Isolation and Molecular Characterization of Chromium Reducing Bacterial Strains from Selected Chrome Contaminated Tannery Waste Sites in Nairobi, Kenya

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DECLARATION

This thesis is my original work and has not been submitted for a degree in any other university.

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"Every finish line is the beginning of a new race" So the saying goes. This applies to research too. I therefore hope that the conclusions drawn from this work will be new beginnings to some other researchers.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

APHA	American Public Health Association
ASAL	Arid and Semi-Arid Land
BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl Trymethylammonium Bromide
DNA	Deoxyribonucleic Acid
EPA	Environmental Protection Agency
GDP	Gross Domestic Product
KES	Kenya Shillings
KIRDI	Kenya Industrial Research and Development Institute
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
NABIR	Natural and Accelerated Bioremediation Research
NCBI	National Centre for Biotechnology Information
NEMA	National Environmental Management Authority
NER	National Environmental Regulation
OSHA	Occupational Safety and Health Authority
rRNA	Ribosomal Ribonucleic Acid
WHO	World Health Organization

ABSTRACT

The leather industry a key agricultural sub-sectors in Kenya with a high potential towards contributing to economic growth, creation of wealth and employment. The Kenyan government is currently encouraging the setting up of tanneries to boost processing of leather. However, one cannot wish away the Cr^{6+} pollution that comes with tanneries. Hexavalent chromium is non-biodegradable and is listed as a Class A human carcinogen by the US Environmental Protection Agency (USEPA). Bioremediation is an evolving and promising technology for treatment of environmental wastes although the application of this technology is uncommon in Kenya and other developing countries. This study aims to isolate Cr^{6+} resistant bacteria and determine levels of parameters that encourage conversion of Cr^{3+} to Cr^{6+}. Levels of hexavalent chromium were found to be below NEMA's allowable limits while total Cr was found to be higher than NEMA's allowable limits. The percentage of fats was found to be low as well as the pH in the tannery waste. Three isolates capable of reducing Cr^{6+} were obtained from tannery waste. Isolates CRB01, CRB02 and CRB03 showed the ability to reduce different concentrations of Cr^{6+} to different extents and exhibited Minimum Inhibitory Concentration levels of 60 mg/L, 80 mg/L and 80 mg/L respectively. Morphological, biochemical and molecular sequence analysis using amplified 16S rRNA genes of these isolates identified CRB01, CRB02 and CRB03 to be *Lysinibacillus pakistanensis* NCCP 54, *Bacillus pumilus* SAFR-032 *and Bacillus safensis* strain NBRC 100820 respectively. This study demonstrates the ability of microorganisms to biodegrade chrome polluted tannery waste. The isolates can be used in the biodegradation of chrome wastes by tanneries which will enable them meet the set maximum

CHAPTER ONE

INTRODUCTION

1.1 Background

Unchecked urbanization and push for industrialization has led to serious pollution problems due to the dumping of sewage and industrial effluents into water bodies. The leather industry is a major industry on an international scale and also of national economic importance. Four percent of Kenya's agricultural Gross Domestic Product comes from hides, skins and the leather industry and this constitutes 1.5% of the overall GDP (Gathii, 2011). Value addition in the livestock sector has however been minimal and most of Kenya's exports have been in the form of unprocessed, raw hides and skins. Recently, however, the government has laid out strategies to develop the leather industry. This springs from its Vision 2030 Programme which promotes industrialization and value addition in key sectors. This is because hides, skins and leather industry is one of Kenya's main agricultural sub-sectors that can contribute to economic growth through expanding exports of both semi-processed and finished leather goods as well as job creation (Bekele *et al.,* 2008).

There is however, a worldwide concern of the leather processing sector as a major polluter to the environment (Famielec and Wieczorek-Ciurowa, 2011). Traditionally, the public has always been concerned with odors associated with tanneries as well as water pollution from untreated discharges from tanneries. Important pollutants associated with tanneries include Chlorides, tannins, Chromium Sulphate and sulphides. In addition to these, trace organic chemicals and increasing use of synthetic chemicals such as pesticides, dyes and finishing agents, as well as from the use of newer processing chemical solvents are also associated with tanneries (Mwinyihija, 2010).

There are two main possible processes of tanning namely chrome tanning and vegetable tanning. Vegetable tanning is carried out in a series of vats that have increasing

concentration of tanning liquor. Vegetable tannins are polyphenolic compounds which form hydrogen bonds with the peptide bonds of the protein chains (Riedl and Hagerman, 2001). Chrome tanning on the other hand uses chromium III as the active ingredient. The chromium tanning process is based on the cross-linkage of chromium ions with free carboxyl groups of the collagen which makes the hide resistant to bacteria and high temperature (Sharaf *et al*., 2013). Chrome tanning is preferred because the process is relatively cheap with respect to time since it only takes a day and produces a highly versatile leather suited to most industries while vegetable tanning can take as long as forty days (Krishnamoorthy *et al*., 2013). Chromium used in tanning is the more stable Cr^{3+} species which is considered nontoxic as compared to Cr^{6+}. Recently however, detection of significant levels of toxic Cr^{6+} in water bodies in various parts of the world have raised questions regarding disposal of chromium containing wastes (Förstner and Wittmann, 2012). Although Cr^{3+} is considered less toxic, the presence of certain naturally occurring minerals, especially MnO_2 oxides, and fats can enhance oxidation of Cr^{3+} to Cr^{6+} in the soil environment. Hexavalent chromium is bio available, and it is this form that is highly mobile and therefore poses the greatest risk of groundwater contamination (Avudainayagam *et al*., 2003).

Heavy metals such as Chromium, copper, lead, zinc, nickel, mercury, and cadmium are well-known to be powerful inhibitors of biodegradation activities (Deeb and Altalhi, 2009). These metals cannot be degraded. When Cr^{6+} accumulates in soils, the high levels become toxic to plants, animals, humans and aquatic life (Zayed and Terry, 2003).

Currently, chromium wastes are managed by recycling either directly or indirectly. In Kenya, heavy metals including Chromium are removed from waste water by flocculation and precipitation (Ruhiu *et al*., 2009). Flocculation is a method of removing suspended particles from waste water by addition of alum. When alum is added to waste water, the suspended particles in water coagulate to form bigger heavier particles which then sink to the bottom to form sludge. Due to the large quantities of suspended solids, high quantities of sludge are produced which become a problem since the sludge has to

be disposed somewhere. After flocculation waste water is then let into another stage of treatment called maturation ponds. Maturation ponds are designed such that ultra violet rays from sunlight can penetrate and kill harmful microorganisms. It is also in maturation ponds that the remaining non-biodegradable particles including heavy metals precipitate to the bottom of the pond as part of sludge (Cavallini, 1996). Indirect recycling involves the precipitation and separation of the chrome from the float containing residual chrome followed by re-dissolving it in acid for re-use. The direct form entails spent float being recycled directly to the chrome tanning processing for re-use. The big challenge to tanning industries is to adopt and practice technologies that are more efficient in recovering Chromium (Ludvík, 2000).

