 µl of MHB only, while positive control wells contained 100 µl of MHB and 10 µl of the bacterial suspension without nanoparticles. Additional wells containing only MHB and nanoparticles were used as blanks. Plates were incubated at 37 °C for 24 h and the optical density was measured at 630 nm using an ELISA reader. The lowest concentration showing no visible bacterial growth (zero absorbance) was recorded as the MIC. To determine the

MBC, 10 µl from wells showing no visible growth were sub cultured onto TSA plates supplemented with glucose and incubated at 37 °C for 24 h. The lowest concentration showing no bacterial colony growth was defined as the MBC, indicating 100% bacterial killing (Andrews, 2001).

Qualitative and Quantitative Assessment of Bio-film Formation in *S. Epidermidis*

Biofilm formation by *Staphylococcus epidermidis* was assessed by means of a 96-well microliter plate-based method. Briefly, individual bacterial colonies were transferred into tryptic soy broth supplemented with glucose (TSBG) and cultivated at 37 °C for 18 h. For the biofilm assay, 100 µl of TSBG was dispensed into each well of a sterile 96-well microliter plate. The overnight bacterial culture was standardized to a turbidity equivalent to 0.5 McFarland and subsequently diluted 1:100 in TSBG, yielding a final bacterial concentration of approximately 1.5×10^6 CFU/ml. An aliquot of 10 µl of the resulting suspension was then inoculated into each well. The plates were maintained at 37 °C for 24 h to facilitate biofilm development.

Following incubation, the contents of the wells were carefully removed and the wells were washed several times with sterile physiological saline to eliminate planktonic and loosely attached bacterial cells. The remaining surface-associated biofilms were fixed by treatment with 99% methanol for 10 min and subsequently stained with 0.03% crystal violet for 10 min. Excess crystal violet was removed and the wells were washed four times with sterile physiological saline before being allowed to air-dry. To quantify the retained biofilm, 100 µl of 70% ethanol was added to each well to solubilize the bound crystal violet. The optical density of each well was subsequently determined at 570 nm using an ELISA reader, with the measured absorbance serving as an indicator of biofilm biomass (Wilson et al., 2017).

Evaluation of the Anti-Biofilm Activity of Nanoparticles

The anti-biofilm activity of the synthesized nanoparticles was evaluated using a microtiter plate-based assay with 0.03% (w/v) crystal violet staining. An overnight culture of the test strain was prepared in Tryptic Soy Broth supplemented with glucose (TSBG). For the assay, 100 µl of TSBG was dispensed into each well of a sterile 96-well microtiter plate. Subsequently, 100 µl of the nanoparticle suspension at an initial concentration of 16,000 mg/L was introduced into the wells of the first column in six replicates. Twofold serial dilutions (1:2) were then performed across the plate to obtain final nanoparticle concentrations ranging from 16,000 to 15.6 mg/L, following the same dilution procedure used for MIC determination.

A 10 µl aliquot of the diluted bacterial suspension, containing approximately 1.5×10^6 CFU/ml, was added to all wells except those designated as negative controls and blanks. Positive control wells consisted of 100 µl of TSBG and 10 µl of the bacterial suspension without nanoparticles. Two rows containing different concentrations of CuO and ZnO nanoparticles prepared in TSBG were included as blank controls. The negative control wells

contained only 100 µl of TSBG, whereas the treatment wells contained TSBG, the respective nanoparticle suspension and the bacterial inoculum.

The microliter plate was incubated at 37 °C for 24 h under static conditions. Following incubation, the contents of the wells were carefully removed and each well was washed three times with sterile physiological saline to eliminate non-adherent bacterial cells. The plate was then air-dried at room temperature. Subsequently, 200 µl of 99% methanol was added to each well to fix the attached biofilm. After 10 min, the methanol was removed and the plate was allowed to dry again.

Each well was then treated with 200 µl of 0.03% crystal violet solution and incubated at room temperature for 10 min. Excess stain was removed by gently washing the plate under running water, followed by air-drying. To solubilize the crystal violet retained by the biofilm, 200 µl of 70% ethanol was added to each well. The optical density (OD) was subsequently measured at 570 nm using a microplate (ELISA) reader.

The percentage of biofilm inhibition was calculated according to the following equations (Wilson et al., 2017):

$$100 \times \frac{OD_{570} \text{ (sample with nanoparticles)}}{OD_{570} \text{ (positive control)}} = \text{Biofilm growth (\%)}$$

$$\text{Biofilm inhibition (\%)} = 100 - \text{Biofilm growth (\%)}$$

Evaluation of the Ability of Nanoparticles to Disrupt Established Bio-film Structures

The ability of CuO and ZnO nanoparticles to disrupt pre-established biofilms was evaluated by first allowing bacterial biofilms to develop and subsequently exposing them to different concentrations of the nanoparticles. Positive-control, negative-control and nanoparticle-control wells were included in the assay, consistent with the control scheme used for the minimum inhibitory concentration (MIC) determination. Briefly, individual colonies of *Staphylococcus epidermidis* were inoculated into Tryptic Soy Broth supplemented with glucose (TSBG) and incubated at 37 °C for 18 h. Following incubation, 100 µl of TSBG was dispensed into each well of a sterile 96-well microliter plate. The bacterial culture was adjusted to a turbidity equivalent to the 0.5 McFarland standard and subsequently diluted 1:100 in TSBG to obtain a final concentration of approximately 1.5×10^6 CFU/ml. A 10 µl aliquot of the diluted suspension was then added to each well and the plate was incubated at 37 °C for 24 h to facilitate biofilm formation.

