ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

HEAVY METAL REMOVAL CAPABILITY OF BACTERIAL ISOLATES FROM AWASH TANNERY EFFLUENTS AND WASTEWATER SOURCES OF SELECTED RIVERS IN ADDIS ABABA

BY:

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A Thesis Submitted to the School of Graduate Studies, Institute of Biotechnology, Addis Ababa University, in Partial Fulfillment of the requirements for the Degree of Master of Science in Biotechnology

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AUGUST, 2024

Acknowledgements

First, I sincerely, would like to thank my adviser, Dr. Addis Simachew, for all of his assistance (comments, suggestions, and energetic guidance) throughout this research project. I am particularly appreciative to Dr. Bayable Atnafu, my co-advisor, for his constructive comments, unwavering advice, and financial support. I also credit Mr. Getnet Belay, my co-advisor, for his technical assistance with the thesis paper during the laboratory work, as well as his wise advice and constructive comments.

A special thanks goes to Ms. Tirunesh Shiferaw and Mr. Solomon Enquahone in particular for aiding me with my laboratory work. They assisted by supplying the chemicals and equipment required for my experiment, which were available both within and outside the laboratory.

I am also very grateful to Prof. Tesfaye Sisay for providing me a variety of antibiotic discs to explore experiments related to antibiotic susceptibility and resistance. Special thanks to Mr. Temesgen Aragaw for his support in working on the BOD and COD activities in their laboratories. I would like to express my appreciation to W/t Addis, the biotechnology laboratory custodian for her unlimited cooperation and help with laboratory cleaning.

It is an honor for me to thank the Institute of Biotechnology, Addis Ababa University for enrolling me as a graduate student and letting me use all the available resources. A great tribute to Ethiopian Forestry Development (EFD) for sponsoring me throughout the study period.

I want to offer heartfelt thanks and blessings to my love, W/o Workenesh Alelign. She has given me unreserved love and honorable encouragement that energized me during the challenging study periods, especially when she was pregnant and shortly after giving birth to my cherished little girl, Eleni Smegnew.

Finally, but just as importantly, I want to send my love to my family, friends, colleagues, and everyone else who helped me in one way or another to finish the study.

Above all, the Almighty God and his holy mother, St. Merry, have my highest praise and gratitude!

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Abbreviations

AAR	Addis Ababa Rivers
AARW	Addis Ababa River Water
AAS	Atomic Absorption Spectrometer
AMU	Atomic Mass Unit
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATSDR	Agency for Toxic Substances and Diseases Registry
ATE	Awash Tannery Effluent
BOD	Biological Oxygen Demand
CLSC	Clinical and Laboratory Standards Institute
COD	Chemical Oxygen Demand
EC	Electrical Conductivity
EEPA	Ethiopian Environmental Protection Authority
EPS	Extracellular Polymeric Substances
ESA	Ethiopian Standards Agency
EU	European Union
FAO	Food and Agricultural Organization
LAR	Little Akakai River
MCC	Mixed Culture Consortium
MIC	Minimum Inhibitory Concentration
NAM	Nutrient Agar Medium
NAM-SHMs	Nutrient Agar Medium Supplemented with Heavy Metals
PCR	Polymerase Chain Reaction
ppm	Parts Per Million
TDS	Total Dissolved Solid
TSS	Total Suspended Solid
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

Abstract

*Heavy metals from various industrial processes for different purposes pollute soil and groundwater worldwide. They have toxic, mutagenic, and carcinogenic effects, which pose health risks to the natural biota. Microbe-based biological treatment methods are an environmentally benign and more efficient heavy metal removal technology than conventional ones. The objective of this study was to evaluate the heavy metal removal capability of naturally occurring heavy metal-resistant bacteria both in NAM-SHM and in the actual wastewater, which was collected from ATE and selected tributaries of AARW. The result showed that four of the 130 heavy metal-resistance isolates were identified based on their morphological and molecular characteristics. Isolates S. xylosusT14Cd and B. cereusT4Zn were obtained from the Cd and Zn metal-supplemented NAM of the ATE, while S. xylosysW1Cr and S. xylosusW12Pb were obtained from the Cr and Pb of the selected tributaries of AARW. For all those isolates, 30°C and pH 8 were the optimum growth conditions. Isolates S. xylosusW1Cr, S. xylosusT14Cd, S. xylosusW12Pb, and B. cereusT4Zn were shown to have MICs of 3500, 4000, 5000, and 7000 ppm against Cr^{6+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}, respectively. Isolates' resistance order to a mixture of heavy metals Pb^{2+}, Zn^{2+}, Cr^{2+}, and Cd^{2+} in 3:3:1:1 (50-800 ppm), respectively, was MCC>S. xylosusT14Cd>S. xylosusW1Cr>S. xylosus*W12Pb>*B. cereusT4Zn. On the NAM-SHM inoculation, isolates S. xylosusT14Cd, S. xylosusW12Pb, and B. cereusT4Zn gave the maximum removal efficiency for Cd^{2+} (82.19%), Pb^{2+} (84.24%), and Zn^{2+} (80.94%) metals in 8 days, respectively, and Cr^{6+} (69.9%) was recorded for S. xylosusW1Cr in 6 days of incubation. Whereas, for MCC Cr^{6+} (79.36%), Cd^{2+} (88.66%), and Zn^{2+} (90.22%) in 8 days, and Pb^{2+} (93.44%) in 6 days, removal efficiency was recorded. The maximum removal efficiency of S. xylosusW1Cr and S. xylosusW12Pb was* 83.00 *and 79.60% in 6 days, while S. xylosusT14Cd, B. cereusT4Zn, and MCC was 84.88, 76.05, and 87.98% of Cr^{6+} in 8 days of incubation on the ATE. Moreover, the maximum removal efficiency of S. xylosusW1Cr was 89.75, 98.33, 97.57, and 99.46% of Cr^{6+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}, respectively, on the selected tributaries of AARW in 6 days of incubation. After 6 days of incubation, S. xylosusT14Cd was able to remove the maximum concentration of metals from AARW, with values of 89.76, 99.89, and 99.08% of Cr^{6+}, Cd^{2+}, and Pb^{2+}, respectively, and 99.12% of Zn^{2+} after 8 days. S. xylosusW12Pb was able to remove up to 99.03% of Zn^{2+} on same medium in 8 days of incubation and also 88.70, 98.93, and 97.77% of Cr^{6+}, Cd^{2+}, and Pb^{2+}, respectively, in 6 days. Similarly, the maximum removal efficiency of B. cereusT4Zn was also 80.48 and 97.25% of Cr^{6+} and Zn^{2+}, respectively, in 8 days of incubation, while 98.35 and 83.28% of Cd^{2+} and Pb^{2+}, respectively, in 6 days. Whereas, for MCC Cr^{6+} (90.29%) and Zn^{2+} (99.99%), in 8 days, while Cd^{2+} (99.97%) and Pb^{2+} (99.37%), in 6 days of incubation, removal efficiency was recorded. In conclusion, the four tested isolates are highly efficient for the removal of heavy metals from the NAM-SHM and actual wastewater samples. Hence, using these identified and tested bacteria for heavy metal bioremediation is recommended for tannery factory effluents and other industries.*

Keywords*: Biological treatment, Heavy metals, Heavy metal-resistant bacteria, Wastewater*

1. Introduction

The environment encompasses both biotic and abiotic factors that collectively create favorable conditions for the survival and development of all living organisms. The most important element in the environment is water, which is essential to both human society and the natural world. For the reasons that agriculture, human consumption, industry, and recreation all depend on the availability and supplies of fresh and adequate water (Sharma and Bhattacharya, 2017). However, it is polluted by different contaminants, which directly or indirectly affect living organisms. The drivers for these contaminants are increasing human populations, industrialization, and consequent urbanization, which leads to water contamination (Kumar *et al*., 2019) and seriously affects human health and other biological organisms (Sivaram and Barik, 2018).

Among the different contaminants, heavy metals are the most common pollutants and a global concern in recent years. Globally, an estimated 300-400 million tons of environmental pollutants, such as heavy metals, solvents, toxic sludge, and other waste, are discharged into water bodies annually by various industries (Palaniappan *et al*., 2010). Consequently, the concentration of heavy metals increased worldwide from decade to decade, especially in developing continents such as Africa, Asia, and South America (Zhou *et al*., 2020). As an example, the current concentration of heavy metals in different African countries is increased in water, fish, soils, vegetables, and food animals from time to time and exceeds the international limits described by Yabe *et al*. (2010). Ethiopia was also included in this study, and the results indicated that the various irrigated vegetables had heavy metal concentrations higher than suggested by irrigation water guidelines. The reason for the presence of high concentrations of heavy metals in this area is that farmers use large volumes of untreated and partially treated wastewater dumped into water bodies to irrigate vegetable crops (Desta Woldetsadik *et al*., 2017).

The two sources of heavy metals are natural processes and anthropogenic activities. The natural sources include volcanic eruptions, metal corrosion, woodland fires, geological weathering, and others. Anthropogenic activities are the major causes of heavy metals, which include metal mining and smelting, agricultural farming (fertilizer, insecticides, and pesticides), textile factories, construction projects, municipal and home activities, etc. (Briffa *et al*., 2020). Heavy metals including arsenic (As), cadmium (Cd), lead (Pb), copper (Cu), chromium (Cr), nickel (Ni), and

zinc (Zn), from various industrial sources pose major environmental challenges worldwide. These contaminants are difficult to remove from the environment due to their toxicity, persistence, and bioaccumulative nature (Ren *et al*., 2009; Jacob *et al*., 2018; Ali *et al*., 2019).

Apart from the aforementioned industries, the leather industry is one of the most important manufacturing industries, and it extremely pollutes water bodies because it uses tanning agents that generate highly turbid, colored, and unpleasant-smelling wastewater. Tannery wastewater is characterized by high contents of pollutants such as organic, inorganic, nitrogenous compounds (e.g., ammonia), hydrogen sulfide, chromium, sodium sulfide, chrome salts, calcium hydroxide, suspended solids, and dissolved solids (Seyoum Leta *et al*., 2004). Among the pollutants, the heavy metal Cr is one of the components in the tannery wastewater that can undergo oxidation from Cr (III) to Cr (VI) states, and Cr (VI) is toxic and can cause carcinogenic, mutagenic, and teratogenic effects on humans and other animals (Tadesse Alemu *et al*., 2017). While Cr(VI) exists in the forms of CrO_4^{2-}, $HCrO_4^-$, and $Cr_2O_7^{2-}$, Cr(III) mostly exists in the forms of $Cr(OH)^{2+}$, $Cr(OH)_3$, $Cr(OH)_4^-$, and $Cr(OH)_5^{2-}$ (Chen and Tian, 2021).

Currently, environmental pollution and ecosystem deterioration are becoming major challenges worldwide, especially in developing countries like Ethiopia, particularly in Addis Ababa city. This is because of rapid economic and industrial activities coupled with inadequate management of industrial wastewater (Milkiyas Petros and Timar Petros, 2019; Banchamlak Tegegne *et al*., 2021). In Addis Ababa city, several industries are mainly located along watercourses and coastal wetlands. As a result, these industries directly discharge large amounts of wastewater into rivers downstream or natural water bodies. Numerous studies also demonstrate that all forms of domestic wastewater and more than 90% of the country's industries discharge their effluents into neighboring agricultural farms and aquatic bodies without any treatment (Temesgen Eliku, 2018). Consequently, this effluent contaminates the surrounding downstream water bodies, local communities, and the environment (Abrha Gebrekidan *et al*., 2009; Fitsum Dechasa and Fikirte Demissie, 2014). Therefore, appropriate and economically feasible technologies that could manage the wastewater from several industries are urgently needed to alleviate the adverse impacts on the environment.

There are various treatment methods for the removal of organic and inorganic contaminants from wastewater before its disposal. Conventional treatment approaches including physical and

chemical treatment methods like lime coagulation, chemical precipitation, ion exchange, soil washing, membrane technology, electrolysis, ultrafiltration, adsorption, and oxidation have been used to treat organic and inorganic contaminants in wastewater (Jacob *et al*., 2018; Xu and Chen, 2020). Even though the above methods are easy, quick, and effective in removing and recovering heavy metals, they have several drawbacks, such as partial metal removal, high reagent and energy requirements, and the production of secondary poisonous sludge (Gosa Girma, 2015; Azizi *et al*., 2016; Xu and Chen, 2020). Also, conventional methods are costly, less efficient, and time-consuming (Kumar *et al*., 2019).

Today, biological treatment methods using bacteria, fungi, algae, yeasts, and even plants are emerging, innovative techniques that are attractive and an alternative option to the present conventional methods of treatment. These biological biomasses can remove heavy metals by metal biosorption, bioaccumulation, bioleaching, biotransformation, bioaugmentation, detoxification, and other mechanisms (Jeyakumar *et al*., 2023). Biological treatment methods have low cost, high efficiency, minimization of chemical or biological sludge, regeneration of biosorbents, the possibility of metal recovery, environmentally friendly technology, a high volume of wastewater removal potential, and a short operating period (Gouda and Taha, 2023). The biological treatment method uses various potential isolates including *B. cereus*, *Bacillus* and *Staphylococcus* (Varadhan and Mohan, 2017) and *B. cereus, Enterobacter cloacae and Enterobacteriaceae bacterium* (Ben *et al*., 2019) isolated from tannery effluents were an ideal for removal of heavy metal and other contaminants.

Therefore, the present study has been undertaken with the objective of screening the naturally resistant bacterial isolates from Awash tannery effluent and selected tributaries of AARW for bioremediation of heavy metals, especially chromium, lead, cadmium, and zinc. The selection of these four heavy metals was based on their classification as the most often recorded heavy metal pollutants in water, as well as their high concentration in AAR tributaries, as reported by numerous academics.

1.1. Statement of the problem

In Ethiopia, previous studies have indicated that metals such as Cr, mostly from tannery effluents, and Pb, Cd, Zn, Hg, and As from other industrial wastewater are released into water bodies above the safe limit of FAO and EEPA (Temesgen Eliku and Seyoum Leta, 2016). Also, various similar studies have showed that the concentrations of Cr are above the standard limits (Seyoum Leta., 2003) and reported 32.2 ± 5.7 mg/L from Mojo tannery effluent; (Aberha Gebrekidan *et al*., 2009) reported 7.82 mg/L from Sheba tannery effluents; and (Assefa Wosnie and Ayalew Wondie, 2014) reported 3.54 ± 0.55 mg/L in Bahir Dar tannery effluent.

Moreover, in AAR tributaries of LAR (one of the polluted rivers), several studies have been reported the presence of potentially toxic heavy metals (Minbale Aschale *et al*., 2015; 2016; Deshu Mamo *et al*., 2021). The authors' findings indicated that most of the heavy metal concentrations, including Cr, Mn, Sb, B, Fe, Sr, Zn, Cu, and Pb, exceeded the permissible limits of drinking water guidelines, while Cr, Mn, and Sr exceeded the irrigation water guideline limits.

In Ethiopia, biological wastewater treatment was reported by (Temesgen Oljira *et al*., 2018) and the result indicated that the bacterial species *Aeromonas sp*., *Pseudomonas sp*., and *Bacillus sp*. were capable of reducing different contaminants (BOD, COD, TN, TP, TSS, TS, and TDS) from the brewery wastewater. Melkamu Tamiru and Adugna Mosisa (2019) also reported the resistance properties of bacteria, and recommended that the bacterial isolates from heavy metal-contaminated soil can be used as bioremediation for heavy metals.

Even though, some reports are present; they are limited on the removal of heavy metals from the tannery and other industries especially in Addis Ababa city. Therefore, the objective of this research is to alleviate the problem of heavy metal contamination by isolating, characterizing and identifying potential bacterial isolates from Awash tannery effluent and selected tributaries of AARW and evaluating its capability for heavy metal removal.

1.2. Objectives

1.2.1. General objective

- To investigate and evaluate potential bacterial strains capable of heavy metal removal, isolated from Awash tannery effluents and selected tributaries of AAR water sources.

1.2.2. Specific Objectives

- To isolate heavy metal-resistant bacterial strains from the Awash tannery and selected AAR water samples.
- To characterize and identify the isolated bacterial strains using morphological and molecular techniques.
- To evaluate the ability of isolates for heavy metal removal both in nutrient agar medium supplemented with heavy metals and in real wastewater samples.

2. Literature Review

2.1. Heavy metal overview

Heavy metals are constituents and naturally occurring elements of the earth's layer. Heavy metals are metals that exhibit both metal and non-metal properties, also known as metals and metalloids. They have different definitions of heavy metals based on their chemical (atomic number and density) and biological properties. Chemically heavy metals refer to metals that have an atomic weight ranging from 63.5 to 200.6 amu, a high atomic number (>20), and an atomic density (>5 g/cm^3) compared to water (Laghlimi *et al*., 2015; Li *et al*., 2019; Taha *et al*., 2023). From a biological perspective, heavy metals are described as a series of metals or metaloids that, even in low concentrations, can be toxic to animals and plants (Kebede Nigussie *et al*., 2012; Li *et al*., 2019). Most of the time, they are used to maintain body metabolism but are toxic to the body when they are present in higher concentrations (Sonone *et al*., 2020).

2.1.1. Sources of heavy metal

The two main sources of pollution with heavy metals in the water bodies are natural and anthropogenic sources. The natural sources of heavy metals emanate from volcanic eruptions, metal corrosion, geysers, woodland fires, sea-salt sprays, wind-borne soil particles, metal evaporation from soil and water, erosion of soil and geological weathering (Briffa *et al*., 2020). Heavy metals can be present in nature in the form of hydroxides, oxides, sulfides, sulfates, phosphates, silicates, and organic compounds, and they can be released into the environment under different and specific environmental conditions (Masindi and Muedi, 2018).

Whereas, the anthropogenic sources, also known as the human activities of heavy metal pollution sources, include industrial discharge, agricultural chemicals, inadequate burning of fossils, pharmaceutical activities, domestic effluents, and atmospheric sources, as well as metal refining, mineral extraction, land filling, livestock and chicken manure, storm runoff, painting and coating enterprises, military operations, etc. (Selvi *et al*., 2019; Briffa *et al*., 2020). Additionally, toxic heavy metals, including As, Cd, Cu, Hg, Pb, Zn, etc., are generated from mines, sewage, smelters, battery industries, dyes, alloys, and electronic factories (e-wastes) in the form of wastewater (Kanwar *et al*., 2020). For instance, lead is released by car exhaust; copper, zinc, and arsenic are

released during smelting; pesticides release arsenic; and burning fossil fuels releases nickel, vanadium, mercury, selenium, and tin (Sonone *et al*., 2020).

Here, the contribution of anthropogenic activities to environmental contamination is greater than that of natural sources because these activities release large amounts of heavy metals as well as petroleum hydrocarbons into the natural ecosystem and disrupt physiological functions in biological systems (Jacob *et al*., 2018). Therefore, the influence of anthropogenic sources of heavy metals on environmental pollution is greater owing to the everyday manufacturing of goods to meet the demands of the large population (Masindi and Muedi, 2018). Generally, the sources of heavy metals are illustrated in Fig. 1.

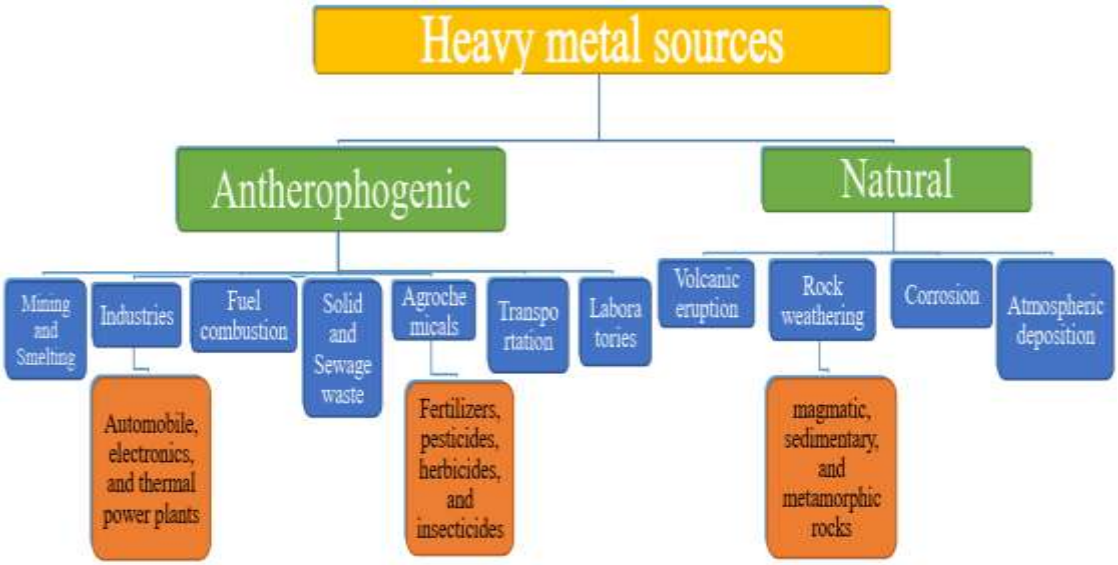


Figure 1: Sources of heavy metals (Kanwar *et al*., 2020; Sonone *et al*., 2020)

2.1.2. Types of heavy metal

Heavy metals are classified as essential or nonessential depending on their role in biological systems (Ali *et al*., 2019). The essential heavy metals are significant for living organisms in their bodies in low concentrations and include Mn, Fe, Cu, and Zn. Whereas the nonessential one has no known biological role in living organisms and includes Cd, Pb, and Hg, which are toxic for living organisms. On the other hand, some elements known as micronutrients, including Mn, Fe, Co, Ni, Cu, Zn, and Mo, are essential for cell growth, resistance to stress, biosynthesis of biomolecules, and secondary metabolite production in plants (Appenroth, 2010). Even though the lists of essential heavy metals may be different for different groups of organisms, i.e., one is

essential for a given group of organisms but nonessential for another, either deficiency or excess of an essential heavy metals leads to diseases or abnormal conditions (Ali *et al*., 2019).

Heavy metals are also classified as macro-nutrient elements (cobalt and iron), micro-nutrient elements (copper, nickel, chromium, iron, manganese, and molybdenum), highly toxic elements (mercury, cadmium, lead, silver, gold, palladium, bismuth, arsenic, platinum, selenium, tin, and zinc), precious elements (platinum, silver, gold, palladium, and ruthenium), and radio nuclides (uranium, thorium, radium, cerium, and praseodymium) (Selvi *et al*., 2019). Other studies also classified heavy metals into four major groups based on their requirements and toxicity (Mahmood *et al*., 2012). These include essential heavy metals or micronutrients (Cu, Zn, CO, Cr, Mn, and Fe), non-essential metals (Ba, Al, Li, and Zr), less toxic (Sn and Al), and highly toxic (Hg and Cd).

2.1.3. Effects of heavy metals

Heavy metals have several economic significance in different manufacturing industries and vital roles in cell growth and metabolic functions within the cell of different organisms (Bandela *et al*., 2016; Igiri *et al*., 2018). However, today they are the most pollutants/toxicants throughout the whole world and are toxic for the cell when the concentration of them are above their tolerance limit (Bandela *et al*., 2016; Igiri *et al*., 2018). Heavy metals are one of the seriously toxic, non-biodegradable elements and metal ions such as lead, cadmium, mercury, copper, chromium, zinc, nickel, and cobalt have very huge environmental impact (Gouda and Taha, 2023). Some of them such as Hg, Pb, and Cd are included in the list of U.S. Environmental Protection Agency's as the main concern pollutants (De *et al*., 2008). In addition the United States Environmental Protection Agency (USEPA) listed heavy metals including Pb, Cr, As, Zn, Cd, Cu, Hg, and Ni, which are the most widespread in the environment and the major heavy metals that are toxic to public health and other organisms (Selvi *et al*., 2019; Singh *et al*., 2019) and they are detected in wastewater even at low concentrations. Furthermore, among the several oxidation states of Cr, Cr (VI) is considered the most toxic form of Cr that is recently classified as one of the 17 most toxic chemicals by the Agency for Toxic Substances and Diseases Registry (ATSDR) and listed as a grade 'A' human carcinogen by USEPA (Baldiris *et al*., 2018). The toxicity of Cr is due to its strong oxidation potential, higher solubility in water, and rapid permeability through biological membranes.

Exceeding of the heavy metals may lead to detrimental adverse reactions in various organs, including the lungs, liver, and kidneys, obstruct the biological function of the central nervous

system and reproductive system, and result in birth defects in humans (Bandela *et al*., 2016) as illustrated in Table 1. Then, at excess levels, both essential and nonessential heavy metals could alter the enzyme specificity, disturb cellular functions, and damage DNA or RNA structures by binding to the cell membranes. The mechanisms of toxicity of heavy metals include breaking fatal enzymatic functions, reacting as redox catalysts in the production of reactive oxygen species (ROS), destructing ion regulation, and directly affecting the formation of DNA as well as protein (Molalgn Medfu *et al*., 2020). Therefore, today, the toxicity and bioaccumulation of heavy metals in environments are the major problem to the whole biological creatures.

In general, the aforementioned toxic heavy metals, which are obtained from numerous industrial activities, can affect aquatic life, human health, food security, and nutrition quality when they enter soil, air, and water bodies (Fig. 2). In addition to this, they affect soil properties, inhibit plant growth when they are highly accumulated (Fekede Terefe *et al*., 2020), and affect all the spheres of the environment, including the hydrosphere, lithosphere, biosphere, and atmosphere (Masindi and Muedi, 2018). As a result, ingestion (consumption of food or drink), inhalation (air), and skin contact during agricultural practice, manufacturing, pharmaceutical, industrial, and/or residential settings are the three main entry points of heavy metals into human bodies (Timothy and Tagui Williams, 2019).

