

BIOREMOVAL AND BIOREMEDIATION OF HEAVY METALS BY METAL-RESISTANT BACTERIA ISOLATED FROM HEAVY METAL-CONTAMINATED SITES

Submitted in partial fulfilment for the award of the degree of
Bachelor of technology

By

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**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE A UNIVERSITY)**

Accredited with Grade "A++" by NAAC

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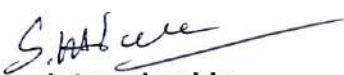
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of Priya Dharshini. A (Reg. No. 40230035) who carried out the project "**BIOREMOVAL AND BIOREMEDIATION OF HEAVY METALS BY METAL RESISTANT BACTERIA ISOLATED FROM HEAVYMETAL CONTAMINATED SITES**" under our supervision from August-2023 to April-2024.


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I, Priya Dharshini.A hereby declare that the Project Report entitled “**Bio removal and Bioremediation of Heavy Metal by Metal Resistant Bacteria Isolated from Heavy Metal Contaminated Sites**” done by me under the guidance of Dr S Usha Nandhini (Internal) and Dr Stalin dhas.T (External) at Sathyabama Institute of Science and Technology, Chennai, Tamil Nadu, India, is submitted in partial fulfilment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

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ABSTRACT

An increase in heavy metals ruins the ecosystem by accumulating and interfering with the food chain. The accumulation of toxic metals like mercury (Hg), cadmium (Cd), selenium (Se), and arsenic (As) causes potential threats to both aquatic and terrestrial environments. These metals invade aquatic environments by leaching the bodies of water bodies through mining operations, petroleum refineries, improper municipal waste disposal, and natural mineral deposits, and the presence of these toxic substances makes them importable for both human and animal consumption and living. Metal biosorption by bacteria has received significant attention due to the safe, productive, and feasible technology for heavy metal-containing wastewater treatment. These metal-tolerating bacteria can bind the cationic toxic heavy metals with negatively charged bacterial structures and live or dead biomass components. Due to the large surface area-to-volume ratio, these bacterial biomasses efficiently act as biosorbents for metal bioremediation under multi-metal conditions. In this study, we aim to isolate heavy metal-resistant bacteria from heavy metal-contaminated sites. The isolated strains will be identified, and their tolerance to various heavy metals will be evaluated. The analysis will be performed for the evaluation of metal biosorption. This study could prove valuable for in situ bioremediation or the removal of toxic heavy metals from the environment.

Keywords: Heavy metals, biomineralization, bioremediation, degradation, contaminants

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LIST OF ABBREVIATIONS

	ABBREVIATION
TEM	Transmission electron microscope
EDX	Energy Dispersive X-ray
EDS	Energy dispersive spectroscopy
MgNH ₄ PO ₄ · 6H ₂ O	Magnesium ammonium phosphate
Ca ₅ (PO ₄) ₃ OH	Calcium apatite
XPS	X-ray photoelectron spectroscopy
UV- vis	Ultraviolet–visible spectroscopy
GPPS	General Purpose Polystyrene
FRP	Fiber Reinforced Plastic
HDPE	High-Density Polyethylene
mL	Milliliter
μL	Microliter
Na ₂ SeO ₃	Sodium selenite
ZnSO ₄ · 7H ₂ O	Zinc heptahydrate
Cd (NO ₃) ₂ · 4H ₂ O	Cadmium citrate
Pb (NO ₃) ₂	Lead nitrate
SeO ₄ ²⁻	Selenate ion
SeO ₃ ²⁻	Selenite
nm	nanometer
mM	millimolar
HNO ₃	Nitric acid
rpm	Revolution per minute
OD	Optical density
AAS	Atomic absorption spectroscopy

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CHAPTER-1 INTRODUCTION:

Metallic elements with a density higher than that of water are referred to as heavy metals (Paul et al., 2012). The earth's crust naturally contains heavy metals, but uncontrolled human activity has significantly changed the geological cycles and biological balance of these elements (Reena et al., 2011). Assuming that toxicity and heaviness are correlated, metalloids that can cause toxicity at low exposure levels, like arsenic, are also considered heavy metals (Paul et al., 2012). Trace elements such as chromium, lead, and nickel have been detected from industrial effluents collected in and around the contaminated site (Jagdish et al. 2006). Several microorganisms are investigated for their potential to remediate soil affected by metals because of their resistance to and detoxification from metals (Roane et al., 1998). Among other things, waste disposal, metallurgical industry operations, mining and smelting of metalliferous materials, corrosion of metals in use, agriculture, and petroleum exploration release heavy metals into the environment (Mohammad et al., 2017).



Fig 1.1: Heavy metal contamination in water bodies through waste dumping

Exposure to non-essential metals (NEMs) like arsenic, cadmium, mercury, and lead can cause chlorosis, inhibition of photosynthesis, imbalanced water and nutrient assimilation, lowered biomass accumulation, reduced soil fertility, and decreased growth and productivity plants (Zango et al., 2020). These metals invade aquatic environments by leaching into water bodies through mining operations, petroleum refineries, improper municipal waste disposal,

and natural mineral deposits, and the presence of these toxic substances makes them importable for both human and animal consumption and living (Lopes et al. 2017).

These metals accumulate in the organs of organisms via the gills, body surface, and digestive tract by ingestion of metal-accumulated food particles, which alters and affects the sustainable living of aquatic animals (Diep et al. 2018). In humans, these types of metals relocate to cells and prevent them from performing functions that affect vascular damage, immune system dysfunction, birth defects, and cancer (Ying et al. 2018).



Fig 1.2: Heavy metal contamination from industries

In recent years, the proliferation of heavy metal pollutants in various environmental matrices has emerged as a pressing concern worldwide. Heavy metals, owing to their persistence and toxicity, pose significant threats to ecosystems, human health, and agricultural productivity. Traditional methods of heavy metal remediation often prove to be costly, labour-intensive, and sometimes environmentally disruptive. However, considering advancements in biotechnology, there has been a growing interest in exploring sustainable and eco-friendly approaches to mitigate heavy metal contamination (Rajendran et al.2022).

A promising approach called "bioremediation" uses microorganisms' metabolic processes to change or immobilize heavy metal contaminants, making them less dangerous or eliminating them from the environment. Bacteria are a class of microorganisms that have

attracted a lot of attention because of their wide range of metabolic activities and environmental tolerance (Hirak et al., 2012). Bacterial-mediated bioremediation offers numerous advantages, including cost-effectiveness, specificity, and minimal environmental impact compared to conventional remediation techniques. Bacterial bioremediation works because of the metabolic activities that these microbes engage in. Some species of bacteria are innately able to precipitate, sequester, or metabolize heavy metals utilizing biotransformation, bioaccumulation, biosorption, and precipitation. These activities lessen the harmful impact of heavy metals on ecosystems and human health by reducing their bioavailability and facilitating their conversion into less toxic or insoluble forms (Rahman et al., 2019).

This study aims to explore the potential of bacterial-mediated bioremediation as a sustainable and efficient approach to mitigate heavy metal contamination in various environmental settings. By elucidating the underlying mechanisms of bacterial metal tolerance and remediation, as well as optimizing operational parameters, this research seeks to contribute to the development of innovative biotechnological solutions for addressing heavy metal pollution challenges (Bala et al., 2022).

When significant concentrations of heavy metals are introduced into the environment, most microflora are killed, which puts pressure on selected organisms to develop that are resistant to the metals. Through a variety of mechanisms, including variations in the uptake or transport of the toxic metal, these resistant strains contribute to the process of self-recovery of the contaminated habitat. In other instances, the metal may undergo enzymatic transformation by oxidation, reduction, methylation, or demethylation into chemical species that may be less toxic or more volatile than the parent compound. These methods can occasionally be found encoding antibiotic resistance genes near plasmid genes, which makes it easier for toxic metal resistance to spread from one cell to another and for heavy metal tolerance.

CHAPTER – 2

REVIEW OF LITERATURE:

2.1 MECHANISMS OF BIOREMEDIATION:

The bioremediation of heavy metals is efficient when the right bacterial strains are chosen. It uses certain mechanisms to adapt to, integrate with, and link with the environment. This reduces tensions in the area and prepares it for the mineralization of heavy metals. (Sreedevi et al., 2022) These include bioaccumulation, which involves intracellular and extracellular sequestration, as well as precipitation of phosphates and sulfides, which results in leaching (Feng Lia et al., 2018) and (Lianwen et al., 2018).

