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Evaluation of the Inhibitory Effect of Copper Nanoparticles Synthesized by Lactobacillus sp. on Pathogenic Bacterial Strains

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Abstract. Lactobacillus sp. against the pathogenic bacteria Staphylococcus aureus and Pseudomonas aeruginosa. The nanoparticles were produced using the bacterial cell-free filtrate, and their physicochemical properties were analyzed using several characterization techniques, including X-ray diffraction (XRD), UV–Visible spectroscopy, Fourier-transform infrared spectroscopy (FTIR), energy-dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), and transmission electron microscopy (TEM). The results confirmed the successful synthesis of pure, spherical CuNPs with particle sizes ranging from 40 to 110 nm. The antibacterial potential of the synthesized nanoparticles was assessed using the well diffusion method at five concentrations: 1000, 500, 250, 125, and 62.5 µg/mL. The concentrations of 1000 µg/mL and 500 µg/mL demonstrated notable antibacterial effects, with inhibition zones averaging 12 mm and 10 mm against Staphylococcus aureus, and 10 mm and 8 mm against Pseudomonas aeruginosa, respectively.

Highlights:

1. CuNPs were successfully synthesized using Lactobacillus sp. in an eco-friendly method.
2. Particles were spherical, 40–110 nm in size, confirmed by various techniques.
3. Higher concentrations effectively inhibited S. aureus and P. aeruginosa.

Keywords: Copper Nanoparticles, Lactobacillus sp., Antibacterial, Staphylococcus aureus, Pseudomonas aeruginosa

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Introduction

In light of the growing challenges posed by bacterial resistance to antibiotics, nanotechnology has emerged as one of the promising solutions to combat this phenomenon, Copper nanoparticles (CuNPs) are among the nanomaterials with high efficacy against a wide range of pathogenic bacteria, having demonstrated their ability to inhibit bacterial growth through multiple mechanisms, including the generation of reactive oxygen species (ROS) and interaction with vital bacterial cell component[1].

With the increasing interest in environmentally friendly methods for nanoparticle synthesis, biological approaches have emerged as sustainable and safe alternatives. In this context, certain bacterial species, such as Stenotrophomonas sp., have shown the ability to synthesize copper oxide nano.particles (CuONPs) with antibacterial, antioxidant, and even anticancer properties [2].

Lactobacillus are beneficial microorganisms widely used in various industrial and medical applications. Studies have shown that some copper-based nanomaterials, such as copper-containing metal-organic frameworks (MOFs), exhibit antibacterial activity against various species, including Escherichia coli and Lactobacillus [3].

This study aims to evaluate the effect of copper nanoparticles synthesized by Lactobacillus bacteria on certain pathogenic bacterial isolates by examining their ability to inhibit the growth of these isolates. This research contributes to the development of alternative and more effective strategies to combat microbial infections, especially in the face of challenges associated with antibiotic resistance.

Method

A. Biosynthesis of Silver Nanoparticles

Lactobacillus was cultured in a flask containing MRS broth and incubated in a shaking incubator at 200 rpm at 37°C for 20 hours. After incubation, the broth containing the bacteria was filtered using filter paper. Then, 10 mL of the Lactobacillus filtrate was added to 90 mL of a 1 mM CuSO4 solution, and the pH of the mixture was adjusted to 7. Two control flasks were also prepared: the first contained only the bacterial solution without the copper solution, while the second contained only the copper solution without the bacterial filtrate.

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The mixture was then placed on a magnetic stirrer for 35 minutes. A black precipitate was observed, indicating the formation of nanoparticles. The precipitate was collected and washed three times using deionized water, followed by centrifugation at 10,000 rpm for 10 minutes. The resulting precipitate was then dried in a hot air oven at 105°C for 5–6 hours until fully dry, to obtain the copper nanoparticles [1].

B. Diagnostic Techniques of Prepared Silver Nanoparticles

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to evaluate the characteristics of electrokinetic nanoparticles, while silver nanoparticles were thoroughly investigated using ultraviolet and visible spectroscopy (UV-Vis), atomic force microscopy (AFM), Fourier infrared transformation spectroscopy (FT-IR), and X-ray scattering (EDX).