After biofilm development, the planktonic cells were removed by carefully discarding the contents of the wells, followed by several washes with sterile physiological saline. Fresh TSBG (100 µl) containing different concentrations of the nanoparticles (15.6–8000 mg/L) was then added to the test and nanoparticle-control wells. The plate was subsequently incubated at 37 °C for an additional 24 h to allow interaction between the nanoparticles and the pre-formed biofilms.

Following nanoparticle exposure, the contents of the wells were removed and the wells were washed several times with sterile physiological saline. The remaining biofilms were fixed with 99% methanol for 10 min and subsequently stained with 0.03% (w/v) crystal violet for 10 min. Excess stain was carefully removed and the wells were washed four times with physiological saline before being allowed to air-dry. Subsequently, 100 µl of 70% ethanol was added to each well to solubilize the biofilm-associated crystal violet. The absorbance was then measured at 570 nm using a microplate (ELISA) reader. The percentage of biofilm degradation was calculated according to the following equations (Mu et al., 2016):

$$\frac{OD_{570} \text{ (in presence of nanoparticles)}}{OD_{570} \text{ (positive control)}} = \text{Biofilm formation (\%)}$$

$$\text{Biofilm degradation (\%)} = 100 - \text{Biofilm formation (\%)}$$

All experiments were conducted in triplicate to ensure reproducibility. For complementary confirmation, after nanoparticle-mediated biofilm inhibition, one well from each nanoparticle concentration was carefully washed and transferred into sterile vials. The samples were then subjected to ultrasonic treatment, vortexed for 20 s and 100 µl of the homogenized suspension was spread onto TSB agar plates. The plates were incubated at 37 °C for 24 h and the number of viable colonies was counted to determine the extent of biofilm cell disruption and recovery (Taglietti et al., 2014).

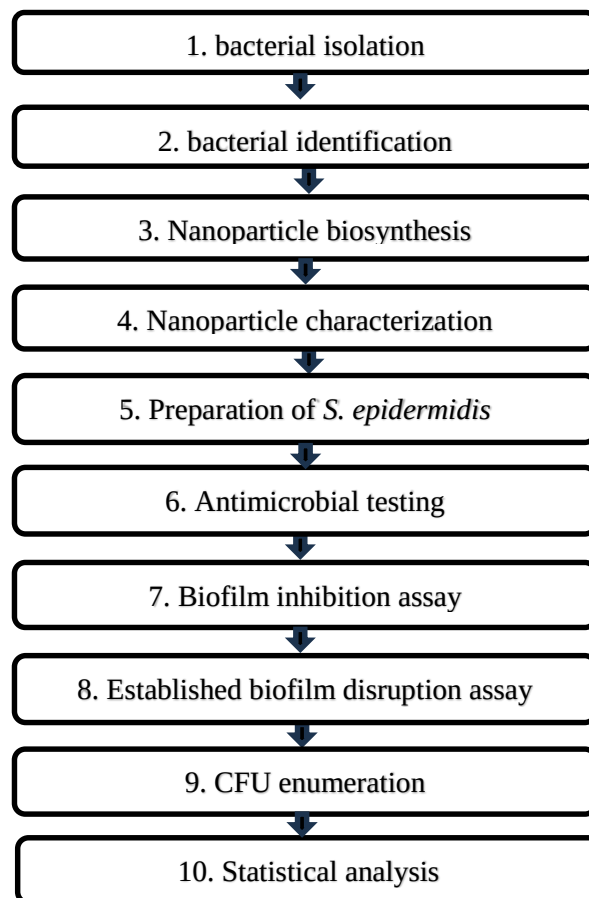


Figure 1. Study workflow chart

FINDINGS

Biosynthesis of ZnO and CuO Nanoparticles by Salt-Tolerant Bacteria

The resistance of 44 cold-resistant and *Streptomyces* strains to zinc sulphate, zinc nitrate, copper sulphate and copper nitrate at 2.5–50 mM was evaluated. All strains tolerated concentrations above 5 mM. Based on initial screening and MTC results, two cold-resistant and two *Streptomyces* strains that showed higher resistance to sulphate than nitrates were selected and 10 mM zinc sulphate was used for nanoparticle synthesis. *Streptomyces* strains were subsequently excluded due to slow growth. Among the remaining isolates, strain OSNP₁₃ showed the highest sediment formation and supernatant color change after autoclaving. It was therefore chosen for ZnO and CuO nanoparticle purification and characterization. Copper oxide and zinc oxide nanoparticles were produced using bacterial culture supernatants as reaction media under controlled thermal conditions. After incubating the bacterial cultures, we collected cell-free supernatants and mixed them with metal salt precursors. The reaction mixtures were heat-treated to promote nanoparticle formation, then washed and calcined to obtain purified metal oxide nanoparticles. This approach utilizes microbial supernatants as a supportive matrix rather than an enzymatic biosynthesis mechanism. NADH-dependent enzymes play key roles in electron transfer during biological nanoparticle synthesis. To our knowledge, this is the first report describing ZnO and CuO nanoparticle production by cold-resistant strains under such conditions. Figure 1.