2.2 BIOSORPTION:

Biosorption in simple terms is put forth as the removal of contaminants from water especially those that contain non-biodegradable materials like metals and dyes. (Gupta et al., 2000). Which broadly interacts with these types of bio adsorption like physical, ion exchange, and complexation (Ibrahim et al., 2016).

2.2.1 PHYSICAL ADSORPTION:

This method of adsorption is like the condensation of gases into liquids and is called the van der Waals force or natural attractive force. This method acts as a multi-layer bed for adsorbent materials on the surface of adsorbent materials when an appropriate temperature and pressure are provided. It is characterized by adsorption of the surface of gases subjected to all gases, with higher physical adsorption due to higher liquefaction capacity. Porous material is considered to have the best physiologic sorption because of its high surface area. (Ibrahim et al., 2016).

2.2.2 ION EXCHANGE ADSORPTION:

In this process, the exchange of binary metal ions which are present on the surface of the absorbent works on purification. Absorption of (Cd) is studied. This reduces concentration and removes the presence of heavy metals. Cation-ion exchange of a few functional groups is identified like Carboxyl, phosphate, and hydroxyl groups. Removal of (Cd) and (Pb) by exchange with K^+ , Na^+ , Mg^+ , and Ca^+ are reported (Bashir, et al., 2018).

2.2.3 COMPLEXATION:

This process is composed of complex or coordinated compounds, which are poly-atomic and comprise positive, negative, or neutral charges. A chelating complex is formed when a coordination ligand is connected to a coordinating atom and a compound atom. The complexation of certain heavy metals like mercury, cadmium, and zinc is reported by *Pseudomonas syringae* and *Pseudomonas fluorescens* (Sreedevi et al.,2022). (Cr) is complexed with hydroxyl carboxyl and amino groups of proteins on the extracellular matrix of *Shewanella putrefaciens*. The efficiency is determined by the oxidation of metal ions and the physical and chemical parameters of the required medium. Metal recovery could control temperature, pH, and time. Biosorption is the most prevalent defence mechanism of heavy metals, which is commonly exhibited by mesophilic bacterial strains (Nisha et al., 2017).

2.2.4 ADVANTAGES OF BIOSORPTION:

- High Affinity,
- Naturally abundant materials are used so produced at low cost
- Multiple metals are handled at the same time
- Hazardous wastes are reduced in great quantity

2.3 BIOACCUMULATION:

Bioaccumulation is the process by which heavy metals are absorbed by plants and microorganisms. The toxic properties of the metal depend upon the environmental conditions and the metal's characteristics (Moiseenko et al.,2020). It diffuses into the cell cytoplasm using energy-dependent or independent means. The toxic metals present in water are dependent on factors like pH and the concentration of organic ligands.

2.3.1 INTRACELLULAR SEQUESTRATION:

It is a process of the formation of a combination of metal ions with cellular cytoplasmic compounds. *B. megaterium* has a dense granule inside the cytoplasm of the cell, which involves the intracellular accumulation of lead in this mechanism. This accumulation process for heavy metals is rarely observed. It involves certain strategies, such as pulling metal ions

out of the cell or preventing metal ions from entering the cell. The resistivity is broadly classified into two categories: metal-dependent and independent. The process carried out by bacteria that are isolated from the environment executes the objective of the diversity of lead resistance mechanisms (Roane et al., 1999).

2.3.2 REDUCTION OF METALS:

Bacteria generate energy from electron donors and acceptors of metals and metalloids. Toxicity is reduced by the transformation of the oxidation state of metal ions. The bacterial strains that relate to aromatics, lactic acid, organic substrates, and acetic acid report the reduction mechanisms of mercury and selenium (Nancharaiyah, et al., 2015). The metal reduction process of sulfate removal bacteria is analyzed by transmission electron microscopy (TEM), energy dispersive spectroscopy (EDS), and energy dispersive X-ray spectroscopy (EDX) (Kiran et al., 2017). Demethylate MeHg Sequential Mercuric reductase enzyme (MerA) and organomercurial lyase (MerB) reduce Hg, which is removed from the cell and extends resistance to MeHg to Hg (II). Ian N. Krout, Thomas Scrimale, and Daria (Vorojeikina Eric S. et al., 2022). Bacterial strains of *Bacillus*, *Pseudomonas putida*, and *Pedomicrobium* reduce Mn (Mohanty et al., 2018).

2.3.3 METHYLATION OF METALS:

Methylation is a distinct pathway that involves various bacterial species and the oxidation state of metal ions. Due to its specific features, it dominates the pathways oxidation state of metal and metalloid function in aquatic environments. Bioaccumulation of neurotoxin methylmercury is represented by the methylation of Hg by anaerobic bacteria. Sulfate-reducing bacteria are used to reduce Hg (II) metal cations (Schaefer et al., 2014). The only organism that has a partially defined methylation pathway is *D. desulfuricans* LS (Cynthia et al., 2011). The increase in Hg (II) can be removed by SRB-enriched sludge and SORB-1-enriched sludge. This method can be broadly used in mercury immobilization and environmental bioremediation of mercury, especially in aquatic environments (Wang., et al., 2018).

2.3.4 ADVANTAGES OF BIOACCUMULATION:

Site-specific accumulation in intraspecies and interspecies identifies specific bioaccumulation sites. A non-steady state of bioaccumulation can be explored. Temperature is the key factor in this process. Toxicity at different levels is studied. (Wang et al., 2019).

2.4 BIOPRECIPITATION:

Bioprecipitation generally means the removal of heavy metals by sulfate-reducing bacteria. The ability of microorganisms to adapt and resist contamination with heavy metals. The sedimentation process is used to separate precipitated compounds from the environment. The metal immobilization of microorganisms is facilitated through carbonate precipitate (Janyasuthiwon et al., 2017). Microbial-induced phosphate propagation (MIPP) also immobilizes metals present in the environment.

2.4.1 REDUCTION:

The reduction process is already discussed under bioaccumulation. The strains such as *Lactobacillus plantarum* reduce (Ni) and (Cr), *Bacillus megaterium* carries out (Pb) and (Cd) reduction and *pseudomonas taiwanensis* carries out the reduction of (As) and (Cd) (Sreedevi et al., 2022). *Staphylococcus epidermidis* can mineralize high amounts of Pb and Cr through carbonate mineralization. The redox reaction, also called the "oxidation-reduction" reaction, can be bio-precipitated (Cr) in both organic and inorganic form (Rahman., 2020).

2.4.2 METAL SULFIDE PRECIPITATION:

Sulfate-reducing bacteria are heterotrophic. It reduces sulfate to sulfide, which forms metal sulfides with cells in the environment. At low solubility, the metal sulfide precipitate is formed by reacting with heavy metals, which react with sulfides. To remove (Tl) Thallium from wastewater, sulfide precipitation with Tl^{II+} and Tl^{+3} is formed to produce Tl_2S precipitate. An increase in pH level up to 8–12 is seen by the removal of Tl. By this method, pH plays a key role in the bioremediation of metals (Huosheng et al., 2019). *Salmonella typhimurium* reports enzymatic sulfide with thiosulfate reductase (Rahman et al., 2020).

2.4.3 METAL PHOSPHATES PRECIPITATION:

To facilitate the transformation of soluble phosphate ions into solid, insoluble forms, phosphate bio-precipitation depends on the metabolic activities of microbes, mainly bacteria. The PAOs release the stored polyphosphate as insoluble phosphate minerals when the environment changes, such as a drop in oxygen levels or an increase in pH. Depending on the precise circumstances, these minerals can take various forms, such as struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3(\text{OH})$), or others. The environment's phosphate concentrations are decreased because of the released phosphate minerals, which take the form of solid particles that can easily settle out of the water or soil. This method in the bioremediation process indicates that a decrease in pH will affect the removal of phosphate (Nagoya et al., 2019).

2.4.4 ADVANTAGES OF BIOPRECIPITATION:

Microbial growth creates efficient metal removal and recovery. It can treat different ranges of wastewater that contain a high amount of salt concentration and particulates. Sulfide precipitation is one of the most effective methods of bioprecipitation (Cindy et al., 2013).

2.5 BIOLEACHING:

The process of biodegrading heavy metals from sludge is called bioleaching. Bioleaching eliminates a larger amount of acid from the environment than the chemical method, so it's both an efficient and sustainable process. Microorganisms like *At. thiooxidans* and *At. ferroxidans* perform bioleaching. This process can be affected by temperature, oxygen concentration, oxidation, and reduction potential, which can oxidize insoluble particles of sulfide and convert them to sulfate. The substrate which is being used in bioleaching is also important because it not only limits the quantity but also affects the removal efficiency of the process and makes it cost more than usual (Yang et al., 2020).