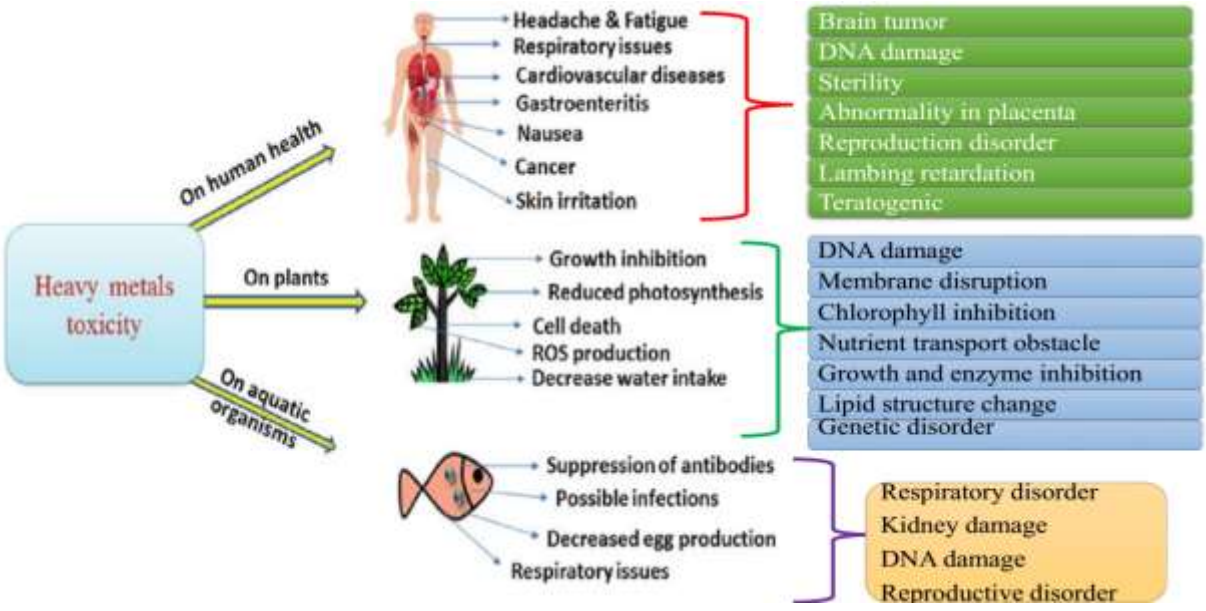


Figure 2: The major adverse effects of heavy metals on living organisms (Selvi *et al*., 2019; Kanwar *et al*., 2020; Vijaya Bhaskar Reddy *et al*., 2021)

Table 1: Selected heavy metals, sources and their effects

NO	Heavy metal types	Sources of heavy metal	Effects of heavy metal	References
1	Lead	Aerial emissions from the combustion of leaded fuel, electroplating, waste from steel, batteries, mining, insecticides, and herbicides.	Bones, liver, kidneys, brain, lungs, spleen, immunological, reproductive problems, blood disorders, hematological, cardiovascular, neural damage, gastrointestinal diseases, and a decrease in male sperm count in men, and abortions in women.	García-Niño and Pedraza-Chaverrí, 2014; Verma and Sharma, 2017; Baby *et al*., 2019; Jeyakumar *et al*., 2023
2	Chromium	Tanneries, fly ash, electronics, steel industries, automobiles, etc.	Skin, lungs, kidneys, liver, brain, pancreas, tests, gastrointestinal, reproductive, allergic reaction, neurotoxic effect, cancer of the respiratory tract, and stomach damage.	García-Niño and Pedraza-Chaverrí, 2014; Baby *et al*., 2019; Sonone *et al*., 2020
3	Cadmium	Paints, smelting, pigments, mining, electroplating, plastic stabilizers, and phosphate fertilizers.	Bones, liver, kidneys, lungs, testes, brain, renal failure, cardiovascular, hyperactivity, slowed growth, chronic anemia, and cancer of the prostate.	Verma and Sharma, 2017; Baby *et al*., 2019; Sonone *et al*., 2020
4	Cupper	Pesticides, chemical fertilizers, biosolids, ore mining, and smelting.	Liver, brain, kidneys, cornea, gastrointestinal, lungs, immunological, and hematological problems.	García-Niño and Pedraza-Chaverrí, 2014; Verma and Sharma, 2017
5	Mercury	Au-Ag mining, smelting, coal combustion, and medical waste.	Brain, lungs, kidneys, liver, carcinogenic effect, cardiovascular, endocrine, reproductive effects, cognitive disorder, gastrointestinal toxicity, neurotoxicity, and nephrotoxicity problems.	García-Niño and Pedraza-Chaverrí, 2014; Verma and Sharma, 2017; Baby *et al*., 2019

6	Arsenic	Pesticides, wood preservatives, biosolids, ore mining, and smelting.	Skin, lungs, brain, kidneys, liver, muscle weakness, cancer, immunological, diabetes, endocrine, miscarriages, and neural problems.	García-Niño and Pedraza-Chaverrí, 2014; Baby *et al*., 2019; Sonone *et al*., 2020
7	Nickel	Industrial effluents, kitchen appliances, surgical instruments, automobile batteries.	Dermatitis, myocarditis, hair loss, encephalopathy, dermal toxicity and irritation, cancer risk, nasopharyngeal tumors, and reduced sperm count.	Bandela *et al*., 2016; Verma and Sharma, 2017; Sonone *et al*., 2020
8	Zinc	Batteries, paints, fuel, pigments, alloys and solders, glass, refineries, fertilizers, and pesticides.	Stomach cramps, fever, vomiting, nausea, neurologic disturbance, hyperactivity, respiratory system influence, psychical dysfunction, and diarrhea effects.	Baby *et al*., 2019; Sonone *et al*., 2020

2.2. Wastewater

Wastewater is any water that contains 99.9% water by weight and the rest of 0.1% suspended or dissolved material such as oils, grease, plastics, heavy metals, sands, etc. that can pollute the environment (Samer, 2015). It may also refer to the water that does not fulfill the minimum criteria of water quality, depending on the World Health Organization (WHO), and is not suitable for drinking due to a change in its physical, chemical, and biological characteristics (Gangaraju *et al*., 2021). This change in water characteristics is mostly owing to wastewater produced from different treated or untreated municipal, industrial, agricultural, and other industries in the form of effluent.

Among the various industry, the leather industry is a very old manufacturing sector in general that can produce large amounts of goods such as leather footwear, leather bags, leather garments, and so on used by people in daily life (Sivaram and Barik, 2018). In the leather industry, tanning is one of the primary units of leather processing. Leather processing involves three major series of steps, including pre-tanning, tanning, and post-tanning, and follows numerous consecutive steps, including soaking, liming, unhearing, reliming, fleshing, deliming, bating, pickling, tanning, neutralization, retaining, dyeing, fat-liquoring, and fixing in these three processes (Christopher *et al*., 2016). At each stage (starting from preservation up to finishing), numerous chemicals and a lot of water are used for the production and treatment processes of leather (Agegnehu Alemu *et*

al., 2021). Hence, the leather industry, which is characterized by complex mixtures of organic and inorganic chemicals, produces different pollutants that are toxic and hazardous to the environment.

Even though the leather industry is a fast-growing traditional industry, on the contrary, it is the most polluting industrial sector for the environment and challenging for management (Christopher *et al*., 2016). In Ethiopia, the modern manufacturing method of the leather industry was started in the 20th century (Tesfaye Admasu *et al*., 2021). It is one of the pillars of the country's economic development and foreign exchange earnings by manufacturing shoes, bags, footwear, garments, and furniture, as well as one of the foreign exchange earnings of the country. However, the wastewater discharged from leather and other related industries is the main source of pollutants for the environment due to poor treatment technologies and inadequate handling methods.

Among the pollutants, heavy metal chromium are the most toxic because 80–90% of the world-wide tanneries usually use the oxidation state of Cr (III) in the form of chromium sulfate ($Cr_2(SO_4)_3$ in their tanning processes (Melaku Tafese, 2021). The chromium salts used in tanning processes are not completely used by hiding tissues, so excess effluents of chromium discharge, thereby polluting the environment and ecosystem (Adugnaw Maru *et al*., 2021). For instance, 1000 kg of rawhide is converted to 200 kg of leather product containing 3 kg of chromium, 250 kg of non-tanned solid waste, 200 kg of tanned waste containing 3 kg of chromium, and 50 000 kg of wastewater effluent containing 5 kg of chromium (Sivaram and Barik, 2018). About, 20% of the finished leather product and more than 60% of the solid and liquid waste, including the highly carcinogenic heavy metal chromium," are produced from these raw materials. Therefore, Cr concentration in tannery wastewater ranges from 2000-5000 mg/l, which is why tannery industries are the major sources of Cr contamination compared to other industries (Baldiris *et al*., 2018).

2.3. Wastewater treatment technologies

As we previously discussed, the presence of heavy metals produced by various sources is either directly or indirectly incorporated into the environment and affects people, animals, and plants. Because of this handling of heavy metals, high exposure to them has major negative effects on one's health as well as the environment and ecosystems, which are the key concerns of scholars. Therefore, using quick, efficient, straightforward, and appropriate technology for wastewater treatment is indispensable. This is because wastewater treatment aims to: protect public health; ensure that wastewaters are handled effectively on a reliable basis without causing annoyance or

offense; recycle and recover the valuable components available in wastewater; and afford practical treatment processes and disposal options (Samer, 2015). All of these goals are achieved by transforming the materials present in the wastewater into secure end products that can be safely disposed of in domestic water without having any negative environmental effects. There are numerous conventional and biological treatment techniques available to get rid of these dangerous heavy metals from the environment.

Several conventional (physical and chemical) treatment methods, including ultrafiltration, desorption, reverse osmosis, electro-dialysis, ion exchange, chemical precipitation, excavation, and stabilization, are used for the removal of heavy metals from contaminated environments (Verma and Sharma, 2017; Goswami *et al*., 2022). Granular activated carbon, electro-dialysis, membrane filtration, thermal treatment, soil replacement and washing are examples of physical remediation techniques, while chemical remediation techniques include SO_2, $Na_2S_2O_5$, $FeSO_4$, Na_2SO_3, $BaSO_3$, lime, and limestone treatments (Chen and Tian, 2021). Additionally, carbon adsorption, evaporation, redox, chelation, wastewater coagulation, and electrochemical heavy metal removal procedures are used (Gouda and Taha, 2023). However, these are not a permanent cure for the problems owing to being expensive for a large-scale operation, releasing secondary waste, being risky for continual monitoring, becoming non-environmentally friendly, and eliminating non-target but important microbes, for instance nitrogen-fixing bacteria and other fauna species (Ali *et al*., 2019; Gouda and Taha, 2023).

Therefore, the current solution to alleviate these limitations is using biotechnological methods, also known as biological treatment methods. The biological treatment methods using bioremediation are an important technique for removing or metabolizing harmful heavy metal wastes into safe, less toxic, and less destructive substances with the help of biological systems in an eco-friendly way without creating secondary pollutants (Verma and Sharma, 2017).

2.4. Bioremediation Mechanisms

Today, environmental biotechnology using bioremediation techniques is a potential or idyllic alternative method for environmental restoration. The term "bioremediation" refers to the employment of microorganisms to remove pollutants from a polluted environment (Priyadarshanee and Das, 2021). In other words, the removal of toxic pollutants (converting high-valent toxic heavy metals into less toxic ions, in the case of heavy metals) by exploiting various

biological agents is known as bioremediation (Jeyakumar *et al*., 2023). *Pseudomonas putida's* breakdown of crude oil in 1981 led to the first patent registration for the idea of environmental restoration utilizing microorganisms (Abo-Alkasem *et al*., 2022). Bioremediation is the most promising and least expensive way to offer the opportunity to eliminate or reduce different toxic contaminants, including heavy metals, using biological agents (Ahemad, 2012).

Bioremediation is a proficient, revolutionary, and economically viable technique that is strongly advised to alleviate the limitations of conventional treatment approaches (Gouda and Taha, 2023; Verma and Sharma, 2017). It is a holistic approach and/or technique that uses biological diversity, directly or indirectly, to convert toxic pollutants into less toxic ones (Abo-Alkasem *et al*., 2023). There are several sources of organisms or biological agents (bacteria, yeast, fungi, algae, and plants) for the remediation of heavy metals from the contaminated site. The removal efficiency of these biological agents, even in harsh environments, is due to the presence of several binding sites, including a large surface area to volume ratio, strong binding affinity, and high photosynthetic efficiency (restricted to photosynthetic microbes and plants) (Jeyakumar *et al*., 2023). In general, these biological agents use several bioremediation techniques (Fig. 3) for the removal of heavy metals, and their mechanisms of action to tolerate and reduce heavy metals are described in Fig. 4.

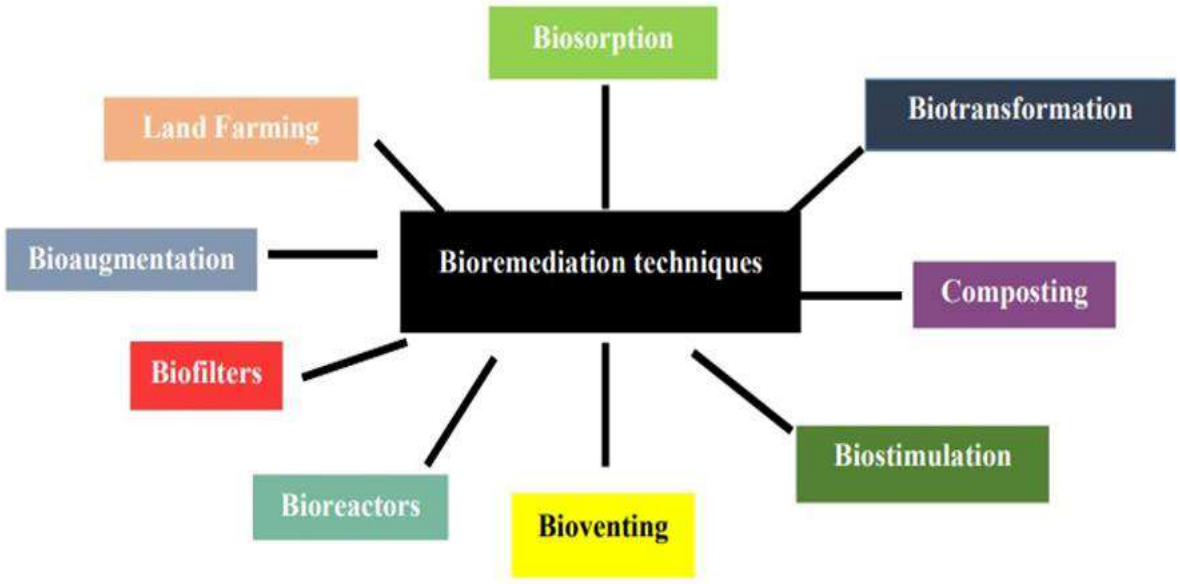


Figure 3: Heavy metal bioremediation techniques using different organisms (Taha *et al*., 2023)

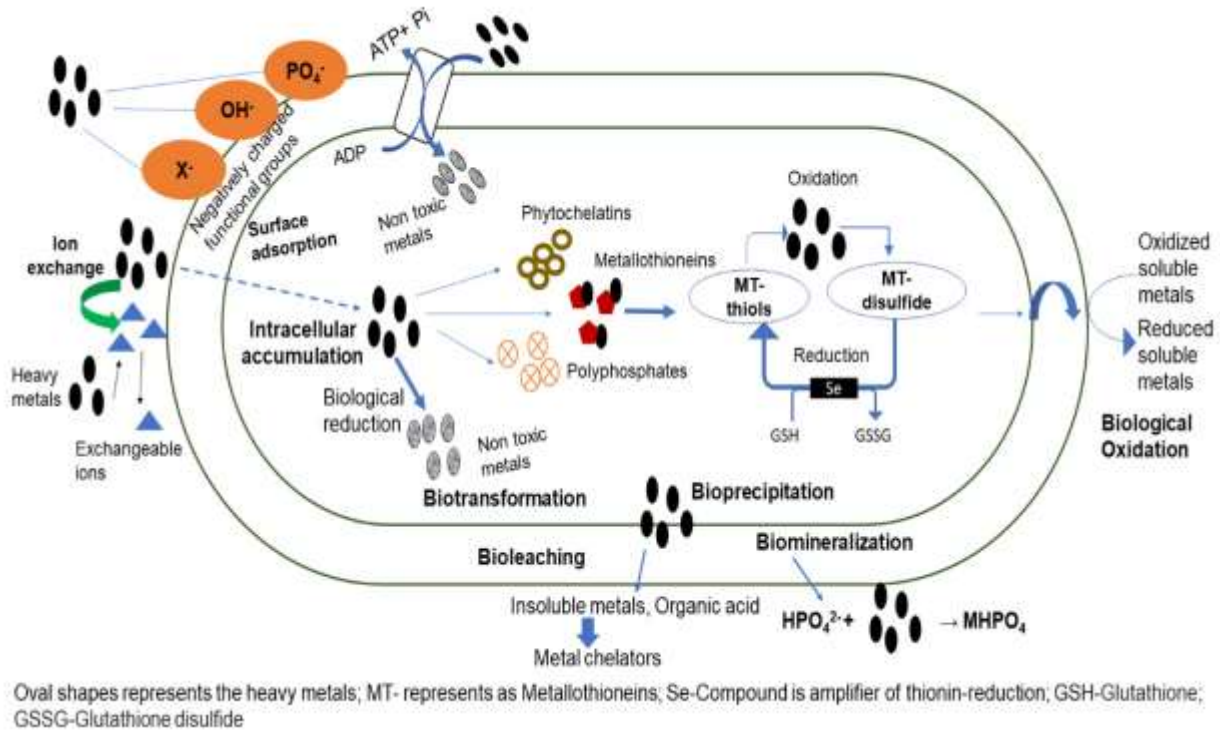


Figure 4: Bioremediation mechanisms of various organisms (Jeyakumar *et al*., 2023)

2.5. Heavy metal removal mechanisms of microbes

Microbes are omnipresent and can thrive in environments with high concentrations of heavy metals. They can break down environmentally harmful substances to eco-friendly or acceptable levels through microbial remediation processes in the soil, water, sludge, and underground water. Microbes have certain mechanisms to tolerate the presence of heavy metals, including biosorption, bioaccumulation, biotransformation, metal-microbe interaction, and bioleaching (Jeyakumar *et al*., 2023). In addition to this, precipitation of metals as phosphates, carbonates, and sulfides, volatilization by methylation, physical exclusion of electronegative components in membranes, extracellular polymeric substances (EPS), and energy-dependent metal efflux systems are the other mechanisms of microbes for heavy metal treatment (De *et al*., 2008). They use various methods such as metal transport across the cell membrane, biosorption to the cell walls, immobilization and binding of metals, intracellular sequestration with low molecular weight and cysteine-rich proteins, complexation, and oxidation-reduction reactions to successfully remediate polluted environments (Dixit *et al*., 2015; Igiri *et al*., 2018; Yuliani *et al*., 2019).

The reason that microbes can remove the various heavy metals is the presence of anions on the outermost layer of their cellular structures (Gupta and Tamrakar, 2023). These anions enable them to attach to the metallic cations of the heavy metals and then involved in heavy metal removal. A metal binding ability can be acquired on or within the cell wall by the total negative charge caused by anionic functional groups such as alcohol and/or hydroxyl (-OH), amine ($-NH_2$), carboxyl (-COOH), thioether (-S-), phosphoryl ($-PO_3H$), ester (-COO-), sulfonate ($-SO_3H$), and sulfydryl or thiol (-SH) that exist in microbes (Sharma *et al*., 2022). For instance, carboxyl groups can bind cadmium to the surface, and amino groups have proven effective in binding and electrostatic interaction, eliminating chromium.

The interaction of microbes with their surroundings, particularly with ions containing heavy metals, greatly depends on the presence of microbial surface structure or cell wall. In general, for efficient remediation, the microbes are governed by biomass character and concentration, characteristics of the target metal, the type of binding sites involved in metal sequestration, operational parameters (temperature, pH, contact time, concentrations of metal ions), and the characteristics of the metal solution (Gouda and Taha, 2023; Jeyakumar *et al*., 2023).

Microbial cells are greatly interested in bioremediation agents because they can achieve different transformation and immobilization processes by conducting heavy metal incorporation (absorption) inside the cell and adsorption on the cell surface by different mechanisms (Afzal *et al*., 2017). Natural occurrences, cheap production, and easy accessibility to treat large volumes of wastewater are the other advantages of such microbes for bioremediation of heavy metals.

Contrasting to other microbial forms, bacteria are unique due to their large surface area, rate of faster growth, robustness, and high resistance to toxic heavy metals (Ahemad, 2012). They have also special heavy metal removal characteristics, including their ubiquity nature, size, ability to grow under controlled conditions, and resilience to environmental conditions, as well as their high surface-to-volume ratios, and potential active chemisorption (chemical adsorption) sites on the cell wall (Molalign Medfu *et al*., 2020). They can remove heavy metals by keeping the metal ions away from the target site (exclusion), pushing out the metal ions from the cell through chromosomal or plasmid-mediated events (extrusion), or the formation of complexes by metal-binding proteins (accommodation), e.g., metallothienins, low molecular weight proteins, or other cell components (Ahemad, 2012). Also, they reduce the toxic metals to less toxic forms (bio-transformation)

through methylation and demethylation processes. The reason that bacteria are especially useful for heavy metal removal is that the role of metals in various metabolic processes, either directly or indirectly, makes the bacteria grow on toxic heavy metals (Nanda *et al*., 2019). The bacterial strains have developed several pathways for managing heavy metals, which include efflux pumps, redox reactions and complex formations, extra- and intra-cellular sequestrations, accumulation on the cell wall, and in certain cases, the metal ions are used as electron acceptors in oxidative phosphorylation (Ahemad, 2012).

Even though the removal of heavy metals has been done in various bacterial species, the majority of them that have the greatest potential alternatives for biological remediation of toxic metals belong to the genera *Streptomyces* and *Pseudomonas* (Gupta and Tamrakar, 2023). There is several evidence that shows the reduction of heavy metals, especially Cr(VI), by using various types of bacteria isolated from tannery effluents, such as *Stenotrophomonas maltophilia* (Baldiris *et al*., 2018), *Bacillus sp.* (Princy and Prabagaran, 2019), *Staphylococcus sciuri* (Elahi and Rehman, 2019), *Cellulosimicrobium funkei* (Karthik *et al*., 2017). Moreover, other studies have been conducted on different bacterial species that are used for bioremediation purposes. For instance, *Flavobacterium*, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Micrococcus sp*., *Acinetobacter sp*., *Arthrobacter sp*., *Micrococcus luteus*, etc. have been tested for removal via detoxification, transformation, and bioaccumulation of heavy metals like Pb, Cr, and Cd in tannery effluent and other industries (Molalign Medfu *et al*., 2020).

In general, Gram-positive bacteria bioaccumulate a higher concentration of heavy metals on their cell walls than Gram-negative bacteria because the former have a thicker peptidoglycan layer and teichoic and teichuronic acids (Ahemad, 2012).

2.5.1. List of mechanisms for heavy metal removal using bacteria

Biosorption: refers to the removal of pollutants from contaminants using biological materials via uptake onto microbial cells in the case of heavy metals. It is a metabolically rate-independent, quick phenomenon, and passive process that allows heavy metal ions to be selectively sequestered by both live and dead cells extracellular (Sharma *et al*., 2022). It is a cost-effective, easily available, and eco-friendly mechanism, so it is considered an innovative technology and strategy for the removal or reduction of heavy metal concentrations from contaminated sites (Jacob *et al*., 2018). In addition, it has many anticipated features, such as rapid kinetics of adsorption and

desorption, to selectively remove heavy metals from various wastewater effluents due to the metal-binding abilities of biological materials even at low concentrations at a wide range of temperature and pH (Migahed *et al*., 2017). Therefore, biosorption has a multi-step process for its mechanisms of action, including surface adsorption by physical and chemical interaction, complexation, diffusion, or precipitation (Jeyakumar *et al*., 2023).

Adsorption or extracellular sequestration, is the affinity between cellular components of the periplasm and the metal ion by passive physical interaction without requiring energy expenditure (Abo-Alkasem *et al*., 2023). Extracellular polymeric substances (EPS) associated with the bacterial cell wall are composed of polysaccharides, mucopolysaccharides, and proteins. EPS contains various functional groups (hydroxyl, carboxyl, amine, and phosphoric groups) that play a significant role in metal adsorption by the interaction of metallic cations with negatively charged functional groups of EPS (Abo-Alkasem *et al*., 2023). After absorption, the adsorbed substance (metal ion) on the microbial surface could be released out of the cytoplasm to the periplasmic region to defend them against the toxic effects of heavy metals by forming an outer protective material (efflux system) (Dixit *et al*. 2015). The binding of copper by carboxyl groups of peptidoglycan on the bacterial cell wall was reported in *Streptomyces pilosus*, and other bacterial species (*Klebsiella aerogenes*, *Pseudomonas putida*, and *Arthrobacter viscosus*) have been reported to bioaccumulate metal cations extracellularly (Nanda *et al*., 2019).

Apart from adsorption, biosorption can be accomplished by ion-exchange methods that are facilitated by different functional groups present on the surface of microbial cell walls to remediate heavy metals (Gouda and Taha, 2023). Moreover, biosorption can be assisted by complexation mechanisms in which cations present in heavy metals attract the anions that have a free electron pair by chelation and electrostatic interaction. Complexation means the aggregation of two or more species, namely the metal ion and functional groups, on the surface of the cell (Priyadarshanee and Das, 2021). *Bacillus, Pseudomonas*, and *Streptomyces* are among the dominant and various bacterial species that were reported for heavy metal biosorption (Pham *et al*., 2022).

Bioaccumulation: is a complex and energy-dependent process that involves the accumulation of heavy metals inside the cellular components. The process is intracellular (occurs in live cells only), slower, and has a higher substrate-specific sequestration mechanism compared to biosorption (Sharma *et al*., 2022). In this process, some microbes produce siderophores (chelating agents) that

mediate the ability of microbes to utilize low-water-soluble metals using energy dependent processes (Abo-Alkasem *et al*., 2023). During the bioaccumulation process, ion pumps, protein channels, endocytosis, carrier-mediated transport, complex permeation, and lipid permeation are the main entry mechanisms of the heavy metal across the bacterial membranes (Verma and Sharma, 2017). In this method, the heavy metal is transported across the phospholipid bilayers of living cells in two phases (Priyadarshanee and Das, 2021). These are attaching to the cell surface of the microbes following the internalization of metal ions or slow diffusion to the periplasm and then to the cytoplasm, like the uptake of essential nutrients (Na^{2+}, K^{+}, and Ca^{2+}). Many bacterial strains, including *Pseudomonas putida*, *Bacillus subtilis*, *Thiobacillus ferrooxidans*, *Rhizopus arrhizus*, *Micrococcus luteus*, *Saccharomyces cerevisiae*, and *Aspergillus niger*, were reported to accumulate cadmium, chromium, silver, lead, mercury, strontium, uranium, and thorium, respectively (Jeyakumar *et al*., 2023).

