STUDY OF NANOPARTICLES SYNTHESISED FROM HALOPHILIC BACTERIA

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GOA UNIVERSITY

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Examined by: Seal of the School

DECLARATION

I hereby declare that the data presented in this Dissertation report entitled, **"STUDY OF NANOPARTICLES SYNTHESISED FROM HALOPHILIC BACTERIA"** is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth Ocean Atmospheric Sciences, Goa University under the Supervision of Dr. Chanda V. Berde 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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CONTENTS

I ABBREVIATIONS

gms	Grams
°C	Degree Celcius
mm	Milli metre
R.T.	Room temperature
Min	Minute
Hrs	Hours
NA	Nutrient agar
NB	Nutrient broth
D/W	Distilled water
ml	Milli litre
µg	Microgram
mg	Milligram
ppm	Parts per million
NaCl	Sodium chloride
Ag	Silver
Pb	Lead
$AgNO_3$	Silver nitrate
rpm	Revolutions per minute
ppt	Precipitate
Wt	Weight
No	Number
UV- Vis spectroscopy	Ultraviolet-visible spectroscopy
SEM	Scanning electron microscope
XRD	X-ray Diffraction
TEM	Transmission electron microscope
EDAX	Energy dispersive X- Ray analysis

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CHAPTER 1

INTRODUCTION

1.1 Halophiles

Halophiles are extremophilic microbes and thrive in saline environments such as marine and estuarine environments, solar salterns, lakes, and brines. They cannot survive in absence of salt (mainly NaCl). They include mainly eukaryotic and prokaryotic microorganisms. Halophiles can balance the osmotic pressure of the environment. The cytoplasm of halophiles is developed in such a way that it is isotonic with the environment. They can store organic compounds in the cytoplasm which helps the halophiles to live in stressful halophilic conditions (Vijayakumar, 2021).

Halophiles cover three domains put forth by Carl Woese, i.e. Archaea, Bacteria, and Eukarya. Halophiles are classified based on the amount of salt required for growth. They are categorised into three essential types. Many of the halophiles are slight halophiles which grows with 2.5-3% NaCl in seawater. Moderate halophiles optimally grow with 3- 15% of salt. Extreme halophiles require and grow with 25% salt (Vijayakumar, 2021).

Halophile organisms follow one of the 2 strategies to survive in saline environments. In a compatible solute strategy, cell maintain low concentration in their cytoplasm by balancing osmotic potential by synthesising organic compounds. This is seen in moderately halophilic and halotolerant bacteria. The salt-in strategy is adopted by true halophiles, i.e., halophilic archaebacteria and extremely halophilic bacteria. They do not synthesise organic compounds to maintain osmotic balance with the environment and may not survive when the salinity of the medium is lowered (Tiquia-Arashiro *et al*., 2016).

1.2 Nanotechnology

Nanotechnology is an emerging science and its elements are at nano-scale (1-1000nm). When the dimension of any element is decreased, its physical, chemical and photo- electrochemical and electronic properties also change. Compared to macroparticles, nanoparticles have unique features such as a high surface-to-volume ratio and quantum effects. Conversion of macroparticles to nanoparticles requires lot of expense. Also, nanoparticles developed from chemicals have contaminants from pre-used chemicals. The chemicals and physical methods are used to make it more toxic for human use. Addition of stabilizing agents are required for maintaining the size of nanoparticles developed from chemical methods.

There are growing applications of nanoparticles in various industrial sectors including food, electronics, pharmaceuticals, fuels, chemicals, polymers, and environmental health.

Nanoparticles even developed from physicochemical methods are expensive and are hazardous to human as well as environmental health (Ramsden, 2005).

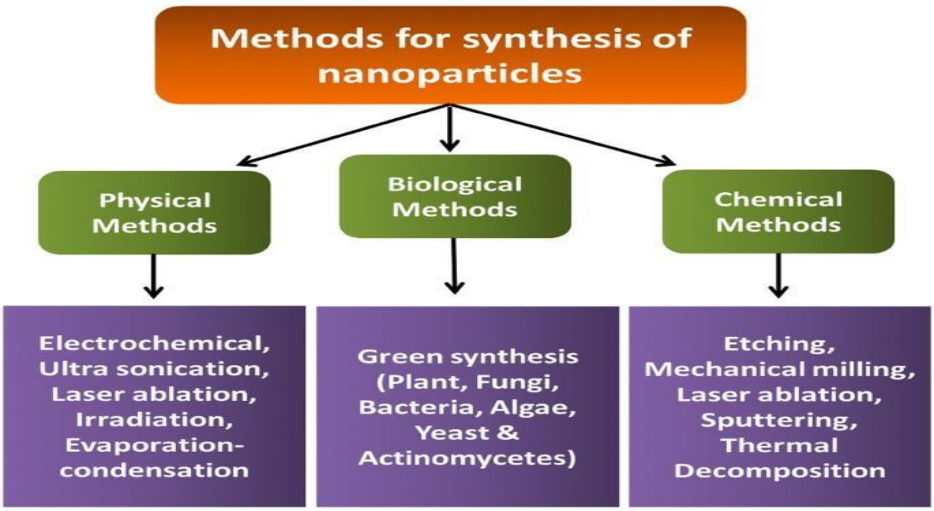


Fig 1: Different methods of synthesis of metal nanoparticles (Khandel et al., 2018).

1.3 Biologically synthesised nanoparticles

Biologically synthesised nanoparticles are clean, non-toxic, and eco-friendly. They get developed in normal temperature and pressure, do not require toxic precursors and may be stabilised by cellular metabolites.

Metallic nanoparticles synthesised with micro-organisms have similar properties of chemically synthesised nanoparticles. The nanoparticles synthesised by this process have higher catalytic activity and greater surface area.

During cell processes, during the synthesis of nanoparticles microorganisms seize target ions from the environment and turn metal ions into elemental metal ions.

They are further classified into intracellular and extracellular activities according into which region the nanoparticles are formed. In intracellular method, nanoparticles, metal ions are trapped into microbial cell to form nanoparticles in the presence of enzymes. In extracellular method, nanoparticle synthesis, the metal ions are trapped on the surface of the microbial cell.

The biosynthesised nanoparticles have variety of applications in medical, food and technology sectors (Li *et al*., 2011).

1.4 Characterisation of nanoparticles

Characterisation refers to study of particles such as size, shape and various physical properties. Nanoparticle properties of size and shape vary significantly.

Scanning electron microscopy (SEM) is a widely used method to check the surfaces of particles which can be characterise nanoscale materials. It allows to detect size, shape and surface morphology of nanoparticles (Mourdikoudis *et al*., 2018).

UV- vis Spectroscopy- It is a technique which helps in optical study of the materials. Ultraviolet and visible absorption spectroscopy allows to measure the attenuation of light which passes through a sample. This follows the principle of Beer-Lambert law.

Fourier transform infrared (FTIR) method is a type of spectroscopy which can detect changes in the total composition of biomolecules by determining changes in functional groups (Eid, 2022) .

1.5 Silver nanoparticles

They show effective antimicrobial activity against Gram-positive and Gram-negative bacteria. Microbes reduce the Ag^{+} ions to metallic Ag^{0} atoms. The Ag^{0} atoms are followed by agglomeration into oligomeric clusters. These clusters further lead to formation of Ag nanoparticles which are spherical in shape. When these colloidal particles are smaller than the wavelength of visible light, the solution have a yellow colour with an intense band in the 300400 nm range (Sharma *et al*., 2008).

1.6 Lead Oxide Nanoparticles

They show antimicrobial activity against some bacteria. When lead nanoparticles are formed it has distinguishing white colour. They also have characterising hemispherical shape (Khalil *et al*., 2017).

1.7 Copper Nanoparticles

It has antimicrobial activity against bacteria as well as fungi. The unique properties of copper nanoparticles allow being used as catalysts, lubricants and specially in technology industry. When copper nanoparticles are formed, they give a distinguishing green colour to the supernatant (Shantkriti *et al*., 2014).

1.8 Applications of Nanoparticles

- **Anti-bacterial activity against Nanoparticles**
  Bacteria are oldest and simplest forms with prokaryotic cellular organisation. There are two different type of cell walls in bacteria. Gram-positive and Gram-negative bacteria. Gram-positive bacteria have thick and multi-layered peptidoglycan layer and it retains the crystal violet stain during gram staining. Gram-negative bacteria have thin and single-layered peptidoglycan and do not retain crystal violet stains during gram staining (Longano *et al*., 2012).

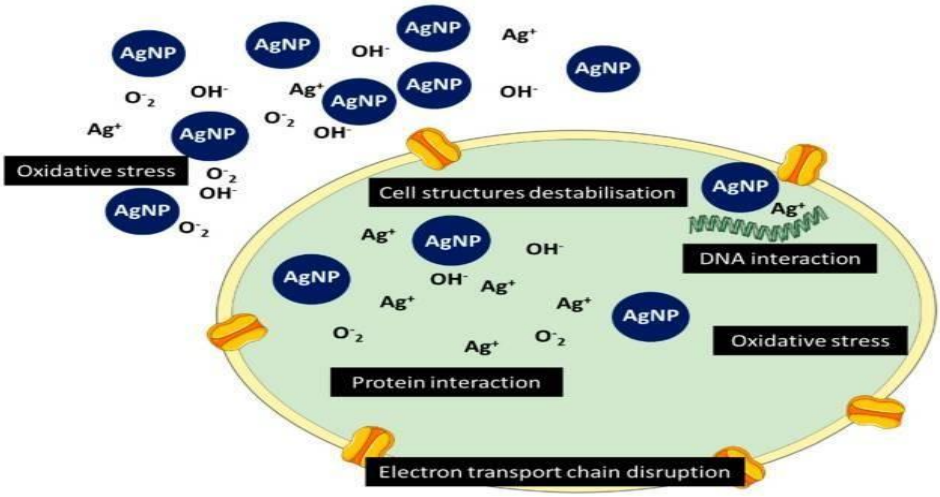


Fig 2: Schematic representation of silver nanoparticles mechanism of antimicrobial activity (Lopez *et al*., 2020). The antimicrobial action of nanoparticles occurs by 4 main mechanisms: -

i. Adhere to the bacterial surface.
ii. It de-stabilises the bacterial wall and cell membrane making it easy to penetrate inside the cell.

iii. It creates oxidative stress and creates toxicity to the cell material by the generation of ROS.
iv. Modules signal transduction pathways (Lopez *et al*., 2020).

The nanoparticles which are resistant and inhibit the growth of bacteria can be used as surfactants or disinfectants in laboratories. The swabs can be prepared to clean the surfaces which are more viable for those bacteria.

- **Anti-fungal Activity**

  The fungus is a member of eukaryotes and can be present whenever they get required amount of nutrition. Fungi also decompose organic matter and play important role in nutrient cycle. Some species of fungi produce bioactive compounds such as mycotoxins which are toxic to human health.

  Surfactants can be prepared to inhibit growth of fungus.

- **Cytotoxicity of Mosquito larvae**

  Mosquitoes are common flying insects living in most parts of the world. They belong to arthropod and can transmit several fatal diseases and affect human health. Adult female mosquitoes transmit the disease by bites when they obtain blood meals from their host. Dengue has been serious as it difficult to control disease as well as the vector mosquito (Gunathilaka *et al*., 2021).

  Culex quefasciatus, transmits West Nile virus and is a causative agent of lymphatic filariasis. During growth, the larvae shed skin four times. The stages between sheds are called instars.

  Materials can be prepared to inhibit the larvae.

- **Inhibition of Biofilm formed from** *Pseudomonas* spp

  Pseudomonas spp is a ubiquitous Gram-negative bacterium. It causes fatal diseases and it is difficult to treat infection as it forms a biofilm attaching to the surface which is difficult to remove them (Tam Tran Thi *et al*., 2020).

  Biofilm is an assembly of microbial cells which adhere to the surface by secreting polysaccharide materials. The solid-liquid medium interface, provides an ideal environment for the attachment and growth of bacterium (Donlan, 2002).

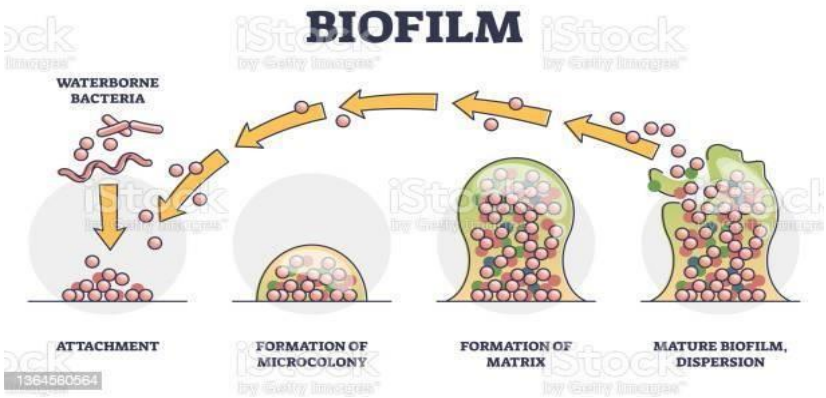


Fig 3: Biofilm Formation stages

Nanoparticles are promising tools against biofilms. The antibacterial effect of bacteria is not effective against nanoparticles.