2.5.1 BIOLEACHING OF SULFIDES:

Acidophilum, *Arthrobacter*, *Acetobacter*, *Trichoderma*, and *Pseudomonas* are heterotrophic bacteria that produce effective organic acids for the process. (Kamizela et al., 2020). Bacterial leaching results in the formation of metal sulfides, which diffuse. Sulfate-

reducing bacteria, *Sulfobacillus thermosulfidooxidans*, *Sulfolobus* sp., *S. acidophilus*, and *Thiobacillus denitrificans* are used in the remediation process (Yang et al., 2020).

2.5.2 BIOLEACHING OF PYRITE:

Acidithiobacillus ferrooxidans oxidizes nonmetal sulfides into soluble metal sulfate. Oxidation of iron from mine water drainage is carried out by *Gallionella ferruginea* (Sreedevi et al., 2022). Characterization of this method is done by various methods, like X-ray diffraction (XDR), which is used for phase detection in the sample. X-ray photoelectron spectroscopy helps in understanding mechanisms and composition. The parameters, like different sulfuric acid concentrations, different temperatures, and different mass ratios in pyrolusite to pyrite, determine the effective leaching process (Shunda et al., 2020).

2.5.3 HETEROTROPHIC BACTERIAL LEACHING:

This method first requires the identification and isolation of heterotrophic nitrifying bacteria, which are extracted from mining sites. Samples are collected and dissolved in water, then grown on a culture medium. This is done twice or three times to identify pure strains of bacteria. The number of strains is observed by absorbance at 600 nm using equipment called UV-visible spectroscopy. The removal of nitrogen ammonia is co-related with strain growth concentration. These organisms produce organic acids, which help in producing chelating agents for compounds that help in the solubilization of metals. *Sphingomonas desiccabilis* and *Bacillus subtilis* reportedly enhanced vanadium extraction (Jingang et al., 2022)

CHAPTER 3

AIM AND OBJECTIVE:

- Isolation of heavy metal tolerant bacteria and evaluation of its tolerance.
- To Identify the specific heavy metals present and their concentrations.
- To Identify bacterial absorbance of heavy metal.

CHAPTER – 4

MATERIALS AND METHODS:

4.1 STUDY AREA AND SAMPLE COLLECTION:

Water, soil and fouled plastics samples are collected from River mouth of Kovalam and CMFRI zone Kovalam ($12^{\circ} 47' 24.6948''$ N $80^{\circ} 15' 7.9992''$ E) which is surrounded by waste dumps such as GPPS (General Purpose Polystyrene), FRP (Fiber Reinforced Plastic) and HDPE (High-Density Polyethylene) which pollute the area. The samples are collected in Plastic zip locks and bottles with forceps and spatula. The collected plastic samples, soil and water samples are stored at 4°C . The image captions sample 1 indicates a fouled sample, and samples 2 and 3 indicate soil and water (Deborah et al.,2016).

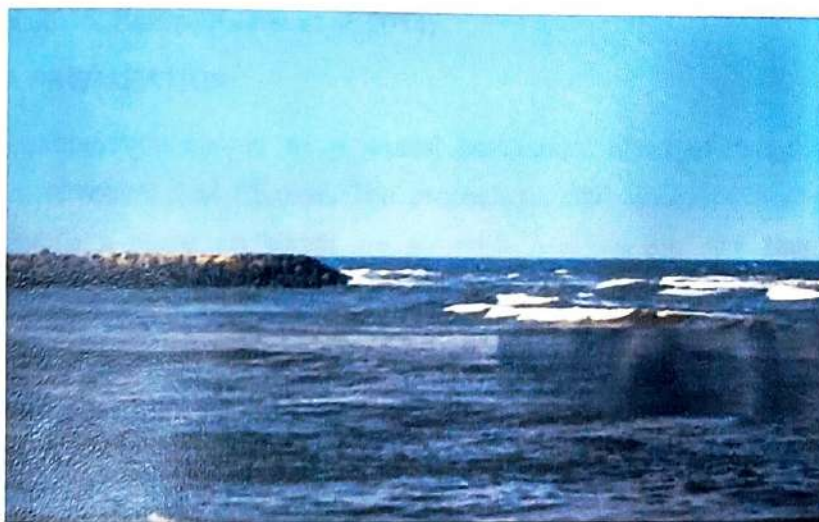


Fig 4.1: Sampling site for analysis

4.2 ISOLATION OF MICROBES:

4.2.1 PREPARATION METHODOLOGY FOR SAMPLE ANALYSIS:

The three different samples were diluted by three different methods and prepared for tenfold serial dilution. For fouled plastic samples, the fouled layer is scrapped out and diluted in $1000\ \mu\text{L}$ of sterile water and vortexed until the sediments are completely dissolved; for soil samples, $0.5\ \text{g}$ of soil is dissolved in $5\ \text{mL}$ of sterile water; and for water samples, $1\ \text{mL}$ of water is dissolved in $5\ \text{mL}$ of sterile water used for serial dilution.

4.2.2 SERIAL DILUTION:

Serial dilution is commonly used to enumerate microorganisms present in samples. One popular method for counting the microorganisms in soil samples is serial dilution. We can spread diluted samples overgrowth media and count the number of colonies that emerge by diluting the sample to a series of predetermined concentrations. This gives an approximation of the original sample's microbial population density.

Serial dilution is a technique used in environmental research to measure the number of pollutants (such as pesticides or heavy metals) in soil samples. The concentration of pollutants present can be ascertained chemically by the researchers by diluting the sample to a range of known concentrations.

The sterile glass tubes are prepared with 4.5 mL of deionized water and sterilized at 121°C and 500 µL of prepared sample is pipetted into the tube labelled as 10^{-1} to 10^{-10} and flushed and the process continued up to ten tubes and the samples are ready for plating (S. Deborah et al.,2016, Raman Kumar et al.,2014)

4.2.3. MEDIA PREPARATION:

Media preparation serves as a crucial component of experimental methodology, particularly in microbiological Studies. The process typically begins with the selection of appropriate media formulations which are available commercially and give the required composition of nutrients. In this method we have used agar and Nb is our standard media for the isolation process. The number of components used is measured accurately as per the company's label and dissolved completely in deionized water and sterilized.

4.2.4 PLATING TECHNIQUES:

Autoclave the nutrient agar solution at 121°C (250°F) for 15 minutes under 15 psi pressure to sterilize the media and kill any microorganisms present after it comes down to 50- 60°C add fluconazole 0.1 x WSS is added as required for prevention of fungal growth and poured in sterile plates. Then 50 µL of diluted sample is pipetted out onto the plate and spread using the sterile L-rod. The plates are then incubated for 24 hrs at 32°C. After the incubation period isolated colonies are picked and subcultured by the quadrant streaking method for pure and isolated colonies. This plating method is also carried out in Nutrient agar medium and incubated at 32°C for 24 hrs and stored at 4°C. (Jabbari Nezhad Kerman et al.2010).

4.3 MICROSCOPIC IMAGING:

Microscopic imaging for bacteria is an essential tool in microbiology, allowing us to observe and analyze bacterial morphology, behaviour, interactions, and more.

Light microscopy: This technique uses visible light to illuminate specimens, enabling the observation of bacterial cells. Brightfield microscopy is the most common method, where bacteria appear as dark objects against a bright background. Phase contrast and differential interference contrast (DIC) microscopy enhance contrast and reveal cellular details.

The sample preparation for microscopic viewing is carried out by smear prepared in ethanol-washed slides and followed by gram staining done by crystal violet stain, Iodine, Decolorizer and safranin after washing the slides are dried completely before viewing in the microscope. Micrography, taking an image using a microscope, provides a means of permanently recording an image for scientific purposes. The slides are viewed in 10X, 40X and 100X magnification (Timberley M. Roane et al. 2019).

4.4 BIOCHEMICAL TEST:

Biochemical tests help identify and classify bacteria into different groups or species. Different species of bacteria have unique metabolic pathways and enzyme profiles, which can be detected through specific biochemical reactions. The broth is prepared with 3 mL in each tube and 300 μ L of the 24 hrs culture is pipetted and incubated for 6 hrs at 32 °C. Then after 6 hrs of incubation 50 μ L bacterial culture is suspended into the respective wells and incubated for 24 hrs at 32°C. After incubation time the reagents are added into the first 3 wells and the color change is observed and interoperated (Kepel et al., 2021).