Bacteria have developed several types of mechanisms to tolerate the uptake of heavy metal ions to survive under metal stressed conditions. These mechanisms include the efflux of metal ions outside the cell, and reduction of the heavy metal ions to a less toxic state (Spain and Alm, 2003). Studies have found various species like *Bacillus* and *Pseudomonas* capable of remediating heavy metals. However, they need favorable environmental factors (Smith *et al*., 1998; Boopathy, 2000).

The main sources of river water pollution in Kenya are industrial discharge, sewage, seepage from waste sites and illegal solid and liquid wastes disposals. The tanning industry in Kenya releases large quantities of effluents and sludge rich in chromium (Cr) and salts into the environment (Wu *et al.*, 2015).

1.2 Statement of the problem

Tannery effluent containing Cr^{6+} is one of the major problems in the leather industry. The waste water containing chromium from tanning process is usually discharged, without proper treatment, into the sewerage system causing potential serious environmental and health impact. Trivalent chromium used in tanning has the potential to be converted to Cr^{6+} that causes adverse effects to the human health where Cr^{6+} inducesacute and chronic toxicity, dermatotoxicity, neurotoxicity, genotoxicity,

carcinogenicity, immunotoxicity, and general environmental toxicity (Bagchi *et al*., 2002). It has been found to be mutagenic in several bacterial systems (Saha *et al.*, 2011). The waste water containing both Cr^{6+} and Cr^{3+} also has the potential of contaminating surface water and ground waters. It is through this route that humans can get in contact with Cr^{6+}.

1.3 Justification of the study

One of the key agricultural sub-sectors in Kenya is the leather industry. The industry has a high potential towards contributing to economic growth, creation of wealth and employment. Kenya earns approximately KES 4 billion from the export of hides, skins, leather, leather goods and footwear annually (Gathii, 2011). This can be improved with value addition and for this reason, the government of Kenya is committed to improving this industry for the country to meet its goals as set in the recently launched vision 2030 mega initiative to industrialization. The government is currently encouraging entrepreneurs to set up tanneries to boost processing of leather (Mwinyihija, 2010). While this is good news, one cannot wish away the pollution that comes with tanneries because they are known to pollute the environment with chromium metal especially the Cr^{6+} species.

Hexavalent chromium is among priority pollutants and listed as a Class A human carcinogen by the US Environmental Protection Agency (USEPA, 1998). This means that there is adequate human data to indicate that Cr^{6+} causes cancer. Therefore, Cr^{6+} toxicity and associated pollution should be treated with concern. Heavy metals including Cr^{6+} and Cr^{3+} are currently removed from waste water in Kenya through flocculation where chemicals such as Alum are used to precipitate the contaminants. Though effective, this method is expensive and not sustainable by small tanneries. Additionally, the method produces a huge amount of sludge which again needs to be disposed (Onyuka, 2014). It is therefore imperative to develop a system that is cheap, efficient and sustainable. Bioremediation is one of the evolving and promising methods that can

be considered. Bioremediation is a process where microorganisms are used to reduce toxic compounds to less toxic products (Fiorenza and Rifai, 2003).

Reduction and bioaccumulation can be employed in the economic removal of chromium from the tannery effluent using bacterial biomass. The advantage offered by the use of microorganisms in metal detoxification is that they are easy to grow and hence lead to rapid biomass production. In addition, they are part of the natural environment (Faryal *et al*., 2007).It is therefore beneficial to develop a bioprocess utilizing selected indigenous microbes that are both Cr^{6+} - resistant and Cr^{6+} – reducing to Cr^{3+}.

This study aims at determining the heavy metal resistance patterns of bacteria that will be isolated from tannery waste water. Identifying these bacteria will help improvise ways of reducing heavy metals that are produced as waste from industries hence industries will achieve proper treatment of raw tannery effluents which will have a positive impact on the environment. This method will be cheaper than conventional methods like the use of precipitating agents like alum which is expensive and leads to formation of large sludge. Biological treatment of Cr^{6+}-contaminated waste is limited because microorganisms lose viability in the presence of high concentrations of Cr^{6+}. This implies that the isolation of microorganisms capable of reducing high Cr^{6+} concentrations would, thus, be very useful.

1.4 Hypothesis

There are no phylogenetically related Cr^{6+}reducing bacterial strains from chrome contaminated tannery effluent which has bioremediation potential from native bacteria isolated from industrial effluent.

1.5 Objectives

1.5.1 General objective

To isolate and characterize Cr^{6+} reducing bacterial strains from chrome contaminated tannery wastes sites and to determine their phylogenetic relationship.

1.5.2 Specific objectives

1. To determine physiochemical parameters such as percentage of fat, pH levels, total and Cr^{6+} concentration in chrome shavings from tannery waste sites in Nairobi, Kenya.
2. To isolate and characterize Cr^{6+} reducing bacterial strains from chrome shavings from tannery waste sites in Nairobi, Kenya.
3. To determine the bioremediation potential using native Cr^{6+} reducing bacteria isolated from chrome contaminated tannery waste sites in Nairobi, Kenya.
4. To determine the phylogenetic relationship of bacterial strains isolated from chrome contaminated tannery waste site in Nairobi, Kenya.

CHAPTER TWO

LITERATURE REVIEW

2.1 Leather industry in Kenya

Kenya has a large livestock resource base with 17.5 million cattle, 27.7 million goats, 17.1 million sheep and 3.0 million camels. This resource combined with emerging livestock such as Nile perch and crocodiles is what the leather industry in Kenya depends on (Rakotoarisoa *et al*., 2008). Currently, the industry provides employment to more than 22,540 people in the country both directly and indirectly. The sector's contribution to Kenya's economy stands at KES 10.6 billion. There are 14 tanneries and another 8 are yet to be completed which will increase the number to 22. Some of the tanneries include Aziz Tanneries Limited, Leather Industries of Kenya, Dogbone Limited and Nakuru Tanneries to mention a few. In Nairobi there are 5 tanneries that are operational and out of 1000 kg of raw hide, nearly 850 kg is generated as solid wastes in leather processing. Only 150 Kg of the raw material is converted in to leather. A typical tannery generate huge amount of waste where 35-40% is chrome shaving, chrome splits and buffing dust (Onjala, 2010).

More than 80% of Kenya comprises of Arid and Semi-arid land (ASAL). The ASALs are home to about 14 million people and 70% of the national livestock herd. This means that most of the land is naturally dry (Mortimore, 2009). The advantage of the leather industry, however, is that it flourishes throughout the year. This means that if the government increases efforts in promoting the leather industry then a lot can be achieved in terms of employment opportunities and increase in the GDP. Indeed the government in its Vision 2030 which aims to transform Kenya into an industrialized, clean and secure middle income country has as part of its objective, to revive the leather industry and that is why more tanneries are being set up as mentioned before (National Planning Commission, 2013).

