Antibacterial activity of copper nanoparticles synthesized using halophilic bacteria

A Dissertation

Course code and Course Title: MBO 381 & Dissertation

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Submitted in partial fulfilment of Master's Degree

In Marine biotechnology

By

Sanjana Sanjiv Gaikwad

21P050009

Under the Supervision of

Dr. Meghanath Prabhu

School of Biological sciences and Biotechnology

Goa University

April 2023

COMPLETION CERTIFICATE

This is to certify that the dissertation report "**Antibacterial activity of copper nanoparticles synthesized using halophilic bacteria**" is a bonafide work carried out by Ms. Sanjana Sanjiv Gaikwad under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Masters of science in the Discipline Marine biotechnology at the School of biotechnology and biological sciences, Goa University.

31/5/2023

Signature and Name of Supervising Teacher/ Mentor for internship
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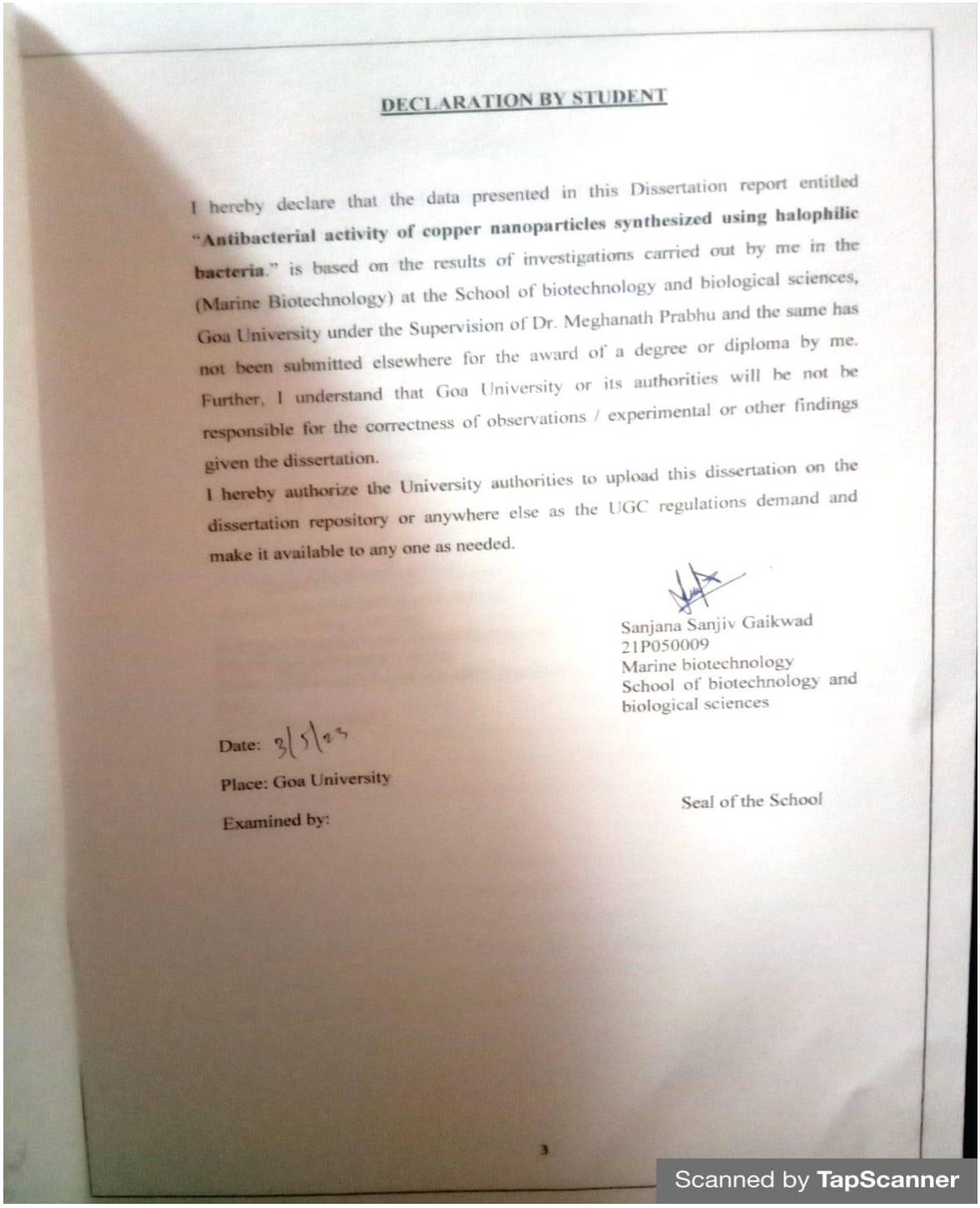

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled "**Antibacterial activity of copper nanoparticles synthesized using halophilic bacteria**." is based on the results of investigations carried out by me in the (Marine Biotechnology) at the School of biotechnology and biological sciences, Goa University under the Supervision of Dr. Meghanath Prabhu and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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3

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Name of Discipline

Date:

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INTRODUCTION

Nanoparticles are the material which scale assemblies of atom. Thus, they have dimensions ranges between 10nm to 100nm (Shobha, 2014). Nanoparticles are solid particles that have all three dimensions in the nanoscale. Physical and chemical properties of nanoparticles are controlled by its exact 3D morphology, structure and composition (Olson, 2000). In recent years nanoparticles have received for it's potential to improve many economic sector such as biomedicine, pharmaceuticals, antimicrobial agents and agriculture (Shobha, 2014). The improvement of nanotechnology have ventured up of researchers to form that have preferences in different regions of pharmaceuticals and medicine (Siddiqi, 2020).

Depending on the origin, there are three types of nanoparticles natural, incidental, engineered (Monica, 2009). Natural nanoparticles are an intriguing topic of research due to their unique properties and potential applications. Natural nanoparticles have been found to possess high surface area, reactivity and biocompatibility, making them useful in various fields such as drug delivery, food industry and environmental remediation (Khan, 2019). Engineered nanoparticles have potential benefits of efficient drug delivery and enhanced imaging contrast. Despite their potential benefits, engineered nanoparticles also raised concerns about their potential toxicity and environment impact. Therefore the development of engineered nanoparticles also requires a comprehensive understanding of their potential risks and regulatory oversight to ensure their safe and effective use.

Nanoparticles can be classified as organic which include dendrimers, liposome and micelles and carbon based materials including fullerene, graphene, carbon nanotubes and carbon black. They exhibit unique physical, biological and chemical properties as compared to their particles at higher levels (Ealia, 2017).

Metallic nanoparticles have gotten interest of many scientist and now they are heavily utilized in biomedical science and engineering (Mody VV, 2010). Metallic nanoparticles span across many areas such as aerospace, medicine and textile . Nanoparticles have also found their use in agricultural fields as nanozinc, nanocopper as growth enhancers by the Indian farmers fertilizers

co- operative limited. Moreover copper nanoparticles and their oxides have found their use in biosensors, dye degradation , antifungal, antimicrobial and nematicidal properties (Mohamed, 2020).

Copper is one of the most abundant elements on the earth. Therefore, copper has been important in research because of its role in living organism (Vimbela G. V., 2017). Copper nanoparticles have obtained research interest due to their mechanical, electrical, magnetic and thermal properties (Mohamed, 2020). An advantages of copper nanoparticles is that copper is cheap and widely available, thus it is cost effective to obtain copper nanoparticles. Copper nanoparticles have shown promising results in antibacterial and antioxidant activity. Wastewater treatment has been possible as a disinfectant property of copper nanoparticles (Ruparelia, 2006).

Metal nanoparticles can be synthesize by using green, chemical and physical methods. Green synthesis of metallic nanoparticles is widely used. It is harmless method. Microorganism like bacteria, fungi are used as a reducing agent It is difficult to preserve cell culture to obtain nanoparticles, therefore many green synthesis of nanoparticles use plant extract to synthesize nanoparticles (Asghar M. A., 2018). Biological synthesis of nanoparticle is mostly used to synthesize inorganic nanoparticles and has become an important method. Biological agents for production of materials at nanometre scale are used to synthesize nanoparticles (J.M.Sloik, 2005). Bacteria agents for production of materials at the nanometre scale are used for synthesis of copper nanoparticles, as they have immense potential to synthesize nanoparticles, as they have short generation time and are easy to manipulate at the genetic level (Saif Hanson, 2008).

The major metallic nanoparticles copper nanoparticles are the most beneficial from its bulk counterparts such as gold or silver. They can be produced by either of the three methods easily viz. Physical, chemical and biological methods. They can exist in both cupric (Cu^{2+}) and cuprous (Cu^{1+}) state or they can exist in the form of oxides (CuO/Cu_2O) owing to their stability (Prajapati, 2021). Green synthesis of nanoparticles is referred to as the biological synthesis of nanoparticles wherein no physical methods or chemicals are applied . These help in assuring environment friendly,

nontoxic and cheaper ways to synthesize those nanoparticles are relatively low when compared to methods available for gold and silver nanoparticles. Few notable methods are the use Arabic gum , plant extracts, ascorbic acid and micro-organisms for reducing Cu^{2+} ions (Cheirmadurai, 2014).