4.5 METAL INFUSED MEDIA INOCULATION:

The metal salt-infused media is prepared in 3 different concentrations between (0-300 mM) the metal salts used are Na_2SeO_3 , $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Pb}(\text{NO}_3)_2$. The plating media for metals includes NB, Agar and respective metal salt after autoclaving pour it into petri plates and solidify. Broth-infused metal salt media is prepared by NB and respective metal salts and sterilized (Yuhua Duan et al 2022 Sharma, Sushil K et al., 2011).

4.5.1 INOCULATION OF BACTERIA:

The selected bacterial strains are inoculated in NB, which is prepared with deionized water and the required amount of NB as per the instruction label, and autoclaved. After it

reaches room temperature, inoculate the bacteria from plates to tubes, label them, and incubate them for 24 hours at 32 °C.

4.5.2 METAL MEDIA INCULCATION FOR Se:

The salt media is prepared in 3 different concentrations (0-300 mM). To examine the Se absorbance by bacteria, the media is spiked with Na_2SeO_3 . The salt is measured as per concentration in grams 0.51882 g (300 mM), 0.34588 g (200 mM) and 0.17294 g (100 mM) completely dissolved in deionized water and then NB and agar and plugged with cotton plug and sterilised at 121°C. Due to the oxidation reaction taking place the media colour changes from normal pale yellow to reddish brown. Oxidation in sodium selenite after autoclaving occurs due to the exposure of the selenite ions to high temperatures and pressure during the autoclaving process. This exposure can lead to the oxidation of selenite ions (SeO_3^{2-}) to selenate ions (SeO_4^{2-}), resulting in the loss of selenium in its lower oxidation state. Autoclaving involves subjecting substances to elevated temperatures and pressures, which can promote chemical reactions, including oxidation (Han-Ming SHEN et al., 1999). Therefore, the oxidative conversion of sodium selenite can occur because of these conditions, leading to changes in the chemical composition of the solution. Inoculation of bacterial species in Se media is done by swabbing the grown culture on the plate using a sterile swab. (Yuhua Duan et al., 2022).

4.5.3 METAL MEDIA INOCULATION FOR Zn:

The salt media is prepared in 3 different concentrations (0–300 mM). Commonly used basal media is nutrient agar, supplemented with the appropriate 0.8626 g (300 mM), (200 mM) and 0.0287 g (100 mM) of zinc salt to achieve the desired zinc ion concentration. The media were sterilized using autoclaving to eliminate any microbial contaminants and ensure the integrity of the zinc salt composition. Following sterilization, the media were poured into sterile Petri dishes and allowed to solidify (Sharma, Sushil et al., 2011).

4.5.4 METAL MEDIA INOCULATION FOR Cd:

The salt media is prepared in 3 different concentrations which contain (0-50 mM). The preparation typically involves dissolving 0.3084 g (10 mM), 0.06169 g (20 mM) and 0.15424

g (50 mM) cadmium nitrate heptahydrate. Once the cadmium salt-containing solution is prepared, it can be combined with agar and nutrient broth for specific growth requirements. The media should then be sterilized by autoclaving, to ensure that it is free from microbial contamination.

4.5.5 BACTERIAL MAINTENANCE IN BROTH CULTURE:

The picked bacterial strains are maintained in the metal spiked broth media by inoculating the bacterial strains from the metal spiked media plates to the freshly prepared broth at room temperature then incubating it for 24 hrs at 32°C and stored at 4°C. By maintaining bacterial cultures in metal media, we can optimize the conditions for bacterial growth and metal remediation. This can lead to improved bioremediation efficiency, remediation rates, and higher metal removal capacities compared to non-tailored growth media (Siobha'n O'Brien et al., 2014).

4.6 GROWTH KINETICS:

To apply Monod kinetics in bioremediation, one typically measures the initial concentration of contaminants and monitors their decrease over time. To predict the growth of both normal culture and Se spiked culture and comparison is made for interoperating the results of biosorption. For growth curve measurements 96 well plates were taken and sterilized in UV light for 15 mins, then 24 hrs bacterial culture was prepared freshly in both metal spiked and normal nutrient media. The well plate is triplicated with NB and Se as control and 100 µL of broth and 10 µL of culture is added into the wells readings are observed in the multi-mode reader for 24 hrs at 600 nm and observations are noted in the Excel sheet. Average and Standard deviation is calculated, and the graph is plotted (Venkata Rao Krishnamurth et al., 2020).

4.7 ATOMIC ABSORPTION SPECTROSCOPY SAMPLE PREPARATION:

Atomic Absorption Spectroscopy is performed to identify the traces of heavy metals. The 24-hour bacterial culture in all three concentrations is prepared and centrifuged for 15 mins at 5000 rpm, the supernatant is discarded, and the pellet is collected and weighed. The bacterial pellet which weighs about 0.52 g is flushed with 500 µL of metal broth transferred

and followed by acid digestion with 15 mL of HNO_3 added into a beaker containing the flushed bacterial pellet and stirred for 15 mins at 100°C in a hot plate stirrer until the sample is completely digested. Transfer the sample after it reaches room temperature. Samples are submitted for AAS analysis (Mardiyono et al., 2019).

CHAPTER- 5

RESULT AND DISSCUSION:

