

Original Paper

Biosynthesis of Copper Oxide Nanoparticles Using *Streptococcus salivarius* Cell-Free Filtrate and Evaluation of Their Antibacterial Activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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ABSTRACT

Copper oxide nanoparticles (CuO NPs) are promising antimicrobial agents, and their biological synthesis offers an environmentally friendly alternative to chemical methods. In this study, CuO NPs were biosynthesised using the cell-free filtrate of *Streptococcus salivarius* as the reducing and capping agent, with copper sulfate (1 mM) as the precursor, at room temperature and pH 7 for 35 min. The product was characterised by Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX), and its antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was evaluated by the agar well-diffusion method at 1000, 500, 250, 125 and 62.5 µg/mL. FTIR revealed bands of proteins and carbohydrates derived from the bacterial filtrate (3428, 2926, 1642, 1453, 1235 and 1044 cm⁻¹) together with the Cu–O stretching band at 618 cm⁻¹. XRD peaks at 2θ = 35.5°, 38.7°, 48.8°, 61.6°, 66.2° and 68.1° matched monoclinic CuO (JCPDS 48-1548) with no secondary phases. SEM showed quasi-spherical, partly agglomerated particles of 44–75 nm, and EDX gave 80.43 wt% Cu and 19.57 wt% O, corresponding to a Cu:O atomic ratio of approximately 1:1. The nanoparticles inhibited both bacteria in a concentration-dependent manner (p ≤ 0.05), with maximum inhibition zones of 16.67 ± 0.58 mm (*S. aureus*) and 17.67 ± 0.58 mm (*P. aeruginosa*) at 1000 µg/mL. *P. aeruginosa* remained susceptible down to 62.5 µg/mL (7.33 ± 0.58 mm), whereas *S. aureus* was inhibited only at ≥ 250 µg/mL. *S. salivarius*-mediated synthesis is thus a simple, rapid and green route to antibacterial CuO NPs, with the Gram-negative pathogen showing the greater susceptibility.

KEYWORDS: Antibacterial activity, Biosynthesis, Copper oxide nanoparticles, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus salivarius*.

1-INTRODUCTION

The steady rise of antimicrobial resistance has reduced the efficacy of conventional antibiotics against many clinically important pathogens and has stimulated the search for alternative antimicrobial agents. Nanomaterials are attractive candidates because their small size, high surface-to-volume ratio and distinctive physicochemical properties allow them to interact with bacterial cells through mechanisms that differ from those of classical antibiotics [1,2].

Among metal-oxide nanomaterials, copper oxide nanoparticles (CuO NPs) have received particular attention because copper is inexpensive, chemically stable and active against a broad range of Gram-positive and Gram-negative bacteria [3,4]. CuO NPs can be produced by physical, chemical and biological routes. Biological (green) synthesis, in which microbial cells or their extracellular metabolites act as reducing, nucleating and capping agents, avoids toxic reagents and harsh reaction conditions and yields particles coated with biomolecules that may improve colloidal stability and biocompatibility [5,6]. Extracellular synthesis using cell-free culture supernatants is especially convenient because the nanoparticles can be recovered without disrupting the cells [7].

The antibacterial action of CuO NPs is mediated by several complementary mechanisms, including electrostatic attachment to the cell envelope, disruption of membrane integrity, release of Cu^{2+} ions, generation of reactive oxygen species (ROS) and oxidative damage to proteins, lipids and DNA [4,8]. The magnitude of the effect depends on particle size, morphology, surface chemistry and concentration, as well as on the structure of the bacterial cell envelope, so that Gram-positive and Gram-negative bacteria may respond differently to the same material [8–10]. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are widely used as representative Gram-positive and Gram-negative pathogens in this type of study because both are leading causes of nosocomial infection and both readily acquire multidrug resistance [1,4].

Streptococcus salivarius is a Gram-positive, non-pathogenic commensal of the human oral cavity that secretes a range of extracellular proteins and metabolites; to our knowledge, its use as a biological agent for the synthesis of CuO NPs has not previously been described. The aims of the present study were therefore (i) to biosynthesise CuO NPs using the cell-free filtrate of *S. salivarius*, (ii) to characterise the product by FTIR, XRD, SEM and EDX, and (iii) to evaluate its antibacterial activity against *S. aureus* and *P. aeruginosa* by the agar well-diffusion method.

