ISOLATION AND SCREENING OF SELECTED HEAVY METAL RESISTANT BACTERIA FROM KOMBOLCHA TANNERY INDUSTRY EFFLUENT

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We hereby certify that we have read and evaluated this thesis entitled '**Isolationand Screening of Selected Heavy Metal Resistant Bacteria from Kombolcha Tannery Industry Effluent''**prepared under our guidance by **BeleteAbera**. We recommended that it was submitted as fulfilling the thesis requirement

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DEDICATION

This thesis manuscript is dedicated to the all members of my family, my motherBeletu Mohammed, my father Abera Fentaye, my wife Mekiya Jemal and my children, who had signi ficant contribution to the execution of this work.

STATEMENT OF THE AUTHOR

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged through citations. This thesis has been submitted in partial fulfillment for the requirement of the degree of master science in biotechnology. This thesis has been deposited at the Haramaya university library to be made available to borrowers under the rules of the library. I solemnlydeclare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma or certificate.

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BIOGRAPHICAL SKETCH

The author was born on January 15, 1983 at Sayint Woreda in South Wollo Zone of Amhara National Regional State. He completed primary and secondary school at Ewa Primary School and Sayint Comprehensive Secondary School, respectively. Following the completion of his high school education, he joined Jimma University-Ambo College (Ambo University College under Jimma University) in November 2003 and completed his Diploma studies in Biology laboratory Technician in July, 2006. Right after graduation, he was employed in Amhara region, South Wollo, at Akesta Comprehensive Secondary School as a biology laboratory technicianand worked there from 2007 to 2010. He also workedat Wollo University as a biology laboratory technician and joined the same University in 2012 and graduated with BSc in applied biology in August2014. In 2016 hejoined the School of Biological Science and Biotechnology in Haramaya University to pursue hispost graduate studies in Biotechnology,as a Ministry of Education sponsored student . Currently, he is working as a Graduate Assistant (GA III) in Wollo University.

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LIST OF ACRONYMS/ABBREVIATIONS

ANOVA	Analysis of Variance
BOD	Biological Oxygen Demaned
CFU	Colony Forming Unite
COD	Chemical Oxygen Demand
CNS	Central Nervous System
DIW	De-ionized Water
FAAS	Flame Atomic Absorption Spectrophotometer
MIC	Minimum Inhibitory Concentration
MR	Methyl Red
NA	Nutrient Agar
NB	Nutrient Broth
OD	Optical Density
PNS	Peripheral Nervous System
Ppm	Parts Per Million
PS	Primary Screening
Rpm	Round per Minutes
SS	Secondary Screening
TEWW	Tannery Effluent Waste water
TVBC	Total Viable Bacterial Count
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

TABLE OF CONTENT

TABLE OF CONTENT (Continued)

TABLE OF CONTENT (Continued)

TABLE OF CONTENT (Continued)

LIST OF TABLES

LIST OF FIGURE

LIST OF FIGURE INAPPENDICES

ISOLATION AND SCREENING OF HEAVY METAL RESISTANT BACTERIA FROM TANNERY INDUSTRY EFFLUENT

ABSTRACT

This study was aimed to replace the chemical and physical treatment with biological treatment to save the environment. It was concerned with the isolation, screening, and charact erization of heavy metal-resistant bacteria isolated from Kombolcha tannery industry effluent. Different ways were used to select the maximum of isolates resistant to heavy metals. Based on, morphological, gram stain and biochemical tests six resistance bacteria were isolated by using free nutrients agar medium and nutrient broth.The isolates showed 2225 ppm Cr, 1100 ppm Hg and 340 ppm Pb of MIC.The isolates are, Klebsiella sp. (B-02), Pseudomonas sp. (B-03),E. Coli (B-15), Enterobacter sp. (B-25), Salmonella sp.(B-51)and Clostridium sp. (B-54). From the six isolated bacteria, Klebsiella spp. and Salmonella sp. were resisted up to 2200 ppm Chromium. Whereas, Pseudomonas sp.and E.coli were resisted .Lead up to 340 ppm and Enterobacter spp.and Clostridium sp. were resisted Mercury i.e. up to1050 ppm. The study on the morphology of resistant isolates showed that the contour and the size of colonies would change in the presence of heavy metals. The concentrations of heavy metals (Crand Pb) in the isolated bacteria (B-03 and B-51) and tannery effluent were determined by using flame Atomic Absorption spectrometer .Concentration of Lead and Chromium in the bacteria were found to be in the range: 0.015-0.0156, and 0.0182-0.0185, respectively and 0.02-0.023 and 0.003-0.00325 in the effluent sample. The TEWW had contained maximum Iron and minimum Chromium.Depending on the results, all resistant isolates allowed determining the order of toxicity of the different metals was as follows: Pb>Hg > Cr. The redaction capacity of Chromium, Mercuryand Lead resistance bacteria were 90%, 85% and 80% respectively. Increasing of metal concentrations on the isolate showed a significant toxic effect for the bacterial growth and metabolism. Chromium in the original effluent sample was by far less th an the permissible limit; these shows, the microbes in the tannery effluent have a capacity of bioremediation of heavy metals and other wastes.

Keywords: Atomic Absorption spectrometer, Bioremediation, Heavy metal, Isolation, Tannery effluent, Resistant bacteria.

1. INTRODUCTION

Industrialization and technological advancement have put an increasing burden on the environment by releasing large quantities of hazardous waste, heavy metals (cadmium, chromium, and lead) and metalloids (elements with intermediate properties between those of typical metals and non-metals, such as arsenic and antimony), and organic contaminants that have inflicted serious damage on the ecosystem. The build-up of heavy metals and metalloids in soils and waters continues to create serious global health concerns, as these metals and metalloids cannot be degraded into non-toxic forms, but persist in the ecosystem. Contamination of the environment with heavy metals has increased beyond the recommended limit and is detrimental to all life forms (Gaur *et al*., 2014, Dixit *et al*., 2015 and Tak *et al*., 2013).

Heavy metal is a general collective term that applies to the group of metals and metalloids with atomic density greater than 4000 Kgm-3, or 5 times more than water (Garbarinoet al., 1995). It is natural components of the earth's crust and, increasingly found in habitats dominated by microbes due to some natural and anthropogenic factors.Some of such heavy metals that turn out to be toxic to living organisms when they are in higher concentrations includes Iron (Fe), Cadmium (Cd), Nickel (Ni), Copper (Cu), Molybdenum (Mo), Selenium (S), Vanadium (V) , Zinc (Zn), Chromium (Cr), Lead (Pb), Arsenic(As), Mercury(Hg), etc. (Jorge *et al*., 2011).

Heavy metals are amongst the most persistent as they are not easily degraded naturally. Thus, removal of these pollutants is a challenge to environmental management. The removal and recovery of heavy metals from contaminated water and wastewater is important in the protection of the environment and human health. In Ethiopia, tanning industries usually discharge their waste water into nearby rivers. Thus, the aquatic environment is more susceptibleto pollution by their wastes including heavy metals. People in the downstream of these rivers use water polluted by tannery industries wastes for cultivation of crops and vegetables, leading the transfer of heavy metals to humans through plants(Sinha *et al*., 2008).

Especially organic pollutants (OPs) are problematic because of their high lipid solubility, which leads to their bioaccumulation in tissues (Guzzella *et al*., 2005)

It enters the body through food and water and gets transferred to all the trophic levels of the ecosystem.In humans, these are highly toxic and have a wide range of chronic effects such as endocrine disrupting activities, mutagenicity, and carcinogenicity (Lee *et al*., 2006).

Water pollution is a major problem in the global context. Water bodies are the main target sites for disposal of industrial waste water(Cheng, 2003). It has been suggested that it is worldwide leading cause of diseases and deaths accountings for more than 14,000 human deaths per a day. Heavy metal pollution to wastewater is of great concern to environmentalist. Heavy metals present in the waste keep on accumulating in the environment and eventually accumulates in food chain therefore causing long lasting health and ecological problem (Carlos and Chuken, 2011).The maximum permissible concentration of some heavy metals in water, as stated by the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), USA, is 0.01, 0.05, 0.01, 0.015, 0.002, and 0.05 mg/L for Ar, Cd, Cr, Pb, Hg, and Ag respectively(Chaturvedi *et al.*,2015).

The main sources of pollutions by heavy metals are usually linked with areas of intensive industrial wastes and automobiles exhausts. Industrial development usually results in the generation of effluent wastes, which are heavily loaded with different types of organic and inorganic pollutants that will be discharged into environments including water bodies. Common sources of heavy metal pollutants are mainly discharges from industries such as Tannery industries, electroplating, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes (Zouboulis *et al*., 2004).

Instead of Physico-chemical treatment biotechnological approaches have received a great deal of attention as an alternative tool in recent years. Microorganisms have the capability of binding of heavy metals and concentrating themfor easy disposal. Microbes have mechanisms to tolerate the presence of heavy metals either by refluxing or through reduction of them by using them as terminal electron acceptors in anaerobic respiration (Gadd, 1992; Nies and Silver, 1995). Which retaining suitable concentrations of essential metals such as Copper, while rejecting toxic metals like Lead, Cadmium, Chromium, and Mercuryis probably one of

the main challenges of living cells (Gatti *et al*, 2000). The first response to toxic metal contamination is reducing though microbial activity (Badar *et al*., 2000).This is confirmed by the fact that habitats that harbor high levels of metal contamination for years still have microbial populations that are less active than the microbial populations inhabiting uncontaminated habitats.

Biosorption is a process of potential use of microbes for treating heavy metal pollution.Bacteria are good biosorbents of metals and they may be in the near future a good alternative for the removal of heavy metals pollutants (Errasquin and Vazquez, 2003).Various types of bio-agents such as bacteria, fungi, algae, seaweeds, etc., have been were studied for their potential of remediating extensively heavy metal pollution. Some bacterial strains are resistant to heavy metal pollutions and by virtue of their resistance to these toxic metals; they can be used to remediate the environments from such pollutions. Resistant bacterial strains solve these problems by a careful regulation that results from the interaction between chromosomally determined action transport systems and metal resistance systems that are mostly determined by plasmids (Brown *et al*., 1999).The quality of Kombolcha tannery industry effluent were not assessed.whereas, the peoples at the dawnstream used for different purpses.

Resistant strains are diverse, and although there have been some studies conducted so far, more needs to be examined. Therefore, this study was designed to look for bacterial strains resistant to heavy metals pollutants of tannery effluents with the following general and specific objectives

General Objective:

 To isolate and screen heavy metal tolerant bacteria from Kombolcha tannery Industry effluent

Specific objectives:

 To isolate heavy metal tolerant bacteria from Kombolcha tannery industry effluent

 To determine the level of selected heavy metals (Chromium, Lead, Copper and Mercury) form Kombolcha tannery effluent

 To evaluate the capacity of bacteria to reduce heavy metal concentrations

 To determine the minimum inhibitory concentrations of heavy metal in contrast to the bacterial isolate

2. LITERATURE REVIEW

2.1. Heavy Metals

Heavy metals are considered one of the most common and hazardous pollutants in industrial effluents that might cause serious problems to human and animal health. It is currently a major environmental problem because metal ions persist in the environment due to their non-degradable nature.Unlike organic contaminants, heavy metals cannot be broken down by chemical or biological processes. Hence, they can only be transformed into less toxic species.The majorities of heavy metals are toxic at low concentrations and are capable of entering the food chain, where they accumulate and inflict damage to living organisms. All metals have the potential to exhibit harmful effects at higher concentrations and the toxicity of each metal depends on the amount available to organisms, the absorbed dose, the route and the duration of exposure (Mani and Kumar, 2014).

Many of the heavy metals are toxic even at very low concentrations; arsenic, cadmium, chromium, copper, Lead, Mercury, Nickel, Selenium, Silver, Zinc etc. are not only cytotoxic but also carcinogenic and mutagenic in nature (*Salem et al*., 2000).According to some current estimates, approximately 1.8 million people, 90 percent of whom are children under 5, still die every year from diarrheal diseases mostly in developing countries (WHO, 2008).

It is estimated that in industrialized countries, 60 percent of diarrheal disease is attributed to unsafe water, sanitation, and hygiene, whereas in developing countries as much as 85-90 percent of diarrheal illness can be attributed to these causes (Keusch *et al*., 2006).These elements are usually transition metals. They have high densities (>5 g cm^{-3}) when compared with other materials (Baird and Cann, 2005). They are non-biodegradable and tend to accumulate in the tissues of living organisms, a process called bio concentration (Baird and Cann, 2005; Kobya *et a*l., 2005). However, they include some elements that are essential for living organisms at low concentrations such as Iron (Fe) and Zinc (Zn) as well as toxic metals like Cadmium (Cd), and Mercury (Hg)(Alloway, 1990).

Heavy metals have an adverse effect on human physiology and other biological systems (Bailey *et al.,* 1999; Kobya *et al.,* 2005). They show a great affinity for other elements such as sulphur and disrupt enzyme functions in living cells by forming bonds with these groups.

Cadmium, lead and mercury ions have the ability to bind to cell membranes, interfering with the cell transport processes (Manahan, 2008). Heavy metals may also stimulate the formation of free radicals and reactive oxygen species, which may lead cells into oxidative stress (Dietz *et al*., 1999).

2.2. Environmental and Health Risks of Heavy Metals

Heavy metals are considered one of the major sources of water and soil pollution which exerts toxic effects on water and soil microorganisms and hence results in the change of the diversity, population size and overall activity of the microbial communities (Ashraf and Ali, 2007) .The metal uptake of plants from soils at high concentrations may result in a great health risk and subsequent accumulation along the food chain is a potential threat to human health (Jordao *et al*.,2006).

The consumption of heavy metal contaminated food can seriously deplete some essential nutrients in the body that are further responsible for decreasing immunological deference, growth retardation, disabilities associated with malnutrition and high prevalence of upper gastrointestinal cancer rates. Heavy metals containing Industrial effluent and agricultural runoff enter in aquatic environment it may toxic to aquatic plants and animals (Khan *et al.*,2008).

2.2.1. Chromium

Chromium (Cr) is the 10^{th} abundant element in the earth's mantle andit is an essential nutrient for plant and animal metabolism; however, the increasing accumulation of chromium in the environment from industrial outputs has caused great concern. Chromium exists in III (+) and VI (+) oxidation states as all other oxidation states are not stable in aqueous solutions. Both valences of chromium are potentially harmful although the latter is more toxic (Dakiky *et al.*, 2002). It was being a strong oxidizing agent, corrosive, soluble in alkaline and mildly acidic water, toxic and potential carcinogens. The toxicity of Cr (VI) derives from its ability to diffuse through cell membranes and oxidize biological molecules (Shaffer *et al.*, 2001).

Conventionally, Cr (VI)-containing industrial effluents are treated by chemical means that are relatively chemical-intensive and energy-intensive and that may be a source of potential metal pollution from the resultant metal-containing chemical sludge. Methods to remediate these sites include excavation, to pump and treat, in situ vitrification and chemical treatment with a reductant (Vermeul *et al*., 1995). Biological reduction of Cr (VI) usually occurs at a neutral pH-range and generates insignificant quantity of chemical sludge as well as offers potential cost-effective remediation strategy (Mahesh *et al*., 1997).

2.2.2. Cadmium

Cadmium is the most dangerous metal ion characterized by high stability and toxicity. It is not degradable in nature and will thus, once released to the environment, stay in circulation and has high toxicity at low concentrations (Karnachuk *et al*., 2003). The wastewaters from the industries and sewage sludge applications have permanent toxic effects to human and the environment. Cadmium is known to bind with essential respiratory enzymes (Nies, 2003) causing oxidative stress and cancer (Banjerdkji *et al*., 2005). Cadmium can affect the kidney, causing renal dysfunction, especially in the proximal tubular cells as it is the main site of cadmium accumulation. It can also cause bone demineralization, either directly by damaging the bones or indirectly as a result of renal dysfunction (Blessy, 2015; Bernard, 2008).

In plants, toxic levels of Cd may cause stunting and chlorosis. The solubility product of Cd is 1.4×10^{-29} but 2.91×10^{-25} for ZnS (Weast, 1984). Thus, cadmium is more toxic (Ragan, 1990) than zinc. As far as cadmium is concerned, it has been estimated that, the anthropogenic emissions of Cd are in the range of 30,000 tons per year.

Cadmium is most commonly accumulated by agriculturally important crops and leads to a decrease in root and shoot growth, and a decrease in nutrient uptake and homeostasis D Toppi and Gabrielli, 1999). Thus, when these crops are consumed by organisms, it may cause severe health effects. Similarly, soil contamination with cadmium leads to loss of biodiversity and activity of soil microbial communities (McGrath 1994). Under acidic condition,cationic metals are generally found as free ionic species or soluble oregano-metals. Sandrin and Maier.(2002) showed that cadmium solubility and speciation varied with a change of pH. At pH 4, ionic

cadmium ($Cd^{2+)}$ measured 44mg/l and at pH 7 measured 4mg/l, producing insoluble cadmium phosphate $Cd_3(PO4)2$ and small amounts of monovalent hydroxylated cadmium ($CdOH^-$).

2.2.3. Lead

Lead (Pb) is physiological and neurological toxic to humans. Acute Pb poisoning may results in a dysfunction in the kidney, reproduction system, liver and brain resulting in sickness and death (Odum *et al.,* 2000). Pb heads the threats even at extremely low concentrations (Kazemipuor *et al*., 2008). Lead poisoning also causes inhibition of the synthesis of haemoglobin, cardiovascular system and acute and chronic damage to the central nervous system (CNS) and peripheral nervous system (PNS).

Moreover, lead can causes difficulties in pregnancy, high blood pressure, muscle and joint pain (Odum *et al*., 2000). Other effects include damage to the gastrointestinal tract (GIT) and urinary tract resulting in bloody urine, neurological disorder and can cause severe and permanent brain damage. While inorganic forms of lead, typically affect the CNS, PNS, GIT and other bio systems, organic forms predominantly affect the CNS. Lead affects children; particularly in the 2-3 years old range by leading to the poor development of the grey matter of the brain, thereby resulting in poor intelligence quotient (IQ). Its absorption in the body is enhanced by Ca and Zn deficiencies (Duruibe *et al*., 2007).

2.2.4. Copper

Copper (Cu) is an essential element in mammalian nutrition as component of metallo-enzymes in which it acts as an electron donor or acceptor. Conversely, exposure to high levels of Cu can result in a number of adverse health effects. Exposure of humans to Cu occurs primarily from the consumption of food and drinking water(Stern *et al*, 2007).

Acute Cu toxicity is generally associated with accidental ingestion; however, some members of the population may be more susceptible to the adverse effects of high Cu intake due to genetic predisposition or disease (Stern *et al*, 2007). Excessive human intake of Cu may leads to severe mucosal irritation and corrosion, widespread capillary damage, hepatic and renal damage and central nervous system irritation followed by depression. Severe gastrointestinal

irritation and possible necrotic changes in the liver and kidney can also occur. The effects of Cu exposure vary from skin irritation to damage to the lungs, nervous system, and mucous membranes (Argun *et al.*,2007).

2.2.5. Mercury

The metallic mercury is a naturally occurring metal which is a shiny silver-white, odorless liquid and becomes colorless and odorless gas when heated. Mercury belongs to heavy metal which is toxic to living creatures. Major sources of mercury pollution include anthropogenic activities such as agriculture, municipal wastewater discharges, mining, incineration, and discharges of industrial wastewater (Chen *et al*., 2012).