Biotransformation: Biotransformation is the process of reducing and/or oxidizing toxic heavy metals into their nontoxic forms by enzymatic reactions (oxidation, reduction, methylation, and demethylation) or metabolic processes of microorganisms (Molalign Medfu *et al*., 2020). For instance, various bacteria have been reported to have Cr(VI) reduction potential, such as *Salipaludibacillus agaradhaerens*, *Bacillus sp*., *Bacillus cohnii* SR2 and *licheniformis* SR3, *Oceanobacillus oncorhynchi*, *Stenotrophomonas maltophilia* and *Cellulosimicrobium funkei* (Karthik *et al*., 2017; Baldiris *et al*., 2018; Zeng *et al*., 2019; Tan *et al*., 2019; Sarankumar *et al*., 2020; Abo-Alkasem *et al*., 2022).

Bioprecipitation and biocrystallization: also called biomineralization, which transforms soluble heavy metals into insoluble forms (Kapahi and Sachdeva, 2019). Therefore, the process involves the precipitation of metal in the form of hydroxide, phosphates, carbonates, and sulfur dioxide via anions released by microbes (Verma and Sharma, 2017). This process takes part in biogeochemical cycles like forming microfossils, deposing iron and manganese, and mineralizing silver on the surface or inside of the cell by the direct activity of enzymes (Molalign Medfu *et al*., 2020). For instance, the removal of heavy metal Cd^{2+} by using *Pseudomonas aeruginosa* and *Klebsiella planticola* was reported in the bioprecipitation process (Kapahi and Sachdeva, 2019).

Bioleaching of metals: It is a bio-hydrometallurgical technique in which microbes are used to oxidize and solubilize sulfur or ferrous ion-containing sediments to extract heavy metals (Verma and Sharma, 2017). This process transforms toxic metals into less toxic metals through the secretion of low-molecular-weight compounds; e.g., the leaching of Cd is promoted by the secretion of organic acids by some microorganisms (Abo-Alkasem *et al*., 2022). In this process the secreted bacterial metabolic compounds interact with toxic metal ions, which results the increased solubilization of metals. Although this method is mainly industrially applicable for Cu, Au, and U leaching worldwide, there are also opportunities to recover other metals such as Zn, Co, Pb, Ni, etc. using microbes for metal leaching from sulfide and oxide minerals (Molalign Medfu *et al*., 2020). For instance, *Streptomyces albidoflavus*, has been reported to leach out different metals (Zn, Ni, Ca, Cu, Al, Cd, Ag, Pb, and Fe) with varying efficiency from printed circuit boards (Kaliyaraj *et al*., 2019). In addition to this the bacterial genera *Sulfobacillus*, *Ferromicrobium*, *Acidiphilum*, and *Acidithiobacillus* are well known in bioleaching of heavy metals ions (Khattak *et al*., 2020).

The general mechanisms of heavy metal sequestration via microbes and the four metal resistance mechanism are presented in Fig. 5-9.

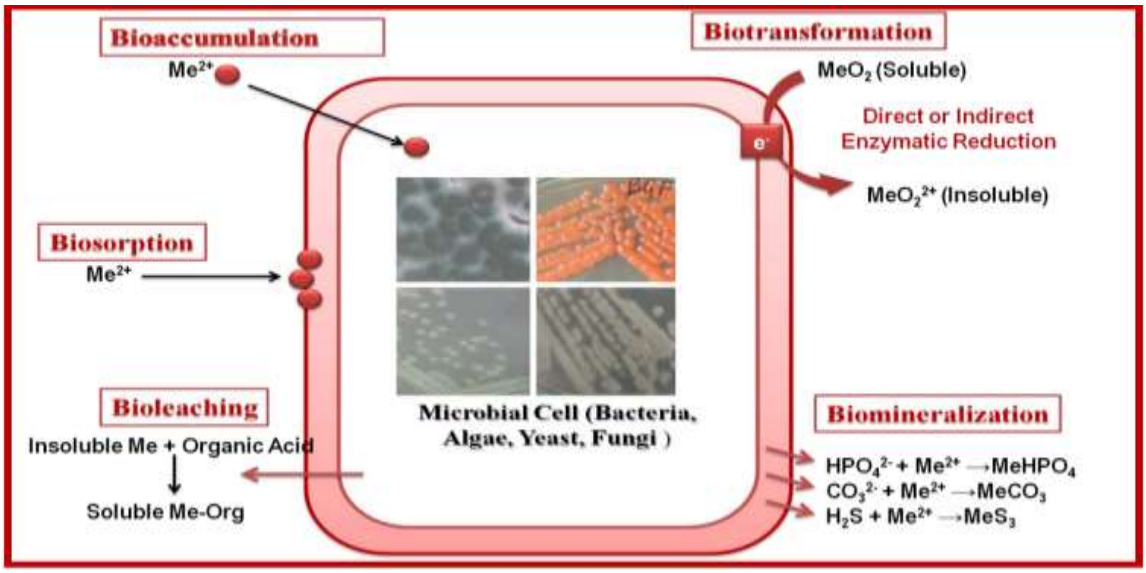


Figure 5: Heavy metal sequestration processes in a microbial cell (Verma and Sharma, 2017).

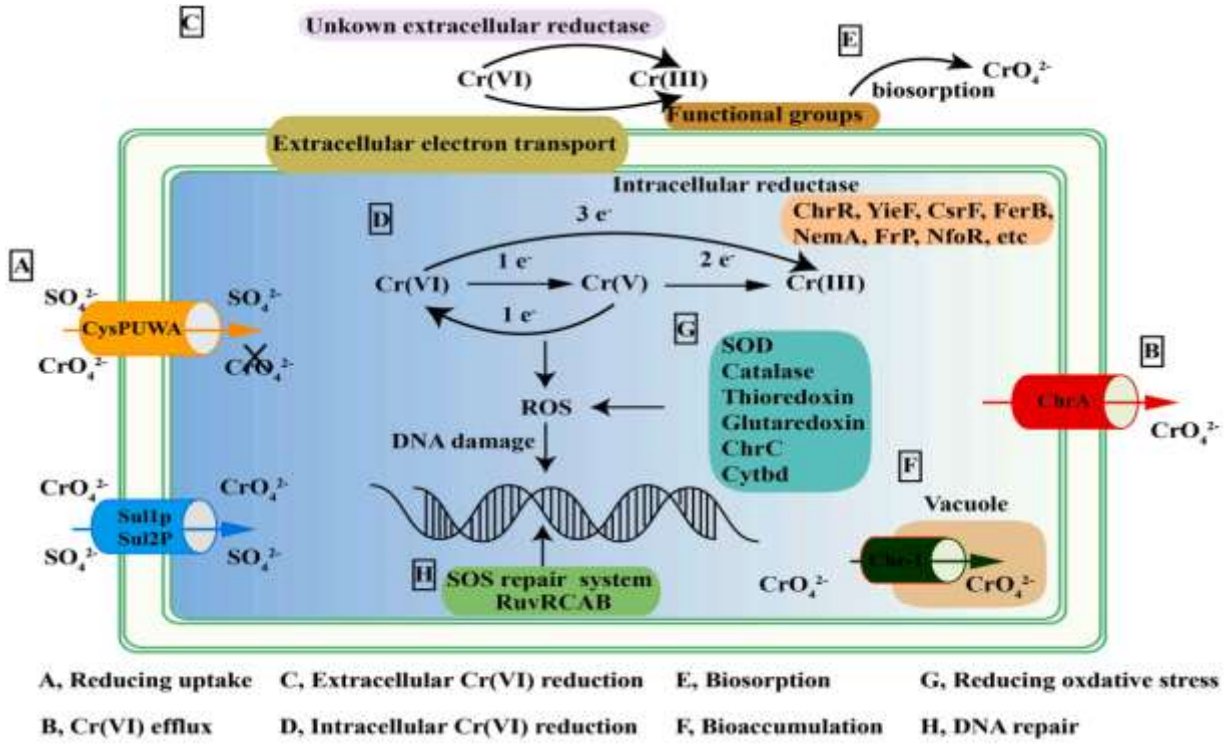


Figure 6: The proposed resistance mechanisms of bacteria to chromium (Cr^{6+})

(A) reducing uptake by closing the SO_4^{2-} transporter; (B) extracellular Cr(VI) efflux by ChrA; (C) extracellular reduction mediated by extracellular reductases, functional groups and proteins in membrane; (D) intracellular Cr(VI) reduction mediated by reductases such as ChrR, YieF, CsrF, FerB, NemA, FrP, NfoR, etc.; (E) biosorption by some membrane functional groups; (F) bioaccumulation in vacuoles; (G) the oxidative stress induced by Cr(VI) can be reduced by SOD, catalase, thioredoxin, glutaredoxin, ChrC and Cytbd; (H) the damaged DNA can be repaired by SOS repair systems, RuvRCAB and other repair proteins (Xia *et al*., 2021)

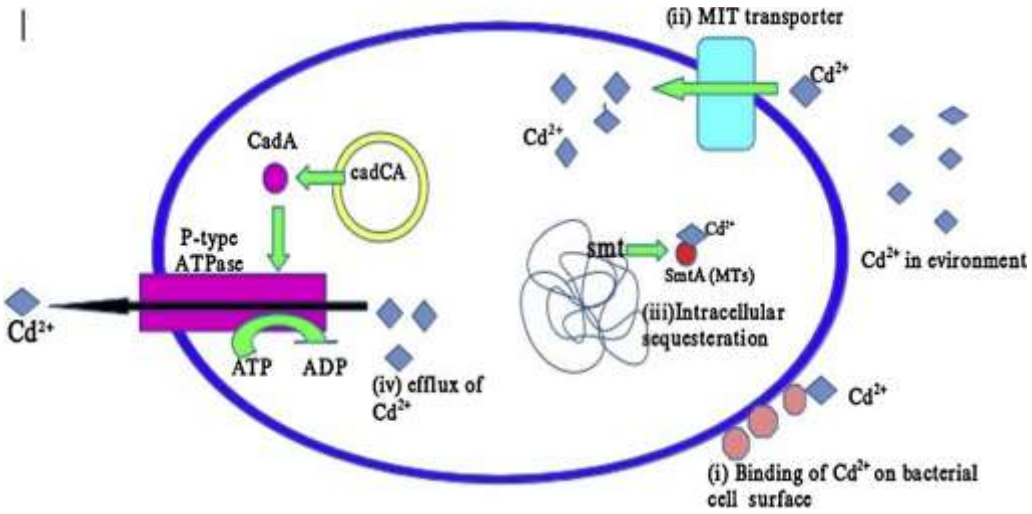


Figure 7: The proposed resistance mechanisms of bactera to cadmium (Cd^{2+})

(I) Binding of Cd^{2+} on the bacterial cell surface (II) MIT metal ion transporter: allows the entry of Cd^{2+} into bacterial cells (III) SmtA (MTs) protein: used for intercellular sequestration and tolerance of Cd^{2+} and its expression controlled by the SmtB gene (IV) CadA transporter (P-type ATPase): is a plasmid-borne resistance system that actively pumps Cd^{2+} out of bacterial cells, and its expression is regulated by CadC gene (Nanda *et al*., 2019)

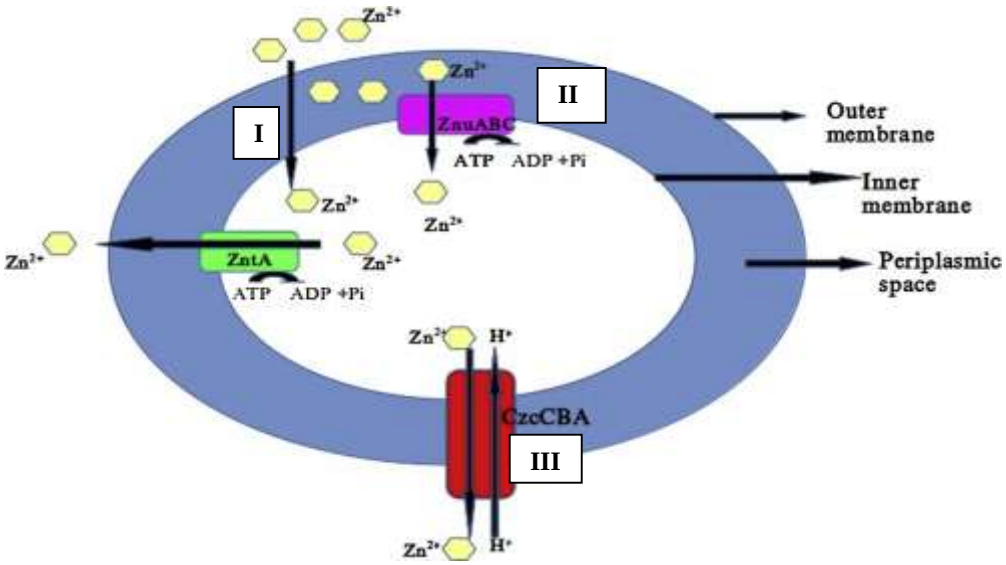


Figure 8: The proposed resistance mechanisms of bacteria to zinc (Zn^{2+})

(I) ZntA is a P-type ATPase that allows the efflux of zinc ions across plasma membrane by an ATP-driven active transport (II) ZnuABC is a periplasmic binding protein-dependent zinc transport system (III) Czc system is a plasmid-borne operon system that used as cation/proton antiporter (effluxes cations from the cell) (Nanda *et al*., 2019)

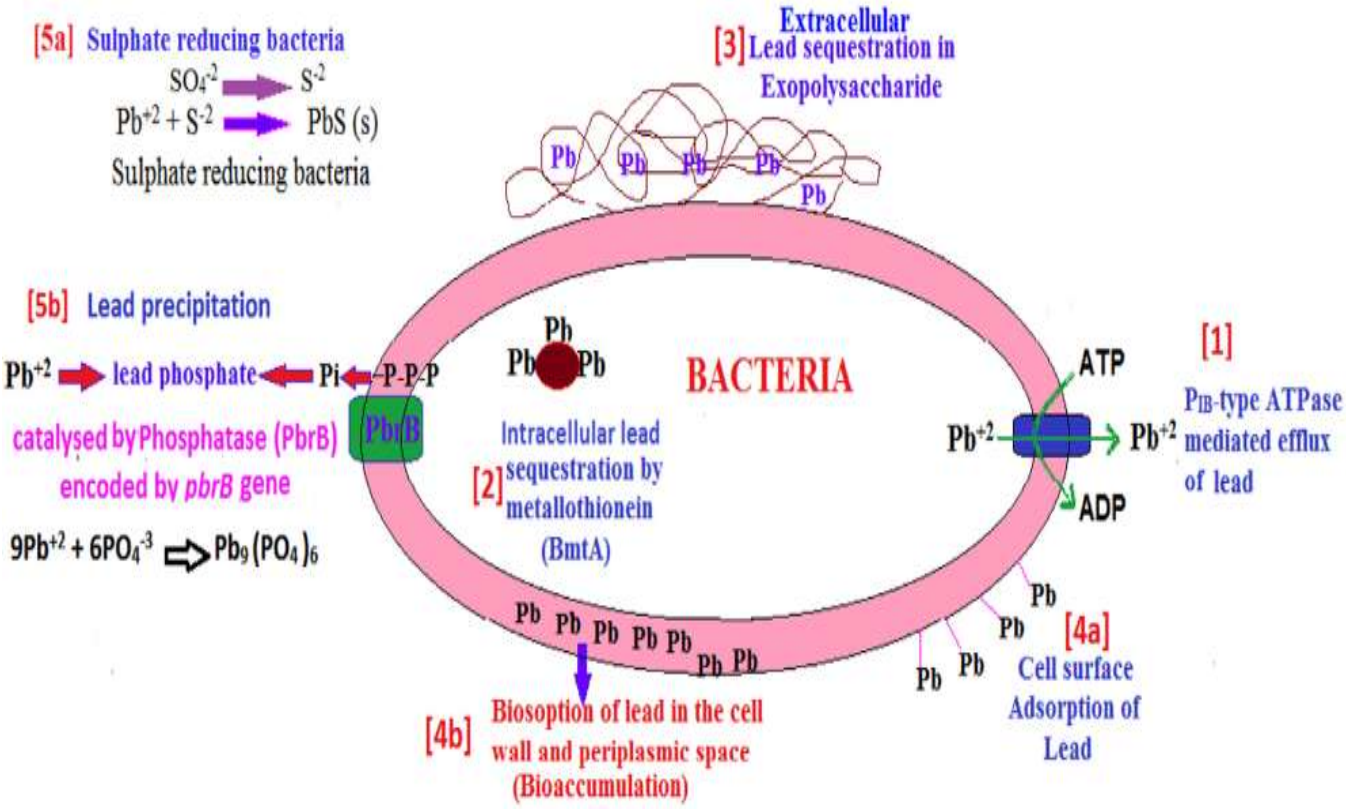


Figure 9: The proposed resistance mechanisms of bacteria to lead (Pb^{2+})

(1) PIB-type ATPase mediated efflux of lead, (2) Lead sequestration by metallothionein (BmtA), (3) Lead sequestration in exopolysaccharide, (4a) Cell surface adsorption of lead, (4b) Biosorption of lead in cell wall and periplasmic space, (5a) Lead precipitation by sulfate reducing bacteria, (5b) Lead precipitation catalyzed by phosphatase enzyme (PbrB) (Naik *et al*., 2013).

3. Materials and Methods

3.1. Description of study area and sample site

Wastewater samples were collected from the two sites: the Awash leather industry located in Akaki Kality Sub-City, and four selected tributaries of AARW (Hana-Mariam, Mekanisa, Kera, and Kebena Rivers). Due to the presence and improper disposal of large amounts of chromium into river water from various tannery industries, the selection criteria for Awash tannery were purposefully added (Temesgen Eliku and Seyoum Leta, 2016). So, potential isolates might be obtained that are used for heavy metal removal. Whereas the criteria for other AAR water sample were based on the presence of high concentrations of heavy metals, data reported by Minbale Aschale *et al*. (2015; 2021) and Deshu Mamo *et al*. (2021). Additionally, the selection was that most of the industries, including tanneries, textiles, woodworks, pharmaceuticals, breweries, wineries, rubber and plastic, national alcohol and liquor factories, abattoirs, and metal products, are releasing inadequately treated wastewater directly into the river (Minbale Aschale *et al*., 2015; Yared Worku and Mekonnen Giweta, 2019). Moreover, a few of the chosen rivers-like the Kebena River-pass through the central parts of the city and are greatly affected by urban development, which is to say, a high population settlement (Yared Worku and mekonnen Giweta, 2019).

The first wastewater samples were collected from Awash leather industry effluents. Awash Leather Industry is one of the Ethio-leather P.L.C. groups in "Nifas Silk Lafto Sub City" of Addis Ababa, Ethiopia. The factory is located on a 123, 000 m^2 site, of which buildings, raw materials, and chemical storage facilities cover about 4,344 m^2. The geographical coordinates of the sample are 8°56.996' N and 38°46.089' E. The tannery processes 3,600,000 sheep and goat skins and 396, 000 hides annually, and it generates 1000-5000 m^3 per day of liquid waste.

The second wastewater sample was collected from four selected AAR namely; Hana-Mariam, Mekanisa, Kera, and Kebena Rivers. Hana-Mariam (Hana Maryam) River is found in Addis Ababa, "Nifas-Silk Lafto" sub city and the geographical coordinates of the sample are 8°55.842' N and 38°45.433' E. The Mekanisa River is found in Addis Ababa, "Nifas-Silk Lafto" sub city and the sample was taken at the geographical coordinates of 8°58.479' N and 38°43.971' E. Kera River is found in "Nifas-Silk Lafto sub city" and it is an intermittent stream located at an elevation of 2215 meters. The sample was taken at the coordinates of 8°58.490' N and 38°44.121' E.

Kebena River also known as Grand Kabana or Great Kabana River, is a stream located in Addis Ababa, Ethiopia and estimate terrain elevation 2162 meters. The samples were collected from "Yeka sub city" near German Embassy with the geographical coordinates of 9°02.012' N and 38°47.458' E. The study area map and the sampling sites along the tannery and river are shown in Fig. 10 below. The Awash tannery effluent is represented by S1, while the tributaries of AAR are represented by Hana-Mariam (S2), Mekanisa (S3), Kera (S4), and Kebena (S5).

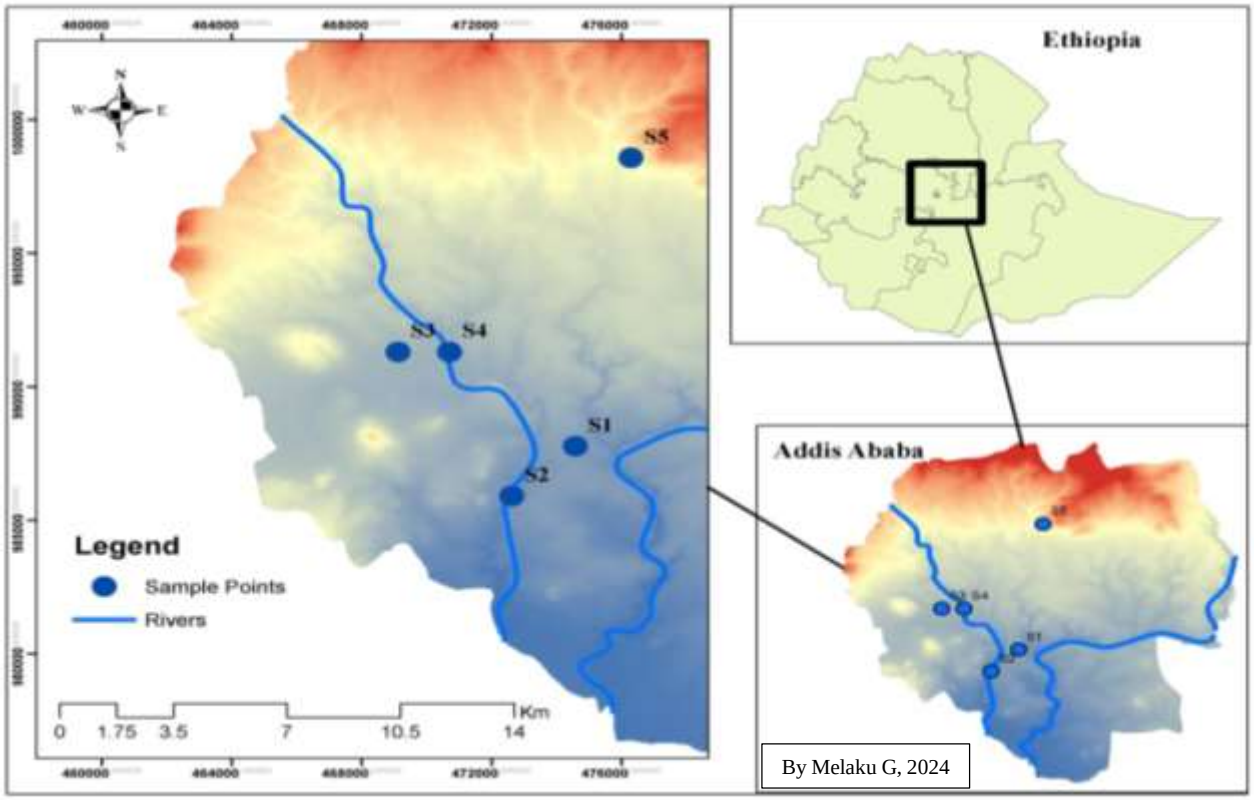


Figure 10: Sampling sites of the studied area along Awash tannery effluent and Addis Ababa River

3.2. Sample collection and analysis

The first sample was collected from Awash tannery wastewater the primary treatment (aerobic and homogenization tank), secondary treatment (biological oxidation tank), and outlet or final discharge tank and all were well mixed (Fig. 11). Awash Tannery has a distinct chromium recovery system in its wastewater treatment facility, along with aeration, sedimentation, and sulfur oxidation ponds. The second wastewater sample was collected from four selected tributaries of AAR (Hana-Mariam; **ሃና-ማርያም**, Mekanisa; **መካኒሳ**, Kera; **ቄራ**, and Kebena; **ቀበና**), and all were mixed

well (Fig. 12). Grab sampling techniques were used to collect samples at different sampling points and mixed them into composite samples for each of the two sampling sites.

The two samples were collected on April 19, 2022, which is commonly the dry season in Ethiopia. For physicochemical, molecular, and heavy metal analysis, as well as bacterial isolation, a total of six wastewater samples (each of 1000 mL) were collected from the selected sites by following a triplicate process. A polyethylene plastic bottle (250- and 1000-mL size for heavy metal and other parameters analysis, respectively) was used for wastewater sampling. It was cleaned with dilute nitric acid, rinsed with de-ionized water, and again washed three times with the wastewater as described by Tariq *et al*. (2019). Temperature, pH, and electric conductivity (EC) were measured on the sampling site with a thermometer and a conductivity meter (BANTE 520 portable conductivity meter, China) and the pH of the sample measured by a pH meter (HINOTEK PH-013, China). The rest samples were transported to laboratory with an ice box, and preserved at 4°C before analysis for up to 24 hours. All the laboratory activities were performed at the Microbial and Molecular Biotechnology laboratory in the Institute of Biotechnology, Addis Ababa University, Ethiopia.