The government has set up strategies that are aimed at discouraging the export of hides and skin with the intention of encouraging value addition before export. These strategies include increase in export duty on all hides and skin to up to 80%. The promotion of value addition is what is encouraging the setting up of tanneries both by the public and the private sectors (Mokhothu and Wanjau, 2013). With these tanneries come negative impacts to the environment because of the environmental pollution associated with tanneries. However, business people and manufacturers in this sector are required to comply with the regulations set by the National Environmental Management authority.

2.1.1 Tannery Waste Site in Nairobi

One of the largest dumpsite in Africa is the Dandora Municipal Dumping Site in Nairobi. It is the main dumping site for most of the solid waste generated by the residents and industries within Nairobi. Dumping at the site is unrestricted. Industrial, agricultural, domestic and medical wastes including used syringes - are strewn all over the site. Plastics, rubber and lead paint treated wood, hazardous wastes containing poisonous chemicals have been found on the dumpsite. The Nairobi River passes by the dump and some of the waste makes its way into the river, which carries these environmental and health risks to communities near the dump and downstream who may be using the water for irrigation of food products and in their homes. A study commissioned by UNEP found high levels of lead and other heavy metals in the blood of area children, who also suffer from respiratory diseases, including chronic bronchitis and asthma. The results also showed dangerously high levels of heavy metals, especially lead, mercury and cadmium, at the dumpsite, in the surrounding environment and in local (Kimani, 2010)

In another study, ground water in Dandora was also studied and it was found to be contaminated by heavy metals including chromium. The study found that there was a high correlation between contamination of wells and distance of the dumpsites (Henry *et al*., 2006). Several studies have further established that the Nairobi river basin is on the

receiving end of effluents. About 80% of these effluents are from manufacturing and service enterprises, making it one of the most polluted rivers in the country (Budambula and Mwachiro, 2006). Assessment of levels of Cr at 6 sites along the Nairobi River found that the levels exceeded 3 times the World Health Organization (WHO) recommended guidelines for drinking water (World Health Organization, 1996). Poor treatment of tannery effluents and wastes may have far reaching health impacts, not only on drinking water, but also the entire food chain through irrigation.

2.2 Chromium

Chromium (Cr) metal is a steel-gray solid with a high melting point. It is considered one of the world's most critical and highly soluble metal pollutants that have a wide range of application in the chemical and metals industries (Mertz, 1969). In the metal industry for instance, Chromium is used in production of stainless steel and in the production of non-iron alloy that is used for plating metals. In the chemical industry it is used in the development of pigments, production of catalysts, refractories and surface treatments. Chromium is also used in the leather industry as a tanning agent (Das and Mishra, 2008). It can exist in six oxidation states, 0, II, III, IV, V and VI. These characteristics create a challenge for the bioremediation of chromium. Trivalent (Cr^{3+}) and hexavalent (Cr^{6+}) are the most common chromium species found in the environment. Trivalent is the most stable form and its compounds are often insoluble in water. Hexavalent chromium is the second most stable form, and the most toxic. Most Cr^{6+} in the environment is created by human activities. Some of the human activities that contribute to the increase in Cr^{6+} in the environment include combustion of coal and oil in which 0.2% of the Chromium produced is Cr^{6+} (Darweesh, 2012). Chrome plating also produces Chromium where 100% is Cr^{6+} (ATSDR, 2000). Waste incineration, pulp and paper mills are also some of the industrial activities that are sources of Cr^{6+} (Su *et al.*, 2010).

2.2.1 Trivalent Chromium (Cr^{3+})

The presence and concentration of Cr^{3+} in the environment is dependent on several factors such as complexation, hydrolysis, adsorption and redox reactions. Trivalent chromium exists as hexa-aquachromium (3^{+}) $[Cr(H_2O)_6]^{3+}$ and its hydrolysis products in the absence of complexing agents other than H_2O or OH^- (Kotaś and Stasicka, 2000). Trivalent chromium can be oxidized to Cr^{6+} via different processes such as oxidation by dissolved oxygen or oxidation by manganese oxide. Oxidation of Cr^{3+} by dissolved oxygen without any intermediate species has been reported to be negligible, while mediation by manganese oxides has been found to be the effective oxidation pathway in environmental systems negligible (Ghosh *et al*., 2013). Oxidation of Cr^{3+} to Cr^{6+} proceeds as shown below (Darweesh, 2012).

$$2Cr^{3+} + 4H_2O + 3/2O_2 \longrightarrow Cr_2O_7^{2-} + 8H$$

2.2.2 Hexavalent Chromium (Cr^{6+})

Hexavalent chromium is the second most stable form, and the most toxic. Most Cr^{6+} in the environment is created by human activities like Chromium plating, use in evaporative cooling towers and chemical manufacturing of chromium (ATSDR, 2000). Hexavalent chromium is much more toxic than Cr^{3+} and mutagenic to most organisms and humans (Laxman and More, 2002). This has mainly been attributed to its rapid permeability through biological membranes and subsequent interaction with intracellular protein and nucleic acids (Poornima, *et al*., 2010). This species of chromium only occurs in environments that are (sub) oxic. Sub oxic environments are those that have very little oxygen or environments where nitrates and nitrites are (meta) stable. When dissolved in water, hexavalent Chromium exists in anionic form which could be $HCrO_4^-$ and CrO_4^{2-}. The anion is called chromate (Sharma *et al.,* 2008).

Chromate (CrO_4^{2-}) is a strong oxidizing agent that is reduced intracellularly to Cr^{6+} and reacts with nucleic acids and other cell components to produce mutagenic and

carcinogenic effects on biological systems (McLean and Beveridge, 2001). Trivalent chromium can readily be converted into Cr^{6+} under natural conditions through various oxidation processes. For example, during treatment of water that contains Cr^{3+}, the water disinfectants used can oxidize Cr^{3+} to Cr^{6+} (Belay, 2010).

2.2.3 Natural Sources of Chromium

Chromium was discovered later than other metals because of its relatively low concentration in the earth's crust. In addition, it is strongly bonded to minerals in which it occurs and it doesn't appear terrestrially as a native metal. The concentrations of chromium in the environment are due to natural processes or man-made activities. The former may be due to leaching of the metal from rocks into water bodies which is the most important natural source or wet precipitation and dry fallout from the atmosphere. It may also be from run-off from the terrestrial systems. This contributes 30-40% of chromium in atmosphere (Seigneur and Constantinou, 1995).

2.2.4 Anthropogenic Sources of Chromium

Chromium and many of its compounds are important in common life. This is because it can be used in very many industries. For example, it is used in electroplating because of its resistance to corrosive agents at room temperature. It is also used in the manufacture of ferrous and non-ferrous alloys. However, it is applied in ferrous alloys more. The other application of Chromium is in the manufacture of ink and pigments and in the textile industry as well. The percentage Chromium used in the three industries mentioned above: metallurgical, refractory and chemical is different and is shown in figure 2.1. All these industries that make use of Chromium contribute to the man-made sources of Chromium. With regard to the leather industry, it has been reported that 80–90% of leather is tanned with chromium chemicals (Johnson *et al*., 2006). About 40% of this is then discharged in the effluent as Cr^{3+} and Cr^{6+} (Saha *et al*., 2013).