The interaction between the biofilm and the nanoparticles process by 3 stage mechanism: -

i. The nanoparticles are placed close to the biofilm.
ii. They attach to the biofilm.
iii. They migrate towards biofilm and destruct it (Shkodenko *et al*., 2020).

- **Antibiotic assay**

  The test is also called an antibiotic sensitivity test. It allows to check how sensitive the bacteria are to different antibiotic medicines. Very few bacteria are targeted by Narrow-spectrum antibiotics. Broad-spectrum antibiotics aim many types of bacteria.

CHAPTER 2 LITERATURE REVIEW

Surve *et al*,. (2012) in their study, halophilic bacteria were isolated from solar salt pans of Ribander, Goa. Morphological, Biochemical, Antibiotic sensitivity, physiological and enzymatic characteristics of the isolates were compared. 2 halophilic bacteria were similar to *Alkalibacillus* HSD 20 and *Virgibacillus panthotheticus* species in an evolutionary tree.

Saleh *et al*,. (2020) in their study, silver nanoparticles from bacteria *Klebsiella pneumonia.* They were synthesised extracellularly, by culture supernatant of the bacteria. The characterisation of the nanoparticles was studied using SEM, FTIR and UV-Visible spectroscopy analysis. The effect of synthesis of nanoparticles at different concentration 1mM, 2mM and 4mM silver was seen. Effect of synthesis at different pH and temperature was also seen. They also checked antimicrobial activity against nanoparticles using bacterial strains *E. coli, S. aureus, P. aeruginosa* and *B. ceruis.* The test was done by 6mm well diffusion method.

Srivastava *et al*,. (2013) in their study, silver nanoparticles were synthesised using haloarchaeal isolate *Halococcus salifodinae* $BK_{3.}$ The nanoparticles synthesised were around 50 and 10nm in size when they were synthesised in NaCl Tryptone Yeast Extract broth and Halophilic nitrate broth. They have reported intracellular synthesis. They have also characterised silver nanoparticles preparation using UV-visible spectroscopy, XRD, TEM and EDAX. Disc diffusion method was used to determine the antimicrobial activity of the nanoparticles against test organisms like Gram negative *E. coli* MTCC 2345, *Pseudomonas aeruginosa* MTCC 2582

and Gram positive *S. aureus* MTCC 737 and *Micrococcus luteus.* Sterile disc of 6mm diameter were dipped in silver nanoparticle suspension of concentration 10 and 50µg/disc.

Longano *et al*,.(2012) in their study, copper nanoparticles were synthesised by electrochemical and physico- chemical methods. As bulk copper materials provide excellent antimicrobial activity against wide range of microorganisms and hence they were properly synthesised at nano scale. The synthesised nanoparticles were tested against microorganisms. They selected Gram negative *E. coli* strain, Gram positive *S. aureus,* yeast *S. cerevisiae* and some common microorganisms like algae *Chlamydomonas* sp. Strain CDI Red and marine diatoms, *Phaeodactylum tricornutum* CCMP 1327. Copper nano antimicrobials proved to effectively kill the microorganisms or significantly inhibits its goal.

Shamseldean *et al*,. (2022) in their study, efficacy of nanoparticles were seen to control mosquito, *Culex quinquefasciatus.* The mosquito larvae were reared in the laboratory. Chitoson, silver nanoparticles and their combinations were tested against larvae. The mortality was seen after 24 hours and 48 hours. Insect larvae were immersed in 100ml of each dilution.

Lahiri *et al*,. (2021) in their study, microbiologically synthesised nanoparticles and studies the role of quorum sensing to inhibit growth of biofilm. Silver nanoparticles, Gold nanoparticles were tested against the biofilm.

CHAPTER 3 AIMS AND OBJECTIVES

The main aim of the dissertation is to study halophilic bacteria for the synthesis of nanoparticles.

Objectives

The project objectified the synthesisation of nanoparticles from halophilic bacterial cultures.

The characterisation of nanoparticles was also seen.

Optimization of nanoparticle were carried out.

Applications of nanoparticles are studied which can be used in many fields.

CHAPTER 4 MATERIALS AND METHODS

2.1 Bacterial cultures used:

Halophilic and halotolerant 150 bacteria which were isolated from salt pans of Goa in 2022 for dissertation purpose, and stored in Marine Microbiology Laboratory refrigerator were used (Yellapu, 2022).

Chlorpyrifos degrading 18 bacteria which were isolated from field of St. Cruz Bandh inlet from 2 different point in 2022 for the dissertation purpose, and stored in Marine Microbiology Laboratory refrigerator were used. (Vaigankar, 2022)

2.2 Revival of bacterial cultures on respective media:

i. Salt pan 150 cultures which were transferred on 10% Rocksalt Nutrient Agar with 2% Agar Agar plates (Appendix 1) and Nutrient agar (Appendix 1) plates prepared both media using distilled water. Incubated for 72 hours at Room temperature 28°C ± 1°C. ii. Chlorpyrifos degrading 18 bacteria were transferred on Zobell Marine Agar plates (Appendix 1) prepared using distilled water. Incubated at for 48 hours at Room temperature (RT) 28°C ± 2°C.

2.3 Subculturing bacterial cultures on slants:

i. Cultures were selected for their better growth and were revived on 10% Rock salt Nutrient agar slants prepared using distilled water. Incubated at RT 28°C ± 2°C for 72 hours.

ii. Cultures were sub-cultured on Zobell Marine Agar slants prepared using distilled water.

Incubated at RT 28°C ± 2°C for 48 hours.

2.4 Screening for Halophilic cultures on NaCl Tryptone Yeast Extract Agar:

NaCl Tryptone Yeast Extract Agar (Appendix 1) was weighed and added to clean conical flask and autoclaved for 30min at 121°C, then poured in cleaned autoclaved petri-plates. After the agar was set, the said 118 cultures were transferred on the agar plates. The plates were kept for incubation at 28°C ± 2°C for 168 hours.

2.5 Detect growth in liquid medium:

Selected 32 cultures from previous test were further tested. 25% NaCl Nutrient Broth (Appendix 1) with distilled water was prepared. The broth along with loopful of selected cultures were inoculated in cleaned autoclaved test-tubes. The test tubes were incubated for 168 hours at 28°C ± 2°C.

2.6 Salt Tolerance of cultures:

Selected 23 cultures from previous test were further tested.The test was performed to check the growth of cultures after keeping for incubation at different time interval. 5%, 15% and 25% NaCl Nutrient agar plates were prepared using distilled water. The cultures were transferred on those plates and kept for incubation for until they showed positive results(growth) at 28°C ± 2°C.

2.7 Lysis test to confirm bacterial cultures:

Selected 23 cultures from previous test were further tested.The test was performed to distinguish between archaebacteria and bacteria. The wavelength of colorimeter was set at 620nm. Distilled water was used as blank. To test the cultures, the test cuvette was filled with distilled water. A loopful of culture from the NaCl Tryptone Yeast Extract agar was mixed with distilled water. Absorbance was recorded for 0second, 1minute, 2minute, 3minute, 4 minute and 5minute. If absorbance remained stable throughout it was considered Bacteria while decrease of absorbance and then reaching to 0 then it was Archaebacteria.

2.8 Synthesis of Nanoparticles by using bacteria:

Selected 21 cultures from previous test were further tested.

Said 2 Methods were used for synthesis of the nanoparticles using metal solutions.

a. Method 1(Srivastava *et al*., 2013)

   In cleaned 50mL flask, 10mL 25% NaCl Nutrient Broth prepared with Mili Q water was prepared and autoclaved. A loopful of culture was added to the medium and 0.5mM of 100mM Silver Nitrate was added. 2 common Controls for all 21 cultures were maintained, Control 1 flask with only 10mL 25% NaCl Nutrient Broth prepared with Milli Q water and Control 2 flask with 10mL 25%NaCl Nutrient Broth and 50µl of 100mM Silver nitrate. The experimental flasks along with control flasks were incubated at 37°C for 168 hours in dark. Checked for colour change.

b. Method 2

   In cleaned 50mL of flask, 10mL of 25% NaCl Nutrient Broth using Milli Q water was prepared and autoclaved. A loopful of culture was inoculated in experimental flask. A Control was maintained which had 10mL of 25% NaCl Nutrient Broth. All the experimental flask and control flask were incubated at 37°C for 168 hours in dark. The content was then centrifuged using Microcentrifuge for 10 minutes at 5000 rpm. The supernatant was stored separately while pellet was suspended in 10mL of Milli Q water for further use.

i. In cleaned test tube 1mL of suspended pellet with Milli Q water was taken and to it 9ml of 0.5mM of 100mM of silver nitrate was added.

ii. In cleaned test tube 1mL of supernatant was taken and 9mL of 0.5mM of 100mM of Silver Nitrate was added. iii. A control was maintained to 1mL of Milli Q water, 9mL of 0.5mM of 100mM of Silver Nitrate was added in a test tube.

Same protocol 2 was repeated for lead nanoparticles and copper nanoparticles. For Lead nanoparticles 0.5mM of 100mM of Lead acetate trihydrate was used and for copper nanoparticles 0.5mM of 100mM of copper chloride was used.

The experimental test tubes along with control test tube were kept for incubation for 48 hours at 37°C. After formation of nanoparticles, silver nitrate solution develops deep brown colour, Lead acetate trihydrate solution develops white colour while Copper Chloride develops green colour.

2.9 Synthesis of Nanoparticles at different metal concentrations:

Method 2 was adapted from previous (2.8.b) step. 14 cultures were selected for further analysis.

To test for different metal concentration for synthesis of nanoparticles (10mM, 50mM, 100mM metal concentration were used) 0.9mL of 10mM of Metal i.e., Silver Nitrate, Copper Chloride and Lead Acetate Trihydrate were taken in clean Eppendorf tubes and to it 0.1mL of pellet suspension and supernatant were added separately. A control of each metal was maintained in one Eppendorf tube containing 0.1mL of Milli Q water and 0.9mL of 10mM of Metal solution. The experimental Eppendorf tubes and controls were kept for incubation for 48 hours at 37°C in dark. If there was formation of nanoparticles, the selected nanoparticles were then synthesised with 10mL overall volume (9 ml metal suspension and 1mL supernatant or pellet suspension) the suspension was centrifuged at 5000 rpm for 10 minutes and washed thrice with Milli Q water.

2.10 Characteristics of Nanoparticles:

The nanoparticles formed were then dried by placing into petri plates and drying at 37°C for 48 hours.

- 14 nanoparticles were selected for further analysis. The excitation spectra of the silver nanoparticles and Lead Nanoparticles dried powder which was suspended in distilled water was recorded in the range of 200-800 nm and 200-400 nm respectively using UV- Visible spectrophotometer with distilled water as the blank. (Srivastava *et al*., 2013)

- Nanoparticles were selected for further analysis. The 3 nanoparticles used further were 4Ag, 8Ag and 8Pb (2 were silver nanoparticles and 1 lead nanoparticles). The nanoparticles were then placed in the pre-weighed petri-plate and kept for drying at 37°C for 48 hours and their weight were recorded.

- Monochrome staining using Phase Contrast Microscope- The dried nanoparticles was placed on clean slide made a suspension with Milli Q water. Heat fixed and drained them with Crystal Violet stain. Waited for 1 minute rinsed with water, air dried and watched the nanoparticles under Phase Contrast Microscope at 100x.

- Nanoparticles were tested for further analysis. SEM analysis- The morphology, particle size distribution and shape of the nanoparticles powder was determined using Scanning Electron Microscopy.
- Nanoparticles were tested for further analysis. FTIR- The Fourier transform infrared method is a type of spectroscopy which detect changes in the nanoparticle composition.

2.11 Optimisation of nanoparticle synthesised using bacteria:

• <u>Effect of Temperature on nanoparticle synthesis</u>

For the test, 100mL of 100mM of silver nitrate solution and 50mL of 100mM of Lead acetate trihydrate solutions were prepared using Milli Q water. In 10 cleaned 15mL centrifuge tubes 9mL of the 100mM silver nitrate along with 1mL pellet suspension (prepared using Protocol 2) was added. In 5 cleaned 15mL centrifuge tubes 9mL of 100mM lead acetate trihydrate added along with 1mL of supernatant (prepared using Protocol 2)

was added. The tubes were labelled and kept for incubation at (-16°C, 4°C, 28°C ± 2°C, 37°C, 60°C) for 48 hours. The solutions were centrifuged at 5000 rpm for 10 minutes and washed thrice using Milli Q water. The nanoparticles were placed in labelled pre weighed petri-plates and dried at 37°C and then recorded their weight. The nanoparticles were mixed with distilled water, the O.D of lead nanoparticles were recorded at 250nm and O.D of silver nanoparticles were recorded at 430nm. Distilled water used as blank for U.V spectroscopy.