5.1 ISOLATED BACTERIA:

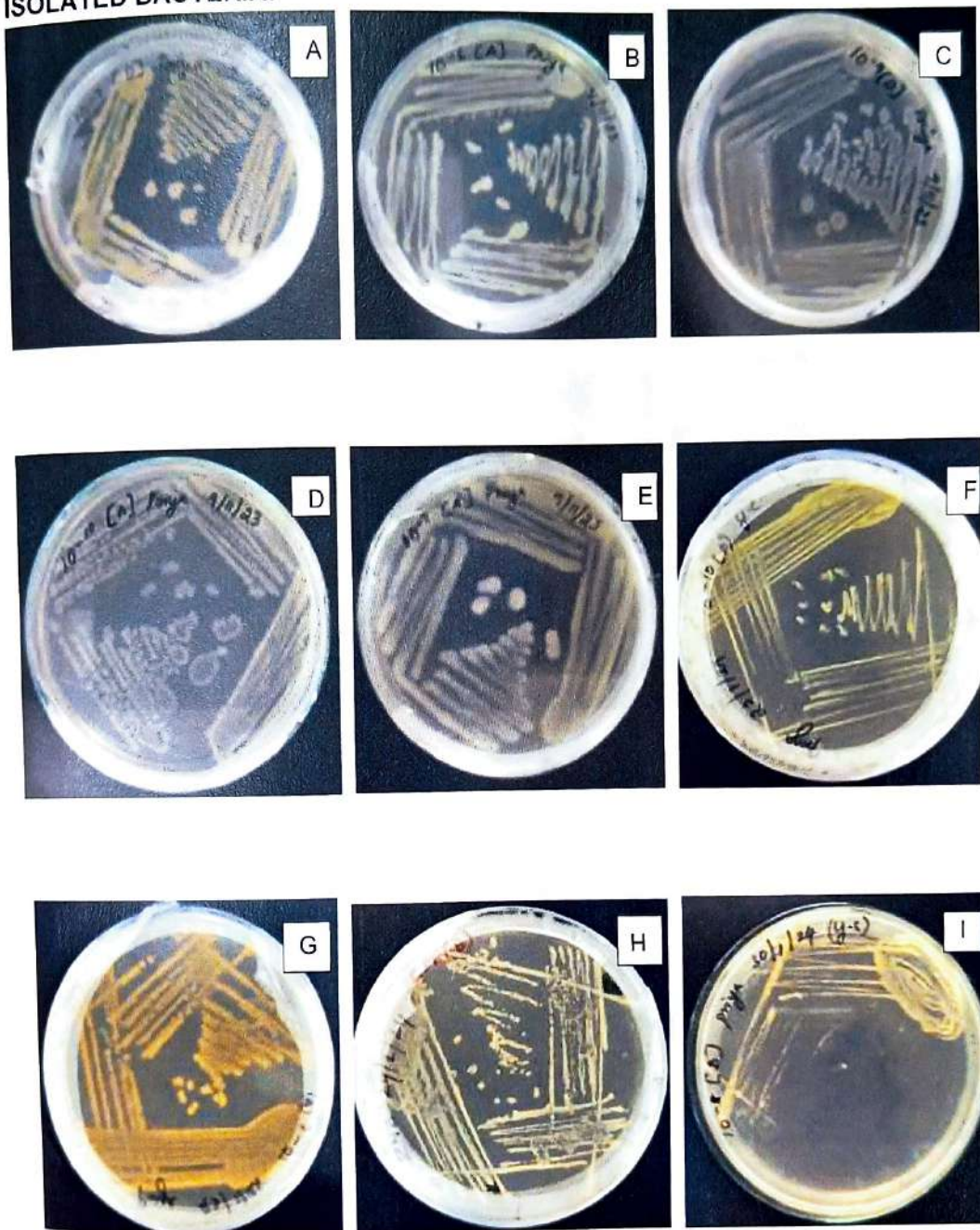


Fig 5.1 Bacterial isolates from sample 1

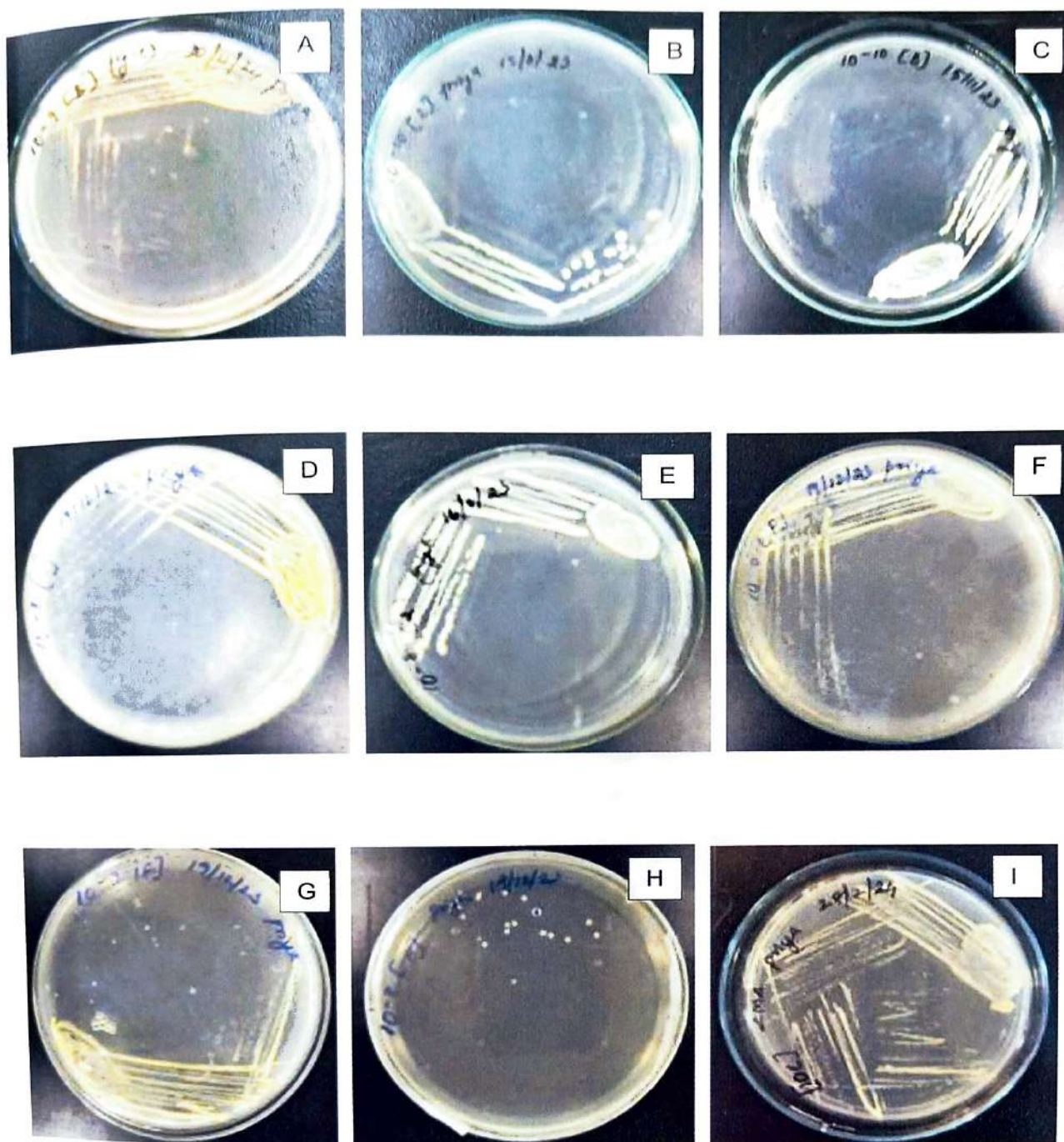


Fig:5.2 Bacterial isolates from sample 2

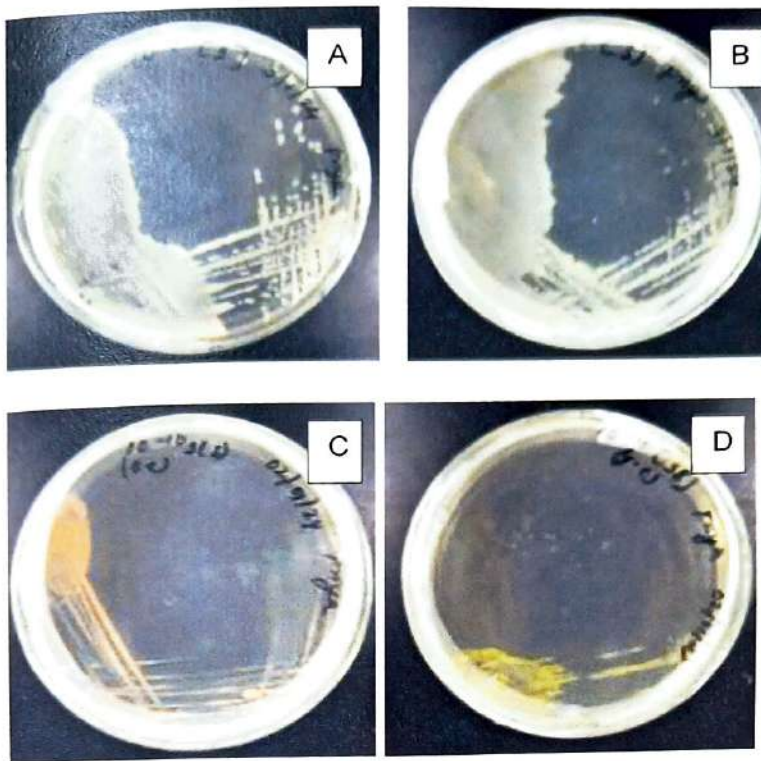


Fig:5.3 Bacterial isolates from sample 3

All isolated strains are stored at 4°C to arrest further growth. In the above image captions sample 1 indicates fouled sample, and samples 2 and 3 indicate soil and water. Isolating bacteria is used to study individual species or strains. By separating bacteria from complex microbial communities we can identify, classify, and characterize them based on their morphology and physiology (Bhumblaet al., 2020).

5.2 MICROSCOPIC IMAGES:

These images can help illustrate findings, support hypotheses, and provide visual evidence for the research conducted.

