The Role of Bacteria in Bioremediation of Heavy Metals

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DECLARATION BY THE STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "The Role of

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This is to certify that the dissertation report "Bioremediation of Heavy Metals Using Bacteria" is a bonafide work carried out by Ms. Anushka Arjun Kaskar under my supervision in partial fulfilment of the requirements for the award of the degree of Master's in Science in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

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1. Introduction

Bioremediation is the process of utilizing naturally occurring microorganisms to convert toxic pollutants into less toxic forms. Heavy metal compounds are often toxic, carcinogenic, or mutagenic, even in small concentrations. While there are several methods for removing heavy metals, such as chemical precipitation, oxidation or reduction, and membrane technology, these methods are often ineffective when the concentrations are less than 100mg/L. (Ahluwalia, S.S, & Goyal, 2007)additionally, heavy metal salts are often watersoluble and cannot be separated through physical methods. (Hussein & Moawad, 2004) In cases where physio-chemical methods are not feasible due to low concentrations or high costs, bioremediation using microbes is a viable and eco-friendly alternative. This technique is a sustainable remediation approach that can restore soil to its natural state. (Kapoor, 1995)

Introducing heavy metals into soil or water causes significant changes in the microbial community. (Doelman & Jansen, 1994) This is mainly due to the obstruction of crucial functional groups, displacement of vital metal ions, or modification of active conformations of biological molecules. (wood, J.M, & Wanng, 1983)The response of microbial communities to heavy metals is determined by the type of metal, the characteristics of the medium, and the species of microbes present. (Akinci G)Additionally, the concentration and accessibility of heavy metals also play a crucial role in these modifications. (Li, F, & Tan, 1994)

In today's world, with the development of countries, there is rapid industrialization that results in the discharge of a large amount of industrial waste containing heavy metals into soil and water. The release of heavy metals into water systems poses a significant health concern as these metals accumulate and cannot naturally breakdown into non-toxic forms. Therefore, it is crucial to identify organisms and strategies for bioremediation of specific metals. Many metals released by industries are toxic even at low concentrations, such as arsenic, cadmium, chromium, copper, lead, mercury, nickel, selenium, silver and zinc. These metals are not only cytotoxic but also have carcinogenic and mutagenic properties. (Salem, H.M, & Eweida, 2000)

species that help in bioremediation of heavy metals are *Flavobacterium,Pseudomonas, Arthrobacter, Methosinus, Bacillus, Corynebacterium, Rhodococcus, Mycobacterium* etc. They posses the capability to adapt to and withstand heavy metal toxicity in contaminated areas.

Bacteria carry out the process of bioremediation of heavy metals through various mechanisms, such as redox processes, adsorption, complexation, ion-exchange, precipitation, and electrostatic attraction, Bioremediation is made possible by converting or transforming an insoluble and immobile form of an element in sediments into a mobile and soluble form. (Perfumo, IM, & R, 2007) Heavy metal biomethylation is an important process in soil and water that can modify the toxicity, volatility, and mobility of heavy metals. Microbes excrete metabolites like carboxylic acids and amino acids that serve as chelators of metal ions, which is also considered an important mechanism in bioremediation.

Microorganisms have the ability to immobilize metals and serve as a reservoir for them through various mechanisms such as biosorption, bioaccululation, bioconversion, and inter/intracellular precipitation (such as the formation of oxalates of Zn, Cu, Co, Cd, Ni) that can be implemented in-situ or ex-situ (Gadd, 2000) (Lim, P.E., & Mohamed, 2003) (A, 2004). (Lin & C.C., 2005) These mechanisms operate in different ways where microbes can actively uptake heavy metals through bioaccumulation or passively bind them through adsorption. (Hussein, H, & Krull, 2001)

1.1 Sources of heavy metals in the environment :

Heavy metals are present in the environment both naturally, from weathering of parent materials, and through human activities, as shown in Fig.1. The most significant natural sources are weathering of minerals, erosion and volcanic activity. Anthropogenic sources, on the other hand, are caused by human activities such as smelting, mining, electropolating, use of pesticides and phosphate fertilizer discharge, as well as the application of biosolids. (Wuana & R.A., 2011) (Modaihsh & Mahjoub, 2004) (Chehragani & Malayeri, 2007) (Fulekar, Singh, & Bhaduri, 2009) Human interference with the natural geochemical cycle of metals results on the accumulation of one or more heavy metals in soil and water. At levels exceeding defined thresholds, this accumulation poses a risk to human health, plants , animals, and aquatic biota.

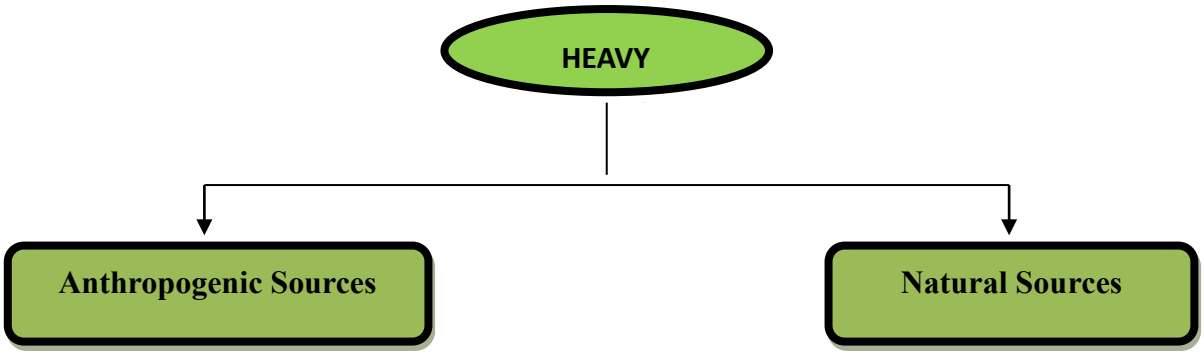

Fig.1: Heavy metal sources

1.2 Bioremediation: Introducing Microbe Based Clean Up System

Bioremediation is a process that uses microbes to clear or immobilize contaminants like hydrocarbons, agrochemicals, and other organic toxicants in metal- contaminated areas such as soil, sediments, and water. However microbes are not capable of breaking down inorganic toxic compounds like heavy metals into harmless ones so they need to be used based on their specialization for the type of contaminants. The effectiveness of bioremediation for heavy metals relies on the ability of microorganisms to metabolize them. Different microorganisms have varying requirements for heavy metals as essential micronutrients for their growth and development. For instance, Fe^{+3} is essential for all bacteria, while Fe^{+2} is important for anaerobic bacteria. (Ahmed & M, 2014) Nonetheless, the adsorption capacity of bacteria is influenced by the microbial total biomass and the geochemistry of the system. (Garbisu, C, & Alkorta, 2001)

In some cases, certain oxyanions of metals do not interact with microbes, and their bioremediation relies on catalyzed redox conversion to insoluble forms. The ability of microbes

to perform reduction or oxidation reactions is determined by their enzymatic activity and biomass concentration. Microbes have immense potential for remediating soil pollutants, particularly those in the rhizosphere. To restore soil health, rhizospheric microbes should be selected based on their ability to adsorb and mobilize heavy metals and trace elements in the soil. Microorganisms can also act as metal accumulators, which is a novel property for the remediation of toxic metals in soil. Microbial remediation is a safe, easy, and effective technology for detoxifying and rehabilitating contaminated soil. Native soil microorganisms have been studied and isolated for their ability to remove or detoxify various toxic products resulting from human activities like mining of ores, organic solvents, pesticides, pigments, plastic, oil and gas extraction, fuel and industrial processes. However, successful execution of this technology is limited by the lack if knowledge about how microbes interact with and utilize trace element and heavy metal pollutants. (Garbisu, C, & Alkorta, 2001)

1.3 Mechanisms of Bioremediation

Microorganisms are present everywhere and dominant in heavy metal-contaminated soil and water, capable of converting heavy metals into non-toxic forms. In bioremediation processes, microorganisms break down organic contaminants into end-products like carbon-dioxide and water, or metabolic intermediates that serve as primary substrates for cell growth. Microbes can produce degradative enzymes for target pollutants as well as resistance to relevant heavy metals, providing two-way defense. Different bioremediation mechanisms include biosorption, metal-microbe interactions, bioaccumulation, and bioleaching. Microbes remove heavy metals from soil and water by utilizing chemicals for growth and development. They are capable of dissolving metals and oxidizing or reducing transition metals. Microbes can restore the environment by oxidizing, binding, immobilizing, volatilizing, and transforming heavy metals. By understanding the mechanisms controlling microorganism growth and activity in contaminated sites, their metabolic capabilities, and their response to environmental changes, bioremediation can be successfully implemented through a designer microbe approach. Many contaminants like organic solvents can disrupt membranes, but cells may evolve defense mechanisms such as outer cell-membrane protective material or hydrophobic or solvent efflux pumps. (Sikkema, J., Bont, & J.A., 1995)For example, plasmid-encoded and energy-dependent metal efflux systems involving ATPases and chemiosmotic ion/proton pumps have been reported for As, Cr, and Cd resistance in many bacteria. (Roane, T.M., & Pepper, 2000)

1.3.1 Bioremediation by adsorption

Microbes can absorb heavy metals by binding them to specific sites within their cellular structure, without expending cellular energy. (Guine; V; Spadini; L; Sarret;, 2006) One important component involved in this process is the extracellular polymeric substance (EPS) found in the bacterial cell walls. EPS is known to have a significant impact on the adsorption of heavy metals due to its acid-base properties. Research on the metal binding behaviour of EPS has shown that it can effectively complex heavy metals through various mechanisms, such as proton exchange and micro-precipitation. (Comte; Guibaud;, 2008) (Fang, L.C., & Huang, 2010) Biosorption by bacteria is an inexpensive and efficient technique for removing pollutants, including non-biodegradable elements like heavy metals, from wastewater or polluted water. The efficiency of biosorption depends on the type of heavy metal and the bacterial species involved, which have different cellular structures that contain functional groups capable of binding heavy metal ions. These functional groups include carboxylate, hydroxyl, amino and phosphate groups, which are present in the bacterial cell wall along with polysaccharides, lipids, and proteins. The bacterial cell wall is the primary physical contact point linking metal ions to the bacterial biomass, and the anionic functional groups present in both Gram-positive and Gram-negative bacteria, such as amine, hydroxyl, carboxyl, sulphate and phosphate, give the cell wall its metal binding capacity.

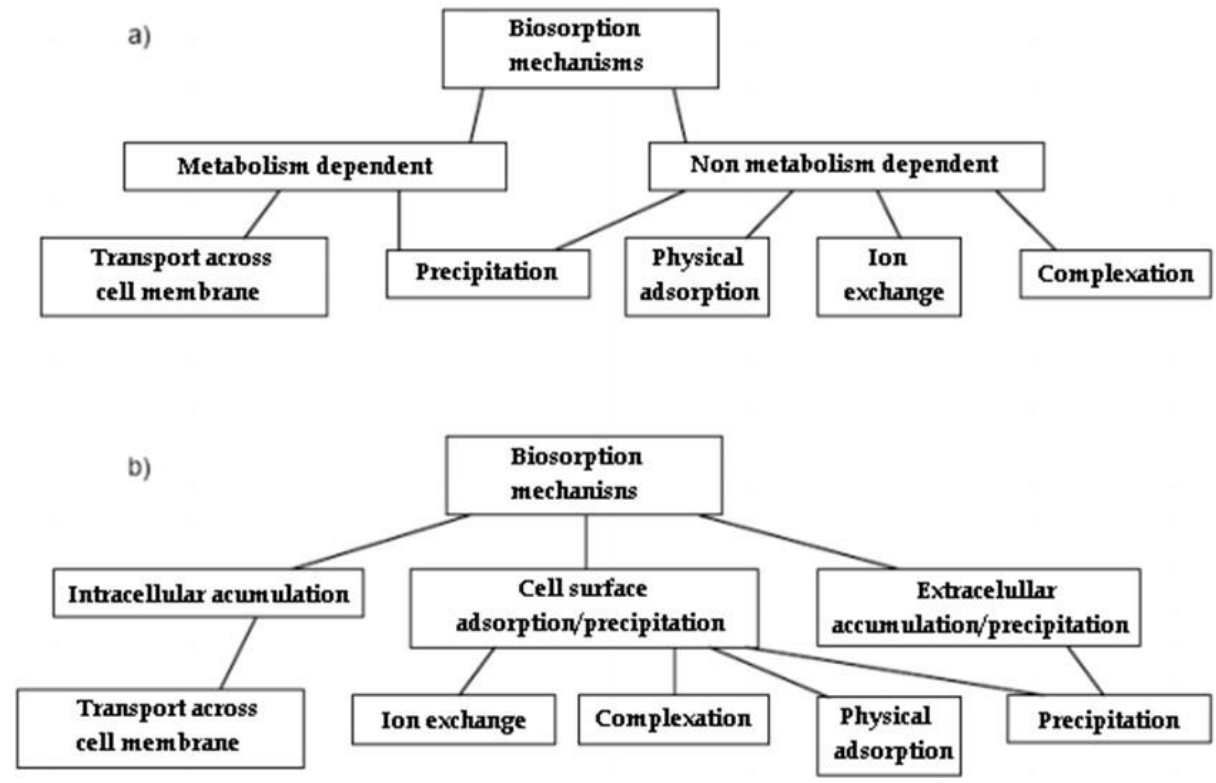

Fig. 2: Mechanism of Biosorption ((Aquino, 2011)

1.3.2Bioremediation by Physio-Bio-Chemical Mechanism

Bioremediation is a process that involves a biosorbent having a higher affinity for metal ions (sorbate). (Das & Vimala, 2008)This affinity continues until an equilibrium is reached between the two components. For instance, *Saccharomyces cerevisiae* can act as a biosorbent for removing Zn (II) and Cd (II) through ion exchange. (Chen, C, & Wang, 2007) (Talos, K, & Pager, 2009) Energy is required for the cell metabolic cycle during heavy metal degradation. The combination of active and passive modes of toxic metal bioremediation is called bioaccumulation. (brierly & C.L., 1990) Microbes can take up hydrophobic contaminants by secreting biosurfactants and directly associating with the cell contaminant. Biosurfactants form stronger ionic bonds with metals due to their low interfacial tension and form complexes before being released from the soil matrix into the water phase. (Thavasi & R., 2011)

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Bioremediation can include either aerobic or anaerobic microbial activities. In aerobic degradation, oxygen atoms are introduced into the reactions through various enzymes, such as monooxygenases, dioxygenases, hydroxylases, oxidative dehalogenases, or chemically reactive oxygen atoms generated by enzymes such as ligninases or peroxidises. Anaerobic degradation of contaminants includes initial activation reactions followed by oxidative catabolism mediated by anoxic electron acceptors. Immobilization is a technique used to reduce the mobilization of heavy metals from contaminated sites by changing their physical or chemical state. Solidification treatment involves precipitation of hydroxides or mixing of chemical agents at the contaminated sites. (Evanko, C.R., & Dzombak, 1997) Microbes can mobilize heavy metals from contaminated sites through leaching, chelation, methylation, and redox transformation of toxic metals. Although heavy metals cannot be completely degraded, the process transforms their oxidation state or organic complex, making them less toxic and water-soluble, and precipitating them. (Garbisu, C, & Alkorta, 2001) Microorganisms utilize heavy metals and trace elements as terminal electron acceptors or reduce them through detoxification mechanisms, which remove metals from the contaminated environments. Microorganisms remove heavy metals through mechanisms employed to obtain energy from metal redox reactions or to deal with toxic metals through enzymatic and non-enzymatic processes. The two main mechanisms for developing resistance in bacteria are detoxification,

which involves transforming the toxic metal state to make it unavailable, and active efflux pumping of the toxic metal from cells. (Silver, 1996)
In soil, microorganisms participate in redox reactions with toxic metals where they act as oxidizing agents and cause the metals to lose electrons. The electrons are then accepted by other electron acceptors like nitrate, sulphate and ferric oxides.

Under aerobic conditions, oxygen acts as an electron acceptor, while under anaerobic conditions, microorganisms use alternative electron acceptors to oxidize organic contaminants. Microorganisms obtain energy for growth by oxidizing organic compounds with electron acceptors such as Fe (II) or Mn (IV). (Lovely, D.R., & Phillips, 1988) Anaerobic degradation of organic contamination can be enhanced by increasing the availability of Fe(III) as an electron acceptor for microbial reduction. (D.R. & Coates, 1996) (Spormann, A.M., & Widdel, 2000)This process is referred to as dissimilatory metal reduction. (Lovely & D.R., Dissimilatory metl reductiom: from early life to bioremediation, 2002).Microorganisms can also reduce the oxidation state of metals, thereby changing their solubility, and facilitate the biodegradation of chlorinated contaminants through reductive dechlorination. In this process, the contaminants, such as chlorinated solvents, act as electron acceptors during respiration by microorganisms. (Lovely, D.R., & Philips, Microbial reduction of uranium., 1991)

Various defense mechanisms are employed by microorganisms to alleviate the stress caused by toxic metals, including exclusion, compartmentalization, complex formation, and synthesis of binding proteins and peptides. (Gomez & Moliternib, 2011) (Cobbett & Goldsbrough, 2002). The accumulation of heavy metals by microorganisms can be analyzed by examining the expression of metal binding proteins and peptides, such as phytochelatins and metallothioneins. These metal binding proteins are known to play a role in hormone and redox signalling processes in response to exposure to toxic metals like Cd, Zn, Hg, Cu, Au, Ag, Co, and Bi. For example, *Synechococcus* sp., a type of cyanobacterial strain, has been shown to express the smtA gene and produce metal-binding proteins. (Huckle, J.W., & Morby, 1993) In *Escherichia coli*, the expression of different proteins and peptides regulates the accumulation of cadmium. (Mejare & Bulow, 2001). Microorganisms also possess natural pathways to resist heavy metals, such as Hg and Ar, which are regulated by metalloregulatory proteins. (Singh, Kang, & Mulchandani, 2008)

Following are some of the heavy metals used in this study:

1.4 Copper

Copper (Cu) is an important micronutrient for all living organisms, yet it can become toxic at low concentrations. Human activities like mining and use of fungicides have led to copper contamination in soil, water, and sediment at levels that can exceed the safe limit. Therefore, it is important to use bioremediation to reduce the environmental risk of copper contamination. (Kumar & Varma, 2016)

Microorganisms, particularly bacteria, can mobilize or immobilize Cu through different mechanisms such as :

- Bioleaching for the recovery of copper from copper- bearing solids • Bioimmobilization to prevent the leaching of Cu into groundwater, and
- Biosorption of Cu by microorganisms.

1.5 Zinc

Zinc and its compounds are commonly found in the earth's crust, rocks, minerals, and carbonate sediments. Through weathering, soluble forms of zinc may be released into water. (Drinking water and health, 1980)Metallic zinc has low bioavailability and does not pose much risk to the environment. However, when zinc reacts with certain substances like oxygen and acids, it can create compounds that are harmful to living organisms. (GJ, 1990)Therefore, it is important to conduct bioremediation of zinc to mitigate the potential environmental hazards associated with its contamination. (A, 2004)

1.6 Cobalt

Cobalt is a silver-grey metal that can exist in different oxidation states, but in aquatic environments, the +2 and +3 valence states are more common and form various organic and inorganic salts. (Handbook of Chemistry and Physics, 1968) In freshwater, cobalt is typically found in forms such as Co^{+2}, carbonate, hydroxide, sulphate, and adsorbed forms, as well as in oxide coatings and crystalline sediments. (Durum & W.H., 1960) In marine water, cobalt usually exists as Co^{+2}, chloride, carbonate, and sulphate. Many marine algal species, including diatoms, chrysophytes, and dinoflagellates, require cobalt for their growth, and microorganisms

need it for nitrogen fixation in legumes. (Smith & Carson, 1981) However, excessive amounts of cobalt can be harmful to humans, as well as to aquatic and terrestrial animals and plants. Therefore, it is essential to reduce its concentration to avoid pollution in water systems.

1.7 Manganese

Manganese is not found in its elemental form in nature, but rather in the form of oxides, carbonates, and silicates in over 100 natural minerals. While manganese has 8 possible oxidation states, only the +2 state can exist as a free ion in aqueous solutions and natural water systems (H.J.M., 1979). It is an essential microelement for microorganisms, plants, and humans, but overexposure to high levels can make it toxic. Actual weathering of rocks and anthropogenic sources such as metal processing and chemical industries can cause manganese contamination (O'neal & Zheng, 2015) (Crossgrove & Zheng, 2004). Various techniques, including ion exchange and biological oxidation, are currently being used to remove manganese from water sources. Biological methods are preferred due to their eco- friendliness and low cost. These biological processes involve the biological oxidation of soluble Mn(II) to Mn(III) and Mn(IV), which precipitate as biogenic Mn oxides (Beukes & Schmidt, 2012) (Barboza & Amorim, 2015). Both pure cultures and mixed consortia of bacteria can remove manganese, with some bacterial strains showing high Mn removal efficiency even at low pH levels. (Akob & et.al, 2014)

1.8 Iron

Iron is abundant in the earth's crust and its presence in natural water varies depending on the local geology and chemical composition of the water. The most common oxidation states of iron in water are Fe^{+2} and Fe^{+3}, although other forms may be present in polluted water. In surface waters, iron is mostly in the ferric state, while ferrous forms may be present in reducing waters. While iron is an essential trace element for plants and animals, high concentrations in water can be toxic to aquatic insects. Acute toxicity has been on served at iron concentrations ranging from 320 to 16,000µg/L, indicating the need for removal if present in high concentrations.

2. Literature Review

Environmental pollution caused by heavy and toxic metals from industries such as mining and metallurgy is a serious problem for both humans and the environment. A study was conducted by (Chang & jung, 2016) to see how a mixture of bacteria could help clean up contaminated soils containing lead, cadmium and copper. The bacteria mixture was found to be more effective than using a single strain of bacteria. The bacterial mixtures showed higher growth rate, urease activity, and resistance to heavy metals. Four bacterial strains were isolated and identified from bacterial mixtures, and they were found to be effective in removing heavy metals from contaminated soils. The bacterial mixtures were able to remove 98.3% of lead, 85.4% of cadmium, and 5.6 % of copper after 48 hours. The use of bacterial mixtures is a useful way to clean up heavy metals from contaminated environments. This method is effective, efficient, and economical, and it does not disturb the target environment.

Heavy metals, a treasure of nature, turns to be toxic at high concentrations in water. Among several methods adopted to alleviate heavy metal pollution, bioremediation is considered to be a sustainable, cost-effective technology. According to (P.R. & Sreedevi, 2022). Bioremediation largely relies on bacteria, apart from other microbes and plants. The inherent and adaptive mechanisms evolved in bacteria to defend the metal toxicity include bioadsorption/biosorption, bioaccumulation, bioprecipitation and bioleaching. Heavy metal resistant bacterial strains are easy to culture and maintain, and even dead cell biomass display high heavy metal remediation potential in solution. All the heavy metal remediation mechanisms exhibited by bacteria in water is comprehensively reviewed with recent research outputs and in-situ and ex-situ techniques. The cellular mechanisms of heavy metal remediation are discussed, considering efficient bacterial strains, physiochemical parameters, nutrient supplementation and design of

novel microbial techniques. Research at omics level would effectuate further manipulation of the cellular process and increase its efficiency. Bacterial heavy metal remediation technique provides double benefit of metal recovery and water purification, along with reuse prospects for both water and metal resources. Technological intervention could meet the challenges of process acceleration, resist biofouling, compete with native wild bacterial species in wastewater, design for commercialization. Industrial translation of the technology is the pivotal avenue to be tackled. Ultimately, understanding of bacterial heavy metal remediation process is essential for the implementation of this promising technology to safeguard the environmental health.