2-MATERIALS AND METHODS

2.1. Bacterial strains

S. salivarius (used for biosynthesis), *S. aureus* and *P. aeruginosa* (test organisms) were previously identified isolates obtained from the culture collection of the College of Applied Sciences, University of Samarra. The identity of the isolates was confirmed by conventional biochemical tests prior to use. Strains were maintained on nutrient agar slants at 4 °C and subcultured before use.

2.2. Preparation of the cell-free filtrate

S. salivarius was inoculated into Brain Heart Infusion (BHI) broth and incubated at 37 °C for 24 h. The culture was centrifuged at 10,000 rpm for 10 min to remove the cells, and the supernatant was passed through a sterile 0.22 µm membrane filter. The resulting cell-free filtrate was used immediately as the biological reducing and capping agent.

2.3. Biosynthesis of CuO nanoparticles

Ten millilitres of cell-free filtrate were added to 90 mL of 1 mM copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution, the pH was adjusted to 7.0 and the mixture was stirred at room temperature for 35 min. A change in the colour of the reaction mixture indicated nanoparticle formation. The precipitate was collected by centrifugation at 10,000 rpm for 10 min, washed three times with distilled water to remove unreacted ions and medium components, and dried at 105 °C for 5–6 h. A control containing CuSO_4 solution and sterile BHI broth without bacterial filtrate was processed in parallel under the same conditions. The dried powder was stored in a desiccator until characterisation.

2.4. Characterisation

Fourier-transform infrared (FTIR) spectra of the dried powder were recorded in the range 4000–400 cm^{-1} (Shimadzu Corporation, Japan) to identify the functional groups of the biomolecules involved in reduction and capping. The crystalline phase was examined by X-ray diffraction (XRD; Shimadzu XRD-6000, Japan) using Cu K α radiation ($\lambda = 0.15406 \text{ nm}$) over a 2θ range of 20–80° with a step size of 0.02°. Surface morphology and particle size were examined by scanning electron microscopy (SEM; ZEISS EVO 10, Carl Zeiss Microscopy, Germany) after sputter-coating; particle diameters were measured directly on the micrographs using the instrument software. Elemental composition was determined by energy-dispersive X-ray spectroscopy (EDX) coupled to the SEM.

2.5. Preparation of nanoparticle suspensions

A stock suspension of 1000 µg/mL was prepared by dispersing 1 mg of dried CuO NPs in 1 mL of dimethyl sulfoxide (DMSO) and sonicating for 30 min. Two-fold serial dilutions (500, 250, 125 and 62.5 µg/mL) were prepared from the stock in DMSO immediately before use.

2.6. Antibacterial activity (agar well-diffusion assay)

Antibacterial activity was assessed by the agar well-diffusion method [11]. Overnight cultures (18–24 h) of *S. aureus* and *P. aeruginosa* were adjusted to a turbidity equivalent to the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) and swabbed uniformly over the surface of Mueller–Hinton agar plates. Wells of 6 mm diameter were cut with a sterile cork borer, and 60 μ L of each nanoparticle concentration was added to separate wells. Plates were kept at room temperature for 30 min to allow diffusion and then incubated at 37 °C for 24 h. The diameter of the inhibition zone (including the well) was measured in millimetres with a ruler. Each concentration was tested in triplicate.

2.7. Statistical analysis

The experiment followed a completely randomised design with three replicates. Data are presented as mean $\pm$ standard deviation (SD). Differences among concentrations within each bacterial species were analysed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). Differences were considered significant at $p \leq 0.05$.

3-RESULTS

3.1. Biosynthesis of CuO nanoparticles

Addition of the *S. salivarius* cell-free filtrate to the copper sulfate solution produced a visible colour change within the 35-min reaction period, indicating reduction of Cu^{2+} and formation of a colloidal product, and a dark precipitate was recovered after centrifugation. No precipitate formed in the filtrate-free control. These observations indicate that extracellular metabolites present in the bacterial filtrate participated in the biosynthesis and stabilisation of the CuO nanoparticles, consistent with previous reports of bacterial supernatant-mediated synthesis of metal and metal-oxide nanoparticles [5–7].