When microorganisms are used for the synthesis of nanoparticles, the reduction can either be intracellular or extracellular (Usha, 2010). In these both the capping and reducing agents are secreted by the microbe itself and this in turn decides the size, shape and agglomeration of that particular nanoparticle which will further describe its chemical and nanometric properties (Honary, 2012).

Characterization of copper nanoparticles is crucial to understand their properties and potential application because of their properties depend on their size, shape and surface chemistry, various techniques such as UV-Vis, X-ray diffraction, Transmission electron microscopy and Fourier transform infrared spectroscopy are commonly used (Khanna, 2005).

Antimicrobial resistance is not a new subject, Bacteria have always been able to adopt and become resistant to antimicrobial molecules. It forced medicine field to research for new antimicrobial molecules (Mchaela Corina Crinson, 2022). Nanoparticles can reduce or stop the evolution of resistant bacteria because nanoparticles target multiple biomolecules at once (Slavin Y. N., 2017). Research has focused on several metal nanoparticles; gold nanoparticles have antimicrobial activity against pathogenic bacteria like *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* (G Ren, 2009).

The study aims to synthesize nanoparticles from halophilic bacteria and evaluate the inhibitory effect of copper nanoparticles on a range of bacterial strains.

LITERATURE REVIEW

Nanoparticles have a high surface area-to-volume ratio compared to bulk materials, which makes their surface area an important factor in their properties and interactions with biological systems. As the size of nanoparticles decreases, their surface area increases, which can lead to enhanced reactivity, surface energy, and surface charge. This increased surface area can also enhance their adsorption capacity and interactions with biomolecules, such as proteins and lipids, which can influence their biological activity and toxicity. Silver nanoparticles were synthesized using a sonochemical route with two reducing agents. The size and morphology of the nanoparticles depended on the strength of the reducing agent, with a strong reducing agent producing spherical nanoparticles with an average size of 10 nm, and a weak reducing agent producing smaller nanoparticles with a narrow size distribution (Wani, 2011).

Metallic nanoparticles have immense potential in nanotechnology and are heavily utilized in biomedical sciences and engineering due to their unique physicochemical properties. They can be synthesized and modified with various chemical functional groups, allowing them to be conjugated with antibodies, ligands, and drugs for targeted drug delivery and diagnostic imaging. Various imaging modalities require contrast agents with unique physiochemical properties, leading to the development of nanoparticulated contrast agents such as magnetic nanoparticles (Fe3O4), gold, and silver nanoparticles (Mody, 2010) The study of metallic nanoparticles has led to a deeper understanding of quantum size confinement and the unique physical properties (Lue, 2001).

The use of noble metals such as gold and silver in the synthesis of nanoparticles has been widely studied, but recently there has been a shift towards the use of more economical metals like copper and nickel. These metals have also been found to have antibacterial properties, making them promising candidates for use in various applications (Chaudhary, 2019). While studies have shown that Cu and Ni nanoparticles have bactericidal activity, they have not yet been synthesized in aqueous solutions without the use of stabilizers such as polymers, ligands, or salts. This can potentially affect their properties and hinder their effectiveness (Ferrando, 2008).

Biological synthesis of nanoparticles is of importance since it is cost effective and does not possess any effects that are held by chemicals such as contamination from precursor chemicals, use of toxic solvents, low productivity and generation of hazardous by product (Singh P. , 2016). One promising approach to achieving this goal is to leverage the wide array of biological resources available in nature. In recent years, a variety of biological resources, including plants, algae, fungi, bacteria, and viruses, have been utilized for the production of low-cost, energy-efficient, and non-toxic metallic nanoparticles (Thakkar, 2010). On the other hand, physical synthesis such as sputter deposition, laser ablation, attribution and pyrolysis are all energy intensive and slow (Srivastava, 2020).Thus the development of reliable, non-toxic and environment- friendly methods for synthesis of nanoparticles is important (Singh J. , 2018).

'Green synthesis' has been an emerging field for nanoparticles synthesis over the decades and hence in contrast different organisms are being used for this synthesis. Many bacteria fungi and plants have shown the ability to synthesize metallic nanoparticles. In addition to this animals of lower and higher taxonomic group have been experimented and re-evaluated for their natural potential to reduce the metallic ions into neutral atoms with no expense of hazardous and toxic chemicals (Das, 2017). The use of biogenic reduction for the synthesis of nanoparticles offers a sustainable, eco-friendly, and low-cost alternative to traditional physico-chemical methods. It also provides an opportunity for the discovery of new and novel approaches to the synthesis of nanoparticles, opening up new possibilities for their use in various applications such as in the medical and biological fields (Hussain, 2016).

The study reported on an eco-friendly and rapid process for the synthesis of silver nanoparticles using the aqueous seed extract of *Jatropha curcas*. A green method was developed to synthesize silver nanoparticles using the aqueous seed extract of *J. curcas*. *Jatropha* seed extract was used as both the reducing and stabilizing agent, and the size of the particles was controlled by varying the concentration of AgNO3. The silver particles were characterized using HRTEM, XRD, and UV-Vis spectroscopic techniques, which showed that they were crystalline with a face- centred cubic geometry. This eco-friendly method could have potential applications in various fields (Bar, 2009).

Silver nanoparticles with a size range of 2-5 nm were synthesized by a silver-tolerant yeast strain called MKY3 when it was exposed to 1 mM soluble silver during the log phase of growth. A new method was developed to separate the nanoparticles from a dilute suspension, which was based on differential thawing of the sample. Investigations using optical absorption, transmission electron microscopy, x-ray diffraction, and x-ray photoelectron spectroscopy confirmed that the nanoparticles were composed of metallic (elemental) silver. The extracellular synthesis of nanoparticles has several advantages, including the potential for large-scale production and easy downstream processing (Kowshik, 2002).

The new, rapid method for synthesizing metallic silver nanoparticles was reported. The method involved using the culture supernatants of *Klebsiella pneumonia, Escherichia coli, and Enterobacter cloacae* to reduce aqueous Ag+ ions, resulting in the formation of silver nanoparticles within 5 minutes of contact between the cell filtrate and silver ions. It was found that the culture supernatants of different bacteria from Enterobacteriacae were potential candidates for the rapid synthesis of silver nanoparticles, and the new method required significantly less time than previous biological methods. Additionally, the study revealed that piperitone could partially inhibit the reduction of Ag+ to metallic silver nanoparticles by Enterobacteriacae (Shahverdi, 2007).

Copper, one of the most abundant elements on Earth, has played a significant role in history due to its various properties such as good electrical and thermal conductivity, high corrosion resistance, and increased malleability. Since the early 14th century, copper has been utilized in ornaments, weapons, and coins. (Vimbela G. V., 2017). Copper can be found in more than 30 types of protein, and it plays an important part in living organisms' metabolism. Numerous enzymes containing copper contribute to different body functions, such as oxygen transportation and iron homeostasis (Vimbela G. V., 2017). In addition, copper is also found in skin, bones and different body organs (Al-Hakkani, 2020).

A study was conducted to analyse the thermal oxidation of copper nanoparticles measuring 20 nm in a 20% oxygen-nitrogen atmosphere at different temperatures. The study found that the mass gain equilibrium of the copper particles varied for

each temperature, and a threshold temperature was identified. The oxide product formed on the copper nanoparticles was primarily Cu_2O below the threshold temperature, but above the threshold temperature, Cu_2O was rapidly formed initially and then changed to CuO (Yabuki, 2011) (Pacioni, 2013).

Synthesize copper nanoparticles (CuNPs) using *Eucalyptus camaldulensis, Azadirachta indica, Murraya koenigii, Avicennia marina, Rosa rubiginosa* and *Datura stramonium* plant extracts. The copper nanoparticles were characterized using various spectroscopic techniques, and TEM images showed that copper nanoparticles with mean sizes ranging from 48 to 29 nm were produced using different plant extracts. The synthesized copper nanoparticles exhibited potent antibacterial activity against various pathogenic strains, with relatively low values of MIC between 15 µg/ml and 60 µg/ml. The most effective antibacterial activity was observed for copper nanoparticles synthesized using *Azadirachta indica,* (Asghar M. A., 2020).

The properties and various applications of copper nanoparticles (Cu-NPs) in the field of pharmaceuticals had attracted significant attention from researchers. In a recent study, copper nanoparticles were biologically synthesized using the aqueous extract of the flower *Millettia pinnata*, and their characteristics were investigated through UV–visible spectroscopy, XRD, FT-IR, SEM, TEM analysis. The synthesized particles were highly durable, spherical, and had an average particle size in the range of 23 ± 1.10 nm. The Cu-NPs demonstrated greater inhibition of DPPH radical and nitric oxide scavenging activities. They were also receptive to both Gram-negative and Gram-positive bacteria (Thiruvengadam, 2019).