Heavy metals from industrial wastewater can pollute the environment, but a new study by (Sharma & Jasrotia, 2021) shows that a mixture of two bacterial strains, *Bacillus cereus* and *Bacilluss pumilus* can remove almost all of the lead and nickel ions from the wastewater. These bacteria form complexes with the metal ions and absorb them, leading to their removal from the water. The study monitored factors such as pH and metal concentration to better understand the process.This approach could be used commercially to clean up heavy metal pollution in wastewater. The mixture of bacteria worked together to efficiently remove metal ions, resulting in upto 95.93% removal of lead and 95.54 % removal of copper.

(Monika, Priyadarshanee, & Das, 2021) in her artilcle discusses the issue of heavy metal pollution caused by industrialization and the need for safe and effective methods for heavy metal removal and recovery. The conventional remediation methods are expensive and produce toxic by products, which are harmful to the environment. Therefore, the use of biological agents like bacteria, algae, yeasts and fungi has gained more attention due to their high removal efficiency, low cost, and availability. Among these agents, bacterial biosorption is considered the safest treatment method for toxic pollutants, especially heavy metals that are not easily biodegradable.

Metal tolerating bacteria are capable of binding cationic toxic heavy metals with negatively charged bacterial structures and live or dead biomass components. These bacterial biomasses acts as efficient biosorbents for metal bioremediation under multimetal conditions due to their large surface area to volume ratio. The review discusses the biosorption potentials of bacterial biomass towards different metal ions, cell wall constituent, biofilm, extracellular polymeric substances (EPS) in metal binding, and the effect of various environmental parameters influencing the metal removal.

The artcle also explains suitable mathematical models of biosorption and their application to understand and interpret the adsorption process. Additionally, the review summarizes different

desorbing agents and their utilization in heavy metals recovery and regeneration of biosorbent . Overall, the article provides a comprehensive review of biosorption and removal of toxic heavy metals by metal tolerating bacteria for bioremediation of metal contamination. (Feliphe & Julio, 2017) examines the use of bacterial bioremediation as a promising strategy to address metal pollution resulting from anthropogeic activities. The study contains the comparison and discussion of the scientific advancements in the potential of bacteria for metal bioremediation in aquatic ecosystems from 2000 to 2016 using PubMed, MEDLINE, and SciELO databases. The review highlights the importance of bioremediation for metal- contaminated industrial wastewater discharge sites, with biosorption being the most commonly studied bioremediation mechanism. The study also identifies Gram negative *Pseudoman aeruginosas* and Gram positive *Bacillus subtilis* bacteria as having the greatest potential for metal bioremediation. The review notes that the most studies focused on understanding the microbial mechanisms involved in metal metabolism and/ or resistance, with *Chromobacterium violeceum* being the most studied microorganism. The findings of this review demonstrates the relevance of bacterial bioremediation for reducing metal contaminant loads in impacted areas and the growing importance of biotechnological solutions to environmental issues.

(M.H., Fulekar, & Sharma, 2012) aims to investigate the potential of a biostimulated microbial culture in remediating heavy metal contaminants from a waste disposal site located in Bhayander (east) Mumbai, India. The physicochemical and microbial characteristics, including heavy metal contaminants, were assessed at the site, and microorganisms adapted to the heavy-metal contaminated environment were isolated, cultur and biosmulated in a laboratory bioreactor under aerobic conditions. The efficacy of te biostimulated microbial consortium in remediating heavy metals such as Fe, Cu and Cd was studied in a bioreactor, with concentrations of 25, 50, and 100µg/mL, over a period of 21 days. The results showed that the biostimulated microbial consortium was effective in remediating heavy metals, with upto 98.5%, 99.6% and 100% mg/L. The study also monitored environmental parameters such as pH, total alkalinity, electronic conductivity, biological oxygen deman, and chemical oxygen demand during the bioaccumulation of heavy metals by microorganisms. The pilotscale study suggests that this approach could be applied to remediate heavy metals from waste disposal sites, thereby contributing to environmental cleanup efforts.

(Oh, 2023) evaluates the feasibility of applying passive treatment methods to treat mine water discharged from the sulphur mine site in South Korea, which contains copper and zinc exeedng the permissible discharge limit. Bench-scale experiment using columns simulating successe alkalinity-producing systes (SAPS) and bioreactors were conducted with limestone and spent

mushroom compost (SMC) as substrate materials, and their structures and mixing ratios varied. The efficiency of metal removal for each column was evaluated and the results showed that SAPS and bioreactors exhibited high Cu removal efficiencies, whike Zn removal efficiencies were high except for re-dissolution events. Bacterial sulphate reduction (BSR) reactions facilitated by the pH increase caused by limestone dissolution were effective at removing metal in the SAPS and bioreactor columns, with sulphide present in the effluent as evidence. Columns with greater SMC ratios had higher removal efficiencies and alkalinity, demonstrating the importance of SMC in metal removal. The study suggests that waste materials such as SMC can be reused in treating Cu and Zn rich mine water through bacterial metal reduction reactions, and the potential lifespan of the treatment facility should be considered. Overall, this literature review provides valuable insights into on-site treatment methods for mine water contaminated with heavy metals.

(Hanjun & Luo, 2010) focused on the bioremediation pontial of the multimetal resistant endophytic bacterium *Bacillus* sp. L14, isolated from the cadmium hyperaccumulator Solanum nigrum L. The study found that EB L14 exhibited hormesis, a beneficial response, in the presence of divalent heavy metals (Cu(II), Cd(II), and Pb (II)) at a relatively lower concentration (10mg/L). The abnormal activity increases of ATPase, which provides energy to help EB L14 reduce the toxicity of heavy metals by exporting the cations, led to this hormesis effect. Within 24 hours of incubation, EB L14 demonstrated high specific uptake efficiencies for Cd(II), Pb(II), and Cu (II), but not for chromium. The mechanism study suggested that inhibiting ATPase activity could significantly enhance the remediation efficiencies of this endophyte. The excellent adaptation abilities and promising remediation efficiencies of EB L14 make it a potential candidate for developing efficient metal removal systems for low concentrations of heavy metals.

Old nonferrous factories have left many areas in Europe contaminated with metals like Zn,Cd,Cu, and Pb. Different methods have been developed to clean up the soil, but most result in the soil being turned into sand. This review by (L. & Diels, 1999) presents a new method for cleaning up sandy soil contaminated with heavy metals, using a bacterium called *Alcaligenes eutrophus* CH34. This bacterium can make the metals more soluble and absorb them into its biomass, which can then be separated from the water using a process called flocculation. The method was successful in reducing the concentration of Cd,Zn, and Pb in the soil, while also decreasing the bioavailability of the metals as measured by biosensors. (Pal & Paul, 2008) focuses on the exreacellular polymeric substances (EPS) of microbial origin, which are complex mixtures of biopolymers that comprise polysaccharides, proteins, nucleic acids, uronic

acids, humic substances, and lipids. EPS formation occurs in a wide variety of free-living bacteria as well as microbial aggregates such as biofilms, bioflocs, and biogranules, resulting from bacterial secretions, shedding of cell surface materials, cell lysates, and adsorption of organic constituents from the environment. EPS may be loosely attached to the cell surface or bacteria may be embedded in EPS. Compositional variation exists among EPS extracted from pure bacterial cultures and heterogenous microbial communities, which are regulated by the organic and inorganic constituents of the microenvironment.

Functionally, EPS aid in cell- to-cell aggregation, adhesion to substratum, formation of flocs, protection from dessication, and resistance to harmful exogenous materials. EPS also serve as biosorbing agents by accumulating nutrients from the surrounding environment and play a crucial role in biosorption of heavy metals. EPS is polyanionic in nature, forming complexes with metal cations that result in metal immobilization within the exopolymeric matrix. These complexes generally result from electrostatic interactions between the metal ligands and negatively charged components of biopolymers. Moreover, enzymatic activities in EPS assist in the detoxification of heavy metals by transformation and subsequent precipitation in the polymeric mass.

Although the core mechanism for metal binding and/ or transformation using microbial exopolymer remains identical, the existence and complexity of EPS from pure bacterial cultures, biofilms, biogranules, and activated sludge systems differ significantly, affecting the EPS- metal interactions. This paper presents the features of EPS from various sources, aiming to establish their role as central elements in bioremediation of heavy metals.

AIM : To isolate and identify the bacteria responsible for carrying out bioremediation of heavy metals such as Cu, Co, Zn, Mn, and Fe and to apply it to the metal polluted environment inorder to reduce the metal concentration present within the environment that may be toxic to living organisms.

OBJECTIVES:

1. To isolate the bacterial cultures from the sample
2. To carry out metal assay of the isolated cultures
3. To select the bacterial culture which shows highest amount of metal tolerance
4. To extract the enzyme responsible for bioremediation of heavy metal
5. To carry out protein profiling of the extracted bacterial cultures
6. To check the growth rate of the cultures in presence of metals

7. To check the mechanism of bioremediation carried out by the selected bacterial cultures
8. To apply this isolated culture into the polluted area inorder for the bioremediation process to take place.

3. Materials and Methods

3.1 Sample collection

Mangrove soil and water sample were collected at 4 different points of Old Goa mangroves. Water samples were collected in clean bottles and soil samples at depth of around 2-5cm from the surface were collected in sterile zip lock bags using sterile spatula. Samples at 4 different locations within the Mantry Villa Old Goa mangrove, (15°29'59.5"N and 73°52'55.3"E) were collected and brought to the marine microbiology laboratory, SEOAS, Goa University and stored in refrigerator until further use

Fig. 3: Location of the sampling site

3.2 Isolation of bacterial strains

After getting the samples to the laboratory, it was labelled as sample 1, 2, 3 and 4 for the soil sample and A, B, C and D for the water sample depending on the 4 different locations. Serial dilutions of all the soil samples were carried out using 0.5gm of soil in 4.5mL of saline. 10 fold dilutions were carried out and the last 3 dilutions were spread plated on the nutrient agar plates. For water sample, 0.1mL of the sample was directly spread plated on nutrient agar plates.

3.3 Selection of bacterial strains

After 24-48 hours, the growth was seen on all the plates. The number of colonies on each plates were counted using viable count method. Fifty one different colonies showing different colony characteristics were selected from the plates to carry out further analysis.

3.4 Purification of the bacterial strains

Subculturing of the selected bacterial strains were carried out on nutrient agar slants. 35 bacterial strains were streaked on the agar slants and 16 bacterial strains which showed spreaded growth were streaked on nutrient agar plates (quadrant streaking). Purification was done by subculturing the strains twice.

3.5Screening for metal tolerance

3.5.1 Screening in broth

Screening of bacterial strains for metal tolerance were carried out using $CoCl_2.6H_2O$ (Cobaltous Chloride Hexahydrate), $ZnCl_2$ (Zinc Chloride), $FeCl_3.6H_2O$ (Ferric Chloride Hexahydrate), $CuCl_2.2H_2O$ (Copper II Chloride dehydrate), and $MnCl_2.4H_2O$ (Manganese II Chloride tetrahydrate) as the metal solutions. 10% of each of the metal stock was prepared by adding 1g of metal to 10mL of autoclaved distilled water each. 550mL of Zobell Marine Broth (ZMB) was prepared by adding 22.1375g of ZMB powder to 550mL of distilled water, autoclaved and dispersed 10mL each in 51 autoclaved testubes under sterile conditions. 10µL (0.01%) of metal stock is added to the 51 tubes. The tubes were then inoculated with the bacterial cultures from the subcultured slants, and incubated for 24 hours under room temperature. Same protocol was followed for all the 5 different types of metals. After 24 hours, the growth was checked and bacterial strains which showed good growth in the presence of metal were selected for further analysis. Out of 51 cultures, 17 cultures were selected (4 spreading type and 13 circular) which showed high turbidity in the text tubes.

3.5.2Screening on agar

20mL of Zobell Marine Agar was prepared in 6 different conical flasks each and autoclaved. Different concentrations of metal solution from the 10% metal stock were added to each of the flask containing ZMA. The different concentrations added were 0.01%, 0.1%, 0.5%, 1% and 2% and one control was maintained with no metal solution added to it. This mixture was then pour plated on Petri plates and left to solidify overnight. Next day plates were checked to make sure there is no contamination. The selected 17 bacterial strains were then spot inoculated on each agar plates having different concentrations of metals and incubated for 24

hours at room temperature to carry out further screening of the bacterial strains and to check their metal tolerance at high concentrations. Same protocol was followed to check the tolerance in presence of all the 5 metals. After 24 hours, the plates were checked for growth and compared with the control plate. Any difference in colony colour or texture was noted down.

3.6 Microscopy of the bacterial isolates

All the 30 plates from the above experiment was taken and the colonies present on each plate were observed under phase contrast microscope to check the presence the metal accumulation around the colony. Experimental plates were also compared to the control plate which did not have presence of any metal in it. Based on the growth and microscopy results, bacterial strains showing good tolerance to metal were selected. Out of 17 strains, 6 bacterial strains were selected for further analysis.

3.7 Atomic Absorption Spectrophotometry (AAS) analysis for Metal Quantification

AAS was carried out to select the bacterial strain which shows maximum reduction of the metal concentration in the solution i.e., which accumulates metal at highest amount. 150mL of nutrient broth was prepared and autoclaved . 20mL of broth was suspended in 7 autoclved conical flasks separately. 50µL (0.05%) of the 10% metal stock was added to each of the conical flask containing nutrient broth and a loopful of the selected 6 bacterial strains were inoculated into the 6 different conical flasks. 1 flask containing metal and nutrient broth was maintained as the control. The flasks were incubated for 24-48 hours at room temperature. Same protocol was carried out for each metal. Cu and Co flasks did not show growth of the bacterial strains

even after keeping it for 48 hours hence the experiment for Cu and Co was repeated by adding the metal stock of 10µL (0.01%) instead of 50µL. These cultures were further transferred into 50mL centrifuge tubes and centrifuged at 5000rpm for 5 minutes at room temperature. The supernatant was transferred to another clean centrifuged tubes and the pellet was discarded. 0.5:50 dilution was carried out of the supernatant using MilliQ distilled water. 0.5:50 dilution for Cu,Co and Zn, 2.5:50 dilution for Fe and 0.1:50 dilution for Zn. These samples were then analysed using Flame AAS by Thermo Fisher Scientific and concentration of metal in control sample and the one containing bacterial strains were determined and the bacterial strains which showed the best result were selected for further analysis. Among 6 bacterial strains , 3 bacterial strains were selected for further analysis.

3.8 Extracellular Polymeric Substances (EPS) Extraction and Quantification

300mL of nutrient broth was prepared and autoclaved and 20mL of each was added to 30 autoclaved conical flasks of 50mL. 10µL (0.01%) of each of the 5 metals from 10% stock was added to 6 conical flasks each . bacterial strains were inoculated into the 5 different metals and incubated at room temperature for 48 hours. The culture was then centrifuged at 5000rmp for 5 minutes at room temperature. The supernatant was transferred to another clean centrifuge tubes and the pellet was discarded. 4mL of the supernatant was taken and 10mL (3 times) of chilled ethanol was added to it and mixed well and allowed to settle for the formation of the precipitate. Once the precipitate is formed, the mixture was transferred to the previously weighed centrifuge tubes, centrifuged again at 5000rpm for 5 minutes at room temperature. The supernatant was discarded and the wet weight of the centrifuge tube was determined. The amount of EPS released by the bacteria was determined by calculations.

3.9 Scanning Electrom Microscopy (SEM) Analysis of Metal Tolerant Bacterial Strains

Nutrient broth of 100mL was prepared and autoclaved. 10mL each was transferred in an autoclaved tubes. 0.01% of Cu and Co was transferred to 2 test tubes repectively and 0.05% of Zn, Mn and Fe was transferred to three test tubes respectively. The selected bacterial strains were inoculated into nutrient agar containing metals and control containing only the nutrient broth and the bacterial strain was maintained for each strain separately. This was incubated for 48 hours at room temperature. Culture broth was then centrifuged at 4000rmp for 5 minutes at

room temperature and the pellet washed with phosphate buffer. This washed pellet was then transferred onto a coverslip. The coverslip was immersed in 4% glutaraldehyde prepared in 50mM phosphate buffer saline(pH7)and incubated overnight at room temperature. Next day, the coverslip was washed with phosphate buffer saline and dehydrated using 30%,50%,70% and 90% ethylalcohol for 10 minutes each followed by 100% of ethylalcohol for 30 minutes. This mixture was then air-dried and taken for scanning electron microscopy where it was gold coated using blue Quorum Model SC720 and then analysed using ZEISS EVO 18 Scanning Electron Microscope. (D'costa, Kunkolienkar, & Naik, 2019)

3.10 Growth Curve of the Metal Tolerant Bacterial Strains

1mL of bacterial culture previously grown in nutrient broth was taken and added to 150mL of nutrient broth containing 0.01% of metal concentration in it. This was performed for all 5 metals and all 3 different bacterial strains. Also control for the three bacterial strains was maintained which did not contain any metal in it. Once the bacterial culture was inoculated, immediately the OD at 620nm was taken in a colorimeter and this was marked as zero reading . The blank used was sterile nutrient broth. The colorimeter was set to auto zero using blank and then the reading of each of the experimental flasks was taken. OD at 620nm was taken after every 1 hour to determine the growth curve of the bacteria. OD was taken until 48 hours i.e., until the reading was static. The graph of absorbance at 620nm v/s Time in hours was plotted and the difference between control flask reading and experimental flask reading was determined.

3.11 Antibiotic Sensitivity Testing of the Selected Bacterial Isolates

40mL of nutrient agar was prepared and pour plated onto sterile periplates and left overnight. Simultaneously, a loopful of culture of the 3 bacterial strains i.e., strain no.6,9 and 33 were inoculated in 10mL nutrient broth separately and incubated for 24 hours at room temperature. Next day, 0.1mL of the bacterial strains were spread plated onto the 3 petriplates each . Sterile antibiotic discs of HiMedia Laboratories (refer appendix 6) were placed onto the plates (10 different antibiotic discs was placed) and incubated overnight at room temperature. Next day, the plates were checked for zone of inhibition and the diameter of the zone of inhibition was

measured using a ruler and the bacterial sensitivity towards the provided antibiotics was determined using zone interpretative chart provided by HiMedia Laboratories.(Appendix 6)

3.12 Quantification of the amount of protein present in the metal tolerant bacterial isolates using Folin-Lowry Method ()

3.12.1 Sample preparation

Culture no. 6, 9 and 33 was grown in the presence of Fe, Zn and Co, Cu, Mn respectively and one control of each culture was grown in 10 mL nutrient broth. All these 8 cultures together was then centrifuged at 4000 rpm for 5 minutes at room temperature. After centrifugation, the supernatant was discarded and the pellet was mixed with 2mL of 25% SDS solution and boiled for 10 minutes in boiling water bath. The total protein content from these samples were then determined by Folin-Lowry method.

3.12.2 Folin-Lowry method

BSA stock solution, reagent A (2% Na_2Co_3 in 0.1% NaOH), reagent B (0.1% $CuSo_4$ in 1% Sodium-Potassium Tartarate), reagent C (Reagent A + Reagent B) and Folin-Ciocalteau reagent was prepared (appendix). Protein standards of 200µg/mL, 400µg/mL, 600µg/mL, 800µg/mL and 1000µg/mL was prepared using Standard BSA solution as given in the following table. 0.5mL of the 8 unknown samples was mixed with 1.5 mL of distilled water and other instructions given in the following addition table was followed. Absorbance at 660nm was taken after incubating the mixtures in a dark room for 20 minutes. Graph of OD vs concentration of protein was plotted to determine the total amount of protein present in the sample. (Appendix 7) (quantitative analysis).

Table no. 01: Addition table for Folin Lowry reagents

Conc. Of proteins (µg/mL)	Vol. of standards (mL)	Vol. of diluent (mL)	Volume of alkaline $CuSo_4$		Volume of FC Reagent		OD at 660 nm

Blank	-	-	5.0		0.5		
1	20	0.2	5.0	Mix	0.5	Incubation	
2	40	0.4	5.0	and	0.5	for 50 minutes in dark	
3	60	0.6	5.0	wait	0.5		
4	80	0.8	5.0	for	0.5		
5	100	1.0	5.0	1	0.5		
Unknown 1	0.5	1.5	5.0	minute	0.5		
Unknown 2	0.5	1.5	5.0		0.5		
Unknown 3	0.5	1.5	5.0		0.5		
Unknown 4	0.5	1.5	5.0		0.5		
Unknown 5	0.5	1.5	5.0		0.5		
Unknown 6	0.5	1.5	5.0		0.5		
Unknown 7	0.5	1.5	5.0		0.5		
Unknown 8	0.5	1.5	5.0		0.5		

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3.13 Estimation of proteins present in the metal tolerant bacterial isolates by Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.13.1 Culture media

Culture no. 6, 9 and 33 was inoculated in 10 mL nutrient broth containing Fe, Zn and Co, Cu, Mn respectively and one control of each culture was also inoculated in 10 mL nutrient broth and incubated at room temperature for 24 hours. Cell material was harvested after 24 hours by centrifugation at 4000rpm for 10 minutes at room temperature and the pellet obtained was used for further protein extraction.

3.13.2 Sample preparation

The pellet was mixed with 2mL of 25% SDS solution and boiled for 10 minutes in boiling water bath. Sample was taken from this boiled material and added to staining buffer then from the above sample ~100µL was taken and loaded in SDS-PAGE.

3.13.3 Preparation of slab gels

Thoroughly clean and dried glasses were assembled in the gel casting assembly. The two glass plates were sealed with the help of tygon, tubing, clamped and the whole assembly was placed

in an upright position. Various components of resolving gel were mixed. The gel solution was poured into the mould in between the clamped glass plates ensuring that there are no air bubbles. Distilled water was overlaid on top as gently as possible and left for 30 minutes for gel to settle. When the gel was polymerized, the water layer was removed and rinsed with stacking gel buffer. The stacking gel components were mixed in the same way as described above for resolving gel. The stacking gel was poured and immediately plasticcomb was inserted in the stacking gel ensuring no air bubbles. The gel was allowed to polymerize for about 40 minutes, then the comb was removed without distorting or damaging the shapes of the wells. The reservoir buffer was poured in the lower and upper chambers. Table no. 02: Chart for preparation of separating gel and stacking gel

	Separating gel (mL)	**Stacking gel (mL)**
Gel concentration	10%	5%
Distilled water	4.1	3.45
30% acrylamide	3.3	0.83
1.5M tris, pH 8.0, 0.4% SDS	2.5	0.63
10% APS	0.1	0.05
TEMED	0.004	0.005

3.13.4 Electrophoresis of the sample:

Around 100µL sample was loaded in the sample wells, also the molecular weight marker proteins was loaded in one of the wells. The current was switched ON and maintained at 80 volts for until the samples have travelled through the stacking gel. Then it was increased to 100 volts until the bromophenol blue dye reaches near the bottom of the gel slab. After the electrophoresis was completed the current was switched OFF and the power supply was disconnected and the gel slab was carefully removed from between the glass plate and the gel was transferred into a tray.

3.13.5 Staining and destaining of gel

Around 80 mL of staining solution was added to the tray containing the gel. The gel was kept overnight in the staining solution for visualization of the bands. Next day the stain was removed and the gel was rinsed with distilled water till a considerable amount of stain reaches out from

the gel. Again the destaining solution was added with constant shaking until distinct bands were observed.

3.14 Bioremediation of metal polluted Soil and Water sample

3.14.1 Sample collection

Collection of soil sample: Polluted soil sample from Shikeri Goa (old mining site) was collected in a ziplock bag and brought to the marine microbiology laboratory. This sample was collected at different sites : part A : containing high concentration of metal, part B: containing a little lesser concentration of metal. These samples were labelled as soil sample C (concentrated) and soil sample D (less concentrated)

Collection of water sample: Water sample was collected in a clean plastic bottle from two different locations i.e., Bicholim and Cortalim (Ferry area) and brought into the marine microbiology laboratory to carry out the experiment.