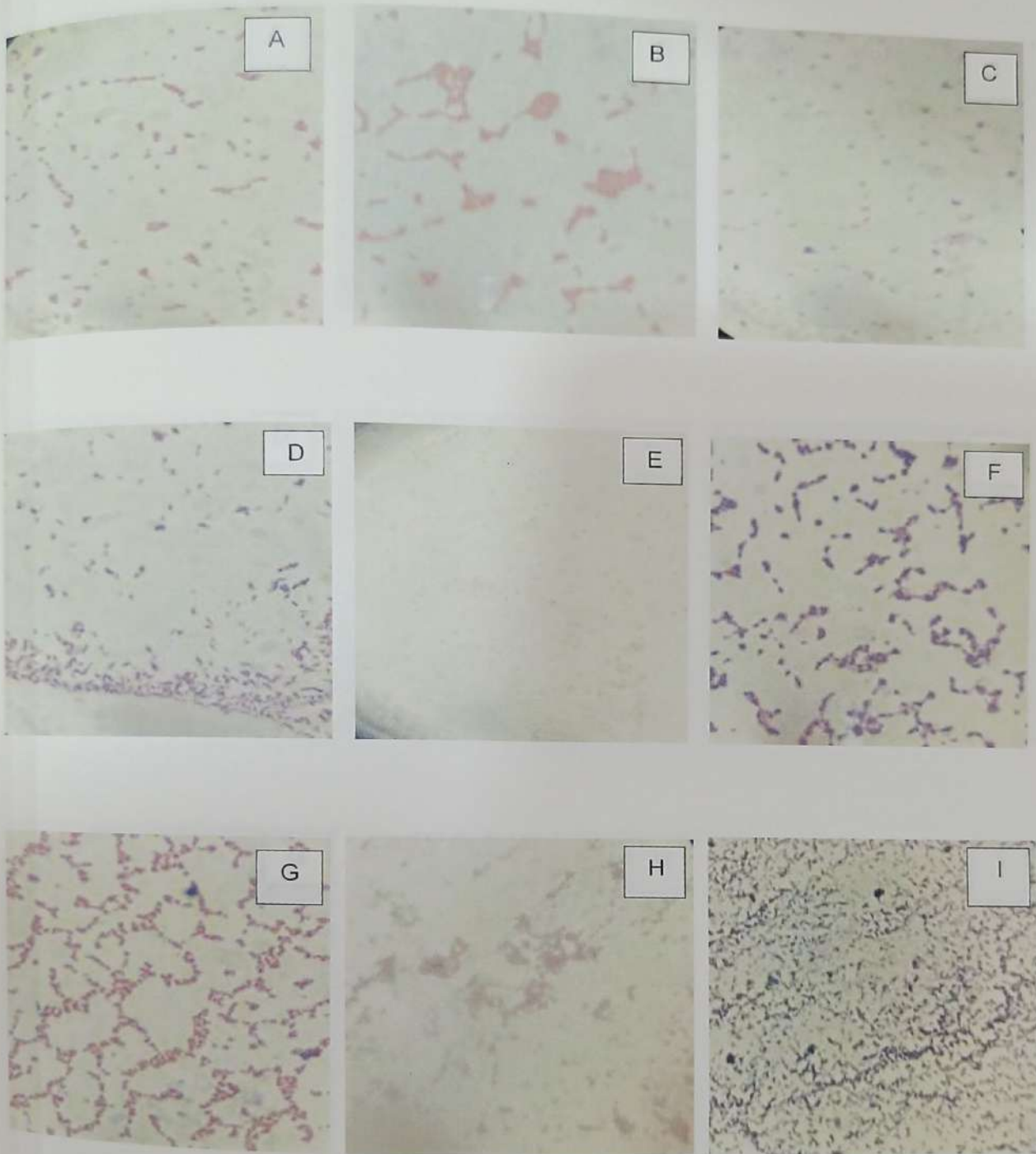


Fig 5.4: Gram staining of sample 1 isolates

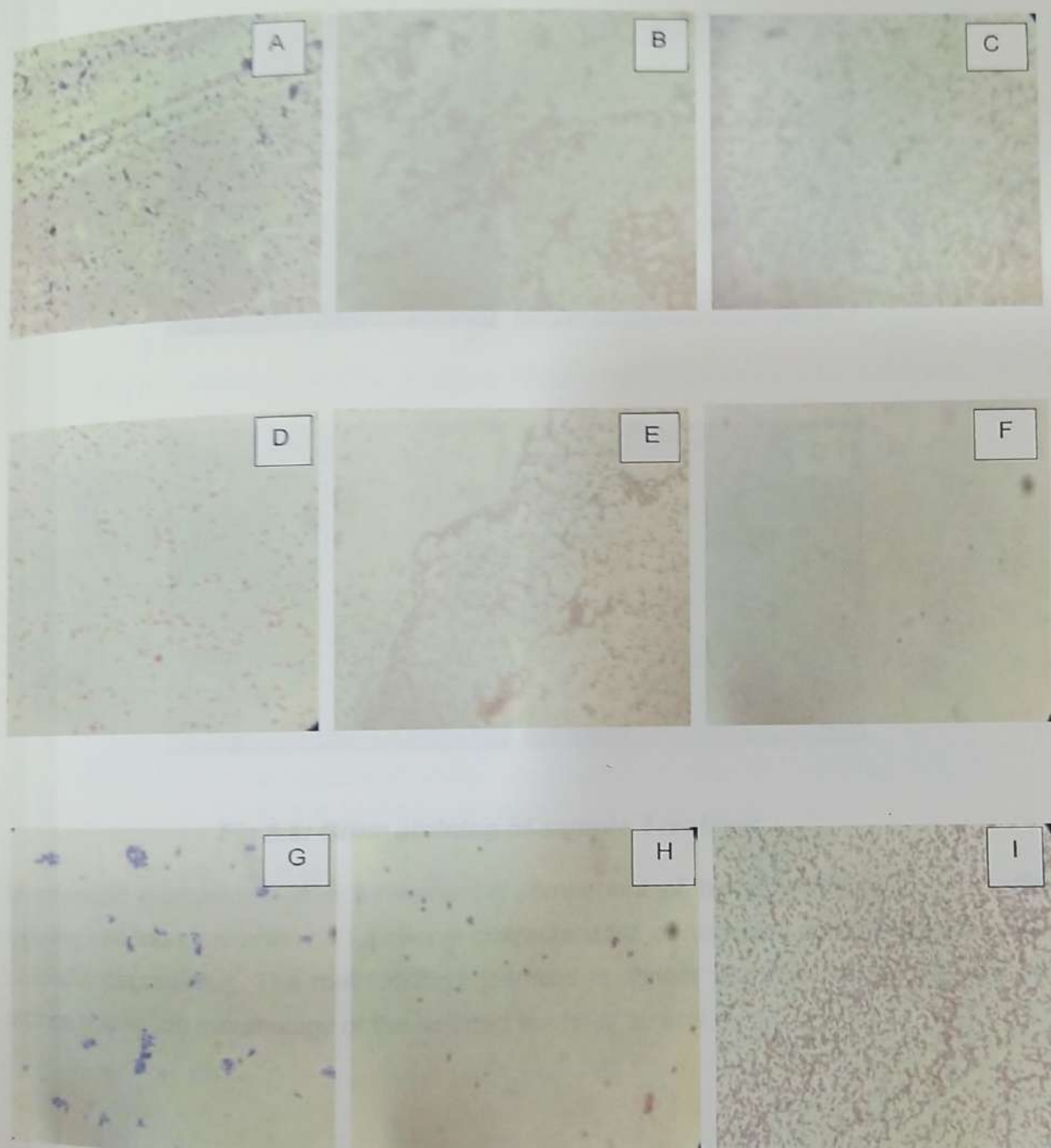


Fig 5.5: Gram staining of sample 2 isolates

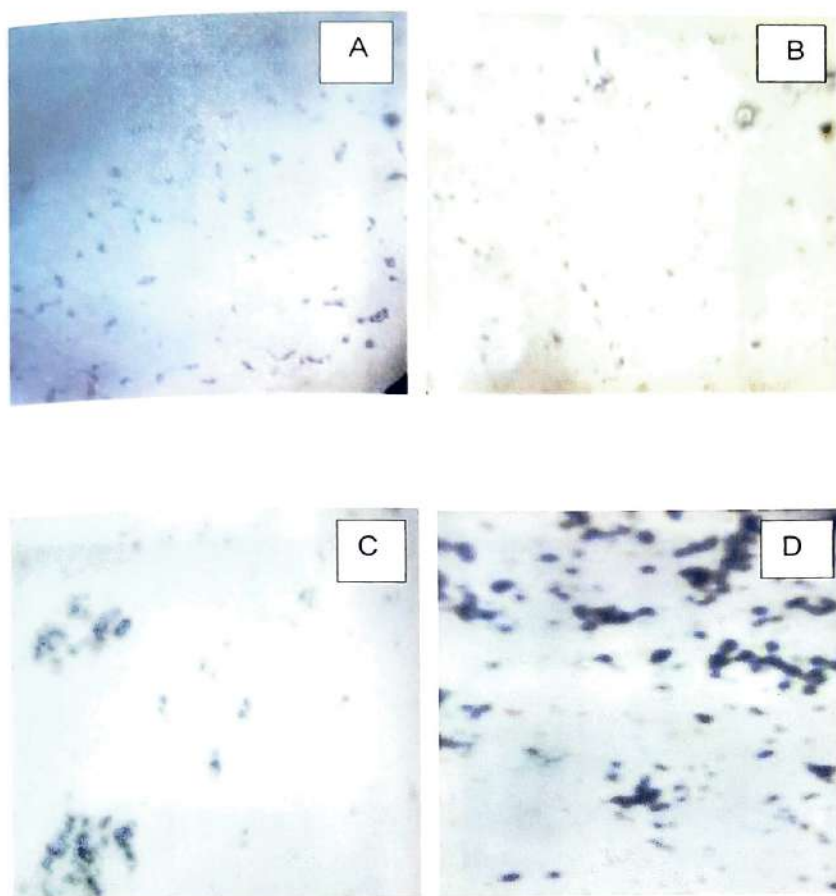


Fig 5.6: Gram staining of sample 3 isolates

Microscopic examination is a fundamental component of bioremediation studies, offering insights into the structural and functional characteristics of microbial communities involved in pollutant degradation. The methodology followed is similar and the result obtained in the picture shows the morphology of the isolated bacterial strains.