Mercury can attack the arrangement of central nervous and causes memory loss, tremors and decreases motion capability. It is the most toxic of the heavy metals (Gerlach, 1981) and occupies the sixth position in the list of hazardous compounds (Nascimento and ChartoneSouza, 2003). Mercury utilizing in golden mining could produce waste, which contains mercury and causes environment pollution. Poisoning causing destruction of a fetus has been detected. Miniamatadecease in Japan is the example of mercury poisoning (Chowdury *et al*., 2012).

Mercury exists mainly in three forms: metallic elements, inorganic salts and organic compounds, each of which possesses different toxicity and bioavailability. A World Bank document in the year 2000 on the National Thermal Power Corporation (NTPC) showed results for mercury concentrations in coal analysis done by NTPC in the range of 0.11 to 0.14 ppm while another study of coal analysis, done by the Roorkee University, India, showed mercury to be in the range of 0.8 to 11.4 ppm (Agrawal *et al*., 2008).

2.2.6. Zinc

Zinc (Zn) is considered to be relatively non-toxic, especially if taken orally. However, excess amount can cause system dysfunctions that result in impairment of growth and reproduction. The clinical signs of zinc toxicosis have been reported as vomiting, diarrhea, bloody urine,

icterus (yellow mucus membrane), liver failure, kidney failure and anemia (Duruibe*et al.*, 2007).

2.3. Removal Methods of Heavy Metal from Effluents

Several technologies are available for the treatment and removal of heavy metals from contaminated site. The selection depends on contaminant and site characteristics, regulatory requirements, costs and time constraints (Riser-Roberts, 1998).

2.3.1. Physicochemical Method

2.3.1.1. Isolation of Containment

Heavy metal contaminants can be isolated and contained, to prevent their movement and reduce their permeability. To accomplish this physical barriers made of different materials are used for capping, and vertical and/or horizontal containment. Capping has been used with good results to reduce the water intake, whereas, vertical barriers are used to reduce the movement of groundwater. Solidification/stabilization techniques are very common in the United States, because they contain the contaminants, lowering the labor and energy costs (Mulligan *et al*., 2001).

2.3.1.2. Mechanical Separation

This method aims at the removal of larger clean particles from smaller polluted particles. This method has been used in mineral ore processing and now in remediation of heavily contaminated soils (Mulligan *et al*., 2001).

2.3.1.3. Chemical Treatments

Chemical reactions such as oxidation and reduction can be used to minimize the mobility of heavy metal contaminants. This is commonly used in treatment of contaminated water. This method involves the addition of chemicals such as potassium permanganate, hydrogen peroxide, or chlorine gas. Chemical treatments have the advantage of being performed in situ, but also may add a new source of contamination (Mulligan *et al.,* 2001).

2.3.1.4. Electro Kinetics

This technique involves passing low intensity electric currents between a cathode and an anode inserted in the contaminated site. An electric gradient generates movement by electro migration, and electrophoresis. The metals can be removed by electroplating or precipitationor recovering the metals by pumping the waste from which it originated. This technique has been used in Europe (Mulligan *et al*., 2001).

2.3.1.5. Ion Exchange

This is one of the more common techniques for heavy metal removal. In this process ions of a given species are displaced from an insoluble exchange material by different ions in solution. The materials used for the exchange include zeolites, chelating resins, microbial and plant biomass. Ion-exchange techniques are highly pH dependent. A drawback to this technique is the high operating costs (Metcalf and Eddy, 2003).

2.3.2. Biological Method

2.3.2.1. Bioremediation of Heavy Metal

Bioremediation is a state-of-the-art technique used for heavy metal removal and/or recovery from polluted environments. The technique utilizes inherent biological mechanisms to eradicat e hazardous contaminants using microorganisms and plants, or their products, to restore pollut ed environments to their original condition (Dixit *et al*.,2015, Mani and Kumar,2014 and Akcil *et al*., 2015). Remediation process is the solution for the problem of heavy metals contamination (Abioye, 2011). It is an environmentally friendly and cost-effective technique for heavy metal removal/recovery, when compared to the conventional chemical and physical techniques, which are often more expensive and ineffective, especially for low metal concentrations (Abioye, 2011).

Bioremediation are effective only when the environmental conditions permit to the growth and activity of microorganism and this always involves the handling of the environment and allow the growth of microorganism (Vidali, 2001). Microbial remediation is described as the use of microorganisms to perform the absorption, precipitation, oxidation, and reduction of heavy

metals in the soil (Su,2014). Microorganisms possess astonishing metabolic pathways which utilize various toxic compounds as a source of energy for growth and development, through respiration, fermentation, and metabolism. Due to their characteristic degradative enzymes for a particular contaminant, they have evolved diverse mechanisms for maintaining homeostasis and resistance to heavy metals, in order to adapt to toxic metals in the ecosystem (Brar*et al*.,2006 and Wei *et al*., 2014).

Strategies developed by microorganisms for continued existence in heavy metal polluted environments, include mechanisms such as bioaccumulation, bio mineralization, biosorption, and biotransformation. These mechanisms are exploited for in situ (treatment at the site of contamination), or ex situ (the contaminated site can be excavated or pumped and treated away from the point of contamination), remediation. Owing to these abilities, they have been effectively used as biosorbents for heavy metal removal and recovery. The majority of heavy metals disrupt microbial cell membranes, but microorganisms can develop defense mechanisms that assist them in overcoming the toxic effect. Thus, the response of microorganisms to heavy metal toxicity is of importance for re-establishing polluted sites.

Recently, different species of *Aspergillus*, *Pseudomonas, Sporophyticus*, *Bacillus* and *Phanerochaete* have been reported as efficient chromium, Lead and Nickel reducers (Yan and Viraraghavan, 2003,). Two Cadmium (Cd) resistant strains i.e. *Bacillus megaterium* H3 and *Neorhizobium huautlense* T1-17 were investigated for metal immobilization and provide safe way to produce rice in Cd-contaminated soils (Li *et al.,* 2017). Moreover, complete genome sequence of the heavy metal resistant bacterium*Agromyces*aureusAR33 has been submitted recently (Correttoet *al*., 2017). Anderson and Cook 2004, reported strains of *Aeromonas, Exiguobacterium, Acinetobacter, Bacillus* and *Pseudomonas,* that can tolerate high concentrations of arsenic species (up to 100 mM arsenate).

2.3.2.2. Bioremediation in Industrial Effluents

Bioremediation is an innovative technique for the removal and recovery of heavy metal ions from polluted areas, and involves using living organisms to reduce and/or detoxify heavy metal pollutants into less hazardous forms, using the activities of algae, bacteria, fungi, or plants. It has been employed for the removal of heavy metals from contaminated wastewaters and soils. This method is an appealing alternative to physical and chemical techniques, and the

use of microorganisms play a significant role in heavy metal remediation. Similarly, the use of microorganisms to remediate polluted environments is sustainable and helps to restore the natural state of the contaminated environment with long term environmental benefits and cost effectiveness (Dixit *et al*., 2015).

Industrial effluents are the most important sources of toxic contaminants in the environment (Mohana *et al.,* 2008). Rapid industrialization and urbanization have enhanced the level of organic contaminants in the environment (Trupti *et al*., 2009). The application of cyanobacteria showed immense potential in waste water and industrial effluent treatment, bioremediation of aquatic and terrestrial habitats, chemical industries, tannery industry, Bio fertilizers, food, feed, fuel, etc. Monika *et al*. (2010) studied microbial load in tannery and textile effluents and their receiving rivers of Dhaka and reported that the total viable bacterial count (TVBC) in both tannery and textile effluents were slightly higher in dry season than rainy and summer.

Alteromonas sp. is osmophilic and alkali tolerant which can be used for bioremediation applications in paper and pulp mill effluents (Murugesan, 2003). Dubey (2011) studied about the potential use of cyanobacteria species in bioremediation of industrial effluents and reported that *Oscillatoria* sp., *Synechococccus* spp., *Nodularia* sp. and *Cyanothece* spp. are a group of *Cyanobacterial* spp. *Psedomonasaeruginosa* and *Brevibacilluschoshinensis* isolated from the textile industrial effluent which decolorize the effluent in the presence of 10% glucose very effectively within 7 days (Annika *et al*., 2012).

Mahmood *et al*. (2013) reported *Bacillus subtilus, Bacilluscereus, Bacillusmycoides, Pseudom onas* sp. and Micrococcus spp. can be the best biological tool for textile and tannery effluent treatment. Bacterial species *Pseudomonas putida* and *Bacillus licheniformes* has a potential application for the bioremediation of heavy metals from domestic and industrial waste water with moderate concentrations of heavy metals (Kamika and Mumba, 2012). *Pseudomonas* spp. is important in the balance of nature and also in the economy of human affairs. It is globally active in aerobic decomposition and biodegradation, and hence they play a key role in the carbon cycle.

Hanel (1986) reported that the chloride concentrations lower than 5 g/l, do not apparently affect the COD removal and the nitrification process. *Acenetobactercalcoaceticus* exhibited significant reduction in BOD and COD from ossein effluents (Manoharan and Subramanian,

1993). *Pseudomonsputid*a and *Acienetobatercalcoaceticus* were studied for degradation of black liquor from a Kraft pulp and paper mill in a continuous reactor.

Psedomonas putida, Citrobacter sp. and *Enterobacter* sp. not only decolorized effluent up to 97% but reduced BOD, COD, phenolic and sulfide up to 96.63, 96.80, 96.92 and 96.67 % respectively within 24 h of growth and the heavy metals were removed up to 82 - 99.8%. The TSS and TDS were removed up to 82 - 99.80%. The TSS and TDS were sharply reduced due to degradation (Chandra, 2001). Maghsoodi*et al*. (2007) suggested that highest COD reduction in dairy plant effluents was obtained by Coccus and Bacillus with an efficiency of 70.7 and 69.5%, respectively.

Sangita *et al*. (2012) studied biodegradation of tannery effluent by using tannery effluent isolate and reported that the decrease in level of COD indicates the reduction of biologically oxidesable and inert organic materials as a result of the degradation by the *Pseudomonas* sp. *Pseudomonas* spp. isolated from the effluent is efficient enough to degrade the tannic components and it is useful to make the effluent non-toxic after treatment, and these waste water can be reused and certainly this biodegradation study will be helpful to some extent for making a pollution free environment.

Krishnaveni *et al*. (2013) studied bioremediation of steel industrial effluents using soil microorganisms and reported that 95% reduction in COD and BOD was observed by using naturally occurring consortia of microorganisms like Bacillus, Pseudomonas, Arthrobacter, and Micrococcus species. Buvaneswari *et al*. (2013) studied isolation and identification of predominant bacteria to evaluate the bioremediation in sugar mill effluent and observed that *Staphylococcus aureus, Bacillus cereus, Klebsiella pneumonia, Enterobacter aeruginosa* and *Escherichia coli* as the major bacterial groups found in sugar mill effluents and also they observed the maximum degrading potential in the case of *Staphylococcus aureus.*

2.3.2.3. Bioremediation of Heavy metals by Microorganisms

These organisms help to neutralized hazardous components in the environment. The process can function naturally or can be improved through the addition of electron acceptors, nutrients, or other factors.Various factors influence the microbial remediation of metals. They include the bioavailability of the metal to the microbe, concentration of pollutants, electron acceptors, moisture content, nutrients, osmotic pressure, oxygen, pH, redox potential, soil structure,

temperature, and water activity. The bioavailability of each metal in soil is influenced by factors such as the buffering capacity, cation exchange capacity, clay minerals content, metal oxide, and organic matter (Tak *et al*.,2013; Mani *et al*., 2014 and Brar *et al*., 2006). In general, remediation of heavy metal is achieved through the removal of the metal ion from substratum to reduce the risk posed by exposure to such heavy metals.

2.3.2.4. Bioremediation of Heavy metal by Bacteria

It is known, that microorganisms accumulate heavy metals in polluted water systems and soil. In order to survive in heavy metal polluted environments, many microbes have developed mea ns of resistance to toxic metal ions. Detoxification can occur through the valence transformatio n mechanism. This is particularly applicable in the case of metals whose different valence states vary in toxicity. In mercury-resistant bacteria, organomercuriallyase converts methyl mercury to Hg(II), which is one hundred-fold less toxic than methyl mercury (Siddiquee*et al*., 2015). The reduction of Cr(VI) to Cr(III) is widely studied, with Cr(III) having less mobility and toxicity. Other detoxification mechanisms of heavy metals are accomplished through metal binding, vacuole compartmentalization, and volatilization (Wu *et al*., 2010). .

Volatilization mechanisms involve turning metal ions into a volatile state. This is only possible with Se and Hg, which have volatile states. Mercury-resistant bacteria utilize the MerA enzyme to reduce Hg(II) to the volatile form Hg(0) (Siddiquee *et al*., 2015). The reduction of Se(V) to elemental Se(0) has been employed to remediate contaminated waters and soils. The metabolic processes of these organisms help to transform pollutants in the environment (Wu *et al*., 2010).

Most microorganisms are known to have specific genes for resistance to toxic ions of heavy metals. Mostly, the resistant genes are found on plasmids or on chromosomes (Nies, 1999). Bacteria were used as biosorbents because of their small size, their ubiquity, their ability to grow under controlled conditions and their resilience to a wide range of environmental situations (Urrutia, 1997). Table 1 lists out the name of some bacteria which may be used for bioremediation in heavy metals contaminated environment. They can remove heavy metals from contaminated sites either by bioaccumulation, precipitation or biosorption.

Table 1 Potential bacteria used for heavy metal remediation

Potential Bacteria	Heavy metal	Reference
Aeromonascaviae	Cd,Cr (IV)	Loukidouet al., 2004
Alcaligeneseutrophus	Cd	Mahvi and Diels, 2004
Bacillus coagulans	Cr (IV)	Srinath.,*et al*., 2002
Bacillus megaterium, and		
Bacillus firmus	Pb,Zn, Cu	Salehizadeh and Shojaosadati, 2003
Bacillus licheniformis,	Cd, Pb, Zn	Basha and Rajaganesh, 2014
Escherichia coli		
Salmonella typhi ,Bacillus	Cu, Cr, Fe	Samarth *et a*l., 2012
licheniformis		
Bacillus licheniformis	Cr (IV)	Zhou et al., 200
Pseudomonas stutzeri	Cr (IV)	Sahin and Ozturk, 2005
Bacillus thuringiensis	Ni	Ozturk, 2007
Corynebacteriumglutamicum	Pb	Choi and Yun, 2004
Nostocmuscorum	Cr (VI)	Gupta and Rastogi, 2008
Pseudomonas aeruginosa	Pb	Lin and Lai, 2006
Sphaerotilusatans	Cu	Beolchini *et al*., 2006
Sphingomonaspaucimobilis	Cd	Tangaromsuk *et al*., 2002
Staphylococcus xylosus	Cd, Cr (IV)	Ziagova *et al*., 2007
Streptomyces coelicolor	Cu	Ozturk *et al*., 2004
Streptomyces rimosus	Fe (III)	Selatnia *et al*., 2004
Thiobacillusferrooxidans	Zn	Liu *et al*., 2003

2.3.2.5. Bioleaching

It is a method of heavy metal mobilization through the excretion of organic acids or methylation reaction. In this method either bacteria or fungi are involved. *Thiobacillus* sp. are responsible for the oxidation of inorganic sulphur compounds (Mulligan *et al*., 2004), forming sulphuric acid. This can be utilized for desorbing the metals in the contaminated site by substitution of protons. *Aspergillus niger* offers also a promising alternative due to its citric

and gluconic acid production, these particular acids can act as chelating agents for heavy metal removal (Mulligan *et al.,* 2001; 2004). These methods include bioleaching, oxidation/reductio n reactions, bioaccumulation and biosorption.

2.3.2.6. Bioaccumulation

This technique is a substrate specific process, driven by ATP (Errasquin and Vazquez, 2003) and is an active process of heavy metal uptake. Three mechanisms of metal transport into the bacterial cell are known: passive diffusion, facilitated diffusion and active transport. Some of the active transport systems are metal selective. A disadvantage of bioaccumulation is the recovery of the accumulated metal which has to be done by destructive means leading to damage of the bio sorbent structural integrity (Ansari and Malik, 2007).

2.3.2.7. Biosorption

This method is another promising biochemical process, which can be applied for the removal of low concentrations of heavy metals in water (Mulligan *et al*., 2001).The complex structure of microorganisms implies that there are many ways for the metal to be taken up by the microbial cell.The choice organism must develop resistance towards metal ions as it comes into contact with the heavy metal pollutant to achieve the goal of remediation. The organism of choice may be native to the polluted environment or isolated from another environment and brought to the contaminated site (Sharma *et al*., 2000).

Advances in the understanding of metabolic pathways of microorganisms are responsible for metal sequestration, improving microbial survival rates, and their stability. This has led to the manipulation of metal adsorption (Gavrilescu *et al*., 2004). Adsorption is the physical adherence of ions and molecules onto the surface of another molecule. The material accumulated at the interface is the adsorbate and the solid surface is the adsorbent. If adsorption occurs and results in the formation of a stable molecular phase at the interface, this can be described as a surface complex. Most solids, including microorganisms, possess functional groups like –SH, –OH, and –COOH on their surfaces, that helps in the adsorption of metals (Gadd *et al*., 2009).

It has been reported that a microbial cell develops resistance to heavy metals through the excretion of metal chelating substances, or through a problem in a particular transport system, which results in a reduced cell accumulation of the metal ion. Another resistance mechanism includes the binding of a metal ion to intracellular molecules, such as metallothionein, vacuole, or mitochondria, which results in changes in the distribution of metal ion (Siddiquee *et al*., 2015). Microorganisms interact with metal ions through cell wall associated metals, intracellular accumulation, metalsiderophore, extracellular polymeric reactions with tr ansformation, extracellular mobilization or immobilization of metal ions, and volatilization of metals (Siddiquee*et al*., 2015).Extracellular Accumulation involves the removal of heavy metals by passive binding to non living biomass (Chen *et al*., 2005). Wherwas intracellular acc umulationis transport of the metal across the cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism.

The environmental conditions, prehistory, and pre treatment required for the removal of heavy metals need to be established in order to select the most appropriate biosorbents for a specific situation, from the extremely large pool of organisms that are readily available. Sometimes, the interest may be to recover a specific metal regardless of equilibrium concentration attained, or on the other hand, the interest may be to curtail levels of pollution in the effluent, in order to fall within the acceptable containment limit. Also, priority may be given to the recovery of a large quantity of metal, while also achieving low equilibrium concentrations. Whatever the case, the biosorbents used should have a high sorption capacity (Romera *et al.*, 2007).The biosorption mechanisms are various and are not fully understood. They may be classified according to various criteria.

3. MATERALS AND METHODS

3.1. Description of the Study Area

Tannery effluent waste was collected from Kombolcha Tannery Industry and laboratory studies were conducted at Wollo University Microbiological laboratory and at Haramaya University central laboratory.Kombolcha is located in Wollo.It is located between latitude and

longitude of 11°8′N and 39°38′E, respectively, and has an elevation that ranges from 1835 and 1843, meters above sea level.