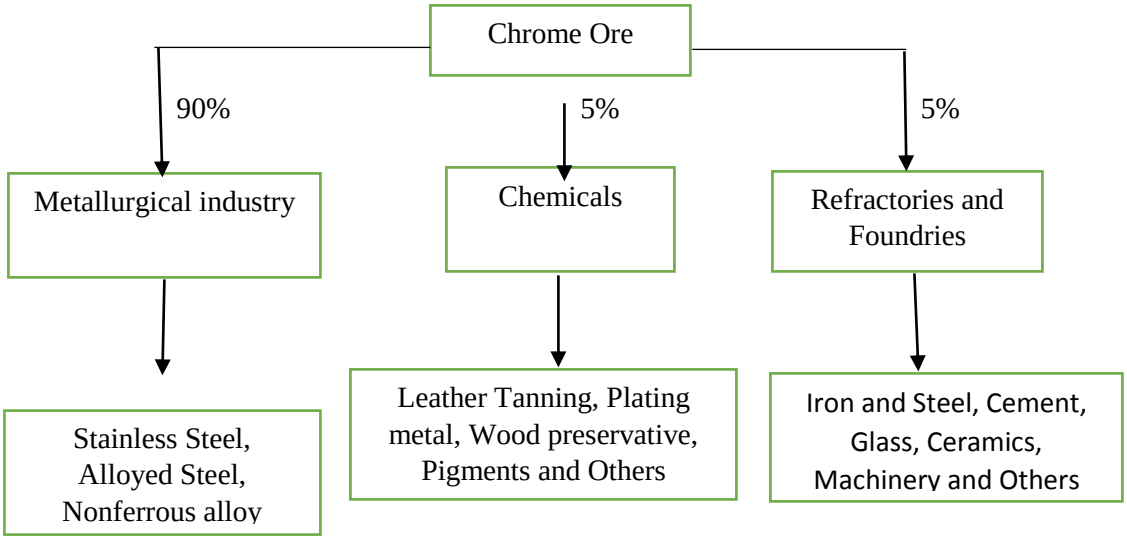

Figure 2.1: Percentage use of Chromium in different industry (adapted from Dhal *et al*., 2013).

2.3 Route of exposure to Chromium

There is little information available with regard to environmental exposure of Cr^{6+} to humans. One of the routes of exposure is through breathing ambient air. The air we breathe is often polluted by various pollutants among them Cr^{6+}. Sources of this pollutant in the air include activities like cigarette smoking, windblown soil, and road dust. Smoking cigarettes indoors increases the concentration of Cr^{6+} in the indoor air substantially. The chromium content of cigarette tobacco from the USA has been reported to be 0.24-6.3 mg kg^{-1} (Smith *et al*., 1997).

Exposure may also occur via skin contact with certain consumer products containing chromium such as some wood preservatives, cement, cleaning materials, textiles and leather tanned using chromium (Alfridi *et al*., 2006). Workers in industries that use chromium can be exposed to higher levels of chromium than the general population. Workers in tanneries employing chrome for tanning for instance are exposed to between 0.001 - 0.005 mg m^{-3} of Cr^{6+} (Huang, 1999).

2.4 Impacts of Cr^{6+}

Hexavalent chromium has several negative impacts on humans and plants alike. Its impact on the environment also affects plants and humans as well.

2.4.1Toxicity of $Cr^{6+}$and Its Impact on Human Health

Chromium III is an essential micronutrient in the diet of animals and humans, as it is crucial for the normal metabolism of lipid, proteins and sugar of mammals (Mordenti and Piva, 1997). Therefore, its deficiency in the system would lead to alteration of the metabolism of sugar, lipids and protein. Elevated levels of Chromium are however toxic depending on the oxidation state.

It is mutagenic in bacteria, mutagenic and carcinogenic in humans and animals (Losi *et al*., 1994), and is also involved in causing birth defects and the decrease of reproductive health (Kanojia *et al*., 1998). Adsorption from Cr^{6+} compounds is higher than Cr^{3+} compounds because chromate (CrO_4^{2-}) enters the cells by facilitated diffusion (Zhitkovich, 2011). By passive diffusion and phagocytosis, adsorption of Cr^{3+} atoms can also take place (ATSDR, 2000).Once chromium enters the bloodstream, its compounds can be distributed to all organs of the body. Hexavalent chromium is unstable in the body and is reduced ultimately to Cr^{3+} by many substances like ascorbate and glutathione (ATSDR, 2000). Once this reduction occurs, excretion can occur through urine, hair, and nails. However, hair and nails provide minor pathways of excretion. It is suggested that toxicity effects of Cr^{6+} compounds result from the destruction of cellular components by generation of free radicals (ATSDR, 2000).

Cellular membranes appear to be impermeable to most complexes of Cr^{3+} compared to Cr^{6+}. This makes Cr^{6+} ten to a hundred times more toxic than Cr^{3+} (Cheung and Gu, 2007). Despite being considered less toxic, intracellular Cr^{3+} forms *in vivo* amino acid nucleotide complexes, whose mutagenic potential is not fully understood (Roundhill and koch, 2002). According to Cheng *et al*, (2012), prokaryotes are more resistant to Cr^{6+} than eukaryotes.

Chromium in general is not classified as a carcinogen by the OSHA (Occupational Safety and Health Authority) and is fairly unregulated, but is considered toxic, level 3. While chromium III is essential for regular operation of human vascular and metabolic systems as well as combating diabetes, too much chromium III may result in severe skin rash, or other more serious symptoms (Sneddon, 2012). Hexavalent chromium is known to be more toxic than Cr^{3+} because Cr^{6+} is the more readily absorbed both by inhalation and oral routes (Baysoy *et al.*, 2013).

The genotoxicity of hexavalent chromium induces a wide variety of effects including chromosomal aberrations, DNA damage, sister chromatid exchange, gene mutation, cell transformation, and dominant lethal mutations. Hexavalent chromium compounds have caused developmental effects in rodents in the lack of maternal toxicity following oral administration (Das and Mishra, 2008).

Hexavalent chromium can enter the cell because of its structural similarity to phosphate which is transported into all types of cells. Once Cr^{6+} enters the cell, it is chemically transformed to the more stable Cr^{3+}. The process by which Cr^{6+} is reduced to Cr^{3+} can cause many forms of DNA damage like oxidative DNA lesions such as strand breaks and chromium-DNA adducts administration (Das and Mishra, 2008). In the cell, Cr^{6+} is reduced to Cr^{3+} and this process gives rise to intermediates that are redox-active. These intermediates, for example sulphur radicals, oxygen radicals or chromium radicals have the potential to induce DNA damage via oxidative stress. The dominant reducer of Cr^{6+} in the cell is ascorbate which is an anti-oxidant. Additionally, an intermediate Cr^{6+} is formed that may form a Cr-DNA adduct (WoŸniak and Błasiak, 2002). The DNA adducts are DNA molecules that are covalently bonded to a cancer causing agent. The DNA adducts can be repaired by the body into the original DNA strand or they can be repaired incorrectly leading to a mutation (Hang, 2010). Three mechanisms by which Cr^{6+} induces carcinogenesis have been postulated and these are through indirect free radical DNA change, direct metal-mediated oxidative DNA damage and direct metal-DNA binding. The consequences of these processes include aneuploidy (abnormal

chromosome number) mutations and even alteration of gene transcription (Sedman *et al.*, 2006).