Figure 11: Sampling site from Awash Tannery (Picture was taken March 10, 2023)

Figure 12: Sampling sites from selected Addis Ababa River: Hana-Mariam (A), Mekanisa (B), Kera (C), and Kebena (D) (Picture was taken March 10, 2023)

3.3. Analyzing physicochemical parameters of samples

Selected physicochemical parameters such as temperature, pH, electrical conductivity (EC), chemical oxygen demand (COD), biochemical oxygen demand (BOD), total dissolved solids (TDS), total suspended solids (TSS), sulfate, sulfide, ammonia, ammonium, phosphate, and heavy metals (cadmium, chromium, lead, and zinc) in mg/L were analyzed following standard protocol (APHA, 2005). The data for temperature, pH, and EC were recorded on site during sampling, whereas other parameters were measured at the laboratory. The BOD_5 and COD was done by Winkler's Modified method. Briefly, to test BOD_5, a wastewater sample was incubated in a BOD incubator (Germany) at 20°C for five days, followed by measuring the difference in oxygen content before and after incubation by titrating using a digital titrator (Japan). To test COD, the sample was digested (DRB200, Germany) in a sealed vial with potassium dichromate and sulfuric

acid at 150°C for 2 hours, and the vials were read in a spectrophotometer (DR6000, Germany) to determine the results. The other parameters were analyzed by reagent tablets (ammonia No 1 and No 2, phosphate, sulphate, and sulphide) by using Palintest photometer (Photometer 7500).

3.4. Heavy metals analysis and operated conditions

To avoid biodegradation and volatilization, 1000 mL wastewater samples were preserved with 5 ml of 68% HNO_3, during sample collection and it was brought to laboratory, and kept in a refrigerator at 4 ℃ until they were further processed and analyzed. For determination of heavy metal concentrations, the wastewater samples were digested following standard procedures (APHA, 2005).

The preserved wastewater samples were well mixed and then 100 mL of samples were digested using 5 ml of conc. HNO_3 (68%). The sample was filtered through Whatman filter paper No. 42, and the filtrate was transferred to a pre-cleaned and sterilized falcon tube. The sample was analyzed using an atomic absorption spectrometer (AAS) (analyticjena-novAA 400p, Germany) for Cr, Cd, Pb, and Zn elements. The AAS machine was adjusted and calibrated according to the manufacturer's instructions. Calibration curves were prepared using a standard solution of 0.0, 0.5, 1.0, 1.5, and 2.0 ppm for Cr and Zn. Also, 0.0, 0.1, 0.5, 0.75, and 1.0 ppm for Cd and 0.0, 2.0, 4.0, 8.0, and 16.0 ppm for Pb metals were performed. The concentrations of these metals were recorded at a wavelength of 357.9, 228.8, 217.0, and 213.9 nm for Cr, Cd, Pb, and Zn, respectively. The concentration was calculated on the linear graph of the standard concentration, and the calibration curves showed linearity with a correlation coefficient ($R^2 \geq 0.997$) for four quantified elements in this study. Each collected water sample was examined in triplicate, and samples were performed for every batch of 20-30 samples as shown in Appendix VIII.

3.5. Media preparation

The nutrient agar (NA) medium containing 0.5% peptone, 0.15% yeast extract, 0.15% beef extract, 0.5% sodium chloride, and 1.5% agar was prepared and sterilized. Also, the heavy metal stock solutions were prepared as shown in Table 2. Briefly, the metal ions Cd^{2+}, Cr^{6+}, Pb^{2+}, and Zn^{2+} were used in the salt forms of $K_2Cr_2O_7$, $3CdSO_4.8H_2O$, $Pb(NO_3)_2$, and $ZnSO_4.7H_2O$, respectively. Stock solutions of 1000 ppm were prepared by dissolving the aforementioned analytical-grade metal salts in distilled water as described in Appendix I. The working solutions were prepared

from the stock solutions and filter sterilizing through 0.22 µm pore-size Millipore membranes. Then, Cr and Cd at a concentration of 70 ppm, while Pb and Zn at a concentration of 100 ppm were supplemented on separately sterilized NA medium and well mixed just before pouring in a Petri Dish (Salih *et al*., 2021).

Table 2: Preparation of heavy metal stock solutions

No	Metal	Salt used	MW of salts (gram)	MW of Heavy metals (gram)	Purity of the salts (%)	Amount for 1000 ppm stock solution (gram)
1	Cr	$K_2Cr_2O_7$	294.2	103.99	99.90	2.83
2	Cd	$3CdSO_4.8H_2O$	769.51	337.23	99.00	2.26
3	Pb	Pb $(NO_3)_2$	331.33	207.2	99.00	1.58
4	Zn	$ZnSO_4.7H_2O$	287.55	65.38	99.00	4.35

3.6. Morphological identification and isolation of the bacteria

To isolate the bacteria, serial dilution from 10^1 to 10^5 was made in a normal saline according to Sanjay *et al*. (2020). Briefly, 1 ml of the sample wastewater was aseptically transferred to the 1st test tube making the dilution 10^{-1}, and then mixed well, and 1 ml from this tube was aseptically transferred to the next tube, making it 10^{-2}. Similar transfers were made until five dilutions were achieved. Then 0.1 ml from 10^{-1}-10^{-5} dilution samples were taken and spread on NA medium supplemented with each heavy metal according to Abd-Alkhadim and Abbas (2020). The culture plates were incubated aerobically at 30°C for 24-48 hrs. and the total viable bacterial colonies were counted by colony counter and calculated as Colony Forming Unit (CFU) using the following equation:

$$\frac{CFU}{ml} = \frac{No.of\ coloniesxTotal\ dilution\ factor}{Volume\ of\ culture\ plated\ in\ ml} \qquad \text{Equation (1)}$$

After 24 hrs. incubation, morphologically distinct bacterial colonies were picked from the plates based on their morphology (shape, color, texture, size, arrangement, elevation, margin, opacity). The bacteria were subsequently sub-cultured until pure culture was obtained on NA supplemented with the aforementioned metal salt concentrations, and these isolates were partially identified according to Bergey's Manual of Systematic Bacteriology (Brenner *et al*., 2005).

3.7. Preliminary isolation of heavy metal-resistant bacterial isolates

Preliminary isolation of the bacteria was conducted on nutrient agar medium (NAM) containing each of four heavy metals with their respective isolates according to (Khan *et al*., 2015). Briefly, a loop-full of bacterial colonies that were obtained from 70 ppm Cr and Cd-supplemented NAM were again tested and spread into another NAM supplemented with $K_2Cr_2O_7$ and $3CdSO_4.8H_2O$ concentrations ranging from 50 to 1200 mg/L. Also, bacterial colonies obtained from 100 ppm Zn-supplemented NAM were tested and spread into NAM supplemented with $ZnSO_4.7H_2O$ concentrations ranging from 100 to 1200 mg/L. Similarly, colonies that were obtained from 100 ppm Pb-supplemented NAM were tested and spread into another NAM supplemented with $Pb(NO_3)_2$ concentrations ranging from 100 to 2000 mg/L. Finally, growth performance was observed on the plate after each plate containing the isolates was incubated at 30℃ for 24-48 hrs. Isolates that could resist up to 1200 ppm for Cr, Cd, and Zn and up to 2000 ppm for Pb were selected for further study.

3.8. Screening of the most heavy metal-resistant bacterial isolates

The most heavy metal-resistance bacterial isolates were screened with a broth medium according to Pandit *et al*. (2013). Briefly, nutrient broth (NB) medium containing 0.5% peptone, 0.15% yeast extract, 0.15% beef extract, and 0.5% sodium chloride at pH 7.6 ± 0.2 was prepared separately for each of four heavy metals. Next, each flask containing NB medium was filled with a concentration of 1200 ppm Cr, Cd, and Zn and 2000 ppm Pb heavy metals. Then, a loop-full colony of each isolate obtained from the preliminary isolation was inoculated in their respective heavy metal-containing NB medium flask and shaken with a 120-rpm rotary shaker at 30℃. The optical density (OD) value of the incubated isolates was measured in 24-hour intervals at 0, 24, 48, 72, 96, and 120 hr. by reading the absorbance at 600 nm using a UV spectrophotometer (JENWAY 6300 spectrophotometer, UK) (Zhenggang *et al*., 2018). Finally, isolates that had a high OD value on the aforementioned concentrations of each heavy metal were taken for further study.

3.9. Characterization of heavy metal-resistance bacterial isolates

The bacterial isolates were characterized both by morphological (Gram-staining) and molecular methods following the protocol (Garcha *et al*., 2016; Begum *et al*., 2017; Orji *et al*., 2021).

3.9.1. Molecular identification

3.9.1.1. Genomic DNA Extraction

The genomic DNA was extracted using the DNeasy blood and tissue extraction kit (QIAGEN, Valencia, USA) according to the manufacturer's instruction. Briefly, a loop-full of overnight culture was taken into clean Eppendorf tubes followed by adding 80 µL of phosphate buffer saline (PBS) and vortexing it. Then, 80 µL Lysis Solution (AL) buffer and 20 µL Proteinase K was added and vortexed well and then incubated on a thermostat plus incubator overnight at 56ºC. After that, vortex and centrifuged overnight sample for 1 min followed by 200 µL supernatant was taken and transferred to a new Eppendorf tube, and 200 µL ethanol was added and homogenized by vortexing it. The mix was transferred to a filter column tube and centrifuged for 1 min then the collection tube was discarded and the filter was pull-on a new receiver tube. Then 500 µl wash buffer 1 (AW1) was added to the column and centrifuged for 1 min and then the flow through was discarded without discarding the receiving tube. Five hundred µL wash buffer 2 (AW2) was added to the column and centrifuged for 3 min and then the collection tube was emptied. Then the emptied column with the collection tube was centrifuged for 1 min to remove residual ethanol. The spin column was placed in a clean Eppendorf tube and 50 µL zymo extraction kit elution buffer was added to the filter and incubated at 56ºC for 10 min. The filter was centrifuged for 1 min and the eluted sample was pipetted back to the filter and centrifuged for 1 min.

The quality and quantity of the genomic DNA were determined by using a Nanodrop (NanoDrop™ 2000/c) spectrophotometer. The DNA concentration ranges from 15 to 60 ng/µL and the quality range from 1.57 to 2.13 were taken, and finally, the resulting DNA was stored at -20°C.

3.9.1.2. PCR Amplification of 16S rRNA genes

For amplification of 16S rRNA gene fragment, a pair of universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used to generate a 1500bp PCR product (Belay Tilahun *et al*., 2020). The PCR reaction contained a total volume of 50 µL (5 µL PCR buffer, 2.6 µL dNTPs, 1 µL of each forward and reverse primers, 1 µL Taq polymerase, 5 µL of template DNA, and the remaining nuclease-free water). Reaction conditions were performed at initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 55°C for 30 sec, and 30-sec chain extension

at 72°C. Final extension was done at 72°C for 5 min. The PCR reaction was run using a thermal cycler (CFX-96^{TM} Real-time system, Singapore).

Amplified DNA products were analyzed by electrophoresis through 0.8% agarose gel in 1X TBE (Tris 108 g, EDTA 7.44 g, H_3BO_3 55 g, and H_2O 1000 ml) buffer at 170V with 35-40A for approximately 1:45 hr. The molecular weight of the PCR products was estimated by using a 1 Kb plus DNA ladder to estimate the approximate size of the amplified fragment. The gel was visualized under a gel documentation system (Gel DOC$^{TM\ XR}$, Bio-Rad, USA) with the aid of an ethidium bromide staining agent with the help of UV light and a camera connected to it.

3.9.1.3. Restriction digestion of the PCR amplified product

The restriction endonuclease enzyme TaqI (5′-T^CGA-3′) and (3′-AGC^T-5′) was used for endonuclease digestion or cleavage of the amplified DNA products. The total volume of 7.0 µL of a reaction mixture containing 0.4 µL of TaqI (1U/µL) enzyme and 1.5µL of 10X Hibuffer TaqI (Himedia, India), and 5.1µL of ultrapure sterilized H_2O was prepared in PCR tubes and 10 µL (~ 5µg) every amplified PCR product was added into each reaction mixture. Then the PCR tubes were incubated at 65°C for 1hr and the enzyme being inactivated at 85°C for 15 min on a thermocycler. The whole digested fragments were run through gel electrophoresis in 3% agarose gel (w/v) at 100 V for 1:45 hr. in the presence of 4 µL ethidium bromide, creating a 'unique' banding pattern. As a size marker 1 Kb plus DNA ladder was used, and the image was taken. Amplified Ribosomal DNA Restriction Analysis (ARDRA) patterns were determined for sequencing (Birhanu Mekuaninte., 2010; Solomon Enquahone, 2018). Finally, microbial diversity and genetic identity were observed based on the ARDRA pattern, and the PCR products of pure isolates were cleaned using the GenElute PCR Clean-Up Kit (Sigma) according to the manufacturer's recommendations for further sequencing purposes.

3.9.1.4. Sequencing and Phylogenetic Analysis

PCR products of each bacterial isolate were sent to Holland, through the collaboration of MRC-ET advanced molecular diagnostic laboratory which is found in Ethiopia, and sequenced by the Sanger DNA sequencing method. The raw DNA sequences were obtained and edited using the BioEdit software package, version 7.7.1, and the sequences were also assembled by CAP contig assembly program. To compare classification resemblances, sequences were aligned with previously published bacterial 16S rRNA sequences in the NCBI DNA databases using BLAST

search tool (http://www.ncbi.nlm.nih.gov/BLAST/) (Kalaimurugan *et al*., 2020). Based on the scoring index, final sequences were aligned with other sequences of representative bacterial 16S rRNA regions using CLUSTAL W in MEGA 11 alignment explore (Nokman *et al*., 2019). To generate a phylogenetic tree, the aligned sequences were run by a Neighbor-Joining method (Tamura *et al*., 2021). The percentage of replicate trees in which the associated taxa clustered together in the 1000 replicate bootstrap values and the evolutionary distances were computed using the Maximum Composite Likelihood method. In this analysis, 14 nucleotide sequences were examined; the final dataset contained 1450 positions in total, and all ambiguous positions were removed for each sequence pair (pairwise deletion option). The DNA sequence submissions were sent to GenBank, and accession numbers were obtained for all four bacterial isolates.

3.10. Determination of optimum growth conditions

The optimal temperature and pH were determined for the maximum growth of heavy metal-tolerant bacterial isolates according to Batool *et al*. (2021) and Jahan *et al*. (2016). To determine the optimum temperature, 50 mL of NB broth medium was prepared on a 250 mL flask by adjusting the pH to 7.6±0.2, and then a loop-full of inoculum was inoculated on it at a temperature of 25, 30, 35, 40, 45, and 50 ℃. Also, to determine the optimum pH each isolate was cultured in 50 mL NB medium containing pH 5, 6, 7, 8, 9, and 10 at 25℃. Optimum growth of the isolate was determined by measuring the growth of isolates at a wavelength of 600 nm in a spectrophotometer.

The growth pattern of the bacterial isolates was determined by culturing in NB broth medium supplemented with each metal according to Elahi *et al*. (2019) and Elahi and Rehman (2019). Briefly, 250 mL flasks containing 50 mL of NB medium were supplemented with all four metals that contained 50 ppm Cr and Cd and 100 ppm Pb and Zn. One hundred µL of overnight culture was inoculated into four flasks and the fifth flask was only culture as a control and then agitated on a rotary shaker at 120 rpm (Pandit *et al*., 2013). Growth was checked as a function of biomass by measuring the absorbance at 600 nm using a spectrophotometer at different time intervals (2, 4, 6, 8, 10, 24, 48, 72, and 96 hr.).

3.11. Minimum inhibitory concentration (MIC)

The heavy metal resistances of the bacterial isolates (WlCr, T14Cd, W12Pb, and T4Zn) were determined by MIC. It is the minimum concentration of heavy metals that are able to inhibit

bacterial growth and it is the primary technique in order to check the resistance of bacterial strains towards various heavy metals in order to build up a suitable heavy metal waste remediation system. The resistance of bacterial isolates against heavy metals (chromium, cadmium, lead, and zinc) was determined using both the plate dilution method described by Khan *et al*. (2015) and broth dilution (Pandit *et al*., 2013). Briefly, the bacterial isolates were grown on the plate by increasing the heavy metal concentrations, which ranged from 50 to1200 mg/L for Cr, Cd, and Zn isolates and from 100 to 2000 mg/L for bacterial isolate resistant for Pb. Also, the bacterial isolates were inoculated into the broth medium with increased heavy metal concentrations, which ranged up to 4000, 4500, 5500, and 7500 ppm against Cr, Cd, Pb, and Zn isolates, respectively, and then incubated at 30°C for 24-48 hours. Then, a loop-full colony was taken from the incubated broth medium and streaked on the NA medium. After the incubation period, growth was determined by physically observing the agar plates. For no growth plates, it was considered the MIC for each isolate.

3.12. Determination of antibiotic susceptibility

Antibiotic susceptibility test was done to determine the ability of bacterial isolate against various antibiotics. The Kirby-Bauer disc diffusion method on a Muller-Hinton (MH) agar plate was used for antibiotic sensitivity-resistance test of bacterial isolates (Begum *et al*., 2017; Tariq *et al*., 2019). The 18-hour-old fresh 100 µL bacterial cultures were spread on an MH agar plate. Commercially available antibacterial discs (OXOID Ltd, Hampshire, UK) including ampicillin (AMP, 10µg), penicillin-G (P, 10µg), tetracycline (TE, 30µg), streptomycin (S, 25µg), nalidixic acid (NA, 30µg), chloramphenicol (C, 30µg), ciprofloxacin (CIP, 5µg), erythromycin (E, 15µg), gentamycin (GEN, 10µg), neomycin (NE, 10µg), norfloxacin (NOR, 10µg), and oxytetracycline (OT, 30µg) were used and placed on MH agar plate at 37℃ for 24 hr. The sensitivity of individual antibiotics was determined by measuring the clear zone diameter (mm) around each antibiotic disc. The sensitivity or resistance categories of the isolates were verified by the standard chart of Hi-Media (CLSI) (Clinical and Laboratory Standards Institute) standards (CLSC, 2014). All the experiments were carried out in triplicate.

3.13. Tolerance tendency of bacteria isolates to heavy metals

Tolerance of the four selected bacterial isolates was determined using the broth dilution method as described previously (Oaikhena *et al*., 2016). Each isolated bacterium was cultured into a 250 mL flask containing 50 mL of NB at 30℃ for 24 hrs. One hundred microliters of overnight cultured

bacterial isolates were taken and each was inoculated into a NB containing different concentrations of heavy metals; 100, 200, 400, 600, 800, and 1000 ppm for Cr, Cd, Pb, and Zn. They were grown at 30℃ for 48 hrs. To know the tolerance tendency of bacterial isolates, growth was determined by measuring the absorbance using a spectrophotometer at 600 nm. The blank was NB containing the same concentration of heavy metals but without bacterial colonies.

3.14. Determination of multi-metal resistance of bacterial isolates

Each bacterial isolate was tested against a mixture of heavy metals (Cr^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}) at concentrations of 50, 100, 200, 400, 600, and 800 ppm in 3:3:1:1 for lead, zinc, chromium, and cadmium, respectively (Saini and Pant, 2016). Briefly, all four isolates were cultured in a mixture of heavy metals as a mixed culture. That is, five hundred microliters (500 µL) of the cultures were inoculated into a 250 mL flask containing 50 mL of NB supplemented with the aforementioned concentrations of heavy metals and grown at 30°C for 48 hrs. The inhibitory concentrations were measured using a spectrophotometer at 600 nm. The blank was NB containing the same concentration of heavy metals but without bacterial colonies.

3.15. Capability of bacterial isolates for heavy metal removal

3.15.1. Evaluation in nutrient agar medium supplemented with heavy metals

The heavy metal removal ability of bacterial isolates was evaluated through batch experimental system in triplicate according to Zhenggang *et al*. (2018). Each isolate was cultured in 50 mL of broth with 100 ppm Cr and Cd and 300 ppm Pb and Zn for 24 hrs. To four 1000 mL sterile conical flasks, 200 mL of the sterilized NB medium supplemented with 100 ppm of each heavy metal was added separately; however, the fifth flask was supplemented in 3:3:1:1 for Pb, Zn, Cr, and Cd. The first, second, third, and fourth flasks were each inoculated with 2 ml of a 24-hour NB culture of W1Cr, T14Cd, W12Pb, and T4Zn, respectively. The fifth flask was inoculated with 2 ml of culture at a 1:1:1:1 ratio (equal amount) for four isolates as a mixed culture consortium (MCC). All flasks were incubated at 30℃ and shaken at 120 rpm. The experiment was left to stand for 8 days. An aliquot of 5 mL was collected in 2-day intervals (2, 4, 6, and 8 days of growth). Samples were spun down at 6000 rpm (Universal 320R, Hettich, Germany) for 15 min and supernatants were filtered through sterilized 0.22 µm membrane filters. The filtrates were used for heavy metal estimation by an atomic AAS (analyticjena-novAA 400p, Germany) (Elahi and Rehman, 2019).

The percent reduction of heavy metal was calculated according to (Oaikhena *et al*., 2016) in triplicate as follows:

$$RC = \frac{HMi - HMf}{HMi} \times 100\% \qquad \text{Equation (2)}$$

…where RC is the removal capability of heavy metal, HMi is the initial concentration of heavy metal (ppm), and HMf is the final residual concentration of heavy metal (ppm).

3.15.2. Evaluation of heavy metal resistance bacterial isolates in the actual wastewater

The isolates were also evaluated in the actual wastewater in batch experiments. To six 1000-mL sterile conical Erlenmeyer flasks were prepared and 200 mL of wastewater from Awash tannery sample was added to each flask and sterilized using an autoclave at 121°C for 20 min. After autoclaving, 10 ppm Cr was supplemented to each flask by filtering (Salih *et al*, 2021). The first flask was not inoculated with bacterium culture (control); the second, third, fourth, and fifth were each inoculated with 2 mL of a 24-hour NB culture of W1Cr, T14Cd, W12Pb, and T4Zn, respectively. The sixth flask was inoculated with a mixed culture consortium (MCC) of all four bacteria in equal concentration. All flasks were cultured at 30°C at 120 rpm shaking incubator. An aliquot of 5 mL was withdrawn from each flask after regular intervals (2, 4, 6, and 8 days of growth). Samples were spun down at 6000 rpm for 15 min, and filtered supernatants were used for heavy metal estimation by AAS (Elahi and Rehman, 2019) in triplicate and the heavy metal removal capacity of each isolate was calculated. Similar procedures was followed except, 10 ppm Cr, Cd, Pb, and Zn (equal concentration) were used for the AAR tributary samples.

3.16. Data analysis

The figures were analyzed by Origin Pro (version 2019b) of the standard method and the mean value was determined by the standard deviation values. The table values were presented as mean ± SD that were analyzed by one way ANOVA. Most of the data were displayed by using tables and graphs. The MEGA 11 software was used to analyze the phylogenetic tree of bacterial isolates and Bio-Edit software was used to generate consensus DNA sequence (contig).

4. Results

4.1. Physicochemical characteristics of the wastewater samples

For physicochemical analysis of the wastewater from the two sources, the EEPA (discharge limits for inland water), ESA, WHO, and EU (discharge limits for drinking water), and FAO (discharge limits for irrigation water) were used. The physicochemical parameter concentrations of the wastewater samples from the two sources were analyzed and results presented in Table 3. Temperature and pH of both sample sites were found to meet the listed standard guidelines. The COD, BOD_5, EC, TDS, TSS, SO_4^{2-}, and chromium concentration of the Awash tannery sample was above the permissible limit of the standard guidelines, except $SO42^-$ within the permissible limit of EEPA as shown in Table 3. Whereas NH_3-N, NH_4^+-N, S^{2-}, and PO_4^{3-} were within the permissible limit of these standards, except the phosphate concentration was above the permissible limit of EU and WHO.

In addition, the COD, BOD_5, TSS, NH_4^+-N, NH_3-N, S^{2-}, and PO_4^{3-} concentrations of selected tributaries of AARW sample was above the permissible limit of the standard guidelines as shown in Table 3. Similarly, EC was on the permissible limit of EU but above the standard guidelines permissible limit of WHO and FAO. TDS was on the permissible limit of EEPA, ESA, and FAO, but it was above the limit of EU and WHO. Except FAO, SO_4^{2-}, was within the permissible limit of other listed standard guidelines. Except the EEPA standard guidelines, the heavy metals chromium, cadmium, and lead were above the permissible limit of the standard guidelines of ESA, EU, WHO, and FAO as shown in Table 3.

Table 3: Physicochemical parameters of Awash tannery effluent and selected Addis Ababa river water sample (Mean ± SD)

N. O.	Parameters type	Wastewater Sample Sources		Standard Guidelines				
		Awash Tannery	AAR Tributaries	EEPA	ESA	EU	WHO	FAO
1	pH	7.89±0.01	7.74±0.02	6-9	6.5-8.5	-	6.5-8.5	-
2	Temperature	25.33±0.33	22.33±0.88	<40	<40	-	-	-
3	COD	1396±4.73	492±3.21	250	250	125	200	120
4	BOD_{5d}	436.67±2.40	227.5±1.58	80	80.0	75.0	80.0	40.0
5	EC	18.85±0.07	2.70±0.01	-	-	2.50	3.00	3.00
6	TDS	5853.93±18.4	683.33±2.60	3000	1000	500	600	2000
7	TSS	1349.77±7.06	338.23±1.28	100	-	30.0	30.0	100
8	Ammonia (NH_3-N)	1.12±0.01	10.02±0.35	5.0	1.50	-	1.50	-
9	Ammonium (NH_4^+-N)	1.31±0.02	20.28±0.57	5.00	1.50	0.50	-	-
10	Sulfate (SO_4^{2-})	364.33±2.60	116.33±1.45	1000	250	250	250	20.0
11	Sulfide (S^{2-})	0.34±0.01	10.57±0.43	2.00	2.00	-	-	-
12	Phosphate (PO_4^{3-})	3.01±0.01	23.91±0.13	5.0	5.00	0.5	-	2.00
13	Chromium	11.4±0.07	0.122±0.001	2.00	0.05	0.05	0.05	0.10
14	Cadmium	NT	0.026±0.002	1.00	0.003	0.005	0.003	0.01
15	Lead	NT	0.110±0.001	0.50	0.01	0.01	0.01	5.00
16	Zinc	NT	0.21±0.002	5.00	5.00	-	-	2.00

Note: NT= not tested, - = not given. The units of all parameters were expressed by mg/L except temperature (℃) and EC (mS/cm). pH has not unit.