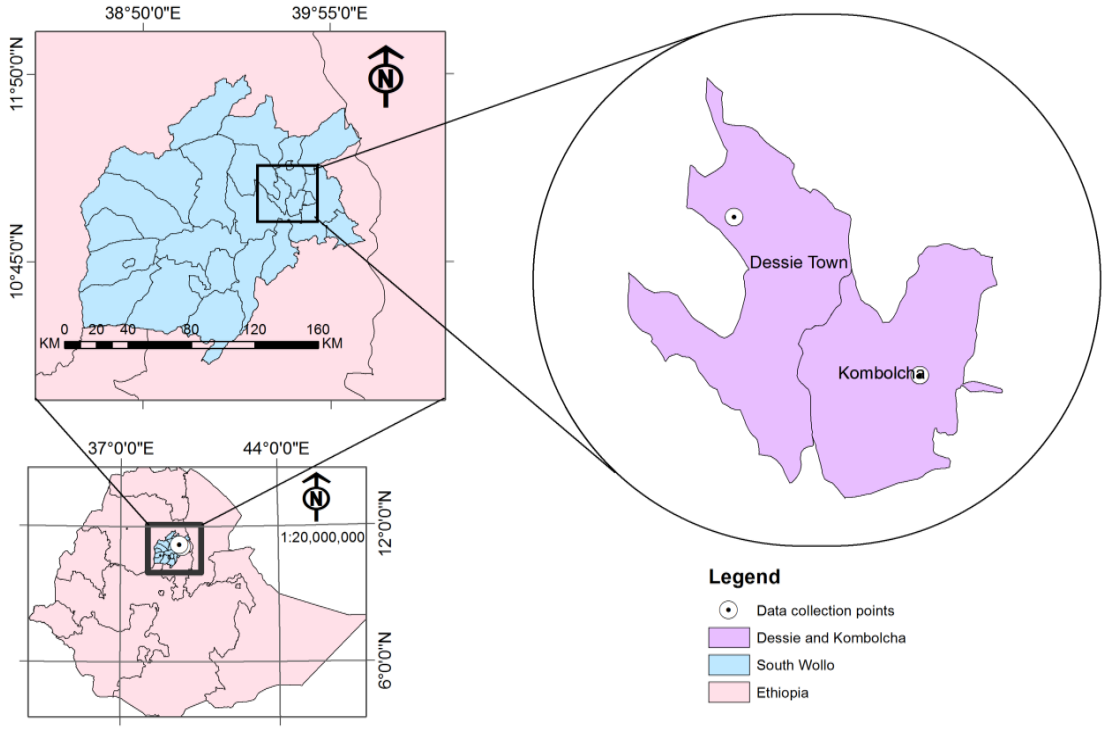


Fig. 1: Map of the study area

Figure1.Map of the study area

3.2. Tannery Effluent Sample Collection

Tannery effluent sample (500ml) was collected from industry's waste disposal areas in sterile bottle with stopper and brought to the Wollo University microbiological laboratory in ice box and stored in a refrigerator at 4^0C until analysis for different parameters including bacterial isolation, heavy metal content determination, etc.

3.3. Physico-Chemical Analysis of Tannery Effluent

The effluent water samples were determined for their Physico-chemical properties such as such as color, odor, pH, temperature, electrical conductivity (EC), total dissolved solids

(TDS), BOD (Biological Oxygen Demand), COD (Chemical Oxygen Demand) following standard methods for comparison with standard limits (WHO, 2011; US-EPA, 2009),(table.2).

3.4. Determination of the Levels of Heavy Metals in the Effluent Sample

The effluent waste water sample collected from Kombolcha tannery industry wasacidified immediately with HNO3 in order of 2 ml of HNO3 per liter of water and preserved in refrigerator at 4oC for laboratory analysis (Dhungan and Yadav, 2009). Samples were put in 250 ml beaker and digested using mixture of concentrated hydrochloric acid and concentrated nitric acid of 5ml and 2ml in volume respectively in a ratio of (5:2) decompose organic compounds. Then, the sample was evaporated to nearly dryness on a hot plate, cooled and further digested by adding the same acid mixture and heated till a clear solution was obtained (APHA, 1998).

The walls of the beaker were washed down with deionized water and samples were filtered through Whatman no. 1 filter paper (Whatman Inc., NJ, USA) into a 100 ml volumetric flask to remove silicate and other insoluble materials. Then each sample was made up to the mark of the flask with deionized water. The heavy metals in the digestate were then determined using atomic absorption spectrophotometer (Perkin Elmer model 5000).
Atomic absorption spectrophotometer grade metal solutions (Sigma Aldrich Chemicals, USA) were used as standards for quantification of the metals. The standard solution of the elements Cr, Pb and other metals were prepared by pouring the required amount of the solution from the stock solution, manufactured by Fisher Scientific Company, USA. Working standards were commonly prepared from stock solution at 1000 mg/L leveled using micropipettes. These solutions were prepared from their pure metal turnings and pure compound using acid mixture. Working standard and blanks were be acidified to the same extent as samples.

The atomic absorption instrument will be set up and flame condition and absorbance were To examine the heavy metal (Cr, Pb and Hg) tolerance of the isolated strains, a loopful of cells of overnight grown NA cultures were inoculated on nutrient agar plates supplemented with different concentrations of heavy metals (Chromium, in potassium dichromate, lead in lead

acetate, and mercury in mercuric chloride)Agar plates with no salts were used as negative controls.

3.5. Serial Dilution of the Effluent Waste Water, and Media Preparation and Bacterial Isolation

After clarifying filtration using Watmann filter paper No. 1, the TEWW samples were serially diluted in phosphatebuffered saline. The collected effluent water sample (1 ml) was serially diluted up to 10^{-6}. A 0.1 ml of each sere was spread plated in duplicate onto the Nutrient medium. Then all plates were incubated at 35±2.5°C for 24 hrs. P^H of the medium was 6.8 and final counts of colonies were noted. For isolation, morphologically distinct bacterial colonies were picked from 10^{-4}, 10-^{5} and10^{-6} Protocol for serial dilution (CFUs) (Alef and Nanninpieri, 1996).

For serial dilutions 1 ml of the tannery effluent sample was added in 9 ml of sterile 0.85 % (w/v) saline solution to make a one in 10 dilution (10^{-1}), and then one ml of this dilution was added to 9 ml 0.85 % (w/v) saline solution to make a one in 100 dilution (10^{-2}). This procedure was repeated until 10^{-6} dilution was reached. Triplicate samples of 0.1ml of each dilution were spread onto agar plates for microbial counts (Alef and Nanninpieri, 1996). Finally,number of bacteria (CFU) per milliliter calculated as:-

Number of colony (CFU) = $\frac{Number\ of\ colonies\ counted\ xDF}{Volume\ Plated}$

3.6. Stock Solution Preparation

Analytical grade salts of $K_2Cr_2O_7$, $HgCl_2$ and $Pb(CH_2COOH)_2$were used to prepare 1000 mg/l of stock solution. The exact quantities of the salts were dissolved in deionized distilled water and were filttered by micropipette with filtter paper. The prepared stock solutions were kept at 4°C and were used no longer than one month storage. The working 1000mg/l stok solution of metals were obtained by appropriate dilution in deionized water prior to each experiment.

3.7. Purification, Maintenance and Preservation of Culture

Isolates were purified by repeated streaking on Nutrient Agar plates until it become pure colonies and maintained on nutrient agar slants at 4°C. For long term preservation, 0.1 ml of each purified isolates was inoculated into 10 ml of nutrient broth and allows growing for 24-48 hours at 37^0C. Then1ml culture from these freshly grown log phase cultures were transferred to cryo vials containing glycerol stock media (40% glycerin) and stored at -20^0C. For routine use isolates were streaked on slants of nutrient agar, incubated at 37°C for 24 hours and refrigerated at 4°C.

3.8. Identification of Bacterial Isolates

Different parameters such as morphological examination, motility test, gram staining and biochemical tests were used to identify bacterial isolates cultured on growth media as follows

3.8.1. Gram staining

A loopful of log phase culture was smeared on a clear slide and stained through Gram's kit staining (Himadia Lab Pvt Ltd) and was performed following the instruction manual the manufacturer. The bacteria were stained by Crystal Violet, Iodine Solution, Alcohol (decolorizing agent) and Safranin stain.Upon staining gram-positive bacteriahave retained crystal violet and hence appeared deep violet in color, while Gram-negative bacteria had lost the crystal violet and it was counter stained by the Safraninto appear red in color. The slide was observed under oil immersion objective lens of compound microscope. Gram's reaction, shape and arrangement of the cells were recorded.

3.8. 2. Screening and Isolation of Heavy Metal Tolerant Bacteria

To examine the heavy metal (Cr, Pb and Hg) tolerance of the isolated strains, a loopful of cells of overnight grown NA cultures were inoculated on nutrient agar plates supplemented with different concentrations of heavy metals (Chromium, in potassium dichromate, Lead in lead acetate, and Mercury in mercuric chloride). Agar plates with no salts were used as negative controls.The inoculated plates were then incubated at 37oc for 24 hours.

Bacterial colonies, which showed better growth on nutrient agar plates supplemented with heavy metal solutions, were considered as resistant strains and bacterial colonies were taken and streaked in the nutrient agar slants and stored. Samples from master plates, which failed to develop colonies in heavy metal treated plates, were considered as sensitive bacterial colonies. The isolated and distinct colonies on these selective media were sub- cultured repeatedly on the same media for purification andoptimized for the analyte. Then blank, standards and samples were aspirated into the FAAS (Model- iCE 3000, Thermo Scientific). The analytical procedures were also calibrated against the above standard reference materials (Islam *et al*.,2013).

3.8.3. Morphological Characterization

All the selected isolates tolerant to Cr, Pband Hg were further characterized on the basis of colony morphology as described by Bergey‟s Manuals of Bacteriology(Bergey *et al., 1974).*

3.8.4. Biochemical Characterization of Isolates

3.8.4.1. Triple Sugar Iron Agar TestS

Triple sugar iron agar (slant) tubes were prepared (appendix) and autoclaved. All selected 17 cultures inoculated in TSI tubes and incubated as described (Ponmurugan *et al*., 2012) and (Patel *et al*., 2013).

3.8.4.2. Citrate Utilization Test

The isolates were tested in Simmon’s citrate agar(%, w0.02;ammonium dihydrogen phosphate, 0.1; dipotassium phosphate, 0.1; sodium citrate, 0.2; NaCl, 0.5; bromothymol blue, 0.008; agar, 1.5%; pH 6.8). Bacterial strains capable of utilizing citrate as a sole source of carbon and energy grew well and produced a color shift from greenish to blue due to alkaline pH of the medium that was created as citrate is utilized by the isolates (Bhagat*et al*., 2016).

3.8.4.3. Catalase Test

A loopful of bacteria culture was put on clean slid and drop of 3% of H2O2 reagent was mixed with the culture . Finally the production of bubbled gas showed the +ve results on the slid because H_2O_2 was broken down in to water and oxygen.

3.8.4.4. Lactose Fermentation test

Lactose broth was prepared based on the instruction of the manufacturer and new inoculums were inoculated in the broth with inoculating loop and incubated at 370C for 24-48 hrs.Lactose fermentation was confirmed by yellow color formation.This desipline was used for glucose , fructose and sucrose tests.

3.8.4.5. Manitol Salt Agar (MSA) Test

MSA was prepared on the plates and the fresh isolate was inoculated on MSA media
It was incubated at 370C for 24 hrs and different results were observed.

3.8.4.6. Indoe Test

Tryptone was prepared in to the test tube and the isolated bacteria were inoculated in the browth at 37^0C for 24hrs.15 drops of Kovac's reagent was added.The development of bright red color at the interface of the reagent was an indication of positive result.

3.9. Heavy Metal Resistance Test of TEWW Bacteria

The selected heavy metalresistant bacterial strains were determined for their maximum tolerance against each heavymetal with different concentrations. In order to find out bacteria resistant to Cr, Pb and Hg. Isolates were inoculated into tannery effluent water nutrient, broth treated with different concentrations (50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600,1000 mg/l) of the selected heavy metals.

Bacterial colonies which showed better growth in 50 ppm of $K2Cr_2O_7$, $Pb(CH_2COOH)_2$and $HgCl_2$ treated plates were considered as highly resistant and were selected for characterization and further experiment. (Guo*et al*., 2009).Some reference strains of Gram positive and Gram negative species such as *Escherichia coli* ATCC 25922, *Escherichia coli*

K12 PCG86, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212, with previously determined sensitivity or tolerance to metals at the tested concentrations, were used as controls of the metal activities.

Finally the number of colony forming units (cfu) observed in the plates were adjusted to cfu/ml of the effluent. Cr, Pb and Hg-tolerant cfu/ml percentages were calculated by comparison with the results obtained in the medium without metal using the formula: number of cfu/mL on Nutrient Agar supplemented with metal x 100 / number of cfu/mL on Nutrient Agar.

$$\% \text{ Cr, Pb and Hg-tolerant cfu/ml} = \frac{number\ of\ cfu/ml\ on\ Nutrient\ Agar\ supplemented\ with\ metal\ x\ 100}{number\ of\ cfu/ml\ on\ Nutrient\ Agar}$$

Whereas, the reduction of heavymetal in isolated bacteria was calculated by the following ways (Lolo WalMarzan*et al*., 2017).

$$\% \text{ of heavy metal utilized} = \frac{1}{4}\ X\ \left(\frac{Heavy\ metal\ utilized\ (PPM)}{Heavey\ metal\ added\ in\ nutrient\ broth}\ X100\right)$$

3.10. Determination of minimum inhibitory concentration of Heavy Metals for Selected Isolates

Maximum resistance of the selected isolates against increasing concentrations of Cr, Pb and Hg on NA plates was evaluated until the strains unable to grow colonies on the agar plates. The initial metal concentration used 50 ppm was prepared from 1000mg/l stock solution. The stock solutions, $K_2Cr_2O_7$, Pb $(CH_2COOH)_2$ and1000 mg/l HgCl2 were prepared in sterile deionized water and sterilized though micropippate by filter paper. The culture growing on the last concentration was transferred to nutrient broth. The broth after 24 hours of incubation was spread plated on to next higher concentration. The procedure was repeated until the isolates were unable to grow on the heavy metal incorporated media and MIC value was determined. The culture grow at a given concentration were subsequently transferred to the next concentration

3.11. Instrumental Calibrations

The data qualities obtained from FAAS for metal analysis are highly affected by the calibration and standard solutions preparation procedures. The instrument was calibrated using a series of standard working solutions of each of the metals prepared freshly by appropriate dilution of the intermediate standard solutions. The intermediate standard solutions were prepared from stock solution of each metal. The concentrations of the intermediate standards, working standard solutions and values of correlation coefficients of the calibration graphs for the two metals of interest were showed in Appendix.

3.12. Data Analysis

The data were summarized into tables and graphs by analyzing using (ANOVA) on Microsoft office excel spread sheet (Excel, 2010) and analyzed using Statistical Package for Social sciences (SPSS) version 20. LSD (least significant difference) test were used to identify significant differences among treatment means. P values < 0.05 were considered significant in all cases.

4. RESULTS AND DISCUSSIONS

4.1 The Physico-Chemical Characteristics of Tannery Wastewater

The permissible limits for parameters in the wastewater from an industrial establishment mentioned in the rightmost columns are stipulated by World Health Organization (WHO, 2002).

Table 2: The physicochemical characteristicsof tannery wastewater

Parameter	Tests	Permissible Reference Standards
Color	Brown Color	Colorless
Odor	Foul Smell	Foul smell
Temperature (^{o}c)	27	≤ 35
pH	5.6	5.5-9.0
Electrical conductivity (µs/cm)	114	1200
DO (mg/l)	2.84	4.5
BOD(mg/l)	59.4	300
COD (mg/l)	142	250
TSS (mg/l)	1000	600
TDS (ppm)	1300	1200

BOD = Biological Oxygen Demand, COD = Chemical Oxygen Demand, DO = Dissolved Oxygen, TDS = Total Dissolved Solid, TSS = Total Suspended Solid

The size of a tannery, nature of the chemicals used in it and its final products determine the quality of effluent waste produced discharged.The color of waste water discharged from Kombolcha tannery industry has brown color. When compared with standard reference, these features are below the acceptable quality, suggesting that the effluent it discharges is enriched with huge amounts of pollutants. Moreover, Total Dissolved Solid (TDS) and Total Suspended Solid (TSS) were found beyond the permissible level showing the extent of pollution by its discharge (Table 2).

On the other hand,pH and temperature of the wastewater were recorded to be 5.6 and 27°C which were in the range of standards. The amount of Dissolved Oxygen (DO) was found far below the reference standard (Table 2) showing very high depletion of oxygen in the effluent that may suffocate aquatic organisms if the waste water is discharged into the

nearby water bodies.The fact that BOD, COD and EC were found below the stipulated permissible levels show that the effluent discharged from Kombolcha tannery industry are highly polluted.

4.2.Levels of Heavy Metals in the Tannery Effluent

After the digestion and filtering procedure was completed, the sample was checked by AAs based on the standards of each metal. As a result the highest element was iron which accounts 50.5% and the list was chromium that accounts 0.75%. The heavy metal content of the treated effluent is presented in Table3.

Table 3. Comparison of the parameters of the collected tannery wastewater sample with the permissible limit stipulated by WHO (World Health Organization).

Parameters/Elements	Amount in Waste water (ppm)	Permissible Limit (ppm)	Relative proportion of each metal
Cr	0.25	2	0.75
Pb	1.66	-	4.32
Mg	5.6	0.1	14.76
Fe	17.7	10	50.5
Cd	-	2	-
Ni	10.5	1	27.4
Zn	3.2	3	8.22
Total			100

The chemical qualities of waste water samples from Kombolcha tannery were compared with the standard limits set by various regulatory bodies including World Health Organizat ion (WHO) (2011) and, U.S. Environmental Protection Agency (US EPA) (2009). Except Cr. all other tested heavy metals were found much above the permissible limit in waste water discharged from Kombolcha tannery industry (Table 3).The low level of Cr. which are also highly toxic to life if found beyond the permissible limit may be due to the partial treatment efficiency of wastewater from the industry or because of less amount of usage of these compounds in the processing. However, the fact that all other heavy metals were above the permissible limit suggests more effort to be put to minimizing these metals from being discharged with the effluents. For example, biological treatments through microbes can be an alternative means of removing these pollutants before the waste is discharged into the environment. The microbe based approach for removal and recovery of toxic metals from industrial effluents can be economical and more efficient in comparison to

physicochemical methods for heavy metal removal (Mindlin.*et al*., 2001; Zouboulis*et al*., 2004). Various mechanisms have been postulated for the development of metal resistance in microorganisms (Nwuche*et al*., 2008; Otth *et al*., 2005). However, in general, all these strategies are found either to prevent the entry of metal ions into the cell or to actively pump out from the cell (Percival *et al*., 2005)

4.3. Colony Morphology, Gram Staining and Motility Test

Results of characterization of the isolated bacteria based on their morphology, gram staining and motility tests were given in Table 4 below. In this study, 0.1 ml ot TEWW was spread on 20 % NA plates and incubated at 37^0C for 24 hours. After incubation, the size, shape, color, elevation and margins of the bacterial colonies were observed under high power microscope. Freshly grown bacterial cultures were subjected to Gram staining and were observed under the microscope for their Gram reaction and shape of the bacterial cells. The Stab motility media was prepared and bacteria were inoculated with inoculating needle. The motile bacteria were grown away from the stab line, and test is positive. While, none motile bacteria were only grown along the stab line.