5.3 BIOCHEMICAL TEST:



Fig 5.7: Before and after a colour change in biochemical test analysis

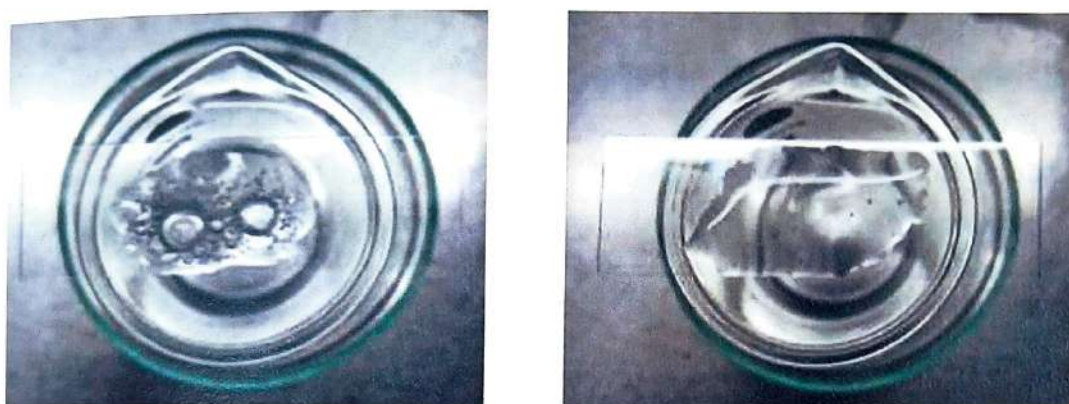


Fig 5.8: catalase enzyme identification testing

Table 5.1 Identification index of metal degrading bacteria:

Test	Indole	Methyl red	Voges	Citrate	Catalyzes
A	-	+	+	-	-
B	-	+	+	-	+

In the results, biochemical testing was conducted to characterize the bacterial strains grown in metal-enriched media broth. Various biochemical assays were employed to identify key metabolic activities and biochemical properties of the bacteria. This included testing for enzyme activities such as catalase, and oxidase, and various substrate utilization tests such as carbohydrate fermentation, indole production, and nitrate reduction. The results of these tests provided insights into the metabolic capabilities and physiological characteristics of the

bacteria under different metal concentrations. Furthermore, biochemical testing helped to classify the bacterial strains and assess their potential for metal bioremediation or other applications. Overall, the results of biochemical testing complemented the growth kinetics data and contributed to a comprehensive understanding of the bacterial responses to metal-enriched media broth (Kepel et al., 2021).

5.4 BACTERIAL GROWTH ON METAL-ENRICHED MEDIA:

The cultivation of bacteria in metal-enriched media represents a significant area of study in microbiology. This approach aims to understand the responses of bacterial communities to metal pollutants and their potential applications in bioremediation. In this the growth dynamics of bacteria in metal-enriched media will be explored, shedding light on how various metals impact microbial proliferation, metabolism, and community composition. Through controlled experiments and molecular analyses, the interactions between bacteria and metals will be elucidated, providing insights into the potential of metal-microbe interactions. Moreover, this research contributes to the development of sustainable solutions for environmental pollution and resource recovery by harnessing the capabilities of metal-tolerant bacteria (Jabbari et al.2010).



Fig 5.10: Absorption of Se in metal-enriched media by A bacteria in all 100 mM, 200 mM & 300 mM Concentrations.



Fig 5.11: Absorption of Se in metal-enriched media by B bacteria in all 100 mM, 200 mM & 300 mM Concentrations.



Fig 5.12: Maintenance of bacterial strains in metal media

5.5 BACTERIAL GROWTH IN METAL-ENRICHED BROTH:

The impact of metal-enriched media broth on bacterial growth. Metals play crucial roles in various biological processes, but their excess presence can be detrimental. Through a series of experiments, we explore how different concentrations of metals in the growth medium influence the growth kinetics, metabolic activity, and gene expression patterns of bacteria. By systematically varying the metal concentrations and analyzing bacterial growth curves, we aim to elucidate the optimal conditions for bacterial growth in metal-enriched media broth. The mechanisms underlying metal toxicity and the adaptive responses of bacteria to metal stress. The conduct of the experiment is to make sure the presence of toxic metals to form siderophores which help in the accumulation of metals we have applied them and the results are similar as the results depict absorption just absorbing the selenium and turning into light brown to yellowish color (Siobha'n O'Brien et al., 2014).



Fig 5.13: Bacterial absorption of Se in broth media

5.6 GROWTH KINETICS:

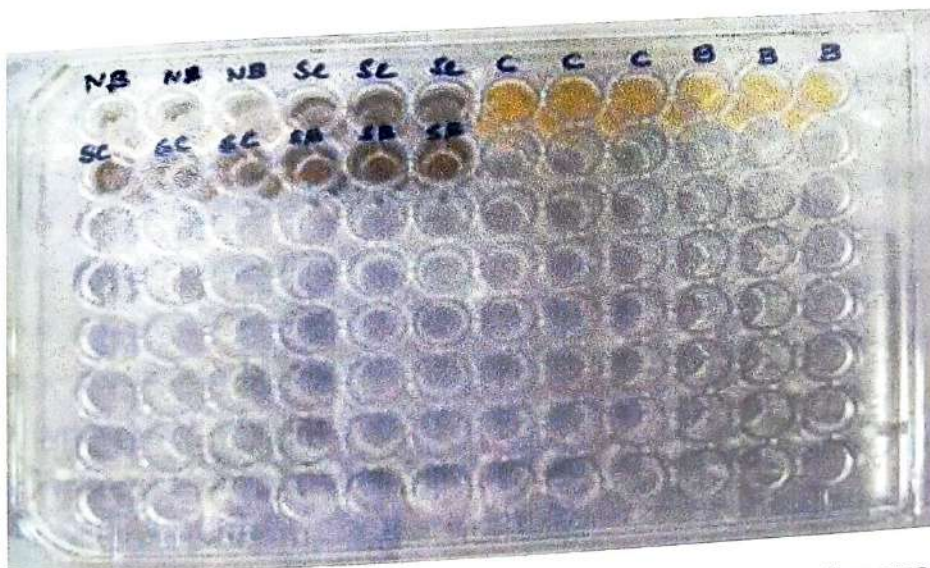


Fig 5.14: Bacterial cultures are suspended in 96 well plates for growth kinetics

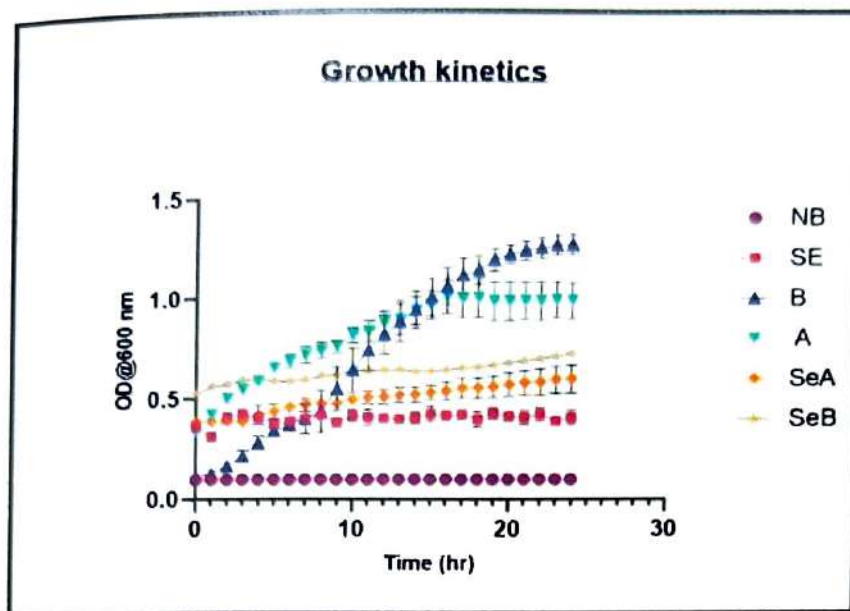


Fig 5.15: Growth kinetic curve for metal degrading bacteria

The growth kinetics of bacteria in metal-enriched media broth were characterized by monitoring optical density (OD) over time using spectrophotometry. Initially, bacterial growth followed a lag phase, during which cells acclimated to the new environment. Subsequently, exponential growth ensued, characterized by a rapid increase in OD as bacteria proliferated. The duration and slope of the exponential phase varied depending on the metal concentration in the media. Higher metal concentrations often led to shorter lag phases and higher growth rates initially, followed by a plateau phase as the media became depleted of nutrients or became toxic due to excessive metal accumulation. These results suggest that metals influence bacterial growth dynamics by modulating nutrient availability, metabolic activity, and cellular stress responses. The growth kinetics of bacteria in metal-enriched media revealed intriguing patterns. Initially, at lower concentrations of metals, bacterial growth followed a typical exponential phase, characterized by rapid cell division and biomass accumulation. However, as the metal concentration increased, we observed a dose-dependent inhibition of bacterial growth. Higher metal concentrations led to prolonged lag phases, reduced growth rates, and ultimately, inhibited growth during the stationary phase. Some bacterial strains exhibited varying degrees of metal tolerance, with certain species