3.14.2 Preparation of bacterial culture

Three cultures which were previously screened, i.e culture no. 6, 9 and 33 was taken. A loopful of these cultures were inoculated into 10mL Nutrient broth each and incubated for 24 hours at room temperature. Next day, these samples were centrifuged at 4000 rpm for 5 minutes at room temperature and the pellet was taken for further analysis.

3.14.3 Sample preparation :

After getting the samples to the laboratory, the water samples from both the sites were autoclaved and the 1g of soil sample each was introduced into a 50 mL conical flask (5 conical flask each) and autoclaved .

3.14.4 Inoculation of the culture into the sample

Control 1:

For water sample : 10mL of water sample was introduced into a sterile 50mL conical flask.

This was then filtered using 0.2µ filter paper and kept ready for AAS analysis.

For soil sample: conical flask containing 1g of soil (autoclaved) was taken and 10mL of normal saline was added to it. This mixture was mixed and allowed to settle for some time.
Then filtered using 0.22µ filter paper and kept ready for AAS analysis.

Control 2:

Same as control 1 but left as it is for few weeks without filtering.

Test:

For water sample: 10mL of water sample was added to a sterile 50mL conical flask and the culture pellet was added to it. Inoculated at room temperature for several weeks so that the culture carries out bioremediation of this contaminated water sample.

For soil sample: culture pellet was mixed with 10mL of saline and this mixture was then added to the sterile conical flask containing 1g of soil sample. Mixed thoroughly and incubated for several weeks so that the culture carries out bioremediation of the contaminated soil sample.(All of these test samples will be then filtered using 0.22µ filter paper and diluted and analysed using AAS along with control samples.)

3.15 Gram staining of all the bacterial isolates

Gram staining was done of all the 51 cultures that were isolated. A loopful of culture was placed on slide and smear was made using normal saline. The cultures were heat fixed on the clean slide. 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 1 minute, washed with water and air dried. A drop of oil was put on the slide and was later viewed under phase contrast microscope at 100x (oil immersion) lens.

4. Results

Water and soil samples from four different spots (shown in fig 4, 5, 6, 7 below) were collected to analyze and isolate metal tolerant bacteria capable of bioremediation. **4.1 Sample collection**

Fig. 4: Old Goa Mangrove Spot A

Fig.5: Old Goa Mangrove Spot B

Fig. 6: Old Goa Mangrove Spot C

Fig. 7: Old Goa Mangrove Spot D

4.2 Selection and subculturing of the bacterial strains

Fifty one different types of colonies were selected and subcultured on nutrient agar plates out of which 35 were circular colonies and 16 colonies showed spreaded growth.

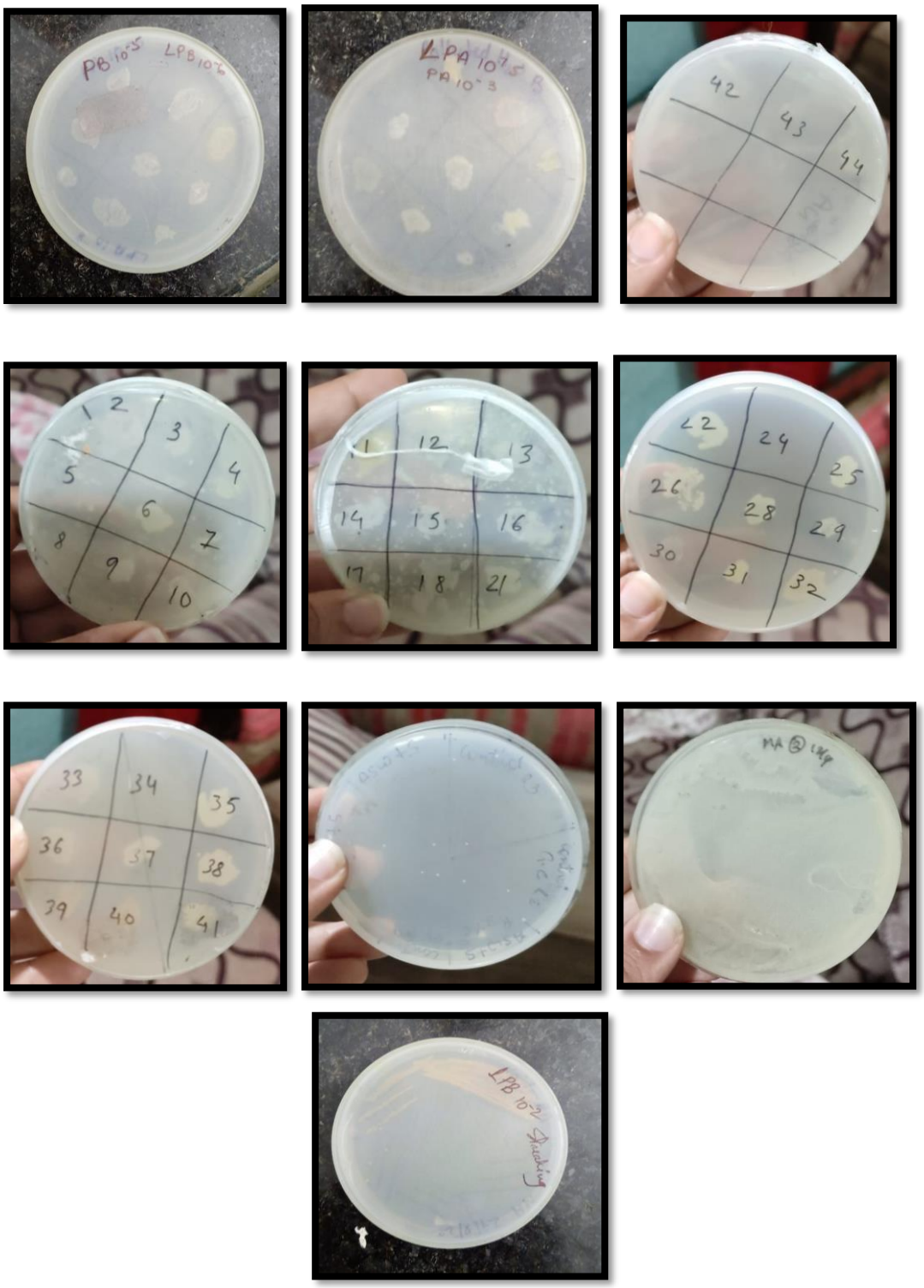


Fig.8: Sub-cultured and streaked colonies on Nutrient agar plates for purification

4.3 Screening for metal tolerance of the bacterial culture in broth

Seventeen cultures from the 51 cultures showed growth in the test-tubes containing 0.01% metal solutions (refer table no. 03 below). Out of these 17 cultures, 4 were from the streaked

plates and 13 were from the sub-cultured plates of circular colonies. These 17 cultures were selected for further analysis.

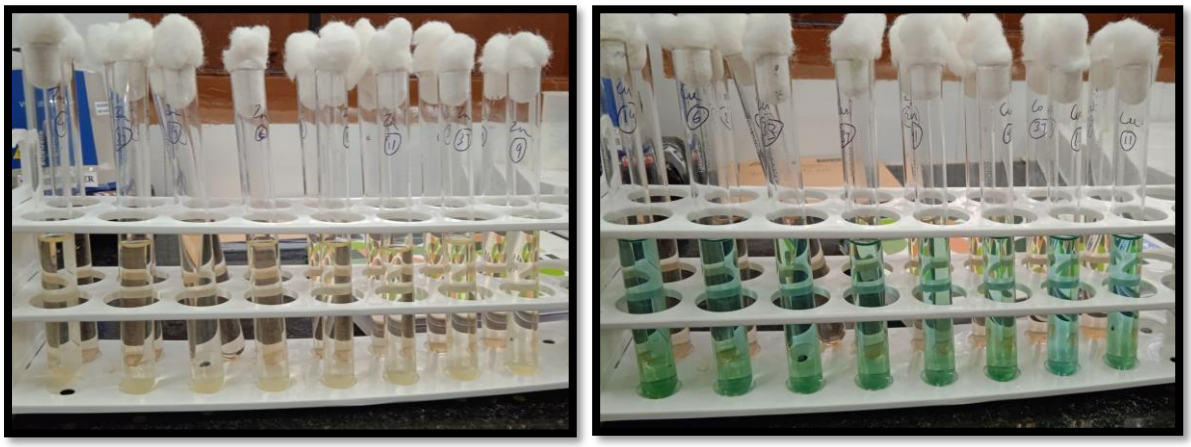


Fig. 9: Screening of the bacterial cultures in broth before incubation for 24 hours

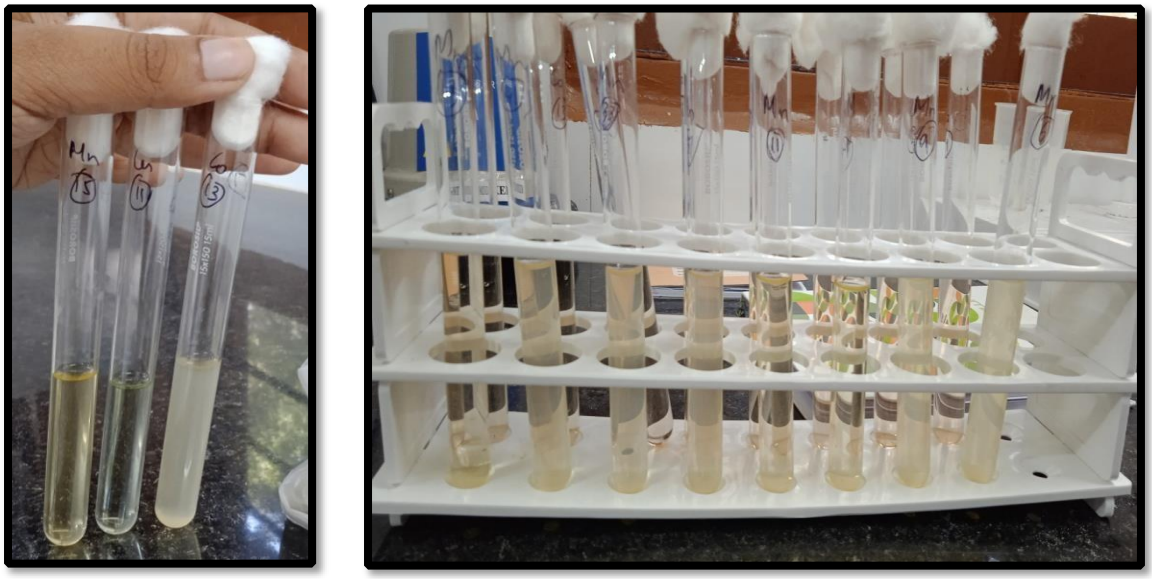


Fig. 10: Growth observed in the test-tubes for screening of the bacterial cultures in broth after incubation for 24 hours

Table no. 3: Growth table of the circular cultures in 0.01% metal conc.

Culture no.	Growth in the medium containing metal conc. Of 0.01%				
	Zn	Mn	Cu	Fe	Co
2	-	-	-	+	-
3	-	-	-	-	-

4	+	+	+	+	+
5	+	-	-	+	-
6	+	+	+	+	+
7	+	+	+	+	-
8	+	-	+	+	-
9	++	+	+	+	+
10	+++	+++	++	+++	+++
11	+	++	+	++	+
12	-	-	-	-	-
13	-	-	-	+	-
14	-	-	+	-	-
15	-		-	+	-
16	-	+	-	-	-
17	+	+	-	+	-
18	+	+	-	-	-
21	+	++	+	++	+
22	++	+	-	+	-
25	+	++	+	++	++
26	+	+	-	+	+
28	++	++	+	++	+
29	++	++	+	++	+
30	-	-	-	-	-
31	+	++	+	++	+
32	+	+	+	+	+
33	+++	++	+	+	+
34	+	+	+	+	+
35	+	+	-	+	+
36	++	+	+	++	+
37	+	++	+	+	+
38	++	+	+	+	++
39	+	+	+	+	+
40	+	+	-	+	+
41	++	++	+	++	+

Key: + → Growth

- → No growth

Table no. 4: Growth table of the spreaded cultures in 0.01% metal conc.

CULTURE NO.	Growth in the medium containing metal conc. of 0.01%

	Zn	Mn	Cu	Co	Fe
1	-	-	-	-	-
2	++	++	+	+	++
3	-	-	-	-	+
4	-	+	-	-	+
5	-	-	-	-	-
6	++	++	++	+	++
7	-	+	-	+	+
8	-	+	+	+	+
9	-	-	-	-	-
10	-	-	-	-	+
11	-	-	-	-	-
12	-	-	-	-	-
13	++	+	+	+	++
14	++	++	+	+	++
15	-	-	-	-	+
16	-	+	-	-	+

Key: + → Growth

- → No growth

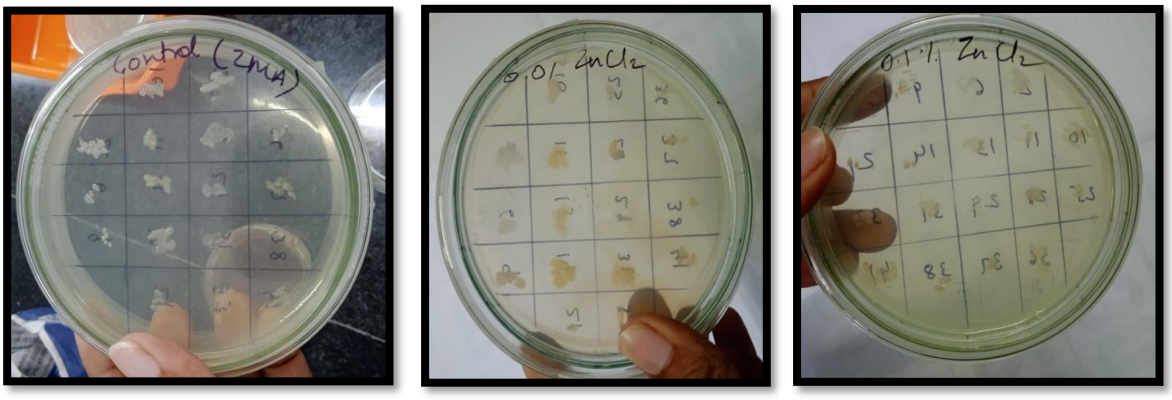
Table no. 5: Colony characteristics of the cultures incubated for 24 hours at room temperature which can tolerate metal concentration of 0.01%

Culture	Shape	Size (mm)	Colour	Elevation	Margin	Opacity	Consistency
2	circular	2mm	yellow	raised	Entire	Opaque	Butyrous
6	Circular	3mm	white	raised	Entire	transparent	Butyrous
9	Circular	2mm	white	raised	Entire	transparent	non sticky
10	Circular	4mm	White	raised	Entire	Opaque	Non sticky
11	Circular	4mm	White	Flat	Entire	Opaque	Sticky
13	Circular	1mm	white	raised	Entire	Opaque	Sticky
14	oval	1mm	white	raised	Entire	Opaque	Sticky
21	oval	2mm	white	flat	Entire	transparent	Sticky
25	Circular	1mm	White	raised	Entire	Opaque	Non sticky
28	Circular	1mm	white	raised	Entire	Opaque	non sticky
29	Cicular	1mm	white	flat	Entire	transparent	Sticky
31	Circular	1mm	white	raised	Entire	transparent	non sticky
33	Circular	1mm	white	raised	Entire	transparent	non sticky
36	Circular	3mm	White	raised	Entire	Opaque	Sticky
37	Circular	Pinpoint	White	raised	Entire	Opaque	Sticky
38	Circular	1mm	White	raised	Entire	Transparent	Non sticky
41	Circular	2mm	white	raised	Irregular	transparent	non sticky

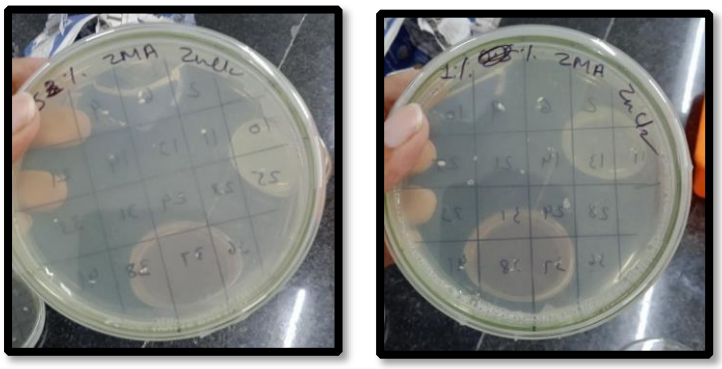
4.4 Screening on agar

Screening of the selected 17 bacterial isolates was done on agar medium containing different concentrations of metal to select the cultures which grew best on increasing concentrations of metals. Eight out of 17 cultures which showed highest metal tolerance were selected for further analysis.

Culture growing on media containing $ZnCl_2$



(A) (B) (C)



(D) (E)

Fig. 11: Bacterial cultures grown on media containing $ZnCl_2$ of different concentrations: **A**: Control, **B**: 0.01% $ZnCl_2$, **C**: 0.1% $ZnCl_2$, **D**: 0.5% $ZnCl_2$, **E**: 1% $ZnCl_2$

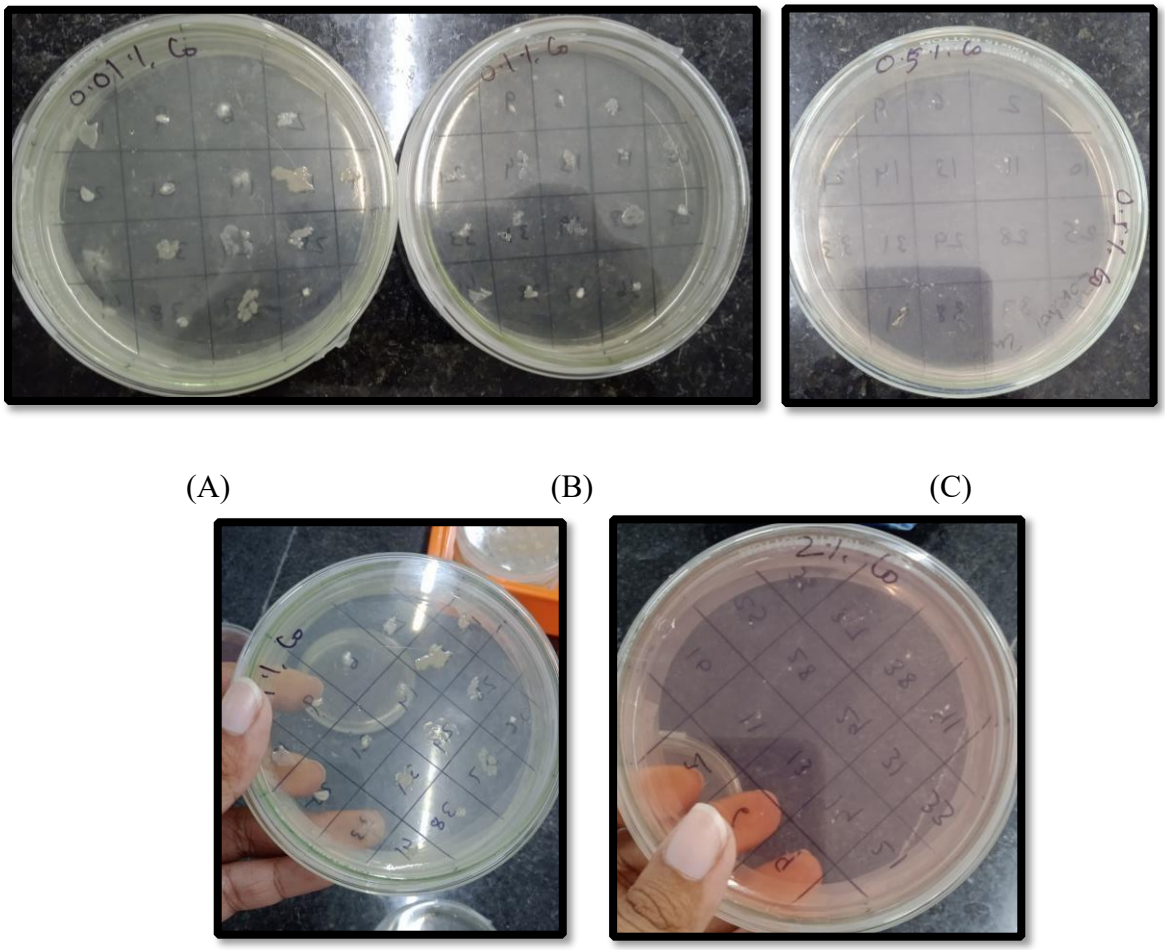
Table no. 6: Growth table of the cultures showing growth at different concentrations of Zn in the agar medium

Colony no.	'arying zinc concentrations				
	0.01%	0.10%	0.50%	1%	2%
2	++	++	-	-	-
6	++	++	+	+	-
9	++	++	+	+	-
10	++	++	-	-	-
11	++	++	+	+	-
13	++	+	+	+	-
14	++	++	-	-	-
21	+	+	-	-	-
25	+	+	+	+	+
28	+	+	+	-	-
29	++	+	+	-	-
31	++	++	+	+	-
33	++	++	+	+	+
36	+	++	+	-	-
37	++	++	+	+	+
38	+	+	-	-	+
41	++	++	+	+	-

Key: + → Growth

- → No growth
-

Culture growing on media containing $CoCl_2.6H_2O$



(A) (B) (C)

(D) (E)

Fig. 12: Bacterial cultures grown on media containing $CoCl_2.6H_2O$ of different concentrations: **A**: 0.01% Co, **B**: 0.1% Co, **C**: 0.5% Co, **D**: 1% Co, **E**: 2% Co

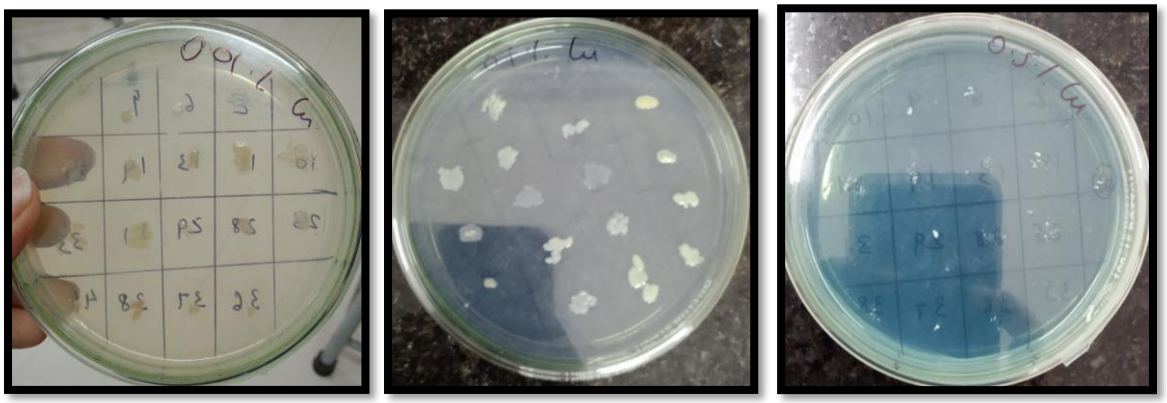
Table no. 7: Growth table of the cultures showing growth at different concentrations of Co

Colony no.	Varying Co concentrations				
	0.01%	0.10%	0.50%	1%	2%
2	++	+	-	-	_
6	++	+	+	-	-
9	+	+	-	-	-
10	++	+	-	-	-
11	++	+	+	-	-
13	++	+	-	-	_
14	++	+	-	-	-
21	+	+	-	-	-
25	+	+	+	+	-
28	++	+	-	-	-
29	++	+	-	-	-
31	++	+	-	-	-
33	++	+	-	-	-
36	+	+	-	-	-
37	++	+	+	+	-
38	+	+	-	-	-
41	++	+	+	-	-