• Effect of pH on nanoparticles synthesis

For the test, 100mM Silver nitrate solution and 100mM lead aetate trihydrate solutions were prepared using Milli Q water. The pH of the both solutions were adjusted to (3, 4, 5, 6, 7, 8, 9). For synthesis of silver nanoparticles, 9mL of 100mM silver nitrate with adjusted pH solution and 1mL of pellet suspension (prepared using protocol 2) was added. For synthesis of lead nanoparticles, 9mL of 100mM lead acetate trihydrate with adjusted pH solution and 1ml of supernatant was added. The solutions were incubated at 37°C for 48 hours. The solutions were centrifuged at 5000 rpm for 10 minutes and washed thrice using Milli Q water. The nanoparticles were placed in labelled pre weighed petri-plates and dried at 37°C and then recorded their weight. The nanoparticles were mixed with distilled water, the O.D of lead nanoparticles were recorded at 250nm and O.D of silver nanoparticles were recorded at 430nm. Distilled water used as blank for U.V spectroscopy.

2.12 Anti-bacterial activity of Nanoparticles:

Known 12 bacteria were tested for anti-bacterial activity against nanoparticles. Bacterial cultures used were *E. coli* 1, *S. aureus* 1, *E. coli* 2, *S. aureus* 2, *S. paratyphi, Klebsiella* spp*, Bacillus* spp, *Proteus* spp 1, *Proteus* spp 2, *Proteus* spp 3, *Proteus* spp 4 and *Pseudomonas* spp. Nutrient Agar plates were prepared using distilled water. All 12 bacteria were inoculated into autoclaved Nutrient Broth test tubes prepared using distilled water. The bacterial cultures were incubated at 28°C ± 1°C for 4 hours. From the broth 50µL was spread on the labelled

Nutrient agar plates. Autoclaved GF/F Filter paper disc with diameter 6mm were used. The disc was dipped in nanoparticle suspension (10mg of nanoparticles in 1mL of distilled water) and

placed on the agar plates. The plates were incubated at 28°C ± 2°C for 24 hours. The diameter of the zones of inhibition were measured and compared.

2.13 Anti-fungal activity of Nanoparticles:

Known 3 fungal species were tested for anti- fungal activity against nanoparticles. The fungal cultures are *Penicillium* spp*, Aspergillus* spp and *S. cerevisiae.* 400mL of Malt extract agar plates with 40mg Ampicillin using distilled water were prepared. *Aspergillus* spp and *Penicillium* spp was inoculated into sterile autoclaved normal saline and used immediately for further analysis while *S. cerevisiae* was inoculated into Malt Extract broth and incubated for 4 hours. From the normal saline and broth 50µL of culture was spread on the labelled Nutrient agar plates. Autoclaved GF/F Filter paper disc with diameter 6mm were used. The disc was dipped in nanoparticle suspension (10mg of nanoparticles in 1mL of distilled water) and placed on the agar plates. The plates were incubated at 28°C ± 2°C for 48 hours. The diameter of the zones of inhibition were measured and compared.

2.14 Cytotoxicity of nanoparticles against Mosquito larvae:

For the experiment, *Culex quinquefasciatus* mosquito larvae at the late 3rd to 4th instar stage were used. 4 Clean autoclaved containers were filled with 100ml of Reverse Osmosis (RO) water. 20 healthy larvae were introduced in each container. One container was labelled as control. While in other 3 containers nanoparticles weighing 10mg was introduced. Mortality was recorded after 1 hour, 24 hours and 48 hours.

2.15 *Pseudomonas* spp Biofilm inhibition by nanoparticles:

In 5 cleaned autoclaved petri-plates having 3 cleaned autoclaved coverslips in each, 20mL Nutrient broth prepared using distilled water was poured. In 3 Nutrient broth plates loopful of *Pseudomonas* spp was inoculated and 5mg of nanoparticles were introduced in each labelled plate respectively. 2 controls were maintained one plate with 20mL nutrient broth, 4 coverslips and loopful of *Pseudomonas* spp. Other control petri plate with only 20mL Nutrient Broth. Day 2 and Day 3 readings were recorded. Each day one coverslip was removed monochrome stained and observed under phase contrast microscope using oil.

2.16 Antibiotic sensitivity test of selected bacterial cultures:

The selected 10 antibiotic disks were selected for the assay, Ampicillin, Chloramphenicol, Erythromycin, Gentamicin, Kanamycin, Nalidixic acid, Rifampicin, Streptomycin, Tetracycline and Vancomycin. The 2 cultures which can synthesise nanoparticles were grown in autoclaved Nutrient Broth which was incubated for 24 hours at 28°C ± 2°C. Nutrient agar plates were prepared using distilled water. 100µL of the culture were spread on the NA plates.

The standardise antibiotic disk were placed on plates. The plates were incubated for 24 hours at 28°C ± 1°C. The zone of inhibition was recorded and compared using standardise chart.

2.17 Gram staining of bacterial cultures:

Gram staining was done of 14 cultures was done to distinguish between Gram-positive and Gram-negative bacterial strains using microscopy. A loopful of culture was placed on slide, smear was made using normal saline. The culture was heat fixed on the clean slide. Primary stain (Crystal Violet) was flooded and kept for 1 minute. The slide was washed with water and air dried. The slide was flooded with Gram's iodine and kept for 1minute. The slide was then flooded with Gram decolouriser kept for 30 seconds and washed with water and then air dried. The slide was then flooded with secondary stain(Saffranin), kept for 1 minute, washed with water and air dried. The slide was later watched under phase contrast microscope at 100x using oil.

CHAPTER 5
RESULTS

3.1 Selection of cultures:

Halophilic and halotolerant 150 bacteria which were isolated from salt pans of Goa in 2022 for dissertation purpose, and stored in Marine Microbiology Laboratory refrigerator were used. (Yellapu, 2022) They were transferred on 10% Rocksalt Nutrient Agar with 2% Agar agar and Nutrient Agar. It was seen that after 72 hours of incubation there was better growth on 10% Rock salt Nutrient agar plates. Total 100 cultures were revived on 10% Rocksalt Nutrient Agar with 2% Agar agar and Nutrient Agar (Fig 4).

Chlorpyrifos degrading 18 bacteria which were isolated from field of St. Cruz Bandh inlet from 2 different point in 2022 for the dissertation purpose, and stored in Marine Microbiology Laboratory refrigerator were used. (Vaigankar, 2022) They were transferred on Zobell Marine Agar plates. After incubation all 18 cultures were revived for further analysis (Fig 5).

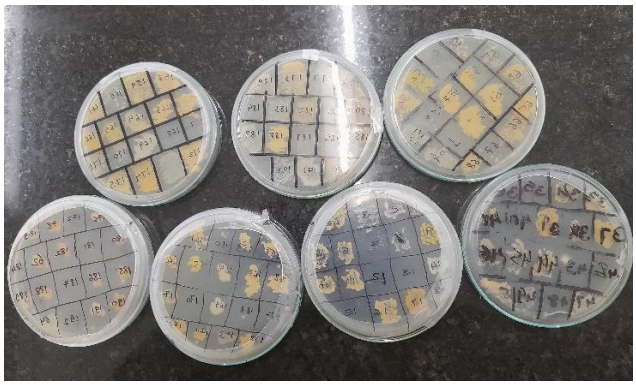


Fig 4: Salt pan cultures revived on 10% Rock salt Nutrient agar plates.

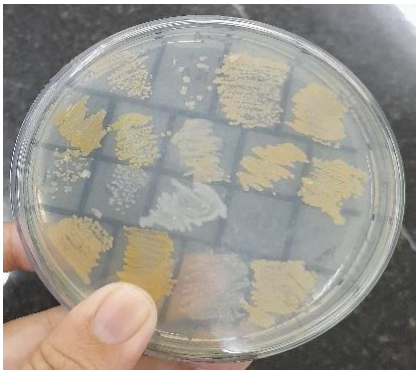


Fig 5: Mangrove cultures were recovered on Zobell Marine agar.

3.2 Screening for halophilic cultures:

After transferring 118 cultures on NaCl Tryptone Yeast Extract Agar plates, only 32 cultures showed positive result after incubation period of 168 hours. Only 23 cultures were able to grow in NaCl Tryptone Yeast Extract Broth after incubation period 168 hours (Fig 6).

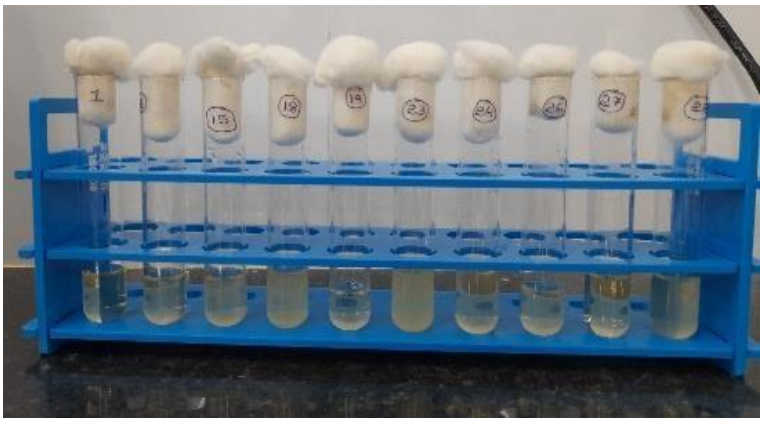


Fig 6: Cultures in NaCl Tryptone Yeast Extract Broth.

3.3 Salt tolerance test of cultures:

23 cultures were spot inoculated on different NaCl concentration Nutrient agar plates. The plates were incubated at 28°C ± 1°C and was checked after what time interval the cultures were grown. All the cultures grew on plates but after different time intervals (Table 1).

Table 1: Table showing hours required for cultures to grow at different NaCl concentrations.

Salt tolerance test (NaCl Concentration)	**5%**	**15%**	**25%**
Cultures	48 hours	96 hours	168 hours

3.4 Lysis test to confirm bacteria:

Milli Q water was used as blank. 21 cultures were bacteria, while 2 were archaea. It was seen that O.D for bacteria remained constant for 5minute while for archaebacteria O.D decreased which then reach 0. (Fig 7)

Fig 7: Auto Photo colorimeter used to differentiate between archaea and bacteria.

3.5 Synthesis of Nanoparticles using bacteria:

Supernatant and pellet suspension prepared by the method 2 (refer methods ana material 2.8.b) of each culture was used for analysis. Table 2 shows Colour differentiating after synthesis of nanoparticles.

There was no synthesis of nanoparticles at concentration of 10mM and 50mM used for any of cultures. The colour remained same as the control after incubation at 37°C for 48 hours. There was formation of nanoparticles at the concentration of 100mM given below in Table 3.

By using method 1 for synthesis of nanoparticles (refer methods and materials 2.8.a.), there was no change in colour of the solution containing 0.5mM of silver nitrate and cultures after incubation for 168 hours. While control remained unchanged. As shown in the Fig 8.

By using Method 2 (Refer methods and materials 2.8.b.)- The supernatant and pellet suspension were separated after centrifugation for 10 minutes at 5000 rpm. As shown in Fig 9.

There was no synthesis of Nanoparticles by using 0.5mM of 100mM of metal concentations. Control remained unchanged.

a b c

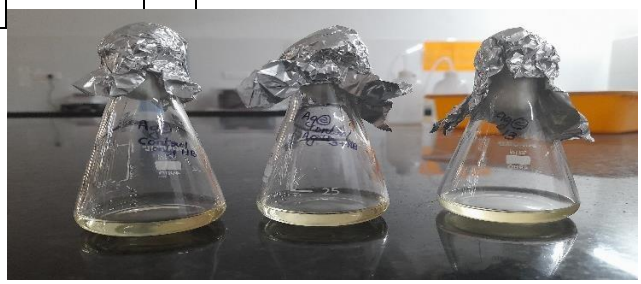


Fig 8: Result of Method 1 after incubation, a) Only 25% NaCl NB, b) 25% NaCl NB + 0.5mM AgNO3, c) 25% NaCl NB + 0.5mM AgNO3 + Culture

a b

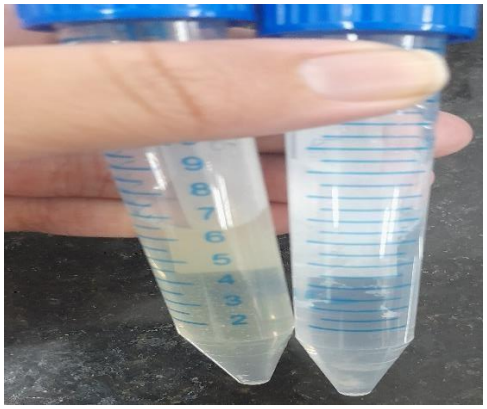


Fig 9: a) Supernatant, b) Pellet suspended in 10ml of Milli Q water.