Table 4: Gram staining and colony characteristics of the isolated bacteria

Isolate	Motility test	Colony color	Gram stain	Shape/size	Elevation	Surface	Spp. may be
B-02	Negitive	Milky	Negative	rod/Small	Convex	Smooth	*Klebsiella* spp.
B-03	Positive	Milky/ whitish	Negative	rod /Large	Flat	Smooth	*Pseudomonas* spp.
B-15	Positive	Golden Yellow	Negative	Rod/large	Convex	Smooth Shiny	*Escherichia Coli*
B-25	positive	White	Negative	Rod	Convex	Smooth	*Enterobacter* spp.
B-51	Negative	White	Positive	Rod/Small	Flat	Smooth	*Salmonella* spp.
B -54	Positive	Milky	Negative	Rod/Large	Flat	Smooth	*Clostridium*sp.

B-02,B-03,B-15, B-25, B-51 and B -54 =Bacterial isolates

4.4. Biochemical Characterization of Isolates

Various micro-organisms that can withstand the toxic condition alone can prevail in the tannery effluent. Likewise many micro-organisms have been isolated by researchers all around the world from tannery effluent for chromium remediation (Shukla*et al*., 2007; Verma*et al*., 2001; Ahmed and Abdul, 2009; Sinha*et al*., 2011).So,biochemical tests were performed to characterize these microorganisms based on their biochemical properties. The biochemical tests performed in the present study were, Catalase test, Indole production test, Starch hydrolysis test, Triple Sugar Iron agar (TSI) test, Methyl Red (MR) test, Citrate utilization test, Oxidation-Fermentation (OF) test, Glucose, fructose and lactose fermentation test were done (Muzaddadi, 2013).The results are shown in Table5.

Table 5: Biochemical characterization of isolates,

Test/Isolate	B-01	B-02	B-03	B-04	B-11	B-14	B-15	B-25	B-28	B-42	B-51	B-54	B-56
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	-	-	+	-	-	+	+	-	-	+	-
Manito Salt Agar	+	-	-	+	+	+	+	+	+	-	+	+	+
Citrate Utilization	-	+	+	-	+	+	-	+	-	-	-	+	+
Methyl Red Test	-	-	-	+	+	-	+	-	-	-	-	-	-
Triple Sugar Iren	K/A	A/G	A/G	A/A	A/A	A/A	A/A	A/A, G	A/A	A/A	K/A, H_2S	A/A	K/A
test	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	-	+	+	-	+
Oxidase test	+	+	+	-	-	+	+	-	+	+	-	+	+
Indole test	-	-	-	-	-	-	+	-	+	-	-	-	-

+ = Positive and - = Negative

K/NC: Alkaline slant/no change in the butt = Glucose, lactose and sucrose non-utilizer (alkaline slant/alkaline butt).

K/A: Alkaline slant/acid butt = Glucose fermentation only.

A/A: Acid slant/acid butt, with gas production = Glucose, sucrose, and/or lactose fermenter

K/A: Alkaline slant/acid butt, H_2S production

A/G: Acid slant/ Gas production

4.5. Isolation of Cr, Pb and Hg Resistant Bacteria

After serial dilution of the tannery effluent, 60 bacterial isolates were selected.Bacterial isolates were marked as B-01, to B-02 toB-60. Further purification was done by streak plate method. Then after, 17 isolates were obtained in nutrient agar containing 50 ppm potassium dichromate, lead acetate and mercuric chloride respectively. Microbial resistance to Chromium [Cr (VI)] , Lead [Pb (II)]and Mercury[Hg(II) were determined by visible growth after 24 hrs in NA and minimal broth supplemented with varying concentrat ions of the respective salts.

The treated isolates were compared with control. Therefore, the presence of metals in the growth medium has as an effect to conserve and to keep the character of resistance in the bacteria, contrary to its or their absence allowed the direct loss of this resistance.Finally, six potential heavy metal degrading isolates (B-02, B-03, B-15, B-25, B-51 and B-54) were characterized based on their cultural, morphological and biochemical characteristics (Table 3). Compared with standard description of Berge's Manual of determinative bacteri ology 9th edition (Bergey *et al*., 1974; Williams and Wilkins, 1994). Six best isolates i.e. *Kl ebsiella* sp., *Salmonella* sp.,*Pseudomonas* sp., *E.coli*, *Enterobacter* sp. and *Clostridium* sp. were selected for subsequent supplement of different concentration of $K_2Cr_2O_7$, Pb(CH_2C $OOH)_2$ and $HgCl_2$ based on the MIC test data**.**

4.6. The Resistance Capacity of Selected Bacterial Isolates

Some of the bacterial isolatesused in this study showed very high-level of resistance against chromium, mercury and lead. The isolated bacteria's (B-02 and B-51) were resisted up to 2200 ppm of Cr,(B-25 and B-54) were resisted up to 1050 ppm of Hg and B-03 &B-15) were resisted 330 and 340 ppm of Pb respectivelly. The maximum biosorption of chromium by B-02 and B-51 (Klebsiella sp. and Salmonella sp.) were 90%, whereas, the maximum biosorption of Mercury by Enterobactersp.andClostridiumsp. (B-25 and B-5) and for lead by Pseudomonas spp. and E. coli (B-03 and B-15) were 85% and 80% respectively. In this study the optimum PH and temperature were 6.8 and 370C, (figure, 2, 3 and 4).

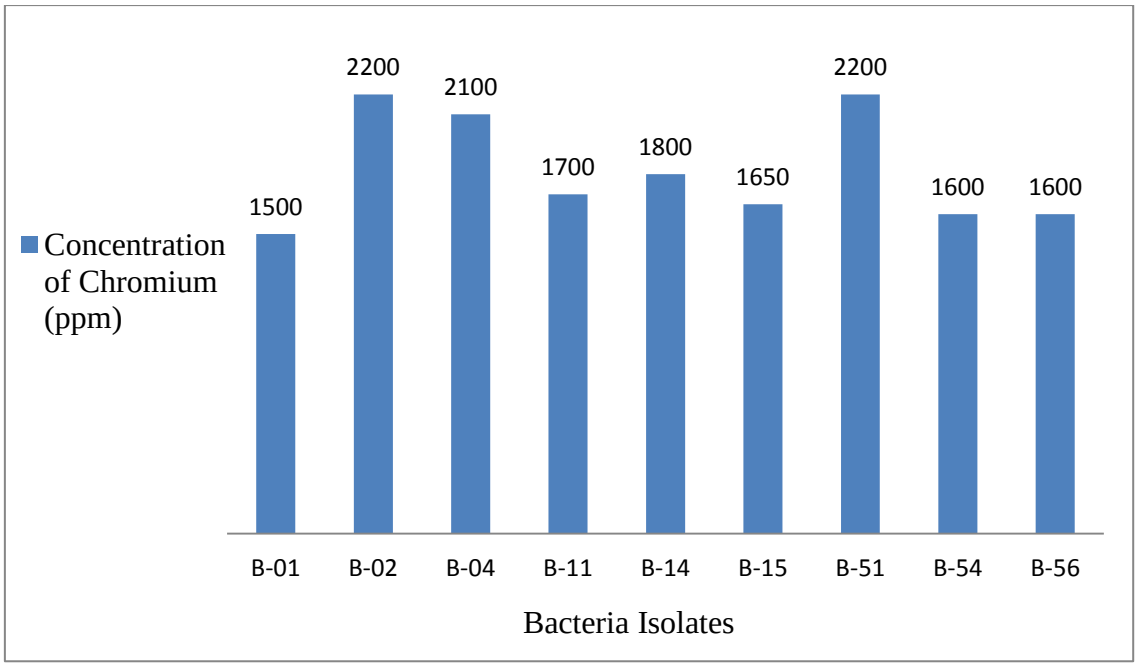


Figure 2. Resistance of bacteria isolates to different concentrations of chromium

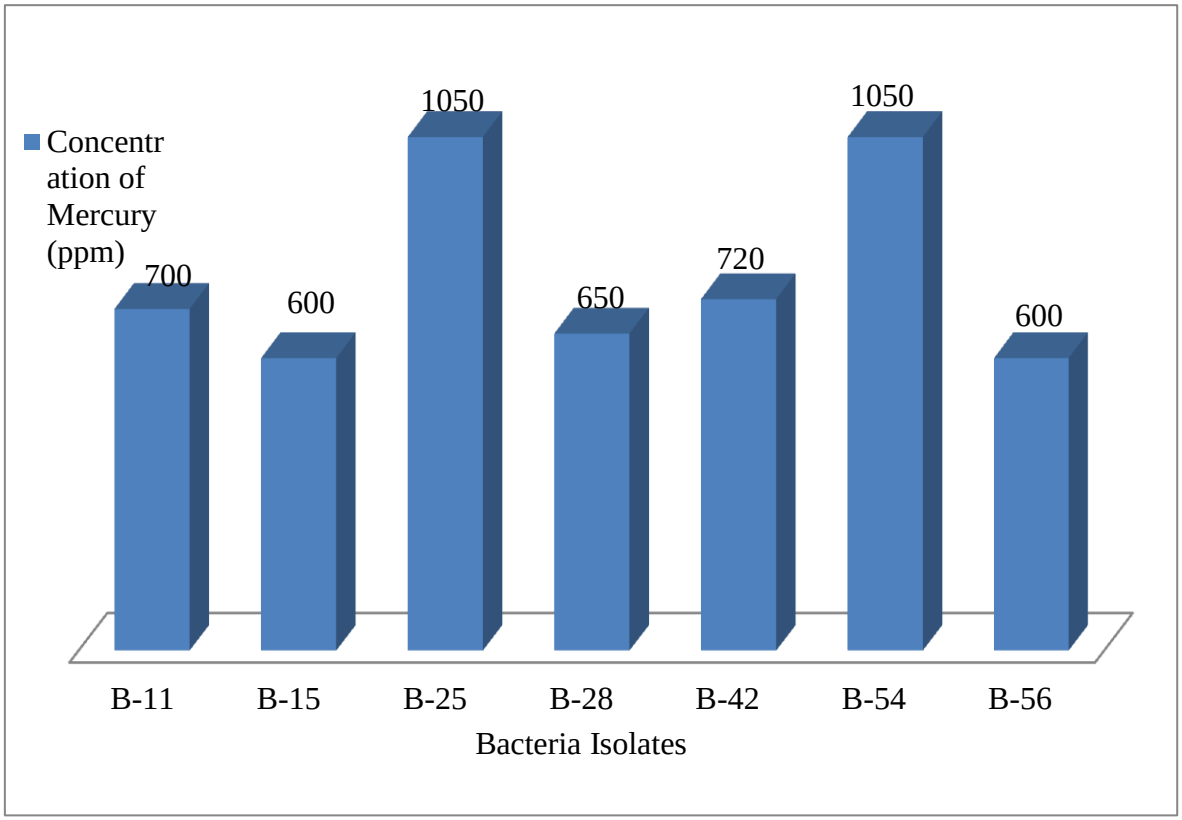


Figure 3.Resistance of bacteria isolates to different concentration of Mercury

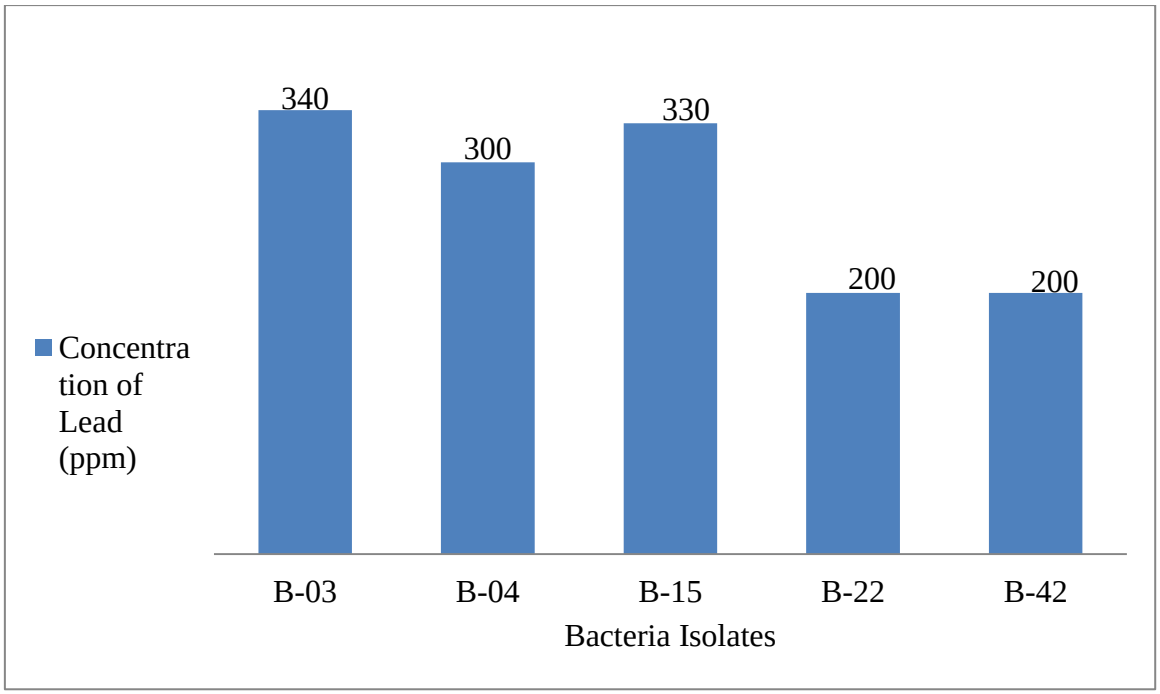


Figure 4.Resistance of bacteria isolates to different concentration of Lead

In these finding ten bacteria isolates were resisted more than 650 ppm of Chromium. Whereas,*Klebsiella* spp. and *Salmonella* spp.(B-02 and B-51) were tolerated up to 2200 ppm of Chromium. On the other hand, seven bacteria isolates were tolerated more than 500 ppm of Mercury. The two best bacteria isolates, which were resisted up to 1050 ppm of Mercury concentration are *Pseudomonas* spp. and *E. col*i (B-03 and B-15) respectively. In the case of Lead, five bacteria isolates were resisted more than 200 ppm concentration. The most Lead resistance bacteria isolate (*Enterobacte*r spp. and *Clostridium* spp. (B-25 andB-54)) were resisted 330 and 340 ppm.

Psedomonasaeruginosa and *Brevibacillushoshinensis* isolated from the textile industrial effluent which decolorize the effluent in the presence of 10% glucose very effectively within 7 days (Annika *et al*., 2012). Mahmood *et al*. (2013) reported *Bacillus subtilus,Bacil lus cereus, Bacillus mycoides, Pseudomonas* spp. and *Micrococcus* spp. can be the best biological tool for textile and tannery effluent treatment.

In the present studies,B-02 and b-51 bacteria have a potential of 90%Chromium (Cr(VI))bio remediation. This was almost similar withHossain (2005).In his study, themaximum biosorption of chromium by *Bacillus subtilis* is found up to 94.25 % with optimum pH of 3.5 and temperature 40^{o}C. Besides, Bacterial species *Pseudomonas putida*and *Bacillus licheniformes* has a potential application for the bioremediation of heavy metals from domestic and industrial waste water with moderate concentrations of

heavy metals (Kamika and Mumba, 2012). The other similar report with the present finding was reported by Congeevaram *et al*. (2007),*Micrococcus* spp. shows maximum removal of Cr (VI) 90% at pH 7.0.

4.7. Levels of Cr, Pb and Hg in Selected Bacteria Isolates

Bacterial isolates were tested for concentration of Cr andPb in their cells using AAS at their corresponding wavelengths viz. 228.8nmand 283.3nm, respectively. The absorbance obtained was related to their corresponding concentration from the concentration standard curves done for each metal. Results show that Cr and Pb concentrations were 0.0155 and 0.0182ppm, respectively. However, it was not possible to determine the concentration of Hg from the bacterial isolates due to lack of facilities. Butthe results showed that Pd and Cr. were able to be sorbed onto bacterial cells and this served as means of removal of these pollutants from the waste**.**

4.8. Determination of the Minimum Inhibitory Concentration of Heavy Metals for Selected Isolates

Bacterial colonies were streaked onto NA agar amended with increasing heavy metal concentrations until the growth completely ceased (Kathiravan*et al*., 2011). The MIC of the six isolates in the present studies was shown in Table 6.

The MIC for all isolates was found to vary between isolates and types of heavy metals and its concentration. MIC of Chromium resistance bacteria were between 500 to 2225 ppm. The bacteria, *Klebsiella* spp. and *Salmonella* spp. (B02 and B-51) were resisted 2200 ppm of Chromium(Cr (VI)).This shows the two bacteria have high resistance to Cr (VI).The MIC in Mercury resistance bacteria isolates were between 450and 1100 ppm.The bacteria, *Enterobacter* sp. and *Clostridium* sp. (B-25 and B-54) was tolerated maximum concentration of Hg, i.e. 1050 ppm each. The two bacteria, in this study indicate higher tolerant capacity of Hg. On the other hand, MIC of Lead resistance bacteria isolateswas between 200 and 340. Two Pb(II) resistant bacteria, *Pseudomonas*sp. *And E.* col*i (B-03 and B-15)*were tolerated 330 and 340 ppm, respectively.

The MIC determined for the three heavy metals were varied depending on the metal and bacterial isolate. This study showed a high incidence of metal resistance for the bacterial isolates. Many bacterial species isolated from industrial effluents were shown to develop

resistance to heavy metals .The increase in the MIC of metals among the bacterial population in any system could be a sign of risk to the ecosystem and all living species.

Table 6 MIC for Chromium, Mercury and Lead against the selected isolates

Isolates	MIC (ppm)		
	Chromium	Mercury	Lead
B-01	1800	ND	ND
B-02	2225	ND	ND
B-03	ND	ND	330
B-04	1950	ND	320
B-11	2000	800	ND
B-14	2100	ND	300
B-15	2000	700	340
B-22	ND	ND	320
B-25	ND	1100	ND
B-28	ND	600	ND
B-42	ND	700	ND
B-43	ND	ND	300
B-51	2225	ND	ND
B-54	2200	1100	ND
B-56	1950	800	250

ND = Not Determined

4.9.Growth Patterns of the Six most Resistant Bacteria under Different Heavy Metal Supplementation

Bacterial isolates B-02 and B-51 showed decreasing trend with increasing concentration of Cr. (Fig, 5).