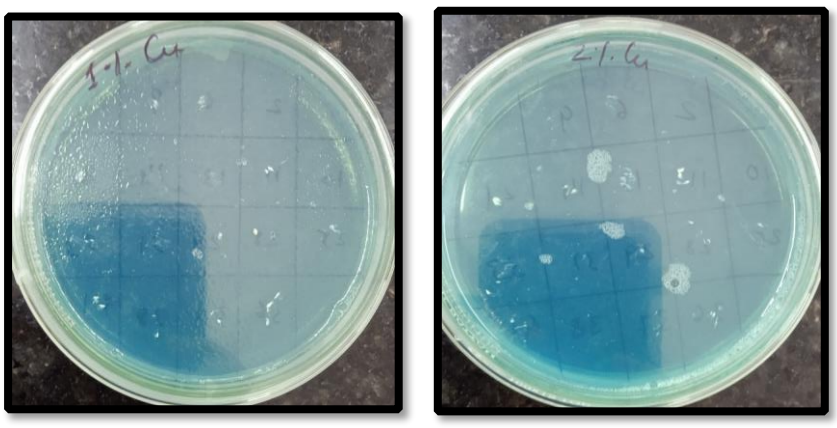
Key: + → Growth

\- → No growth

<u>Culture growing on media containing $CuCl_2.2H_2O$</u>



(A) (B) (C)



(D) (E)

Fig. 13: Bacterial cultures grown on media containing $CuCl_2.2H_2O$ of different concentrations: **A**: 0.01% Cu, **B**: 0.1% Cu, **C**: 0.5% Cu, **D**: 1% Cu, **E**: 2% Cu

Table no. 8: Growth table of the cultures showing growth at different concentrations of Cu

Colony no.	Varying Cu concentrations				
	0.01%	0.10%	0.50%	1%	2%
2	++	++	-	-	-
6	+	+	+	+	-
9	+	+	+	+	-
10	++	++	-	-	-
11	++	++	+	+	+
13	+	++	+	-	-
14	++	+	+	-	-
21	+	+	+	+	+
25	+	+	+	+	+
28	+	+	+	+	-
29	+	++	+	-	-
31	++	+	-	-	-
33	++	+	+	+	-
36	+	+	+	-	-
37	+	+	-	-	-
38	+	+	-	-	-
41	+	++	+	-	-

Culture growing on media containing $MnCl_2.4H_2O$

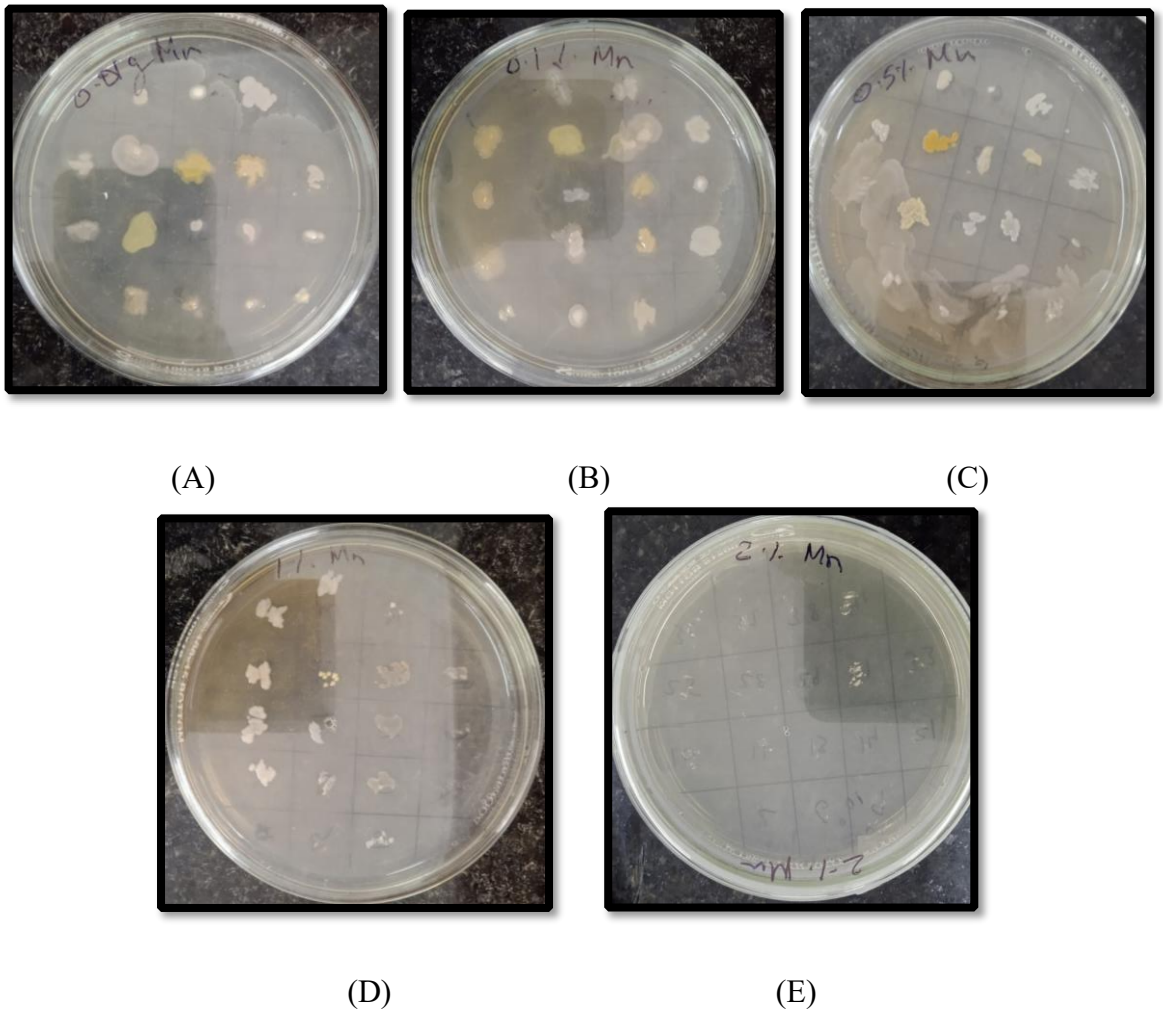


Fig. 14: Bacterial cultures grown on media containing $MnCl_2.4H_2O$ of different concentrations: **A**: 0.01% Mn, **B**: 0.1% Mn, **C**: 0.5% Mn, **D**: 1% Mn, **E**: 2% Mn

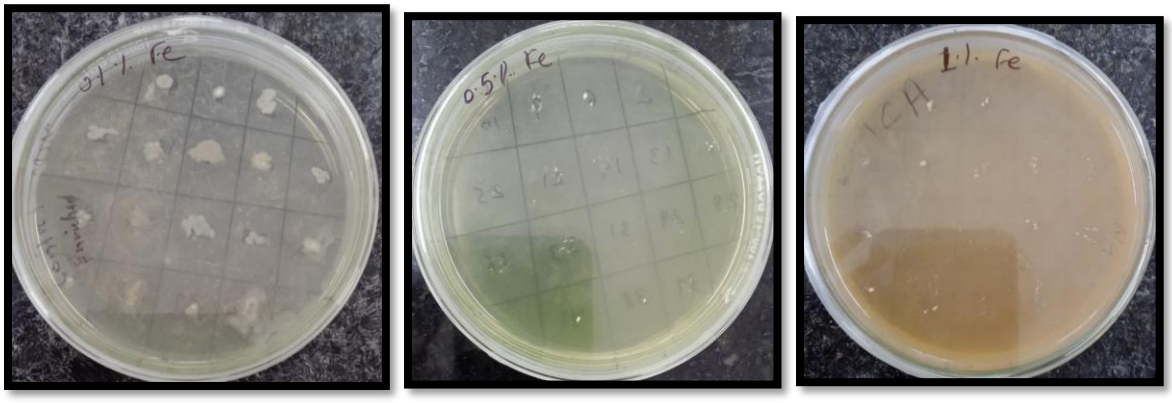
Table no. 9: Growth table of the cultures showing growth at different concentrations of Mn

Colony no.	Varying manganese concentrations				
	0.01%	0.10%	0.50%	1%	2%
2	++	++	+	-	-
6	++	+	+	+	+
9	++	+	+	+	+
10	+	+	+	+	-
11	++	+	+	+	-
13	++	+	+	-	-
14	+	+	+	+	-
21	+	+	+	-	-
25	+	+	+	+	-
28	+	+	+	+	-
29	+	+	+	-	-
31	+	+	+	-	-
33	++	+	+	-	-
36	+	+	+	-	-
37	++	+	+	-	-
38	+	+	+	+	+
41	++	+	+	+	+

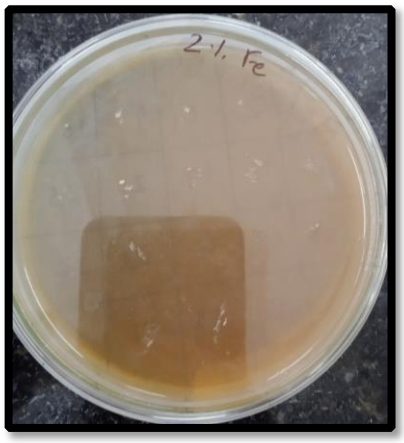
Key: + → Growth

- → No growth

Culture growing on media containing $FeCl_3.6H_2O$



(A) (B) (C)



(D)

Fig. 15: Bacterial cultures grown on media containing $FeCl_3.6H_2O$ of different concentrations: **A**: 0.1% Fe, **B**: 0.5% Fe, **C**: 1% Fe, **D**: 2% Fe

Table no. 10: Growth table of the cultures showing growth at different concentrations of Fe

Colony no.	Varying iron concentrations				
	0.01%	0.10%	0.50%	1%	2%
2	+	+	-	-	-
6	+	+	+	+	+
9	+	+	+	+	+
10	+	+	+	+	-
11	++	+	+	+	-
13	++	++	+	-	-
14	+	+	+	+	-
21	+	+	-	-	-
25	+	+	+	+	-
28	+	+	+	+	-
29	+	+	-	-	-
31	+	+	-	-	-
33	+	+	-	-	-
36	+	+	-	-	-
37	+	+	+	-	-
38	+	+	+	+	+
41	+	+	+	+	+

Table no. 11: Change in pigmentation of isolates during growth on media containing different metals

Culture no.	Control	Cu	Co	Fe	Zn	Mn
2	white	milky white	White	White	white	white
6	white	White	White	White	white	white
9	Creamy	White	yellowish white	yelllowish white	yellow	creamy
10	white	Creamy	creamy	White	creamy	creamy
11	light yellow	Creamy	yellowish white	Yellow	white	light yellow
13	Yellow	White	yellowish white	Yellow	white	white
14	yellow/white	White	White	yellow/white	white	dark yellow
21	white	White	White	White	white	white
25	white	White	Milky	Milky	white	white
28	white	White	White	yellow/white	white	white
29	white	White	White	White	yellow	white
31	yellowish white	yellowish white	White	Yellow	white	yellowish white
33	Milky	yellowish white	yellowish white	White	yellow	yellowish white
36	white	yellowish white	white	White	yellow	milky
37	yellowish white	yelllow/white	yellow/white	yellow/white	yellow/white	yellowish white
38	darl yellow	yellow/white	dark yellow	dark yellow	dark yellow	white

41	yellowish white	Milky	milky	yellowish white	milky	white

Table no. 12: Culture grown in metal concentration of 0.1% to select final 6 metal tolerant bacteria's.

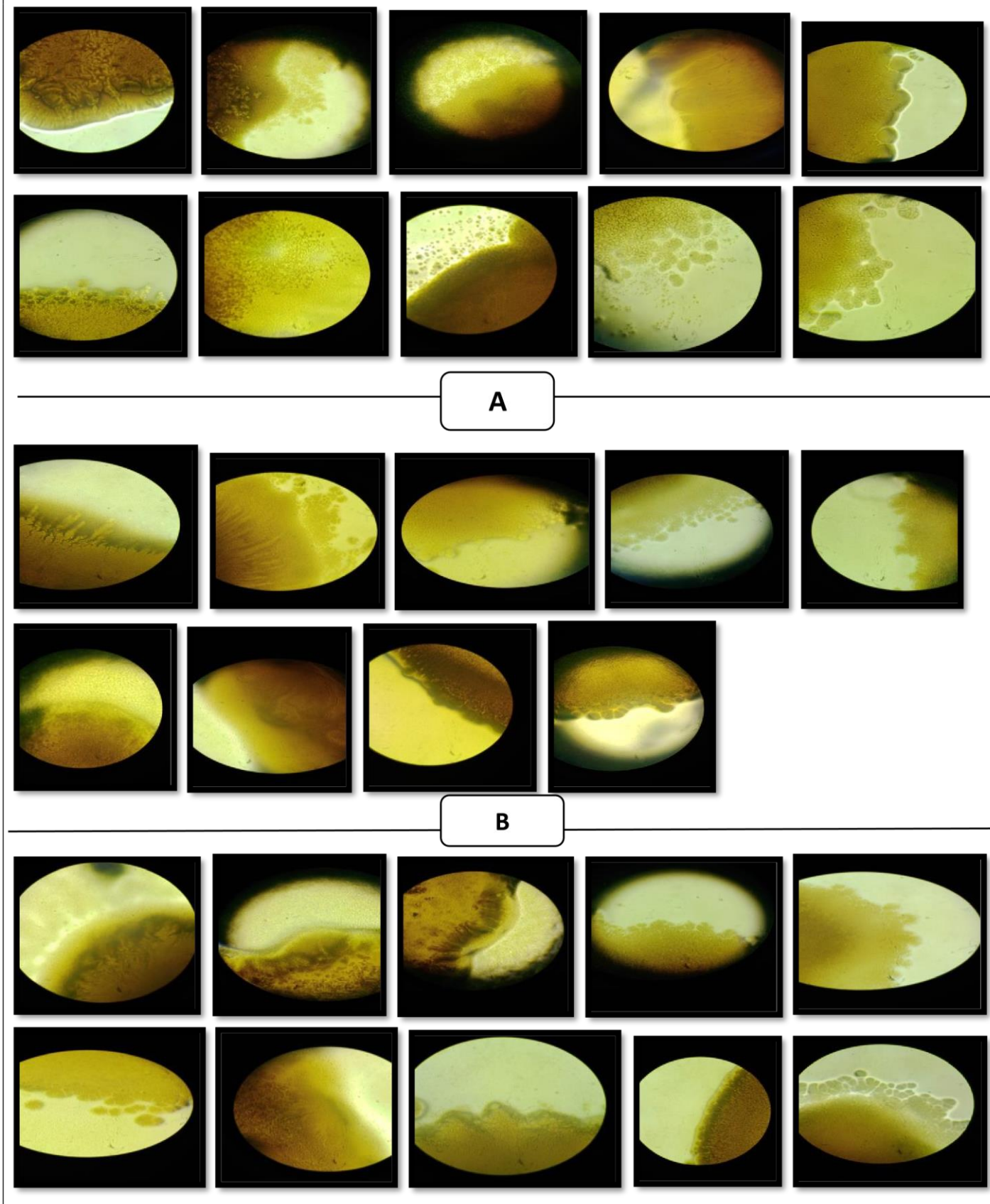
Culture no.	Metals				
	Cu	Zn	Co	Fe	Mn
6	-	-	-	+	+
9	+	-	-	+	+
11	-	+	-	+	+
13	-	-	-	-	-
14	-	-	-	-	-
33	+	+	+	+	+
37	+	+	-	+	+
41	+	+	-	+	+

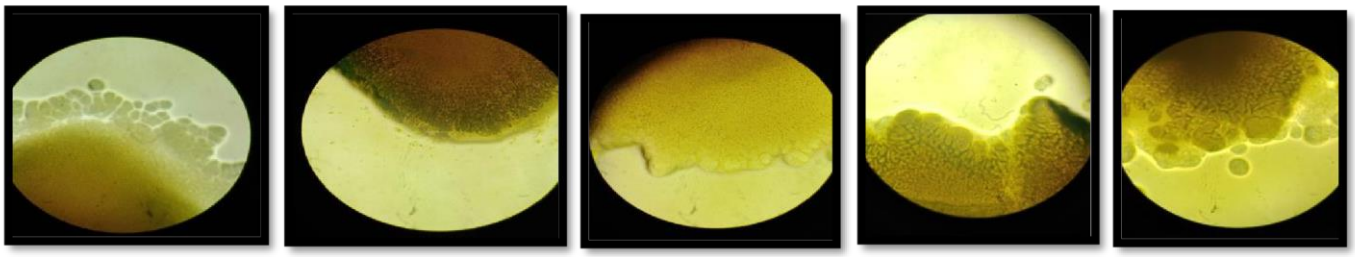
Key: + → Growth

- → No growth

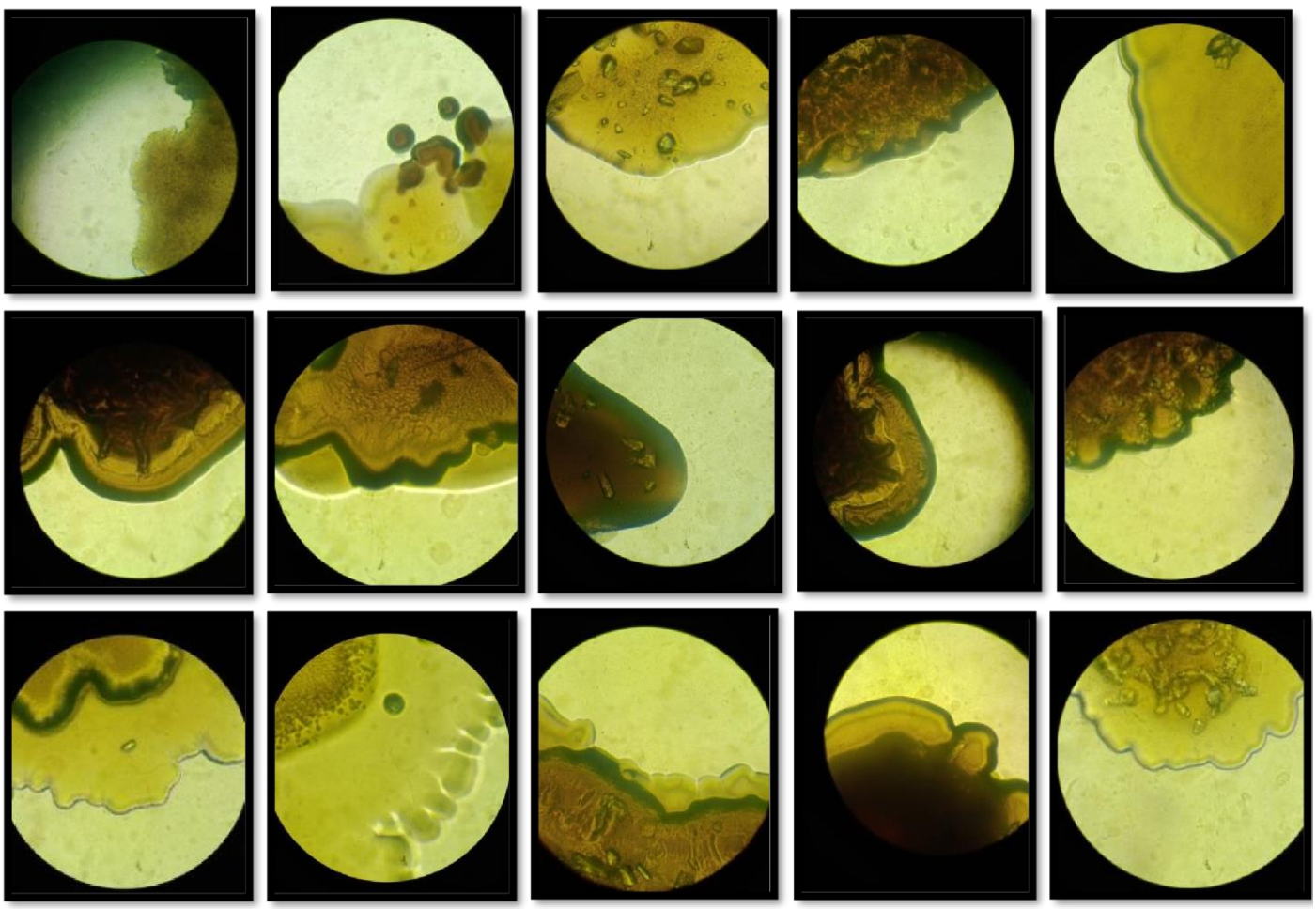
4.5 Microscopy of the bacterial cultures grown on plates using phase contrast microscope

Microscopy of the bacterial cultures grown on the medium containing different metals were carried out using phase contrast microscope and referring to the images, and the results obtained above (refer table no.11) , 6 out of 8 different bacterial cultures were chosen for further analysis.