EEPA = Ethiopian Environmental Protection Authority provisional discharge limits for inland water (EPA, 2011)

ESA = Ethiopian Standards Agency for drinking water (ESA, 2013)

EU = European Union Directives for drinking water guidelines (1998)

WHO = World Health Organization for drinking water guidelines (2011)

FAO = Food and Agricultural Organization for irrigation water (Ayers and Westcot 1985)

4.2. Enumeration and morphological identification of bacterial isolates

In this study, bacterial isolates from ATE and selected tributaries of AARW sample were identified and characterized. Bacterial growth was observed after 24-72 hr. of incubation, and a total of 130 different bacterial isolates were primarily screened morphologically (size, color, shape, margin, arrangement, texture, etc.) from the two sites. In ATE, a total of 65 bacterial isolates were found in the NAM supplemented with the four heavy metals. Hence, 25, 15, 14, and 11 bacteria were isolated from Cr, Cd, Pb, and Zn-supplemented NAM, respectively. In the same manner, 65 bacterial isolates were found in the tributaries of the AARW sample. Hence, the NA supplemented with Cr, Cd, Pb, and Zn gave 27, 13, 13, and 12 bacterial isolates, respectively. As shown in Table 4, the respective dilution factors that the bacterial isolates found were 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions for Cd, Cr, Zn, and Pb-supplemented NAM, respectively, on their cultured plates. The total viable bacterial colonies were counted and calculated as CFU based on Equation 1, and the result is shown in Table 4.

Table 4: Total viable cell counts in each of four heavy metal incorporated nutrient agar medium

Sample sources	**Total viable count (CFU/mL)**			
	Cr	Cd	Pb	Zn
ATE	$1.9x10^5$ (188)	$1.6x10^4$ (160)	$8.0x10^6$ (80)	$1.8x10^6$ (180)
AARW	$8.4x10^4$ (84)	$1.8x10^4$ (180)	$1.1x10^7$ (108)	$1.3x10^6$ (128)
DF of the sample	10^2	10^1	10^4	10^3

Note: ATE: sample from Awash tannery effluent, AARW: sample from selected tributaries of Addis Ababa river water and DF: dilution factor, and the number in brackets indicate the number of colony obtained from the plate.

4.3. Preliminary isolation and screening of the bacteria

As shown in Appendix II, the bacterial isolates were tested up to 1200 ppm for Cr, Cd, and Zn-supplemented isolates, while up to 2000 ppm for Pb-supplemented isolates in both sampling sites. From the two sample sites, 52 different bacteria were isolated and able to tolerate up to 100 ppm concentrations of Cr-supplemented NAM. Similarly, 28 isolates from Cd-supplemented NAM were tolerated up to 100 ppm. Also, 27 bacteria were isolated and able to tolerate up to 800 ppm of Pb-supplemented NAM. On the other hand, 23 isolates taken from both sampling sites tolerated

up to 300 ppm of Zn-supplemented NAM. Out of 130 bacterial isolates, 37 were preliminary screened by the NA plate method, of which 13, 5, 13, and 6 isolates were obtained from Cr, Cd, Pb, and Zn-supplemented NAM, respectively (Table 5).

Table 5: Preliminary isolation and screening of the bacteria from the sample sites

Concentrations of 4 heavy metals (ppm)	**Number of isolates from Cr**	**Number of isolates from Cd**	**Number of isolates from Pb**	**Number of isolates from Zn**
50	52	28	-	-
100	52	28	27	23
200	50	26	27	23
300	47	26	27	23
400	43	19	27	21
500	29	15	27	18
600	24	13	27	16
700	18	8	27	14
800	18	8	27	11
900	18	6	26	9
1000	18	5	22	9
1200	13	5	17	6
1500	-	-	16	-
2000	-	-	13	-
Total isolates	**37**			

Note: - = isolates not tested at this concentrations

4.4. The most heavy metal resistance bacterial isolates

After preliminary isolation, the 37 bacterial isolates were minimized by using M medium supplemented with 1200 ppm of Cr, Cd, and Zn, while 2000 ppm concentrations for Pb heavy metals. The OD value was measured and recorded at 0, 24, 48, 72, 96 and 120 hrs. as indicated in Appendix III. Out of 37, only four isolates (W1Cr, T14Cd, W12Pb, and T4Zn) were selected for further study because they showed the highest resistance ability at the specified concentrations of the respective heavy metals. The morphology of these isolates are indicated in Fig. 13.

Figure 13: Pure cultures of four (W1Cr, T14Cd, W12Pb, T4Zn) bacterial isolates

4.5. Identification of the bacterial isolates

4.5.1. Gram staining

Each of the isolated organisms was identified morphologically using gram staining and their type; shape, and structure were presented in the Fig. 14. The characteristics of the isolate were observed using a binocular microscope, and all four isolates were gram-positive. The isolates W1Cr, T14Cd, and W12Pb were coccus (spherical shape), and white. The isolates W1Cr and W12Pb exist in groups, whereas T14Cd exists alone. The isolate T4Zn was a bacillus (rod shape), yellowish, and single.

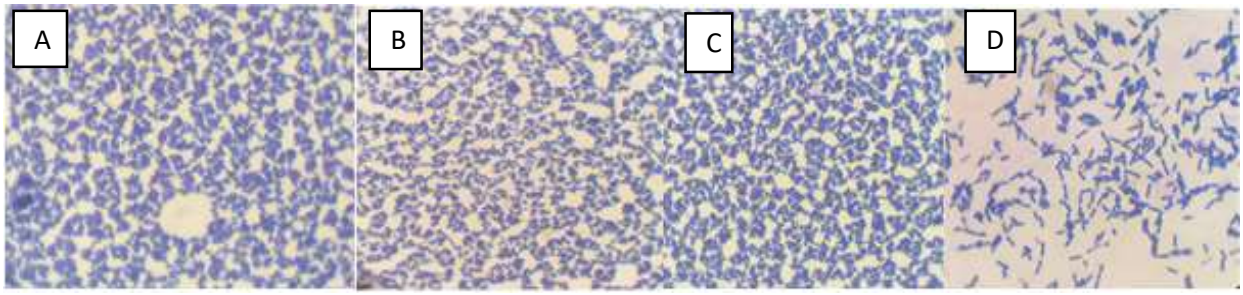


Figure 14: Binocular microscopic characteristics of the four isolates: WlCr (A), T14Cd (B), W12Pb (C) and T4Zn (D)

4.5.2. Molecular identification

4.5.2.1. Genomic DNA extraction and PCR amplification of 16S rRNA

We performed DNA extraction from the bacterial isolate and agarose gel electrophoresis of the genomic DNA was conducted. As a result, all eight bacterial isolate bands were clearly visible, and the resulting image on the gel is presented in Fig. 15A. The 16S rRNA genes were amplified through PCR from the genomes of the selected bacterial isolates. PCR amplifications with the

same oligonucleotide primers were performed, and a single band of similar size (approximately 1500 base pairs) was obtained, as illustrated in Fig. 15B.

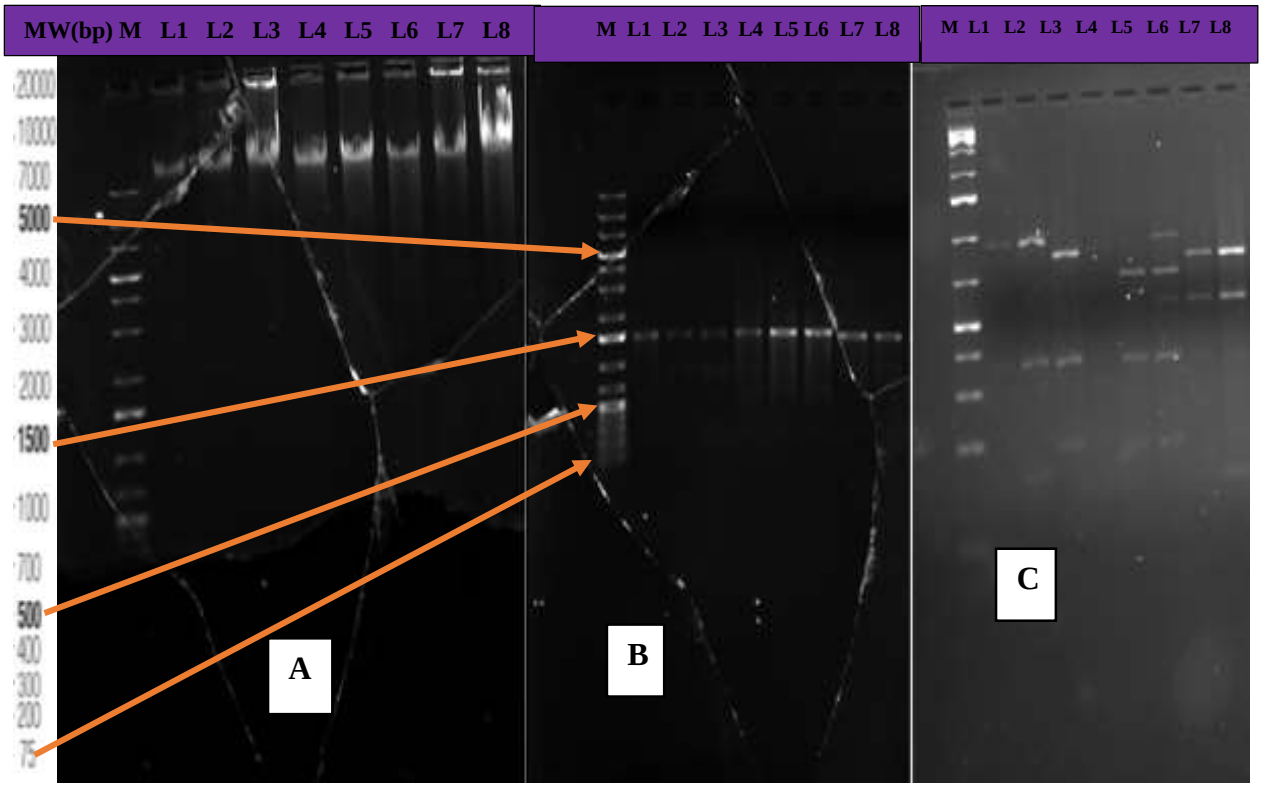


Figure 15: MW (bp)= Molecular weight of the marker; Genomic DNA (A), PCR amplification products of 16S rDNA gene (B), and Restriction digestion of the PCR products of isolates (C). M =1Kb plus DNA ladder, L1= W1Cr, L2=T21Cr, L3=T14Cd, L4=W3Cd, L5=T3Pb, L6=W12Pb, L7=W8Zn, and L8=T4Zn

4.5.2.2. Restriction digest analysis of the 16S rRNA amplified product

In order to differentiate similar isolates that have similar fragments (bands), the eight bacterial isolates were examined by Amplified Ribosomal DNA Restriction Analysis (ARDRA). Following the cleavage of the 16S rRNA PCR amplification products by TaqI enzymes, each of the amplification products showed their own band patterns, as shown in Fig. 15C. Among the eight best isolates, four isolates (T14Cd, W3Cd, W12Pb, and T3Pb) were distinct polymorphic groups based on the ARDRA profile obtained. However, T21Cr and W1Cr and W8Zn and T4Zn had almost similar restriction band patterns or were cut at similar sites by the enzyme TaqI.

4.5.2.3. Sequencing and phylogenetic analysis of 16S rRNA

The PCR products of 16S rRNA of four isolates (W1Cr, T14Cd, W12Pb, and T4Zn) were sequenced and the resulted sequences were edited using Bioedit software package. The consensus sequences obtained were blasted and compared with available sequences in the database using NCBI BLAST. All the four bacterial isolate showed significant similarity >99% in the GenBank. Accordingly, the 3 bacterial isolates were identified and belonging to genera *Staphylococcus* and one genus *Bacillus* as presented in Table 6. Based on this, they were identified as *Staphylococcus xylous strain W1Cr (S. xylous W1Cr), Staphylococcus xylous strain T14Cd (S. xylous T14Cd),* and *Staphylococcus xylous strain W12Pb (S. xylous W12Pb)* and *the Bacillus cereus strain T4Zn (B. cereus T4Zn).* Their accession numbers obtained from the GenBank were PP914113, PP914114, PP914115, and PP914116, respectively. A phylogenetic tree was constructed based on the 16S rRNA gene sequences as presented in Fig. 16.

Table 6: Phylogenetic analysis of bacteria based on similarity to the partial 16S rDNA sequence

NO.	Strains Sequenced	E-value	Identity (%)	Close Relatives	Accession No.
1	*Staphylococcus xylous strain W1Cr (**PP14113**)*	0.0	99.93	*Staphylococcus xylous strain Kl 162*	NR_036907.1
2	*Staphylococcus xylosus strain T14Cd (**PP14114**)*	0.0	99.93	*Staphylococcus xylosus strain KL 162*	NR_036907.1
3	*Staphylococcus xylosus strain W12Pb (**PP14115**)*	0.0	99.89	*Staphylococcus xylosus strain KL 162*	NR_036907.1
4	*Bacillus cereus strain T4Zn (**PP14116**)*	0.0	99.72	*Bacillus cereus ATCC 14579*	NR_074540.1

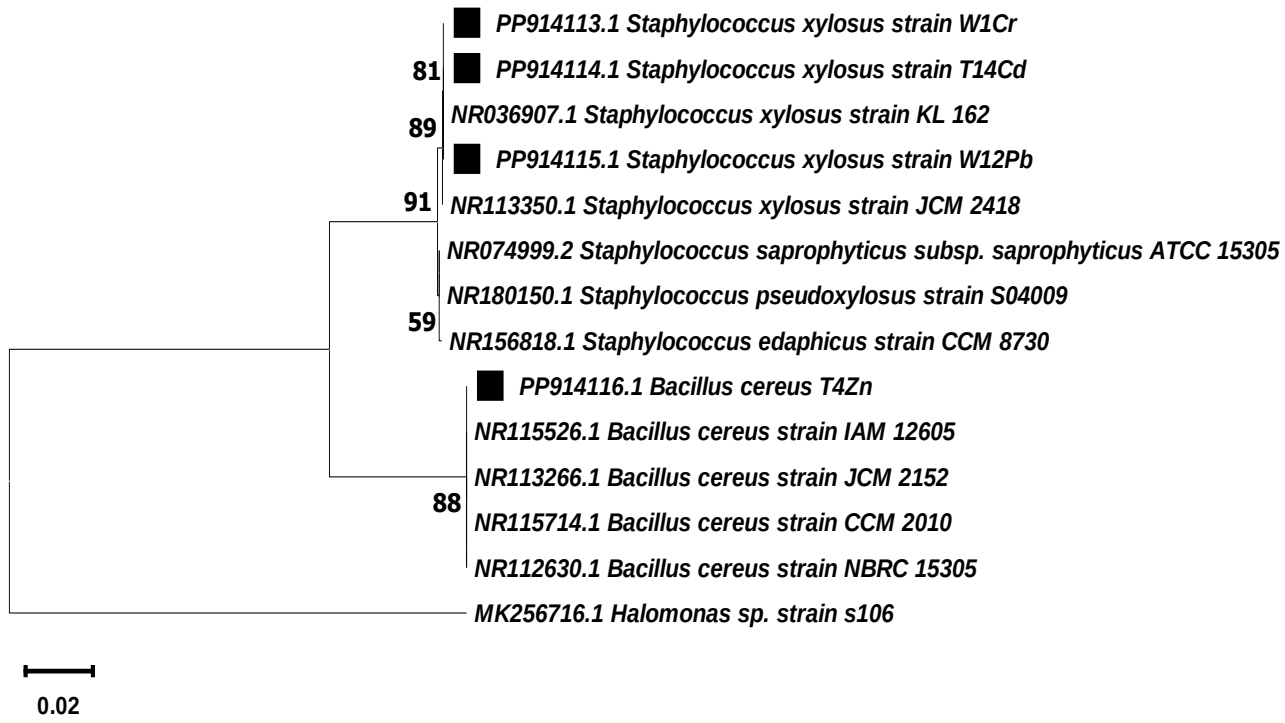


Figure 16: Phylogenetic relationships of heavy metal-resistant isolates with their relatives

4.6. Temperature and pH optimization of the isolates

The temperature optimization values of all the four bacterial isolates were presented in Fig. 17A. The highest growth for all four bacterial isolates were recorded at 30℃ which is the optimal temperature for these isolates. In temperature 25, 35, and 40℃ there was some growth on each bacterium, however at high temperature (50ºC) their growth was inhibited.

In addition, pH optimization values of all the four bacterial isolates were presented in Fig. 17B. The optimum pH that was recorded highest OD value of all four bacterial isolates were pH 8. All four bacterial isolates were grown at pH 6, 7, 9, and 10. However, at pH 5, their growth was very slow, which indicates the acidic pH retarded their growth.

From the bar graph *S. xylosus strain W1Cr* and *W12Pb* isolates for temperature and *S. xylosus strain WlCr* and *B. cereus strain T4Zn* isolates for pH were comparatively fast growing than other isolates.

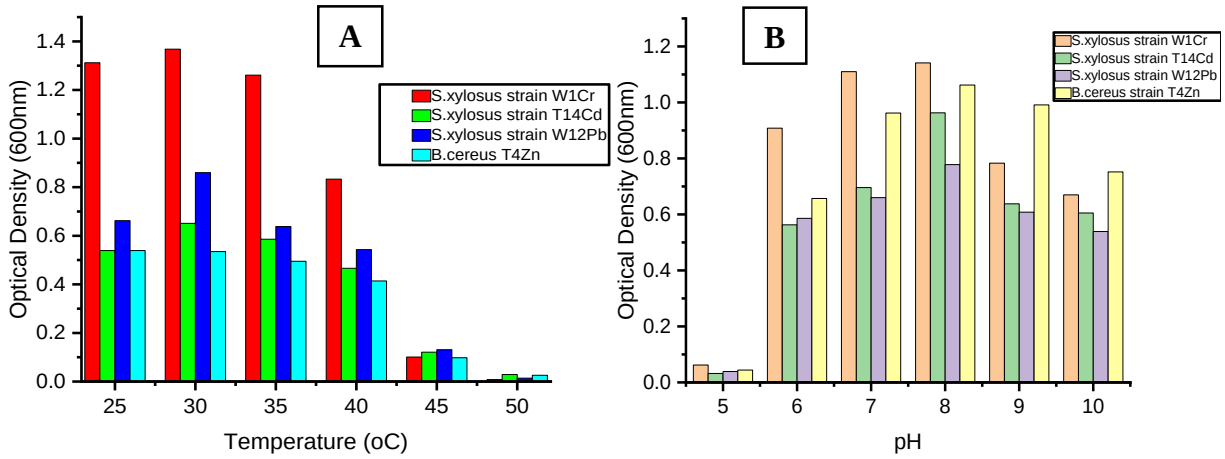
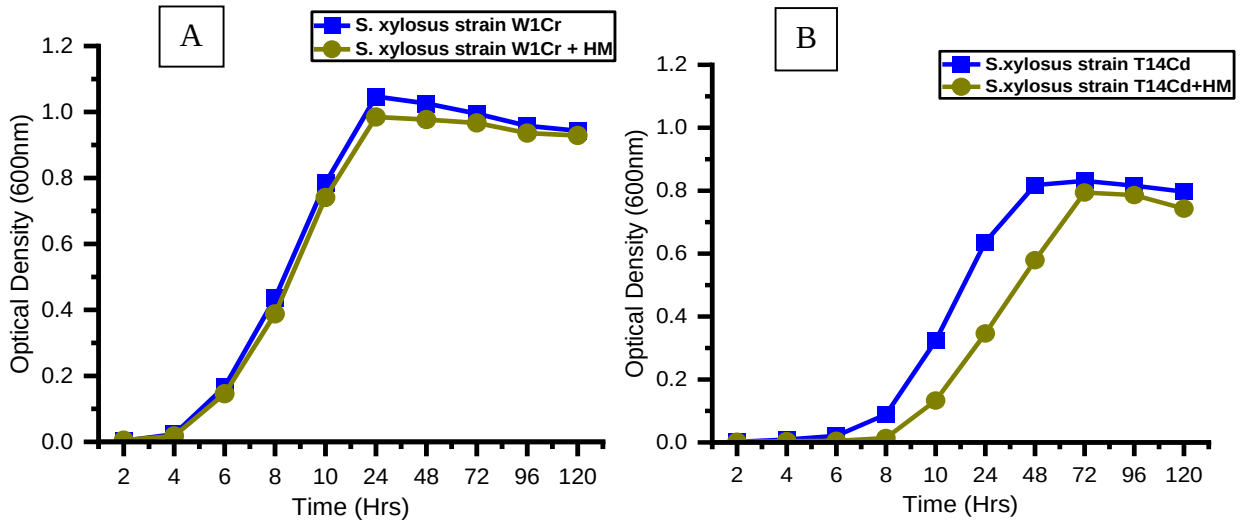


Figure 17: Temperature and pH optimization for four bacterial isolates within 24 hr.

4.7. Effect of heavy metals on the growth of the bacterial isolates

Growth pattern was conducted at 50 ppm for Cr and Cd and at 100 ppm for Pb and Zn metals. Hence, the effects of heavy metal concentrations on the growth of the bacterial isolates were presented in Fig. 18 (A-D) at 2, 4, 6, 8, 10, 24, 48, 72 and 96 hrs. The isolates *S. xylosus strain W1Cr* and *S. xylosus strain W12Pb* had highest OD value at 24 hrs. for both the control (without metal supplemented NA media) and Cr and Pb metal supplemented NA media, respectively. Also, isolate *B. cereus strain T4Zn* had highest OD value at 48 hrs. for Zn metals and the control, whereas for Cd metals and its control it was at 72 hrs. for isolate *S. xylosus strain T14Cd*. Therefore, even though, the metal supplemented media and the control had showed similar growth pattern, there was different absorbance effects on the growth of these respective bacterial strains.



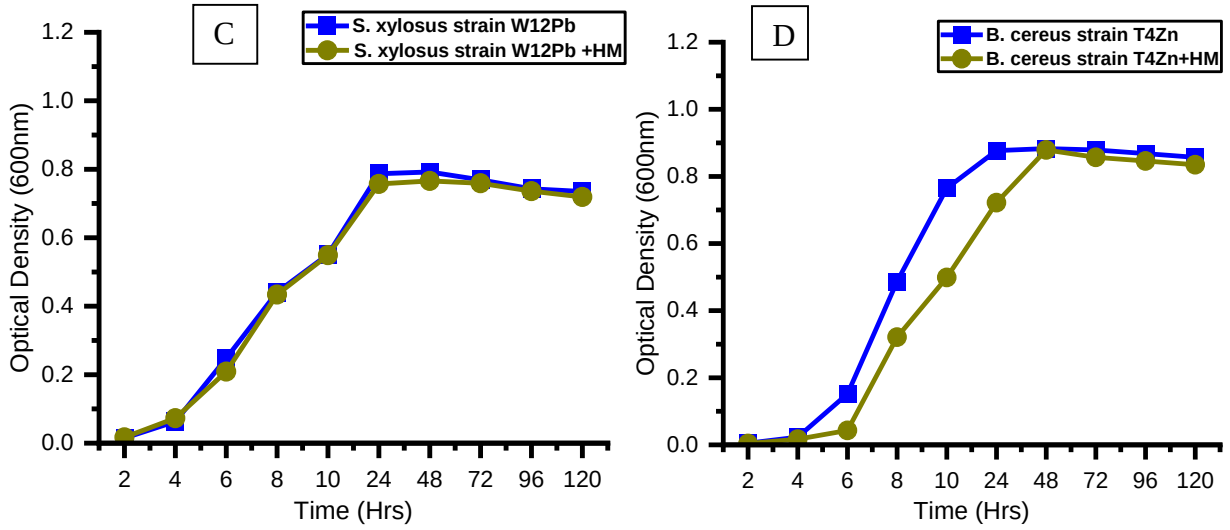


Figure 18: Effect of heavy metal on growth curve of bacterial isolates: A) *S. xylosus strin W1Cr* = isolate on NA and *S. xylosus strin W1Cr* +HM= 50ppm Cr with isolate. B) *S. xylosus* strain T14Cd = isolate only and *S. xylosus* strain T14Cd) +HM= 50ppm Cd with isolate. C) *S. xylosus strain W12Pb* = isolate only and *S. xylosus strain W12Pb* +HM= 100ppm Pb with isolate D) *B. cereus strain T4Zn* = isolate only and *B. cereus strain T4Zn* +HM= 100ppm Zn with isolate)

4.8. Minimum inhibitory concentration

The MIC was done for *S. xylosus strain W1Cr*, *S. xylosus strain T14Cd*, *S. xylosus strain W12Pb*, and *B. cereus strain T4Zn* isolates by regularly increasing the concentration of Cr, Cd, Pb, and Zn, respectively, on NA plates stepwise until the isolate failed to show growth. The MIC that inhibited the growth of bacteria against Cr, Cd, Pb, and Zn was found to be 3500, 4000, 5000 and 7000 ppm for *S. xylosus strain W1Cr*, *S. xylosus strain T14Cd*, *S. xylosus starin W12Pb*, and *B. cereus strain T4Zn* isolates, respectively, presented in Appendix IV. Accordingly, there was a decline in the growth of these four isolates with increased concentration of the respective heavy metals.

4.9. Profiles of antibiotic sensitivity and resistance

The antibiotic sensitivity-resistance was tested for twelve antibiotics and the zone of inhibition in diameter (mm) measurement value is given in Table 7 and the measured value was taken from Appendix V. All bacterial isolates were resistant for Ampicillin and penicillin antibiotics, wheras they were sensitive to tetracycline, streptomycin, chloramphenicol, ciprofloxacin, and erythromycin antibiotics.