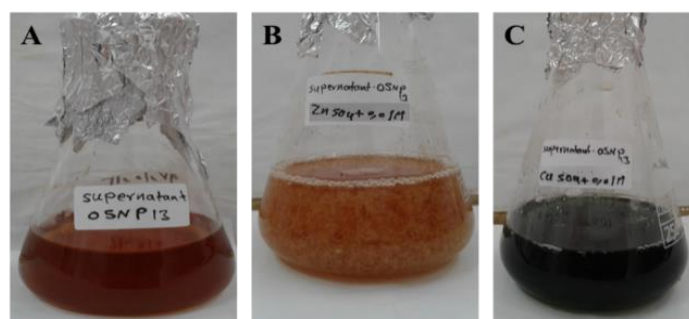


Figure 1. Biosynthesis of copper and zinc oxide nanoparticles in the supernatant of OSNP₁₃ under autoclave conditions. (A–C) Supernatant: (A) initial luminescence, (B) after addition of zinc and copper sulphate, (C) post-autoclaving.

UV–vis spectroscopy (Figure 2) revealed absorption peaks at 269 nm and 288 nm, corresponding to ZnO and CuO nanoparticles, respectively, attributed to their characteristic surface plasmon resonance.

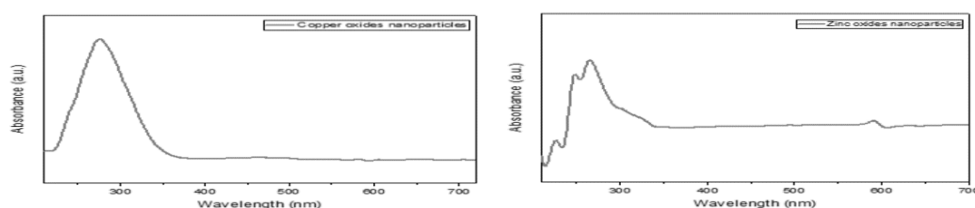


Figure 2. UV–vis spectra of nanoparticles: (A) CuO and (B) ZnO

DLS analysis (Figure 3) determined the nanoparticle size, size-distribution profile and polydispersity index (PDI). The resulting particle-size distribution profiles indicated that the mean particle sizes of the biosynthesized CuO and ZnO nanoparticles were 63.21 nm and 59.16 nm, respectively.

The polydispersity index (PDI) values of the CuO and ZnO nanoparticles at physiological pH were calculated as 0.27 and 0.19, respectively. The PDI is a widely used parameter for assessing the breadth of particle-size distribution and the degree of homogeneity within a colloidal nanoparticle suspension. Generally, lower PDI values indicate a narrower and more homogeneous particle-size distribution, whereas higher values reflect greater size heterogeneity and polydispersity. According to commonly used criteria, PDI values below 0.3 generally indicate a relatively uniform particle-size distribution and good colloidal dispersity. In contrast, values above 0.7 typically indicate a broad and highly heterogeneous size distribution (Honary et al., 2013). Therefore, based on the obtained PDI values, the CuO and ZnO nanoparticles biosynthesized by *Microbacterium* sp. OSNP₁₃ exhibited relatively high size uniformity and acceptable colloidal dispersion.

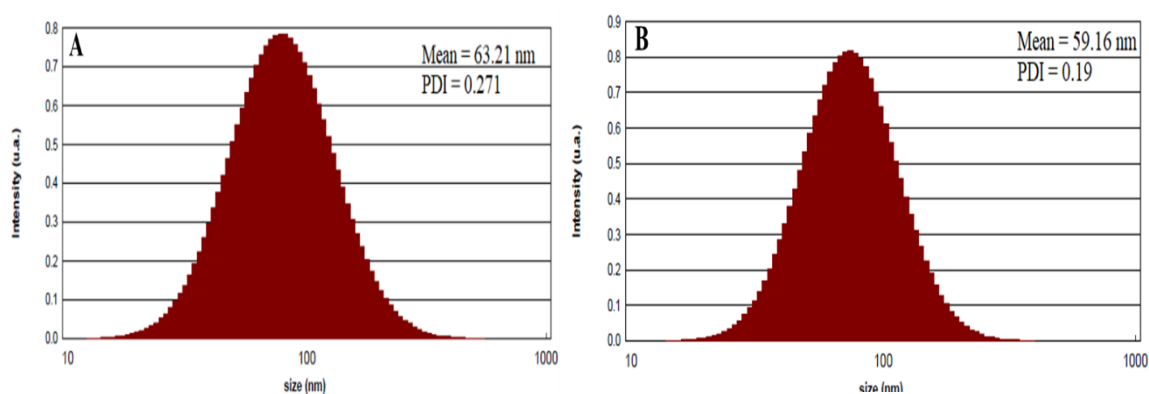


Figure 3. Particle-size distribution of CuO nanoparticles (A) and ZnO nanoparticles (B)

X-ray diffraction is based on the diffraction of X-rays following their interaction with the periodic atomic arrangement of a crystalline material. X-rays occupy a region of the electromagnetic spectrum between γ rays and UV radiation. XRD analysis provides valuable information regarding the crystalline structure, phase composition, crystallinity and lattice characteristics of materials.