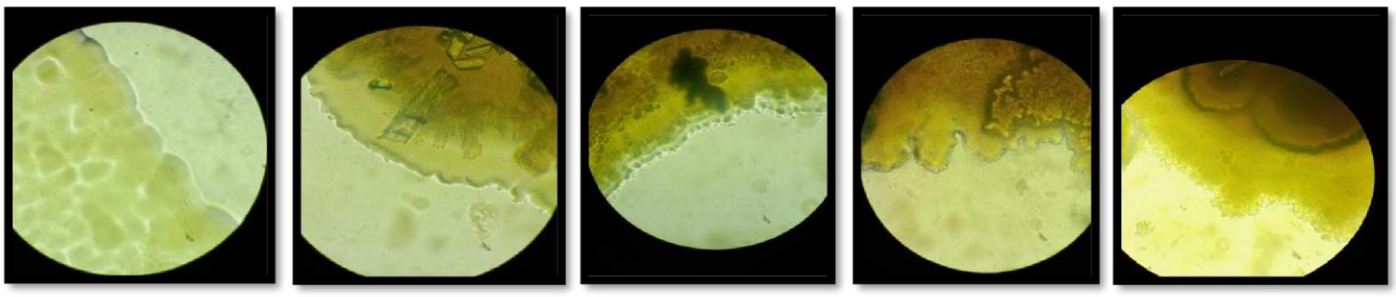

A
B

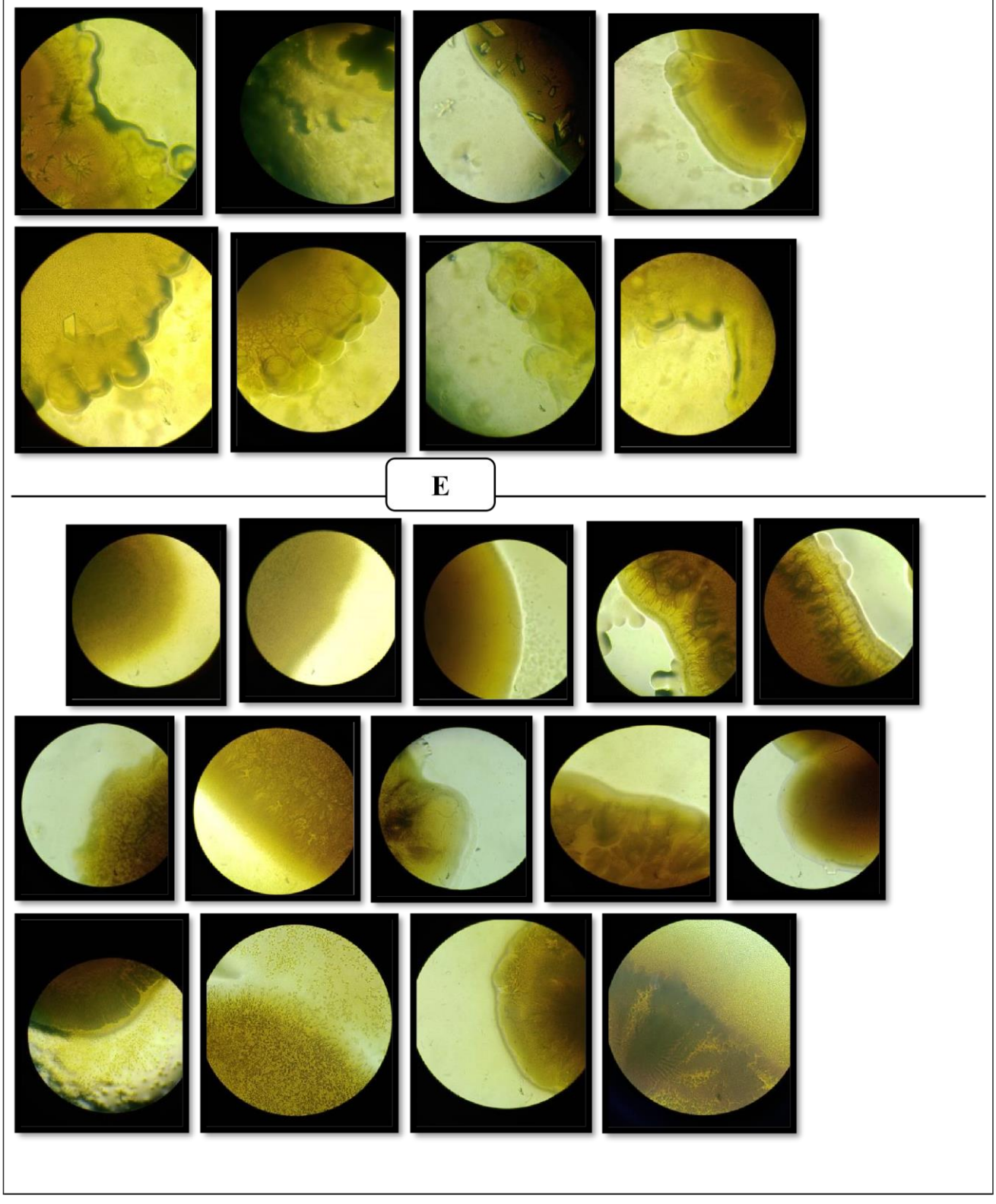


C



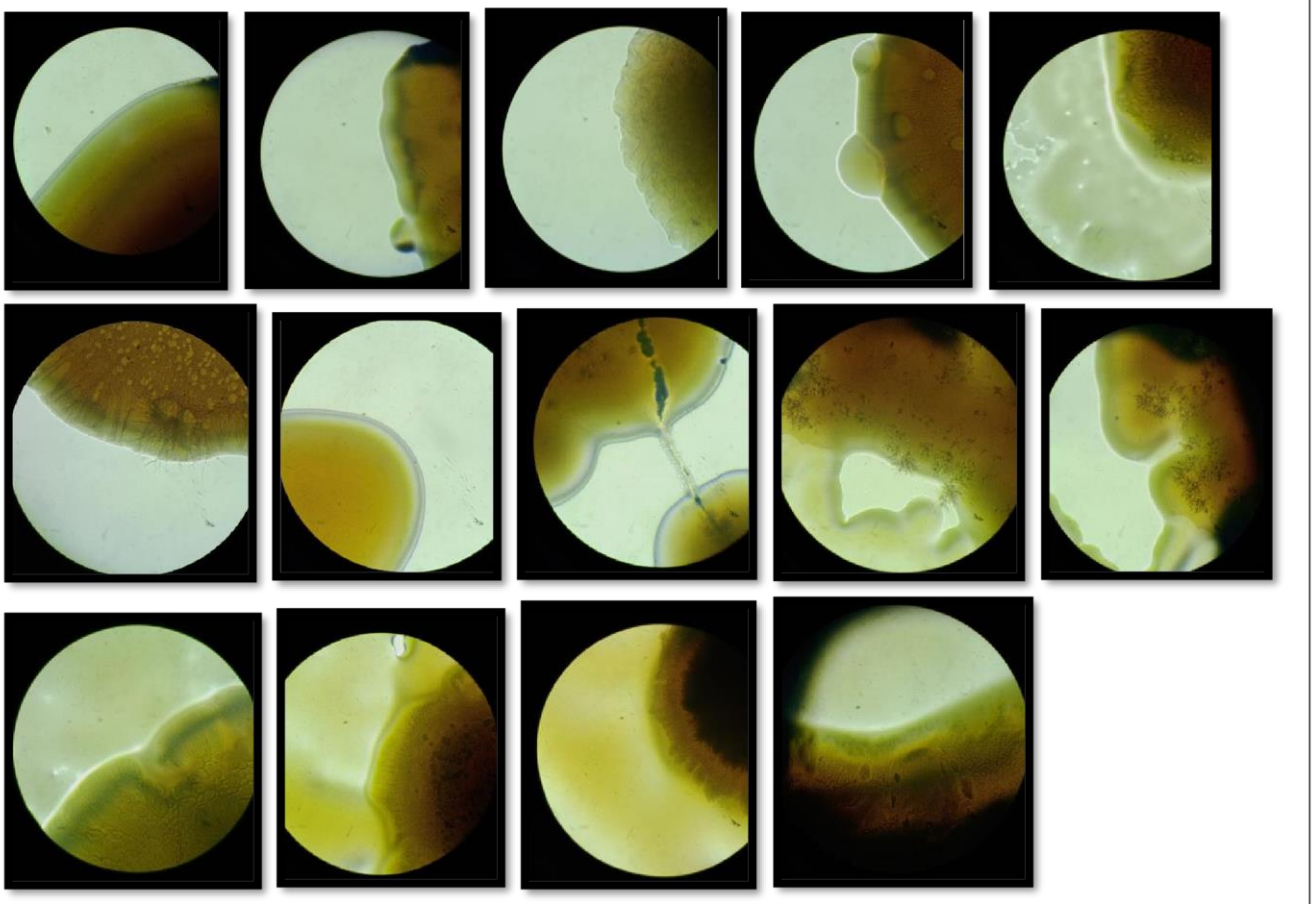
D



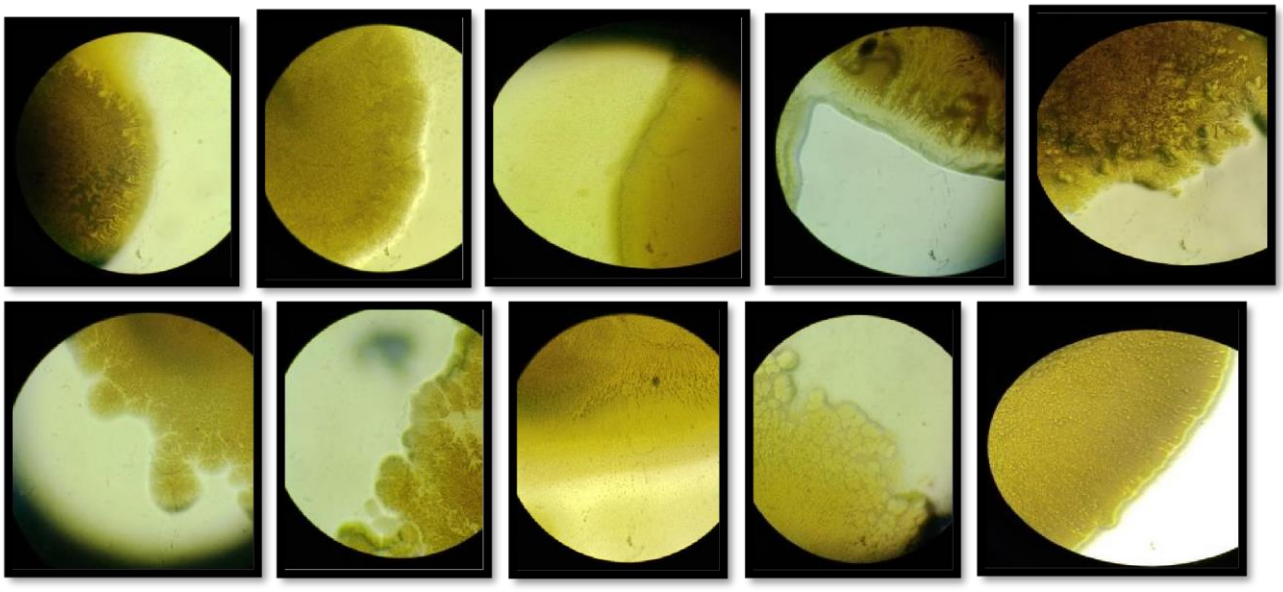


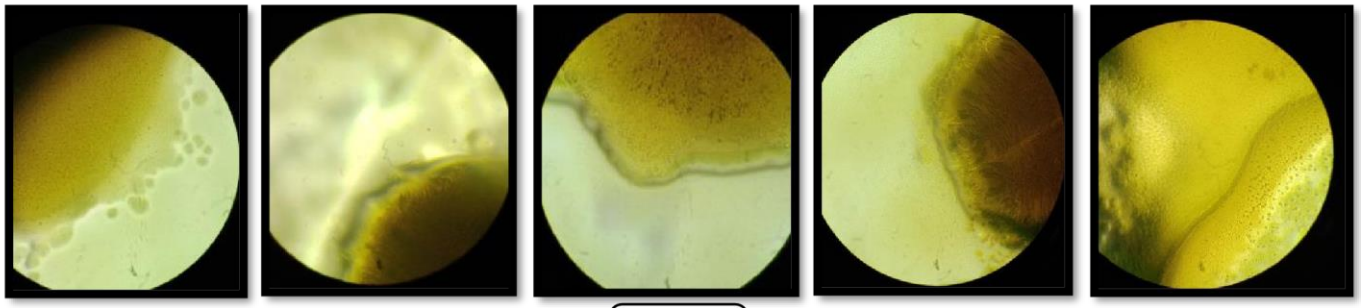
E

F

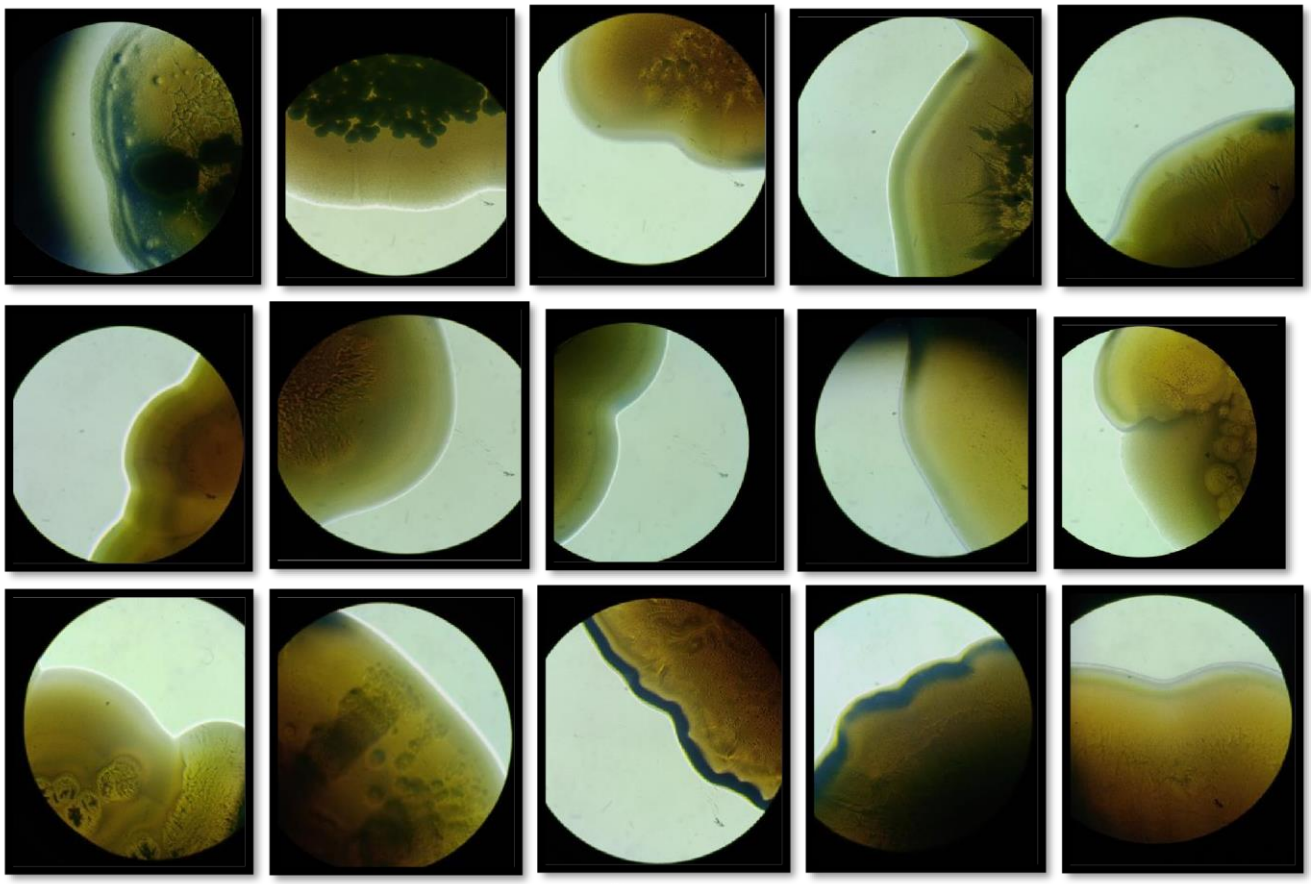


G

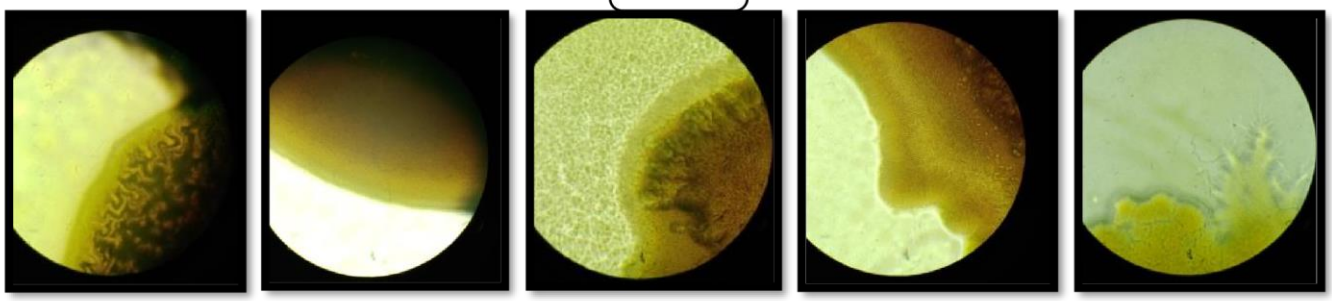


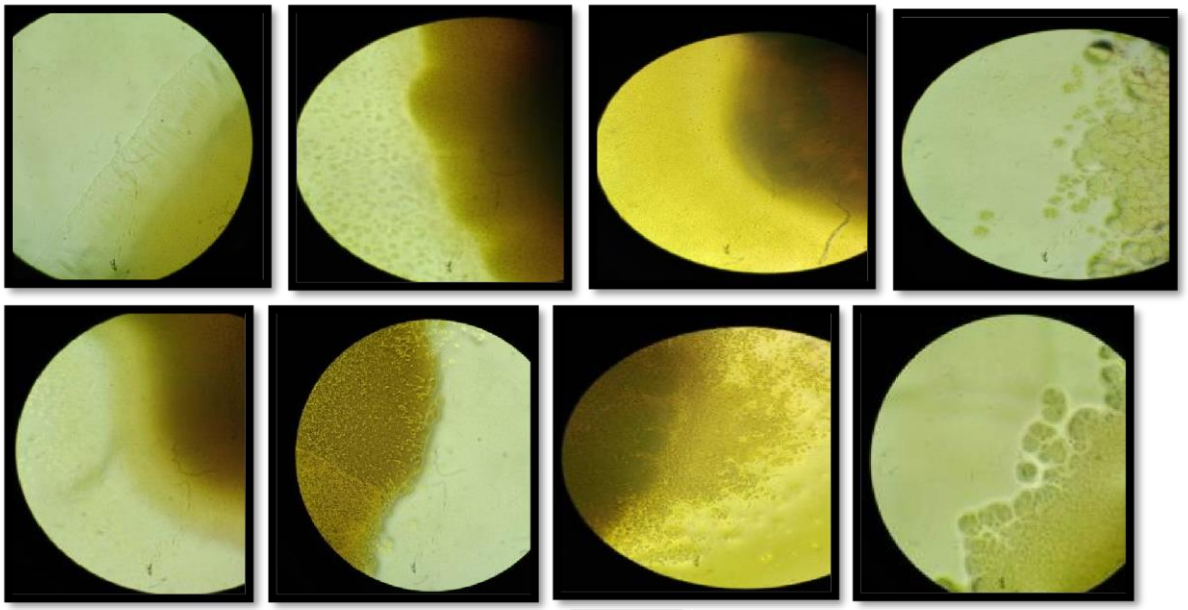


H

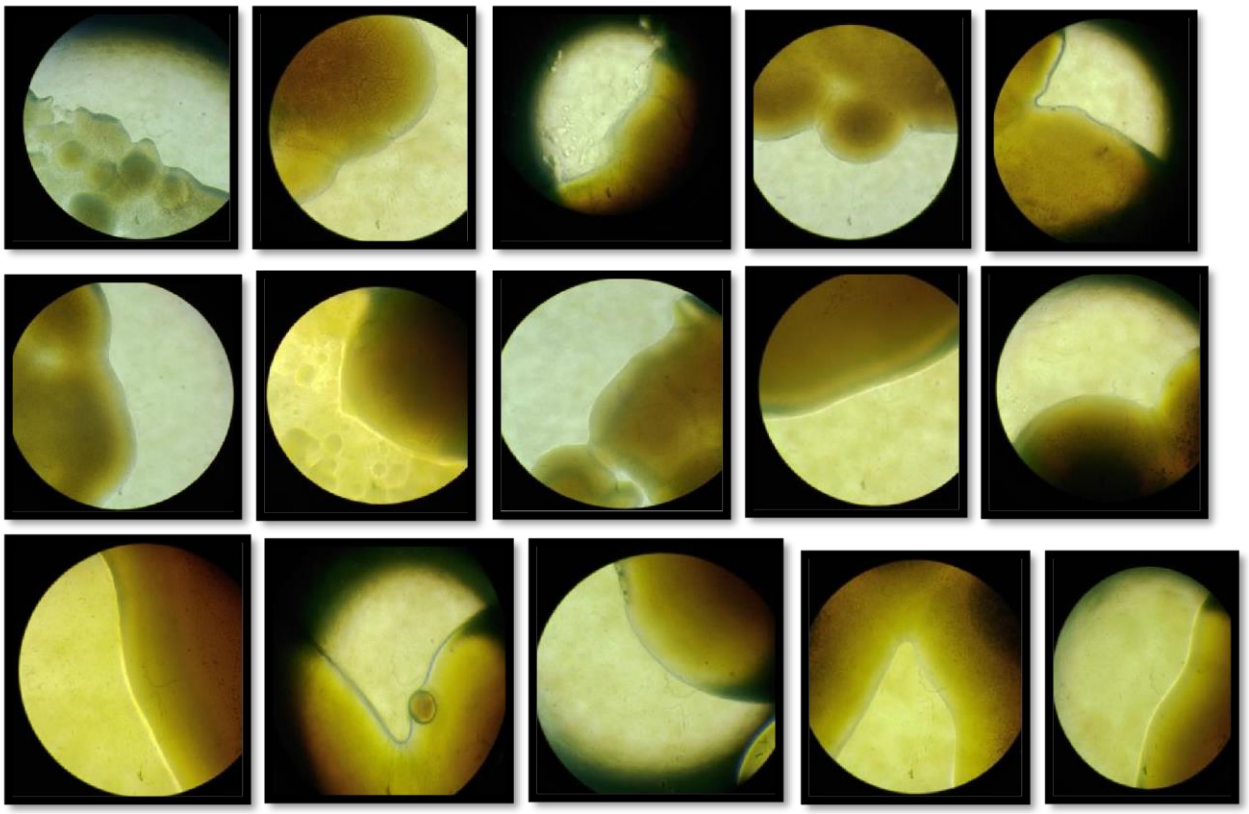


I





J



K

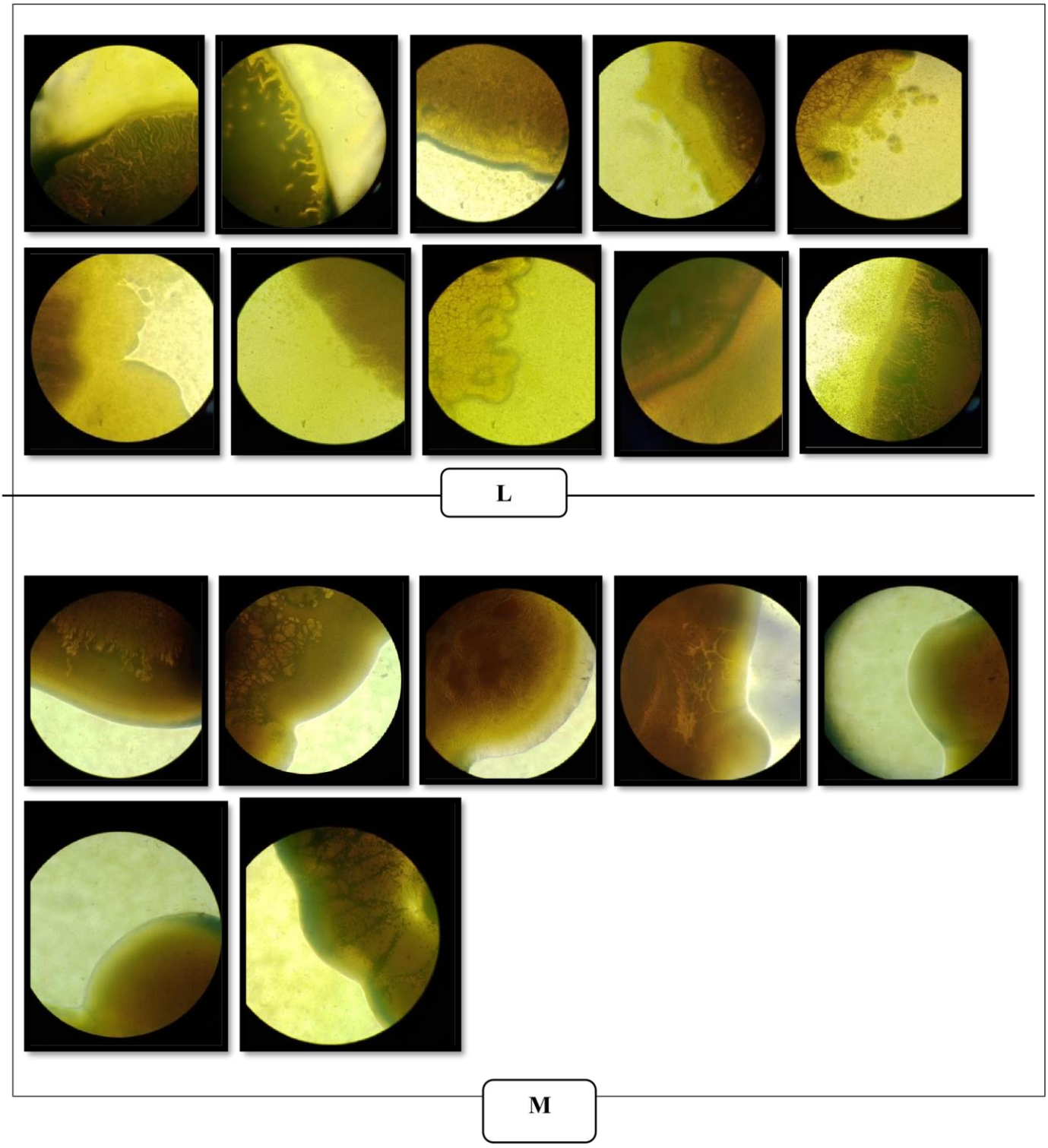


Fig. 16: Microscopy images of the colonies under phase contrast microscope.