Calculation for 0.5mM Silver Nitrate solution

100mM = 1000mL = 16.98gm

100mM=10mL=0.17gm X=

$$\frac{0.5mM}{100mM} \times 10\text{mL}$$

X= 50µL of silver nitrate solution from stock was added in each experimental flask.

Table 2: Colour differentiating after synthesis of nanoparticles.

Metal	Initial colour after addition of metal solution	After synthesis of Nanoparticles
Silver Nitrate	Cloudy	Deep brown
Lead Acetate Trihydrate	Cloudy	White
Copper Chloride	Blue	Green

3.6 Synthesis of Nanoparticles at different concentrations:

According to Appendix 3 metal solutions of different concentrations were prepared.

There was no synthesis of nanoparticles at 10mM and 50mM of metal concentrations. Only by using 100mM silver nitrate and lead acetate trihydrate nanoparticles were synthesised (Table 3).

Table 3: Number of cultures showing positive results using 100mM metal concentration.

Metal	Nanoparticles synthesised using Supernatant	Nanoparticles synthesised using Pellet suspension
Silver Nitrate(100mM)	0	14
Lead acetate trihydrate(100mM)	1	0
Copper chloride(100mM)	0	0

3.7 Characteristics of Nanoparticles:

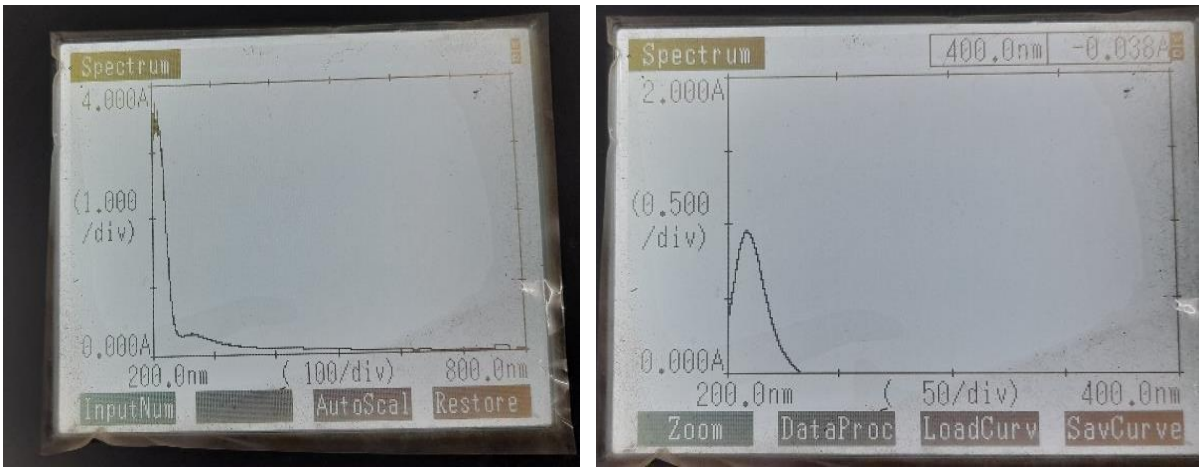
Spectrum of Nanoparticles-

The excitation spectra of the silver nanoparticles and Lead Nanoparticles dried powder which was suspended in distilled water was recorded in the range of 200-800 nm and 200-400 nm respectively using UV-Visible spectrophotometer with distilled water as the blank (Fig 10).

Only 3 nanoparticles showed proper spectrum. 2 silver nanoparticle and 1 lead nanoparticle. 2 silver nanoparticle had strong peak at 430nm and 1 lead nanoparticle had strong peak at 250nm. The selected nanoparticles will be further labelled as:

4 Ag, 8 Ag and 8 Pb.

Culture 8 could synthesise silver nanoparticles 4 Ag, as well as lead nanoparticles 8 Pb. Culture 4 could synthesise only silver nanoparticles 4 Ag.



a **b**

Fig 10: Spectrum of a) Silver nanoparticle in the range of 200-800 nm and b) Lead nanoparticles in the range of 200-400nm.

Weight of nanoparticles-

Nanoparticles were synthesised in 10mL of respective metal 100mM concentration solution.

4 Ag- 0.008g 8 Ag- 0.018g 8 Pb- 0.072g

Monochrome staining-

Monochrome staining of the 3 obtained nanoparticles were done to check if there is any bacterial residue remaining in them. There were no bacterial residue remains in the nanoparticles. The silver nanoparticles appeared dark violetish blackish colour after staining while lead nanoparticles appeared violet in colour (Fig 11).

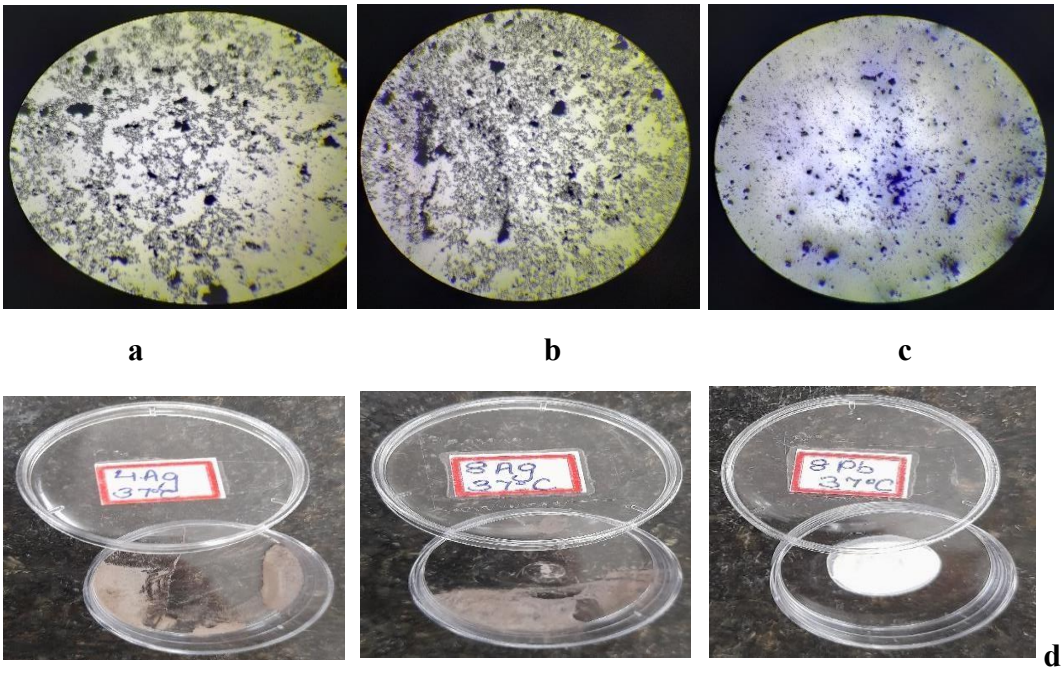
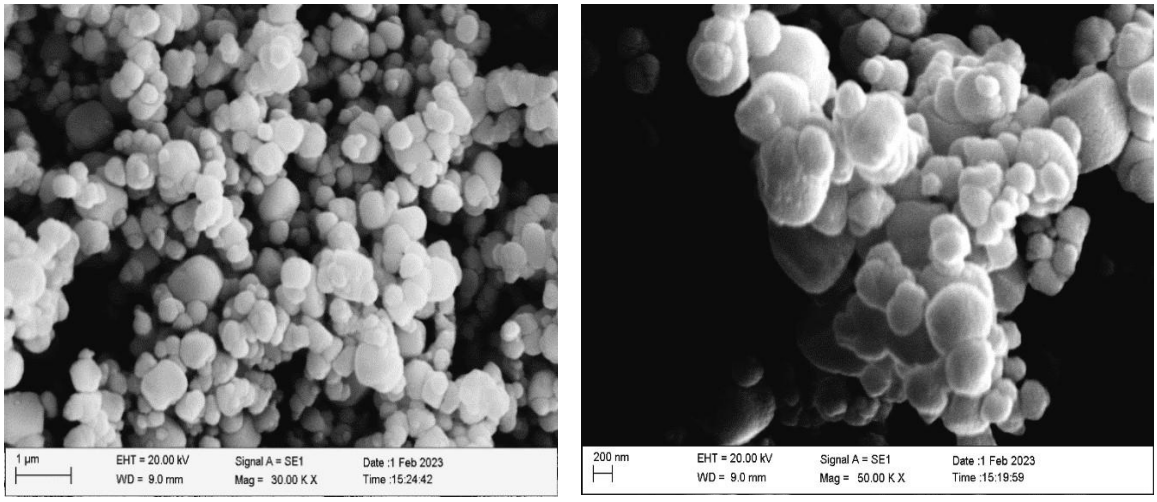


Fig 11: Observed under microscope, stained using monochrome staining a) 4 Ag nanoparticles, b) 8 Ag nanoparticles, c) 8 Pb nanoparticles ; d) Dried 4 Ag nanoparticles, e) Dried 8 Ag nanoparticle, f) Dried 8 Pb nanoparticles.

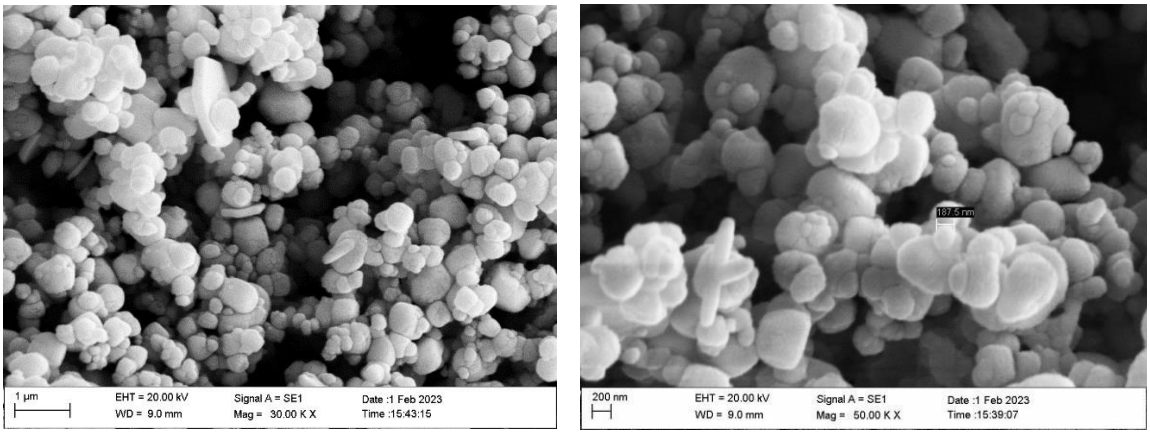
SEM analysis-

The 3 nanoparticles were seen under Scanning electron microscope. The size of nanoparticles was found to be approx. 200nm. The silver nanoparticles appeared spherical shaped while lead nanoparticles were in hemi-spherical shape (Fig 12, 13,14).



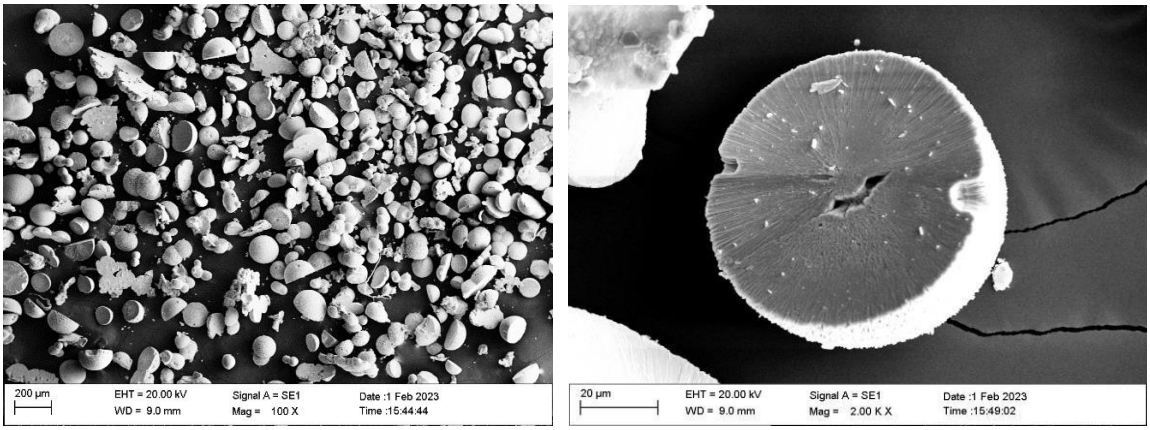
a **b**

Fig 12a and b: 4 Ag nanoparticles seen under SEM was magnified at 30.00 K X and 50.00 K X. The nanoparticles are spherical shaped.



a **b**

Fig 13 a and b: 8 Ag nanoparticles seen under SEM was magnified at 30.00 K X and 50.00 K X. The nanoparticles are spherical shaped.



a **b**

Fig 14a and b: 8 Pb nanoparticles seen under SEM was magnified at 100 X and 2.00 K X. The nanoparticles are hemi-spherical shaped.