In this study, XRD analysis was performed on dried biosynthesized copper oxide and zinc oxide nanoparticles over a 2θ range of 20° – 80° to identify crystalline phases and confirm their respective crystal structures. The XRD patterns were processed and plotted using X'Pert HighScore software. The XRD pattern of the CuO nanoparticles exhibited nine distinct diffraction peaks corresponding to the crystallographic planes (110), (002), (111), (102), (020), (202), (113), (311) and (004), as shown in Figure 4. These characteristic reflections confirmed the crystalline nature of the biosynthesized CuO nanoparticles and were consistent with the JCPDS reference standard No. 048-1548.

Similarly, the XRD pattern of the ZnO nanoparticles produced by *Microbacterium* sp. OSNP13 exhibited characteristic diffraction peaks corresponding to the crystallographic planes (100), (002), (101), (102), (110), (103), (200), (112), (201) and (004). The observed diffraction pattern agreed well with the reference JCPDS standard pattern No. 043-0002, confirming the formation of crystalline ZnO nanoparticles. The diffraction peaks were consistent with the characteristic hexagonal wurtzite crystal structure of ZnO, whereas the CuO nanoparticles exhibited the characteristic monoclinic crystal structure. Overall, the XRD results provided strong evidence for the successful formation and crystallization of the biosynthesized CuO and ZnO nanoparticles (Mallick & Sahu, 2012; Shatnawi et al., 2016).

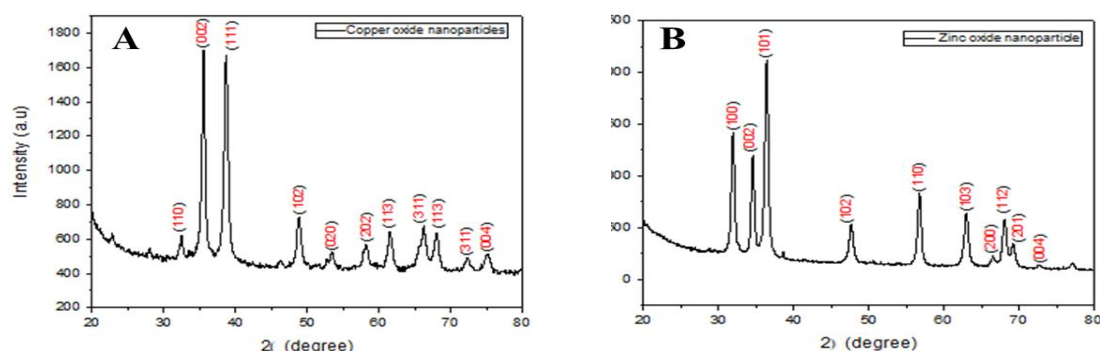


Figure 4. XRD patterns of CuO nanoparticles (A) and ZnO nanoparticles (B).

Investigation of the Anti-Biofilm Activity of Nanoparticles

In this part of the study, *Staphylococcus epidermidis* was selected as the test organism to assess the antibiofilm activity of the biosynthesized nanoparticles. Initially, we evaluated the biofilm-forming capacity of *S. epidermidis* using a 96-well microtiter plate assay (Figure 5).

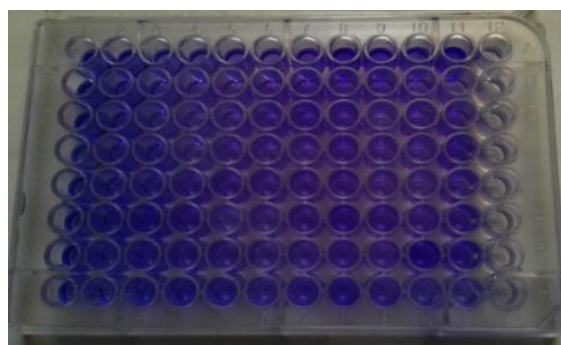


Figure 5. Biofilm formation by *Staphylococcus epidermidis* in 96-well microplates

Subsequently, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CuO and ZnO nanoparticles against *S. epidermidis* was determined using the same methodology described for the antimicrobial activity assay. The MIC and MBC values for CuO nanoparticles were 2000 and 2000 µg/mL, respectively, whereas those for ZnO nanoparticles were 250 and 250 µg/mL, respectively (Figure 6).

Notably, in contrast to the findings obtained from the antimicrobial activity assay, ZnO nanoparticles exhibited a substantially stronger inhibitory effect against *S. epidermidis* than CuO nanoparticles under the experimental conditions employed in this study. The markedly lower MIC and MBC values observed for ZnO (250 µg/mL) compared with CuO (2000 µg/mL) indicate a greater antibacterial potency of ZnO against *S. epidermidis*. These findings suggest that the biosynthesized ZnO nanoparticles may have greater potential than CuO nanoparticles for controlling *S. epidermidis* under the conditions evaluated in the present study.