C. Preparation of Nanoparticles Stock Solutions

Stock solutions were prepared at a concentration of 1000 µg/ml copper nanoparticles by dissolving 1 mg in 1 ml of solvent DMSO and placing the nanoparticle solution 30 minutes in an ultrasonic bath. The remaining concentrations of 1000, 500, 250, 125, and 62.5 µg/ml were then calculated using the dilution law [4].

D. Testing the Effectiveness of Silver Nanoparticles Against Isolates of Bacteria under Study.

The test was carried out using a well diffusion method, where the Müller medium was prepared according to the manufacturer's instructions and then left to harden. The bacterial stranded was prepared at the age of 18-24 hours and a concentration of 1.5×108 cells/cm3 and compared with a solution of the standard turbidity constant McFarland, the bacteria were spread by cotton swabs. The cork drill made drilling and 60 µl of concentrations of 1000, 500, 250, 125and 62.5 µg/ml prepared from the nanoparticles were transferred and placed in the pits and then incubated in the incubator at a temperature of 37°C For 24 hours, the diameter of the information produced by each concentration was recorded [4].

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E. Statistical Analysis

The ANOVA test of complete random design (CRD) was used to statistically assess the findings. The Dunkin' polynomial test was used to compare the arithmetic averages at a 0.05% probability level [5].

Results and Discussion

A. Biosynthesis of CuNPs

The Lactobacillus bacterial solution was used as both a reducing and stabilizing agent in the biosynthesis of copper nanoparticles (CuNPs). The results of the study showed a color change to dark gray for silver, milky white for zinc, and black for copper, which indicates the synthesis of nanoparticles. This color transformation is likely due to the appearance of the surface plasmon resonance (SPR) absorption band, a characteristic feature of metallic nanoparticles such as silver, gold, zinc, and copper [6].

The nanoparticles were obtained in powdered form following a drying process, and their weight was subsequently recorded. Their structural and morphological characteristics were analyzed using various techniques, including UV–Visible spectroscopy, atomic force microscopy (AFM), X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), and scanning electron microscopy (SEM).

The biosynthesis of nanoparticles using microorganisms such as bacteria has gained attention as a promising approach in green nanotechnology, owing to its simplicity, environmental sustainability, and cost-effectiveness compared to traditional chemical and physical synthesis methods [7]. Lactobacillus spp., a member of the lactic acid bacteria group, has emerged as a suitable candidate for the biosynthesis of copper oxide nanoparticles (CuO NPs) due to its biocompatibility and biosynthetic capabilities. These bacteria secrete various bioactive substances, including enzymes, organic acids, proteins, and extracellular polysaccharides (EPS), which serve as natural reducing and stabilizing agents during nanoparticle formation [8]. Studies have demonstrated that Lactobacillus strains can effectively reduce

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copper ions (Cu^{2+}) to CuO nanoparticles through mild, eco-friendly biological processes, eliminating the need for hazardous chemicals and enabling synthesis under moderate conditions [9].

B. FourierTransform Infrared (FT-IR) Spectroscopy of Copper - Nanoparticles Synthesized Using Lactobacillus

Fourier-transform infrared (FT-IR) spectroscopy was performed using a Nicolet 670 spectrometer to identify the functional groups associated with the copper oxide (CuO) nanoparticles synthesized by Lactobacillus spp. The spectra were recorded within the range of 4000–500 cm^{-1}, as illustrated in Figure (1).

The FT-IR analysis revealed distinct absorption bands in the 3100–3680 cm^{-1} region, which correspond to N–H stretching vibrations, indicative of amine groups or amide bonds present in bacterial membrane proteins. A minor absorption peak at 2926 cm^{-1} was associated with C–H stretching vibrations typical of alkenes.