2.4.2 Effect of Cr^{6+} on plant

Chromium compounds are highly toxic to plants and are detrimental to their growth and development. Although some crops are not affected by low Cr concentration (3.8×10^{-4} μM), Cr is toxic to higher plants at 100 μM kg^{-1} dry weight. Hexavalent chromium reduces seed germination in plants and can result to up to 20% reduction of seed germination. This is suspected to be due to root damage. Other studies have indicated retardation of growth, photosynthesis, and enzyme activities in algae due to the presence of Cr^{6+} at concentration as low as 10 ppm (Sharma and Agrawal, 2005).

2.5 Standards for Total Chromium in Drinking Water and in Discharged Effluent

As mentioned before, concentration is of vital importance when it comes to toxicity of heavy metals. Different authorities from different regions of the world have therefore come up with maximum allowable limits of heavy metals in drinking water and effluents. These authorities include the World health Organization (WHO), U.S Environmental Protection Agency (EPA), The European Union (E.U), Kenya Bureau of Standards (KEBS) and National Environmental Management Authority (NEMA). The KEBS standards include those of Cr^{6+} and Cr^{3+} but the others mentioned give limits for total Chromium. The limits are shown in Table 2.1.

Table 2.1: Table showing maximum allowable limits of Total Chromium (µg/L) by different authorities

Authority	Concentration of Cr (µg/L)
Environmental Management and Coordination (water quality regulation) 2006 (Kenya) (KEBS)	50 (Total Chromium)
European Union (Drinking water directive 98/83/EC)	50 (Total Chromium)
WHO (Guidelines for drinking water quality)	50 (Total Chromium)
U.S Environmental Protection Agency (EPA: maximum contamination limit)	100 (Total Chromium)
National Environmental Management Authority (NEMA) Standards for Discharge of Effluent into Water or on Land)	1000(Total Chromium)
National Environmental Management Authority (NEMA) Standards for Discharge of Effluent into Water or on Land)	50 (Cr^{6+})

2.6 Tanning and Cr^{6+} Generation

Tanning is the process of transforming animal skins to leather. The technology of tanning is largely conventional having become popular among the tanners. Currently, there are only two main processes which include vegetable tanning (organic tanning) where agents such as the barks of certain trees are used and chrome tanning (inorganic mineral tanning) where chromium is used (Covington, 2009). The basis of tanning is such that collagen, the main protein of skin interacts and bonds with polyphenols in plant materials or chromium. Vegetable tanning is used to produce sole and other heavy leather while chrome tanning is employed for producing light weight leather like shoe uppers and upholstery. About 90% of leather produced globally is by chrome tanning (Sundar *et al*., 2002). Oxidation of Cr^{3+} to Cr^{6+} by oxygen in air during the processes

carried out at higher pH in leather and footwear manufacturing process is an important cause of Cr^{6+} generation (Kilic *et al*., 2011).

The Chromium used in tanning is the Basic Chromium Sulphate which is the trivalent form of Chromium (Cr^{3+}). This means that ideally, hexavalent Chromium (Cr^{6+}) is not supposed to be found in the tannery. Processing stages where there is a possibility of the generation of Cr^{6+} include neutralization, fat-liquoring, thermal and photo-ageing, and ammonium treatment.

2.6.1 Neutralization

Immediately after tanning, neutralization has to be carried out to remove acids in the leather that had been applied in earlier tanning stages. These acids are removed because they can cause a problem by causing the deterioration of leather during the drying stage. Mild alkalis that have some effect on the Chromium complex like sodium formate are usually used at this stage (Kaul, 2005).

Presence of a strong oxidizing agent in acidic condition promotes oxidation of Cr^{3+} into Cr^{6+}. Oxidation can also occur at higher pH with the help of mild oxidizing agents. The above conditions prevail during neutralization of wet blue leather.

2.6.2 Fat-liquoring

When hides and skins are tanned using Chromium, the material usually dries out to become hard and crusty. This makes it unsuitable for many purposes. To circumnavigate this problem, a process known as fat-liquoring is employed whereby reactive oils known as fat-liquors are used to treat the crusty material. The oils attach themselves to the material's fibrous structure and lubricate them making them soft, flexible and supple (Bajza and Vrcek, 2001).

Fat-liquors play an important role in the development of Cr^{6+}. Sulfated fish oils, sulfited fish oils, fat-liquoring products with single or multiple unsaturated fatty acids either free or esterified are the kind of fat-liquors known to cause the formation of Cr^{6+}. The free radicals that are released by the unsaturated lipids in presence of UV light can

significantly cause the formation of Cr^{6+}. Sulphited vegetable oils were also found to cause Cr^{6+} formation (Babu *et al.*, 2005).

2.6.3 Thermal and Photo-ageing

Ultra violet light is used to dry the leather. Exposure to UV light can induce the formation of large amounts of Cr^{6+}. Natural light or UVA light (UV light produced by lamps of 366 nm) has also been reported to be able to induce the formation of Cr^{6+} (Hedberg *et al*., 2015).

2.6.4 Ammonia Treatment

Before the tanned material can be dyed, it is usually treated with ammonia and sodium bicarbonate. The aim of this step is to improve leveling and penetration of the dye. This may promote the oxidation of Cr^{3+} to Cr^{6+}. Studies have shown that samples that have been treated with ammonia and sodium bicarbonate showed presence of Cr^{6+} when heated to 80 ^{o}C for 24 hours (Candar *et al.*, 2001).

2.7 Removal of Chromium from contaminated effluent

Several methods exist for the removal of heavy metal ions from waste water. These methods include conventional methods like chemical precipitation, membrane filtration, ion exchange, adsorption and photo catalysis. The other method is bioremediation (Barakat, 2011).

2.7.1 Conventional Methods

The ion exchange method uses an ion exchanger to remove heavy metals including chromium from waste waters. An ion exchanger is a solid that is capable of exchanging anions or cations from surrounding materials. It usually contains resins or polymers which are insoluble matrices which is basically a support structure. These resins and polymers are usually in the form of small white or yellowish beads with small diameters made up from organic polymer substrates (Nachod, 2012).

This method has successfully been applied in industries in the removal of heavy metals. However, they come with several disadvantages one of which is the fact that the matrix is easily fouled by the various organics in the waste water and hence it cannot handle

highly concentrated metal solution (Rieman and Walton, 2013). Another drawback of the ion exchange method is that it is sensitive to pH of the solution and is non-selective. Resins used in ion exchange also have to be regenerated every time they get exhausted which complicates operations of the system (Gu and Brown, 2006). The chemical costs are high compared to the unit of metal removed. All these disadvantages make the use of ion exchange an unsuitable method for small tanneries. What is needed is a method that is cheap and sustainable.