Additionally, all isolates were sensitive to gentamycin, neomycin, and norfloxacin antibiotics except isolate *S. xylosus strain T14Cd* which was intermediate. All isolates were intermediate for oxytetracycline antibiotics. Moreover, *S. xylosus strain W1Cr* and *S. xylosus strain W12Pb* isolates were resistant to nalidixic acid antibiotics, while *S. xylosus strain T14Cd*, and *B. cereus strain T4Zn* were sensitive. The classification was based on CLSI (Clinical and Laboratory Standards Institute) standards (CLSI, 2014). Therefore, antibiotic susceptibility for the tested isolates showed different sensitivity and resistivity against the various antibiotics.

Table 7: Antibiotic sensitivity performance of heavy metal tolerant isolates

S.No	Isolates	Types of antibiotics (µg)											
		AMP (10)	P (10)	TE (30)	S (25)	NA (30)	C (30)	CIP (5)	E (15)	GEN (10)	NE (10)	NOR (10)	OT (30)
1	*S. xylosus strain W1Cr*	10	26	28	21	7	29	27	26	24	19	23	23
2	*S. xylosus strain T14Cd*	NZ	NZ	27	20	36	33	37	23	14	15	14	23
3	*S. xylosus strain W12Pb*	11	27	32	26	NZ	31	26	30	26	23	18	27
4	*B. cereus strain T4Zn*	NZ	9	23	24	25	23	27	33	18	16	27	25
	R	≤13	≤28	≤11	≤11	≤13	≤12	≤15	≤13	≤12	≤13	≤12	≤17
	I	14-16	-	12-14	12-14	14-18	13-17	16-20	14-22	13-14	14-15	13-16	18-25
	S	≥17	≥29	≥15	≥15	≥19	≥18	≥21	≥23	≥15	≥16	≥17	≥26

Note: AMP: *Ampicillin*; P: *Penicillin*; TE: *Tetracycline*; S: *Streptomycin*; NA: *Nalidixic acid*; C: *Chloramplicol*; CIP: *Ciprofloxacin*; GEN: *Gentamycin*; E: *Erythromycin*; NE: *Neomycin*; NOR: *Norfloxacin*, and OT: *Oxyteracycline*. NZ= *No Zone of inhibition.*

4.10. Tolerance tendency of bacteria isolates to heavy metals

The effects of heavy metals on microbial growth at various concentrations (100-1000 ppm) were tested, and the results are presented in Fig. 19 (A-D). Hence, even though they had different resistance capacities, all four bacterial isolates tolerated Cr^{6+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} metals up to 1000 ppm. The resistance order of these bacterial isolates was different for each of the heavy metals. The growth of all the bacterial isolates gradually declined as the concentration of heavy metals increased. Hence, the lower the concentrations of heavy metals, the lesser the effect, and the higher one had the most notable effect on the bacterial growth.

As shown in Fig. 19A, the resistance order for Cr metal was *S. xylosus strain W1Cr>S. xylosus strain T14Cd>B. cereus strain T4Zn>S. xylosus strain W12Pb*. Accordingly, this order, Cr had maximum tolerance by the *S. xylosus strain W1Cr* isolate, while it had minimum tolerance by the isolate *S. xylosus strain W12Pb*. Also, the resistance order of Cd metal was *S. xylosus strain T14Cd>S. xylosus strain W12Pb>S. xylous strain W1Cr>B. cereus strain T4Zn*, as presented in Fig. 19B. Accordingly, the metal Cd was highly tolerable by *S. xylosus strain T14Cd*, whereas it had a minimum tolerance by *B. cereus strain T4Zn*. In addition, *S. xylosus strain W12Pb>S. xylosus starin T14Cd>S. xylosus strain W1Cr>B. cereus strain T4Zn* for Cr metals, as shown in Fig. 19C. Hence, lead was highly tolerable by *S. xylosus strain W12Pb* while least tolerable by *B. cereus strain T4Zn*. Moreover, *S. xylosus strain W1Cr>S. xylosus strain T14Cd>B. cereus strain T4Zn>S. xylosus strain W12Pb* for Zn metals, as shown in Fig. 19D. Hence, Zn was highly tolerable by *S. xylosus strain W1Cr* and least tolerable by *S. xylosus strain W12Pb*.

According to the result shown in Fig. 19 (A-D), except for the Zn-supplemented NAM, the rest of the three bacterial isolates had a high tolerance tendency for their respective supplemented heavy metals.

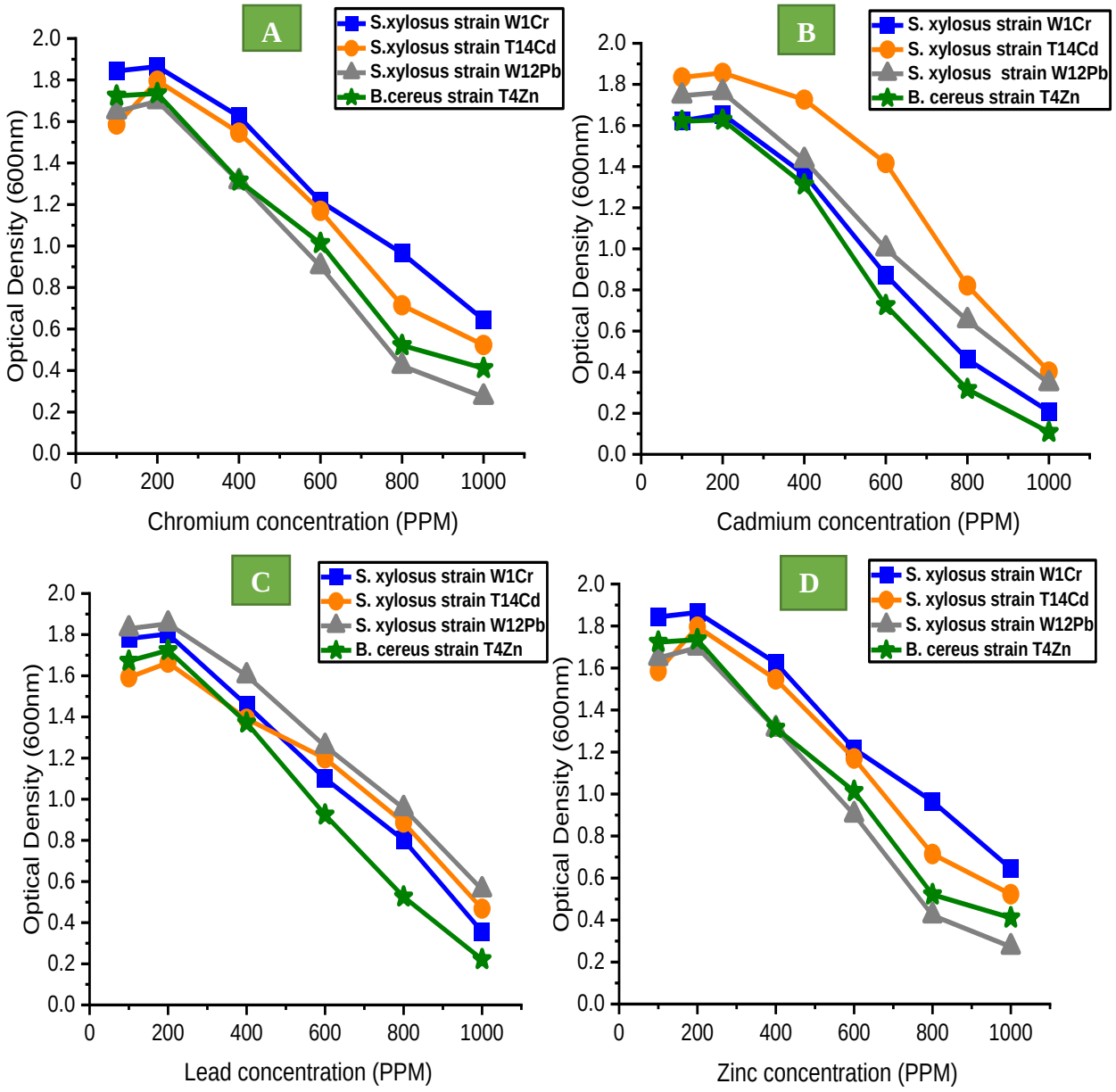


Figure 19: Heavy metal tolerant trend of bacterial isolates at different concentrations of chromium (A), cadmium (B), lead (C), and zinc (D)

4.11. Determination of multi-metal resistance of bacterial isolates

The multi-metal tolerance abilities of the four bacterial isolates and their mixed culture (MC) were indicated in Fig. 20. The four bacterial isolates were assessed for their resistance to a mixture of heavy metals (Cr^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}) at concentrations ranging from 50-800 ppm in a 3:3:1:1 ratio. As a result, the MC and a single isolate were able to tolerate up to 800 ppm concentrations of these heavy metals. Here, the resistance order of the four isolates and the MC were: MC>*S.*

xylosus strain T14Cd>S. xylosus strain W1Cr>S. xylosus strain W12Pb>B. cereus strain T4Zn. As a result, mixed culture demonstrated the highest level of tolerated resistance for a mixture of heavy metal concentrations. In the single-isolate test, *S. xylosus strain T14Cd* isolate was highly tolerable to a mixture of heavy metals, while *B. cereus strain T4Zn* had the least. The microbial turbidity decreased with an increase in the concentration of heavy metals (50-800 ppm).

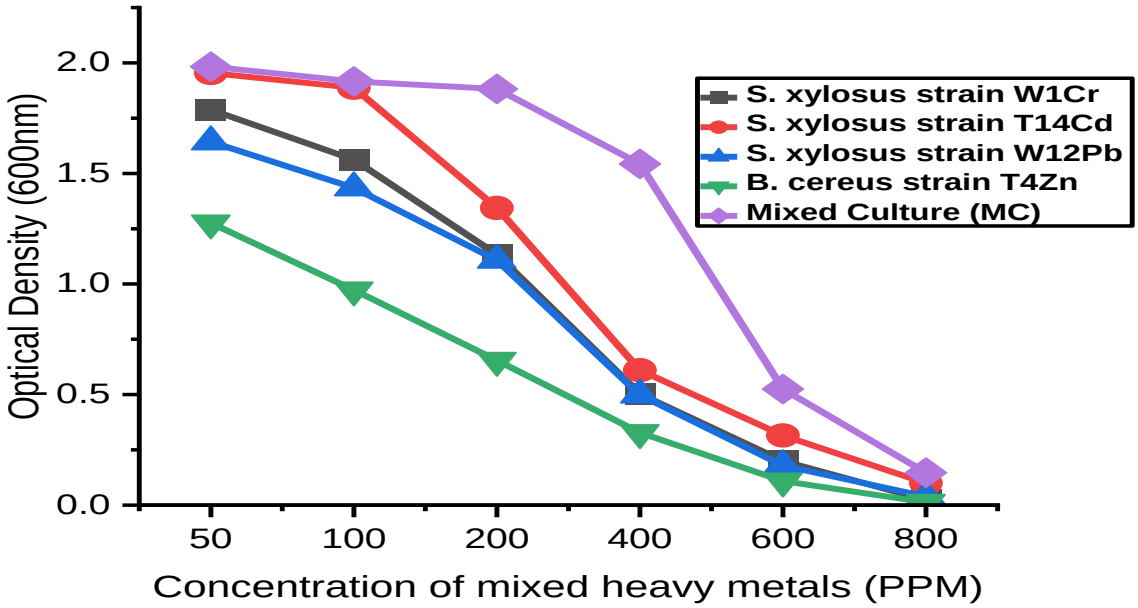


Figure 20: Multi-heavy metal tolerance of the bacterial isolates

4.12. Evaluation of the potential isolates for heavy metal removal

4.12.1. Evaluation in nutrient agar medium supplemented with heavy metals

The four potential bacterial isolates and their mixed culture consortia (MCC) removal efficiency were done in the NAM supplemented with the four heavy metals, and the results are presented in Table 8. Except for the *S. xylosus strain W1Cr* isolates, which maximally removed 69.91% of Cr^{6+} at 6 days of incubation, all the three isolates (*S. xylosus strain T14Cd*, *S. xylosus strain W12Pb*, and *B. cereus strain T4Zn*) maximally removed more than 80% of Cr^{6+} at 8 days of incubation in NAM supplemented with the respective heavy metals. Additionally, the MCC had more than 88% removal efficiency for Cd^{2+} and Zn^{2+} at 8 days of incubation, while it was at 6 days of incubation for Pb^{2+} metal. However, after 8 days of incubation, the removal efficiency of MCC for Cr^{6+} metal was 79.36%. Compared to single isolates the MCC showed a significant removal efficiency for all tested heavy metals.

4.12.2. Evaluation of potential bacterial isolates in the actual wastewater

The four potential bacterial isolates and their MCC removal efficiency of chromium metal in ATE were done, and the results are presented in Table 9 at days 2, 4, 6, and 8 of the incubation periods. The color of wastewater sample before and after treatment was clearly shown in Appendix VIA and Appendix VIB, respectively. Before treatment the sample color was dark while white and turbid after treatment. The bacterial isolates *S. xylosus strain W1Cr* and *S. xylosus strain W12Pb* were removed more than 79% of Cr^{6+} metal from the ATE at day 6 of incubation. The bacterial isolates *S. xylosus strain T14Cd* and *B. cereus strain T4Zn* were removed more than 76% of Cr^{6+} metal from the ATE at 8 days of incubation. The maximum removal efficiency of MCC was 87.98% at 8 days of incubation. Accordingly, the MCC showed a significant removal efficiency for Cr^{6+} heavy metal when compared to the single isolate.

Table 8: Removal efficiency of each isolates in the nutrient agar medium supplemented with heavy metals

Days	**Removal efficiency (%) of heavy metal by different isolates**							
	S. xylosus strain W1Cr	*S. xylosus strain T14Cd*	*S. xylosus strain W12Pb*	*B. cereus strain T4Zn*	MCC			
	Cr	Cd	Pb	Zn	Cr	Cd	Pb	Zn
2	20.30	38.16	56.81	37.82	24.62	64.78	65.92	32.81
4	37.00	49.71	76.23	47.77	40.14	75.81	83.66	50.75
6	**69.91**	74.95	83.29	70.25	54.75	86.54	**93.44**	78.54
8	59.36	**82.19**	**84.24**	**80.94**	**79.36**	**88.66**	91.96	**90.22**

Table 9: Chromium removal efficiency of isolates from Awash tannery effluent

N.O.	**Isolates Name**	**Chromium Removal efficiency (%) at different time (days)**			
		2	4	6	8
1	*S. xylosus strain W1Cr*	39.32	56.93	**83.00**	79.08
2	*S. xylosus strain T14Cd*	40.83	53.45	82.24	**84.88**
3	*S. xylosus strain W12Pb*	38.88	55.63	**79.60**	76.96
4	*B. cereus strainT4Zn*	35.55	49.73	73.58	**76.05**
5	MCC	37.83	63.98	82.22	**87.98**

The study also evaluated the four bacterial isolates and their MCC removal efficiency of chromium, cadmium, lead, and zinc heavy metals in the actual wastewater of selected tributaries of AARW sample. The results are presented in Table 10 at days 2, 4, 6, and 8 of the incubation periods. The wastewater sample color changes before and after treatment are shown in Appendices VIIA and VIIB, respectively. Accordingly, the wastewater color before treatment was dark, whereas after treatment it changed to a yellowish color and created high turbidity, which indicates the presence of microbial growth in the medium. The maximum removal efficiency of isolates *S. xylosus strain W1Cr*, *S. xylosus strain T14Cd*, and *S. xylosus strain W12Pb* was more than 88% of Cr^{6+} metal in 6 days of incubation, whereas isolate *B. cereus strain T4Zn* removed 80.48% in 8 days of incubation. Compared to the single isolate, the MCC had a significant removal efficiency (90.29%) for Cr^{6+} heavy metals at 8 days of incubation. In addition, the four bacterial isolates maximum removal efficiency for Cd^{2+} heavy metal was more than 98% after 8 days of incubation, whereas the MCC had somewhat more than the single isolate, which was 99.97% of Cd^{2+} metal at the same incubation time.

Moreover, the maximum removal efficiency of isolates *S. xylosus strain W1Cr*, *S. xylosus strain T14Cd*, and *S. xylosus strain W12Pb* was more than 97% of Pb^{2+} heavy metal in 6 days of incubation, whereas only 83.28% of this metal was removed by isolate *B. cereus strain T4Zn* at the same incubation time. The maximum removal efficiency of MCC was 99.37% of Pb^{2+} metal in the same incubation period, which was somewhat more than the single isolates of *S. xylosus strain W1Cr*, *S. xylosus strain T14Cd*, and *S. xylosus strain W12Pb*. It had a significant difference compared with *B. cereus strain T4Zn* isolates. Furthermore, the maximum removal efficiency of isolates *S. xylosus strain T14Cd*, *S. xylosus strain W12Pb*, and *B. cereus strain T4Zn* was more than 97% of Zn^{2+} metals in 8 days of incubation. However, 99.46% of Zn^{2+} metal was removed by the isolate *S. xylosus strain W1Cr* at 6 days of incubation. The maximum removal efficiency of MCC was almost 100% at 8 days of incubation, which is somewhat different compared to the single isolate removal efficiency.

Table 10: Heavy metal removal efficiency of isolates in the actual wastewater of selected tributaries of Addis Ababa river

Isolate type	**Removal efficiency (%) of different heavy metals in different time (days)**															
	Cr				**Cd**				**Pb**				**Zn**			
	2	4	6	8	2	4	6	8	2	4	6	8	2	4	6	8
S. xylosus strain W1Cr	60.17	74.79	**89.75**	87.41	81.18	94.29	**98.33**	96.91	68.25	94.14	**97.57**	97.56	71.88	92.58	**99.46**	99.43
S. xylosus strain T14Cd	42.38	55.55	**89.76**	88.43	80.59	94.14	**99.89**	99.22	60.59	64.14	**99.08**	99.65	75.01	90.88	98.02	**99.12**
S. xylosus strain W12Pb	46.47	59.37	**88.70**	88.45	88.98	92.40	**98.93**	98.50	62.17	72.73	**97.77**	96.57	67.10	92.41	97.70	**99.03**
B. cereus strainT4Zn	34.49	46.76	78.50	**80.48**	77.45	90.95	**98.35**	97.53	57.55	64.52	**83.28**	79.93	67.47	86.93	95.73	**97.25**
MCC	38.36	50.82	85.07	**90.29**	75.59	90.75	**99.97**	98.31	71.80	80.28	**99.37**	99.35	68.11	92.41	97.38	**99.99**

5. Discussion

The two physical water quality parameters pH and temperature can influence chemical and biochemical reactions, metabolic activities, rate of microbial degradation, and the amount of dissolved oxygen in water. In this study, both pH and temperature values were found within the permissible limits of EEPA, ESA, and WHO. A comparable findings were reported for Bahir Dar tannery, Ethiopia, for pH (7.15 ± 0.09) and temperature (25.5 ± 2.2℃) (Assefa Wosnie and Ayalew Wondie, 2014). Similar findings were also reported for pH from 6.08 to 8.45 in Modjo River (Temesgen Eliku, 2018). Our study is also comparable to those of the Nile River in Egypt, which is reported to be 7.7-9.0 pH and 20.5-26.8℃, respectively (Abdel-Satar *et al*., 2017).

The COD and BOD are the two important water quality indicators. COD measures the amount of oxygen consumed when the water sample is chemically oxidized, while BOD represents the amount of dissolved oxygen required for microorganisms to degrade organic matter in water. The two parameters in our study exceeded the standard guidelines due to the multiple introductions of organic and inorganic contaminants into Addis Ababa city's surface water. The commonly identified sources of pollutants are, the Addis Ababa city abattoir on Kera site (Zemene Worku and Seyoum Leta, 2017), the Alcohol factory at Mekanisa site, tanning industries at the Hana-Mariam sites (Tesfaye Admassu, 2020), and different domestic wastes, sewage lines connected to the river, etc. at the kebena river sites. Compared to other studies, the average concentrations of COD and BOD in AARW samples were higher than the reported values for LAR (40.33 ± 5.13 to 425 ± 8.0 and 12.34 ± 10.11 to 188 ± 7.07) mg/L), respectively (Deshu Mamo *et al*., 2021). However, our results were lower than the finding for LAR (COD: 260-1373.33) and (BOD: 95.66-555.33) mg/L in Ethiopia (Abrha Mulu *et al*., 2013) and the reported values of Hafede Tannery (BOD:1024) and (COD: 2517.33) mg/L (Fekede Terefe *et al*, 2020). The concentration differences between our study and others might be due to the sources of wastewater contamination that were introduced and the seasonal variation of sampling.

The TDS, TSS, and EC, are important parameters for water quality. Excessive concentrations of these factors may have an impact on aquatic life, light transmission, and irrigation appropriateness. In our findings, these parameters exceeded all standard guidelines listed in Table 3 for the Awash tannery sampling sites. Thus, the high concentration might be due to the sample taken from both treated and untreated sites and the insufficient treatment system. Also, EC concentration was

surpassed the WHO, TDS surpassed EU and WHO, and TSS concentrations exceeded all the guidelines in the AARW sample. Possible reasons might include wastewater from domestic and sewage systems that is released into the river water system, as well as dissolved and suspended solids from Addis Ababa city's abattoir wastes.

Compared to other findings, the concentration of EC in Awash tannery was higher than the reported values of Batu (8890 ± 15.55), Awash (8750 ± 5.97), and Abyssinia (5680 ± 5.11) µS/cm (Bitew Kassaw *et al*., 2022) tanneries, whereas lower than the report by Tesfaye Admassu (2020) that was EC (31.5 ± 0.2 mS/cm) in Batu tanneries. Also, the TSS concentrations were greater than those reported by Belete Abera (2018) and Fekede Terefe (2020) from Kombolcha (1000 mg/L) and Hafede tanneries (135 mg/L), respectively. The concentration of EC and TDS values obtained from AAR in our study was higher than the reported values from the Elgo River in southern Ethiopia (Chan Kujiek and Sahile, 2024) (EC: 182-268 µS/cm and TDS: 192.5-275.5 mg/L), whereas it was lower than the TSS value (680-2774) mg/L. The reason for the difference in concentrations of TDS, TSS, and EC between our study and others might be due to the difference in dissolved solids (minerals, salts, metals,) and inorganic salts (calcium, magnesium, potassium, sodium, sulfates, etc.) that are introduced into water sourced from urban and agricultural runoff.

The presence of excess concentrations of nutrients such as ammonia, ammonium, sulfate, sulfide, and phosphate can affect surface water quality in many ways, including eutrophication, algal blooms, and decreasing dissolved oxygen in water that may affect aquatic life (Deshu Mamo *et al*., 2021). At the AAR sampling site, most of these nutrient parameters exceed almost all the guidelines. The cause may have been inadequately treated wastewater from textile processing factories, garage and hospital wastes, detergents and soaps, pulp factories, and municipal wastes (Hamere Yohannes and Eyasu Elias, 2017; Deshu Mamo *et al*., 2021).

Compared to other findings, Awash tannery was higher than the values reported by Bitew Kassawe *et al*. (2022) for NH_3-N (0.05 ± 0.009), NH_4^+-N (0.05 ± 0.001), SO_4^{2-}(185 ± 11.163), S^{2-}(0.09 ± 0.001), and PO_4^{3-} (1.26 ± 0.010) mg/L in a similar tannery site. However, it was lower than the value of NH_3-N (49.79 mg/L) report found from Hafede tannery (Fekede Terefe *et al*., 2020). Additionally, the concentration of $SO4^{2-}$ in AAR sampling site result was higher than the reported values of the LAR average concentration of $SO4^{2-}$ (54.62 mg/L) (Deshu Mamo *et al*., 2021) and Elgo River $SO4^{2-}$ (17.18–47) mg/L (Chan Kujiek and Sahile, 2024). However, it was lower than

the reported values of Kera slaughterhouses discharged effluent $SO4^{2-}$ (693.333 mg/L) (Abrha Mulu *et al*., 2013) in Addis Ababa, Ethiopia. The difference in concentrations of these nutrients between our study and others might be due to the different nitrogen, phosphate, and sulfate input sources in the surface water.

In our study, the concentration of Cr (VI) exceeded all the guidelines, which is due to insufficient treatment methods and sampling site for this metal in the Awash tannery. In agreement with our finding, Tesfaye Admassu (2020) reported 12.0 ± 2.7 mg/L total Cr from Batu tannery, Addis Ababa, Ethiopia. On the other hand, except for zinc, the concentrations of all four heavy metals in the AARW sample mix exceeded all the guidelines except EEPA guidelines. Our findings were similar to those of Deshu Mamo *et al*. (2021), who found that an average concentrations of Cr (0.012 ± 0.007 to 0.203 ± 0.199), Cd (<0.014 ± 0.0007 to 0.02 ± 0.001), Pb (0.031 ± 0.008 to 0.124 ± 0.034), and Zn (0.048 ± 0.037 to 0.318 ± 0.158) mg/L in different 11 sites of LAR. In the same way, the maximum concentration of Cr (0.031), Cd (0.024), Pb (0.71), and Zn (0.086) in ppm was reported from Tanjaro River, Iraq (Salih *et al*., 2021). The reason might be that most tannery industries, such as Batu, Awash, Abyssinia, and New Wing tanneries, released their waste into the Hana-Maryiam River sampling site without sufficient treatment. In addition, different sources of heavy metals, such as pigments, stabilizers, alloys, detergents, vehicle batteries, battery maintenance, cable covers, garage wastewaters, fuel stations, and agrochemicals, are directly discharged without any treatment along the river sites.

In our findings, the maximum cultivable bacterial isolates from Awash tannery samples were 1.6 x 10^4 and 1.8 x 10^6 CFU/ml for Cd and Zn metal NA-supplemented medium at 10^2 and 10^4 dilution factors, respectively. Accordingly, the CFU value for Cd was lower than that for Zn; this might be due to the toxicity of Cd metal is higher compared to Zn in the NA-supplemented medium. Also, the maximum cultivable bacterial isolates of the AAR water sample were 8.4 x 10^4 and 1.1 x 10^7 CFU/ml for Cr and Pb metal NA-supplemented medium at 10^1 and 10^3 dilution factors, respectively. Hence, the CFU value for Cr was lower than that for Pb; this might be due to the higher level of toxicity of Cr metal than Pb in the NA-supplemented medium. Kalaimurugan *et al.* (2020) reported that bacterial colony count was 1.75 x 10^4 CFU/ml from a 10^{-1} dilution of chromium-contaminated soils; Sanjay *et al.* (2020) reported 1.68 x 10^{-4} CFU/ml from a 10^{-1} dilution from tannery effluent sample; and John and Rajan (2022) reported a bacterial colony count

of 10^{-2} dilutions (3.0 x 10^3 to 4.8 x 10^5 CFU/ml) from tannery effluent influenced-environment. In addition, Harun *et al.* (2023) reported a range of 338-501 x 10^3 CFU from 4 sites in the gold mining areas of Anka, Zamfara, Nigeria. Moreover, Batool *et al.* (2021) in Pakistan reported 2 x 10^{-6} for Korangi sludge and 3 x 10^{-5} Korangi water used for enumeration of bacterial population in industrial waste.