Notably, peaks at 1642 cm^{-1} and 1495 cm^{-1} were attributed to Amide I and Amide II bands, respectively, both of which are characteristic of protein structures found in bacterial membranes. Furthermore, absorption bands observed at 1455 cm^{-1} and 1235 cm^{-1} were assigned to C–N stretching vibrations in aromatic and aliphatic amines.

A weaker band at 1044 cm^{-1} was linked to C–O stretching vibrations, suggesting the presence of carboxyl and alcohol functional groups. Importantly, a prominent peak in the 400–600 cm^{-1} region confirmed the formation of copper oxide (CuO) nanoparticles, reflecting metal–oxygen vibrations specific to CuO.

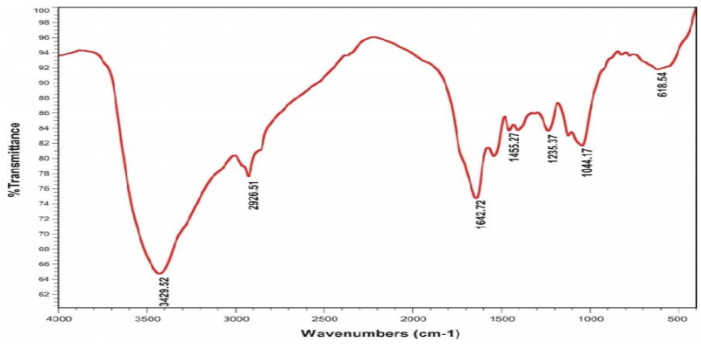

Figure 1. Fourier-transform Infrared (Ft-ir) Spectrum of Copper Nanoparticles (Cuo Nps)

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To Based on the experimental findings, a potential mechanism for the biosynthesis of CuO nanoparticles is proposed. In contrast to chemically synthesized metal nanoparticles, biologically produced nanoparticles tend to exhibit enhanced stability in aqueous environments and reduced aggregation. The FT-IR analysis suggests that carbonyl groups present in amino acids and peptides secreted by bacteria interact with metal ions, forming a stabilizing organic coating around the nanoparticles. This biological capping layer likely prevents nanoparticle agglomeration and enhances their dispersion in solution. These observations support the hypothesis that microbial biomolecules play a dual role in both the formation and stabilization of CuO nanoparticles in aqueous systems.

C. X-Ray Diffraction (XRD) Analysis of Copper Nanoparticles

The crystalline structure of the synthesized nanoparticles was characterized using X-ray diffraction (XRD), as depicted in Figure (2). Prominent diffraction peaks were observed at 2θ values of 35.47°, 38.75°, 48.79°, 61.57°, 66.18°, and 68.05°, which correspond to the crystallographic planes (111), (111), (202), (202), and (220), respectively. These diffraction peaks closely match the reference data provided by the Joint Committee on Powder Diffraction Standards (JCPDS), specifically card no. 041-0254. The alignment of the observed peaks with standard JCPDS data and previously reported literature confirms the crystalline nature of the copper oxide (CuO) nanoparticles synthesized via the biological method.

The average particle size of the CuO nanoparticles was estimated to be approximately 200 nm using the Scherrer equation:

$$D_{hki} = \frac{k \times \lambda}{\beta hki \times Cos\ \theta hki}$$

$$K = 0.94$$

$$\lambda = 1.05418 A^{\circ}$$

$$\beta_{hki} = \deg \times \frac{\pi}{180}$$

Where:

D is the average particle diameter,

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K is the Scherrer constant (commonly ranging between 0.9 and 1),

λ is the wavelength of the X-ray radiation (0.15406 nm for Cu Kα),

β is the full width at half maximum (FWHM) of the diffraction peak (in radians),

θ is the Bragg diffraction angle.