Adsorption is another method used for the removal of heavy metals. Adsorption refers to a mass transfer process whereby a pollutant is transferred from a liquid phase to a solid phase where it is bound by either chemical or physical forces or both (Babel and Kurniawan, 2005). The advantage of this method is that it can be used even when the concentration of heavy metal is low. However, it has the disadvantage of leading to production of extra waste products or secondary pollutants.

Photocatalysis is a very good method of detoxifying waste waters especially with regard to heavy metals with redox potential like Chromium (Ayati *et al.,* 2014). Redox potential refers to the measure of how easily a metal is able to gain or lose electrons. The use of Cr^{3+} in the tanneries is one of the potential sources of Cr^{6+} in waste waters that come from tanneries. Therefore, photocatalysis comes in handy in the reduction of Cr^{6+} to Cr^{3+} which is more environmental friendly. This method involves reduction induced by Ultra Violet (UV) light and sensitized by a photocatalyst like Titanium dioxide (TiO_2) (Hoffmann *et al.,* 1995). The advantage of this method is the production of less harmful byproducts. However, the method takes a long time.

Membrane filtration makes use of permeable membranes that can separate heavy metals and suspended solids from waste water on the basis of pore size. The advantage of this method is that it requires a small space and achieves high separation selectivity. However, drawbacks associated with this method include fouling of the membrane which leads to high operational cost (Kurniawan *et al.,* 2006). This means that it is not a sustainable method for use by small industries.

The most widely used method of heavy metal removal even here in Kenya is chemical precipitation (Crump *et al*., 2004). The mechanism that takes place during chemical precipitation can be summarized as shown below (Corapcioglu and Huang, 1987).

$$M^{2+} + 2(OH)^{-} \longleftrightarrow M(OH)_2$$

In the above equation M^{2+} represents the dissolved heavy metal ion while OH- represent the chemical precipitant. $M(OH)_2$ is the resulting insoluble metal hydroxide. The most common precipitant used is lime and limestone (Mirbagheri and Hosseini, 2005). The main advantage of this method is that it can be used to treat waste waters with heavy metal concentration of up to 1000 mg/L. In addition, this method is simple and the equipment required inexpensive. It is also safe and convenient and this explains why it is widely used. This method however has drawbacks too. For instance, for the heavy metal pollutants to be reduced to acceptable levels, large amounts of chemicals have to be used. The method also leads to the production of excess sludge which requires further treatment. Precipitation of metals is also slow and the excess sludge also has long term impacts on the environment (Aziz *et al.,* 2008).

2.7.2 Bioremediation

Bacteria are known to be able to devise ways of surviving harsh conditions. Some of them produce very resistant spores that help them survive harsh environment while others have developed mechanisms of converting the toxins into lesser toxic products. They are the ultimate waste disposers of nature (Issazadeh *et al.,* 2013). These microbes devour the dead, inert and decomposing materials on the surface of the earth. Humans have discovered how good they are in doing this that they have taken interest in manipulating them to clean up messes created by anthropogenic activities (Rahm *et al*., 2006). This concept is known as bioremediation. Bioremediation therefore, refers to the use of biological agents especially microorganisms like bacteria, fungi or yeast to clean up soils and water contaminated with various contaminants (Strong and Burgess, 2008). To be able to do this, the technology depends on the promotion of the growth of specific microbial consortia that are indigenous to the contaminated sites. Establishing such

microbial consortia is done in various ways such as addition of nutrients, control of moisture, addition of terminal electron acceptor or by control of temperature (Hess *et al.*, 1997).

Before bioremediation can be applied to clean up a contaminated site, it is important to first isolate and characterize the indigenous microorganisms that reside in that site. Genetically modified bacteria with the genetic capability to act on the contaminants may be introduced, a process known as bioaugmentation (Vogel, 1996). The genetically modified bacteria may contain the right genes but they are known to fail due to various factors such as competition from the native microbial consortia. Ethical and moral considerations also make the use of genetically modified bacteria difficult (Huang *et al*., 2002). Indigenous bacteria on the other hand have the advantage of adaptation to the environment of the contaminated site. Additionally, they co-exist with other organisms and have tolerance to the contaminant. Isolation and characterization of the native species that is tolerant to the contaminant is therefore imperative in order to understand the optimum condition at which the species can clean up the contaminant.

Despite the term 'bioremediation' being fairly new, forms of bioremediation have been practiced by humans since the beginning of recorded history. This has been demonstrated by evidence of kitchen middens (ancient household garbage dumps) and compost piles dating back to 6000 B.C. Bioremediation was used over 100 years ago with the opening of the first biological sewage treatment plant in Sussex, UK, in 1891 (NABIR , 2003).

Different microorganisms both prokaryotes and eukaryotes have been found to have the natural capacity to biosorb toxic heavy metals (Singh, 2014). These microorganisms may be bacteria, algae, fungi or yeast. Examples of bacteria include *Bacillus cereus, Pseudomonas veronii,* and *Sporosarcina ginsengisoli* while examples of fungi include*Aspergillus versicolor, Penicillium canescens* and *Aspergillus fumigatus.* Examples of yeast are *Candida utilis* and *Saccharomyces cerevisiae* and that of algae is *Cladophora fascicularis* (Kujan *et al*., 2006). All these microorganisms interact with the

toxic heavy metals differently. Eukaryotes (yeast, fungi and algae) have been found to be more sensitive to toxicity from the heavy metals compared to the prokaryotes (bacteria).

Contaminated sites often contain more than one pollutant and therefore it is essential that an organism that can survive in that environment be chosen to bio remediate the pollutant of choice. Besides, research has shown that microorganisms isolated from the contamination site are able to bioremediate pollutants better than those that were procured (Philip *et al.,* 1998). This means that it is important to obtain a microorganism that is indigenous to that site and this is done by isolation and characterization of the organism from that site.

Bioremediation is slowly being adopted in Kenya. For recently, bacteria capable of degrading Linuron which is a substance found in pesticides have been isolated for determination as to whether the bacteria can be used in bioremediation technologies (Miriti *et al.,* 2014). However, indigenous chromium VI- resistant has not been isolated yet. This study therefore aims to isolate these bacteria in preparation for the realization of Vision 2030 which aims to make Kenya an industrialized country. There are three different types of bioremediation which include biostimulation, bioaugmentation and intrinsic bioremediation.

2.7.3 Biostimulation

Natural bioremediation of contaminated sites is usually limited due to the inadequate availability of nutrients and conditions that are not optimum for the native bacteria present at these sites (Leung, 2004). These limitations can be circumvented by biostimulation which refers to the addition of nutrients and electron acceptors to modify the environment to suit the microorganisms present (Rhykerd *et al.,* 1999). The advantage of this type is that bioremediation will be carried out by microorganisms which are already well suited to that environment and are well distributed spatially at the site. A challenge however exists on how to optimally deliver the nutrients to the microorganisms.