3.2. FTIR analysis

The FTIR spectrum of the biosynthesised CuO NPs (Figure 1) showed absorption bands at 3428, 2926, 1642, 1453, 1235, 1044 and 618 cm^{-1} . The broad band centred at 3428 cm^{-1} is assigned to O–H and N–H stretching vibrations of the hydroxyl and amine groups of proteins and polysaccharides; the band at 2926 cm^{-1} corresponds to aliphatic C–H stretching; the strong band at 1642 cm^{-1} is characteristic of the amide I (C=O stretching) vibration of proteins; the bands at 1453 and 1235 cm^{-1} are attributed to C–H bending and amide III/C–N stretching vibrations, respectively; and the band at 1044 cm^{-1} corresponds to C–O and C–O–C stretching of carbohydrates. The presence of these bands demonstrates that proteins and carbohydrates from the bacterial filtrate are bound to the nanoparticle surface and act as capping agents. The band at 618 cm^{-1} lies in the metal–oxygen region (400–700 cm^{-1}) and is assigned to the Cu–O stretching vibration of monoclinic CuO, providing direct spectroscopic evidence of copper oxide formation. This profile is consistent with FTIR spectra reported for biologically synthesised CuO NPs [5,12–15].

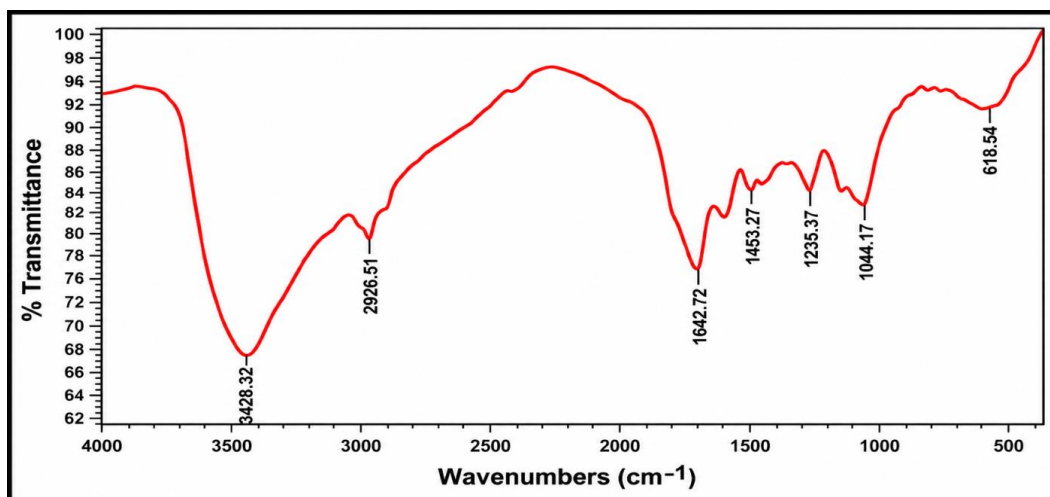


Figure (1): FTIR spectrum of CuO nanoparticles biosynthesised using the cell-free filtrate of *S. salivarius*.

3.3. XRD analysis

The XRD pattern (Figure 2) showed well-defined diffraction peaks at $2\theta \approx 35.5^\circ$, 38.7° , 48.8° , 61.6° , 66.2° and 68.1° , which are indexed to the (-111) , (111) , (-202) , (-113) , (-311) and (220) planes, respectively, of monoclinic CuO (tenorite; JCPDS card no. 48-1548). No reflections attributable to Cu_2O , metallic Cu or $\text{Cu}(\text{OH})_2$ were detected, confirming that the product is single-phase crystalline CuO. The sharp, intense peaks indicate a high degree of crystallinity.

Crystallite size was not estimated from the diffractogram because instrument-corrected full width at half maximum (FWHM) values were not available for a reliable Scherrer analysis; the physical particle size was therefore evaluated from the SEM micrographs (Section 3.4). It should be noted that crystallite size derived from the Scherrer equation represents the size of coherently diffracting crystalline domains and may differ from the physical particle size observed by electron microscopy, because individual particles can contain several crystalline domains and peak broadening is also influenced by lattice strain and instrumental effects [16].