In our study, after preliminary isolation, further identification was conducted using the broth dilution method. The reason is that when the supplemental heavy metal concentration was higher, the NA medium did not fully solidify, which prevented it from streaking the isolate since the medium was just broken. This might be due to the formation of precipitation when a high concentration of heavy metal is supplemented with NA medium. Similarly, Pandit *et al.* (2013) reported that six potential isolates out of 40 were screened from five different sites in Amravati Khadi, (Gujarat, India).

In our investigation, almost all isolates had close relationships (in heavy metal removal efficiency and originated from the same genus or species) to the bacterial strains that were isolated from different tanneries, textiles, and river wastewaters and they also demonstrated >99% 16S rRNA gene sequence similarity. The three isolates in this study, affiliated with members of the genus *staphylococcus*, that were reported from Mandovi estuary, Goa, India (Pereira and Ramaiah, 2019) when they were exposed to chromate (Cr^{6+}), and the tellurite-resistant bacteria reported by Soleimani Sasani *et al*. (2020) were isolated from wastewater, sediments, and residues of Iran. In addition, the strain *Staphylococcus xylosus 222W*, which was isolated from wastewater, was reported by Al-gheethi *et al.* (2017) for the removal of Ni in an aqueous solution. On the other hand, isolate *B. cereus strain T4Zn* was related to the bacterium strain TWSL-5; it was identified as *Bacillus cereus* with the highest metal resistance that was isolated from a textile dyeing factory in Faridabad, Haryana, India (Kumari *et al*., 2021).

To determine the large-scale biomass production for further bioremediation application of these bacterial isolates, optimum growth conditions (temperature, pH, and growth pattern) were conducted. Both high and low temperatures affect bacterial growth because the former can damage structural parts of the plasma membrane with denatured or inhibited enzymes, while the latter slows the pace of metabolism with inactivating enzymes (Ndeddy Aka and Babalola, 2017). In our study, the maximum growth temperature was observed at 30ºC for all four bacterial isolates. In

agreement with our study, Batool *et al*. (2021) reported on Cr-resistant strains S35 and S48, in which the optimum temperature for both strains was 30°C.

The other important parameter is pH, in which the chemistry of the metals in solution, the activity of functional groups on the cell wall, and the competition of metallic ions for the binding site are all impacted by both low and high pH. In our study, all four heavy metal tolerant bacterial isolates showed maximum growth at pH 8. A similar result was found for the Cr^{6+} resistance strain *B. cereus* b-525k, in which the optimum cultivation was observed at pH 8 when incubated at 37°C (Elahi *et al*., 2022).

In our study, the effect of heavy metals on the growth of bacterial isolates were determined. The four isolates were little slower in the presence of those metal than that of control, such type of results also have been reported in the study (Pandit *et al*., 2013). However, the resulted growth pattern was not significantly different from the control growth, this might be due to the low concentration of heavy metals that the isolate treated. Similar result was obtained in the presence of $K_2Cr_2O_7$ for both S35 and S48 strains compared with the control culture (Batool *et al*., 2021).

In our study, the strain *S. xylosus strain W1Cr* failed to grow at 3500 ppm, and it was in agreement with the bacterial strain *B. cereus strain VITSCCr02*, which was found to resist up to 3300 mg/L of Cr (VI) (Sundar *et al*., 2010). However, it was much higher than the study for chromium, which examined it at a range of 100-1600 ppm, and two strains (*B. paranthracis* and *B. paramycoides*) showed growth at 1600 ppm (Batool *et al*., 2021). Our study had a higher MIC than others; this might be due to the characteristics of the isolate (affinity towards heavy metals, its cell wall functional group, and enzyme production during stress) (Al-Gheethi *et al*., 2017).

In our findings, the strain *S. xylosus strain T14Cd* failed to grow at 4000 ppm, and so it was in harmony with *Enterobacter cloacae* bacterial strains, which withstand up to 4000 µg/mL Cd^{2+} (Ghosh *et al*., 2022). However, it was higher than the report by El-Meihy (2018), who found that the MIC of cadmium against *Alcaligenes faecalis* MG257493.1 was 2500 mg/L. Moreover, the results of our investigation were substantially higher than the study's reported MIC of 1100 µg/mL against cadmium (Marzan *et al*., 2017). The possibility that the wastewater sample source from which the isolate was brought had a high cadmium contamination level, explaining their inherent tolerance to this metal, could account for the higher MIC in our investigation compared to others.

In our study, strain *S. xylosus strain W12Pb* failed to grow at 5000 ppm, and it was higher than the reported value, with a MIC of 3500 mg/L against lead using the bacterial strain *Alcaligenes faecalis* MG257493.1 (El-Meihy, 2018). But this result was significantly higher than in another strain of *B. cereus*, BUK-BCH-BTE2, reported with an MIC of 3000 mg/L tolerance of lead (Harun *et al*., 2023). Moreover, it was significantly greater than that reported for the Pb^{2+} bioprecipitation ability (MIC: 1000 mg/L) of *B. cereus strain TWSL5* (Kumari *et al*., 2021). Our study's greater MIC than others may have resulted from the isolate's natural resistance to lead, which was taken from a wastewater sample that was highly contaminated with the metal.

The strain *B. cereus strain T4Zn* failed to grow at 7000 ppm, and it was higher than the report by El-Meihy (2018), which indicated that the MIC of zinc against *A. faecalis* MG257493.1 was 4500 mg/l. In addition, it was much higher than the species, *Paenibacillus dendritiformis*, reported with an MIC of 1200 µg/mL (Shahid *et al*., 2024). Because of the high levels of heavy metal contamination at our sample collection sites, our isolates may be more resistant than those from previous research, which could explain why our results were greater than others.

In our study, even though all three species (*S. xylosus*) were non-pathogenic and susceptible to a wide range of various tested antibiotics, they were resistant to AMP and P. However, most of the non-pathogenic but metal-tolerant bacterial isolates were more susceptible to antibiotics. The reason for these isolates resistance to AMP and P could be related to the occurrence of genes responsible for heavy metal and antibiotic co-resistance (Tariq *et al*., 2019). Our results were in agreement with Sen and Joshi (2016), who reported that *P. aeruginosa HMT16* and *P. aeruginosa HMT7* isolates exhibited resistance to both of these antibiotics (AMP and P). A similar study was investigated for susceptibility to various antibiotics by Kumar *et al.* (2019) and confirmed *E.coli KL* isolate was susceptible to a wide range of antibiotics such as E, GEN, and C.

In our study, *S. xylosus strain W1Cr* has relatively highest resistance for Cr^{6+} and Zn^{2+}, whereas *S. xylosus strain W12Pb* is relatively sensitive for these metals. On the other hand, *S. xylosus strain T14Cd* and *S. xylosus strain W12Pb* have comparatively highest resistance for Cd^{2+} and Pb^{2+}, respectively, whereas *B. cereus strain T4Zn* is comparatively sensitive to these metals. Based on the result, at high concentrations of heavy metal, the growth of bacteria would be inhibited, which exhibit toxic effects on bacterial growth and leading to biomass reduction and a prolonged lag period to acclimatize to a higher stress condition (Zhenggang *et al*., 2019; Mishra *et al*., 2021).

Similar study for Pb, Cr, and Cd was reported by gradually increasing from 100 to1000 µg/L concentrations for *Hafnia*, *Gemalla*, and *Micrococcus* bacterial species, and the results showed the bacteria growth is concentration-dependent (Marzan *et al*., 2017). Olowomofe *et al*. (2020) reported similar heavy metal tolerance tendency of three isolates, *B. Subtilis*, *Serratia liquefaciens*, and *B. cereus*.

In our study, the multi-metal tolerance abilities of the four bacterial isolates and their mixed culture had different resistance abilities. Accordingly, the four isolates' varying levels of tolerance to heavy metals might be due to the resistance mechanisms, including variations in the metal's uptake and transport, the efflux of metal ions outside the cell, and the metals' enzymatic transformation into chemical species that may be more volatile or less toxic than their parent compounds (Olowomofe *et al*., 2020). In addition, their tolerance variation might be due to the presence of a large set of genes in plasmids as compared to others (Saini and Pant, 2016). A similar result was reported by Saini and Pant (2016), who found that the four isolates had varying levels of multi-metal tolerance in a mixture of metal solutions (Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+}) at a concentration of 1 mM. In addition, Muzammil *et al*. (2020) reported that the multi-metal resistance of *B. cereus* GCFSD01 to (Cd: Cr: Pb: Ni, 1:1:1:1) was up to 5.5 mM.

In our study, the maximum removal efficiency of isolate *S. xylosus strain W1Cr* was 69.91% of Cr^{6+} after 6 days of incubation, while *S. xylosus strain T14Cd* was 82.19% of Cd^{2+}, *S. xylosus strain W12Pb* was 84.24% of Pb^{2+}, and *B. cereus T4Zn* was 80.94% of Zn^{2+} after 8 days of incubation in the NAM supplemented with the respective heavy metals. Accordingly, the four isolates had various removal efficiency. This could be because the bacterial isolates developed various resistance mechanisms through exclusion, extrusion, formation of metallothienins or demethylation, and extracellular polymeric substances (EPS) (De *et al*., 2008; Igiri *et al*., 2018; Yuliani *et al*., 2019). The type or toxicity level of heavy metals might be the other reason for their difference. In harmony with our study, John and Rajan (2022) reported that the Cr (VI) removal efficiency of *S. pyogenes*, *P. putida*, and *B. thuringiensis* was 52.18, 51.89, and 50.07%, respectively. Moreover, according to Huang *et al*. (2018), the removal efficiencies of *B. cereus* reached up to 38.3% for Zn^{2+}, 81.4% for Cd^{2+}, and 40.3% for Pb^{2+} at a concentration of 10 mg/L.

The maximum removal efficiency of isolates *S. xylosus strain W1Cr* and *S. xylosus strain W12Pb* was 83 and 79.6% of Cr^{6+}, respectively, from the ATE at day 6 of incubation, while isolates *S. xylosus strain T14Cd* and *B. cereus strain T4Zn* removed 84.88 and 76.06% respectively, at 8 days of incubation. Bacterial strains have developed multiple mechanisms for managing heavy metals, including efflux pumps, redox reactions, complex formations, and extra- and intracellular sequestrations (Ahemad, 2012). This could account for the variation in removal efficiency among the four isolates. In harmony with our study, Elahi *et al*. (2019) reported that *M. testaceum* B-HS2 isolate removed 96% of Cr^{6+} from the real tannery effluent in a pilot-scale experiment after 6 days. Similarly, a bacterium species, *B. cereus*, could successfully remove 100% chromium from an effluent in 7 days of incubation (Kumari *et al*., 2021).

The maximum removal efficiencies of the four isolates were greater than 80, 98, 83, and 97% for chromium, cadmium, lead, and zinc metals, respectively, in the actual wastewater of selected tributaries of AARW sample. The removal percentage difference might be due to the amount and toxicity level of these heavy metals present in the river water sample and the tolerance mechanisms of the isolates (Yuliani *et al*., 2019). In agreement with this finding, Oaikhena *et al*. (2016) reported that the isolates *P. aeruginosa*, *S. aureus*, *E. coli*, *P. vulgaris*, and *K. pneumonia* removed 23.1, 11.6, 21.6, 20, and 15.4% of chromium, while 100, 36.1, 13.1, 65, and 69.6% of cadmium, 53.9, 46.6, 88, 89, and 20% of zinc, respectively. In addition, the highest cadmium removal efficiency of 77% was achieved by the *B. cereus* ATCC 9632 strain at a concentration of 15 mg/L (Trihadiningrum, 2014). Moreover, Gawali *et al*. (2014) found that 45% of lead could be removed by *E. coli* isolated from industrial wastewater. Furthermore, *P. putida* removed 96% of the lead from industrial wastewater mixed-liquor media, according to Kamika and Momba (2013).

The maximum removal efficiencies of MCC were 79.36, 88.66, and 90.22% for Cr, Cd, and Zn metals, respectively, at 8 days of incubation, while 93.44% for lead at 6 days of incubation in the NAM supplemented with the respective heavy metals. Also, the maximum removal efficiency of MCC was 87.98% of Cr^{6+} from ATE, while it was greater than 99% for all tested heavy metals, except chromium, for which 90.29% was removed in 8 days of incubation in the actual wastewater of selected tributaries of AARW sample.

In agreement with our study, chromium (33.14%), zinc (90.1%), and cadmium (100%) were removed by the MCC, comprising five isolates (Oaikhena *et al*., 2016). Additionally, according to Singh *et al*. (2012), the removal efficiency of Cd, Cr, Pb, and Zn with MCC was 85.23, 71.62, 44.75, and 87.66%, respectively. Moreover, a mixture of bacterial strains, *Viridibacillus arenosi* B-21, *Sporosarcina soli* B-22, *Enterobacter cloacae* KJ-46, and *E. cloacae* KJ-47, obtained from an abandoned mine site in Korea, showed a removal efficiency of 98.3 and 85.4% of Pb and Cd, respectively, at 48 hrs (Kang *et al*., 2016).

Accordingly, our study's results demonstrate that in the NAM supplemented with the respective heavy metals, ATE, and AARW samples, the MCC showed a significant removal efficiency when compared to the single isolate for almost all tested metals. The consortia of four bacterial isolates' synergistic metabolic activities may be responsible for mixed cultures' high multi-metal tolerance ability. This is because the variety of enzymatic types among the isolates may have improved the process compared to individual isolates with fewer enzyme types (Oaikhena, 2016).

Various scholars agree that it is better to use mixed cultures for the removal of heavy metals from highly contaminated sites (Camargo *et al*., 2003). The reason to use consortia of cultures for more contaminated or field applications is that bacteria are more metabolically superior, competitive, and are more likely to survive and be stable for removing metals (Joutey *et al*., 2015). The other reason might be due to higher bacterial cell density at high levels of heavy metals (Kang *et al*., 2016). In general, for heavy metal removal, bacterial consortiums are more suitable for field applications, with better metabolic capabilities (Kader *et al*., 2007).

6. Conclusion and Recommendation

6.1. Conclusions

In our findings, the majority of the physicochemical parameter results both in the ATE and selected tributaries of Addis Ababa river water sample were above the permissible discharge limit of EEPA, ESA, and APHA standard guidelines. This shows that, river water in Addis Ababa is highly contaminated by different pollutants that are generated from various industries, so additional treatment systems are required for those factories.

In the preliminary isolation, a total of 130 bacterial isolates were found in the ATE and selected tributaries of AARW sample sites. Among 130 isolates, four potential bacterial isolates used for heavy metal removal were identified by phylogenetic analysis inferred from comparative 16S rRNA gene sequences. Three of them belong to the genus *Staphylococcus*, and the rest one belong to the genus *Bacillus*. The optimal growth temperature and pH for these four heavy metal-resistant gram-positive bacterial isolates were 30°C and pH 8, respectively.

The MICs for each isolate, *S. xylosus strsin W1Cr*, *S. xylosus strain T14Cd*, *S. xylosus strain W12Pb*, and *B. cereus strain T4Zn*, were found to be able to tolerate up to 3500, 4000, 5000, and 7000 ppm concentrations for Cr, Cd, Pb, and Zn metals, respectively. It was found that while the majority of non-pathogenic heavy metal resistance isolates were sensitive to the other 12 antibiotics, all of them were resistant to AMP and P antibiotics. This indicates the presence of genes that are responsible for both heavy metal and antibiotic resistance. The four isolates could tolerate Cr^{6+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} metals up to 1000 ppm, although their tolerance to these heavy metals varied from one another.

Our findings, revealed that river and tannery effluent contain significant amounts of bacterial isolates resistant to heavy metals. Treatment of heavy metals both in the NAM supplemented with the respective heavy metals and actual wastewater by single and consortia isolates was conducted under a batch aerobic experiment system. Based on this, the four bacterial isolates showed various levels of removal efficiencies for Cr, Cd, Pb, and Zn metals. Generally, the mixed culture consortia had the highest heavy metal removal efficiency both in the NAM supplemented with the four heavy metals and actual wastewater (ATE and selected AARW) that were analyzed in this study.

6.2. Recommendations

- While the Awash tannery wastewater treatment plant's efficiency in removing prominent pollutants is promising. But, further work needs to be done to ensure that the final tannery effluent meets the effluent quality standards outlined in the EEPA, ESA, and other international standard guidelines.
- Our finding reveals the isolation, characterization (morphological and molecular), and evaluation of heavy metal-tolerant strains. However, constructing the recovery system needs to be done after the heavy metals have been treated.
- Since the result of this study relies on a laboratory scale using a flask, further study on the treatment of real tanneries and river water on a pilot or field scale should be done.
- Although the removal of heavy metals was achieved in an aerobic batch experimental system, the proposed resistance mechanism of the isolates during removal should be considered.
- Moreover, Fourier-transform infrared (FTIR) analysis that identifies the functional groups present on the bacterial cell surface has not been studied. So, future consideration should be given.
- Furthermore, intracellular uptake of the metal ions and the compound distribution before and after the removal of heavy metals from bacterial cells could be confirmed with scanning electron microscopy (SEM) and energy dispersive X-ray (EDX) analysis. Therefore, future consideration should be assumed.

7. References

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8. Appendices

Appendix I: Preparation of Heavy metal Stock solutions

The stock solutions of chromium, cadmium, lead and zinc heavy metals were prepared from analytical grade salts of potassium dichromate (VI), cadmium sulfate hydrate, lead (II) nitrate and zinc sulfate heptahydrate. Then the respective grams of the salts were weighed into 1000 mL Duran bottle mixed with distilled water to give stock solutions of 1000 ppm.

Preparation of chromium stock solution

It is found in the form of salt as $K_2Cr_2O_7$.

Molecular weight of the salt is 294.18 gm

Molecular weight of Cr_2 is 103.99 gm

Purity of the salt is 99.9%

Stock solution of chromium= (294.18/103.99) x 0.999= 2.83gm

Therefor to prepare 1000 ppm stock solutions of chromium dissolve 2.83 gm of the salt in 1 L distilled water.

Preparation of cadmium stock solution

It is found in the form of salt as $3CdSO_4.8H_2O$.

Molecular weight of the salt is 769.51 gm

Molecular weight of 3Cd is 337.23 gm

Purity of the salt is 99%

Stock solution of cadmium= (769.51/337.23) x 0.99= 2.26 gm

Therefor to prepare 1000 ppm stock solutions of cadmium dissolve 2.26 gm of the salt in 1 L distilled water.

Preparation of lead stock solution

It is found in the form of salt as $Pb(NO_3)_2$.

Molecular weight of the salt is 331.33 gm

Molecular weight of lead is 207.2 gm

Purity of the salt is 99%

Stock solution of lead= (331.33/207.2) x 0.99= 1.6 gm

Therefor to prepare 1000 ppm stock solutions of lead dissolve 1.6 gm of the salt in 1 L distilled water.

Preparation of zinc stock solution

It is found in the form of salt as $ZnSO_4.7H_2O$.

Molecular weight of the salt is 287.55 gm

Molecular weight of zinc is 65.38 gm

Purity of the salt is 99%

Stock solution of zinc= (287.55/65.38) x 0. 99= 4.35 gm

Therefor to prepare 1000 ppm stock solutions of zinc dissolve 4.35 gm of the salt in 1 L distilled water.

Smaller concentrations such as 500 ppm, 400 ppm, 300 ppm etc. was prepared from the stock solution using the general formula: (C1x V1 =C2 xV2) where

C1 = Initial concentration (Stock concentration)

V1 = Initial volume (Volume to be taken for stock)

C2= Final concentration (Required concentration)

V2 = Final volume (Required volume)

Appendix II: Preliminary isolation of bacterial isolates from the two sample sources using four heavy metals

Chromium bacterial isolates from Awash tannery wastewater samples												
Isolates name	**Concentration (PPM)**											
	50	100	200	300	400	500	600	700	800	900	1000	1200
T1Cr	√	√	×	_	_	_	_	_	_	_	_	_
T2Cr	√	√	√	√	√	×	_	_	_	_	_	_
T3Cr	√	√	√	×	_	_	_	_	_	_	_	_
T4Cr	√	√	√	√	√	√	√	×	_	_	_	_
T5Cr	√	√	√	√	√	√	√	√	√	√	√	√
T6Cr	√	√	√	√	√	×	_	_	_	_	_	_
T7Cr	√	√	√	√	√	×	_	_	_	_	_	_
T8Cr	√	√	√	×	_	_	_	_	_	_	_	_
T9Cr	√	√	√	√	√	×	_	_	_	_	_	_
T10Cr	√	√	√	√	×	_	_	_	_	_	_	_

T11Cr	√	√	√	√	√	√	×	_	_	_	_	_
T12Cr	√	√	√	√	√	×	_	_	_	_	_	_
T13Cr	√	√	√	√	√	√	×	_	_	_	_	_
T14Cr	√	√	√	√	×	_	_	_	_	_	_	_
T15Cr	√	√	√	√	√	√	√	√	√	√	√	√
T16Cr	√	√	×	_	_	_	_	_	_	_	_	_
T17Cr	√	√	√	√	√	√	√	√	√	√	√	√
T18Cr	√	√	√	√	√	×	_	_	_	_	_	_
T19Cr	√	√	√	√	√	√	√	×	_	_	_	_
T20Cr	√	√	√	√	√	√	×	_	_	_	_	_
T21Cr	√	√	√	√	√	√	√	√	√	√	√	√
T22Cr	√	√	√	√	√	×	_	_	_	_	_	_
T23Cr	√	√	√	√	√	√	√	√	√	√	√	√
T24Cr	√	√	√	√	√	√	√	×	_	_	_	_
T25Cr	√	√	√	√	√	×	_	_	_	_	_	_
Chromium bacterial isolates from AAR tributaries wastewater samples												
W1Cr	√	√	√	√	√	√	√	√	√	√	√	√
W2Cr	√	√	√	√	√	√	√	√	√	√	√	×
W3Cr	√	√	√	√	√	×	_	_	_	_	_	_
W4Cr	√	√	√	√	√	√	√	√	√	√	√	√
W5Cr	√	√	√	√	√	×	_	_	_	_	_	_
W6Cr	√	√	√	√	√	√	√	√	√	√	√	√
W7Cr	√	√	√	√	√	√	√	√	√	√	√	×
W8Cr	√	√	√	√	√	√	√	√	√	√	√	×
W9Cr	√	√	√	√	√	√	√	√	√	√	√	√
W10Cr	√	√	√	√	√	√	√	√	√	√	√	√
W11Cr	√	√	√	√	√	×	_	_	_	_	_	_
W12Cr	√	√	√	√	√	×	_	_	_	_	_	_
W13Cr	√	√	√	√	√	√	√	√	√	√	√	×
W14Cr	√	√	√	√	√	√	√	√	√	√	√	×
W15Cr	√	√	√	√	√	√	√	√	√	√	√	√

W16Cr	√	√	√	×	_	_	_	_	_	_	_	_
W17Cr	√	√	√	√	√	√	×	_	_	_	_	_
W18Cr	√	√	√	√	√	√	√	√	√	√	√	√
W19Cr	√	√	√	√	√	√	√	√	√	√	√	√
W20Cr	√	√	√	√	√	×	_	_	_	_	_	_
W21Cr	√	√	√	√	√	√	×	_	_	_	_	_
W22Cr	√	√	√	√	√	×	_	_	_	_	_	_
W23Cr	√	√	√	√	√	√	√	×	_	_	_	_
W24Cr	√	√	√	√	×	_	_	_	_	_	_	_
W25Cr	√	√	√	√	√	√	√	×	_	_	_	_
W26Cr	√	√	√	√	√	√	√	×	_	_	_	_
W27Cr	√	√	√	√	×	_	_	_	_	_	_	_
Cadmium bacterial isolates from Awash tannery wastewater samples												
T1Cd	√	√	×	_	_	_	_	_	_	_	_	_
T2Cd	√	√	√	√	√	√	√	×	_	_	_	_
T3Cd	√	√	√	√	√	√	√	√	√	√	×	_
T4Cd	√	√	√	√	√	√	√	√	√	×	_	_
T5Cd	√	√	√	√	√	√	√	×	_	_	_	_
T6Cd	√	√	√	√	×	_	_	_	_	_	_	_
T7Cd	√	√	√	√	√	√	√	×	_	_	_	_
T8Cd	√	√	√	√	√	√	√	×	_	_	_	_
T9Cd	√	√	√	√	√	√	×	_	_	_	_	_
T10Cd	√	√	√	√	×	_	_	_	_	_	_	_
T11Cd	√	√	√	√	×	_	_	_	_	_	_	_
T12Cd	√	√	√	√	√	×	_	_	_	_	_	_
T13Cd	√	√	√	√	√	√	√	√	√	√	√	√
T14Cd	√	√	√	√	√	√	√	√	√	√	√	√
T15Cd	√	√	√	√	√	×	_	_	_	_	_	_
Cadmium bacterial isolates from AAR tributaries of wastewater samples												
W1Cd	√	√	√	√	√	_	_	_	_	_	_	_
W2Cd	√	√	√	√	×	_	_	_	_	_	_	_