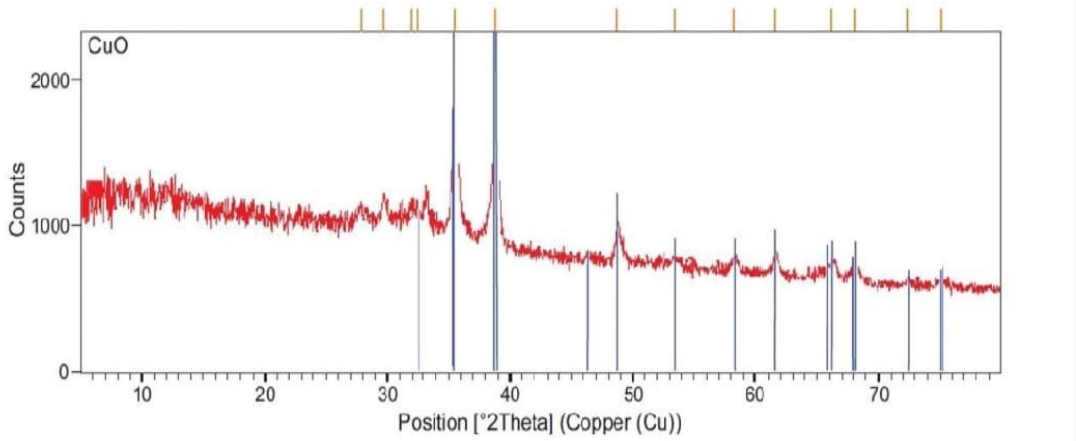

Figure 2. X-ray Diffraction (Xrd) Pattern of Copper Nanoparticles (Cuo Nps)

D. SEM Analysis of Copper Nanoparticles

The morphological characteristics of the biologically synthesized copper oxide (CuO) nanoparticles were examined using Scanning Electron Microscopy (SEM), with a particular focus on Field Emission Scanning Electron Microscopy (FESEM). FESEM was employed to investigate both the surface topography and elemental composition of the samples. Unlike Energy-Dispersive X-ray Spectroscopy (EDX), FESEM enables high-resolution imaging of localized areas, especially at low electron accelerating voltages, making it suitable for analyzing potential contamination at micro- and nanoscale levels.

This technique is primarily used to identify the presence and distribution of elements within the sample. As illustrated in Figure (3), the FESEM images revealed that the CuO nanoparticles were predominantly spherical in shape and evenly distributed, with particle sizes ranging from approximately 30 nm to 75 nm. Moreover, the absence of additional diffraction peaks indicative of impurities confirms the high purity of the synthesized CuO nanoparticles.

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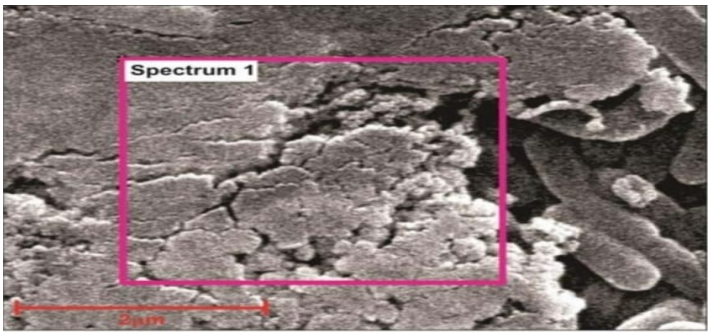

Figure 3. Scanning Electron Microscope (Sem) Image of the Biosynthesized Copper Nanoparticles (Cuo Nps)

E. EDX Analysis and TEM Imaging of Copper Nanoparticles

The elemental composition of the biologically synthesized CuO nanoparticles was determined using Energy-Dispersive X-ray Spectroscopy (EDX). The EDX spectrum (Figure 4) confirmed the presence of copper (Cu) and oxygen (O) as the primary constituents. Quantitative analysis revealed that copper accounted for 80.43% and oxygen for 19.57% by weight, verifying the formation of copper oxide (CuO) nanoparticles with high elemental purity.