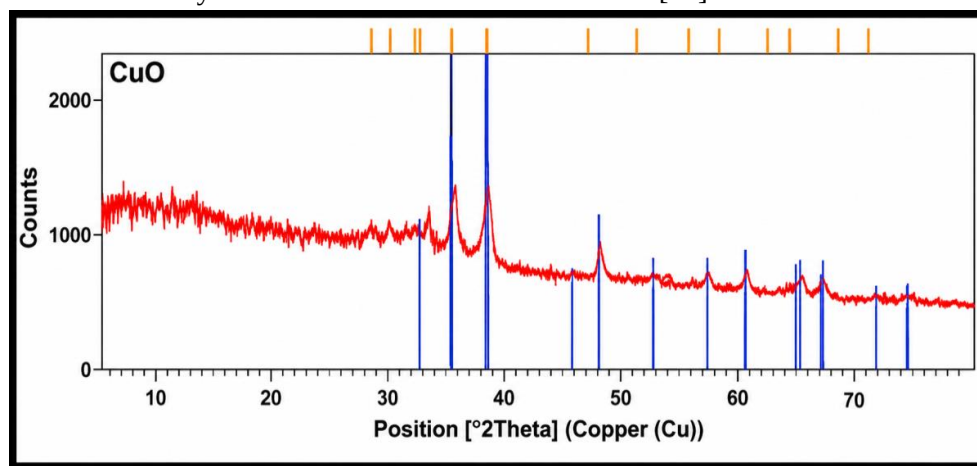


Figure (2): XRD pattern of CuO nanoparticles biosynthesised using *S. salivarius*; the vertical lines mark the reference reflections of monoclinic CuO (JCPDS 48-1548).

3.4. SEM analysis

SEM examination (Figure 3) revealed abundant, roughly spherical to quasi-spherical nanoparticles that were partially agglomerated into clusters. At higher magnification (Figure 3b), individual particles measured directly on

the micrograph ranged from approximately 44 to 75 nm in diameter (measured values of 44.6, 58.9, 70.3 and 75.0 nm). Larger elongated structures measuring approximately 160–500 nm were also observed and are interpreted as particle aggregates and/or residual biological material from the bacterial filtrate. The observed agglomeration may be associated with the high surface energy of nanoscale particles and with interactions or bridging between surface-associated biomolecules originating from the bacterial filtrate. Similar aggregation has been reported for biologically synthesised metal-oxide nanoparticles [5,13].

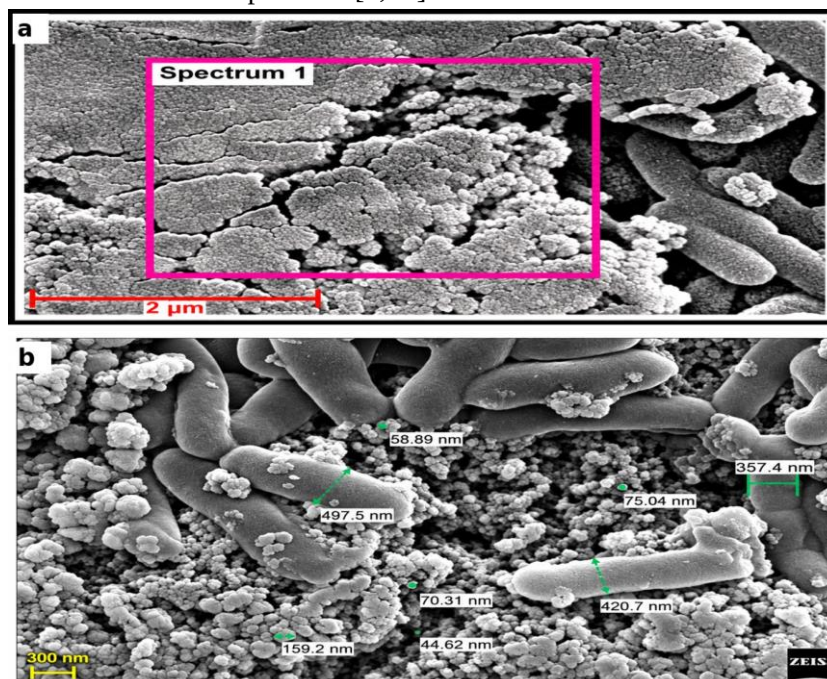


Figure (3): SEM micrographs of CuO nanoparticles biosynthesised using *S. salivarius*: (a) overview showing agglomerated particles and the area selected for EDX analysis (scale bar 2 µm); (b) higher magnification with measured particle diameters (scale bar 300 nm).

3.5. EDX analysis

The EDX spectrum (Figure 4) showed strong signals for copper (Cu L α at 0.93 keV and Cu K α /K β at 8.04 and 8.90 keV) and oxygen (O K α at 0.52 keV). Copper and oxygen accounted for 80.43 and 19.57 wt%, respectively, corresponding to 50.86 and 49.14 at%, i.e. a Cu:O atomic ratio of approximately 1:1, which is the stoichiometry expected for CuO and is in agreement with the XRD phase identification. Minor unassigned peaks are attributable to the biomolecular capping layer and the sample support. Because EDX cannot distinguish the oxidation state of copper, phase identity rests on the XRD result.