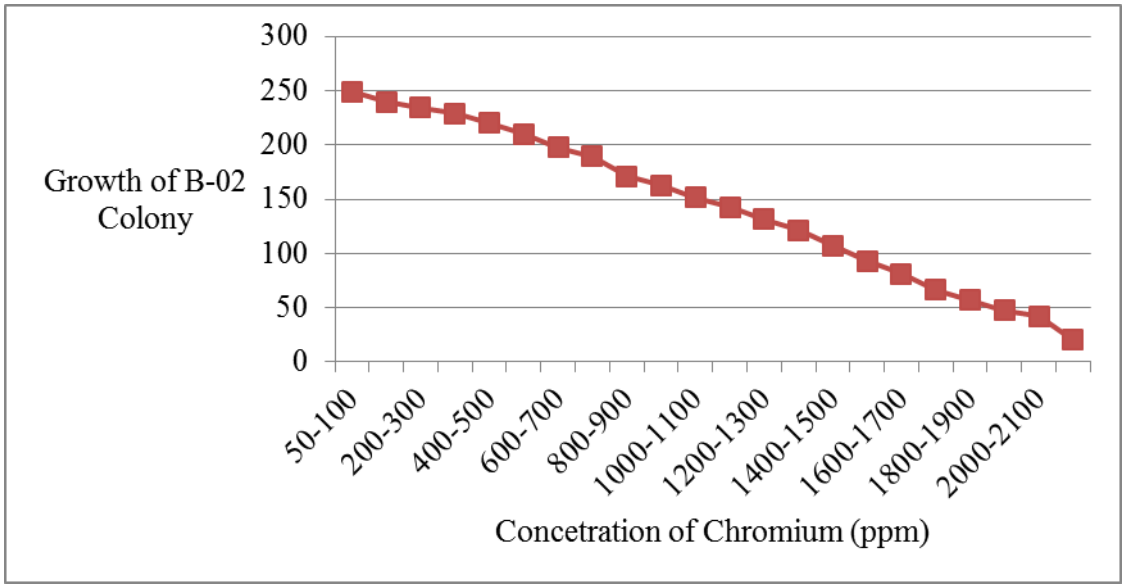


Figure2.The relation between chromium concentrations andgrowth of B-02

(*Klebsiella*sp.)

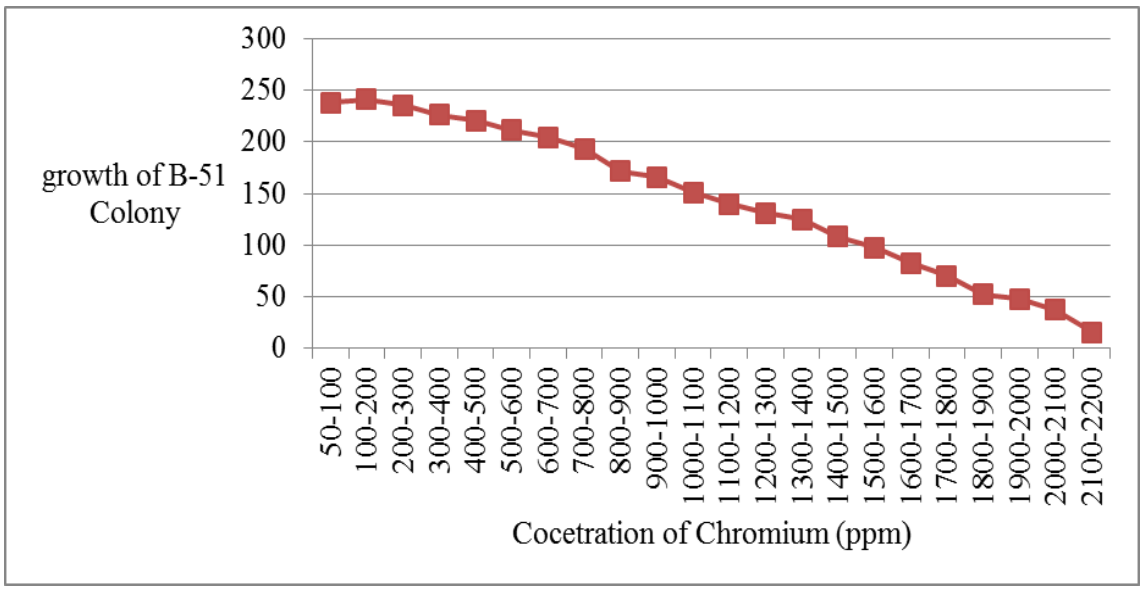


Figure6.The relation between mercury concentration and growth of B-51 (*Salmonella*sp.).

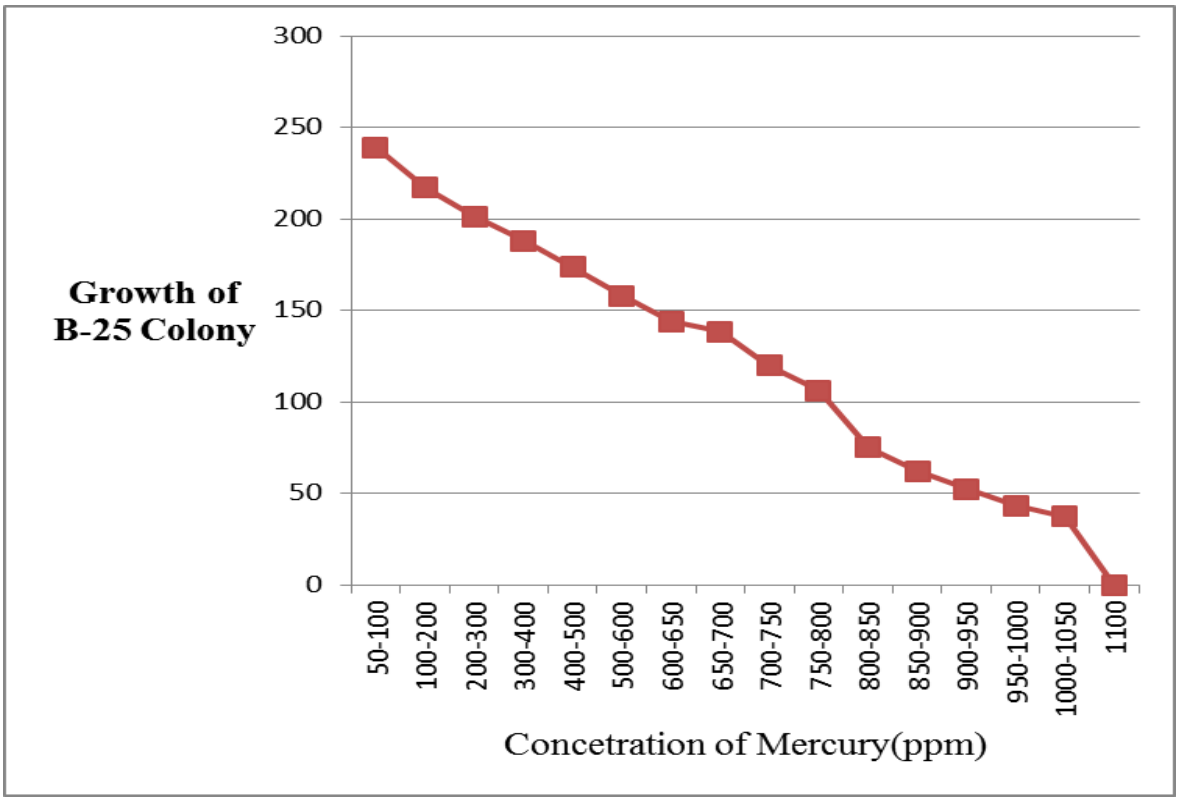


Figure 7.The relation between Mercury concentration andgrowth of B-25 (*Salmonella*sp.)

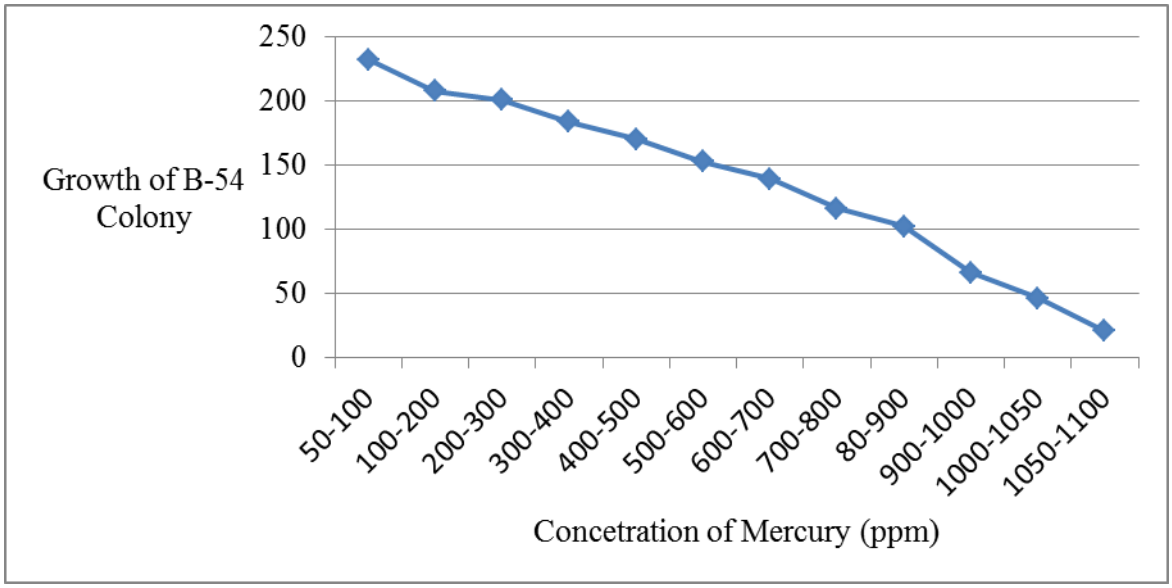


Figure 8.The relation between mercury concentration and growth of B-54 (*Clostridium* sp.

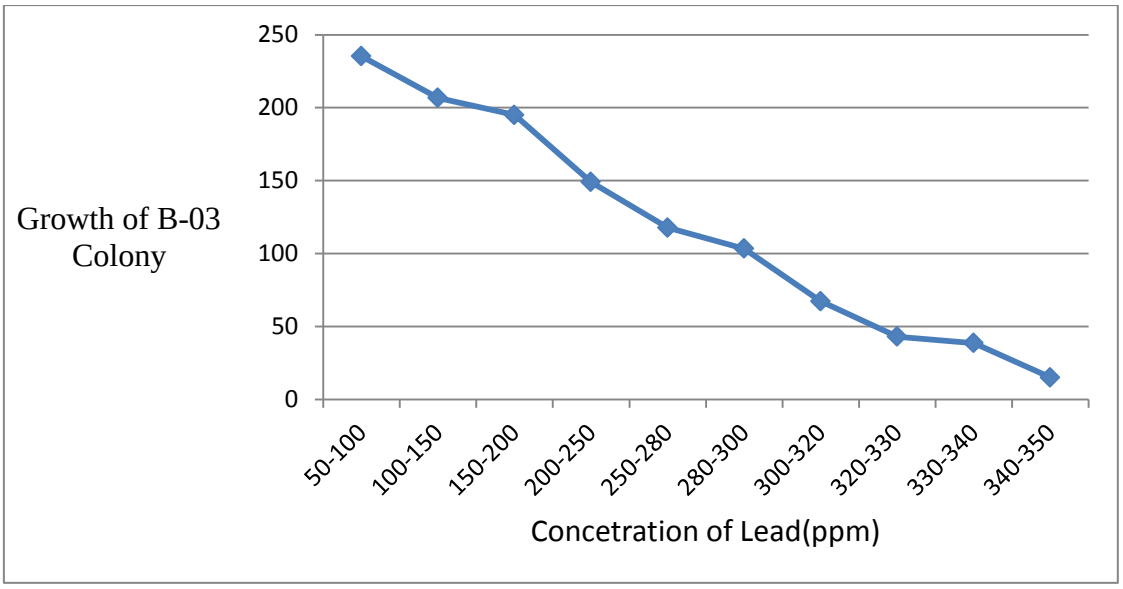


Figure9: The relation between lead concentrations and growth of B-03 (*Pseudomonas* sp.)

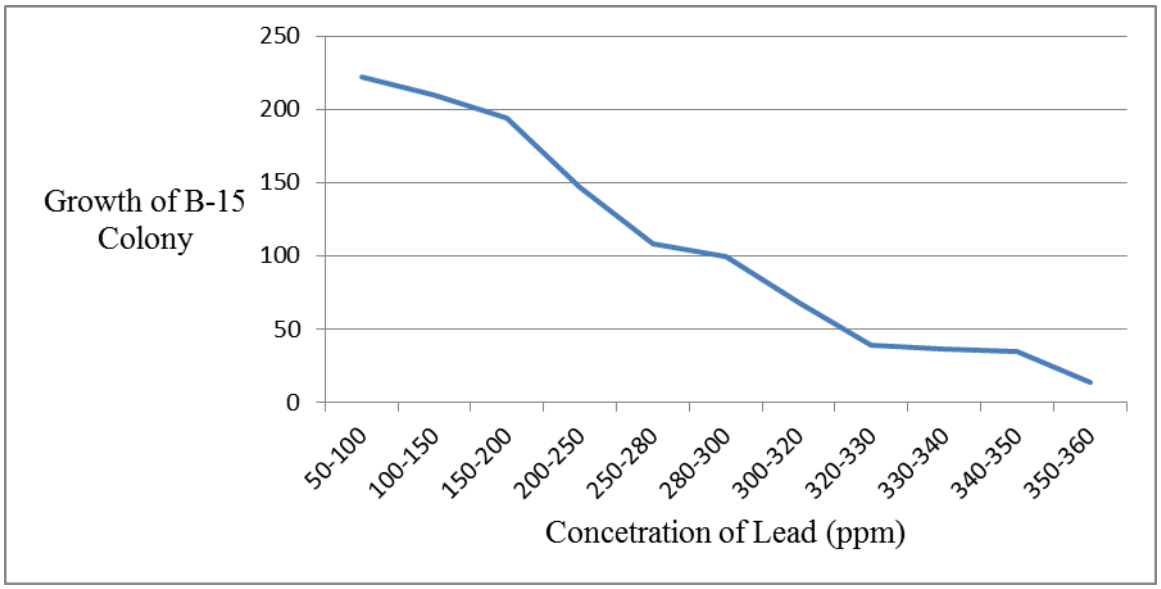


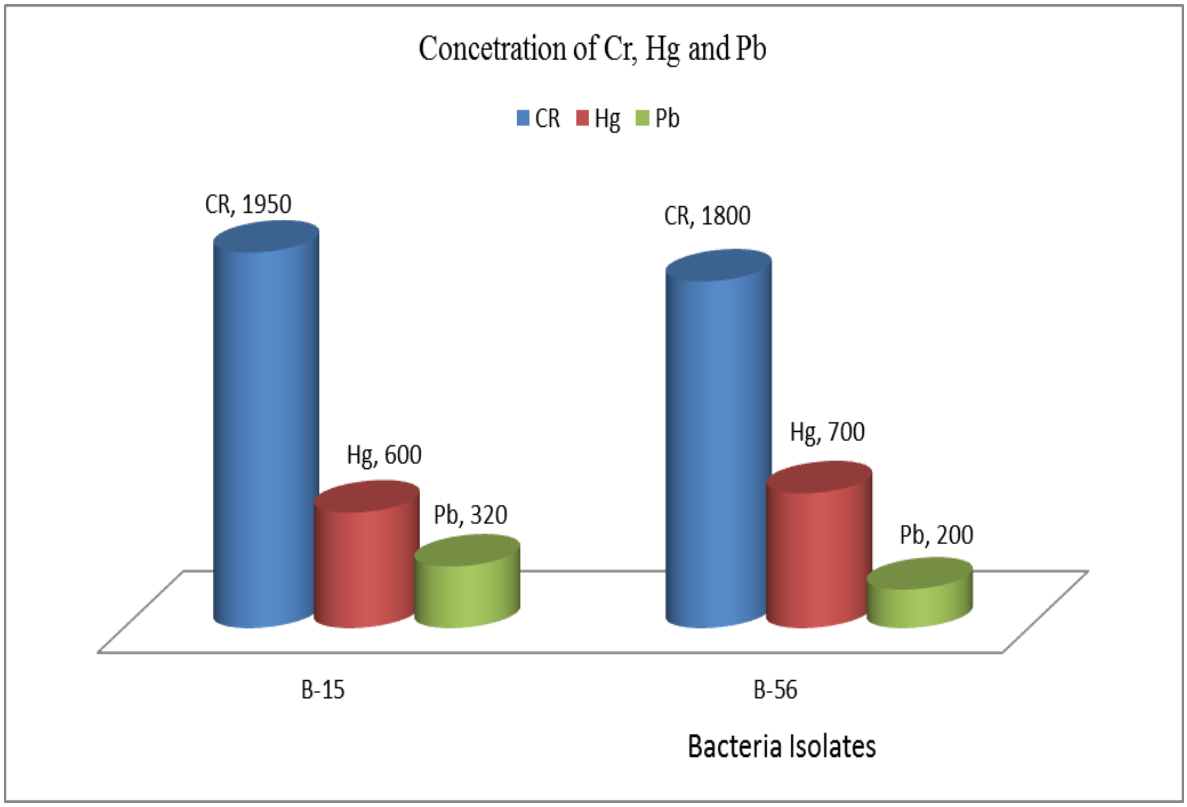
Figure10. The relation between lead concentrations and growth of B-15 (*E.* coli)

In the present study, the relationships of bacteria growth with concentration are opposite.As the concentrations of the heavy metal were increased the growth of the bacteria were decreased. Thus, all the tested heavy metals are toxic for bacteria as concentration increases. In addition, the redaction capacities of the isolates were also decreased as concentrations increased. Therefore, the presence of metals in the growth medium has as an effect to conserve and to keep the character of resistance in the bacteria, contrary to its or their absence allowed the direct loss of this resistance. Different researchers were used different way of sterilization of heavy metal .Some sterilized with

media at 1210c for 15 min and some other used micropipette and filtering methods. The sterilization variation were varied the resistivity and minimum inhibition concentration.

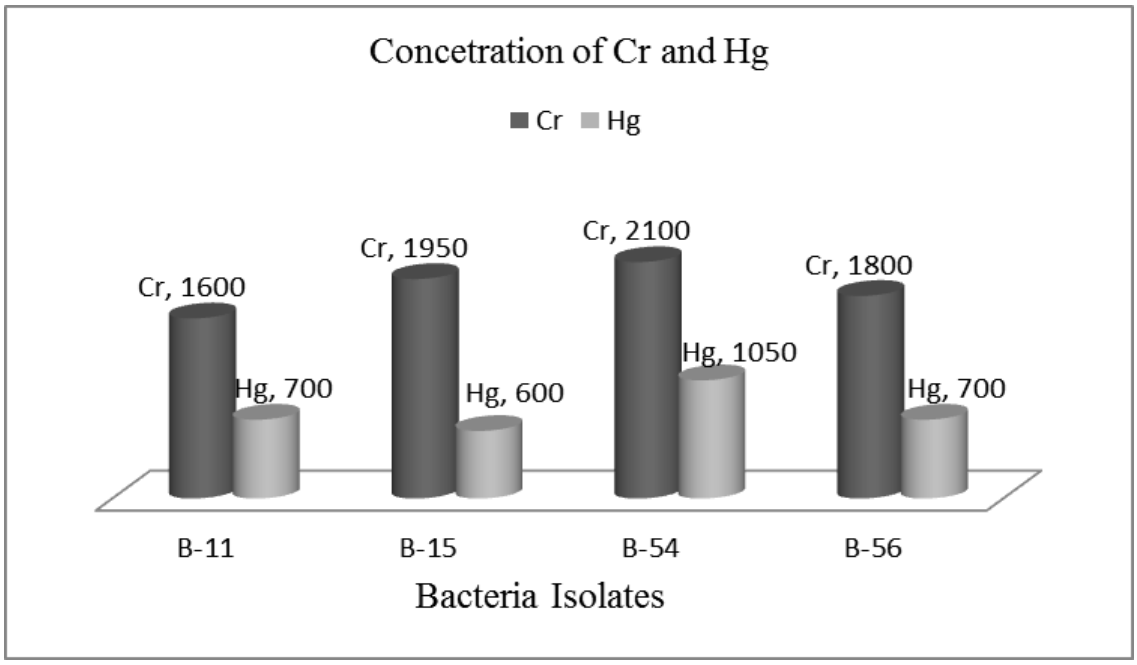
4.10. Resistance of Isolated Bacteria to Multiple Heavy metals

In this study, some isolates were resisted all the three heavy metals and some other were tolerated two heavy metals. The detail was showed on figure 10,11 and 12.