Copper oxide nanoparticles (CuNPs) were synthesized using water-soluble polysaccharides (SPs) extracted from brown seaweed, *Sargassum vulgare.* UV-VIS, SEM, TEM, EDX, FTIR, and XRD measurements were performed to characterize the synthesized SPs-CuNPs. The MIC values for SPs-Copper nanoparticles against *S. aureus* MRSA and MSSA were 250 and 150 µg/ml, respectively. The SPs-Copper nanoparticles exhibited the greatest biofilm inhibition against *S. aureus* MRSA and MSSA at 100 and 50 µg/ml, respectively (Marzban, 2022).

Pure copper nanoparticles were synthesized in the presence of a chitosan stabilizer through chemical means. Different characterization techniques were used to authenticate the purity of the nanoparticles, including UV-visible spectroscopy, transmission electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, and field emission scanning electron microscopy. The antibacterial and antifungal activity of the nanoparticles were investigated using several microorganisms of interest, including methicillin-resistant *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella choleraesuis, and Candida albicans* (Usman, 2013).

According to the literature, it has been observed that Gram-positive bacteria exhibit a higher level of resistance to the mechanism of action of nanoparticles. This is believed to be due to the difference in the structure of their cell walls. Gram-positive bacteria have a thicker cell wall, which provides an additional layer of protection, thereby reducing the interaction between the nanoparticles and the cell wall (Crisan, 2021). The outer layer of Gram-negative bacteria is coated in lipopolysaccharide, which carries a negative charge. When nanoparticles come into contact with these bacteria, they release positive ions that exhibit a strong attraction to the negatively charged molecules in the cell wall. As a result, the positively charged nanoparticles tend to accumulate in the bacterial cell wall and eventually penetrate it, releasing ions that lead to intracellular damage (Slavin Y. N., 2017).

The antibacterial properties of copper have received relatively less attention compared to other materials, owing to its instability caused by oxidation and its tendency to form complexes with water molecules in aqueous environments (Varshney, 2010).

Agarose, a biopolymer of natural origin, is frequently employed to stabilize metal and semiconductor nanoparticles. Upon stabilizing silver and copper nanoparticles within the agarose matrix, a remarkable antibacterial effect against *E. coli* bacteria is observed. Furthermore, the metal nanoparticles dispersed uniformly within the agarose composite films can be conveniently transformed into carbon-metal composites that have promising catalytic potential (Datta, 2008).

The toxicological mechanisms by which nanoparticles cause cell damage typically involve the generation of reactive oxygen species (ROS) and the resulting induction of oxidative stress. ROS-mediated cellular damage induced by nanoparticles is a commonly observed phenomenon (Yang, 2009). CuO nanoparticles have the capability to produce significant amounts of reactive oxygen species (ROS), including O^{2-}, OH, and H_2O_2, even when present in small quantities (Xia, 2007).

With a salinity level of 34.2% as of 2011, the Dead Sea boasts the highest salinity level among known environments, while the salinity level of widely distributed oceans is 3.5%. Organisms in such extreme environments typically maintain low intracellular ionic concentrations to counteract the osmotic imbalance (Feldman, 2015). Bacteria in the Dead Sea have unique properties due to extreme environmental conditions such as high salinity, low nutrient availability, and high UV radiation. They possess adaptive strategies including the synthesis of compatible solutes for osmoregulation and protection against salt-induced stress. The bacterial communities are diverse, with many unexplored species.

The synthesis of copper nanoparticles using halophilic bacteria is an area of research that has not been extensively explored. In this study, the primary objective was to investigate the synthesis of copper nanoparticles using halophilic bacteria and to evaluate their antimicrobial properties. To the best of our knowledge, this study is the first of its kind and can provide valuable insights into the potential of halophilic bacteria in nanoparticle synthesis. The findings of this study could contribute to the development of sustainable and environmentally friendly methods for nanoparticle synthesis.

AIM AND OBJECTIVES

The aim of this study was to synthesize, characterize copper nanoparticles using halophilic bacteria and to test the antibacterial effect of these nanoparticles. The following objectives were designed to fulfil the aim:

1. Biochemical and molecular characterization of bacterial culture.
2. Synthesis of copper nanoparticle using halophilic bacterium.
3. Characterization of nanoparticles synthesized.
4. Antimicrobial activity of copper nanoparticles.

MATERIALS AND METHODS

1. CHARACTERIZATION AND INDENTIFICATION OF BACTERIAL ISOLATES BY GRAM'S CHARACTER AND 16S rDNA ANALYSIS.

The halophilic bacterial culture, GUWSP2 was kindly provided by guide, Dr. M. Prabhu. The culture was previously isolated from the Dead Sea sediment (Tehssena, 2020). The culture was streaked and grown on NTYE agar plates to check for the purity of the culture and to observe the presence of any contamination. Gram staining is a common laboratory technique used to differentiate bacteria into two groups based on the characteristics of their cell walls. Bacteria that stain purple are classified as Gram-positive, while those that stain pink are classified as Gram-negative. The Gram stain reaction provides important information about the structure and composition of the bacterial cell wall, which can help identify the bacterial species. The purity of the culture was further confirmed by Gram staining.

Gram's staining

A smear of the bacterial isolates, prepared on separate glass slides, was subjected to heat fixation. Subsequently, the slides were stained with crystal violet (described in Appendix 2) for one minute and rinsed with distilled water. Gram's iodine (described in Appendix 3) was then added to the slide and allowed to react for one minute, after which the slide was drained and rinsed with distilled water. Next, the slide was rinsed with alcohol kept for approximately 15 seconds, (until the slide turned almost colourless). The slide was then rinsed again with distilled water and counterstained with saffranine (described in Appendix 4) for 20-30 seconds. Rinsed with distilled water, and air-dried. Finally, the slide was observed under the oil immersion objective of a light microscope.

Molecular identification-

For molecular identification of the GUWSP2 culture, services of Hi-Media were employed for the purpose of conducting 16S rDNA sequencing on their bacterial sample. Hi-Media is a renowned company that provides various microbiological services, including DNA sequencing and identification of microorganisms utilizing molecular biology methodologies such as 16S rDNA sequencing. The extensive range of expertise offered by Hi-Media in the fields of microbiology and molecular biology make them an appropriate choice for performing 16S rDNA sequencing on the GUWSP2 culture.

The 16S rDNA partial gene sequence was subjected to BLAST searches in the NCBI gene bank database, a comprehensive repository of genetic information. The search yielded multiple hits, but the top nine sequences were selected based on their maximum identity score. These sequences were then aligned using Clustal W, a widely-used program for multiple sequence alignment, with the assistance of MEGA X software, which provides tools for phylogenetic analysis and evolutionary inference.

2. SCREENING ISOLATES FOR COPPER NANOPARTICLE SYNTHESIS USING CELL-FREE SUPERNATANT AND CELL BIOMASS.

The culture was grown in NTYE broth (described in Appendix 1) at room temperature (30°C) and 140 rpm until the optical density (OD) of the culture reached approximately 0.9 at 600 nm, after 48 hours of incubation. The broth was then divided equally into tubes and centrifuged at 9500 rpm for 22 minutes using a BioEra Model Page centrifuge, in order to separate the cell biomass in the pellet from the cell-free culture broth in the supernatant.

To the cell-free supernatant, an equal amount of 0.01 M $CuSO_4$ was added, and the mixture was incubated on a shaker for 24 hours.

This step was taken in order to obtain copper nanoparticles, which were synthesized using the supernatant as a reducing agent for the CuSO4. The synthesis of copper nanoparticles was monitored using UV-Visible spectroscopy on a Shimadzu UV-Vis spectrophotometer by taking a scan of a reaction mixture between 300-500 nm.

3. CHARACTERIZATION OF SYNTHESIZED COPPER NANOPARTICLES.

UV-Visible spectroscopy

The synthesized copper nanoparticles were analysed via UV-Vis spectroscopy to investigate their optical properties of nanoparticles, including their size and shape. For this purpose, a sample solution of the supernatant was prepared, and 1 millilitre of the solution was dispensed into a cuvette. The cuvette was then placed in a UV-Vis spectrophotometer, and a spectrum was recorded from 300-500 nm to observe the peak absorption by the sample.

Two control experiments were also performed to provide a basis for comparison. In the first control experiment, a solution containing only CuSO4 and the broth was prepared and analysed using the same method as the sample. This control was used to determine the absorbance contribution of the broth and CuSO4 in the absence of any synthesized copper nanoparticles. In the second control experiment, a 0.01 M CuSO4 solution was analyzed using the same method to establish the baseline absorbance of CuSO4 in solution.