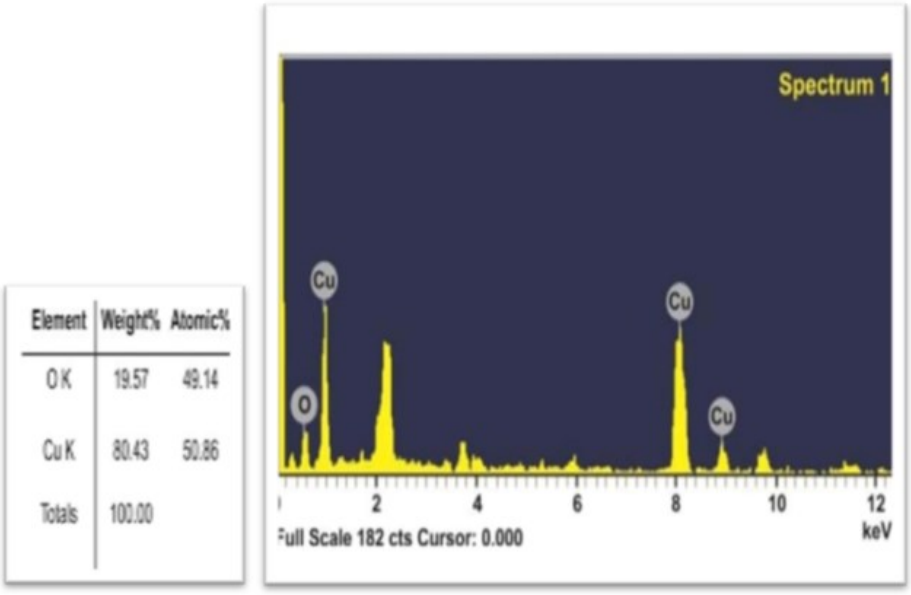

Figure 4. Energy Dispersive X-ray (EDX) Analysis of Copper Nanoparticles

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F. Transmission Electron Microscopy (TEM) Imaging of Copper Nanoparticles

Transmission Electron Microscopy (TEM) was employed to further characterize the morphology and size of the biosynthesized CuO nanoparticles. TEM imaging was performed by analyzing prepared sample grids. As illustrated in Figure (5), the nanoparticles exhibited a predominantly spherical morphology with diameters ranging from 40 to 110 nanometers. The analysis was conducted at a resolution of up to 300 nanometers, with a magnification of 27.8 kilopixels (kX), enabling precise visualization of the nanoparticle structure and distribution.

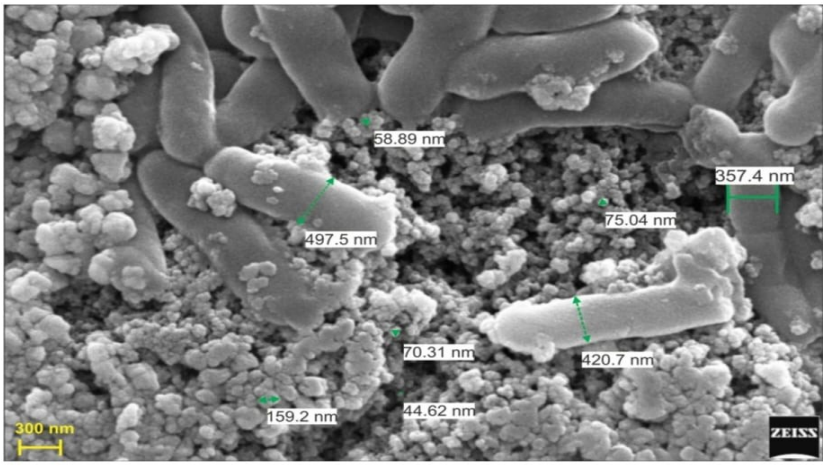

Figure 5. Aggregation of Copper Nanoparticles under Transmission Electron Microscope (TEM)