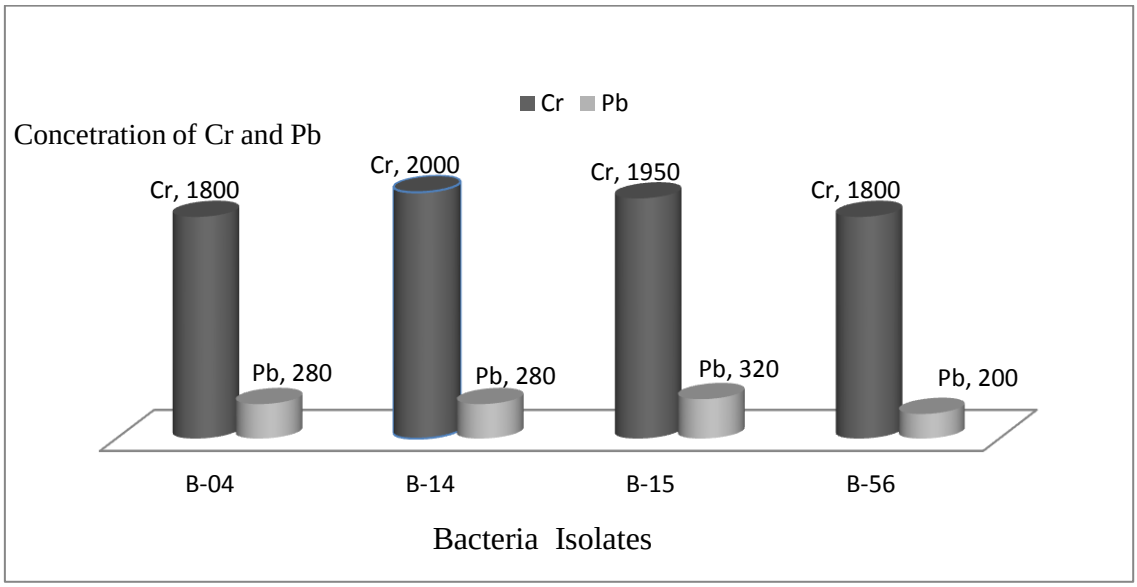
Cr= Chromium, Hg = Mercury and Pb = Lead

Figure11.Multiple heavy metal resistant of isolates



Cr = Chromium and Hg = Mercury

Figure 12.isolated bacteria which are resistant to Chromium and Mercury



Cr = Chromium and Pb = Lead

Figure13. Isolated bacteria which are resistant to Chromium andLead

Some of the bacterial isolates used in this study showed very high-level resistance against potassium dichromate, mercuric chloride and lead acetate in nutrient broth and the results are in concurrence with the results of many scientists. Bacterial tolerance to chromate

($CrO4^{2-}$) has been found in several Pseudomonas strains(Boop*et al*., 1983) and(Basu*et al*., 1997) reported that, isolated chromium resistant bacteria from effluent of tanneries and found that they could resist up to 250 µg/ml of Cr (VI) in the medium. Megharaj*et al.*, 2003 also reported strains, which were isolated from polluted soil, could resist up to 100 µg/ml of Cr (VI). Strains reported by Filali*et al*. (2000)also exhibited different heavy metals resistances.

Resistance of toxic metals in bacteria probably reflects the degree of environmental contamination with these substances and may be directly related to exposure of bacteria to them. However, unpolluted environments may also harbor metal resistant organism or organisms that readily adapt to high concentrations of metals. The incidence of plasmid bearing strains is more in polluted sites than in the unpolluted sites (Malik ,2000).

The three bacterial isolates used in this study were found tolerant to multiple metals. These observations assume great significance because effluent from any metal-related industry contains several metal ions/ contaminants. Furthermore, when there are other metal contaminants, it might be practical to use the heavy metal tolerant bacteria to remove heavy metals (Lovely, 1995).

5. SUMMARY, CONCLUSION AND RECOMFNDATIONS

5.1. Summary and Conclusion

There is a global concern about the treatment of industry wastes due to its ultimate impact on the ecosystem and human health. Based on this concern, the present study was conducted by selecting different parameters relevant to indicate the amounts of pollutants in the tannery effluent and to look other alternatives of treatments rather than physicochem ical treatments. The physicochemical parameters were measured by different instruments. Most were beyond the permissible limits. On the other hand, the level of heavy metal in TEWW was investigated by AAS analysis.

The Kombolcha tannery industry effluent samples were showed the average concentration of Cr and Cd (0.25ppm and 0 ppm) were below the permissible limits, whereas, the average concentration of Fe, Ni, Mg and Zn (17.7, 10.5, 5.6& 3 ppm) were by far above the permissible limits. The various, Physico-chemical parameters and heavy metals content of the treated tannery effluent discharged into the environment were analyzed and the values reveals that it will enhance the pollution load and pose severe threats to human health and environment by ultimately entering the food chain. Thus, the discharged effluent requires further remediation. So, biological treatment especially, bacteria were needed. During the present investigation efforts were made to isolate strains of bacteria which tolerate and accumulate heavy metal salt (chromium, lead and mercury).

Out of 60 bacterial isolates from tannery effluent water sample, 17 isolates were obtained in nutrient agar containing 50 ppm potassium dichromate, lead acetate and mercuric chloride respectively. The isolated bacteria were identified based on, morphology, gram stained and various biochemical tests. Microorganisms that were exposed to TEWW were survived in highly enriched heavy metals environment and they were adapted to this stress by developing various resistance mechanisms. These mechanisms, on the other hand, could be utilized for the treatment and removal of heavy metals from tannery effluent. Six best isolate i.e. *Klebsiella* sp. (B-02), *Pseudomona*s sp. (B-03), *E. coli* (B-15), *Enterobacter* sp. (b-25), *Salmonella* spp. (B-51) and *Clostridium* sp.(B-54) were selected. The future prospect lies in the application of these bacteria for purposes like heavy metal remediation and potential use for removing toxic metals and other pollutants from industrial effluents. The heavy-metal chloride salt of mercury, acetate of lead and chromate salt of chromium at

varying concentrations (50 ppmto1000ppm), (50ppm to 350 ppm) and (50ppmto 2200ppm) were taken for the study.

The minimum inhibitory concentration (MIC) values for the isolated bacteria's were detected. MIC of the heavy metal resistant bacterial isolates were grown on heavy metals incorporated media, against respective heavy metal was determined by gradually increasing the concentrations of the heavy metals, each time on NA plate and NB until the strains are unable to grow on the plate. The starting concentration used was 50 ppm. Heavy metals which were used in different concentrations included Cr (VI) (50–2200 ppm), Hg (50- 1050 ppm) and Pb (50-340ppm). Plates were incubated at 370c for 48 hrs. The MIC of chromium resistant bacteria, B-02 and B-51 were 2200 ppm, for lead, B-03 and B-15 was 330 ppm and for mercury, B-25 and B-54 were 1050ppm.

The maximum biosobtion capacity of the isolate bacteria was 90%, 80% and 85% for each heavy metal respectively. In addition, the isolated bacteria have a capacity to resist multiple heavy metals. For instance, B-15 was resisted 1950ppm Cr, 600ppm Hg 320ppm and Pb whereas B-56 was resisted 1800ppm Cr, 700ppm Hg and 200 ppm Pb. In this investigation, the relationships of bacteria growth with concentration were contrary. As the concentrations of the heavy metal were exceeded the growth of the bacteria were decreased. Thus, all the tested heavy metals are toxic for bacteria as concentration increases. In addition, the redaction capacity of the isolates was also decline as concentrations increased.

5.2. Recommendations

The finding of this research has provided six isolated bacteria which are best resistant of chromium, mercury and lead. It indicates industrial waste treated with biological methods were best rather physical and chemical treatments. Based on this research finding, it is important to set the following points as recommendations.

- In the present study, best heavy metal resistant bacteria were isolated. So, biological treatment methods should be applied in industry instead of physico-chemical treatments. Because, it has a low cost, efficient, no need of sophisticated technology as well as environmentally friendly technology for the treatment of industrial wastewater. It is considered the best choice to ensure high treatment efficiency and performance under metal stresses especially when industrial effluents are involved.

- Natural habitats are generally characterized by the coexistence of a large number of toxic and nontoxic actions and therefore, it is necessary to study multiple metal effects on the physiology and biochemistry of microorganisms (Verma et al., 1995).
- Even though Kombolcha tannery effluent showed less amounts of chromium and cadmium, the other metals (Fe, Ni, Zn, Mg,etc.) And its physicochemical parameters are beyond the permissible limits. Thus, it should be further treated to be safe the ecosystem.
- Molecular identification of the isolated bacteria should be done to investigate the type of chemicals or proteins secreted by the microorganisms in order to gain resistance against heavy metals and other pollutant.
- Mercury was not detected in the resistant bacteria and in the TEWW as well, due to lake of instruments and standards even in Ethiopia. So, immediatealternative should be set.

6. REFERENCES

Abioye and O.Peter, 2011. Biological remediation of hydrocarbon and heavy metals contaminated soil. *Soil Contamination*,7: 127–142.

Agrawal, P., Mittal, A., Kumar, M., and Tripathi, S.K. 2008. Mercury exposure in Indian environment due to coal fired thermal power plants and existing legislations. *Indian Journal of Forensic Medicine and Pathology*, 1(2): 1016-1022.

Ahmed Zahoor and Rehman Abdul (2009). Isolation of Cr (VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater.*Journal of Environmental sciences*, 21(6): 814- 820.

Akcil, A., Erust, C., Ozdemiroglu, S., Fonti, V., Beolchini, F. 2015. A review of approaches and techniques used in aquatic contaminated sediments: Metal removal and stabilization by chemical and biotechnological processes. *Journal of Cleaner Production*, 86: 24–36

Alef, Y., Nannipieri, P., 1996. *Methods in Applied Soil Microbiology and Biochemistry.Academic Press. London.*

Alina, M., Azrina, A., Yunus, M.A.S., Zakiuddin, M.S., Effendi, M.I.H., and Rizal, M.R., 2012. Heavy metals (Mercury, Arsenic, Cadmium) in selected marine fish and shellfish along the straits of Malacca.*International Food Research Journal*, 19(1): 35-140.

Alloway, B.J. 1990. *Heavy metals in soils*. *Glasgow and London: Blackie and Son Ltd*

Annika, A.D., Arvind, R.G., Sayali, R.N. 2012. Decolourisation of textile dyes and biological stains by bacterial strains isolated from Industrial effluents. *Advances in applied science research*, 3 (5): 2660-2671.

Anderson, C.R. and Cook, G.M. 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand.*current micro biology*, 48: 341-347.

Ansari, M.I. & Malik, A. 2007. Biosorption of nickel and cadmium by metal resistant bacterial isolates from agricultural soil irrigated with industrial wastewater. *Bioresource Technology* , 98: 3149-3153.

APHA. 1998. Standard methods for the examination of Water and Waste water. *American Public Health Association*. 20. APHA Press, Washington, DC.

Argun, M.E., Dursun, S., Ozdemir, C., Karatas, M. 2007. Heavy metal adsorption by modified oak sawdust: Thermodynamics and kinetics. *Journal of Hazardous Materials*, 141: 77–85.

Ashraf, R. and Ali, T.A. 2007.Effect of heavy metals on soil microbial community and mung beans seed germination.*Pakistan Journals of Botany*, 39 (2), 629-636.

Bailey, S.E., Olin, T.J., Bricka, R.M., Adrian, D.D. 1999, A review of potentially low-cost sorbents for heavy metals. *Water Research*, 33: 2469-2479.

Baird, C. and Cann, M. 2005. Environmental Chemistry. W.H. Freeman and New York Company

Banjerdkji, P. Vattanaviboon, P. Mongkolsuk,S. 2005. Exposure to cadmium elevates expression of genes in theoxy R and Ohr R regulons and induces cross-resistance toperoxide killing treatment in Xanthomonascampestris. *Applied Environmental Microbiology*, 71 (4): 1843-1849.

Basha, S.A. and K. Rajaganesh. 2014. Microbial bioremediation of heavy metals from textile industry dye effluents using isolated bacterial strains. *International Journal of Current Microbiology and Applied Scienc*e, 3: 785-794.

Basu, M., Bhattacharya, S. and Paul, A.K. 1997.Isolation and characterization of chromium resistant bacteria from tannery effluent. *Bulletin Environtal Contamination and Toxicology* , 58: 535-542

Bergey, D.H. Buchanan, R.E. Gibbons, N.E. 1974. Bergey's manual of determinative bacteriology. Williams and Wilkins C.O. Balti- more, p: 1246.

Bernard, A. 2008.Cadmium and its adverse effects on human health.*Indian Journal of Medicinal Research*, 128(4): 557-564.

Bhagat, N., Vermani, M. and Bajwa, H.S. 2016.Characterization of Heavy Metal (Cadmium and Nickle) Tolerant Gram Negative Enteric Bacteria from Polluted Yamuna River, Delhi. *African Journal of Microbiology Research*, 10(5): 127-137.

Biology online. 2007. Fungus, http://www.biology-online.org/dictionary/FungusDate retrieved: 21/08/09

Blessy, M.B. and Krishnamurthy, N.B. 2015. Health effects caused by metal contaminated ground water. *International Journal of Advanced Science and Research*, 1(2): 60-64.

Brar, S.K. Verma, M. Surampalli, R. Misra, K. Tyagi, R. Meunier, N.; Blais, J. 2006 Bioremediation of hazardous wastes-A review. *Practice Periodical of Hazardous, Toxic and Radioactive Waste Management*, 10: 59–72.

Bopp, LH, Chakrabarty AM & Ehrlich ML. 1983. Chromate resistance plasmid in Pseudomonas aerugines.*Journal of Bacteriology*, 155:1105.

Brown, M.j. and Lester, J.N. 1982. Role of bacterial extracellular polymers in metal uptake in pure bacterial culture and activated sludge effect of metal concentration.*Water Resource*, 16: 1539.

Buvaneswari, S., Muthukumaran, M. Damodarkumar, S. Murugesan, S. 2013. Isolation and Identification of predominant bacteria to evaluate the bioremediation in sugar mill effluent. *International Journal of Current Science*, 5: 123-132.

Carlos, J. and Chuken S. 2011.Biosorption of Metals by Phenol-Resistant Bacterial Isolates from Contaminated Industrial Effluents.*African Journal of Microbiology Research*, 5(18): 2627-2631

Chandra, R. Bharagava, R.N, Kapley, A. and Purohit, H.J. 2011.Bacterial diversity, organic pollutants and their metabolites in two aeration lagoons of common effluent treatment plant (CETP) during the degradation and detoxification of tannery wastewater.*Bioresource Technology*, 102: 2333–2341.

Chaturvedi, A.D. Pal, D. Penta, S. Kumar, A. 2015.Eco-toxic heavy metals transformation by bacteria and fungi in aquatic ecosystem. *World Journal of Microbiology and Biotechnology*, 31: 1595–1603.

Cheng S (2003). Heavy Metal Pollution in China: Origin, Pattern and Control. *Environmental Science and Pollution Reteach*, 10 (3): 192-198.

Chen, C.W., Chen, C.F. and Dong, C.D. 2012. Distribution and Accumulation of Mercury in Sediments of Kaohsiung River Mouth, Taiwan. APCBEE Procedia,1: 153-158.

Chen, J.Z., Tao, X.C., Xu, J., Zhang, T. and Liu, Z.L. 2005a. Biosorption of lead, cadmium and mercury by immobilized Microcystisaeroginosa in a column. *Process Biochemistry*, 40: 3675-3679.

Chhikara, S. Dhankhar, R. 2008. Biosorption of Cr (VI) ions from electroplating industrial effluent using immobilized Aspergillusniger biomass. *Journal of Environmental Biology*, 29, 773–778.

Choi, S.B. and Y.S. Yun. 2004. Lead biosorption by waste biomass of Corynebacteriumglutamicum generated from lysine fermentation process. *Biotechnolgy Letters*. 26: 331-336.

Chowdhury, S. Bala, N.N. and Dhauria, P. 2012. Bioremediation-a natural way for cleaner environment.*International Journal of Phamaceutical, Chemical and Biological Sciences*. 2(4): 600-611.

Congeevaram, S., Dhanarani, S., Park, J., Michael, D. and Kaliannan, T. 2007. Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates.*Journal of Hazardous Materials*, 146: 270–277.

Corretto, E. Antonielli, L., Sessitsch, A., Compant, S., Höfer, C., Puschenreiter, M. and Brader, G. 2017. Abstract Complete genome sequence of the heavy metal resistant bacterium Agro

mycesaureus AR33T and comparison with related Actinobacteria. *Standards in Genomic Sciences*. 12:2-10.

Dakiky, M. K., M. H. Khamis, A. N. Manassra, and M. R. Mereb, 2002. Selective Adsorption of Chromium (VI) in Industrial Waste Water using Low-Cost Abundantly available Adsorbents. *Journal of Advance in Environmental Research*, 6:533-540.

Dell'Amico, E., Mazzocchi, M., Cavalca, L., Allievi, L. &Andreoni, V. (2008). Assessment of bacterial community structure in a long term copper polluted exvineyard soil. Microbiological Research, 163: 671-683.

Dhungana, T.P and Yadav, P.N. 2009. Determination of Chromium in Tannery Effluent and Study of Adsorption of Cr (VI) on Sawdust and Charcoal from Sugarcane Bagasses.*Journal of Nepal Chemical Society*, 23: 93- 101.

Di Toppisanita, L. and Gabbrielli, R. 1999. Response to cadmium in higher plants.*Environmental and Experimental Botany*. 41(2): 105-130.

Dietz, K.J., Baier, M. and Krämer, U. 1999.Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants.*In. Heavy Metal Stress in*. 201: 73

Dixit, R. Malaviya, D. Pandiyan, K. Singh, U.B. Sahu, A. Shukla, R. Singh, B.P. Rai, J.P. Sharma, P.K. Lade, H. 2015. Bioremediation of heavy metals from soil and aquatic environment: *An overview of principles and criteria of fundamental processes. Sustainability*, 7: 2189–2212.

Durga, B. Devi, Thatheyus, A.J. and Ramya D. 2012.Bioremoval of hexavalent chromium, using Pseudomonas fluorescens.*Journal of Microbiology and Biotechnology Research*, 2:727-735.