FTIR analysis-

The 4 Ag nanoparticle showed peaks in the range of 500-4000 cm^{-1} wavenumber. The 4 Ag were observed in the presence of bands due to O-H stretching around 3400 cm^{-1}, aldehyde stretching around 2900 cm^{-1} , -C=C- (cis) stretching around 1650 cm^{-1} . FTIR analysis confirms the presence of O-H stretching which is responsible for reducing metal ions into nanoparticles (Fig 15).

The 8 Ag nanoparticle showed peaks in the range of 500-4000 cm^{-1} wavenumber. The 8 Ag were observed in the presence of bands due to O-H stretching around 3400 cm^{-1}, =C-H (cis) bending around 1400 cm^{-1} , cis-CH=CH- bending out of plane around 600 cm^{-1} . FTIR analysis confirms the presence of O-H stretching which is responsible for reducing metal ions into nanoparticles (Fig 16)

The 8 Pb nanoparticle showed peaks in the range of 500-4000 cm^{-1} wavenumber. At approx 3900 cm^{-1} the transmittance was highest. The peaks were not proper due to constant over lapping of the peaks. The problem behind over lapping of the peaks is caused by similar stretching of vibration mode from different compounds or same identicle peaks from isomers (Fig 17).

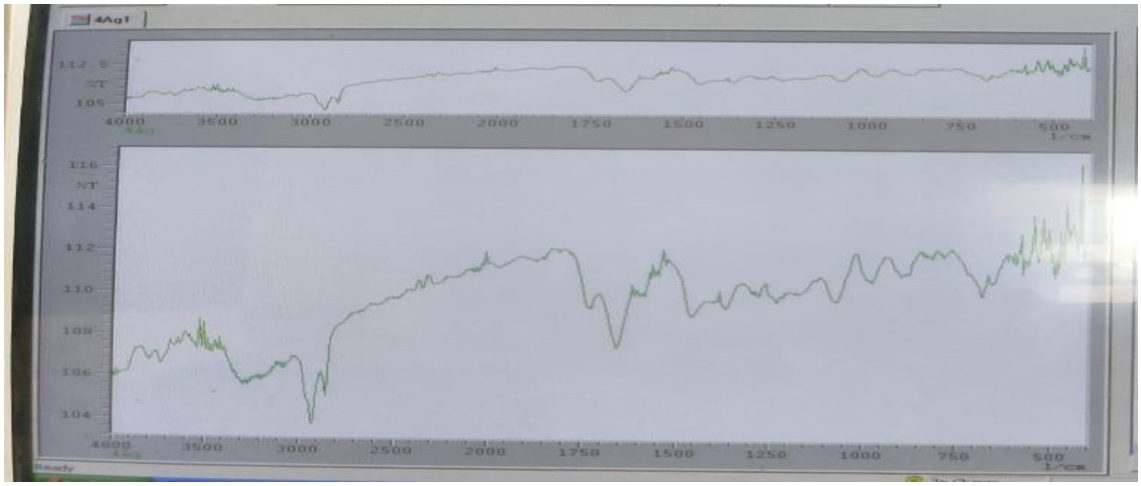


Fig 15: FTIR spectrum of 4 Ag nanoparticles.

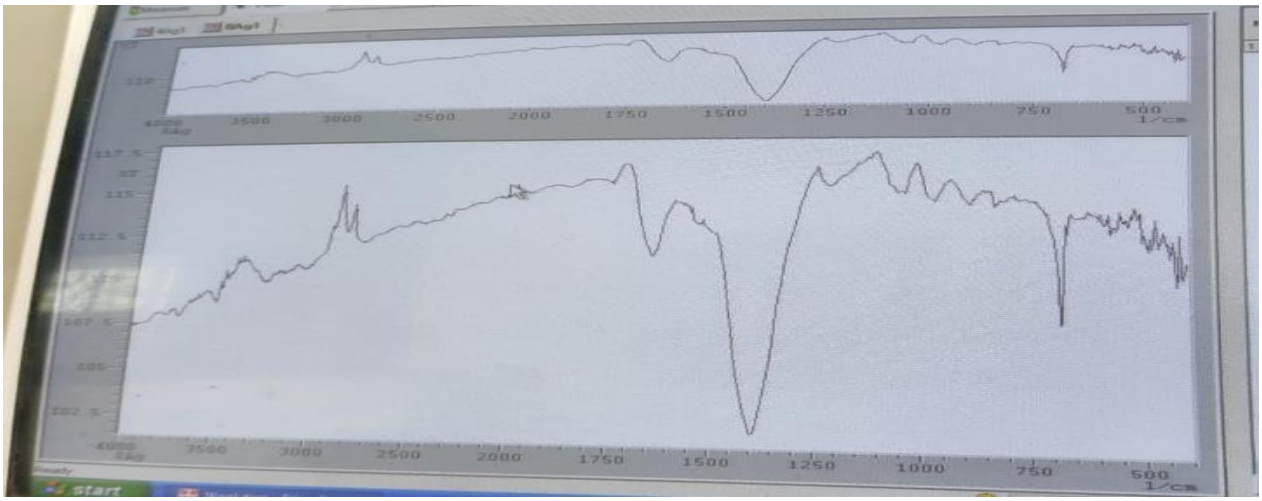


Fig 16: FTIR spectrum of 8 Ag nanoparticles.

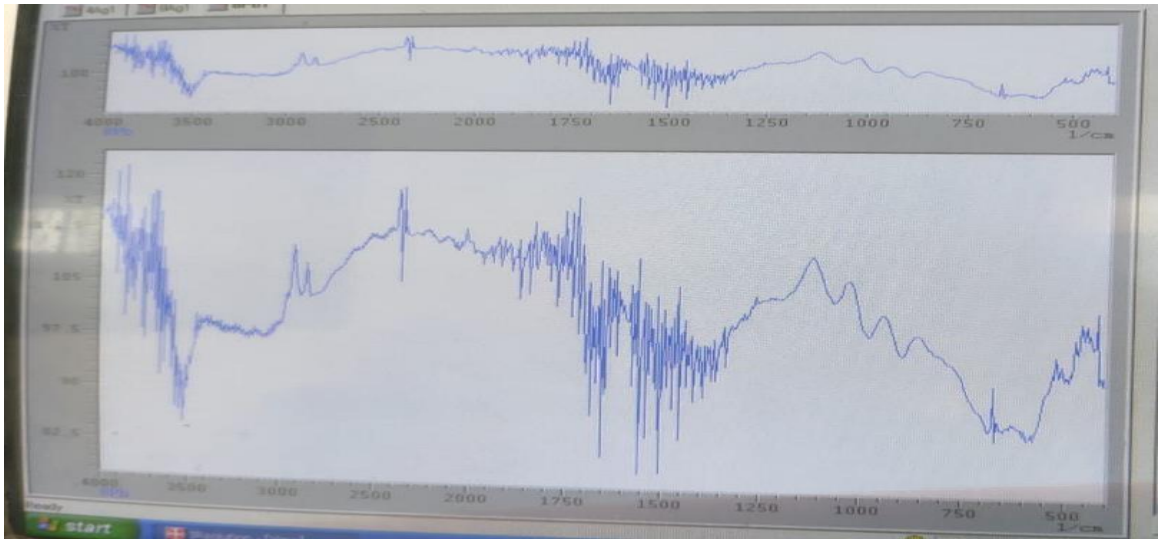


Fig 17: FTIR spectrum of 8 Pb nanoparticles.

3.8 Optimisation of nanoparticles by bacteria:

Effect of temperature on synthesis of nanoparticles-

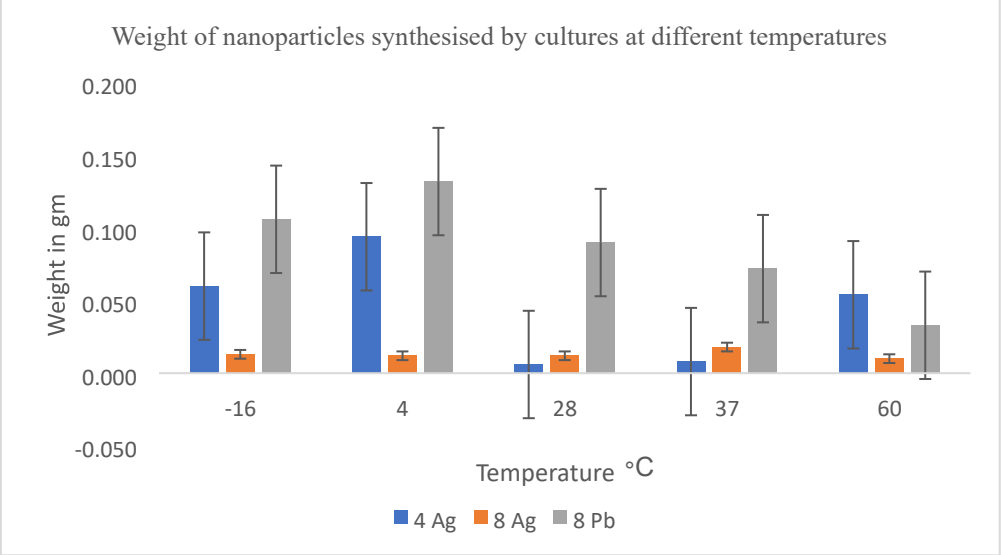
The solutions were kept for incubation at different temperatures for 48 hours. Absorbance of silver nanoparticles(4 Ag and 8 Ag) were recorded at 430 nm wavelenght, while absorbance of lead nanoparticles(8 Pb) were recorded at 250nm wavelenght.

It was seen that the optimum temperature for synthesising of nanoparticles by incubation of solution at 4°C. Also as the temperature for incubation was raised the quantity also decreased (Table 4 and Graph 1).

Table 4: Effect of different temperature on synthesis of nanoparticles, showing weight and absorbance.

TEMP→ Nanoparticle↓	-16°C		4°C		28± 2°C		37°C		60°C	
	Wt in gm	Abs	Wt in gm	Abs	Wt in gm	Abs	Wt in gm	Abs	Wt in gm	Abs
Ag 4	0.060	0.308	0.094	0.328	0.006	0.130	0.008	0.121	0.054	0.314
Ag 8	0.013	0.185	0.012	0.171	0.012	0.311	0.018	0.171	0.01	0.184
Pb 8	0.106	0.216	0.132	0.245	0.090	0.294	0.072	0.214	0.033	0.212

Graph 1: Comparison of weight of nanoparticles synthesised by cultures at different temperatures.



Effect of pH on nanoparticles synthesis-

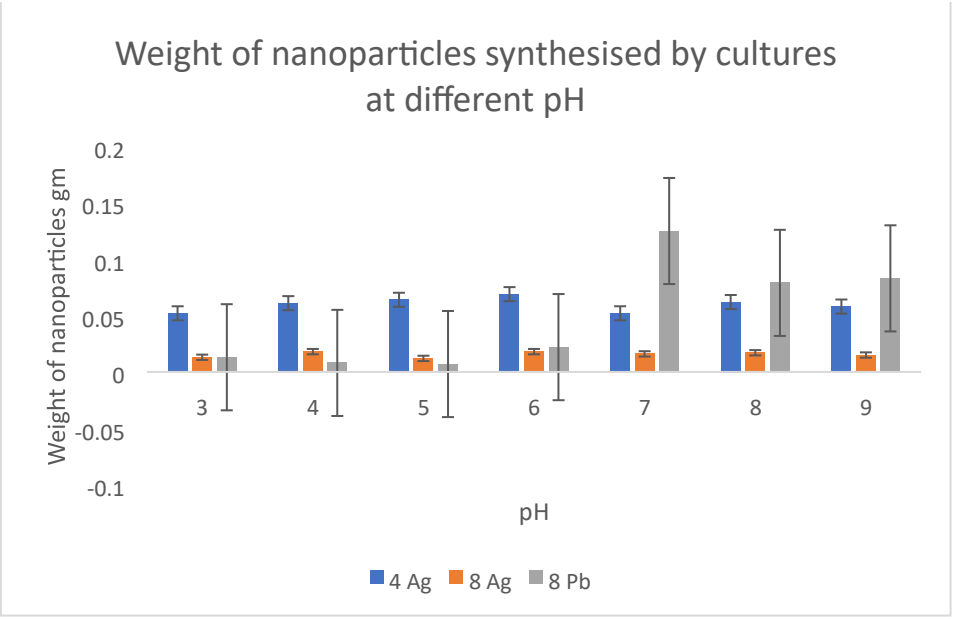
The nanoparticle synthesis solutions were prepared in different pH solutions. Absorbance of silver nanoparticles (4 Ag and 8 Ag) were recorded at 430 nm wavelength, while absorbance of lead nanoparticles (8 Pb) was recorded at 250nm wavelength.