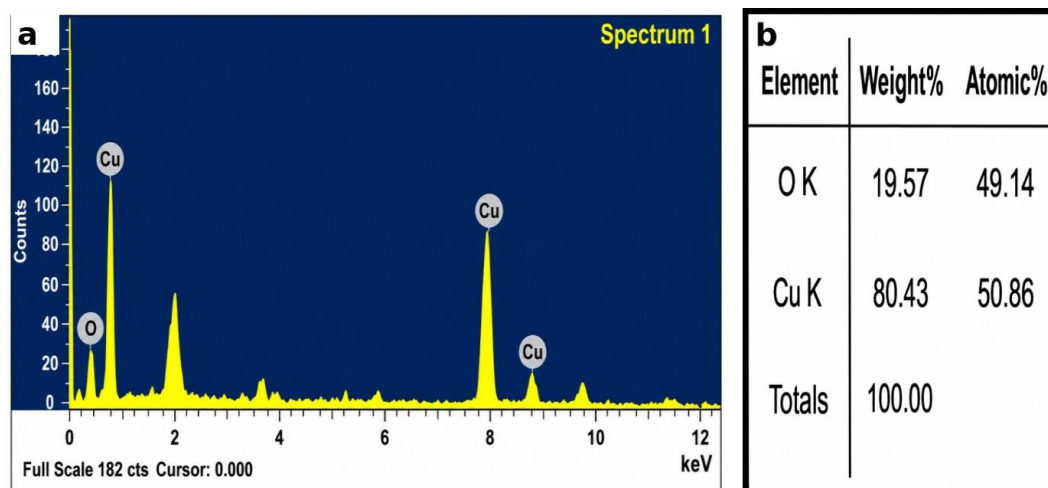


Figure (4): EDX analysis of the biosynthesised CuO nanoparticles: (a) spectrum; (b) elemental composition (weight % and atomic %).

3.6. Antibacterial activity

The biosynthesised CuO NPs inhibited the growth of both test organisms in a concentration-dependent manner (Table 1; Figure 5). Against *S. aureus*, the inhibition zone decreased from 16.67 ± 0.58 mm at 1000 $\mu\text{g/mL}$ to 14.33 ± 0.58 mm at 500 $\mu\text{g/mL}$ and 7.33 ± 0.58 mm at 250 $\mu\text{g/mL}$, and no inhibition was observed at 125 or 62.5 $\mu\text{g/mL}$. Against *P. aeruginosa*, inhibition zones of 17.67 ± 0.58 , 16.33 ± 1.15 , 14.33 ± 1.53 , 9.67 ± 0.58 and 7.33 ± 0.58 mm were recorded at 1000, 500, 250, 125 and 62.5 $\mu\text{g/mL}$, respectively. Within each species, the differences between concentrations were significant ($p \leq 0.05$), except between 1000 and 500 $\mu\text{g/mL}$ and between 125 and 62.5 $\mu\text{g/mL}$ for *P. aeruginosa*. *P. aeruginosa* was therefore inhibited at all concentrations tested, whereas *S. aureus* was inhibited only at ≥ 250 $\mu\text{g/mL}$; at 250 $\mu\text{g/mL}$ the zone against *P. aeruginosa* was almost twice that against *S. aureus*.

Table (1) Inhibition zone diameters (mm, mean $\pm$ SD, n = 3) of biosynthesised CuO nanoparticles against *S. aureus* and *P. aeruginosa* by the agar well-diffusion method

Concentration ($\mu\text{g/mL}$)	<i>S. aureus</i>	<i>P. aeruginosa</i>
1000	16.67 ± 0.58^a	17.67 ± 0.58^a
500	14.33 ± 0.58^b	16.33 ± 1.15^a
250	7.33 ± 0.58^c	14.33 ± 1.53^b
125	0.00 ± 0.00^d	9.67 ± 0.58^c
62.5	0.00 ± 0.00^d	7.33 ± 0.58^c

Values within a column followed by different superscript letters differ significantly (Duncan's test, $p \leq 0.05$). Zone diameter includes the 6 mm well; 0.00 indicates no inhibition.