W3Cd	√	√	√	√	√	√	√	√	√	√	√	√
W4Cd	√	√	√	√	√	√	√	√	√	×	_	_
W5Cd	√	√	√	√	√	√	√	√	√	√	√	√
W6Cd	√	√	×	_	_	_	_	_	_	_	_	_
W7Cd	√	√	√	√	×	_	_	_	_	_	_	_
W8Cd	√	√	√	√	×	_	_	_	_	_	_	_
W9Cd	√	√	√	√	√	√	√	×	_	_	_	_
W10Cd	√	√	√	√	√	√	√	√	√	√	√	√
W11Cd	√	√	√	√	√	√	×	_	_	_	_	_
W12Cd	√	√	√	√	√	×	_	_	_	_	_	_
W13Cd	√	√	√	√	×	_	_	_	_	_	_	_
Zinc bacterial isolates from Awash tannery wastewater samples												
T1Zn	_	√	√	√	×	_	_	_	_	_	_	_
T2Zn	_	√	√	√	√	√	√	√	×	_	_	_
T3Zn	_	√	√	√	√	√	√	√	√	√	√	√
T4Zn	_	√	√	√	√	√	√	√	√	√	√	√
T5Zn	_	√	√	√	√	√	√	×	_	_	_	_
T6Zn	_	√	√	√	√	√	√	√	√	√	√	×
T7Zn	_	√	√	√	√	√	√	√	√	√	√	×
T8Zn	_	√	√	√	√	√	√	√	×	_	_	_
T9Zn	_	√	√	√	√	√	√	√	√	×	_	_
T10Zn	_	√	√	√	√	×	_	_	_	_	_	_
T11Zn	_	√	√	√	√	√	×	_	_	_	_	_
Zinc bacterial isolates from AAR Tributaries wastewater samples												
W1Zn	_	√	√	√	√	√	√	√	×	_	_	_
W2Zn	_	√	√	√	√	√	√	√	√	×	_	_
W3Zn	_	√	√	√	√	√	×	_	_	_	_	_
W4Zn	_	√	√	√	√	√	√	√	√	√	√	√
W5Zn	_	√	√	√	√	√	√	√	√	√	√	√
W6Zn	_	√	√	√	√	√	√	√	√	√	√	×
W7Zn	_	√	√	√	√	√	√	×	_	_	_	_

W8Zn	_	√	√	√	√	√	√	√	√	√	√	√	
W9Zn	_	√	√	√	√	√	√	√	√	√	√	√	
W10Zn	_	√	√	√	√	×	_	_	_	_	_	_	
W11Zn	_	√	√	√	√	×	_	_	_	_	_	_	
W12Zn	_	√	√	√	×	_	_	_	_	_	_	_	
Lead bacterial isolates from Awash tannery wastewater samples													
Isolates name	**Concentration (PPM)**												
	100	200	300	400	500	600	700	800	900	1000	1200	1500	2000
T1Pb	√	√	√	√	√	√	√	√	√	√	×	_	_
T2Pb	√	√	√	√	√	√	√	√	√	×	_	_	_
T3Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T4Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T5Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T6Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T7Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T8Pb	√	√	√	√	√	√	√	√	√	×	_	_	_
T9Pb	√	√	√	√	√	√	√	√	√	√	×	_	_
T10Pb	√	√	√	√	√	√	√	√	√	√	×	_	_
T11Pb	√	√	√	√	√	√	√	√	√	√	√	√	×
T12Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
T13Pb	√	√	√	√	√	√	√	√	√	×	_	_	_
T14Pb	√	√	√	√	√	√	√	√	√	√	×	_	_
Lead bacterial isolates from AAR Tributaries wastewater samples													
W1Pb	√	√	√	√	√	√	√	√	√	√	√	×	_
W2Pb	√	√	√	√	√	√	√	√	√	√	√	√	×
W3Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W4Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W5Pb	√	√	√	√	√	√	√	√	√	√	√	√	×
W6Pb	√	√	√	√	√	√	√	√	√	√	×	_	_
W7Pb	√	√	√	√	√	√	√	√	√	×	_	_	_
W8Pb	√	√	√	√	√	√	√	√	√	√	√	√	√

W9Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W10Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W11Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W12Pb	√	√	√	√	√	√	√	√	√	√	√	√	√
W13Pb	√	√	√	√	√	√	√	√	×	_	_	_	_

Appendix III: Further screening of the most potential isolates using nutrient broth

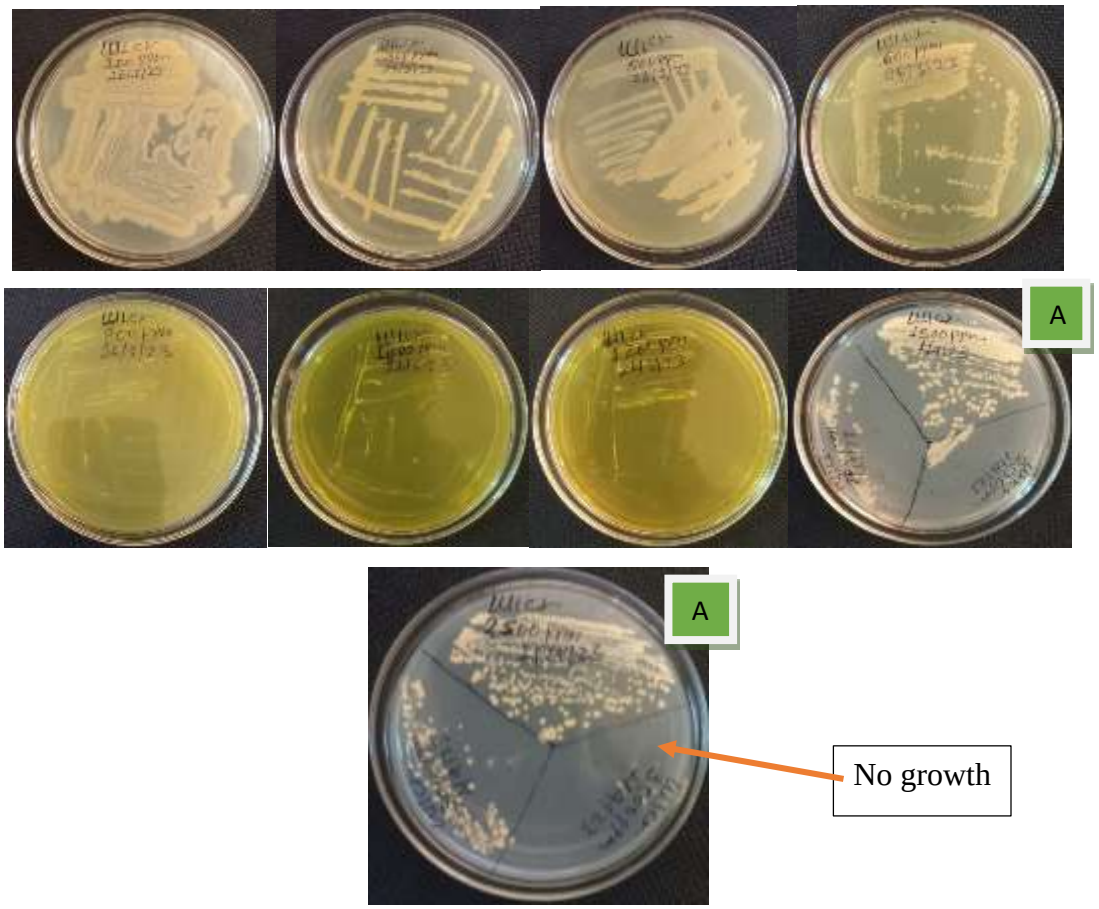
Chromium OD values (Hours) at 1200 ppm						
Isolates	**0**	**24**	**48**	**72**	**96**	**120**
T5 Cr	0.037	0.056	0.071	0.076	0.683	0.625
T15 Cr	0.066	0.076	0.074	0.079	0.084	0.044
T17 Cr	0.069	0.072	0.087	0.096	0.634	0.572
T21 Cr	**0.052**	**0.044**	**0.043**	**0.403**	**0.783**	**0.629**
T23 Cr	0.083	0.082	0.068	0.103	0.119	0.103
W1 Cr	**0.026**	**0.089**	**0.716**	**0.655x2**	**0.514x2**	**0.406x2**
W4 Cr	0.028	0.043	0.045	0.033x2	0.031x2	0.018x2
W6 Cr	0.020	0.063	0.249	0.258x2	0.261x2	0.168x2
W9 Cr	0.090	0.082	0.095	0.042x2	0.055x2	0.034x2
W10 Cr	0.062	0.056	0.072	0.225x2	0.322x2	0.204x2
W15 Cr	0.070	0.062	0.074	0.034x2	0.064x2	0.057x2
W18 Cr	0.098	0.100	0.114	0.446x2	0.399x2	0.318x2
W19 Cr	0.090	0.096	0.413	0.289x2	0.253x2	0.239x2
Cadmium OD values (Hours) at 1200 ppm						
Isolates	**0**	**24**	**48**	**72**	**96**	**120**
T13 Cd	0.022	0.025	0.027	0.029	0.046	0.0
T14 Cd	**0.025**	**0.058**	**0.086**	**0.132**	**0.169**	**0.116**
W3 Cd	**0.010**	**0.013**	**0.09**	**0.015**	**0.034**	**0.0**
W5 Cd	0.001	0.002	0.05	0.004	0.007	0.0
W10Cd	0.007	0.014	0.017	0.023	0.011	0.0
Zinc isolates (OD) values at 1200 ppm						
Isolates	**0**	**24**	**48**	**72**	**96**	**120**

T3 Zn	0.037	0.049	0.069	0.216	0.295	0.264
T4 Zn	**0.035**	**0.034**	**0.048**	**0.239**	**0.300**	**0.286**
W4 Zn	0.033	0.032	0.034	0.042	0.043	0.035
W5 Zn	0.017	0.015	0.011	0.020	0.023	0.012
W8 Zn	**0.054**	**0.062**	**0.054**	**0.063**	**0.067**	**0.045**
W9 Zn	0.036	0.039	0.033	0.058	0.06	0.039
Lead isolates (OD) values at 2000 ppm						
Isolates	**0**	**24**	**48**	**72**	**96**	**120**
T3 Pb	**0.086**	**0.277**	**0.385**	**0.444**	**0.567**	**0.411**
T4 Pb	0.033	0.054	0.058	0.088	0.093	0.072
T5 Pb	0.062	0.156	0.167	0.165	0.157	0.103
T6 Pb	0.023	0.024	0.032	0.065	0.078	0.039
T7 Pb	0.031	0.109	0.115	0.163	0.191	0.147
T12 Pb	0.022	0.25	0.067	0.072	0.111	0.101
W3 Pb	0.072	0.082	0.086	0.104	0.112	0.093
W4 Pb	0.053	0.253	0.297	0.361	0.386	0.215
W8 Pb	0.071	0.236	0.338	0.366	0.403	0.371
W9 Pb	0.066	0.198	0.266	0.409	0.508	0.398
W10 Pb	0.021	0.072	0.091	0.092	0.097	0.073
W11 Pb	0.097	0.189	0.225	0.289	0.324	0.245
W12 Pb	**0.037**	**0.429**	**0.484**	**0.521**	**0.604**	**0.476**

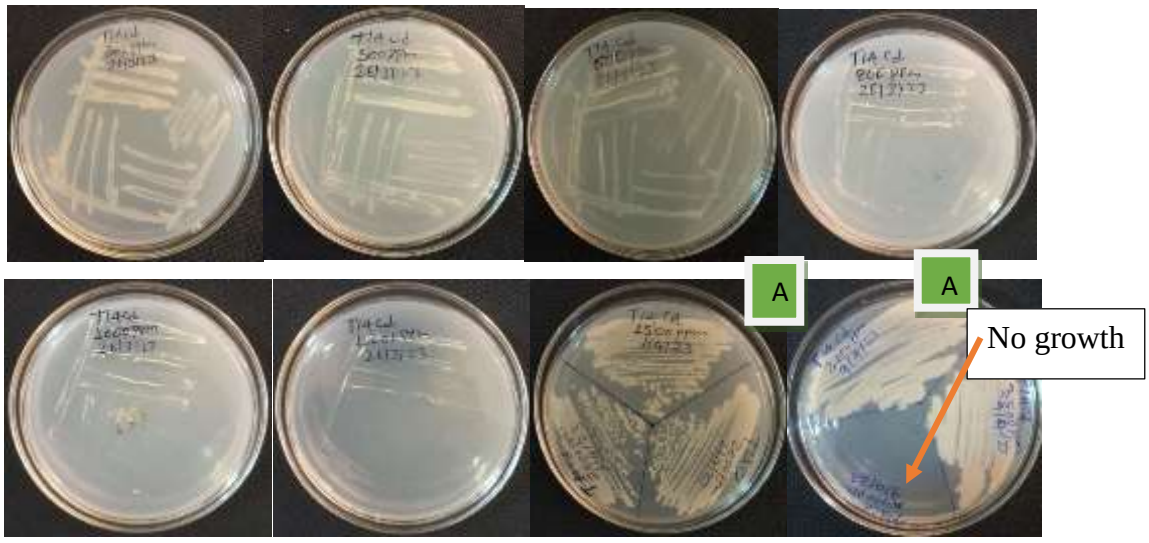
Appendix IV: Minimum Inhibitory Concentration (MIC)

Growth of bacterial isolates on agar plat at different concentration of the respective heavy metal. Letter (**A**) represents bacterial colony grown on NB media and taken from it, then streaked on NA media only, whereas the rest plates were representing bacterial colony directly streaked and grown on the NA media with the respective supplemented heavy metals.

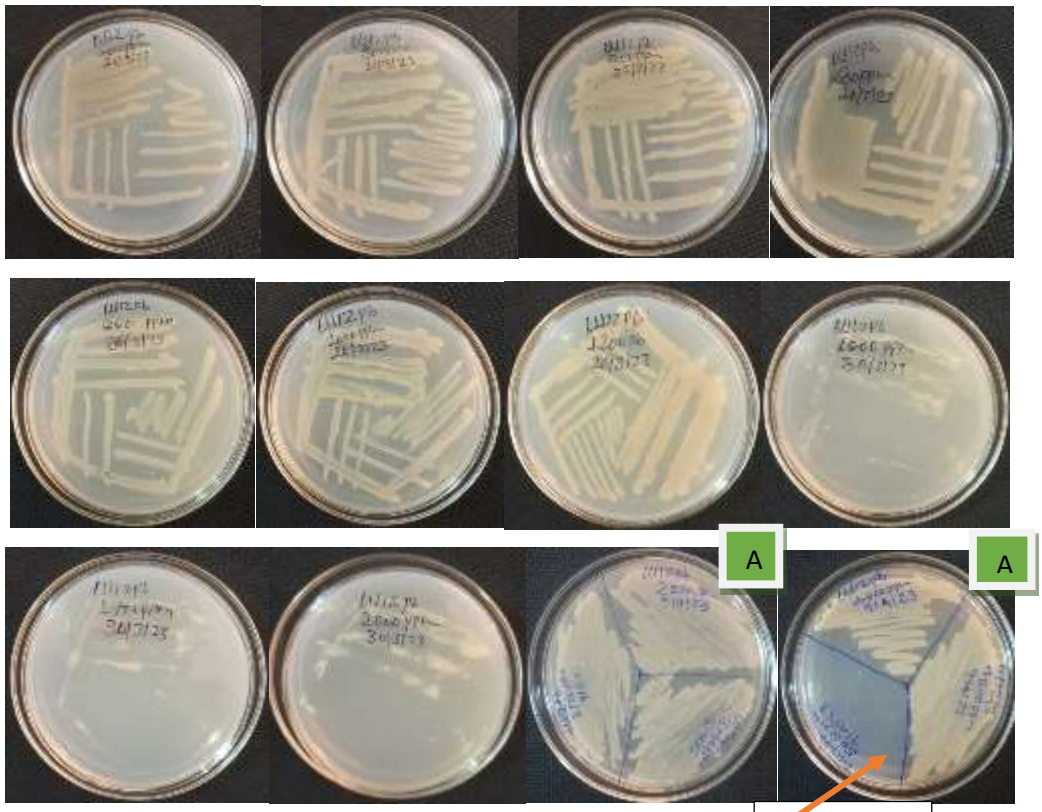
MIC for Chromium metal isolates



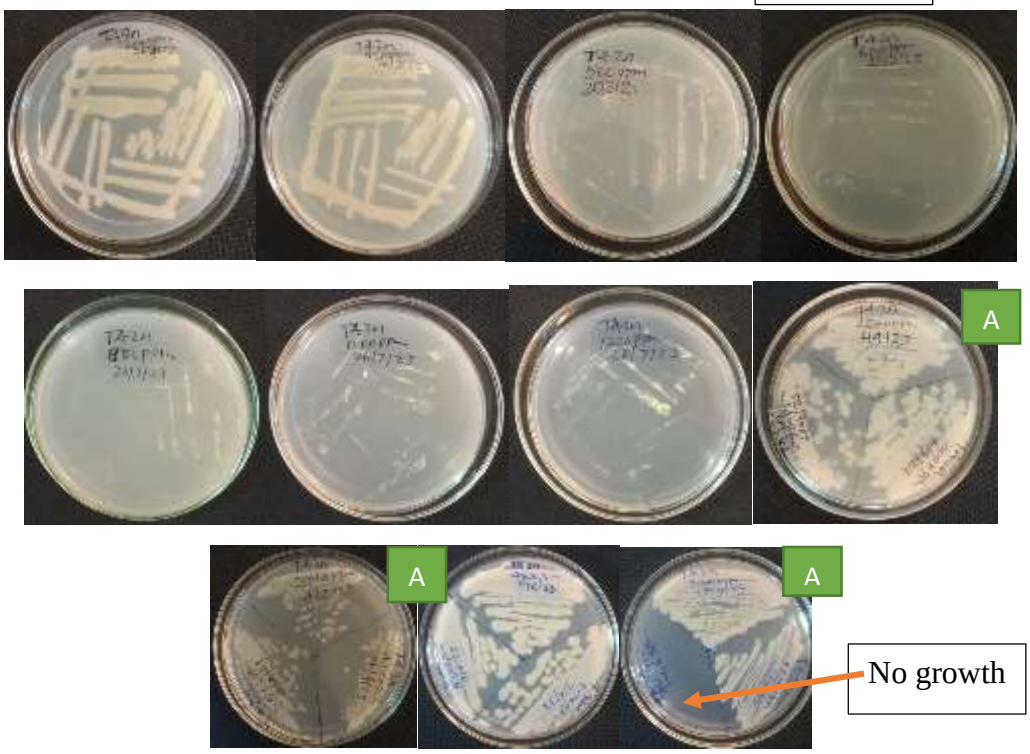
MIC for Cadmium metal isolates



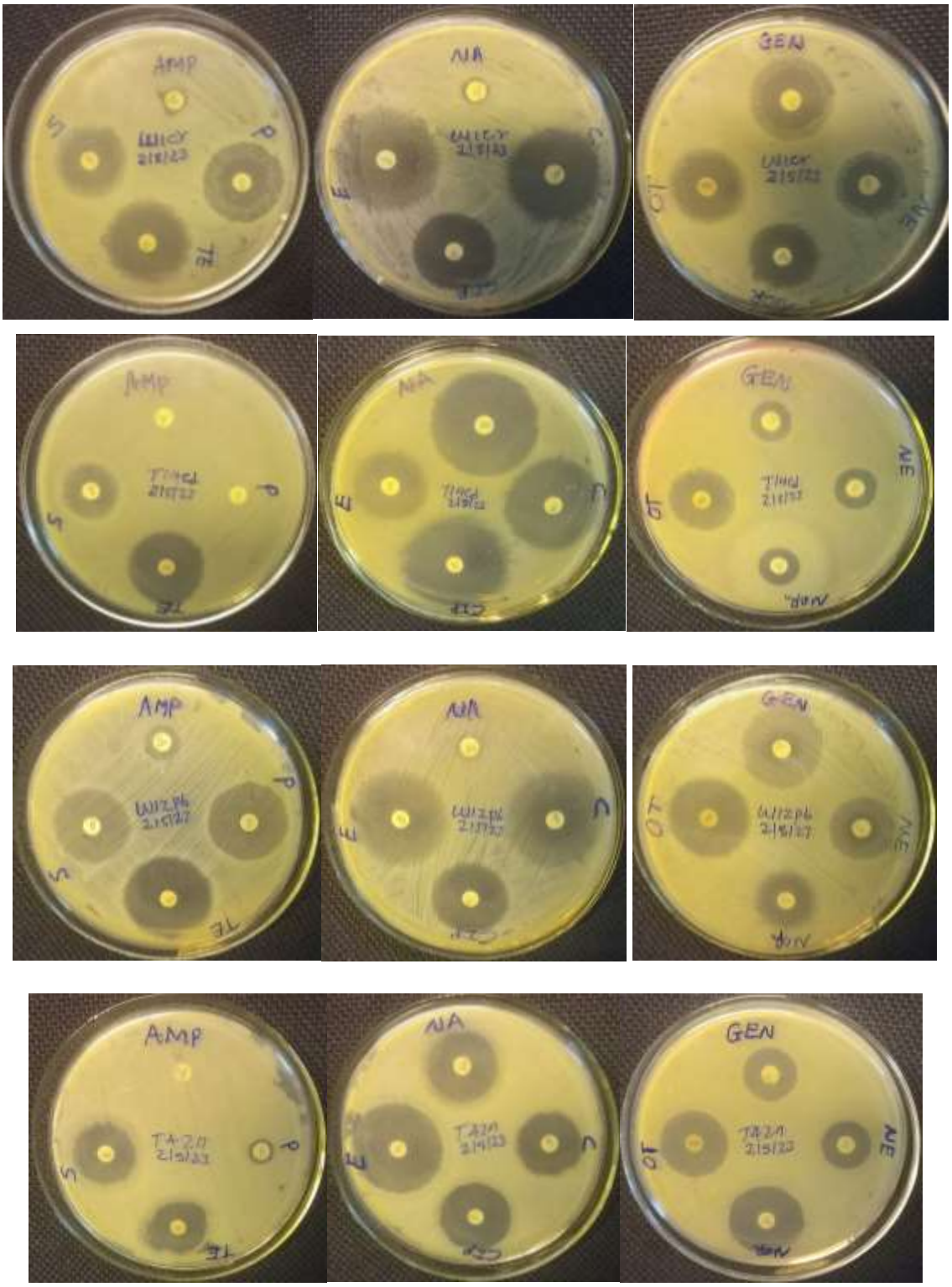
MIC for Lead metal isolates



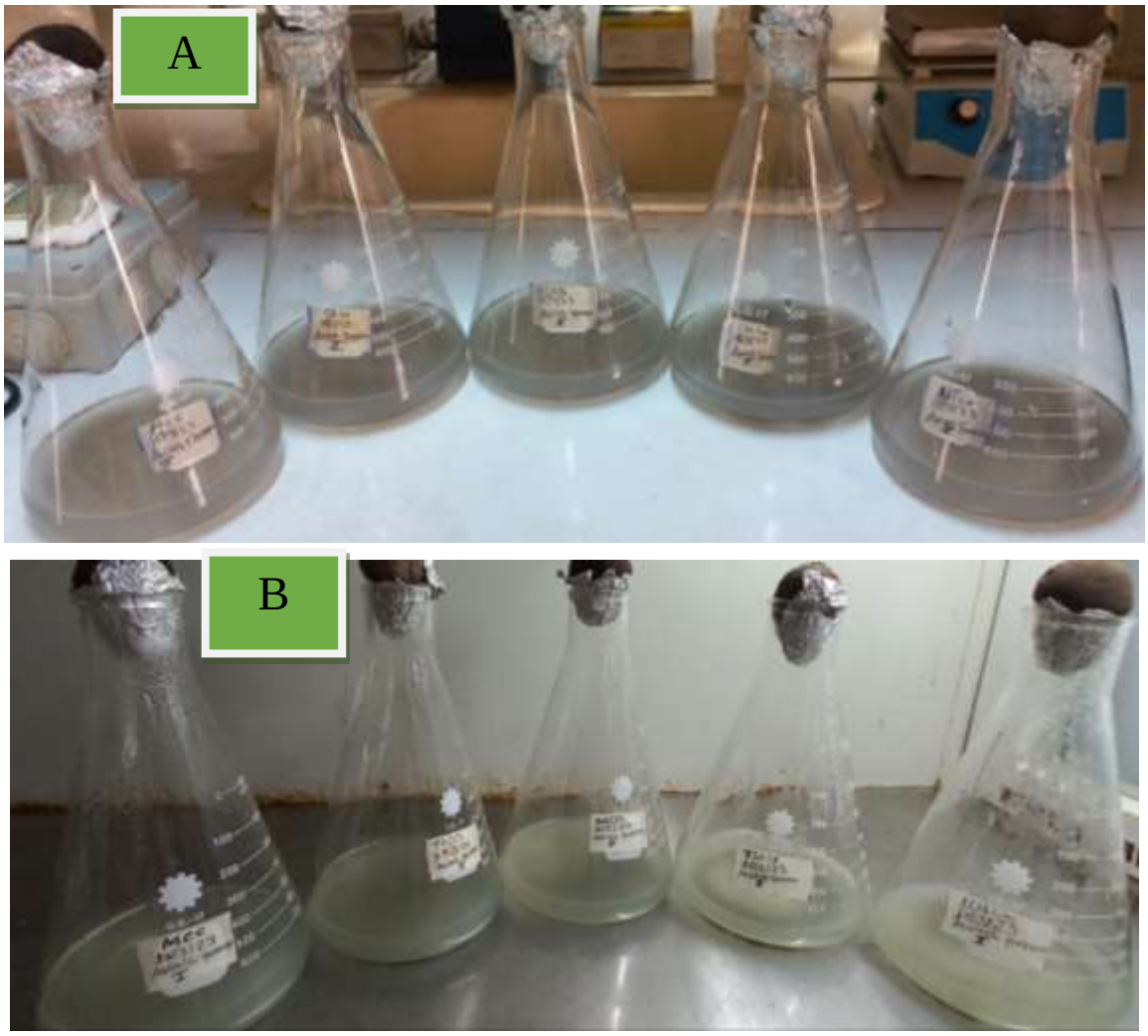
MIC for Zinc metal isolates



Appendix V: Zone of inhibition for tested bacterial isolates in twelve antibiotics



Appendix VI: Color of the media: before (A) and after (B) treatment in the AT wastewater



Appendix VII: Color of the media: before (A) and after (B) treatment in the AAR wastewater

Appendix VIII: Image during heavy metal analysis (AAS analysis)