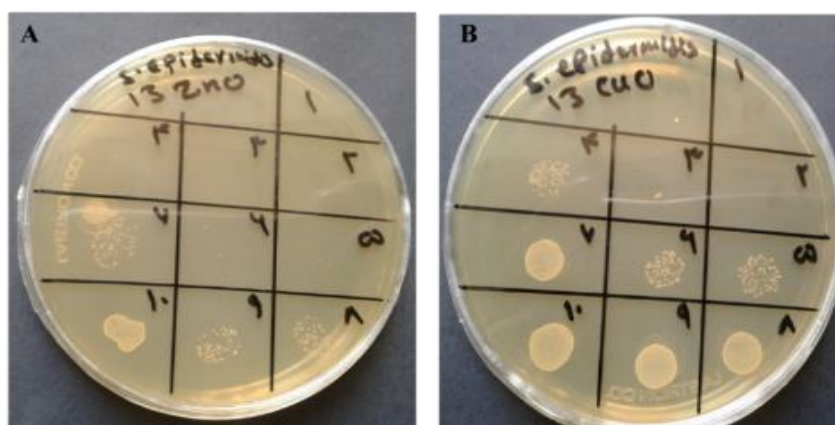


Figure 6. Minimum bactericidal concentration (MBC) assay of zinc oxide (A) and copper oxide nanoparticles (B) against *Staphylococcus epidermidis*. Wells 1–10 correspond to a two-fold serial dilution of the nanoparticles, with final concentrations ranging from 15.6 to 8000 µg/mL

Evaluation of the ability of nanoparticles to inhibit biofilm formation

After determining the MIC values, the ability of CuO and ZnO nanoparticles to inhibit biofilm formation by *Staphylococcus epidermidis* in 96-well micro titer plates was evaluated. The results showed that CuO and ZnO nanoparticles completely inhibited biofilm formation at concentrations of 500 and 250 µg/mL, respectively. ZnO nanoparticles exhibited superior anti biofilm activity, which may be attributed to their smaller particle size.

Table 1. Percentage Inhibition of Biofilm Formation by *Staphylococcus epidermidis* in the Presence of CuO and ZnO Nanoparticles

Concentration (µg/mL)	8000	4000	2000	1000	500	250	125	62.5	31.25	16
ZnO NPs	100%	100%	100%	100%	100%	100%	65%	47%	12%	6%
CuO NPs	100%	100%	100%	100%	100%	12%	10%	10%	4%	5%

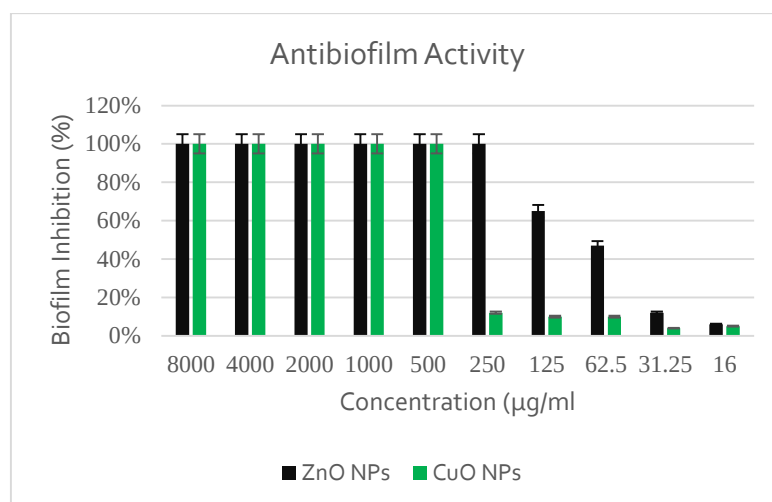


Figure 7. Inhibition percentage of biofilm formation by *S. epidermidis* in the presence of different concentrations of copper oxide and zinc oxide nanoparticles.

Evaluation of the Ability of Nanoparticles to Destroy Biofilm Structures

The ability of the biosynthesized CuO and ZnO nanoparticles to disrupt pre-formed *Staphylococcus epidermidis* biofilms was evaluated using a 96-well microliter plate assay. As shown in Figure 8 and Table 2, both nanoparticles exhibited a concentration-dependent effect on pre-established biofilms. Complete disruption of the pre-formed biofilm was achieved at concentrations of 4000 µg/mL for CuO and 2000 µg/mL for ZnO, indicating that ZnO nanoparticles were more effective in disrupting *S. epidermidis* biofilms at a lower concentration. To further assess the effect of nanoparticle treatment on the viability of biofilm-associated bacterial cells, we carefully washed the treated biofilms and transferred them to sterile vials. The samples were then sonicated and vortexed for 20 s to facilitate the detachment and homogenization of the remaining biofilm-associated cells. Subsequently, a 100-µL aliquot of the resulting cell suspension was spread onto TSB agar plates using the lawn-culture method. The plates were incubated at 37 °C for 24 h, after which the resulting colony-forming units (CFUs) were enumerated to quantify the reduction in viable biofilm-associated bacterial cells following nanoparticle treatment. The percentage of biofilm disruption was calculated using the following equation:

$$100 \times (1 - \text{CFU}_t / \text{CFU}_0) = \text{Biofilm destruction (\%)}$$

where CFU_0 represents the colony count obtained from untreated biofilms and CFU_t represents the colony count obtained after nanoparticle treatment. ZnO nanoparticles produced a greater reduction in viable biofilm-associated bacterial cells at lower concentrations than CuO nanoparticles, further supporting their comparatively higher efficacy against pre-formed *S. epidermidis* biofilms under the experimental conditions used in this study.