The resulting UV-Vis spectra of the sample and controls were analyzed to identify any peak absorbance and to compare the relative intensities of the spectra.

4. ISOLATION AND PURIFICATION OF SYNTHESIZED COPPER NANOPARTICLES:

Following the scan of the reaction mixture, a green-coloured pellet was obtained by centrifugation of the resulting precipitate at 9500 rpm for 22 minutes. Subsequently, a purification step was carried out by mixing the pellet with distilled water, followed by another centrifugation step at 9500 rpm for 22 minutes. The resulting pellet was then transferred to a petri dish, covered with aluminium foil, and dried in a hot air oven at 75°C for 24 hours. The dried sample was ground using a mortar pestle to obtain a fine powder.

5. ANTIBACTERIAL ACTIVITY OF CuNPs

Antimicrobial susceptibility testing of pathogenic bacteria using the well diffusion method

The sensitivity of copper nanoparticles was determined by performing an antimicrobial susceptibility test using the well diffusion method against three pathogenic bacterial strains: *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), and *Staphylococcus aureus* (ATCC 6538). Both gram-negative (*E. coli*) and gram-positive (*B. subtilis and S. aureus*) bacterial strains were used to evaluate the antimicrobial efficacy of the synthesized nanoparticles.

Sterile nutrient agar plates (appendix 4) were prepared by pouring the nutrient agar into petri dishes and allowed to solidify. A loopful of 24 hrs. old grown cultures of gram-positive bacteria, *B. subtilis* and *S. aureus* and gram-negative bacteria, *E. coli* were prepared in 1 ml of 8% sterile saline

solution. A volume of 50 µl of each culture suspension was spread plated on the surface of the agar plates. Copper nanoparticles (CuNPs) were synthesized, and 500 µg of the nanoparticles were weighed and added to 10 ml of distilled water to make a concentration of 50 µg/ml of colloidal solution. The colloidal solution was sonicated to disperse the nanoparticles evenly. Using a sterile pipette, 100 µl of the copper nanoparticle solution (corresponding to 5 µg) was added to each well in the agar plates. The plates were then incubated at 37°C to allow the growth of the bacteria and observe the effects of the copper nanoparticles on their growth. This experiment was performed using the well diffusion method to determine the sensitivity of the pathogenic cultures to the synthesized copper nanoparticles.

Determination of the antibacterial property of copper nanoparticles (CuNPs) was carried out using the Colony Forming Unit (CFU) assay.

Firstly, 1 ml of each culture (OD of 0.9 at 600 nm) was taken in separate microcentrifuge tubes and centrifuged at 5000 rpm for 10 minutes at 4°C. The resulting pellets were then resuspended in 1X phosphate-buffered saline (PBS) (appendix 5) and treated with different concentrations of CuNPs (10 µg/ml, 25 µg/ml, 50 µg/ml, 75 µg/ml, and 125 µg/ml) for 4 hours at room temperature with 20 rpm rotation. Controls were also prepared in the absence of CuNPs.

After treatment, the samples were diluted to 10^{-6}, and 100 µL of each sample was plated on nutrient agar plates. The plates were then incubated at 37°C for overnight in a biological oxygen demand (BOD) incubator for cultures to grow.

The colonies were counted, and the results were used to determine the antibacterial property of CuNPs against *E. coli* and *B. subtilis*. The experiment was conducted in duplicate to ensure accuracy and reproducibility of the results.

LD_{50} Calculation

LD50 refers to the lethal dose of a toxic substance required to cause mortality in 50% of a given population. The arithmetical method for determining the median lethal dose (LD50) was introduced by Reed L.J. and Muench H. in 1938. Various modifications have been proposed for calculating the percentage of bacterial colonies that either survived or died at different dose levels during toxicity testing. An optimal LD50 can be obtained by calculating the average dose that causes 50% mortality and another dose that causes 50% survival (Saganuwan, 2011).

Following is the modified formula were used to calculate LD_{50} for assay performed above-

LD50 by Reed and Muench modified formula.

LD50 = MLD + MSD / 2, MLD = Median lethal dose; MSD = Median survival dose (Saganuwan, 2011).

RESULTS AND DISCUSSION

1. CHARACTERIZATION AND INDENTIFICATION OF BACTERIAL ISOLATES BY GRAM'S CHARACTER.

Gram's character

After conducting Gram staining and microscopic examination of the isolated bacteria designated as GUWSP2, it was observed that the Gram character of the bacteria was Gram negative (Fig 1) indicating that the bacterial cell wall does not retain crystal violet stain during the Gram staining process. In addition, the microscopic examination revealed that the bacteria were rod-shaped bacilli (Fig 1) which suggests that the cells are cylindrical in shape and may appear singly, in pairs, or in chains. Therefore, based on the results of the Gram staining and microscopic examination, it can be concluded that GUWSP2 is a Gram-negative, rod-shaped bacterium.

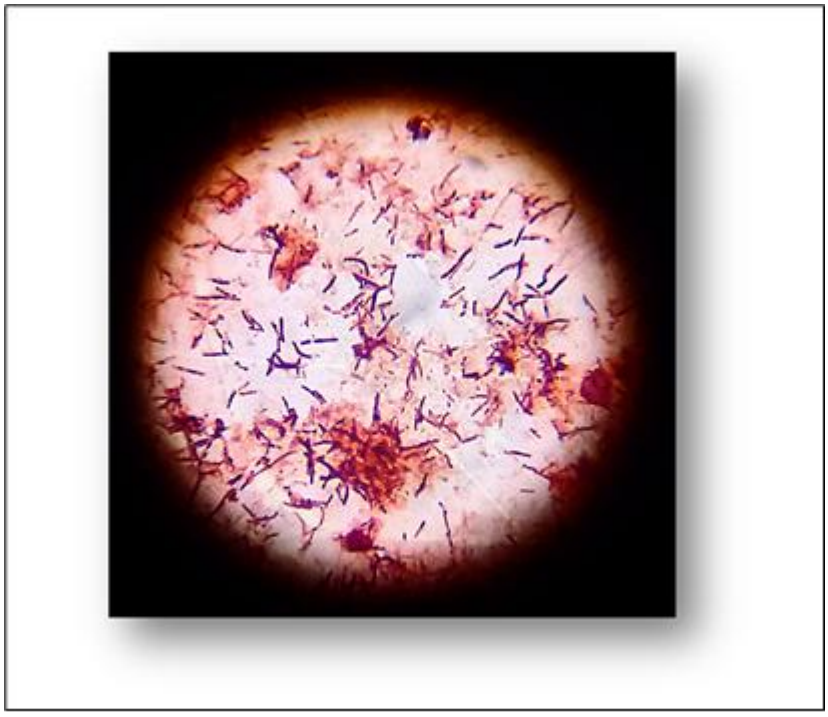

Fig 1: Gram's character of the bacterial isolate, showing Gram negative short-rods morphology.

2. SANGER SEQUENCING OF ISOLATED GUWPSP2

Upon sequencing of 16S rDNA, and subsequently using BLAST, the analysis revealed that GUWSP2 is closely (90%) related to *Cytobacillus horneckiae*. The sequence alignment process helped to identify regions of similarity and difference among the sequences, allowing for further analysis of their evolutionary relationships and potential functions. Overall, this approach provided a powerful tool for exploring the genetic diversity and relationships among the selected sequences phylogenetic tree was constructed. This was achieved using the neighbour-joining method, which is a widely-used algorithm for constructing evolutionary trees based on genetic data. The phylogenetic tree provided insights into the evolutionary relationships between the selected sequences, helping to infer their potential functions and evolutionary origins. Figure 2 shows a graphical representation of the phylogenetic analysis results, which confirmed the close relationship between GUWSP2 and *Cytobacillus horneckiae*.