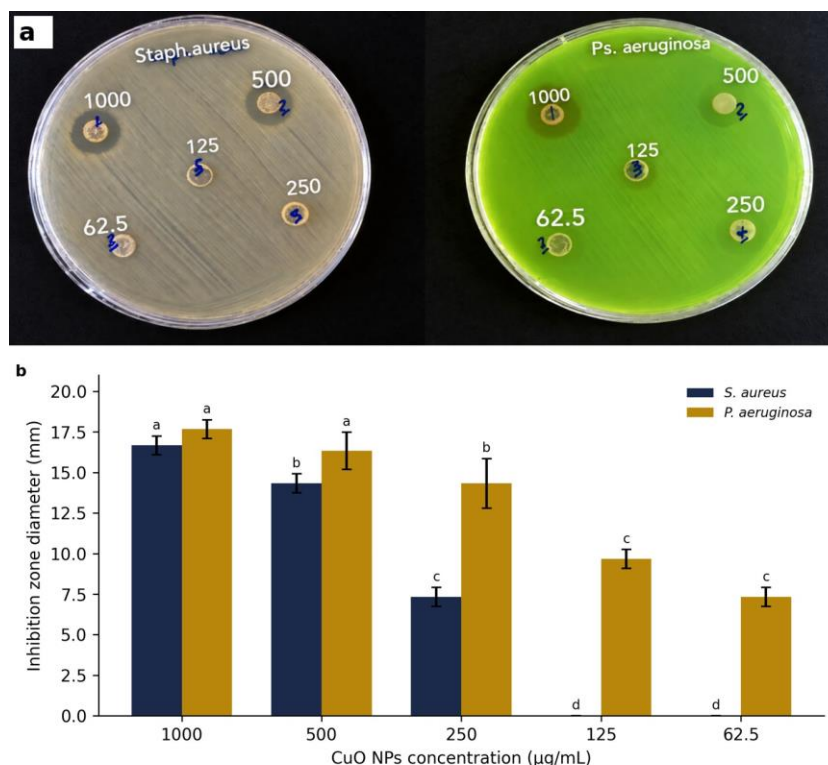


Figure (5): Antibacterial activity of biosynthesised CuO nanoparticles: (a) representative agar well-diffusion plates of *S. aureus* (left) and *P. aeruginosa* (right) with wells containing 1000, 500, 250, 125 and 62.5 µg/mL; (b) inhibition zone diameters (mean ± SD, n = 3); different letters above bars of the same species indicate significant differences ($p \leq 0.05$).

4-DISCUSSION

This study shows that the cell-free filtrate of the oral commensal *S. salivarius* can reduce Cu^{2+} and produce crystalline, single-phase CuO nanoparticles under mild conditions (room temperature, neutral pH, 35 min), without the need for toxic reducing agents or high temperatures. Extracellular biosynthesis using bacterial supernatants has previously been demonstrated for silver and CuO nanoparticles with *Bacillus* spp. and other bacteria [5–7]; the present results extend this approach to a lactic acid bacterium of the genus *Streptococcus*.

The FTIR data indicate that proteins (amide I and amide III bands) and carbohydrates (C–O bands) from the filtrate are adsorbed on the particle surface. Such biomolecules are widely accepted to act both as reducing agents, through electron donation by amine, hydroxyl and carboxyl groups, and as capping agents that limit particle growth and reduce agglomeration [5,13,14]. The strong Cu–O band at 618 cm^{-1} , together with the XRD pattern matching JCPDS 48-1548 and the 1:1 Cu:O atomic ratio obtained by EDX, provides three independent lines of evidence that the product is CuO rather than Cu_2O or metallic copper.

SEM showed quasi-spherical primary particles of 44–75 nm that were partly agglomerated. Particle size and shape are important determinants of the antibacterial efficacy of CuO NPs: smaller particles present a larger surface area for ion release and ROS generation and interact more readily with the cell envelope [9,10]. The sizes obtained here are comparable to those reported for CuO NPs synthesised with *Bacillus* sp. and plant extracts [5,6,13,17], although somewhat larger than the 20–30 nm particles obtained by some microwave-assisted or chemical routes [17]. Any discrepancy between crystallite size from XRD and particle size from SEM is expected, because the Scherrer

equation measures coherently diffracting domains rather than physical particles, and aggregated particles may contain several crystallites [16].

The biosynthesised CuO NPs exhibited clear, concentration-dependent activity against both *S. aureus* and *P. aeruginosa*, with maximum inhibition zones of 16.7 and 17.7 mm at 1000 µg/mL. These values are of the same order as those reported for biologically synthesised CuO NPs against the same species [5,12,14,17,18]. The antibacterial effect of CuO NPs is generally attributed to a combination of mechanisms: adhesion of the positively charged particles to the negatively charged cell surface, disruption of membrane integrity and increased permeability, release of Cu²⁺ ions that bind to thiol groups of proteins and to DNA, and generation of ROS (superoxide, hydroxyl radical and hydrogen peroxide) that cause lipid peroxidation, protein oxidation and DNA damage, culminating in cell death [2,4,8,10].