The differences observed between the crystal violet- and CFU-based measurements may be attributed to the distinct principles underlying the two assays. While crystal violet staining primarily reflects total biofilm biomass, including both cellular and extracellular matrix components, CFU enumeration estimates the viable, culturable bacterial population. In

addition, variations in biofilm detachment, cell recovery and potential cell loss during washing, sonication and vortexing may contribute to differences between the two measurements.

Table 2. Percentage of *S. epidermidis* biofilm disruption at different concentrations of zinc oxide and copper oxide nanoparticles

Nanoparticles Concentration ($\mu\text{g/ml}$)	8000	4000	2000	1000	500	250	125	62.5	31.25	15.6
ZnO NPs	100%	100%	100%	79%	65%	44%	39%	47%	6%	12%
CuO NPs	100%	100%	95%	66%	31%	25%	22%	10%	4%	5%

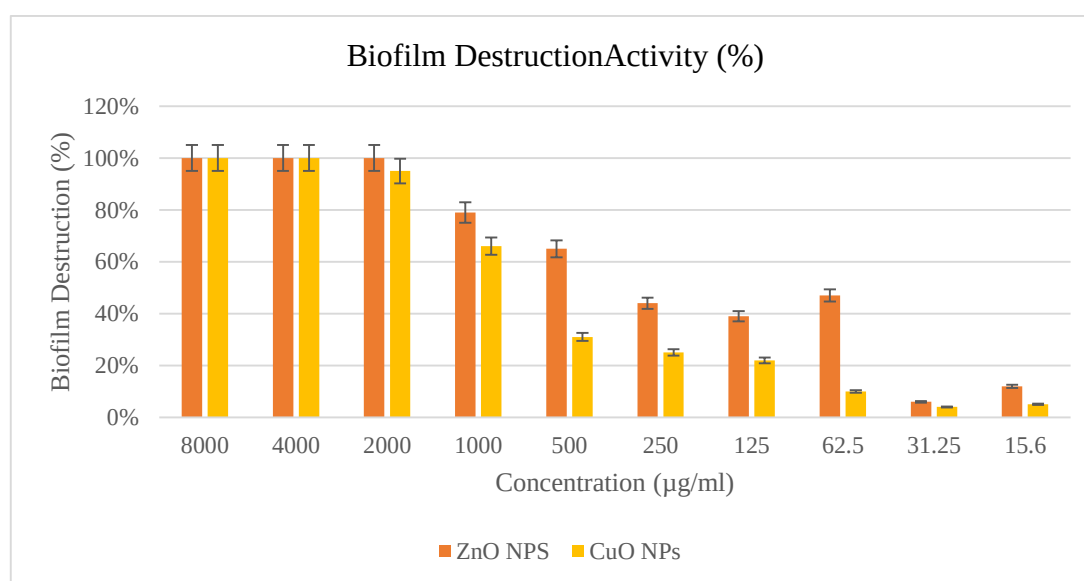


Figure 8. Percentage of biofilm disruption of *S. epidermidis* in the presence of zinc oxide and copper oxide nanoparticles.

The colony-count results are presented in Figures 9 and 10. To enable accurate enumeration of the surviving bacterial population, samples from each nanoparticle concentration were diluted 20-fold before plating. ZnO nanoparticles exhibited pronounced biofilm-disrupting activity, resulting in a reduction of more than 90% in the viable biofilm-associated bacterial population at a concentration of 500 $\mu\text{g/mL}$. In comparison, CuO nanoparticles completely eradicated the detectable viable biofilm-associated population at 2000 $\mu\text{g/mL}$.

The results of this supplementary CFU-based assay showed a slight discrepancy from those obtained in the previous assay for ZnO nanoparticles. This difference may reflect variations in bacterial cell recovery and additional cell detachment or disruption during sample processing, particularly during washing, sonication, vortexing and dilution. Therefore, interpret these findings in the context of the methodological differences between the two assays.

Table 3. Percentage of *S. epidermidis* biofilm disruption measured by CFU assay in the presence of different concentrations of zinc oxide and copper oxide nanoparticles

Nanoparticles Concentration ($\mu\text{g/ml}$)	8000	4000	2000	1000	500	250	125	62.5	31.25	15.6
ZnO NPs	100%	100%	100%	100%	96%	82%	86%	71%	77%	78%
CuO NPs	100%	100%	%	67%	56%	32%	27%	21%	14%	8%