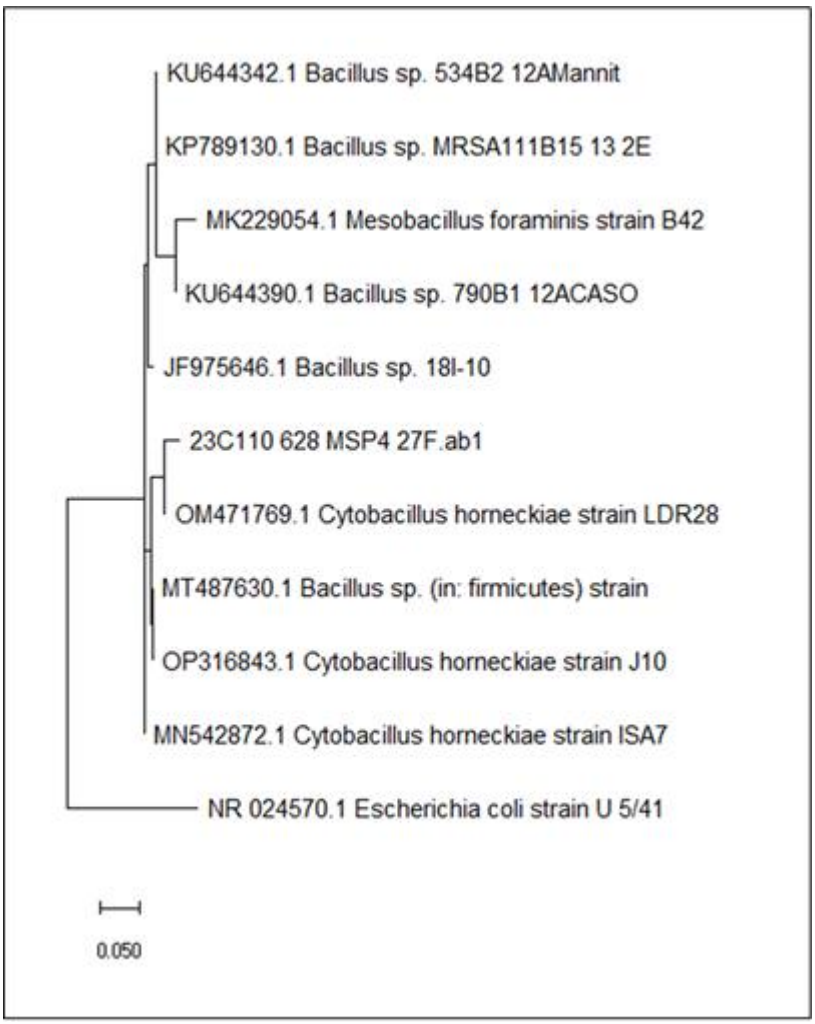

Fig 2: The genetic relation between various strains of *Bacillus* and GUWSP2 is assessed using the neighbour-joining method of molecular phylogenetic analysis based on their 16S rDNA gene sequences.

3. SCREENING ISOLATES FOR COPPER NANOPARTICLE SYNTHESIS USING CELL-FREE SUPERNATANT AND CELL BIOMASS.

Based on the observations made in the experiment, it can be concluded that the synthesis of copper nanoparticles was confirmed visually by observing a colour change in the cell-free supernatant. The initial colour of the reaction mixture was yellow, which changed to bluish-green after being exposed to a 0.01 M copper sulphate solution (Fig 3).

Fig 3: Reaction supernatant and copper sulphate solution. Beaker no. 1 , Control- NTYE broth and 0.01 M copper sulphate solution; beaker no. 2, Colour change to greenish blue-cell free supernatant and 0.01M copper sulphate solution; beaker no. 3, 0.01M copper sulphate solution.

Furthermore, after 24 hours of incubation, more colour change to green was observed in the cell-free supernatant, indicating that the synthesis of particles could have occurred (Fig 4). These observations suggest that the non-pathogenic bacteria used in the experiment were able to synthesize copper nanoparticles. These findings are consistent with a similar study conducted by S. Shankirti in 2014, which used non-pathogenic soil bacteria to synthesize copper nanoparticles. In that study, the colour changes in the supernatant were also reported to conclude the synthesis of particles. Overall, the visual observations made in this experiment provide strong evidence for the extracellular synthesis of copper nanoparticles by the bacteria used in the study.

Fig 4: Greenish blue colour of cell free supernatant after 24 hours of incubation.

In addition to the colour change observed in the cell-free supernatant, which indicated the potential synthesis of copper nanoparticles, the presence of these nanoparticles was further confirmed by analysing the absorption peak spectrum ranging from 300-500nm (Shobha, 2014) of the supernatant. UV-Visible spectra analysis was conducted to confirm the formation of various nanoparticles from different salts, which exhibited characteristic peaks. The characteristic absorption peaks for copper nanoparticles (CuNPs) were observed within the range of (300-500) nm reported by Pérez-Alvarez 2021. In this experiment, the peak absorption of copper nanoparticles (CuNPs) was found to be at 312 nm (Fig 5), which confirmed the successful synthesis and presence of CuNPs.

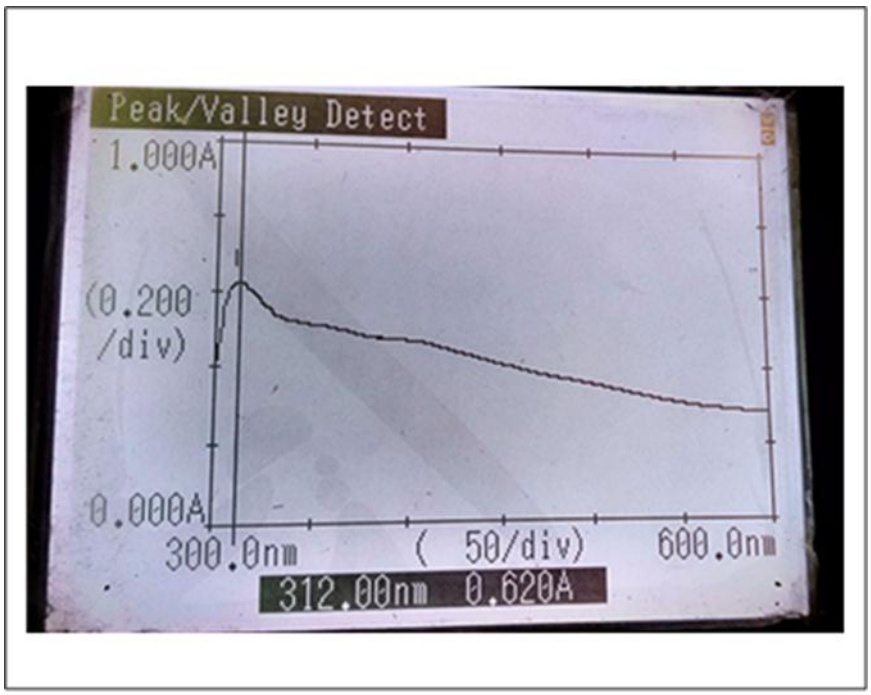

Fig 5: Graph of absorbance v/s wavelength (nm) of synthesized nanoparticles.

The centrifugation of the bacterial culture yielded a green pellet (Fig 6), indicating the presence of copper ions or copper-containing compounds in the culture medium.

Fig 6: Green pellet observed after centrifugation.

The pellet was further processed by washing, and drying to isolate the copper nanoparticles in powdered form (Fig 7).

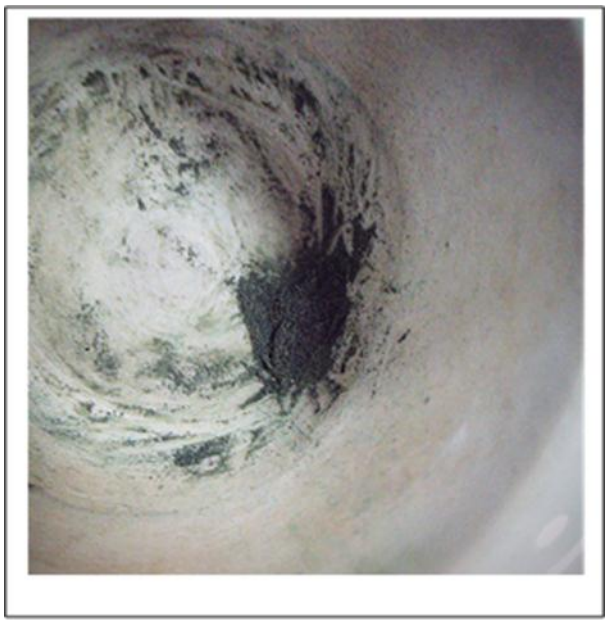

Fig 7: Powdered form of CuNPs

ANTIMICROBIAL ACTIVITY OF COPPER NANOPARTICLES.

Antimicrobial susceptibility testing of pathogenic bacteria using the well diffusion method

The adherence of copper nanoparticles (CuNPs) in the nanoscale range to bacterial cell walls leads to the disruption of the membrane integrity and eventual cell lysis through multiple mechanisms. These mechanisms include electrostatic interactions between CuNPs and the cell surface, effects on the cell membrane and intracellular proteins that result in denaturation, and effects on sulfur and phosphorous-containing entities such as DNA. The overall effect of CuNPs on bacterial cells can be attributed to their ability to adhere to cell surfaces and disrupt their integrity, ultimately leading to bacterial cell death. (Mahmoodi, 2018).Thus, the toxicity level of the copper nanoparticles can be quantified in millimetres based on the size of the zone of

clearance observed. This is dependent on the size of the nanoparticles and their ability to induce cell lysis and create a clear zone around the nanoparticle-treated area.

The antimicrobial activity of the copper nanoparticles synthesized in this study using GUWSP2 was evaluated against three bacterial strains: *E. coli*, *B. subtilis,* and *S. aureus* and the inhibitory zones shown by bacteria due to presence of nanoparticles were measured as 34 mm, 42 mm, and 27 mm, respectively (Table 1) and (Fig. 8 and Fig 9).