Duruibe, J.O., Ogwuegbu, M.O.C. and Egwurugwu, J.N. 2007.Heavy metal pollution and human biotoxic effects.*International Journal of Physical Sciences*, 2: 112-118.

Errasquin, E.L. and Vazquez, C. 2003. Tolerance and uptake of heavy metals by Trichodermaatroviride isolated from sludge. *Chemosphere*, 50:137-143.

FilaliMeknassi, Y. Tyagi, R.D. and Narasiah, KS. 2000. Simultaneous sewage sludge digestionand metal leaching: effect of aeration. Process Biochemistry, 36: 263-273.

Gadd, G.M. 2009. Biosorption: Critical review of scientificrationale, environmental importance and significance for pollution treatment. Journal of Chemical Technology and Biotechnology, 84: 13–28.

Gaur, N., Flora, G., Yadav, M., Tiwari, A. 2014. A review with recent advancements on bioremediation-based abolition of heavy metals. Environ. Sci. Process. Impacts, 16: 180–193.

Gavrilescu, M. 2004. Removal of heavy metals from the environment by biosorption.*Engineering and Life Science*, 4: 219–232.

Gopalan, R. and Veeramani, H. 1994. Studies on microbial chromate reduction by Pseudomonas sp.In aerobic continuous suspended growth.*Biotechnology and Bioengineering*, 43(6): 471-476.

Guo, Z., Megharaj, M. Beer, M. Ming, H. Rahman, M. M. WU, W. and Naidu, R. 2009. Heavy metal impact on bacterial biomass based on dna analysis and uptake by wild plants in the abandoned copper mine soils. *Bioresource technology*, 100: 3831-3836.

Gupta, N. and Kumar, V. 2012. Identification and isolation of heavy metal (copper) resistant bacteria. *Archives of Applied Science Research*, 4(1): 577-583.

Gupta, V.K. and A. Rastogi. 2008. Sorption and desorption studies of chromium (VI) from non-viable cyanobacteriumNostocmuscorum biomass. *Journal of Hazardous Materials*, 154: 347-35

Hanel, K., 1986. Biological treatment of Sewage by the activated sludge process. Ellis Horwood limited, London.

Hetzer, A., Daughney, C.J. and Morgan, H.W.2006. Cadmium Ion Biosorption by the thermophilic Bacteria Geobacillusstearo-thermophilus and G. thermocatenulatus. *Applied and Environmental Microbiology*, 72: 4020-4027.

Irwin, I.J. and Baird, A.W. 2004. Extremophiles and their applications to veterinary medicine.*Irish Veterinary Journal*, 57: 348-354.

Islam, E.U. Yang X. He Z. and Mahnmood Q. 2007. Assessing potential dietary toxicity of heavy metals in selected vegetables and food crocks. *Journal of Zhejiang University Science* 8: 1–13.

Islam, S., Islam, F. Bakar, M.A. Das, S. and Bhuiyan, H.R., 2013. Heavy metals concentration at different tannery wastewater canal of Chittagong city in Bangladesh.*International Journal of Agriculture, Environment and Biotechnology*, 6(3): 355-62.

John, H. 2006. Medical microbiology a brief introduction, university of leeds faculty of biological sciences Uk medicine net 2009.

Jordao, C.P. Nascentes, C.C. Cecon, P.R. Fontes, R.L.F. and Pereira, J.L. 2006. Heavy metal availability in soil amended with composted urban solid wastes. *Environmental Monitoring and Assessment*, 112: 309–326.

Kamika, L. Momba, M.N.B. 2012.Comparing the tolerance limits of selected bacterial and protozoan species to vanadium in waste water systems.*Water Air and Soil pollution*, 223(5): 2525-2539.

Karnachuk, O.V. Kurochkina, S.Y. Nicomrat, D., Frank, Y.A., Ivasenko, D.A., Phyllipenko, E.A. and Tuovinen, O.H. 2003. Copper resistance in Desulfovibrio strain R2. *Antonie Van Leeuwenhoek*, 83: 99-106.

Kazemipour, M. Ansari M., Tajrobehkar S., Majdzadeh M., and Kermani H.R. 2008. Removal of lead, cadmium, zinc, and copper from industrial wastewater by carbon developed from walnut, hazelnut, almond, pistachio shell, and apricot stone. *Journal of Hazardous Materials*, 150: 322–327.

Keusch, G.T., Fontaine, O. and Bhargava, A. 2006. "DiarrheDiseases".

Khan S., Cao, Q., Zheng Y.M., Huang Y.Z. and Zhu Y.G. 2008. Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. *Environmental Pollution*, 152: 686-692

Krishnaveni, R., Pramila Devi, Y. RamgopalRao., S. 2013. Bioremediation of steel industrial effluents using soil microorganisms.*International journal of advanced Biotechnology and Research*, 4(1): 51-56.

Lee, DH, Lee I.K, Song K, Steffes, M. Toscano, W, Baker and Jacobs Dr Jr. 2006. A strong dose-response relation between serum concentrations of persistent organic pollutants and diabetes.*Diabetes care*, 29: 1638–1644.

LI, Y., Pong, H., He. G., Wang. Q. and Sheng, 2017. Cd immobilization and reduce tissue accumulation of rice (Oryza sativa Wuyun-23) in the presence of heavy metal resistant bacteria. *Ecotoxicology and environmental safety*, 138: 56-63.

Lin, C.C. and Y.T. Lai. 2006. Adsorption and recovery of lead (II) from aqueous solutions by immobilized Pseudomonas aeruginosa PU21 beads. *Journal of Hazardous Materian*, 137: 99-105.

Liu, H.L. Chen, B.Y. Lan, Y.,W. and Cheng, Y.C. 2004. Biosorption of Zn (II) and Cu (II) by the indigenous Thiobacillusthiooxidans. Chemical Engineering Journal, 97(2-3): 195-201.

Lolo WalMarzan ,MehjabeenHossain, SohanaAkter Mina, YasminAkter, A.M., Masudul Azad Chowdhury. 2017. *Egyptian Journal of Aquatic Research*, 43: 65–74.

Loukidou, M.X. Karapantsios, Th.D., Zouboulis, A.I. and Matis, K.A. 2004. (i) 'Diffusion kinetic study of chromium (VI) biosorption by Aeromonascaviae. Industrial and Engineering Chemistry Research, 43: 1748–1755; (ii) 'Diffusion kinetic study of cadmium(II) biosorption by Aeromonascaviae'. *Journal of Chemistry, Technology and Biotechnology*, 79: 711–719. 25.

Lovely DR. 1995.Bioremediation of organic and metal contaminants with dissimilatory metal reduction.*Journal of Indian Microbiology*, 14: 85-93.

Maghsoodi, V. Samadi, A. Ghobadi, Z. 2007.Biodegradation of effluents from dairy plant by bacterial isolates.*Iran Journal of Chemistry and Engineering*, 26 (1).

Mahesh, A. James, R. Peterson, N. Johnstone, L. Johnstone, C. Turick, E. David, R. Y. and William, A. A. 1997. Bacterial reduction of hexavalent chromium by Enterobacter cloacae strain HO1 grown on sucrose. *Biotechnology Letters*. 19: 691–694.

Mahmood, R., Shariff.R.Ali,S. Hayyat, M. U. 2013. Bioremediation of textile effluents by indigenous bacterial consortia and its effects on zea mays L.CVC 1415.*Journal of Animal and Plant Sciences*. 23 (4): 1193-1199.

Mahvi, A. H. and Manahan, S.E. 2004. Environmental Chemistry. Boca Raton: CRC Press LLC.

Malik, A. and Jaiswal, R. 2000. Metal resistance in Pseudomonas strains isolated from soil treated with industrial wastewater. *World Journal of Microbiology and Biotechnology*, 16:177-182.

Mani, D. Kumar, C. 2014. Biotechnological advances in bioremediation of heavy metals contaminated ecosystems: An overview with special reference to phytoremediation. *International Journal of Environtal Science and Technology*, 11: 843–872.

Manoharan, C., Subramanian, G., 1993. Feasibility studies using cyanobacteria in ossein effluent treatment. *Indian Journal of Environmental Health*, 35(2): 88-96

Megharaj M, Avudainayagam S &Neidu R. 2003. Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. *Current Microbiology*, 47: 51-54.

McGrath, S.P. 1994. Effects of Heavy Metals from Sewage Sludge on Soil Microbes in Agricultural Ecosystems. In: Ross, S.M. (Ed.), Toxic Metals in Soil-Plant Systems. Wiley, New York, 247-273.

Metcalf and Eddy Inc. (2003). Wastewater engineering: treatment and reuse / revised by georgetchobanoglous, franklin l. Burton and h. David stensel. 4. New York: Mcgraw-Hill Higher Education.

Mohana, S. Shrivastava, S. Divehi, J. Medawar, D. 2008. Bioresource Technology, 99, 562-569.

Monika, D. Kawser, A.M.D. Fauzia, B. Sahana, P. MonirulIslam, M.D. Mahmuda, B. 2010. Microbial load in Tannery and textile effluents and their receiving rivers of Dhaka. *Journal of Biological sciences*, 19 (1): 73-81.

Mulligan, C.N., Kamali, M. & Gibbs, B.F. 2004.Bioleaching of heavy metals from a low-grade mining ore using Aspergillusniger.*Journal of Hazardous Materials*, 110: 77-84.

Mulligan, C.N. Yong, R.N. and Gibbs, B.F. 2001. Remediation technologies for metal contaminated soils and groundwater: an evaluation. *Engineering Geology*, 60: 193-207

Muzaddadi.A.U. 2013.Laboratory techniques of basic microbiology for the beginners.

Narendra Publishing House. New Delhi.

Nascimento, A.M.A and ChartoneSouza, E. 2003.Operon mer bacterial resistance to mercury and potential for bioremediation of contaminated environments.*Genetics and Molecular Research*, 2: 92-101.

Nies, D.H. (2003). Efflux-mediated heavy metal resistance in prokaryotes.*FEMS Microbiology Reviews*, 27: 313-339.

Nies, D.H. 1999. Microbial heavy metal resistance.*Applied Microbiologyand Biotechnology*, 51: 730-750.

Nwuche, C.O. Ugoji, E.O. 2008. Effects of heavy metal pollution on the soil microbial activity.*International Journal of Environtal Scince and Technology*, 5(3): 409-414.

Odum, H.T. 2000. Back Ground of Published Studies on Lead and Wetland. In: Howard T.

Otth, L. Solís, Gabriela; Wilson, M.Fernández, H. 2005. Susceptibility of Arcobacterbutzleri to heavy metals. *Brazil Journalof Microbiology*, 36 (3):286-288.

Ozturk, A. 2007.Removal of nickel from aqueous solution by the bacterium Bacillus thuringiensis.*Journal of Hazardous Material*, 147 (1-2): 518523.

Patel, P.H., Patel, J.P. and Bhatt, S.A. 2013.Characterization and phylogenetic relatedness of Azotobactersalinestris. Journal of Microbiology and Biotechnology Research, 3(1): 65-70.

Percival, S.L. Bowler, P.G. Russel, D. 2005. *Bacterial resistance to silver in wound care. Journal of Hospital Infection*, 60: 1-7.

Pérez,de Mora, A. Burgos, P. Madejón, E. Cabrera, F. Jaeckel, P. and Schloter, M. 2006. Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments. *Soil Biology and Biochemistry*, 38: 327-341.

PiresBouças, P. D. Izumi, E. FurlanetoMaia, L. Sturion, L. Suzart, S. 2010. Effects of environmental and nutritional factors on gelatinolytic activity by Enterococcus faecalis strains isolated from clinical sources. *African Journal of Microbiology Research*, 4 (10): 969-976.

Ponmurugan, K. Sankaranarayanan, A. and Al-Dharbi, Naif Abdullah. 2012. Biological Activities of Plant Growth Promoting Azotobacter sp. Isolated from Vegetable Crops Rhizosphere Soils. *Journal of Pure and Applied Microbiology*, 6(4): 1689-1698.

Ragan, H.A and Mast, T.J. 1990.Cadmium inhalation and male reproductive toxicity. *Review of Environmental Contamination and Toxicology*, 114: 1-22.

Renella, G. Mench., M. Gelsomino, A. Landi, L. and Nannipieri, P. 2005. Functional activity and microbial community structure in soils amended with bimetallicsludges. *Soil Biology and Biochemistry*, 37: 1498-1506.

Riser-Roberts. E. 1998. Remediation of Petroleum Contaminated Soil. *Biological, Physical and Chemical Processes, Lewis Publishers*, Boca Raton, Florida, USA.

Ritesh Mishra, VartikaSinha, Ambrose Kannan, and Raj K. 2012.*Upreti Toxicology Internationa*l, 19: 25–30

Romera, E. Go nzález, F. Ballester, A. Blázquez, M. Munoz, J. 2007. Comparative study of biosorption of heavy metals using different types of algae.*Bio resources and Technology*, 98: 3344–3353.

Sahin, Y. and A. Ozturk. 2005. Biosorption of chromium (VI) ions from aqueous solution by the bacterium Bacillusthuringiensis. *Process Biochemistry*, 40: 1895-1901.

Salehizadeh, H. and S.A. Shojaosadati. 2003. Removal of metal ions from aqueous solution by polysaccharide produced from Bacillus firmus. *Water Resources*, 37: 4231-4235.

Salem, H.M. Eweida, E.A. and Farag, A. 2000.Heavy metals in drinking water and their environmental impact on human health.*In ICEHM*, 542-556.

Samarth, D.P. C.J. Chandekar and R.K. Bhadekar. 2012. Biosorption of heavy metals from aqueous solution using Bacillus licheniformis. *International Journal of Pure and Applied Science and Technology*, 10 (2): 12-19.

Sandaa, R.-A., Torsvik, V. &Enger, Ø. 2001. Influence of long-term heavy-metal contamination on microbial communities in soil. *Soil Biology and Biochemistry*, 33: 287-295

Sandrin, T.R. and Maier, R.M. 2002.Effects of pH on cadmium toxicity, speciation and accumulation during naphthalene biodegradation.*Environmental Chemistry*, 21(10): 2075-2079.

Sangitha, P.I. Aruna .U.K. Maggirwar, R.C. 2012. Biodegradation of tannery effluent by using tannery effluent isolates. *International Multidisciplinary Research Journal*, 2(3): 43-44.

Selatnia, A. Boukazoula, A, Kechid, N. Bakhti, M.Z. and Chergui, A. 2004. Biosorption of Fe3+ from aqueous solution by a bacterial dead Streptomyces rimosus biomass. Process biochemistry, 39(11): 1643-1651.

Shaffer R.E. Cross J.O. Pehrsson S. L. R. and Elam W.T. 2001. Speciation of chromium in simulated soil samples using X-ray absorption spectroscopy and multivariate calibration. *AnalyticaChimicaActa*, 442: 295–304.

Sharma, P.K. Balkwill, D.L. Frenkel, A.;Vairavamurthy, M.A. 2000. A new Klebsiella planticola strain (Cd-1) grows anaerobically at high cadmium concentrations and precipitates cadmium sulfide. *Applied and Environmental Microbiology*, 66: 3083–3087.

Shukla, O.P. Rai, O.N. Singh, N.K. Dubey, Smita and Baghel, V.S. 2007. Isolation and characterization of chromate resistant bacteria from tannery effluent. *Journal of Environment and Biology*, 28(2): 399-403.46.

Siddiquee, S. Rovina, K. Azad, S. Naher, L. Suryani, S. 2015. Heavy metal contaminants removal from wastewater using the potential filamentous fungi biomass.*A review. Journal of Microbiology, Biochemistry and Technology*, 7: 384–393.

Silver, S. Misra, T.K. 1988. Plasmid-mediated heavy metal resistances. *Annual Review Microbiology*, 42: 717-743.

Sinha, Sankar Narayan, Biswas, Mrinal and Paul, Dipak. 2011. Biodegradation potential of bacterial isolates from tannery effluent with special reference to hexavalent chromium. *Biotechnology Bioinformatics and Bioengineering*, 1(3): 381-386.

Sobolev, D. Begonia, M.F. 2008. Effects of heavy metal contamination upon soil microbes: lead-induced changes in general and denitrifying microbial communities as evidenced by molecular markers. *International Journal of Environtal Resources and Public Health*, 5 (5): 450-456.

Srinath, T. Verma, T. Ramteke, P.W. and Garg, S.K. 2002.Chromium (VI) biosorption and bioaccumulation by chromate resistant bacteria.*Chemosphere*, 48(4): 427-435.

Sterrit, R.M. Lester, J.N. 1980. Interactions of heavy metals with bacteria.*Science of the Total Environment*, 14: 5-17. 11.

Su, C. 2014. A review on heavy metal contamination in the soil worldwide: Situation, impact and remediation techniques. *Environ. Skept. Crit*., 3: 24–38.

Tak, H.I. Ahmad, F. Babalola, O.O. 2013.Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals. *International Reviews of Environmental Contamination and Toxicology; Springer*: New York, NY, USA, 33–52.

Tangaromsuk, J. Pokethitiyook, P. Kruatrachue, M. Upatham, E.S. 2002.Cadmium biosorption by Sphingomonaspaucimobilis biomass.*Bioresouce Technology*, 85: 103–105.

Trevors, J.T. Oddie, K.M. Belliveau, B.H. 1985. Metal resistance in bacteria.*FEMS Microbiology Review*, 32 (1): 39-54.

UNE. 2002. Global Mercury Assessment. Inter Organization Programme for the Sound Management of chemicals. Issued by, UNEP Chemicals. Geneva, Switzerland.

Urrutia, M.M.1997. General bacterial sorption processes. In Biosorbents for metal ions.*Ed J. Wase and C. Forster.CRC Press*, UK. 3966.

Vaituzis, Z. Nelson, J.D. Wan, L.W. Colwell, R.R. 1975. Effects of mercuric chloride on growth and morphology of selected strains of mercury resistant bacteria.*Appllied. Microbiology*, 29 (2):275-286.

Verma,S.K. And Singh S.P. 1995. Multiple chemical resistance in the cyanobacterium, Nostocmuscorum. *Bull Environ ContamToxicol*., 54: 614-619.

Verma, T. Srinath, T. Gadpayle, R.G. Ramteke, P.W. Hans, R.K. and Garg, S.K.2001. Chromate tolerant bacteria isolated from Tannery effluent. *Bioresource Technology*, 78 (1): 31-35.

Vermeul, V. R. Gorky, Y. S. and Teel, S. S. 1995.Geochemical, microbiological and hydrologic characterization at the in situ redox manipulation test site .PNL-10633, *Pacific North-West-Laboratory*, Richland, W.A.

Vidali, M. 2001. Bioremediation: An overview. *Journal of Applied Chemistry*, 73 (7): 1163-1172.

Wang, Y. T. and Shen, H., 1995.Bacterial reduction of hexavalent chromium. *Journal Indian Microbiology*, 14: 159–163.

Weast, R.C. 1984. CRC handbook of chemistry and physics, 64 e, CRC Press, Inc.,Boca Raton, Florida, USA.

Wei, W. Liu, X. Sun, P. Wang, X. Zhu, H. Hong, M. Mao, Z.-W. Zhao, J. 2014. Simple whole-cell biodetection and bioremediation of heavy metals based on an engineered lead-specific operon. *Environtal Science. Technology*, 48, 3363–3371.