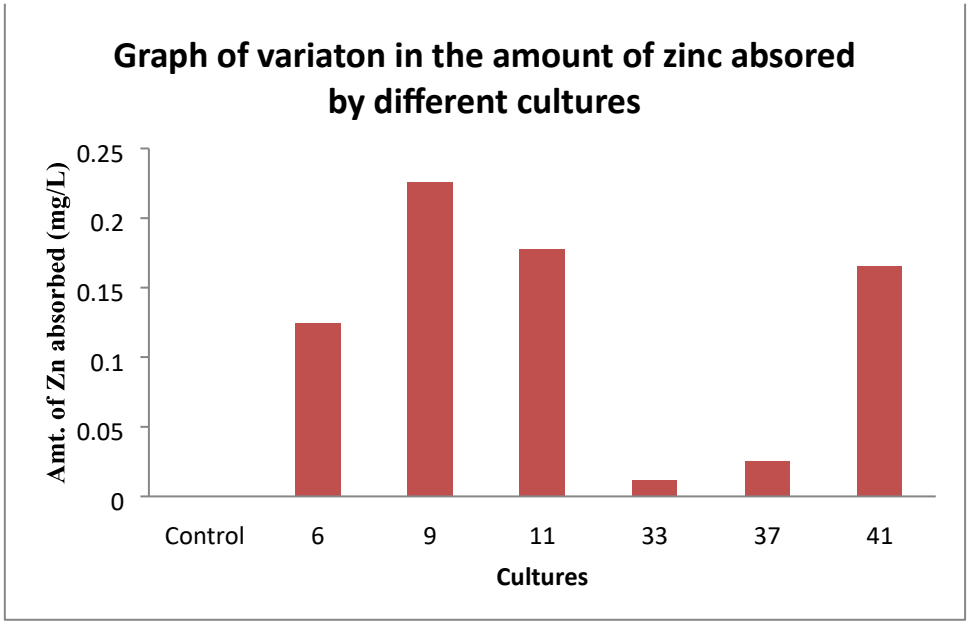
A: control, **B:** 0.1% Fe, **C:** 0.01% Fe **D:** 0.01% Zn, **E:** 0.1% Zn, **F:** 0.01% Co, **G:** 0.1% Co, **H:**0.01% Cu, **I:**0.1% Cu, **J:** 0.01% Mn, **K:**1% Mn, **L:** 0.1% Mn **M:**0.5% Mn

4.6. Atomic Absorption Spectrophotometry analysis of the selected bacterial cultures showing metal tolerance

Bacterial cultures which showed highest absorption of metal was selected by AAS analysis. From the results obtained(table 12,13,14,15,16), it is seen that culture no. 6, 9 and 33 absorbed higher amount of metals as compared to all other cultures therefore out of 6 cultures, these 3 cultures were selected for further analysis.

Table no. 13: Detection of amount of Zn absorbed by the selected bacterial cultures by AAS method

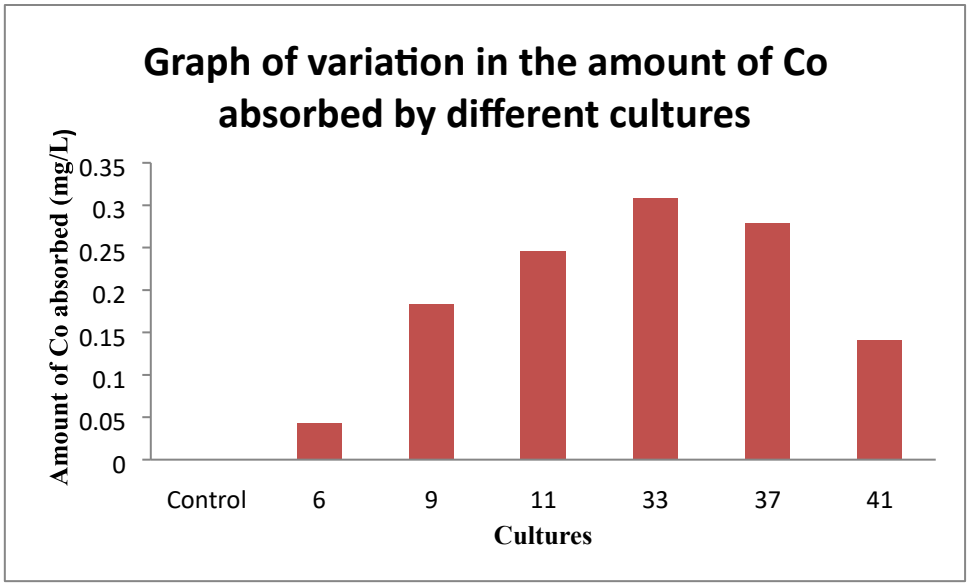
Sample	Conc. (mg/L) detected by AAS (Y)	Amount of metal absorbed by the organism(mg/L) (X-Y)
Control	0.4986 (X)	0
6	0.3742	0.1244
9	0.273	0.2256
11	0.321	0.1776
13	0.4868	0.0118
37	0.4732	0.0254
41	0.3333	0.1653



Graph 1: Amount of Zn absorbed by the bacterial cultures

Table no. 14: Detection of amount of Co absorbed by the selected bacterial cultures by AAS method

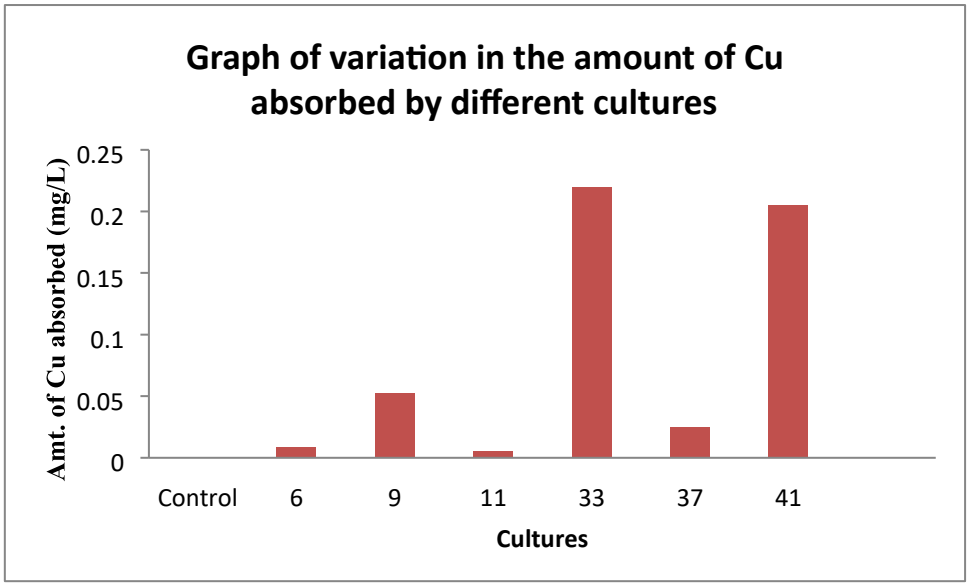
Cultures	Conc. (mg/L) detected by AAS	Amount of metal absorbed by the organism (mg/L)
Control	0.6201	0
6	0.5772	0.0429
9	0.4373	0.1828
11	0.3746	0.2455
33	0.3122	0.3079
37	0.3412	0.2789
41	0.4792	0.1409



Graph 2: Amount of Co absorbed by the bacterial cultures

Table no. 15: Detection of amount of Cu absorbed by the selected bacterial cultures by AAS method

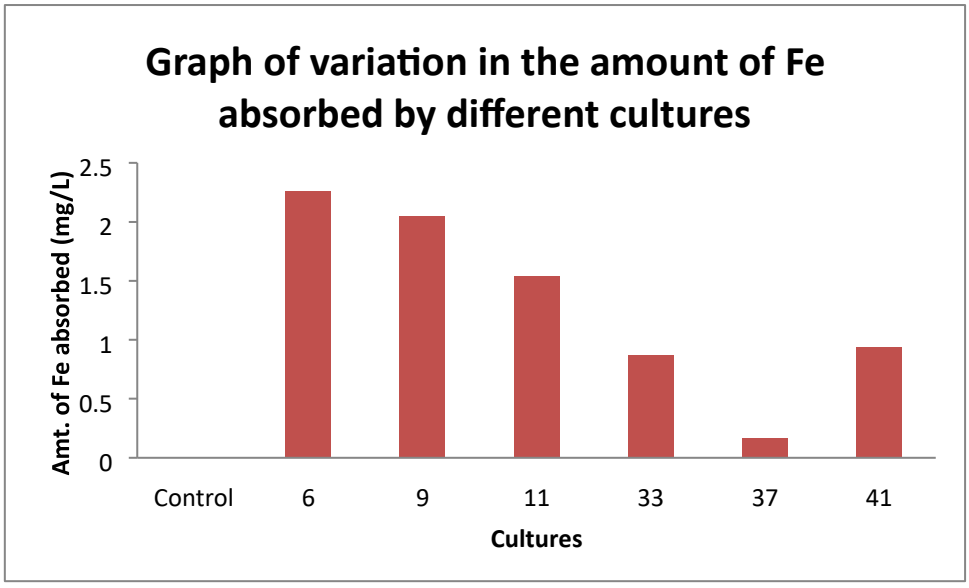
Cultures	Conc. (mg/L) detected by AAS	Amount of metal absorbed by the organism (mg/L)
Control	0.4386	0
6	0.4303	0.0083
9	0.3862	0.0524
11	0.4371	0.0051
33	0.2188	0.2198
37	0.4141	0.0245
41	0.2334	0.2052



Graph 3: Amount of Cu absorbed by the bacterial cultures

Table no.16: Detection of amount of Fe absorbed by the selected bacterial cultures by AAS method

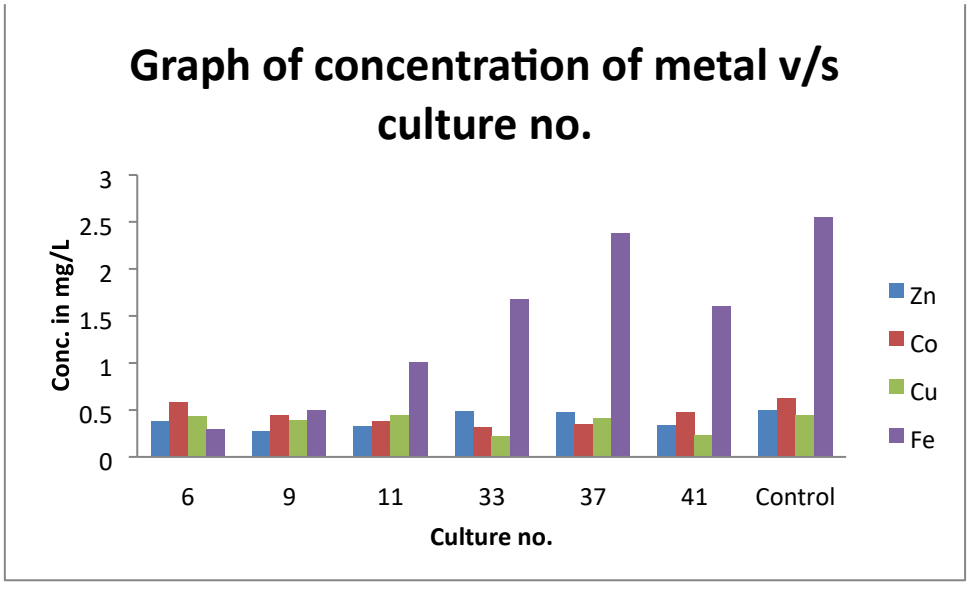
Culture	Conc. (mg/L) detected by AAS	Amount of metal absorbed by the organism (mg/L)
Control	2.545	0
6	0.2873	2.2577
9	0.4959	2.0491
11	1.009	1.536
33	1.6798	0.8652
37	2.3837	0.1613
41	1.6067	0.9383



Graph 4: Amount of Fe absorbed by the bacterial cultures

Table no. 17: Amount of metal detected by AAS in mg/L

Sample	Concentration of metal in mg/L			
	Zn	Co	Cu	Fe
Blank	0	0	0	0
standard 1	0.4	2.5	1	2
Standard 2	0.8	3	2	4
Standard 3	1	6	3	6
Standard 4	1.5	9	5	9
6	0.3742	0.5772	0.4303	0.2873
9	0.273	0.4373	0.3862	0.4959
11	0.321	0.3746	0.4371	1.009
33	0.4868	0.3122	0.2188	1.6798
37	0.4732	0.3412	0.4141	2.3837
41	0.3333	0.4792	0.2334	1.6067
Control	0.4986	0.6201	0.4386	2.545



Graph 5: Amount of metal present detected by AAS

4.6 EPS extraction and quantification

Different concentration of EPS was extracted by each culture grown in the presence of metal (table no. 19 and Graph 6).

Table no. 18: Weight of Eppendorf tubes in the absence of pellet

Culture no.	Weight of Eppendorf in g (Y)				
	Zn	Fe	Mn	Cu	Co
6	1.12	1.138	1.116	1.122	1.135
9	1.099	1.132	1.152	1.113	1.164
11	1.143	1.101	1.147	1.147	1.139
33	1.129	1.155	1.117	1.131	1.117
37	1.117	1.126	1.163	1.13	1.133
41	1.137	1.133	1.118	1.132	1.148

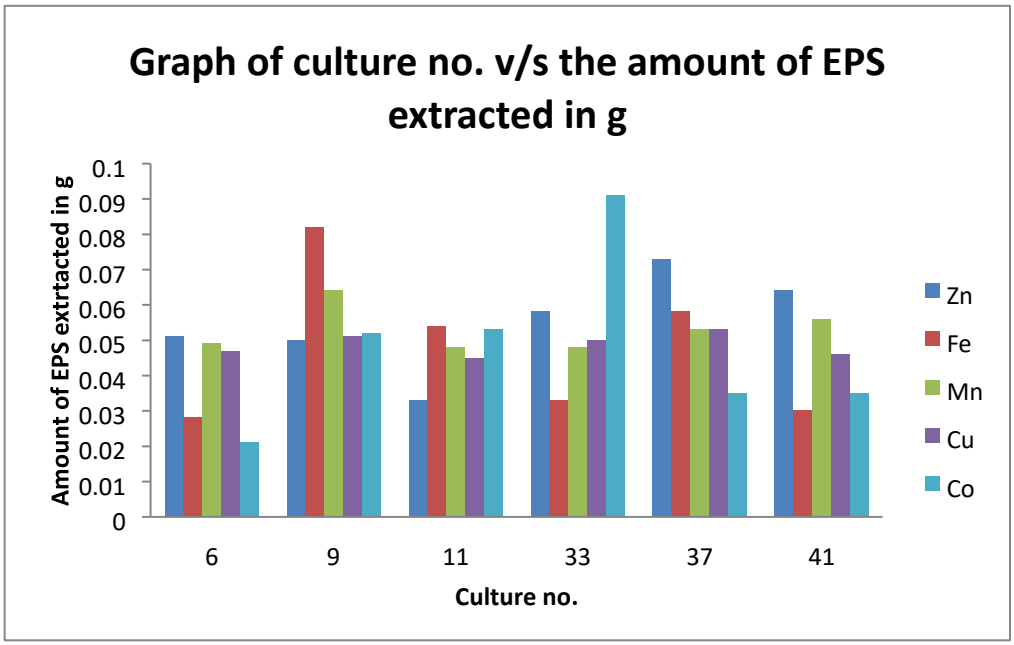
Table no. 19: Weight of Eppendorf tubes along with EPS pellet

Culture no.	Weight of Eppendorf + eps pellet in g (X)				
	Zn	Fe	Mn	Cu	Co
6	1.171	1.166	1.165	1.146	1.156
9	1.149	1.214	1.216	1.164	1.216
11	1.176	1.155	1.195	1.155	1.192
33	1.187	1.188	1.165	1.181	1.208
37	1.19	1.184	1.216	1.183	1.168
41	1.201	1.163	1.174	1.178	1.183

Table no. 20: Amount of EPS extracted from the cultures in the presence of metal concentration of 0.01%

Culture no.	Amount of EPS extracted (X-Y) in g				
	Zn	Fe	Mn	Cu	Co
6	0.051	0.028	0.049	0.047	0.021
9	0.05	0.082	0.064	0.051	0.052
11	0.033	0.054	0.048	0.045	0.053
33	0.058	0.033	0.048	0.05	0.091
37	0.073	0.058	0.053	0.053	0.035

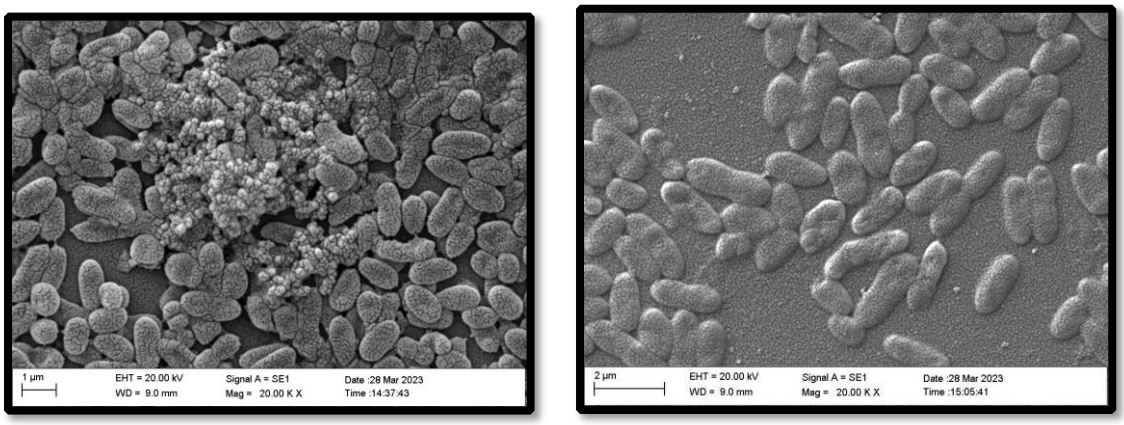
41	0.064	0.03	0.056	0.046	0.035



Graph 6: Different amount of EPS extracted by different cultures when grown in the presence of metals

4.7 SEM analysis of the selected bacterial cultures

Bacterial cultures grown in the presence of metal solution formed clusters with eachother whereas the bacterial cultures grown in the absence of metals did not show any clusters (accumulation).



(A) (B)

Fig.17: SEM image of bacterial culture no.6 grown in the presence and absence of Zn. **A :** Presence of Zn, **B**: Absence of Zn