2.7.4 Bioaugmentation

Sometimes the indigenous species at a certain contaminated site do not have the capacity to remediate the pollutant or the population of that species is low. In such situations, non-native organisms with the capacity to remediate pollutants are introduced to the contaminated site. This kind of bioremediation is called bioaugmentation (Leahy and Colwell, 1990). This kind of bioremediation can also be applied where speed is an important factor because addition of a resistant species will speed up the clean-up process. The success of bioaugmentation depends on the survival of the introduced species because they have to adapt to the environment, compete with the indigenous species and survive the various predators present at the contaminated site.

2.7.5 Intrinsic Bioremediation

Also called natural attenuation or monitored natural attenuation (MNA) is a kind of bioremediation that consists of unassisted biological physical and chemical processes that act to limit the migration and reduce the toxicity, mobility and mass of pollutants in the soil (Alvarez and Illman, 2005). Due to factors like presence of low permeability soils, it is sometimes not technically feasible to remove all contaminants from every soil particle and this is where intrinsic bioremediation becomes important (Rifai *et al.*, 1995). Even though no human intervention takes place, thorough monitoring is carried out. It has an advantage of the ability to be used in conjunction with other methods of remediation. This type of bioremediation is carried out at the site of contamination and is therefore called in situ bioremediation.

2.7.6 Advantages and Disadvantages of Bioremediation

Being a natural process, it is considered by the general public as an acceptable method for the treatment of contaminated materials such as soil. In addition, the residues from the treatment process are usually harmless. Many hazardous wastes can be transformed through bioremediation into less toxic substances especially heavy metals that are non-biodegradable (Boopathy, 2000). This eliminates the probability of future liability that is related with the treatment and disposal of contaminated material. Bioremediation can

also be carried out at the site of contamination unlike cases where contaminated materials have to be carried to other places to be destroyed. This makes it fairly cheap compared to other methods of waste treatment. Carrying out remediation on site is also beneficial because normal activities can continue taking place (Vidali, 2001).

However, this method relies on addition of either new species or additives to enhance a certain species. This may be disruptive to the rest of the microbial community indigenous to that particular site. Extrapolating from bench and pilot studies to field operations is also an uphill task. In addition, there have been concerns that the products of biodegradation may be more toxic or persistent to the environment than the patent compound (Kumar *et al.*, 2011).

2.8 Reduction of Hexavalent Chromium by Microorganisms

Regardless of the toxicity of Cr^{6+}, microorganisms have been shown to be resistant to it. These organisms demonstrated the capacity to reduce Cr^{6+} to Cr^{3+} with the first species, *Pseudomonas* spp, being reported in 1976 (Romanenko and Koren'kov, 1976). Beveridge (2000) isolated and characterized a chromium reducing bacterium from a chromate copper arsenate contaminated site. Reports showed a gram-negative bacterium isolated from a chromium-contaminated site that was capable of reducing hexavalent chromium to an insoluble precipitate, thereby removing this toxic chromium species from solution. Analysis of the 16S rRNA from the isolate revealed that it was a Pseudomonas with high similarity to Pseudomaonas synxantha. The bacterium was tolerant to high concentrations of chromate (500 mg/l) and can reduce Cr^{6+} under aerobic and anaerobic conditions. It also exhibited a broad range of reduction efficiencies under minimal nutrient conditions at temperatures between 480^{o}C and 370^{o}C and at pH levels from 4 to 9. Felix Gutierrez-Corona in (2003) reported Cr^{6+} reduction in a chromate-resistant strain of *Candida maltose* isolated from the leather industry. Resistance of the strain to high Cr^{6+} concentrations and its ability to chemically reduce chromium was studied. When compared to the three laboratory yeasts *Candida albicans*, *Saccharomyces cerevisiae* and *Yarrowia lipolytica*, the *C. maltosa* strain was

found to tolerate chromate concentrations as high as 100 mg/ ml. In addition to this phenotypic trait, the *C. maltosa* strain showed ability to reduce Cr^{6+}. Zhihui Yang *et al.*, (2009) reported Cr^{6+} remediation by indigenous bacteria in soils contaminated by chromium-containing slag at a Steel-Alloy factory in Hunan Province, China. His results showed that when sufficient nutrients were amended into the contaminated soils, total Cr^{6+} concentration declined from the initial value of 462.8 mg kg-1 to 10 mg kg-1 within 10 days at removal rate 97.8%. These results indicate the significance of isolating indigenous bacteria as they are more effective in biodegradation or biotransformation of wastes.

Some of the other bacteria that have since been isolated include strains of *Escherichia* (Shen and Wang 1993), *Bacillus* (Garbisu *et al*., 1998), and *Enterobacter* (Ohtake *et al*., 1990). Some of these bacteria act aerobically while others act anaerobically. An example of bacteria that reduce Cr^{6+} to Cr^{3+} anaerobically is *Desulfovibrio vulgaris* (Lovley and Phillips 1994). The process can be summarized by the following equation.

$$2\ Cr^{6+} + 3\ H_2 \leftrightarrow 2\ Cr^{3+} + 6\ H^+$$

Chrome resistant *Pseudomonas ambigua* strain GI is reported to reduce chromate anaerobically (Horitsu *et al*., 1987). The exact mechanism that these microorganisms use to reduce Cr^{6+} is variable and dependent on the species. For instance, some microorganisms use soluble enzymes to reduce Cr^{6+} while others use Cr^{6+} as the final electron acceptor in the respiratory chain (Bopp and Ehrlich, 1988). The reason why the aforementioned bacteria are able to resist or tolerate Cr^{6+} is because they possess the *chrBCAF* operon. An operon is a group of genes that function as a single unit. These genes confer resistance against Cr^{6+} by coding for the synthesis of ChrA and ChrB proteins which act as chromate-sensitive regulators (Branco *et al.,* 2005). Efflux of chromate ions from the bacteria cell cytoplasm and reduction of Cr^{6+} to Cr^{3+} are the mechanisms that have been properly characterized and will be discussed below.

2.8.1 Efflux

There are organisms that survive conditions of heavy metal toxicity by removal of these toxins from their cells. This is called efflux mechanism. *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* are examples of these organisms (Alvarez *et al.,* 1999). Chromate resistant determinants from pUM505 and pMOL28 which are plasmids of *Pseudomonas aeruginosa* and *Alcaligenes eutrophus* respectively were analyzed and it was reported that ChrA protein, which is a product of the ChrA gene was the one responsible for the resistance of these bacteria to Chromate. It was hypothesized that the ChrA protein conferred resistance through reduced accumulation of Chromate in the two bacteria. However, there was no direct evidence to confirm the above hypothesis although the study showed that vesicles of the membrane of *P. aeruginosa* and *A. eutrophus* could accumulate more than four times more Chromate than bacteria that were not resistant to Chromate. Resistance via efflux mechanism conferred by ChrA protein has also been reported in *P. aeruginosaand Cupravidus metallidurans* (Juhnke *et al.,* 2002).