It was seen optimum pH for the synthesis of nanoparticle was at pH 7. As the pH decreased the amount of nanoparticles synthesised decreased (Table 5 and Graph 2).

Table 5: Effect of different pH on synthesis of nanoparticles, showing weight and absorbance.

Nanoparticle → pH ↓		4 Ag	8 Ag	8 Pb
3	**Wt in gm**	0.052	0.013	0.013
	Abs	1.204	0.722	0.128
4	**Wt in gm**	0.061	0.018	0.008
	Abs	1.206	0.622	0.122
5	**Wt in gm**	0.064	0.012	0.007
	Abs	1.242	0.621	0.118
6	**Wt in gm**	0.069	0.018	0.022
	Abs	1.110	0.444	0.275
7	**Wt in gm**	0.052	0.016	0.125
	Abs	0.121	0.628	0.414
8	**Wt in gm**	0.062	0.017	0.079
	Abs	1.928	0.545	0.542
9	**Wt in gm**	0.058	0.015	0.083
	Abs	0.123	0.456	0.462

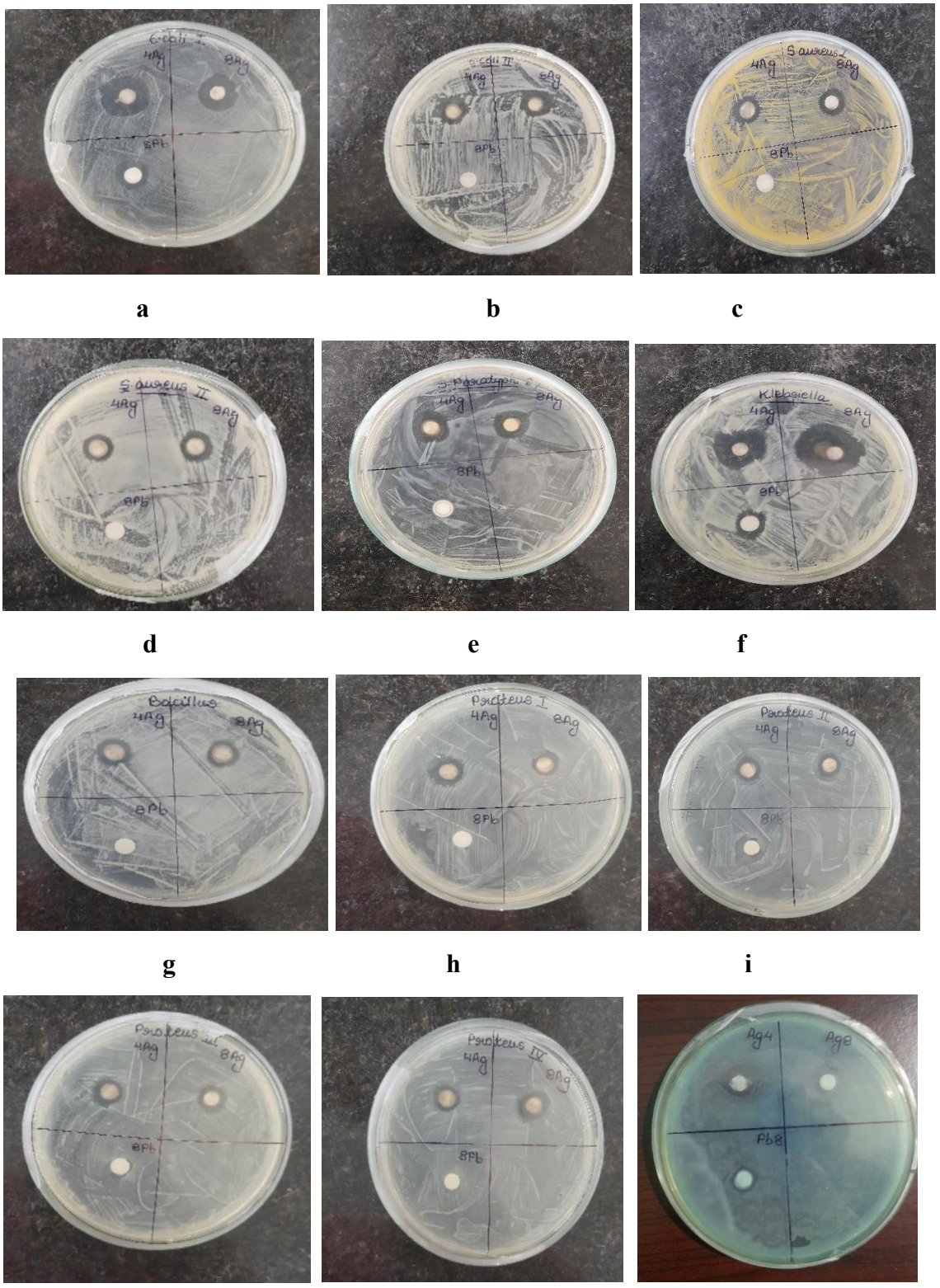
Graph 2: Comparison of weight of nanoparticles synthesised by cultures at different pH.



3.9 Anti- bacterial activity of Nanoparticles:

Nutrient agar plates were prepared. The pure bacterial cultures were inoculated in nutrient broth incubated for 4 hours and spread plate on agar plates. 10mg of nanoparticle was suspended in 1ml of distilled water and used. Disc with 6mm diameter were dipped in suspension and placed on agar plates.Incubated for 24 hours and zone of inhibition of bacterial by nanoparticles were recorded then compared (Fig 18 and Table 6).

It was seen that, nanoparticles could inhibit the growth of *Klebsiella* spp at a greater efficiency, while nanoparticles could inhibit the growth of *E. coli* 1 at a less extend (Graph 3)



a b c

d e f

g h i

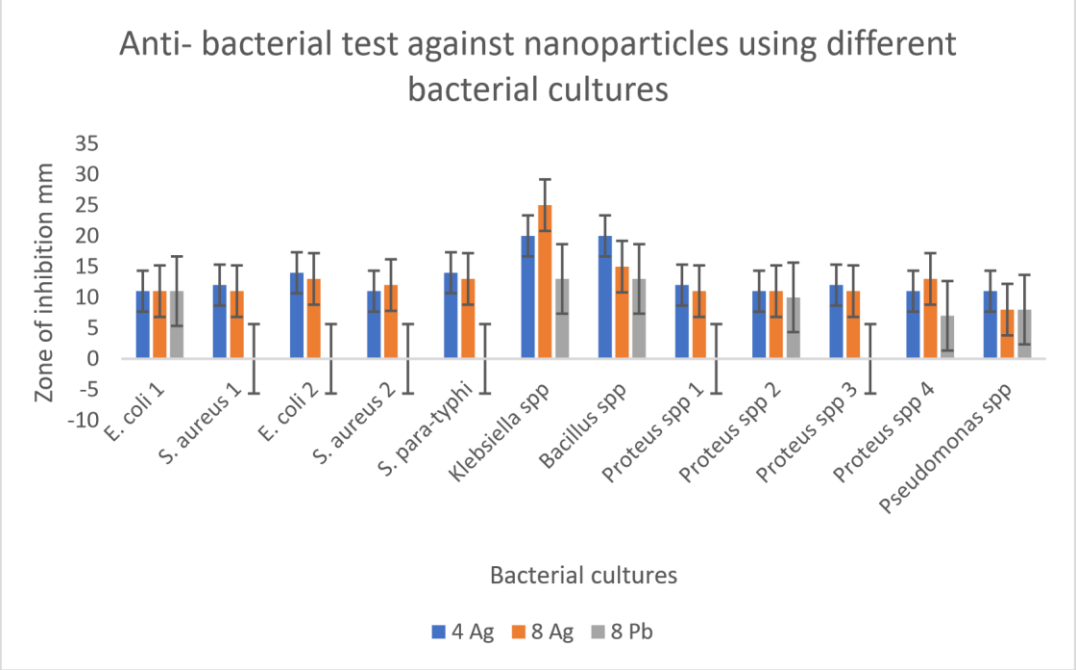
j k l

Fig 18: Nanoparticles tested against following cultures a) *E. coli* 1, b) *S. aureus* 1, c) *E. coli* 2, d) *S. aureus* 2, e) *S. paratyphi*, f) *Klebsiella* spp, g) *Bacillus* spp, h) *Proteus* spp 1, i) *Proteus* spp 2, j) *Proteus* spp 3 k) *Proteus* spp 4, l) *Pseudomonas* spp.

Table 6: Anti- bacterial activity of Nanoparticles using different pure cultures.

Nanoparticles→ Bacteria↓	4 Ag Zone of inhibition mm	8 Ag Zone of inhibition mm	8 Pb Zone of inhibition mm
E. coli 1	11	11	11
S. aureus 1	12	11	-
E. coli 2	14	13	-
S. aureus 2	11	12	-
S. para-typhi	14	13	-
Klebsiella spp	20	25	13
Bacillus spp	20	15	13
Proteus spp 1	12	11	-
Proteus spp 2	11	11	10
Proteus spp 3	12	11	-
Proteus spp 4	11	13	7
Pseudomonas spp	11	8	8

Graph 3: Anti- bacterial test of nanoparticles using different bacterial cultures.



3.10 Anti- fungal test of nanoparticles:

Malt exact agar plates were prepared with Ampicillin. The pure *S. cerevisiae* culture was inoculated in malt extract broth incubated for 4 hours and spread plate on agar plates. While *Penicillium* spp and *Aspergillus* spp was inoculated in normal saline and spread plate on prepared plates. 10mg of nanoparticles was suspended in 1ml of distilled water and used. Disc with 6mm diameter were dipped in suspension and placed on agar plates. Incubated for 48 hours and zone of inhibition of fungul culture by nanoparticles were recorded then compared (Fig 19 and Table 7).

Silver nanoparticles nanoparticles are more effective against *Penicillium* spp while less effective against *Aspergillus* spp. S. cerevisiae had intermediate effect. While lead nanoparticles had no effect against any fungal culture (Graph 4).

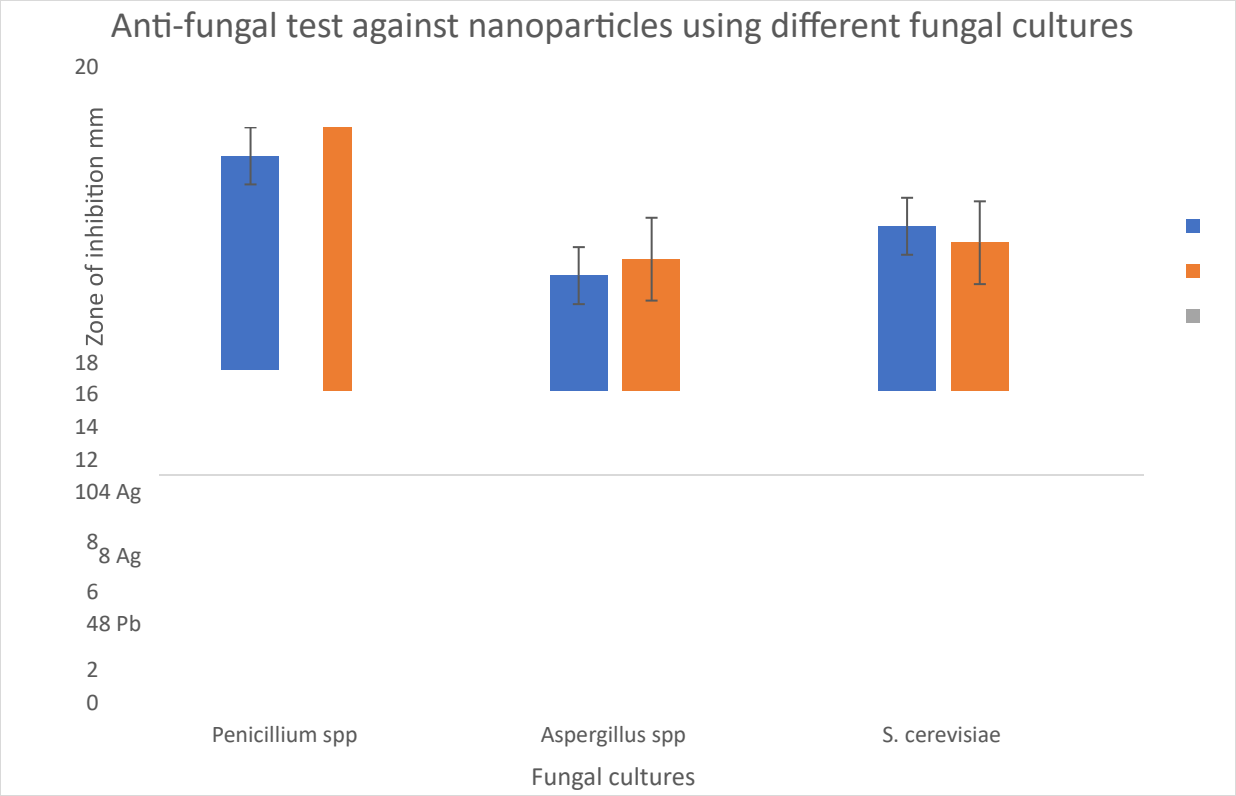
a b c

Fig 19: Nanoparticles tested against following fungal cultures a) *Penicillium* spp, b) *Aspergillus* spp c) *S. cerevisiae.*

Table 7: Anti-fungal test of nanoparticles using different pure fungal cultures.