Table 1: Zone of inhibition (in mm) of copper nanoparticle against various bacterial strains

	Bacterial strains	ZOI (mm)
A	*E.coli*	34mm
B	*B.subtilis*	42mm
C	*S.aureus*	27mm

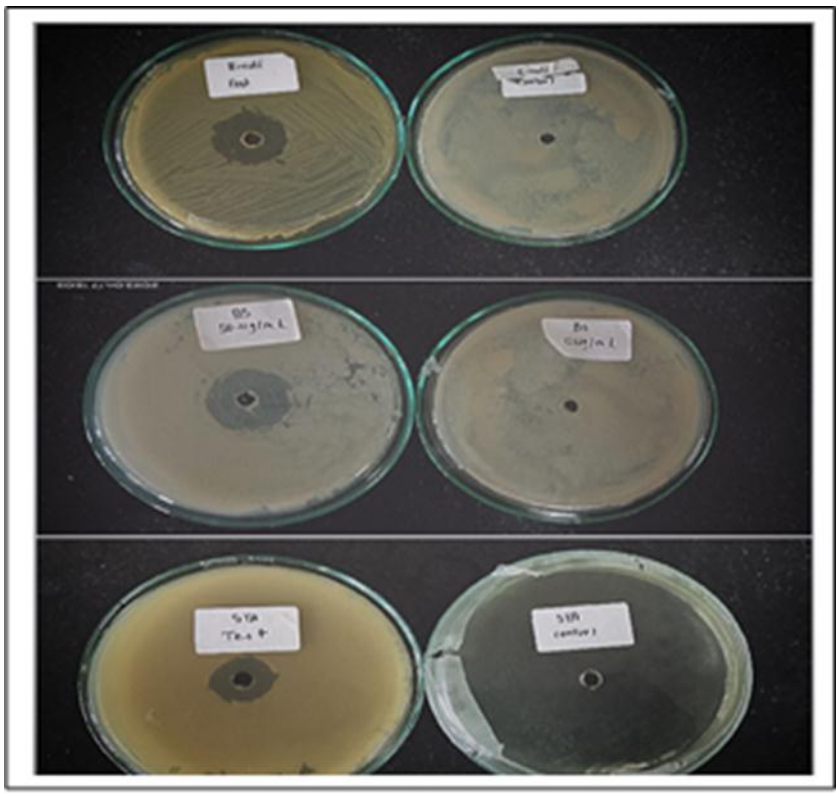

Fig 8: Zone of inhibition showed by 50 µg/ml of CuNPs against various pathogenic culture (Row 1- *E. coli*, test and control, Row 2-*B. Subtilis*, test and control, Row 3-*S. aureus* test and control) .

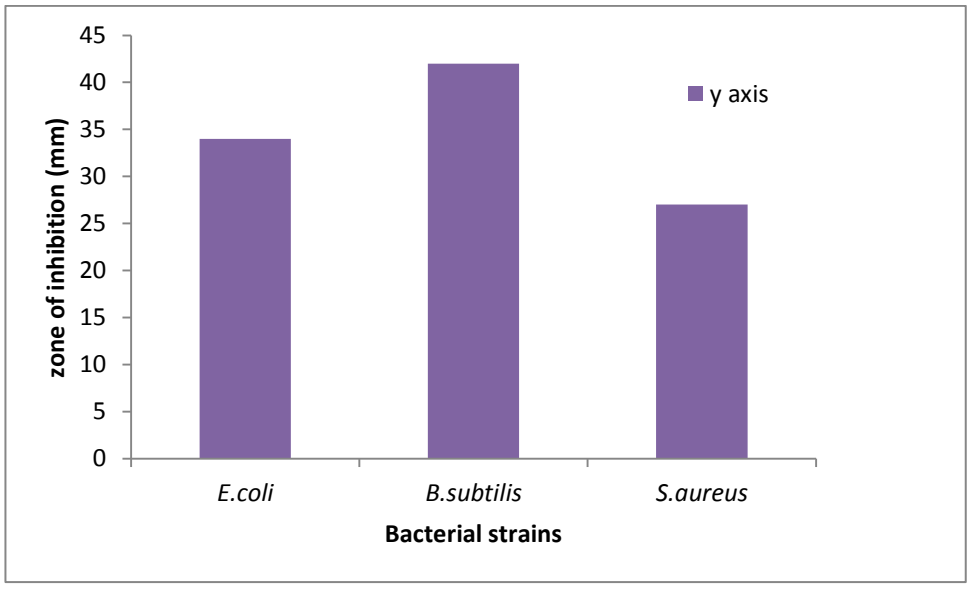

Fig 9- Graphical representation of ZOI

Determination of the antibacterial property of copper nanoparticles (CuNPs) was carried out using the Colony Forming Unit (CFU) assay.

The antimicrobial assay results indicated that the lower concentration of copper nanoparticles led to a higher colony count compared to the lower concentration. Specifically, the CFU assay was performed by inoculating a bacterial culture onto a nutrient agar plate and adding either a low or a high concentration of copper nanoparticles onto the surface of the agar. The plate was incubated under appropriate conditions and the number of visible bacterial colonies was counted. The results showed that the number of colonies on the plate treated with the lower concentration of copper nanoparticles was significantly higher than the number of colonies on the plate treated with the higher concentration of copper nanoparticles. This finding suggests that the lower concentration of copper nanoparticles may not be effective in inhibiting bacterial growth, and may even promote bacterial proliferation. The CFU assay is a widely used method for determining the antibacterial activity of various compounds, including nanoparticles. It involves the counting of colonies that grow on agar plates, which provides an estimate of viable bacterial cells. The assay is simple, reliable, and relatively inexpensive, making it a popular choice for antimicrobial susceptibility testing. The antimicrobial activity of copper nanoparticles (CuNPs) at different concentrations was evaluated against two bacterial strains, Escherichia coli (*E. coli*) and Bacillus subtilis *(B. subtilis*), using the colony forming unit (CFU) assay.

The concentration of CuNPs ranged from 0 to 125 µl/ml. The results showed that the CFU count for E. coli decreased as the concentration of CuNPs increased, with the highest CFU count of decreased as the concentration of CuNPs increased, with the highest CFU count of 27.5×10^9 observed at 0 µl/ml and the lowest CFU count of 0.2×10^9 observed at 125 µl/ml, indicating a dose-dependent antimicrobial effect (Table 2) and (Fig 10 and Fig 12). For *B. subtilis*, the CFU count decreased with increasing concentration of CuNPs up to 50 µl/ml, with

the highest CFU count of 15.5×10^9 observed at 0 µl/ml and the lowest CFU count of 7.5×10^9 observed at 50 µl/ml. However, at higher concentrations (75 and 125 µl/ml), the CFU count increased for *B. subtilis*, suggesting a possible protective effect of CuNPs at those concentrations (Table 2) and (Fig 11 and Fig 12). Notably, at 125 µl/ml, the CFU count was negligible for *E. coli*, indicating a strong antimicrobial effect, but completely inhibited the growth of *B. subtilis*. These findings suggest that the antimicrobial activity of CuNPs is both concentration and strain-dependent, and should be carefully optimized for specific applications. Further studies are needed to elucidate the underlying mechanisms and potential toxicity of CuNPs at different concentrations.

Table 2 – Colony forming unit (10^9) per ml of *E.Coli and B.subtilis* with different concentration of copper nanoparticles

Concentration of CuNPs	CFU(10^9)/ml (*E.Coli*)	CFU(10^9)/ml(*B.subtilis*)
0 µl/ml	27.5	15.5
10µl/ml	15.0	-
50µl/ml	5.5	7.5
75µl/ml	1.5	3.0
125µl/ml	0.2(negligible)	0