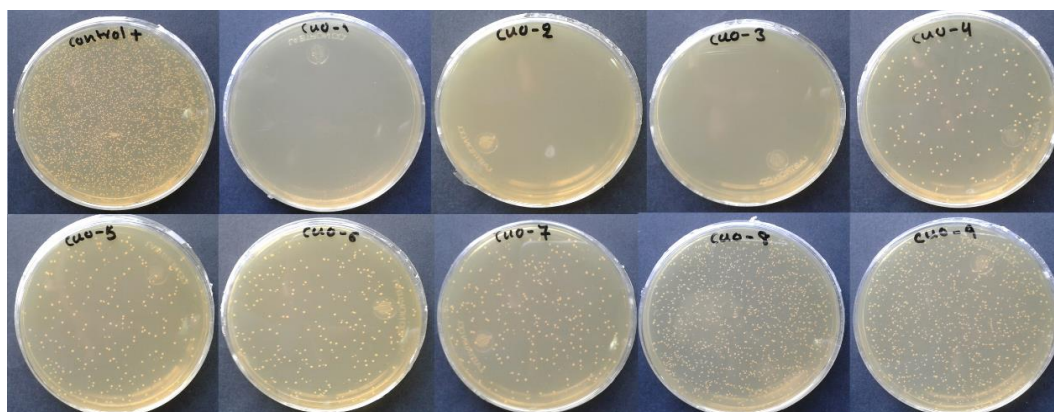


Figure 9. *S. epidermidis* biofilm disruption by copper oxide nanoparticles measured by CFU. Samples were 20-fold diluted at each concentration (Plates 1–9: 8000–31.25 $\mu\text{g/mL}$).

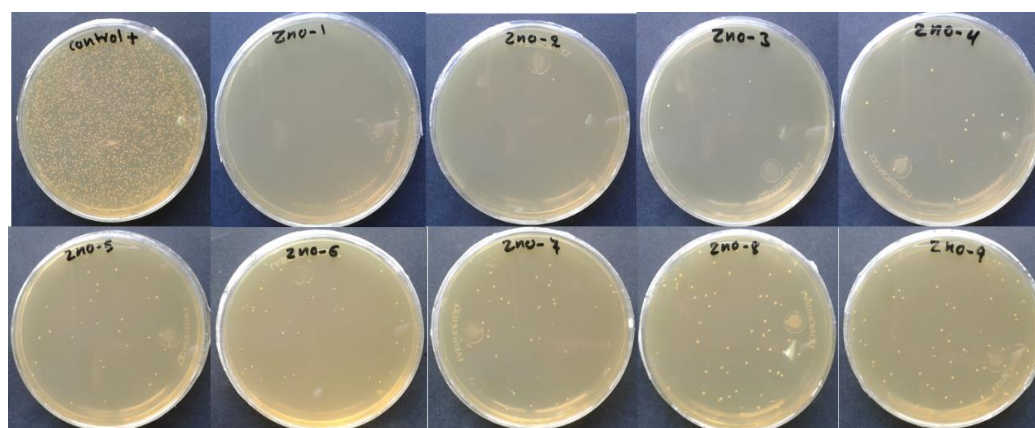


Figure 10. *S. epidermidis* biofilm disruption by Zinc oxide nanoparticles measured by CFU. Samples were 20-fold diluted at each concentration (Plates 1–9: 8000–31.25 $\mu\text{g/mL}$).

DISCUSSION

Biological nanoparticle synthesis represents an attractive alternative to conventional physical and chemical approaches because of its potential environmental and economic advantages. However, the mechanisms underlying microbe-mediated nanoparticle formation and the factors governing nanoparticle physicochemical properties remain incompletely understood. In this study, we evaluated psychrotolerant bacterial isolates for the biosynthesis of CuO and ZnO nanoparticles using autoclave-treated cell-free supernatants. To the best of our knowledge, this approach has not previously been reported for the biosynthesis of CuO and ZnO nanoparticles by cold-resistant bacterial isolates.

Nanoparticle formation was initially indicated by color changes and precipitate formation, with *Microbacterium* sp. OSNP13 exhibiting the highest nanoparticle production

and recovery. Microbial extracellular extracts and cell-free biological components have previously been exploited for nanoparticle biosynthesis, demonstrating that extracellular biomolecules can participate in precursor transformation, nucleation and stabilization. Ghanbari et al. (2018), for example, demonstrated microorganism-assisted extracellular synthesis of silver nanoparticles using *Aspergillus fumigatus* extract under autoclave-assisted conditions. However, because that study involved fungal extract and Ag nanoparticles rather than bacterial cell-free supernatants and CuO/ZnO, it provides general support for the biological synthesis concept rather than a direct precedent for the present system. Thus, the present study may represent a distinct application of cold-adapted bacterial extracellular products for CuO and ZnO nanoparticle synthesis.

UV–Vis spectroscopy showed absorption maxima at 288 nm for CuO and 269 nm for ZnO. The CuO result is comparable to the ~285 nm absorption reported for biologically synthesized CuO nanoparticles by Yugandhar et al. (2017), whereas ZnO nanoparticles have been reported to exhibit optical absorption in the near-UV region, with values varying with particle size, morphology, surface chemistry and synthesis conditions. Fooladsaz et al. (2012) reported UV–Vis absorption behavior of ZnO nanoparticles in the 275–350 nm region. Nevertheless, UV–Vis spectroscopy alone cannot unequivocally establish crystalline phase identity. In particular, particle size, surface states, capping molecules, aggregation and residual biological components may influence the optical response. Therefore, the present UV–Vis findings should be considered preliminary spectroscopic evidence, whereas phase identification was based primarily on XRD analysis.