Nanoparticle→ Fungi↓	**4 Ag Zone of inhibition mm**	**8 Ag Zone of inhibition mm**	**8 Pb Zone of inhibition mm**
Penicillium spp	13	16	-
Aspergillus spp	7	8	-
S. cerevisiae	10	9	-

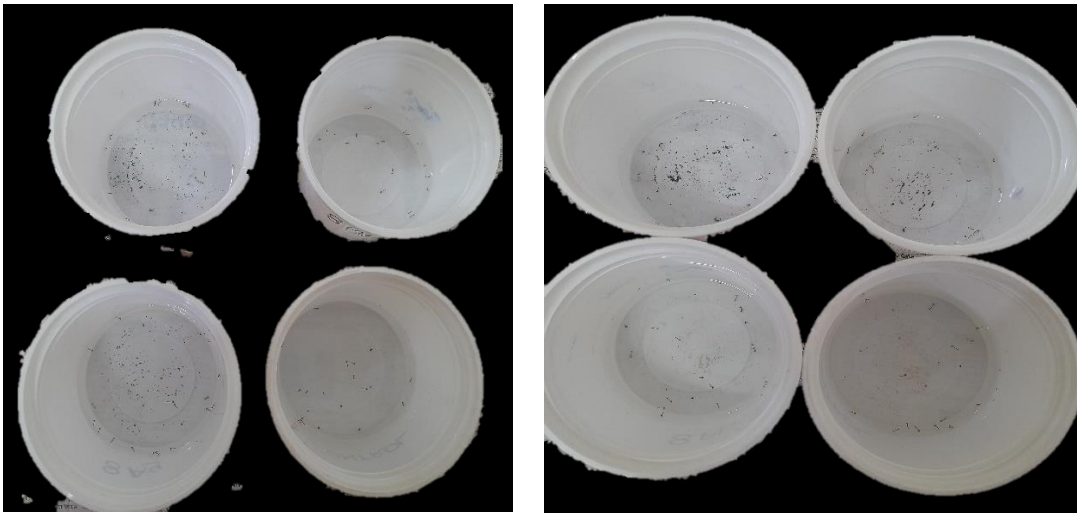
Graph 4: Nanoparticles tested of fungal cultures.



3.11 Cytotoxicity of mosquito larvae:

In control there was death of larvae because of natural death or starvation. 4 Ag nanoparticle had 100% after addition in water containing larvae. Above 20% Mortality in control is considered correct results (Table 8). *Culex quinquefasciatus* mosquito larvae were used which were at late 3rd to 4th instar larval stage (Fig 20,21).

Fig 20: Day 0, Experimental containers containing healthy larvae and 10mg nanoparticles, while control container with only healthy larvae.



a **b**

Fig 21: a) Day 1, after 24 hours ; b) Day 2, after 48 hours.

Calculations of nanoparticles to be added and mortality percentage.

100ppm of nanoparticle

$$\text{Ppm} = \frac{mass\ of\ solute\ mg}{mass\ of\ solvent\ mg} \times 10^6$$

$$100\text{ppm} = \frac{a}{100} \times 10^6$$

a = 10mg in 100ml of RO water

$$\text{Mortality \%} = \frac{dead\ larvae\ after\ 48\ hours}{total\ larvae} \times 100\ \%$$

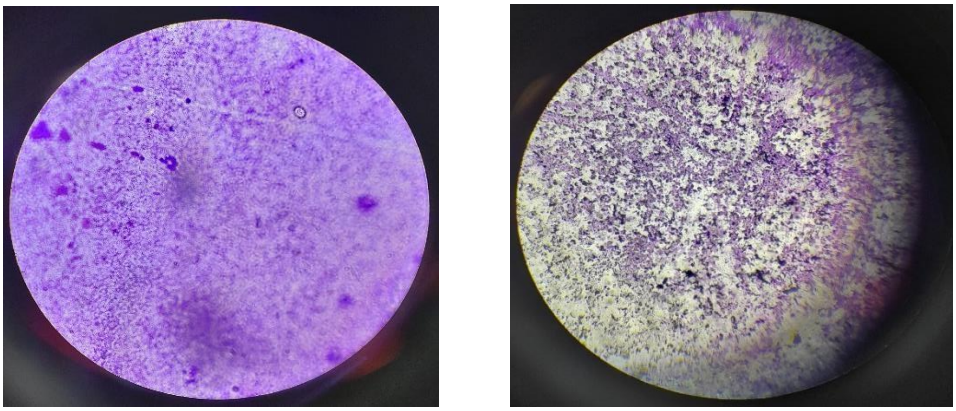
Table 8: Mortality of the mosquito larvae after introduction of 10mg nanoparticles.

Sample	Total larvae	Dead larvae			Mortality %
		After 1 hour	After 24 hours	After 48 hours	
4 Ag	20	1	13	20	100%
8 Ag	20	0	2	12	60%

8 Pb	20	0	2	9	45%
Control	20	0	6	6	30%

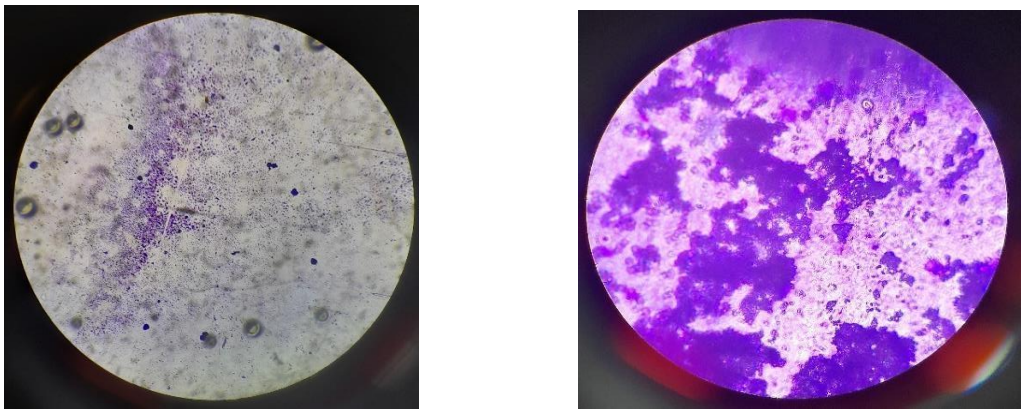
3.12 Inhibition of Biofilm Formed Using nanoparticles:

On Day 2 and Day 3 the coverslips were stained and seen under microscope. It was seen that in the petriplate where nanoparticles were present there was inhibition of biofilm. Control had formation of biofilm (Fig 22,23).



a **b**

Fig 22: a) Control - *Pseudomonas* spp after monochrome staining ; b) *Pseudomonas* spp treated with 4 Ag.



a **b**

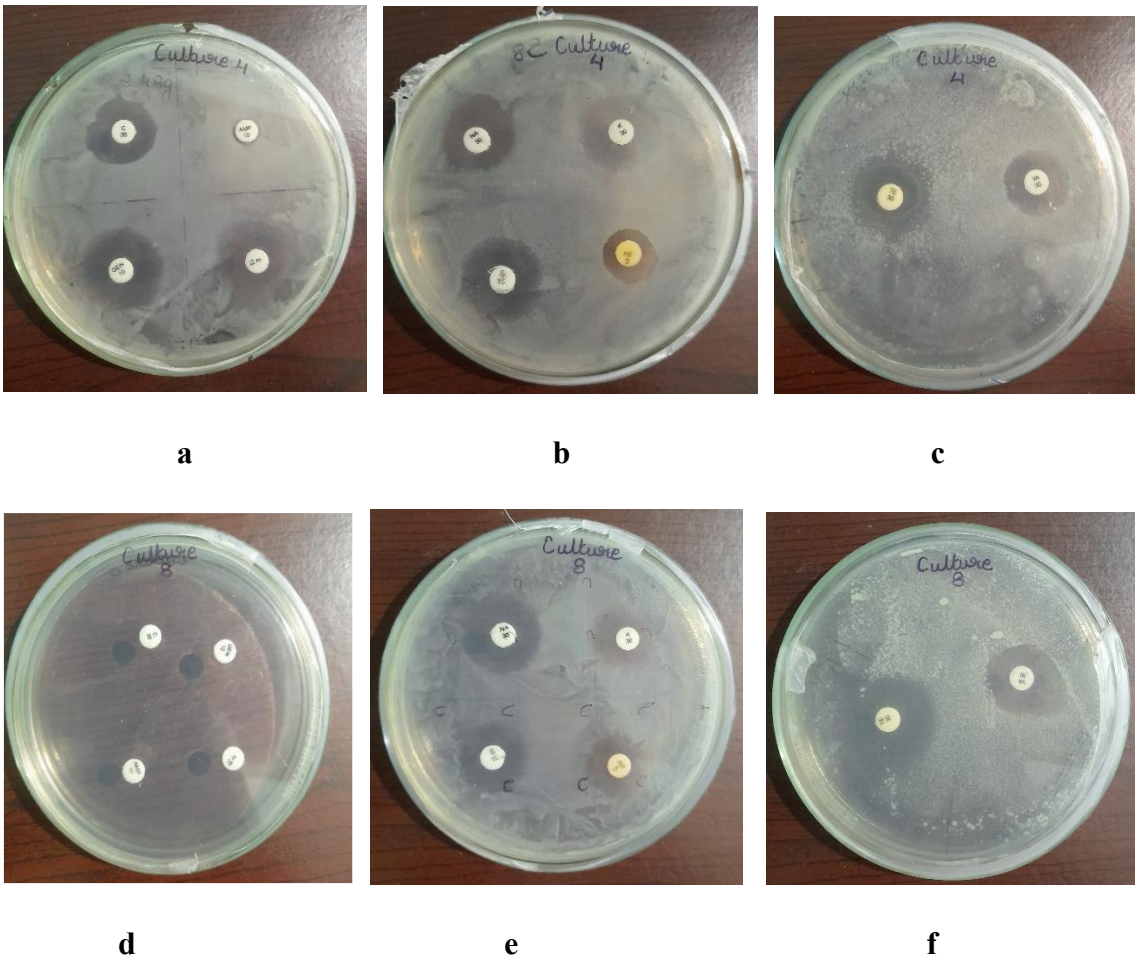
Fig 23: a) *Pseudomonas* spp treated with 8 Ag ; b) *Pseudomonas* spp treated with 8 Pb.

3.13 Antibiotic test against cultures which can synthesise nanoparticles:

The cultures were grown in Nutrient broth incubated at 28°C ± 1°C for 24 hours.

100µL of the culture was spread on Nutrient agar plates.The sterile antibiotic discs were placed on the agar plates. Incubated for 24 hours and zones of inhibition were recorded. The antibiotic discs were of HiMedia (Fig 24 and Table 9).