Whitman, W.B. Coleman, D.C. and Wiebe, W.J. 1998.Procaryotes: the unseen WHO. 2008. “Water Quality Interventions- to Effectiveness” (T.F. Clasen, and L. Hal).

WHO. 2002. Water pollutants: biological agents, dissolved chemicals, none dissolved chemicals, Sediments, Heat, WHO CEHA, Amman, Jordan.

WHO. 2008. “Water quality interventions- to effectiveness” (T.F. Clasen, and l. Hal)

Wu, G. Kang, H.; Zhang, X. Shao, H. Chu, L. Ruan, C .2010. A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities. *J. Hazard. Mate*., 174: 1–8.

Yan, G., and Viraraghavan, T. 2003. Heavy metal removal from aqueous solution by fungusmucorrouxii.*Water Research*, 37(18): 4486-4496.

Yang, X., Feng, Y., HE, Z., and Stoffella, P.J. 2005.Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation.*Journal of Trace Elements in Medicine and Biology*, 18: 339-353.

Yurieva, O., Kholodii, G., Minakhin, L., Gorlenko, Z., Kalyaeva, E., Mindlin, S., Nikiforov, V. 1997. Intercontinental spread of promiscuous mercury resistance transposons in environmental bacteria. *Moecular Microbiology*, 24 (2): 321–329.

Zhou, M., Liu, Y., Zeng, G., Li, X., Xu, W. and Fan, T., 2007.Kinetic and equilibrium studies of Cr (VI) biosorption by dead Bacilluslicheniformis biomass. *World Journal of Microbiology and Biotechnology,* 23(1): 43-48.

Ziagova, M., Dimitriadis, G., Aslanidou, D., Papaioannou, X., Tzannetaki, E.L., and LiakopouloK yriakides, M. 2007. Comparative study of Cd (II) and Cr (VI) biosorption on Staphylococcus xylosus and Pseudomonas sp.In single and binary mixtures.*Bioresource technology,* 98(15): 2859-2865.

Zouboulis, A.I., Loukidou, M.X., and Matis, K.A. 2004. Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal polluted soils. *Process Biochemis try*, 39:909–916.

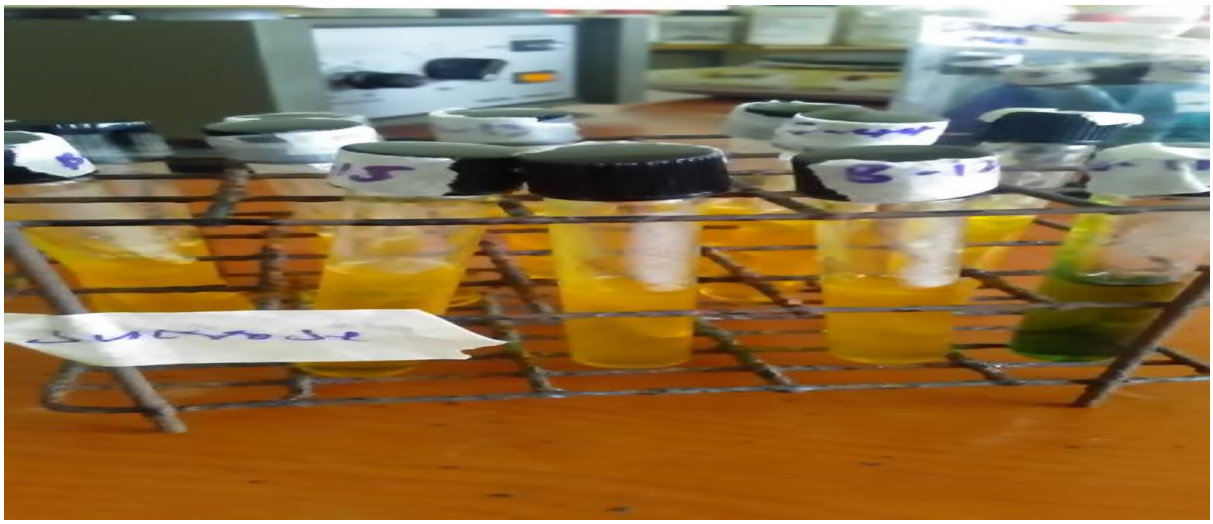
7. APPENDECIES

7.1 .Appendix Figure

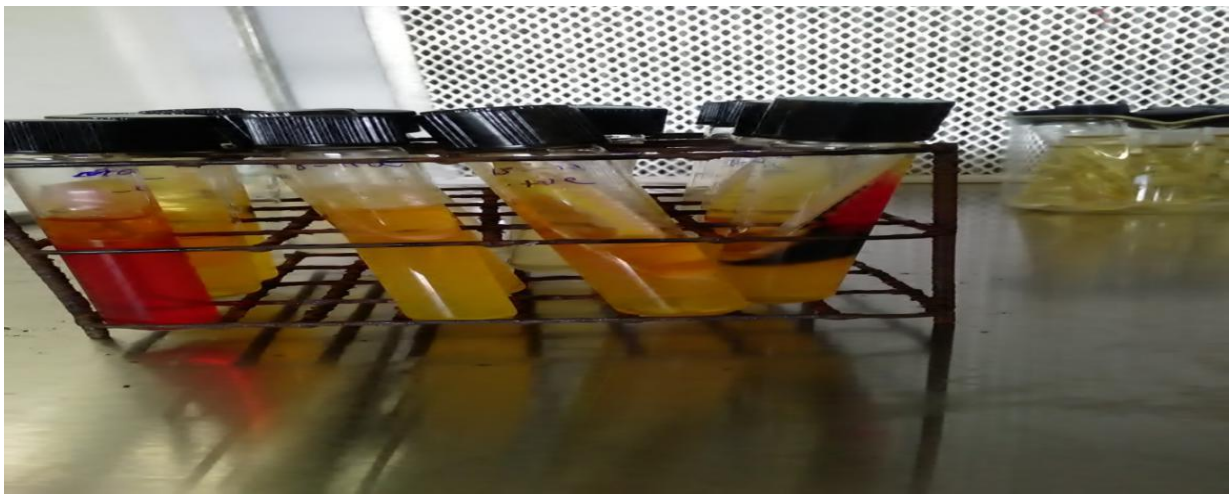
Appendix figure 1.Inoculation of isolates in the safety cabinet

Appendix figures 2.morphological and gram stain examination though the microscope

Appendix figures 3. Citrate utilization tests

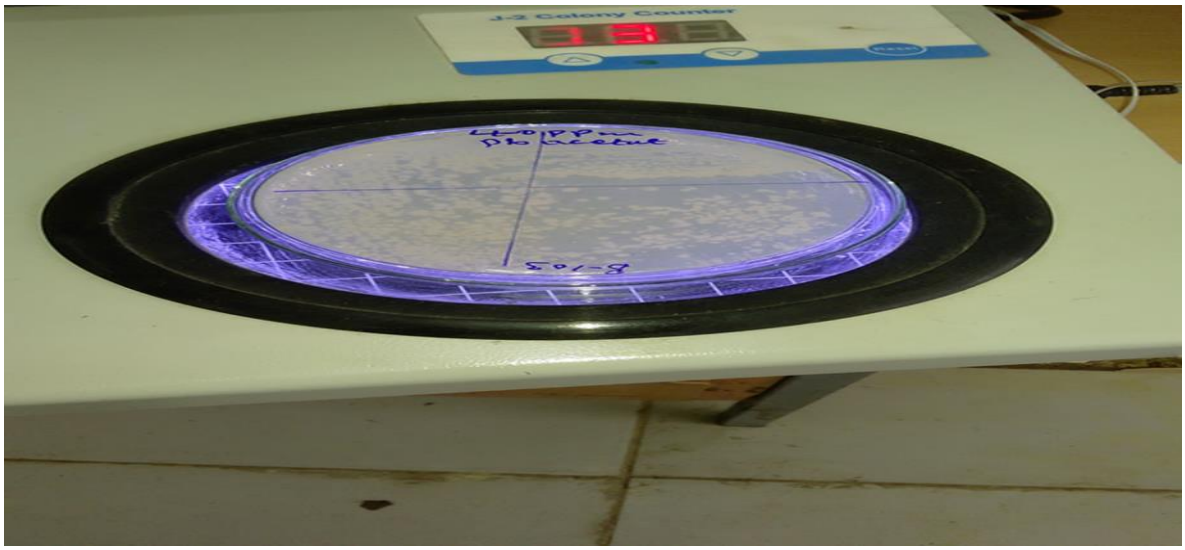


Appendix figures 4.carbohydrate oxidation and fermentation test

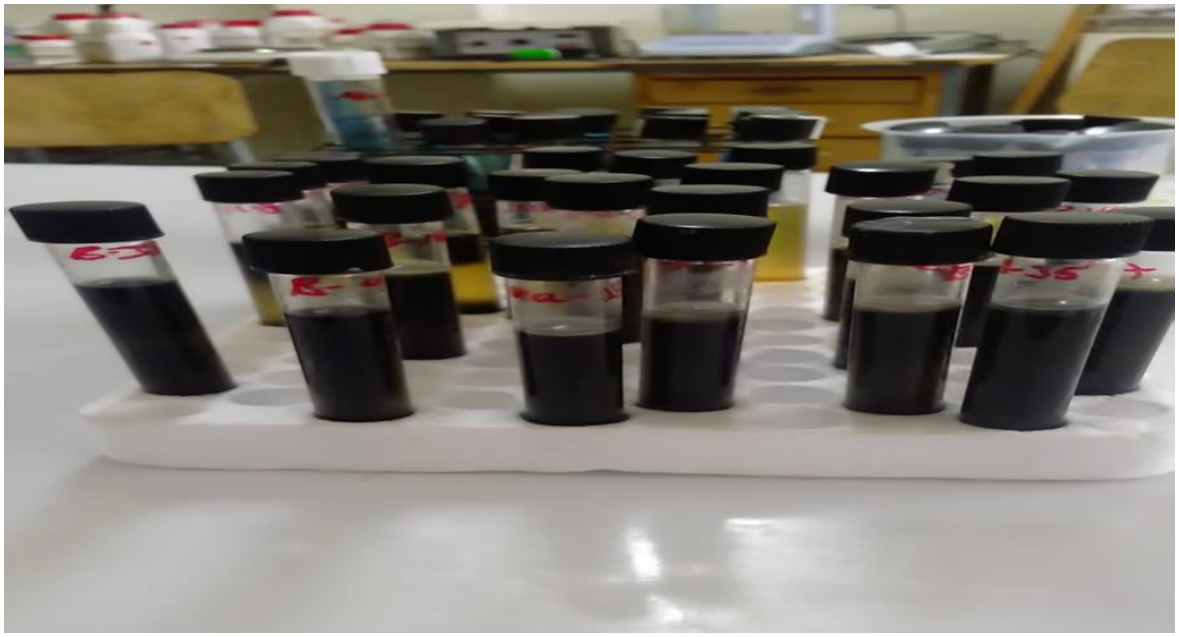


Appendix figures 5.triple sugar iron agar test

Appendix figures 6. Indole tests of isolated bacteria



Appendix figures 7. counting the numbers of bacteria colony by colony counter

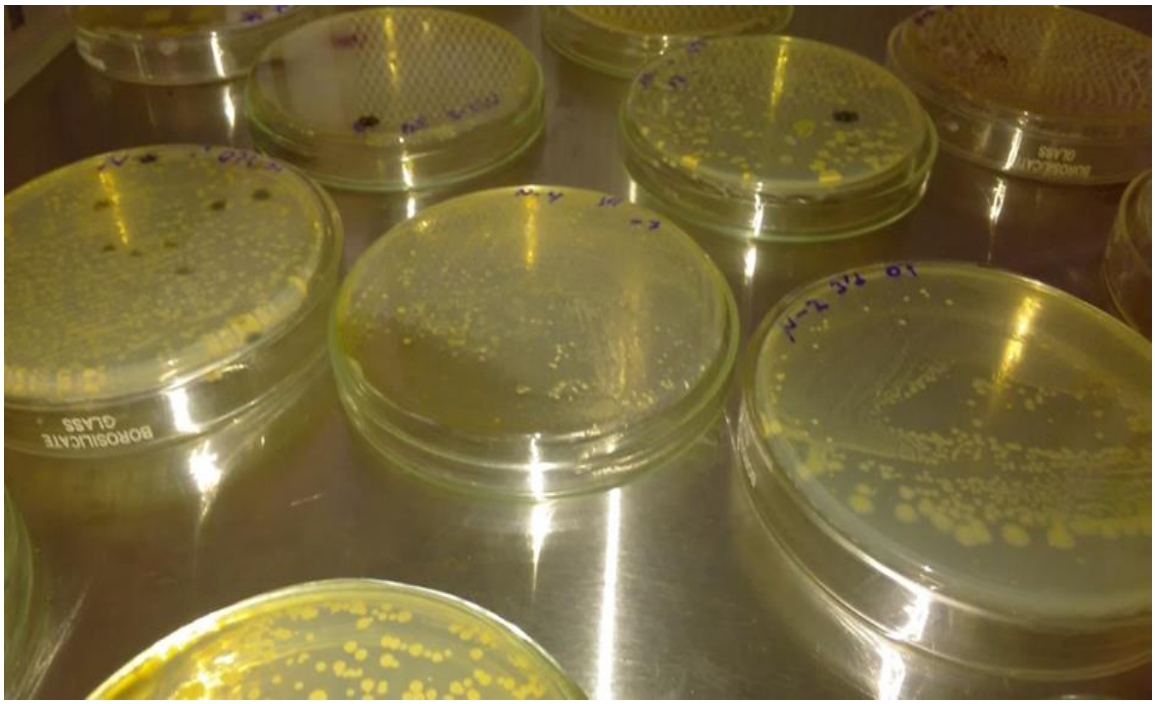


Appendix figures 8. Motility tests of bacteria isolates

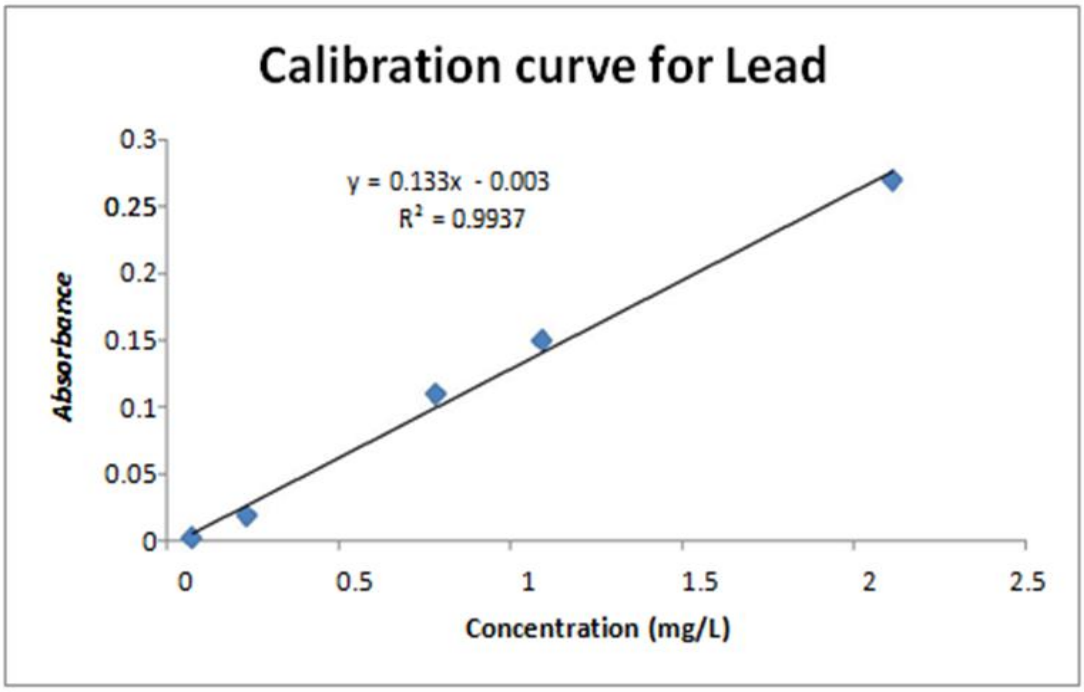
Appendix figures 9.heavy metal standard preparation for AAS analyzation

Appendix figures 10. Recording of bacterial growth in the plate and test tube

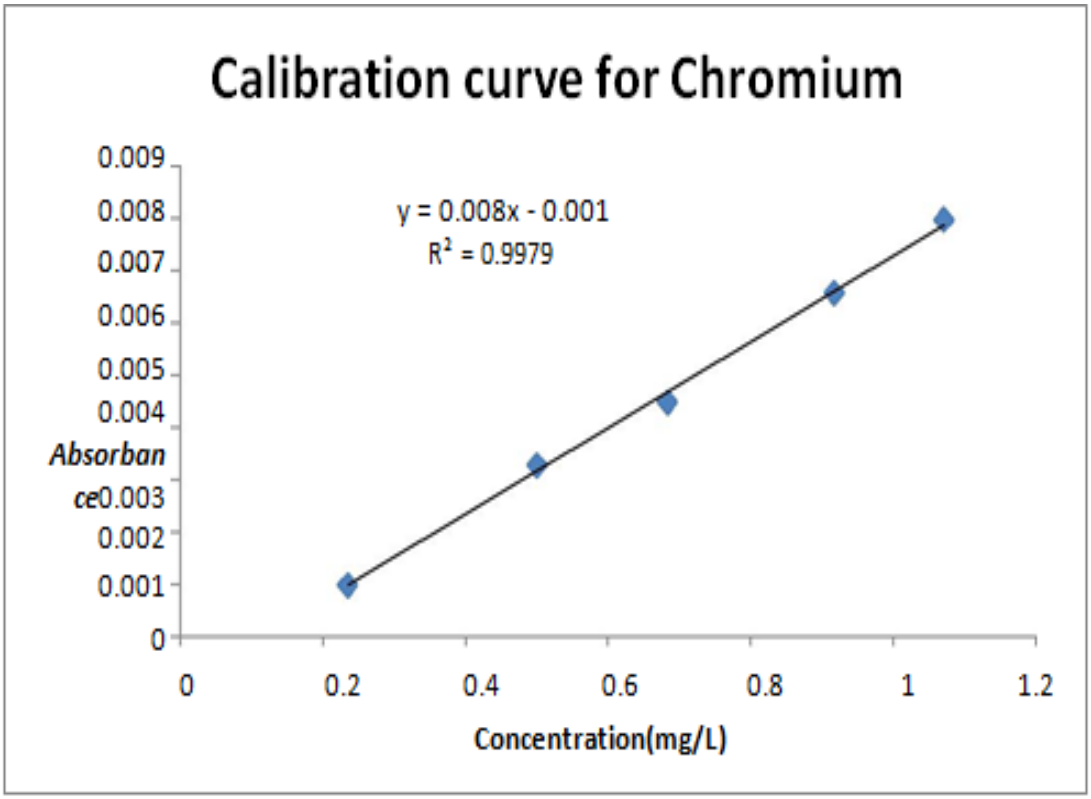
Appendix figures 11. Isolated bacteria without heavy metal (control).).



Appendix figures 12. Isolated bacteria with heavy metal



Appendix figure13. Calibration Curve of Lead



Appendix figure14. Calibration Curve of Chromiu