G. Sensitivity Testing of Copper Nanoparticles Using the Well Diffusion Method

The results shown in Table 1 and Figures 6 and 7 demonstrate that the of antimicrobial activity of biosynthesized copper oxide nanoparticles (CuO NPs), prepared using Lactobacillus, was evaluated against Staphylococcus aureus and Pseudomonas aeruginosa isolates using the well diffusion method at concentrations of 1000, 500, 250, 125, and 62.5 µg/mL,the results demonstrated noticeable inhibitory activity, with inhibition zone diameters for S. aureus reaching 12 mm and 10 mm at concentrations of 1000 and 500 µg/mL, respectively. For Ps. aeruginosa,

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the inhibition zones were 10 mm and 8 mm at the same concentrations. These variations in bacterial response are likely attributed to structural differences in the bacterial cell wall. Ps. aeruginosa possesses an outer membrane rich in lipopolysaccharides (LPS), which provides additional protection against antimicrobial agents, unlike the more permeable cell wall of Gram-positive bacteria such as Staph. aureus [10].

CuO NPs exhibited significant antibacterial activity against a broad spectrum of bacteria, attributed to their high surface area, which facilitates interactions with bacterial membranes, leading to the disruption of cellular structures and essential functions such as damage to DNA, proteins, and critical enzymes [10,11].

Recent studies suggest that CuO NPs cause morphological changes in bacterial membranes, increasing permeability and leading to leakage of cellular contents, ultimately resulting in cell death [12]. The proposed antibacterial mechanism involves the release of copper ions that interact with vital cell components such as DNA and ribosomes, inhibiting key biological processes.

Various studies have reported differing outcomes regarding the antimicrobial efficacy of CuO NPs. For example, Khashan et al. [13] reported that CuO NPs synthesized via laser ablation in liquids displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria. Taran et al. [14] used Bacillus sp. to synthesize CuO NPs and demonstrated their antibacterial effectiveness. Similarly, Abboud et al. [15] observed antibacterial effects of these nanoparticles against Enterobacter aerogenes and Staph. aureus.

Despite the differences in methodologies used to assess microbial growth inhibition, the underlying principle remains the same, CuO nanoparticles carry a positive charge that allows them to interact with the negatively charged bacterial cell surface. This interaction leads to nanoparticle accumulation on the cell membrane, causing alterations in the chemical and physical properties of the membrane, resulting in damage to vital functions such as permeability, osmoregulation, and electron transport [16].

The aggregation of nanoparticles on the plasma membrane can generate reactive oxygen species (ROS), which disrupt membrane integrity and allow the

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nanoparticles to enter the cell. Inside the cell, positively charged ions are released, potentially binding to ribosomes and inhibiting protein synthesis or interfering with microbial DNA replication by binding and damaging the genetic material [17], [18].

Additionally, these nanoparticles can bind to thiol (–SH) groups in proteins, leading to enzyme inhibition and disruption of cellular function, ultimately resulting in bacterial cell death [19]. Furthermore, CuO NPs may interact with the bacterial surface and plasma membrane, altering surface chemistry, reducing ATP levels, and disrupting energy balance, leading to compromised membrane stability.

Nanoparticles can induce the release of metal ions that cause morphological alterations and interact directly with the bacterial cell membrane, leading to a reduction in the transmembrane electrochemical potential. This interaction compromises membrane integrity and ultimately results in cell death. Additionally, nanoparticles are known to generate reactive oxygen species (ROS), which can cause oxidative stress, leading to mitochondrial dysfunction, lipid peroxidation, DNA damage, and membrane disruption. These effects interfere with the activity of essential enzymes, culminating in cellular death. Furthermore, exposure to nanoparticles has been shown to alter the expression of key proteins, significantly affecting bacterial metabolic pathways, including denitrification, active transport mechanisms, and electron transfer processes [20].