Ampicillin was most resistant against both the cultures, while tetracycline was most sensitive against both cultures (Graph 5).



a **b** **c**

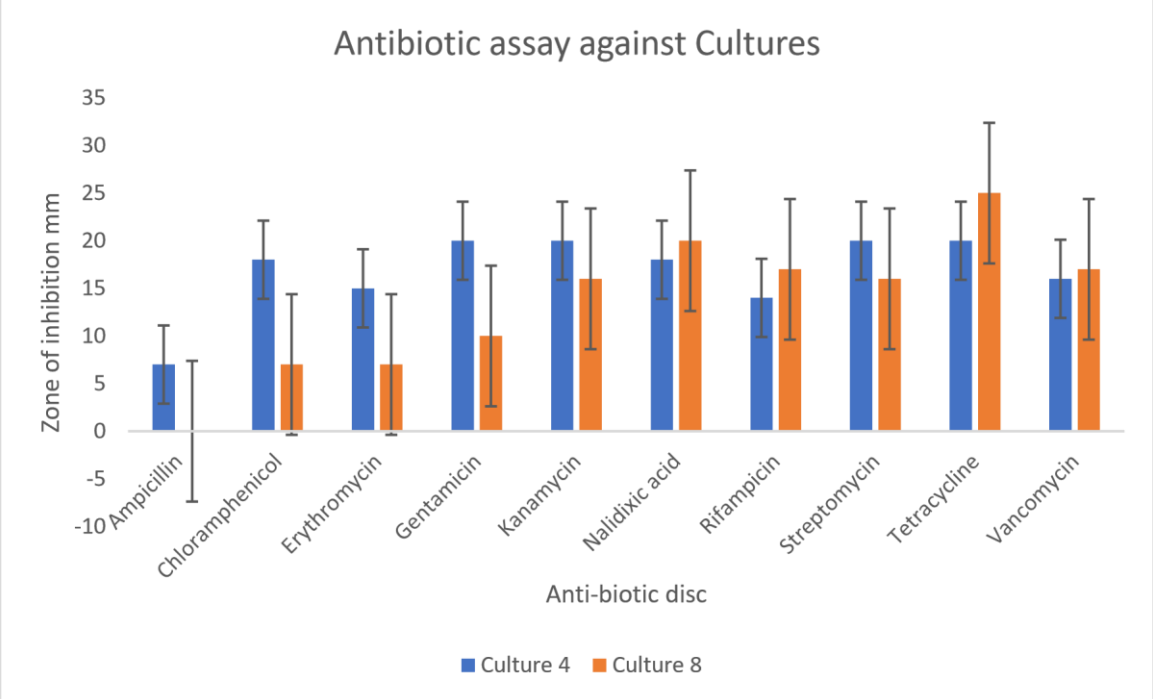
d **e** **f**

Fig 24: Anti-biotic assay against culture which can synthesise nanoparticles. a,b,c) Culture 4 ; d,e,f) Culture 8

Table 9: Cultures with effect against Antibiotic discs.

Culture→ Antibiotic disk↓	Zone of inhibition mm			
	Culture 4		Culture 8	
Ampicillin	7	Resistant	-	Resistant
Chloramphenicol	18	Intermediate	7	Resistant
Erythromycin	15	Intermediate	7	Resistant
Gentamicin	20	Sensitive	10	Resistant
Kanamycin	20	Sensitive	16	Intermediate
Nalidixic acid	18	Not known	20	Not known
Rifampicin	14	Not known	17	Not known
Streptomycin	20	Sensitive	16	Sensitive
Tetracycline	20	Sensitive	25	Sensitive
Vancomycin	16	Intermediate	17	Sensitive

Graph 5: Cultures tested against Antibiotic disc



3.14 Gram Staining of selected 14 cultures

The cultures which could synthesise nanoparticles were Gram Negative rods and Gram Positive cocci (Table 10).

4 Ag- Gram Negative rods

8 Ag and 8 Pb- Gram Positive cocci

Table 10: Gram character of selected 14 cultures.

Culture	Gram character
1	Gram Positive rods
2	Gram Negative cocci
3	Gram Negative cocci
4	Gram Negative rods
5	Gram Positive rods
6	Gram Negative cocci
7	Gram Negative cocci
8	Gram Positive cocci
9	Gram Negative rods
10	Gram Positive rods
11	Gram Positive cocci
12	Gram Negative rods
13	Gram Positive rods
14	Gram Negative cocci

CHAPTER 6
DISCUSSION

There are very few reports on nanoparticles extracted from halophilic bacteria. In our study most selected bacteria could synthesise silver nanoparticles. While lead nanoparticles were produced by very few halophilic bacteria. None of the selected bacteria could produce copper nanoparticles. One of the reason for cultures not producing copper nanoparticles as, they were not able to capture copper to convert into free metal ions. Many studies were done on silver nanoparticles as compared to lead nanoparticles. Weight of nanoparticles attatained by us was approx 0.01gm. Saleh *et al*. (2020) in their study had synthesised silver nanoparticles extracellularly from the supernatant, while in our study we have synthesised silver nanoparticles intracellularly using pellet suspension while lead nanoparticles were extracted extracellularly using supernatant. In most of the papers reported silver nanoparticles are

synthesised extracellularly, but in our paper its intracellularly because of the enzymes present inside the cells were capable to reduce metals into free nanoparticles.

Characteristics of nanoparticles studied by Saleh *et al*,(2020) had similar results that of our results. SEM images of silver nanoparticles showed spherical shape while lead nanoparticles were hemi-spherical shape. Very few studies were done on lead nanoparticles characteristics. The FTIR ananlysis had similar results for silver nanoparticles as studied by Kumari *et al,.* (2013) which had peak at O-H stretching responsible for reducing metal ions into nanoparticles. Lead nanoparticles FTIR results were not that proper because the peaks were overlapping one another, as they had similar functional groups.

Srivastava *et al*, (2013) in their study, they tested silver nanoparticles against very few bacterial strains, while we have tested silver as well as lead nanoparticles against many bacterial and fungal strains. The disc diffussion method for anti microbial test is widely accepted test compared to well diffussion method. Even in Srivastava *et al*, (2013), study she could produce nanoparticles by incubating cultures and metals together but in our study we couldn't achieve because the cultures were incubated in static position and metal concentration was not enough for synthesis of nanoparticles by bacteria as compared to archae studied by them.

Shamseldean *et al*,. (2022) in their study, efficacy of nanoparticles were seen to control mosquito, *Culex quinquefasciatus.* Only silver nanoparticles and gold nanoparticles results has been updated. In our study we have studied effect of silver as well as lead nanoparticles on late 3^{rd} to 4^{th} instar larval stage of mosquito *Culex quinquefasciatus.*

Biofilm inhibition by nanoparticles is hardly studied. The action of nanoparticles on biofilm was studied by us.

Silver concentration in groundwaters and surface waters is generally below 2µg/L. The limited concentration for silver metals in waters should be less then 0.1mg/L.

The limited concentration for lead metal in water should be less then 10µg/L.

The limited concentration of silver and lead is granted by WHO.

CHAPTER 7
CONCLUSIONS

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This study was concluded to synthesise nanoparticles from halophilic bacteria. Here we have synthesised silver and lead nanoparticles successfully.

- Silver nanoparticles was synthesised intracellularly from 2 bacteria using pellet suspension (4 Ag and 8 Ag). Lead nanoparticles was synthesised extracellularly from 1 bacteria using supernatant (8 Pb). It was seen that 1 culture could synthesise both the nanoparticles (8 Ag and 8 Pb).
- The characteristics using SEM, UV-Vis spectroscopy and FTIR were studied. The effect of temperature, pH and varying metal concentration was also studied having highest activity.
- Nanoparticles due to their nanoscale and having distinguishing properties can be used in many fields. 3 application of the nanoparticles were studies.
- Silver nanoparticles were effective against Gram negative bacteria, Gram- positive bacteria and fungal strains as compared to the lead nanoparticles.
- Silver and lead nanoparticles could kill the mosquito larvae *Culex quinquefasciatus* at late 3rd to 4th instar larval stage.
- Silver and lead nanoparticles can also inhibit the biofilm formed by *Pseudomonas* spp.
- Anti- biotic test against 4^{th} and 8^{th} culture was done to characterise the halophilic organisms.

- As compared to lead nanoparticles silver nanoparticles are highly effective and can be used in many fields.

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APPENDIX

APPENDIX 1 Media Composition

1. Nutrient Agar HiMedia

Ingredients	**gm/L**
Peptone	5
Yeast extract	3
Sodium chloride	5
Agar	15
Distilled water	1000mL
pH	6.8-7.2

2. Zobell Marine Agar HiMedia

Ingredients	**gm/L**
Peptone	5
Yeast extract	1
Ferric citrate	0.100
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.240
Calcium chloride	1.800
Potassium chloride	0.550
Sodium bicarbonate	0.160
Potassium bromide	0.080
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016

Disodium phosphate	0.008
Agar	15
Distilled water	1000mL
pH	7.5-7.77

3. NaCl Tryptone Yeast Extract Agar

Ingredients	**gm/100 mL**
NaCl	25
Tryptone	0.5
Yeast Extract	0.3
Agar Agar	2.5
Potassium chloride	0.5
Magnesium sulphate heptahydrate	2
Distilled water	100mL

4. Malt Extract Agar HiMedia

Ingredients	**gm/L**
Malt extract	20
Glucose	20
Peptone	1
Agar	20
Distilled water	1000mL
pH	5.4

5. Saline

Ingredients	**gm/L**
NaCl	0.85
Distilled water	1000mL

APPENDIX 2 Metals

Metal	Molecular weight
Silver nitrate	169.8gm
Lead acetate trihydrate	379.33gm
Copper chloride	170.48gm

APPENDIX 3 Preparation of metal solution

Silver Nitrate 10mM → 10mM = 100mL = 0.17gm in 100mL of Milli Q water

Silver Nitrate 50mM → 50mM = 100mL = 0.85gm in 100mL of Milli Q water

Silver Nitrate 100mM → 100mM = 100mL = 1.7gm in 100mL of Milli Q water

Lead acetate trihydrate 10mM → 10mM = 100mL = 0.38gm in 100mL of Milli Q water

Lead acetate trihydrate 50mM → 50mM = 100mL = 1.9gm in 100mL of Milli Q water

Lead acetate trihydrate 100mM → 100mM = 100mL = 3.8gm in 100mL of Milli Q water

Copper Chloride 10mM → 10mM = 100mL = 0.17gm in 100mL of Milli Q water

Copper Chloride 50mM → 50mM = 100mL = 0.85gm in 100mL of Milli Q water

Copper Chloride 100mM → 100mM = 100mL = 1.7gm in 100mL of Milli Q water

APPENDIX 4 Monochrome stain and Gram staining reagents

1. Crystal Violet HiMedia

Ingredients	Quantity
Ammonium oxalate	8gm
Crystal violet	10gm

Alcohol	100mL
Distilled water	900mL

2. Gram's Iodine

Ingredients	**Quantity**
Iodine	1gm
Potassium iodide	2gm
Distilled water	300mL

3. Decolorizer

Ingredients	**Quantity**
Ethanol	95mL
Distilled water	5mL

4. Saffranin

Ingredients	**Quantity**
Safranin powder	20mg
Distilled water	20mL

APPENDIX 5 Water

- RO water
- Milli Q water

• Distilled water APPENDIX 6 Antibiotic assay standardise chart

Name of antibiotics (dose)	Inhibitory zone diameter to nearest millimeter (mm)		
	Sensitive (S)	Moderately sensitive (MS)	Resistant (R)
Amoxicillin (30 μg/disk)	≥18	14–17	≤13
Cloxacillin (5 μg/disk)	≥25	22–24	≤21
Cephalothin (30 μg/disk)	≥18	15–17	≤14
Cephradine (25 μg/disk)	≥18	13–17	≤12
Cefuroxime (30 μg/disk)	≥23	15–22	≤14
Cefixime (5 μg/disk)	≥19	16–18	≤15
Kanamycin (30 μg/disk)	≥18	14–17	≤13
Streptomycin (10 μg/disk)	≥15	12–14	≤11
Neomycin (30 μg/disk)	≥17	13–16	≤12
Vancomycin (30 μg/disk)	≥12	10–11	≤9
Erythromycin (15 μg/disk)	≥23	14–22	≤13
Azithromycin (15 μg/disk)	≥18	14–17	≤13
Ciprofloxacin (15 μg/disk)	≥21	16–20	≤15
Levofloxacin (5 μg/disk)	≥17	14–16	≤13
Tetracycline (30 μg/disk)	≥15	12–14	≤11
Doxycycline (30 μg/disk)	≥14	11–13	≤10
Cotrimoxazole (25 μg/disk)	≥16	11–15	≤10
Chloramphenicol (30 μg/disk)	≥18	13–17	≤12

Antibiotics	Disc content	Diameter of zone of inhibition (mm)		
		Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Neomycin	30 mcg	12	13-16	17
Gentamicin	10 mcg	12	13-14	15
Vancomycin	30 mcg	14	15-16	17
Ampicillin	10 mcg	13	14-16	17
Bacitracin	10 units	8	9-12	13
Erythromycin	15 mcg	13	14-22	23
Penicillin G	10 units	14	--	15
Streptomycin	10 mcg	11	12-14	15
Chloramphenicol	30 mcg	12	13-17	18