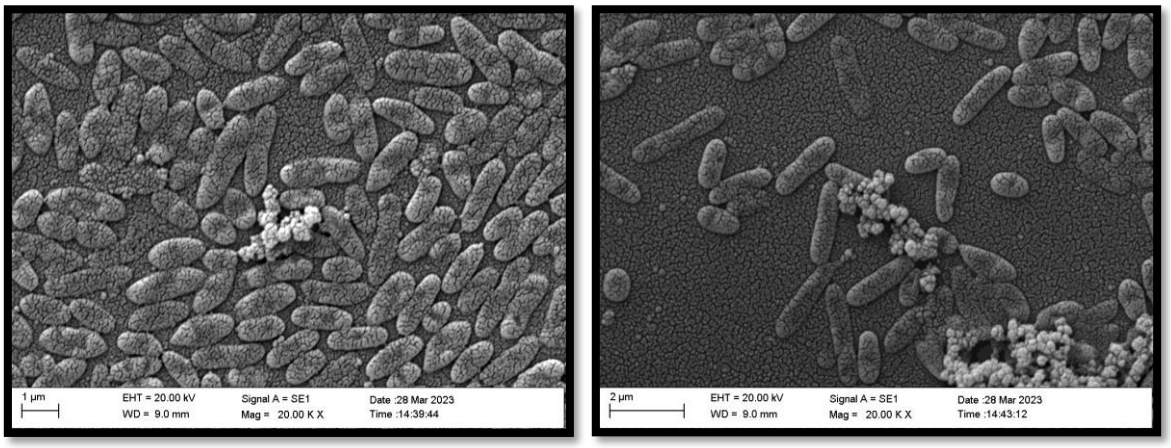


Fig. 18: SEM image of bacterial culture no.33 grown in the presence of Mn

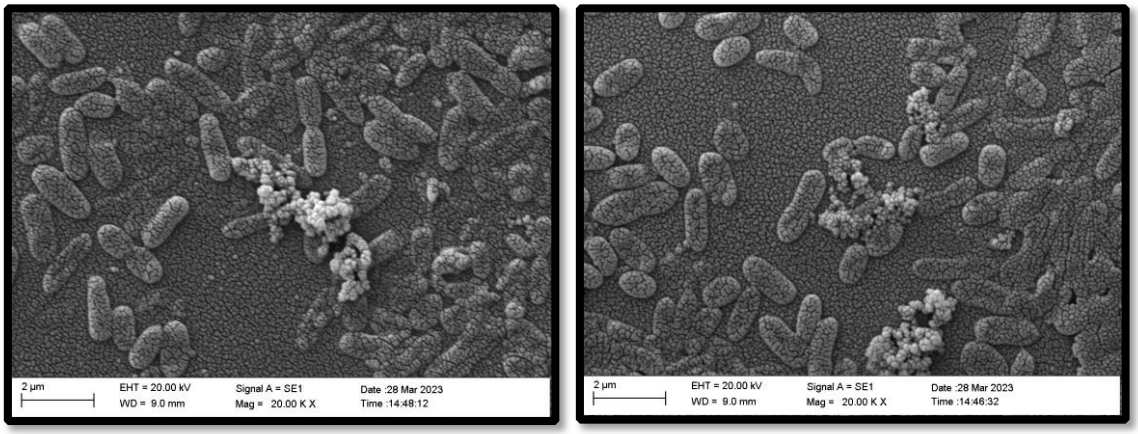


Fig.19: SEM image of bacterial culture no.33 grown in the presence of Cu

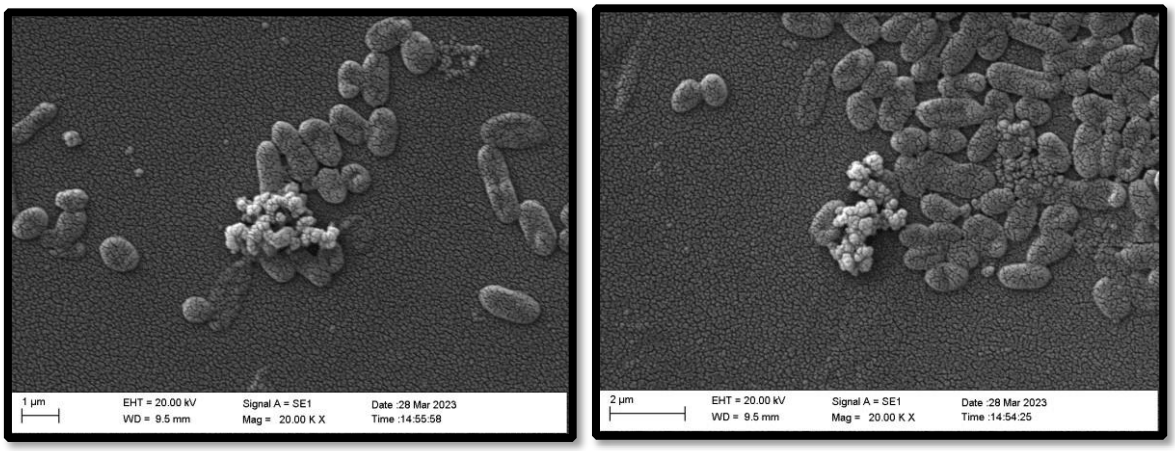


Fig. 20: SEM image of bacterial culture no.33 grown in the presence of Co

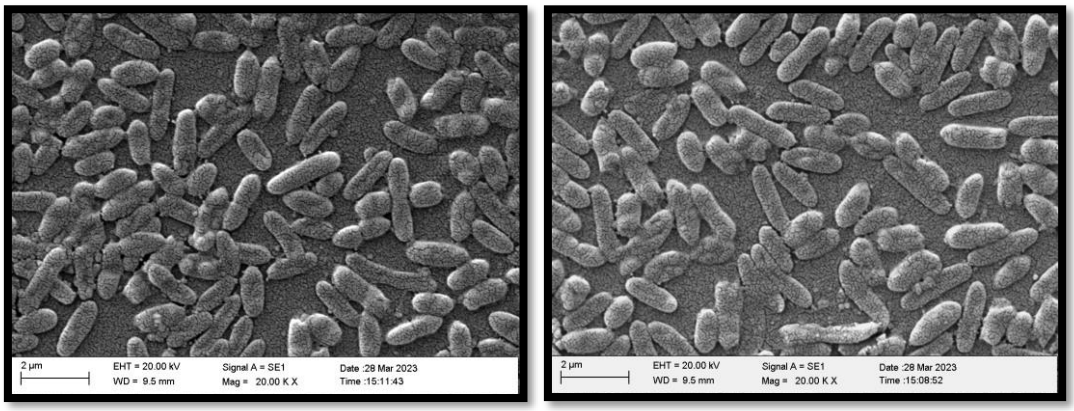


Fig. 21: SEM image of bacterial culture no.33 grown in the absence of any metal

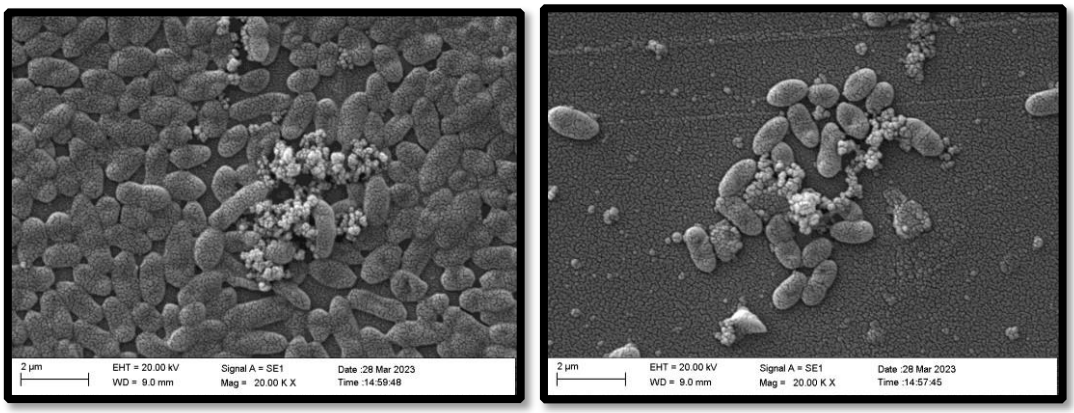


Fig. 22: SEM image of bacterial culture no.6 grown in the presence of Fe

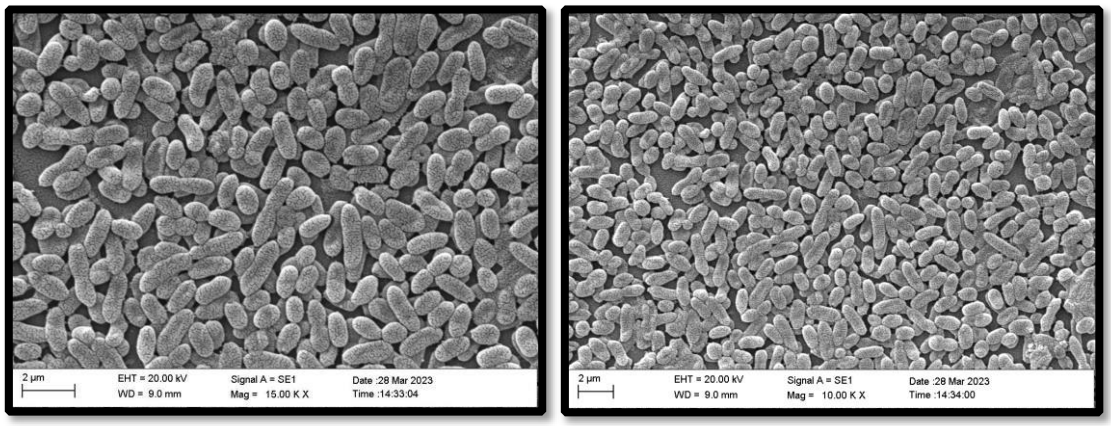


Fig. 23: SEM image of bacterial culture no.6 grown in the absence of Fe

4.8 Growth curve of the bacterial cultures in the presence and absence of metals.

Growth curve of all the three cultures in the presence and absence of their respective

metals was carried out and compared with each other. It was observed that the cultures reach the stationary phase very quickly in the presence of heavy metals. The growth of the culture is very slow and the growth is very less as compared to the cultures grown in the media containing no metal solution.

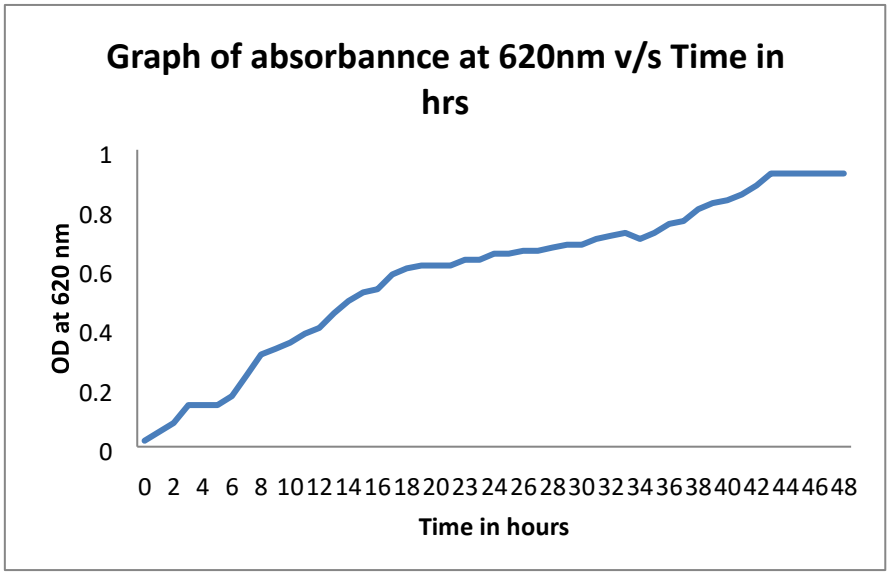
Fig..24: Culture grown in the presence of different metals and the control flasks

Table no.21: Difference in the OD of each bacterial cultures in the presence and absence of heavy metals

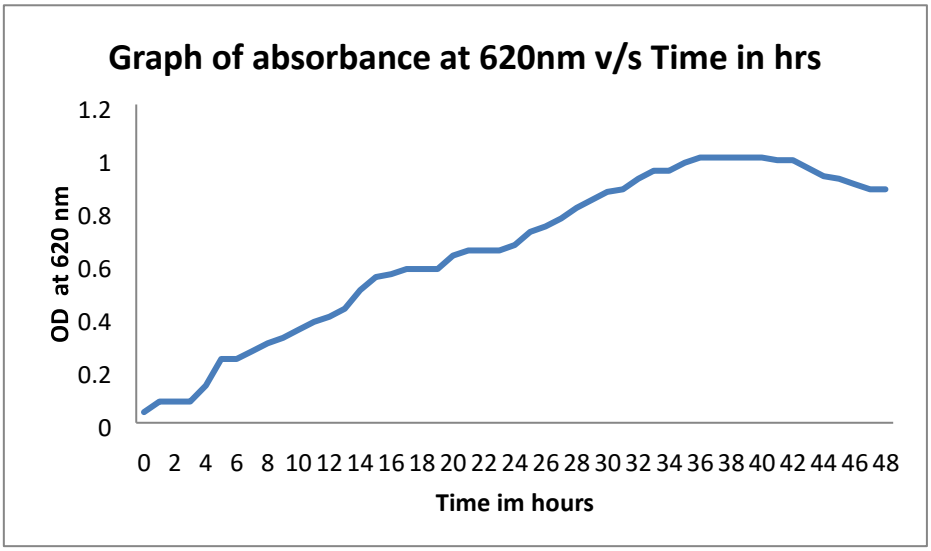
Time	OD at 620nm							
	Culture no. 06		Culture no. 33				Culture no. 09	
	Control	FE	Control	Mn	Co	Cu	Control	Zn

0	0.02	0.04	0.02	0.01	0.02	0.02	0.02	0.02
1	0.05	0.08	0.02	0.02	0.02	0.06	0.02	0.02
2	0.08	0.08	0.02	0.02	0.02	0.06	0.05	0.07
3	0.14	0.08	0.06	0.08	0.04	0.09	0.09	0.1
4	0.14	0.14	0.1	0.14	0.04	0.1	0.16	0.13
5	0.14	0.24	0.14	0.18	0.05	0.12	0.24	0.16
6	0.17	0.24	0.18	0.21	0.06	0.12	0.25	0.17
7	0.24	0.27	0.19	0.25	0.07	0.13	0.27	0.17
8	0.31	0.3	0.22	0.25	0.07	0.14	0.3	0.19
9	0.33	0.32	0.22	0.25	0.08	0.14	0.31	0.2
10	0.35	0.35	0.26	0.29	0.08	0.16	0.34	0.22
11	0.38	0.38	0.27	0.32	0.1	0.17	0.35	0.22
12	0.4	0.4	0.3	0.36	0.11	0.19	0.38	0.24
13	0.45	0.43	0.32	0.38	0.11	0.19	0.38	0.25
14	0.49	0.5	0.35	0.39	0.13	0.22	0.39	0.26
15	0.52	0.55	0.38	0.39	0.13	0.24	0.42	0.27
16	0.53	0.56	0.41	0.4	0.13	0.25	0.42	0.28
17	0.58	0.58	0.42	0.42	0.15	0.25	0.43	0.28
18	0.6	0.58	0.42	0.42	0.15	0.25	0.44	0.29
19	0.61	0.58	0.44	0.45	0.17	0.26	0.45	0.3
20	0.61	0.63	0.46	0.46	0.18	0.27	0.45	0.3
21	0.61	0.65	0.48	0.49	0.18	0.27	0.46	0.3
23	0.63	0.65	0.48	0.49	0.2	0.27	0.48	0.31
23	0.63	0.65	0.49	0.52	0.2	0.29	0.49	0.32
24	0.65	0.67	0.49	0.58	0.2	0.3	0.52	0.33
25	0.65	0.72	0.52	0.53	0.23	0.33	0.55	0.33
26	0.66	0.74	0.55	0.53	0.23	0.34	0.55	0.34
27	0.66	0.77	0.55	0.54	0.25	0.38	0.58	0.35
28	0.67	0.81	0.57	0.57	0.27	0.39	0.62	0.36
29	0.68	0.84	0.59	0.57	0.28	0.39	0.63	0.36
30	0.68	0.87	0.6	0.59	0.28	0.4	0.63	0.38

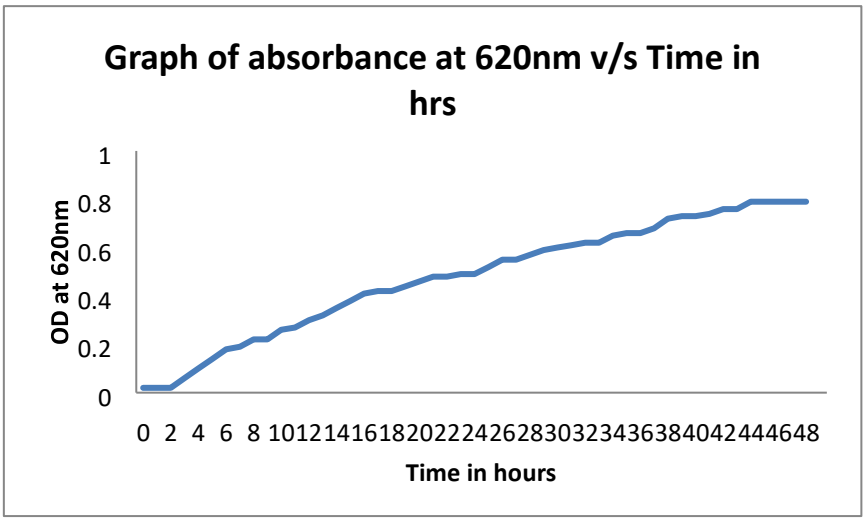
31	0.7	0.88	0.61	0.6	0.3	0.42	0.66	0.41
32	0.71	0.92	0.62	0.63	0.34	0.42	0.67	0.41
33	0.72	0.95	0.62	0.63	0.34	0.43	0.69	0.41
34	0.7	0.95	0.65	0.65	0.38	0.44	0.69	0.42
35	0.72	0.98	0.66	0.67	0.38	0.46	0.69	0.44
36	0.75	1	0.66	0.67	0.39	0.47	0.72	0.44
37	0.76	1	0.68	0.67	0.39	0.49	0.74	0.45
38	0.8	1	0.72	0.69	0.41	0.49	0.76	0.45
39	0.82	1	0.73	0.72	0.41	0.5	0.79	0.45
40	0.83	1	0.73	0.74	0.41	0.5	0.82	0.46
41	0.85	0.99	0.74	0.79	0.43	0.51	0.85	0.48
42	0.88	0.99	0.76	0.79	0.44	0.51	0.88	0.48
43	0.92	0.96	0.76	0.79	0.46	0.52	0.88	0.48
44	0.92	0.93	0.79	0.79	0.46	0.52	0.92	0.5
45	0.92	0.92	0.79	0.79	0.46	0.52	0.95	0.51
46	0.92	0.9	0.79	0.76	0.46	0.53	0.95	0.51
47	0.92	0.88	0.79	0.76	0.48	0.53	0.95	0.51
48	0.92	0.88	0.79	0.75	0.48	0.54	0.95	0.51



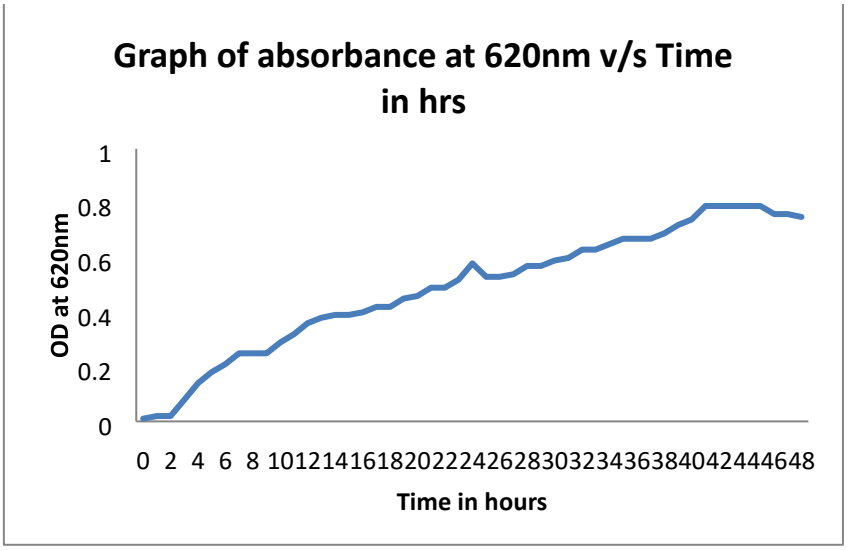
Graph 7: Growth curve of culture no.6 in the absence of Fe



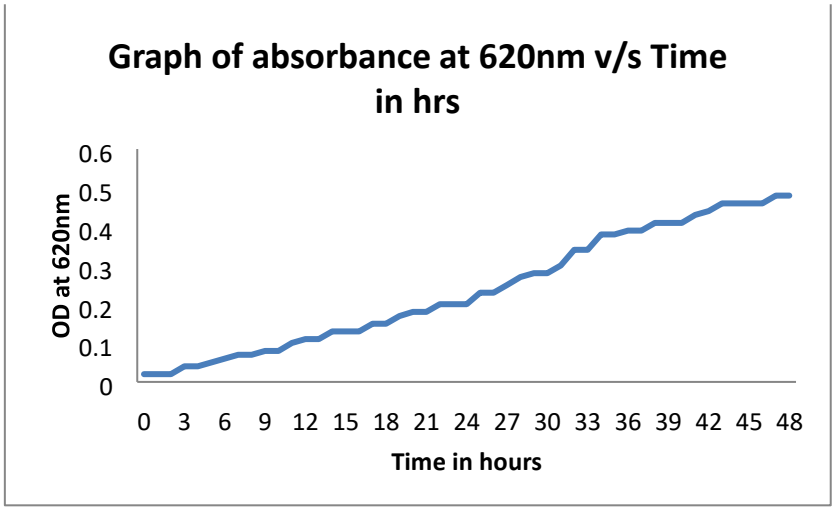
Graph 8: Growth curve of culture no.6 in the presence of Fe



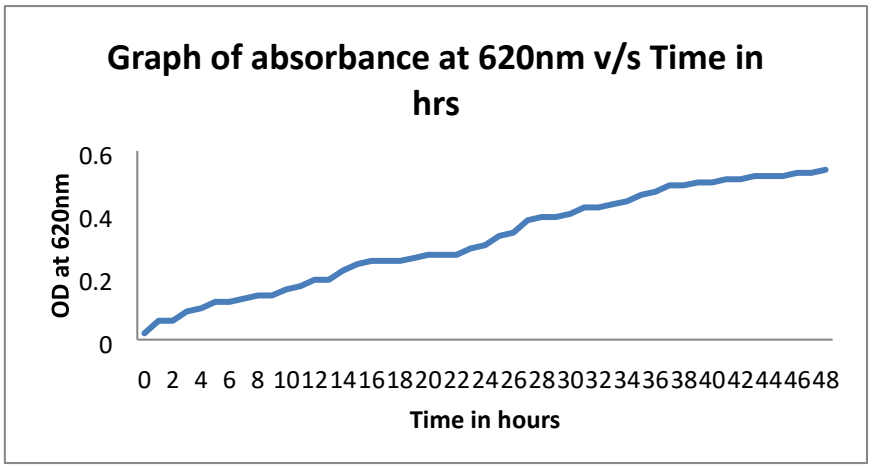
Graph 9: Growth curve of culture no. 33 in the absence of metal



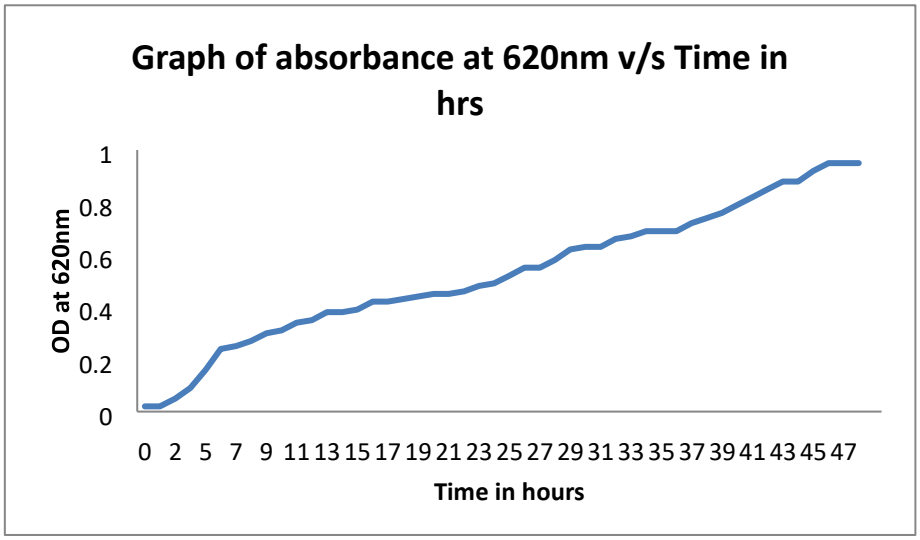
Graph 10: Growth curve of culture no. 33 in the presence of Mn



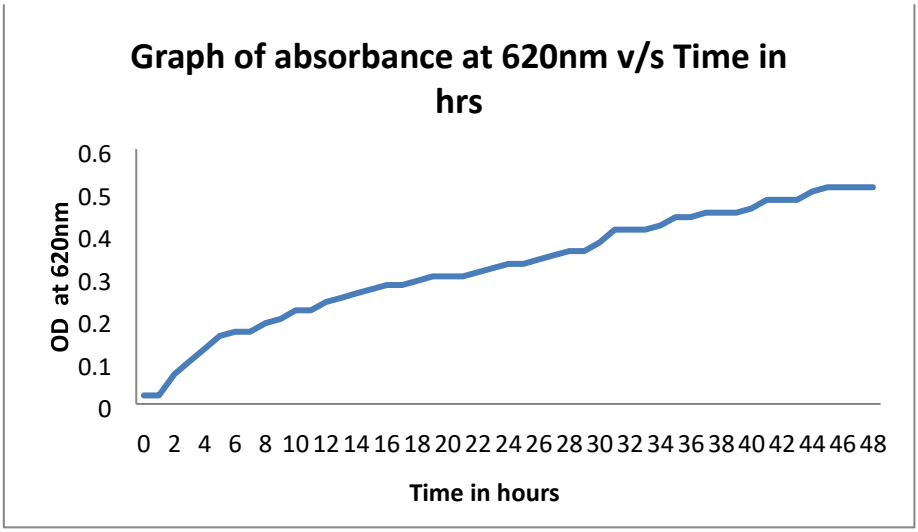
Graph 11: Growth curve of culture no. 33 in the presence of Co



Graph 12: Growth curve of culture no. 33 in the presence of Cu



Graph 13: Growth curve of culture no.9 in the absence of Zn



Graph 14: Growth curve of culture no.9 in the presence of Zn

4.9 Antibiotic sensitivity testing of the bacterial cultures

All the three cultures, i.e., culture no. 6, 9 and 33 was tested in the presence of antibiotics using sterile HiMedia antibiotic disks and the results obtained are given in the following table (table no. 21)

Table no. 22: Sensitivity and resistance of the cultures against particular antibiotics

Antibiotic disk	Zone of inhibition in mm					
	Culture no.6		Culture no.9		Culture no. 33	
Ampicillin	7mm	Resistant	7mm	Resistant	18mm	Sensitive
Chloramphenicol	27mm	Sensitive	28mm	Sensitive	18mm	Sensitive
Erythromycin	11mm	Resistant	16mm	Intermediate	7mm	Resistant
Gentamycin	25mm	Sensitive	28mm	Sensitive	25mm	Sensitive
Kanamycin	22mm	Sensitive	20mm	Sensitive	16mm	Intermediate
Nalidixic acid	20mm	Not known	7mm	Not known	7mm	Not Known
Rifampicin	15mm	Not known	16mm	Not known	12mm	Not Known
Streptomycin	26mm	Sensitive	25mm	Sensitive	22mm	Sensitive
Tetracycline	20mm	Sensitive	26mm	Sensitive	20mm	Sensitive