An interesting observation was the greater susceptibility of *P. aeruginosa*, which was inhibited at all concentrations down to 62.5 µg/mL, whereas *S. aureus* was inhibited only at 250 µg/mL and above. The thick, multilayered peptidoglycan wall of Gram-positive bacteria (20–80 nm) can act as a physical barrier that adsorbs nanoparticles and copper ions before they reach the cytoplasmic membrane, whereas the thin peptidoglycan layer of Gram-negative bacteria and the negatively charged lipopolysaccharide of their outer membrane favour electrostatic attraction and accumulation of Cu²⁺ and CuO NPs at the cell surface [8,9]. Greater or comparable sensitivity of Gram-negative bacteria to CuO NPs has been reported in several studies [9,17,18], although the opposite pattern has also been described, indicating that the outcome depends on the particle characteristics and the specific strains used [4,8]. Strain-related differences in efflux systems and copper-homeostasis genes may also contribute [2].

Some limitations of this study should be acknowledged. The agar well-diffusion assay is a qualitative-to-semiquantitative screening method, and the diffusion of nanoparticles through agar may be affected by particle size and agglomeration, which could influence the observed inhibition zones [11]. A standard antibiotic positive control and a solvent-only (DMSO) negative control were not included in the assay, so no control inhibition-zone values are available for comparison; future work should include appropriate positive and negative controls. Further studies using broth microdilution to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), together with time–kill kinetics and testing against clinical multidrug-resistant isolates, are recommended to validate the antibacterial activity. Additional characterisation by dynamic light scattering (DLS) and zeta-potential analysis would provide information on the hydrodynamic size distribution and surface charge, respectively. Finally, because CuO nanoparticles may exhibit cytotoxic effects toward mammalian cells at concentrations relevant to their antimicrobial activity [19,20], their biocompatibility and cytotoxicity should be evaluated before any biomedical application is considered.

CONCLUSION

Copper oxide nanoparticles were successfully biosynthesised at room temperature using the cell-free filtrate of *Streptococcus salivarius*. FTIR confirmed the involvement of bacterial proteins and carbohydrates as reducing and capping agents and revealed the Cu–O vibration, XRD identified single-phase monoclinic CuO, SEM showed quasi-spherical particles of 44–75 nm, and EDX gave a Cu:O atomic ratio close to 1:1. The nanoparticles displayed concentration-dependent antibacterial activity against *S. aureus* and *P. aeruginosa*, with the Gram-negative organism being inhibited at lower concentrations. *S. salivarius*-mediated synthesis is therefore a simple, rapid and environmentally benign route to antibacterial CuO NPs; determination of MIC/MBC values, evaluation against multidrug-resistant clinical isolates and assessment of cytotoxicity are the next steps toward practical application.

DECLARATIONS

Ethical approval: Not required, because no human or animal subjects were involved; the bacterial isolates were previously identified laboratory strains obtained from the College of Applied Sciences, University of Samarra.

Informed consent: Not applicable.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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Author contributions: S.A.H.: conceptualisation, methodology, investigation, laboratory work, data analysis and writing – original draft. A.M.Y.A.: supervision, characterisation, validation and writing – review and editing. Both authors read and approved the final version of the manuscript.