DLS revealed mean hydrodynamic diameters of 63.21 nm for CuO and 59.16 nm for ZnO, with PDI values of 0.271 and 0.19, respectively (Figure 3), indicating relatively narrow particle-size distributions. Differences from previously reported particle dimensions may reflect differences in precursor concentration, synthesis route, reaction conditions, dispersion state and biological reducing or capping molecules. Tiwari et al. (2014) reported biosynthesized copper nanoparticles with a mean hydrodynamic diameter of 110.9 nm and a PDI of 0.312. Importantly, these particles were copper nanoparticles rather than CuO nanoparticles and therefore provide a comparative reference for biologically synthesized copper-based nanoparticles rather than a direct comparison with the present CuO system. Ghorbani et al. (2015) reported ZnO nanoparticles with a particle size of approximately 30 nm, whereas Kumar et al. (2017) reported CuO nanoparticles with particle dimensions in the range of approximately 43.3–52.5 nm. These differences highlight how synthesis conditions and biological components influence nanoparticle growth and aggregation.

XRD provided the principal structural evidence for phase identification (Figure 4). The CuO diffraction pattern was consistent with monoclinic CuO, whereas ZnO corresponded to the characteristic hexagonal wurtzite structure. The crystallite sizes estimated using the Debye–Scherrer equation were 31.29 nm for CuO and 19.25 nm for ZnO. The smaller XRD-derived crystallite sizes compared with DLS measurements are expected because XRD estimates coherently diffracting crystalline domains, whereas DLS measures the

hydrodynamic diameter of dispersed particles, including the effects of surface-associated biomolecules and possible particle aggregation. Thus, the two techniques provide complementary, not equivalent, measurements. The identification of monoclinic CuO is consistent with previous reports of biologically synthesized CuO nanoparticles, including the study of Yugandhar et al. (2017), while the hexagonal wurtzite structure observed for ZnO is consistent with previous ZnO characterization studies (Anvekar et al., 2017; Fooladsaz et al., 2012; Ghorbani et al., 2015; Raoufi, 2013).

Metal oxide nanoparticles may exert antibacterial effects through multiple, potentially overlapping mechanisms, including direct interaction with the bacterial cell envelope, membrane disruption, reactive oxygen species (ROS) generation, oxidative stress, intracellular damage and metal-ion release. Sirelkhatim et al. (2015) summarized evidence that ZnO nanoparticles can induce oxidative stress and cellular damage through ROS generation, membrane interactions and Zn²⁺ release. These mechanisms may contribute to the antibacterial effects observed in the present study; however, the specific mechanism responsible for the activity of the synthesized CuO and ZnO nanoparticles was not directly investigated.

The biosynthesized nanoparticles also exhibited substantial antibiofilm activity against *Staphylococcus epidermidis*. ZnO completely inhibited biofilm formation at 250 µg/mL, compared with 500 µg/mL for CuO and completely disrupted pre-formed biofilms at 2000 and 4000 µg/mL, respectively. ZnO's greater activity may partly relate to its smaller hydrodynamic and crystallite dimensions; however, the present data do not establish a causal relationship between particle size and antibiofilm activity. Other factors, including surface chemistry, crystal structure, aggregation state, dissolution behavior, ion release, surface charge and ROS generation, may also contribute to the observed difference between ZnO and CuO. These findings are broadly consistent with previous reports describing antimicrobial and antibiofilm properties of copper- and zinc-based nanoparticles. LewisOscar et al. (2015) reported approximately 94% disruption of *Pseudomonas aeruginosa* biofilms following treatment with copper nanoparticles at 100 ng/mL. Importantly, these were copper nanoparticles, not CuO nanoparticles. Hassani et al. (2015) also reported significant inhibition of *P. aeruginosa* biofilm formation by ZnO nanoparticles. In addition, Singh et al. (2015) demonstrated antibiofilm and membrane-damaging activity of cuprous oxide (Cu₂O) nanoparticles against *Staphylococcus aureus*. Because Cu, Cu₂O and CuO represent chemically distinct materials, these studies should be considered comparative evidence for copper-based nanoparticles rather than direct evidence for the activity of CuO nanoparticles. Differences among studies may result from nanoparticle composition and phase, physicochemical properties, particle size, bacterial strain, exposure time, concentration and assay methodology.

Overall, the complementary UV–Vis, DLS and XRD analyses provide convergent evidence supporting the formation of monoclinic CuO and hexagonal wurtzite ZnO nanoparticles by *Microbacterium* sp. OSNP₁₃. The observed antibacterial and antibiofilm activities further

support the potential of these biologically synthesized metal oxide nanoparticles as antimicrobial nanoparticles.

CONCLUSION

This study demonstrated that copper oxide and zinc oxide nanoparticles generated using microbial culture supernatants exhibit in vitro antimicrobial and biofilm-reducing effects against *Staphylococcus epidermidis*. The observed reduction in biofilm biomass was largely associated with concentration-dependent bactericidal activity, with zinc oxide nanoparticles showing greater efficacy. While these findings highlight the potential of metal oxide nanoparticles as anti-biofilm agents, further studies involving nanoparticle characterization, cytotoxicity assessment and evaluation under clinically relevant conditions are necessary before practical applications can be considered.

Authors contributions

1. Bashir Ahmad Aseem: Conducted the practical and laboratory experiments, including nanoparticle biosynthesis and experimental evaluation.
2. Mohammad Arif Ahsas: Wrote the original manuscript, organized the study findings and prepared the manuscript for submission.
3. Hasan Karimy: Performed data analysis and contributed to the interpretation and presentation of the results.
4. All authors reviewed and approved the final version of the manuscript.

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Conflict of Interest Statement

The authors declare that there is no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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