Table 1. Inhibitory Activity of Copper Nanoparticles Against Staph. Aureus and Ps.aeruginosa

Con. µg/ml	**Diameter of Inhibition Zone (mm)**	
	Ps.aeruginosa	***Staph.aureus***
1000	12.33 ± 0.58 a	14.67 ± 0.58 a
500	11.00 ± 1.00 a	10.33 ± 0.58 b
250	0.00 ± 0.00 b	0.00 ± 0.00 c
125	0.00 ± 0.00 b	0.00 ± 0.00 c
62.5	0.00 ± 0.00 b	0.00 ± 0.00 c

*Different letters indicate a significant difference

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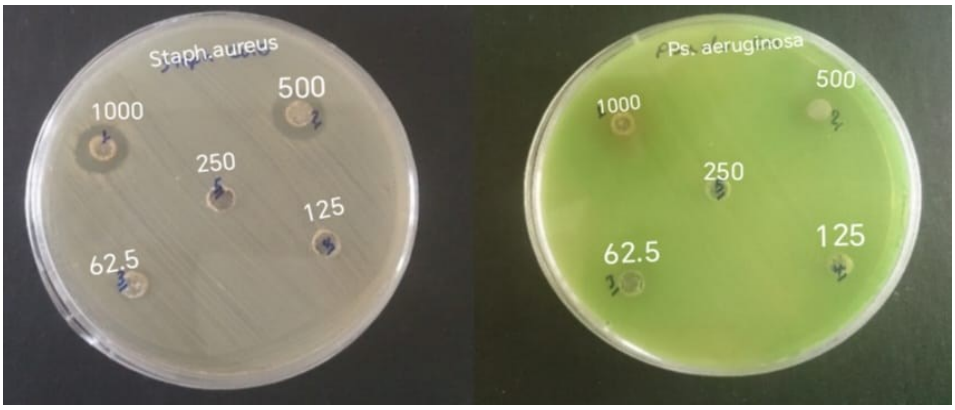

Figure 6. Effect of Copper Nanoparticles on Staph. aureus and Ps.aeruginosa

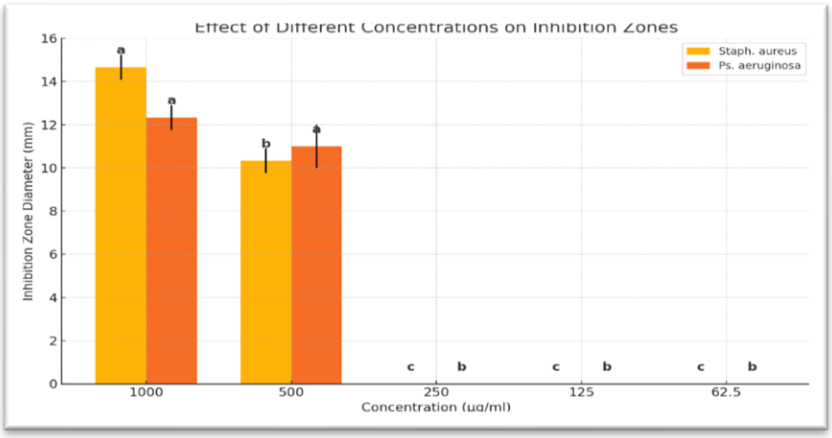

Figure 7. Inhibition Zone Diameters of Staph. Aureus and Ps.aeruginosa at Different Concentrations of Copper Nanoparticles Biosynthesized by Lactobacillus Spp.

Conclusions

The current study demonstrates the biosynthesis of copper oxide nanoparticles (CuO NPs) by Lactobacillus spp. through a green, eco-friendly, and cost-effective route of nanomaterial synthesis. Typical analyses like FT-IR, XRD, SEM, TEM, and EDX confirmed the production of spherical, pure, and crystalline nanoparticles with size 40-110 nm.

Biologically synthesized CuO NPs exhibited high antibacterial activity, particularly at doses of 1000 and 500 µg/mL, against Pseudomonas aeruginosa and Staphylococcus aureus. The differential sensitivity of the Gram-positive Staph. aureus compared to the

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Gram-negative Ps. aeruginosa can be attributed to structural differences between their cell walls.

These findings indicate the promise of Lactobacillus-delivered CuO NPs as effective antimicrobial agents, presenting an expedient alternative to traditional antibiotics and artificial nanoparticles, especially in view of the onset of antibiotic resistance. Future research must tackle the identification of the detailed molecular.

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