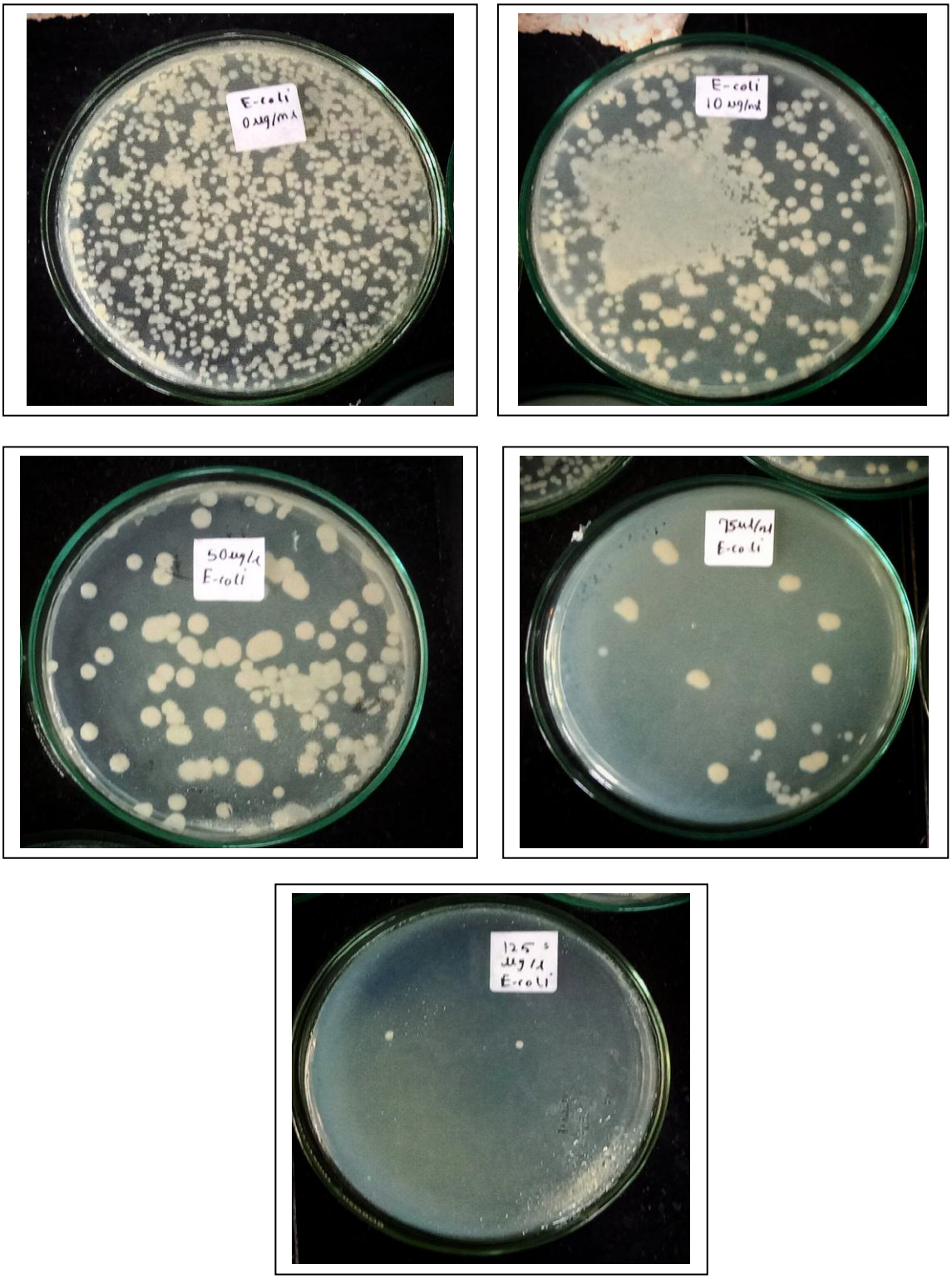

Fig 10- Antimicrobial assay of *E.coli* with different concentration of CuNPs (0ug/ml, 10ug/ml, 50ug/ml, 75ug/ml, 125ug/ml)

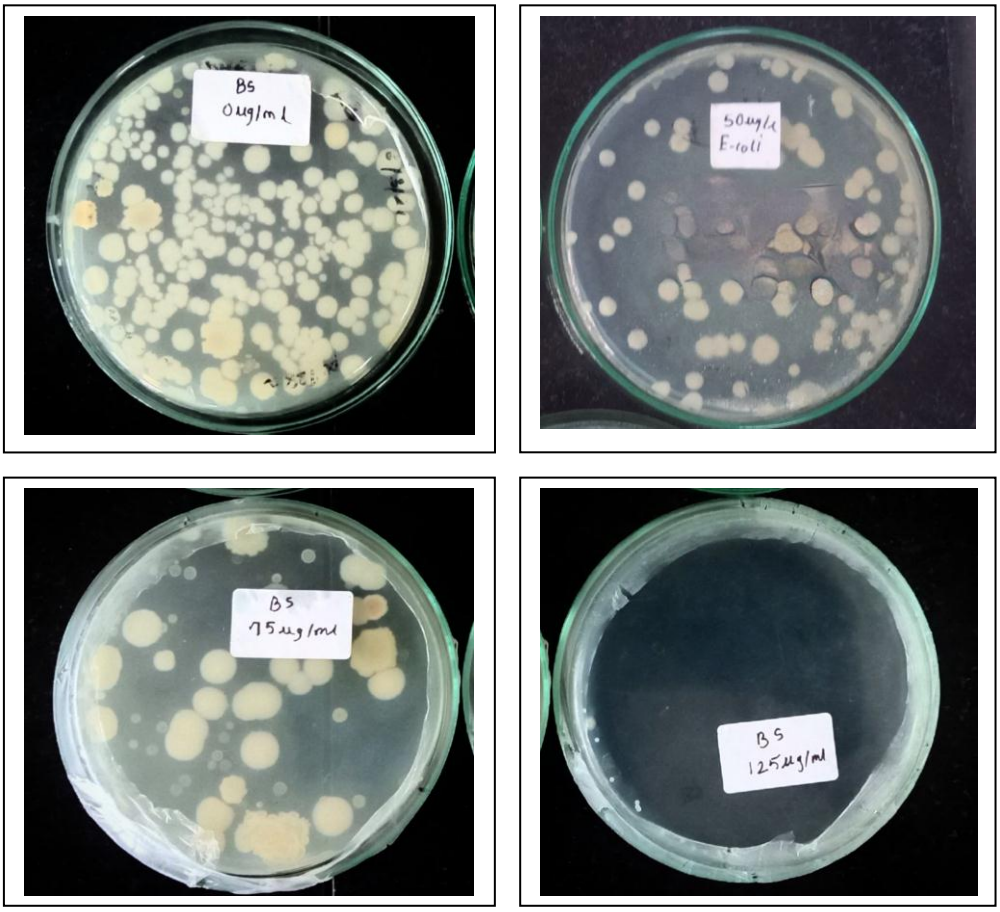

Fig 11- Antimicrobial assay of *B.subtilis* with different concentration of CuNPs (0ug/ml, 10ug/ml, 50ug/ml, 75ug/ml, 125ug/ml)

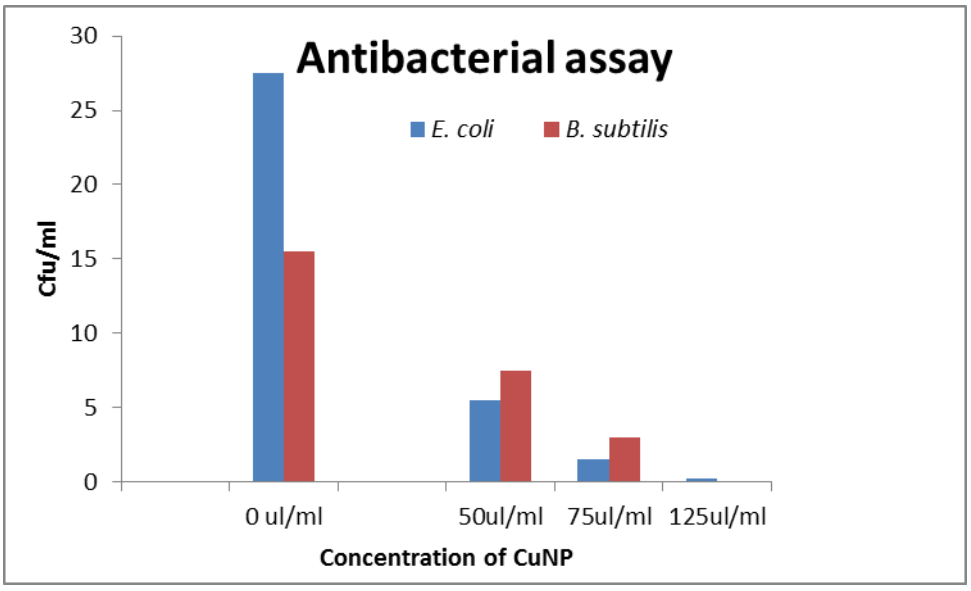

Fig 12 – Graphical representation of CFU per ml, x axis-concentration of CuNPs Y axis-CFU (10^9) /ml.

Table 3: Result of LD_{50} calculation

Sr.no	Con.of CuNPs(µg/ml)	CFU (10^9)/ml	Log con.of CuNPs	% Mortality	% Survival
1.	0	27.5	-	0	100
2.	10	15.0	1	54.5	45.5
3.	50	5.5	1.7	20	80
4.	75	1.5	1.88	5.5	94.5
5.	125	0.2	2.09	0.7	99.03

- Estimation of MLD

  80 – 50/80-45.5 = 30/34.5 = 0.86
  Log difference of CuNPs = 1.7-1 = 0.7
  So that, 0.86 × 0.7 = 0.602
  Now, Antilog of (1 + 0.602) = 46.632

- Estimation of MSD

  54.5 – 50/ 54.5- 20 = 4.5/34.5 = 0.13
  Log difference of CuNPs = 1.7-1 = 0.7
  So that, 0.13 × 0.7 = 0.091
  Now, Antilog of (1 + 0.091) = 1.620

  LD_{50} = 46.632 + 1.620 / 2 = 48.252/2 = 24.12 µg/ml.