Vancomycin	15mm	Sensitive	15mm	Intermedi ate	12mm	Resistant

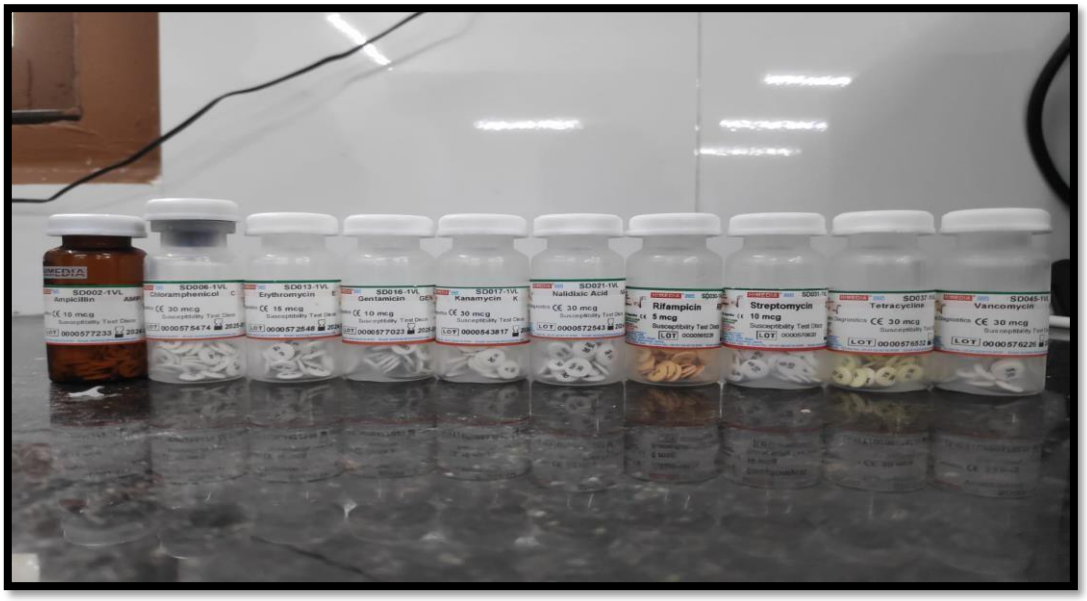


Fig. 25: Antibiotic disks used for carrying out antiobiotic sensitivity testing of the bacterial cultures

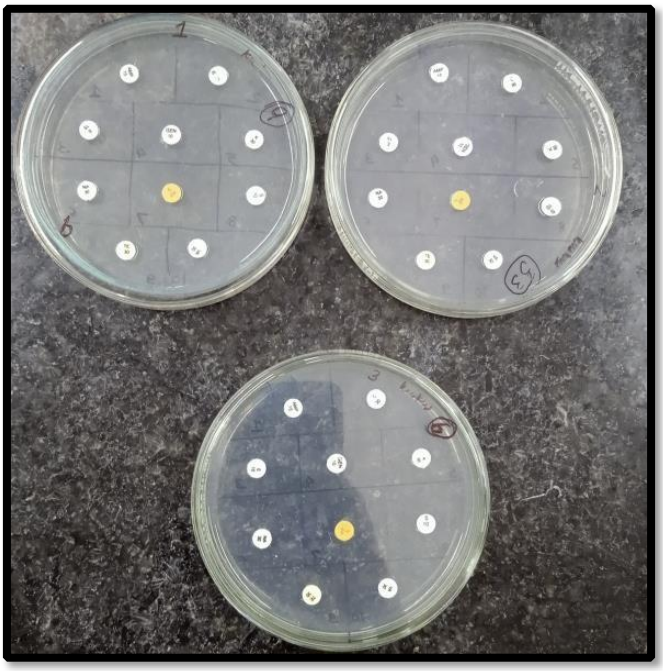
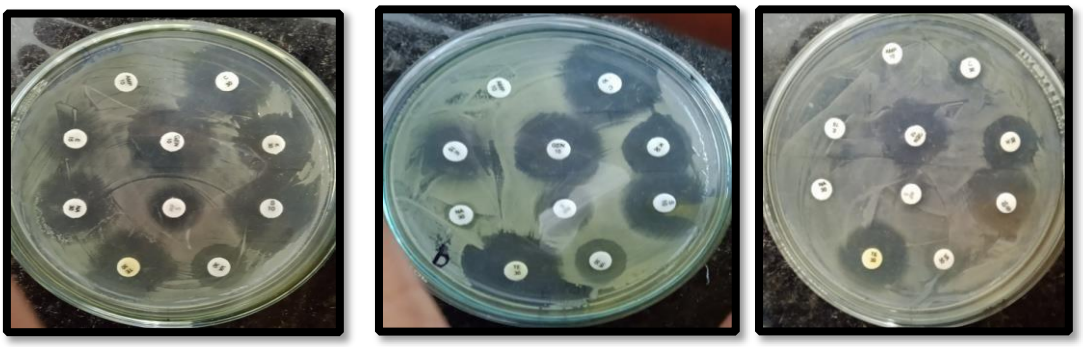


Fig. 26: Spread plating and inoculation of the antibiotic disks into the plates



(A) (B) (C)

Fig. 27: Zone of inhibitions observed on the culture plates.

A: Culture no.6, **B**: Culture no.9, **C**: Culture no. 33

4.10Estimation of amount of protein present in the culture by Folin Lowry Method

The amount of protein present in the cultures were quantified with Folin Lowry method of protein quantification. The changes in the amount of protein present in the presence of metal and absence of metal is given in the following table(table no.22) and the standard graph is attached in the appendix .

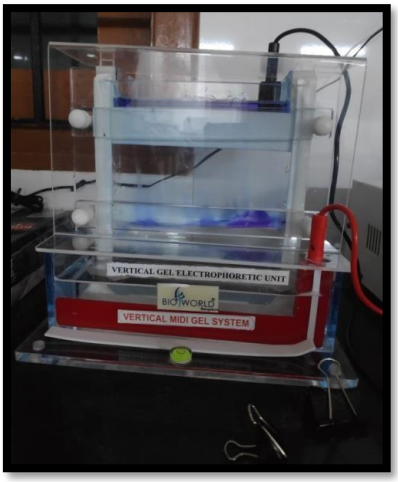
Table no. 23: Concentration of protein present in the cultures.

Conc. Of protein (µg/mL)	OD at 660 nm	Conc. In 500µL (mg/0.5mL)	Conc. In 1mL (mg/mL)
Copper 33	0.632	550	1100
Cobalt 33	0.539	460	920
Manganese 33	0.736	480	1360
Iron 6	0.588	500	1000

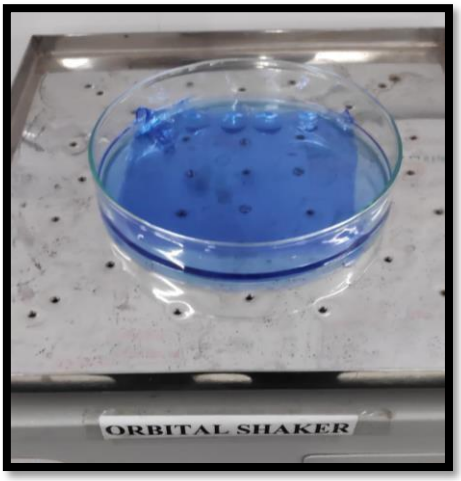
Zinc 9	0.698	600	1200
6 control	0.579	490	980
9 control	0.443	370	740
33 control	0.473	400	800

4.11 Estimation of the protein present in the bacterial isolates by SDS-PAGE method

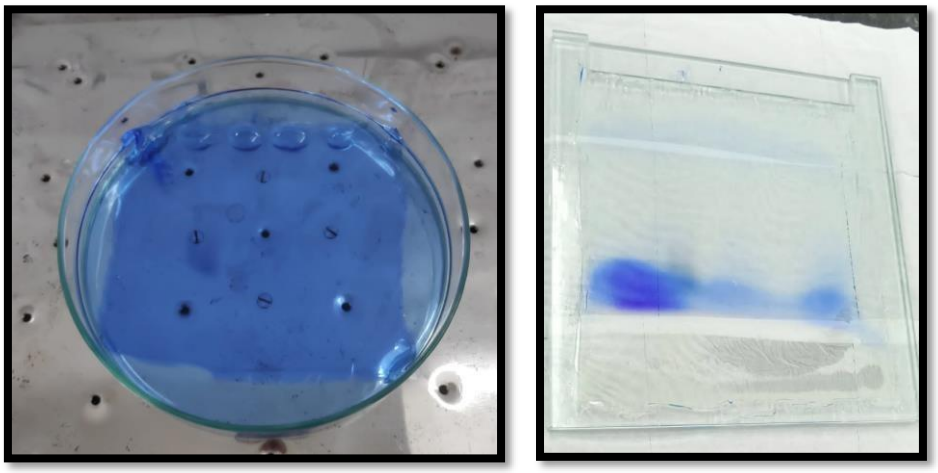
Proteins extracted from the cultures grown in the presence of metals and without metals were run on SDS PAGE to detect the type of protein present. The gel did not show any bands after running, this may be because the staining was not proper or may be because a very less amount of sample was loaded on the gel. Insufficient current or running buffer may also be the possible reasons.



(A)



(B)



(C) (D)

Fig. 28: Set up and running of the SDS-PAGE

A: Running of the gel, **B:** Overnight staining of the gel, **C:** Destaining of the gel, **D:** Gel after running.

4.12 Bioremediation of metal polluted soil and water sample

The three selected and tested metal tolerant bacterial cultures have been inoculated into the raw water and soil sample to check or its bioremediation preoperties.

Fig. 29: Shikeri Mining area (soil sampling site)

Fig. 30: Cortalim (water sampling site)

Fig.31: water samples inoculated with culture 6, 9 and 33 separately

Fig. 32: Soil samples inoculated with culture 6, 9 and 33 separately.

4.13 Gram staining of the bacterial cultures

Gram staining of the bacterial cultures were carried out to determine the gram character of each of the bacterial isolates. Out of the three metal tolerant cultures, one was Gram positive rod (culture no.6) . and two were Gram nehative rods (culture no. 9 and 33)

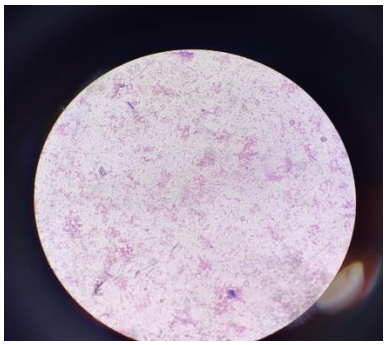
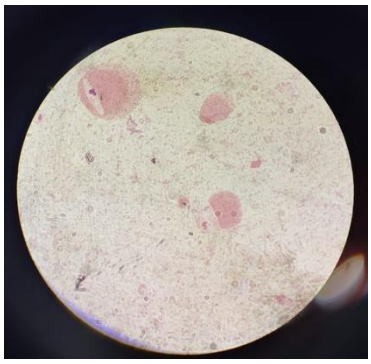
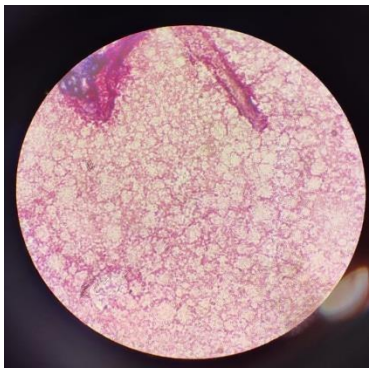
Culture	Microscope Image	Gram Character
6		Gram Positve Rods
9		Gram Negative Rods
33		Gram Negative Rods

Fig. 33 : Microscopy images of the cultures after Gram staining.

5. Discussion

Heavy metals even at very little amount is present everywhere and greater accumulation of it posses a greater threat to aquatic as well as terrestrial life forms. (Chang & jung, 2016) In this study, I worked on 51 different cultures out of which just 6 of them showed good results with respect to tolerating greater concentration of metal. This may be because the bacteria could not survive the heavy metal concentration as we know from (P.R. & Sreedevi, 2022) that higher concentration of the metal is toxic to the bacterial strains. After isolating the cultures in pure form, it was subjected to screening n broth with metal concentration of 0.01% in which 17 cultures showed metal tolerance. These 17cultures were then introduced in different concentration of media in the agar medium which resulted in only eight out of 17

cultures to show metal tolerance. It was noted that the medium containing Fe and Mn had greater number of colonies at higher concentration than Cu and Co. This may be because the environment from which these cultures were isolated already contained some amount of Mn and Fe but did not have any concentration of Co or Cu in it. Hence they are sensitive towards these particular metals. There was also change in pigmentation of isolates during growth on media containing different metals which may be due to binding of the metals to the isolates or effect of metals i.e., change in proteins due to presence of metals in the isolates. Accumulation of metal along the border or margin of the colonies could also be seen when viewed the colonies under phase contrast microscope using 10x objective.

Atomic Absorption Spectrophotometry (AAS) helped to select the bacterial culture which shows greater amount of metal absorption. AAS determines the amount of metal present in the sample to be tested. The culture was selected based on the strategy that lesser the amount of metal present within the sample (smaller the AAS reading in terms of mg/L), the greater the amount of metal is absorbed by the bacterial culture. Hence, it was seen that the culture no. 6 absorbs greater amount of Fe, culture no. 9 absorbs greater amount of Zn and culture no. 33 absorbes greater amount of Cu, Co and Mn. Culture no. 33 alone can be used to bioremediate all these three metal polluted areas together.

The EPS results stated that culture 33 produces higher amount of EPS, implies higher amount of metal can be accumulated or biosorbed by this bacterial culture. (Pal &

Paul, 2008)Therefore, biosorption of metals by culture 33 is to a greater extent and produces good positive results. (Monika, Priyadarshanee, & Das, 2021)When these cultures were subjected to Scanning Electron Microscopy, the difference between the pure culture and the culture grown in the presence of metal was clearly visible. The cultures grown in the presence of metals showed adsorption of metals onto their surface whereas the control cultures were clearly visible as isolated cells. The growth of the cultures in the presence of metals was also slow as compared to the growth of the cultures without any involvement of metals. Especially, the culture growing in the presence of Cu and Co, their growth rate was very less or slow as compared to the cultures growing in other metals.

Culture no. 6, 9 and 33 when subjected to antibiotics, showed resistance towards different types of antibiotics. Culture 6 was resistant against Ampicillin and Erythromycin, culture no. 9 showed resistance against ampicillin only and culture no. 33 was resistant against Erythromycin and Vancomycin. The protein estimation by Folin Lowry method also showed that there was a difference in the amount of protein present within the culture in the presence of metal and in the absence of metal. According to the results, there was increase in the amount of protein within the culture when they were subjected to heavy metals , implies, there might be binding of metals to the cell surface and leading to structural and conformational changes and changes in the concentration of proteins present within the cell. Therefore, we can say that presence of metal does impact the growth rate and protein concentration of a particular bacterial isolate. Since culture no. 6, 9 and 33 showed good results for bioremediation of heavy metals, this culture were then individually applied to raw water and soil samples of Cortalim and Bicholim area so that they bioremediate the heavy metals present in it and convert it into less toxic form. These 3 tested cultures can also be used as a mixture in future, to bioremediate heavy metals present in the metal polluted area since it is known by (Chang & jung, 2016) that bacteria in the form of a mixture bioremediates heavy metals more efficiently than in the form of single isolated culture. All the three cultures obtained are identified to be rods (by Gram staining) out of which culture no. 6 is Gram negative rod and culture no. 9 and 33 are Gram positive rods. Since they are rods in shape and we already know from (Sharma & Jasrotia, 2021) and (Feliphe & Julio, 2017) that the *Bacillus* spp. bioremediate the heavy metals more efficiently, there is a higher possibility that these isolated and tested bacterial cultures might be *Bacillus* spp.

6. Conclusion

Bioremediation using bacteria is a promising and effective approach for the removal of heavy metals from contaminated environments. Bioremediation of heavy metals using bacteria typically involves a two-step process: bioaccumulation and biotransformation. During the bioaccumulation step, bacteria uptake heavy metals from the environment through various mechanisms such as adsorption, ion exchange, and surface complexation. The heavy metals are then stored in the bacterial cell in a process known as biosorption. This helps to remove the heavy metals from the environment and prevent their further spread. In the biotransformation step, the bacteria transform the heavy metals into less toxic or non-toxic forms through various mechanisms such as oxidation, reduction, methylation, and demethylation. This process is facilitated by the production of enzymes by the bacteria that are capable of breaking down or transforming the heavy metals into less harmful forms.

Moreover, the use of bacteria for bioremediation is eco-friendly and cost- effective compared to conventional remediation methods. However, the success of bioremediation largely depends on several factors such as bacterial strains, metal concentrations, environmental conditions, and the availability of nutrients. In order to select a bacterial strain for bioremediation of heavy metals, various experiments were carried out. From all the results obtained, we can conclude that the bacteria which absorbs higher concentration of metal are the one's which are more capable of bioremediation. The bacteria produces EPS which are responsible for the bioaccumulation or bioadsorption of the metal to the bacterial surface. Hence, the greater the amount of EPS produced, greater is the bioremediation efficiency of the bacterial isolate.

It was also seen that as the concentration of these heavy metals increases, the amount of viable bacteria decreases, which implies that even a little amount of these heavy metals such as Zn, Cu, Co, Fe, and Mn can have a large deleterious effect on the living biota and hence it is important to find out the bacterial cultures that will help to bioremediate all these harmful metals. Bacteria which grow in the presence of heavy metals at high concentration are the one's which are responsible for the bioremediation of these heavy metals by the mechanism of either bioadsorbing or bioaccumulating

these metals for their growth and development. Hence, they can convert a large amount of heavy toxic metals into less toxic form. However, the concentration of metal at a very high amount is toxic and only certain bacteria can tolerate the metal upto certain percent and then it becomes sensitive towards the metal after their exposure to high amount of metal concentration. (P.R. & Sreedevi, 2022) Therefore, at higher metal concentration, there are hardly any bacterial isolates growing. Also it is clearly visible that the metals also have an effect on growth rate of the bacterial isolates. The growth rate of the bacteria is also very slow and minimun in the presence of the metals.

Sometimes during the process of bioremediation, the bacteria undergo certain structural and functional changes, this also leads to alteration of certain proteins present in them thereby leading to changes in the functional properties of the bacterial species. But, however, this is said to be the most eco- friendly and cost- effective approach to carry out bioremediation of any metal polluted environment and can be used for any upcoming projects to bioremediate large amount of land or water using easily culturable and low maintainance bacterial cultures which are naturally found in the environment.

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8. 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

1. 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

1. Saline

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

Appendix 2 Metals

Metal	Molecular weight
Cobaltous Chloride Hexahydrate	237.93g
Zinc Chloride	136.30g
Ferric chloride hexahydrate	270.30g
Copper(II) chloride dehydrate	170.48g
Manganese (II) chloride tetrahydrate	197.91g

Appendix 3 preparation of metal stock solution

10% Metal stock	Conc. of metal in g	Volume of distilled water in mL
$CoCl_2.6H_2O$	1	10
$ZnCl_2$	1	10
$FeCl_3.6H_2O$	1	10
$CuCl_2.2H_2O$	1	10
$MnCl_2.4H_2O$	1	10

Appendix 4 Preparation of metal solutions

Conc. of metal	Volume of metal stock	Volume of distilled water
0.01%	0.02mL	20mL
0.1%	0.2mL	20mL

0.5%	1mL	20mL
1%	2mL	20mL
2%	4mL	20mL

Appendix 5 Preparation of SEM reagents

1. Phosphate buffer saline (1X)

Reagents	Concentration in gm/L
NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Distilled water	1000mL

Adjust pH to 7.4

2. Ethanol

Conc. of ethanol	Volume of ethanol	Volume of distilled water
30%	15mL	50mL
50%	25mL	50mL
70%	35mL	50mL
90%	45mL	50mL

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 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

Appendix 7 Protein estimation by Folin-Lowry Method

Standard : 1mg /ml BSA

Diluent : Distilled water

Reagents: Solution A: 2% sodium carbonate in 0.1 N NaOH

Solution B: 0.5% copper sulphate solution in 1% sodium potassium tartarate solution.

Alkaline copper sulphate solution: 50 ml solution A + 1 ml of solution B. Prepare it just before using.

Folin- ciocalteau reagent- 1 part Folin-Phenol [2 N]: 1 part water (*1:1)*

Test tube No.	**Conc. Ug/ml**	**Stock (ml)**	**Diluent (ml)**	**Alkaline CuS04**		**Fc reagent**		**OD at 660nm**
Blank	-	-	1.0	5.0	**Mix And wait for 1 min**	0.5	**Incubate for 50 Min in**	0.000
1	20	0.2	0.8	5.0		0.5		0.397
2	40	0.4	0.6	5.0		0.5		0.625
3	60	0.6	0.4	5.0		0.5		0.685
4	80	0.8	0.2	5.0		0.5		0.775
5	100	1.0	-	5.0		0.5		0.925
Unknown 1	-	0.5	-	1.5		0.5		0.632

Unknown 2	-	0.5	-	1.5		0.5	**dark**	0.539
Unknown 3	-	0.5	-	1.5		0.5		0.736
Unknown 4	-	0.5	-	1.5		0.5		0.588
Unknown 5	-	0.5	-	1.5		0.5		0.698
Unknown 6	-	0.5	-	1.5		0.5		0.579
Unknown 7	-	0.5	--	1.5		0.5		0.443
Unknown 8	-	0.5		1.5		0.5		0.473

Appendix 8 SDS PAGE reagent composition

. **30% acrylamide**: weigh 29g acrylamide, 1g N, N – methylene bis-acrylamide. Add 60 ml warmed deionized water and heat to 37 ℃. Add deionized water to make a final volume of 100ml; filter; Then we have 30% (w / v) acrylamide stock solution; Acrylamide and bisacrylamide were transformed slowly into acrylic acid and double acrylic acid during storage, so the pH of the solution should be no more than 7.0 and it should be placed in a brow bottle at 4 ℃.

2. **10% sodium dodecyl sulfate (SDS)**: weigh 10g SDS and 90ml deionized water; heat to 68 ℃ and add a few drops of concentrated hydrochloric acid until the pH becomes 7.2; then water to 100ml; after the whole processes, we have 10% (w/v) SDS.

3. **Stacking gel buffer (1mol / L Tris-HCl pH 6.8)**: dissolve 12.12g Tris in 80ml deionized water. Adjust the pH to 6.8 with concentrated hydrochloric acid; add deionized water to 100ml and store at 4℃.

4. **Resolving gel buffer** (1.5mol / L Tris-HCl pH 8.8): dissolve 18.16g Tris in 80ml deionized water; adjust the pH to 8.8 with concentrated hydrochloric acid; add deionized water to 100ml; store at 4 ℃.

5. **10% ammonium persulfate (AP)**: ammonium persulfate provides the free radical necessary for the catalysis of the Polymerization of Acrylamide and Bis-acrylamide; Use deionized water to prepare a small amount of 10% (w/v) solution and store at 4 ℃. Since ammonium persulfate will decompose slowly, it should be freshly prepared every other week.

6. **TEMED (N, N, N, N – tetramethylethylenediamine)**: by catalyzing ammounium persulfate to form free radicals, TEMED accelerated the polymerization of acrylamide and bis-acrylamide. Since TEMED only functions in a free base form, the polymerization reaction would be inhibited when the pH is low.

7. **Tris- glycine electrophoresis buffer**: weigh 15.1g Tris and 94g glycine; Dissolve in 900ml deionized water; then add 50ml 10% (w/v) SDS and deionized water to 1000ml. Dilute 5-fold when using. The final concentration would be: Tris, 25mmol/L; glycine, 250mmol/L; SDS, 0.1% and the pH of the buffer is 8.3.