Data availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Hajipour MJ, Fromm KM, Ashkarran AA, Jimenez de Aberasturi D, Ruiz de Larramendi I, Rojo T, et al. Antibacterial properties of nanoparticles. *Trends Biotechnol.* 2012;30(10):499-511. doi:10.1016/j.tibtech.2012.06.004
2. Lemire JA, Harrison JJ, Turner RJ. Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nat Rev Microbiol.* 2013;11(6):371-384. doi:10.1038/nrmicro3028
3. Naz S, Gul A, Zia M, Javed R. Synthesis, biomedical applications, and toxicity of CuO nanoparticles. *Appl Microbiol Biotechnol.* 2023;107(4):1039-1061. doi:10.1007/s00253-023-12364-z
4. Gudkov SV, Burmistrov DE, Fomina PA, Validov SZ, Kozlov VA. Antibacterial properties of copper oxide nanoparticles (review). *Int J Mol Sci.* 2024;25(21):11563. doi:10.3390/ijms252111563
5. Talebian S, Shahnavaaz B, Nejabat M, Abolhassani Y, Rassouli FB. Bacterial-mediated synthesis and characterization of copper oxide nanoparticles with antibacterial, antioxidant, and anticancer potentials. *Front Bioeng Biotechnol.* 2023;11:1140010. doi:10.3389/fbioe.2023.1140010
6. Taran M, Rad M, Alavi M. Antibacterial activity of copper oxide (CuO) nanoparticles biosynthesized by *Bacillus* sp. FU4: optimization of experiment design. *Pharm Sci.* 2017;23(3):198-206. doi:10.15171/PS.2017.30
7. Saravanan M, Vemu AK, Barik SK. Rapid biosynthesis of silver nanoparticles from *Bacillus megaterium* (NCIM 2326) and their antibacterial activity on multi drug resistant clinical pathogens. *Colloids Surf B Biointerfaces.* 2011;88(1):325-331. doi:10.1016/j.colsurfb.2011.07.009
8. Slavin YN, Asnis J, Häfeli UO, Bach H. Metal nanoparticles: understanding the mechanisms behind antibacterial activity. *J Nanobiotechnology.* 2017;15:65. doi:10.1186/s12951-017-0308-z
9. Azam A, Ahmed AS, Oves M, Khan MS, Memic A. Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and -negative bacterial strains. *Int J Nanomedicine.* 2012;7:3527-3535. doi:10.2147/IJN.S29020
10. Laha D, Pramanik A, Laskar A, Jana M, Pramanik P, Karmakar P. Shape-dependent bactericidal activity of copper oxide nanoparticle mediated by DNA and membrane damage. *Mater Res Bull.* 2014;59:185-191. doi:10.1016/j.materresbull.2014.06.024
11. Balouiri M, Sadiki M, Ibensouda SK. Methods for *in vitro* evaluating antimicrobial activity: a review. *J Pharm Anal.* 2016;6(2):71-79. doi:10.1016/j.jpha.2015.11.005
12. Thekkae Padil VV, Černík M. Green synthesis of copper oxide nanoparticles using gum karaya as a biotemplate and their antibacterial application. *Int J Nanomedicine.* 2013;8:889-898. doi:10.2147/IJN.S40599
13. Naika HR, Lingaraju K, Manjunath K, Kumar D, Nagaraju G, Suresh D, et al. Green synthesis of CuO nanoparticles using *Gloriosa superba* L. extract and their antibacterial activity. *J Taibah Univ Sci.* 2015;9(1):7-12. doi:10.1016/j.jtusci.2014.04.006

14. Sivaraj R, Rahman PKSM, Rajiv P, Salam HA, Venckatesh R. Biogenic copper oxide nanoparticles synthesis using *Tabernaemontana divaricata* leaf extract and its antibacterial activity against urinary tract pathogen. Spectrochim Acta A Mol Biomol Spectrosc. 2014;133:178-181. doi:10.1016/j.saa.2014.05.048
15. Velsankar K, Aswin Kumara RM, Preethi R, Muthulakshmi V, Sudhahar S. Green synthesis of CuO nanoparticles via *Allium sativum* extract and its characterizations on antimicrobial, antioxidant, antilarvicidal activities. J Environ Chem Eng. 2020;8(5):104123. doi:10.1016/j.jece.2020.104123
16. Holzwarth U, Gibson N. The Scherrer equation versus the 'Debye-Scherrer equation'. Nat Nanotechnol. 2011;6(9):534. doi:10.1038/nnano.2011.145
17. Ibrahim AA. Microwave-assisted biogenic synthesis of pure copper oxide nanoparticles by *Lepidium sativum* L. leaves extract: characterization and evaluation of antibacterial activity. J Biotechnol. 2025;406:82-90. doi:10.1016/j.jbiotec.2025.07.003
18. Ahamed M, Alhadlaq HA, Khan MAM, Karuppiah P, Al-Dhabi NA. Synthesis, characterization, and antimicrobial activity of copper oxide nanoparticles. J Nanomater. 2014;2014:637858. doi:10.1155/2014/637858
19. Karlsson HL, Cronholm P, Gustafsson J, Möller L. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol. 2008;21(9):1726-1732. doi:10.1021/tx800064j
20. Bondarenko O, Juganson K, Ivask A, Kasemets K, Mortimer M, Kahru A. Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells *in vitro*: a critical review. Arch Toxicol. 2013;87(7):1181-1200. doi:10.1007/s00204-013-1079-4