  The LD50 was determined to be 24.12 micrograms per millilitre (µg/ml).

  In this study, the Reed and Muench modified formula was used to estimate the median lethal dose (MLD) and median survival dose (MSD) of copper nanoparticles (CuNPs). The LD50, which is the average of MLD and MSD, was also determined.
  The MLD was estimated to be 46.632 µg/ml, whereas the MSD was found to be 1.620 µg/ml. additionally, the log difference of CuNPs was calculated to be 0.7. By adding the product of MLD and MSD difference and this log difference, the antilog values of 46.632 and 1.620 were obtained, respectively. Consequently, the LD50 was calculated to be 24.12 µg/ml.

Conclusion

The growth of halophilic bacteria in an 8% concentration environment was observed, and a colony was isolated and designated as GUWSP2. The bacterial isolate was characterized as Gram-negative and rod-shaped. The isolate's 16S rDNA gene sequence was obtained, and a phylogenetic tree was generated and the culture was found similar to *Cytobacillus horneckiae*. . The cultures were found to be capable of extracellular synthesis of copper nanoparticles, which was confirmed visually and by UV-Visible spectroscopy analysis of the supernatant. The absorption peak of copper nanoparticles was found to be at 312 nm. These results suggest that the non-pathogenic bacteria used in the experiment have the potential to be used in the synthesis of copper nanoparticles. Copper nanoparticles synthesized using this bacteria exhibited strong antimicrobial activity against *E. coli, B. subtilis,* and *S. aureus.* And the zones of inhibition were found 34mm, 42mm and 27mm respectively. . These results highlight the potential of these nanoparticles for use as effective antimicrobial agents in medicine, agriculture, and food industries. However, further studies are needed to understand their mechanism of action and potential toxicity. The antimicrobial activity of copper nanoparticles against *E. coli* and *B. subtilis* is concentration and strain-dependent. CuNPs demonstrated a dose-dependent antimicrobial effect on *E. coli*, while for *B. subtilis*, protective effects were observed at higher concentrations. The LD50 value for this antibacterial CFU assay, calculated using the Reed and Muench formula is approximately 24.12 µg/ml of CuNPs. Further studies are needed to optimize CuNPs for specific applications and to investigate their mechanisms of action and potential toxicity. The findings were consistent with previous studies on bacterial synthesis of copper nanoparticles.

Future prospectus

1. Further research could be conducted on the GUWSP2 bacterial isolate to fully understand its capabilities and potential applications. This could include studying its ability to synthesize other types of nanoparticles, as well as investigating its potential as a biocontrol agent in saltwater environments.

2. The potential use of the GUWSP2 bacterial isolate in the synthesis of copper nanoparticles could be further explored. This could include optimizing the conditions for nanoparticle synthesis, such as varying salt concentrations or pH levels, and investigating the potential of using the bacterial isolate in large-scale industrial applications.

3. Antimicrobial applications of copper nanoparticles hold great promise in medicine, food packaging, and water treatment. However, more research is needed to fully understand their potential benefits and risks and develop effective and safe delivery methods and manufacturing processes.

SUMMARY

Given culture of GUWSP2 was maintained on NTYE agar plates supplemented with 8% salt concentration. The culture was characterized by gram staining, revealing it to be a gram-negative rod. Further molecular characterization was performed by Sanger sequencing, sequence blast, and phylogenetic tree construction, which demonstrated that the culture is closely related to *Cytobacillus horneckiae.*

Copper nanoparticles were synthesized using a cell-free supernatant from a culture grown in NTYE broth. The supernatant was mixed with CuSO4 and incubated on a shaker for 24 hours, producing a green pellet of copper nanoparticles. The pellet was dried, ground to a fine powder, and analysed for characterization. . The characteristic absorption peaks of copper nanoparticles (CuNPs) fall within the range of 300-500 nm. The peak absorption of the CuNPs synthesized in the study was determined to be at 312 nm, which conclusively confirmed their successful synthesis.

Copper nanoparticles synthesized in this study using given culture showed a strong antimicrobial effect against three bacterial strains, with inhibitory zones ranging from 27mm, 34mm, 42 mm. Therefore, they have the potential to be used for antimicrobial applications in various fields such as medicine, agriculture, and food industries. The study evaluated the antimicrobial activity of copper nanoparticles (CuNPs) at different concentrations against *Escherichia coli* (*E. coli*) and *Bacillus subtilis (B. subtilis)* using the colony forming unit (CFU) assay. Results showed that CuNPs had a dose-dependent antimicrobial effect on E. coli, with the highest CFU count observed at 0 µl/ml and the lowest at 125 µl/ml.

For *B. subtilis*, CFU count decreased with increasing concentration up to 50 µl/ml, but increased at higher concentrations. At 125 µl/ml, CuNPs completely inhibited the growth of *B. subtilis* and showed a strong antimicrobial effect on *E. coli*. The study highlights the concentration and strain-dependent nature of CuNPs' antimicrobial activity and the need for optimization for specific applications. The LD50 value for this antibacterial CFU assay, calculated was approximately 24.12 µg/ml of CuNPs.

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APPENDIX

1. NTYE BROTH

Magnesium sulphate hepatahydrate	2g
Potassium chloride	0.5g
Calcium chloride dihydrate	0.02g
Yeast extract	0.30g
Tryptone	0.5g
Nacl	8gm
Distilled water	100ml
pH	6.5-7.0

2. NTYE AGAR

Magnesium sulphate hepatahydrate	2g
Potassium chloride	0.5g
Calcium chloride dihydrate	0.02g
Yeast extract	0.30g
Tryptone	0.5g
Nacl	8gm
Distilled water	100ml
pH	6.5-7.0
Agar	2gm

3. CRYSTAL VIOLET

Solution A

Crystal violet	2g
Ethanol	20ml

Solution B

Ammonium oxalate	0.8g
Distilled water	80ml

Mix solution A and solution B to make total 100ml.

4. GRAMS IODINE

Iodine	1g
Potassium iodide	3g
Distilled water	100ml

5. SAFRANIN

Safranin	2.5g
95% ethanol	10ml
Distilled water	90ml

6. NUTRIENT AGAR PLATES

Nutrient Agar	2.8g
Distilled water	100ml

7. 1 X PBS

Sodium chloride	8g
Potassium chloride	0.2g
Sodium phosphate dibasic	1.44g
Potassium phosphate monobasic	0.24g

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1 Antibacterial activity of copper nanoparticles synthesized using halophilic bacteria
2 Introduction
3 Nanoparticles are the material which scale assemblies of atom. Thus, they have dimensions ranges between 10nm to 100
nm (Shobha, 2014). Nanoparticles are solid particles that have all three dimensions in the nanoscale.
Physical and chemical properties of nanoparticles are controlled by its exact 3D morphology, structure and composition (Olson, 2000). In recent years nanoparticles have received for it's potential to improve many economic sector such as biomedicine, pharmaceuticals, antimicrobial agents and agriculture (Shobha, 2014). The improvement of nanotechnology have ventured up of researchers to form that have preferences in different regions of pharmaceuticals and medicine (Siddiqi, 2020). Depending on the origin, there are three types of nanoparticles natural, incidental, engineered (Monica, 2009). Natural nanoparticles are an intriguing topic of research due to their unique properties and potential applications. Natural nanoparticles have been found to possess high surface area, reactivity and biocompatibility, making them useful in various fields such as drug delivery, food industry and environmental remediation (Khan, 2019). Engineered nanoparticles have potential benefits of efficient drug delivery and enhanced imaging contrast. Despite their potential benefits, engineered nanoparticles also raised concerns about their potential toxicity and environment impact. Therefore the development of engineered nanoparticles also requires a comprehensive understanding of their potential risks and regulatory oversight to ensure their safe and effective use.
Tiny particles called nanoparticles can be divided into two categories: organic particles and carbon-based particles. Organic particles include dendrimers, liposomes, and micelles, while carbon-based particles include fullerene, graphene, carbon nanotubes, and carbon black.
These tiny particles have different
physical, biological, and chemical properties
than larger particles (Ealia, 2017). Metallic nanoparticles have gotten interest of many scientist and now they are heavily utilized in biomedical science and engineering (Mody VV, 2010). Metallic nanoparticles
are used in various fields like aerospace, medicine, textiles, and farming. Indian farmers use some nanoparticles like nanozinc and nanocopper to make crops grow better. Additionally, copper nanoparticles and their oxides
4 have been found helpful in many ways, such as sensing devices, removing dyes, and fighting germs, fungus, and pests (