displaying greater resilience to metal toxicity compared to others. Additionally, we observed that specific metals exerted differential effects on bacterial growth, suggesting that the type and concentration of metal ions play critical roles in shaping microbial responses. Overall, we underscore the complex interplay between metals and bacterial growth dynamics. (Venkata Rao Krishnamurth et al., 2020).

The growth curve was plotted using the obtained values. The microplate reader is set to 600 nm for the fluorescent proteins and readings are taken every 10 mins for 16 hrs but the OD value is which was performed was same and the equipment was set to take readings for every 1 hr so there were 25 readings in total.

5.7 AAS:

Atomic Absorption Spectroscopy (AAS) was employed to analyze the metal concentrations in the growth media and elucidate their impact on bacterial growth. Through AAS, precise measurements of metal ions such as zinc, copper, iron, and manganese were obtained, allowing for the characterization of metal-enriched media broth used in bacterial culture experiments. The results revealed significant variations in metal concentrations across different experimental conditions, providing crucial insights into the relationship between metal availability and bacterial growth dynamics. Moreover, AAS analysis facilitated the identification of threshold levels of metal toxicity, beyond which bacterial growth was markedly inhibited. These findings highlight the utility of AAS as a powerful analytical tool for studying metal-microbe interactions and emphasize its importance in elucidating the mechanisms underlying metal toxicity in microbial systems (Mardiyono et al., 2019).

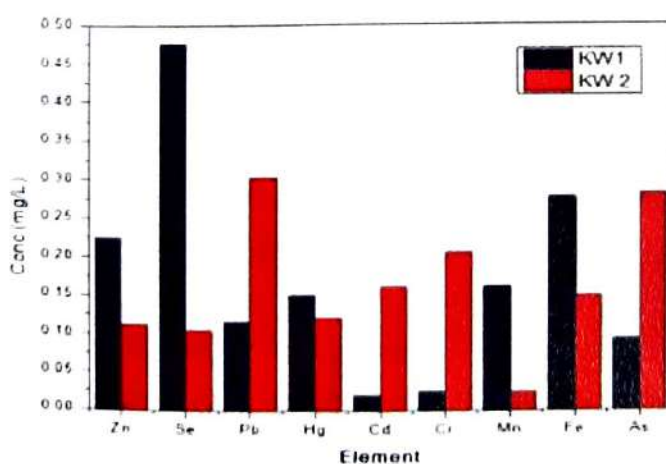


Fig 5.16: Presence of Heavy metal in water

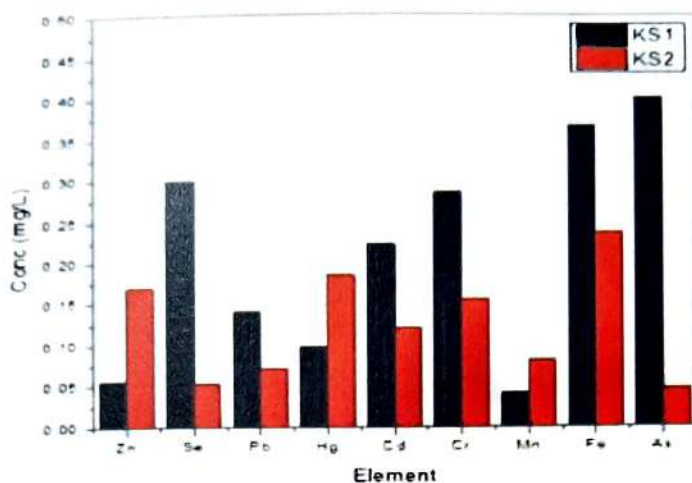


Fig 5.17: Presence of Heavy metal in soil

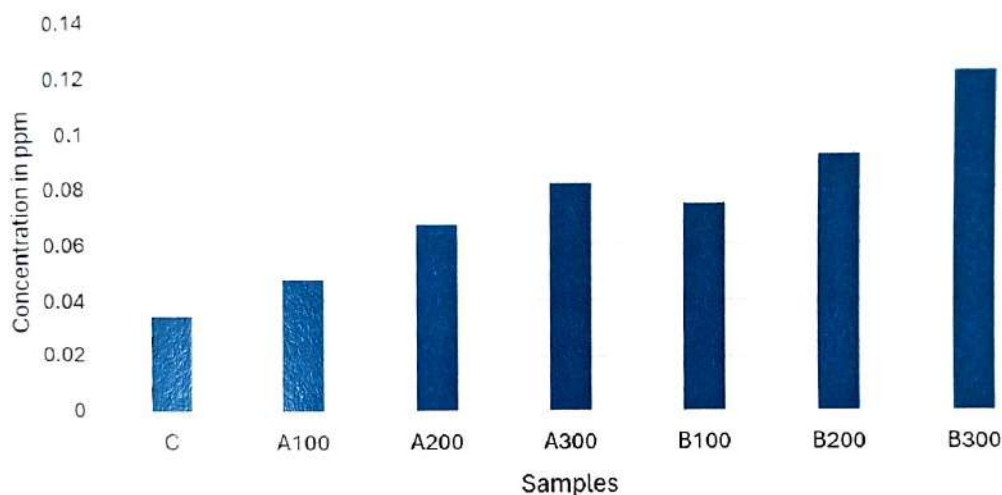


Fig 5.18: Presence of Se in digested sample due to metal absorption by bacteria

The absorption of metals by bacteria has been proven through atomic absorption spectroscopy (AAS), a widely used analytical technique. AAS allows for the quantification of metallic elements in a sample by measuring the absorption of specific wavelengths of light. Through this method, it has been demonstrated that bacteria can indeed absorb metals from their surroundings. This conclusion underscores the pivotal role bacteria play in environmental remediation processes. The objective was to add the wastewater sample to the different concentrations of bacteria, which shows results like before and after remediation percentages of metals. The methodology was modified as required and the results were obtained.

CHAPTER-6

SUMMARY AND CONCLUSION:

The isolation of bacteria from the Kovalam River mouth for bioremediation purposes presents a promising avenue for environmental restoration. The successful isolation of bacteria from such a specific and potentially polluted location underscores their adaptation to local environmental conditions, enhancing their efficacy in bioremediation efforts. Overall, the utilization of bacteria isolated from the Kovalam River mouth holds great promise for mitigating pollution and restoring ecological balance in aquatic ecosystems. The growth kinetics of these bacteria are crucial for optimizing bioremediation processes. In the context of heavy metal bioremediation, the lag phase may be prolonged as bacteria adapt to the toxic metal environment. Once adopted, exponential growth occurs as bacterial populations rapidly increase, utilizing heavy metals as energy sources or sequestering them through biosorption mechanisms. The stationary phase ensues as resources become limited or toxic metal concentrations reach inhibitory levels. Death phase occurs when cells begin to die due to nutrient depletion or metal toxicity. Bioremediation, employing bacteria to mitigate heavy metal pollution, stands as a promising eco-friendly approach to tackle environmental contamination. Atomic Absorption Spectroscopy (AAS) plays a pivotal role in quantifying heavy metal concentrations before and after the bioremediation process. Bacteria possess inherent mechanisms to either immobilize metals or transform them into less toxic forms, thereby rendering them less hazardous to the environment. Moreover, AAS enables the determination of metal uptake rates by bacterial cultures, facilitating the optimization of bioremediation strategies. The application of bacterial bioremediation coupled with AAS analysis offers a sustainable solution for mitigating heavy metal pollution, paving the way towards cleaner and healthier ecosystems.

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