Chromate enters cells through the Sulphate (SO_4^{2-}) uptake systems because it is a structural analog of SO_4^{2-} meaning its structure resembles that of SO_4^{2-}. Therefore, if a Chromium resistant bacteria has intracellular Chromate reductases then Cr^{6+} will be reduced to Cr^{3+} intracellularly (Viti and Giovannetti, 2007). If the bacterium lacks these reductases, then, the Cr^{6+} that has accumulated inside the cell induces the *chr* operon and activates the Chromate efflux pump. This leads to the efflux of Cr^{6+} from the bacteria cell ultimately protecting the bacteria.

2.8.2 Reduction of Chromate

Reduction of Chromate by bacteria can either take place through enzymatic means (direct) or through chemical means (indirect). Compounds like glutathione, cysteine, thiosulfates and sulfites are involved in chemical reduction (Donati *et al.,* 2003). Enzymatic reduction on the other hand takes place through soluble membrane bound reductases that occur in anaerobic, aerobic and facultative bacteria (Ramirez-Diaz *et al.,* 2008). Anaerobic reduction is a slow process compared to aerobic reduction. In some

bacteria species, reduction of chromate is a respiratory process while in others it is cometabolic. In the respiration process, NADH donates electrons to Cr^{6+}. Here, Cr^{6+} serves as the terminal electron acceptor which leads to energy conservation for cell maintenance and growth (Chirwa and Molokwane, 2011). Several enzymes are involved in the reduction of Cr^{6+} and these include Cr^{6+} reductase, cytochrome P450, DT-diaphorase and aldehyde oxidase (Patra *et al*., 2010). Enzymatic reduction therefore either occurs aerobically through soluble chromate reductases found in the cytosol or anaerobically through membrane bound Chromate reductases where Chromate acts as the final electron acceptor. Both of these mechanisms are enhanced by glutathione or NADH which act as enzyme co-factors (Elangovan *et al*., 2006).

2.9 Methods of Analysis

Traditionally, bacteria have been identified on the basis of phenotypic characteristics. This however is not as accurate as identification based on genotypic methods. The 16S rRNA gene is the most established genetic marker used for bacterial identification and classification, mainly because it consists of both highly conserved and hypervariable regions. The conserved regions can serve as universal primer binding sites for the amplification of the whole gene or fragments of the gene, whereas the hypervariable regions contain species-specific sequences that can discriminate between different bacteria and archaea Problems remain in that the sequences in some databases are not accurate, there is no consensus quantitative definition of genus or species based on 16S rRNA gene sequence data, the proliferation of species names based on minimal genetic and phenotypic differences raises communication difficulties, and microheterogeneity in 16S rRNA gene sequence within a species is common.

In the determination of total chromium in samples, using flame atomic absorption spectroscopy (FAAS), several factors contribute to the accuracy of the results. Parameters such as: flame-type, possible interfering effects of common anions and cations and reagents added are especially important. From literature, it is revealed that such interferences can be circumvented by selecting flame conditions and masking

agents. Two types of flame are widely mentioned, air-acetylene and nitrous oxide-acetylene flames. It is suggested that a slightly fuel-rich air-acetylene flame has a higher sensitivity as compared to a lean flame. In such a case, interferences are eliminated by proper masking agents. The nitrous oxide-acetylene flame has a lower sensitivity, but is also less prone to interferences (Krishna *et al.*, 2004)

Spectrophotometry is one of the most widespread analytical techniques due to its simplicity, reliability, and low-cost instrumentation for both direct measurements and coupled to other techniques or processes such as chromatography, electrophoresis and flow analysis. In the determination of Cr^{6+}, the chromium reaction with oxidizes 1.5–diphenyl carbazide is usually free from interferences. However, certain substances may interfere if the chromium concentration is relatively low. To compensate for possible slight losses of chromium during digestion or other operations of the analysis, treat the chromium standards by the same procedure as the sample.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Sample Collection

Chromium contaminated tannery effluent and chrome shavings waste samples were collected from the disposal site around tannery located at Kenya Industrial Research and Development Institute (KIRDI) and also from a tannery in Dandora between November-December 2014. The samples were collected in triplicate. Solid wastes were collected from heaps of the waste from the sites. Three samples were collected from each heap from each site. In one heap, three samples were collected from the top part of the heap, three other samples from the middle part of the heap and three more samples from the bottom part of the heap. The sampled were then mixed based on the area of collection. That is, the three samples from the top part we mixed together and the same done for samples collected from the middle part and the bottom part. The result of these was that the total number of samples was 9. The samples were collected in sterile plastic containers and transported to the laboratory for bacteriological analysis.

3.2 Chemical Analysis of Tannery Waste

Samples were analyzed for a number of parameters such as pH, fat content, total Chromium and hexavalent Chromium. All reagents used were analytical grade.

3.2.1 Determination of Total Chromium

Total chromium was determined using flame atomic absorption spectrometry using the standard test ISO 5398-3:2007 (IULTCS/IUC 8-3). The main principle of this technique is that the chromium that is present in the leather is solubilized in the hexavalent state. The solution was then analyzed by flame atomic spectrometry. Samples, which were solid wastes were prepared by grinding using a Wiley mill. 100 mg of the ground sample was then weighed into a 125 mL conical flask containing 25 mL of 2N HCL. The contents were then filtered using Whatman #1 filter paper into a volumetric flask. The concentrations were then determined using prepared standards. The measurements were done in three triplicates and the mean taken as the final reading

3.2.2 Determination of Hexavalent Chromium (Cr^{6+})

Hexavalent chromium was determined using the standard 1.5–diphenyl carbazide method ISO 17075:2007 (IULTCS/IUC 18). The principle behind this method is that soluble Cr^{6+} is leached from the sample in phosphate buffer at pH 7.0 to 8.0. The Cr^{6+} in the solution oxidizes 1.5–diphenyl carbazide to 1, 5-diphenylcarbazone to give a red/violet complex with chromium which can be quantified photometrically at 540 nm. The results were then read using a curve generated using prepared standards.

3.2.3 Determination of Fat content

The fat content was determined using the standard method ISO 4048:2008 (IULTCS/IUC 4).This method uses a soxhelet apparatus and a solvent that dissolves the fat in the leather. The solvent is then evaporated to leave the fat whose weight is then taken. In this method, samples were prepared by grinding the chrome shavings using a Wiley mill. 9 samples were prepared by grinding and mixing with 25 mL of analytical grade hexane.

3.3 Culture of Chrome Resistant Bacteria from Tannery Waste

The top, middle and bottom heap samples from the two selected locations KIRDI and Dandora were mixed according to section 3.1. The presence of bacteria in the samples was determined by culturing the bacteria in basal peptone water as a diluent. 1 mL of the diluent containing bacteria was then aseptically inoculated on nutrient broth and incubating at 37 ºC for 24 hours. This was followed by platting aseptically on nutrient agar amended with Cr^{6+} as $K_2Cr_2O_7$ to final concentration 40 mg/L using sterile filtered Cr^{6+} stock solutions using the standard plating method described by Robert Koch and incubating at 37 ºC for 24 hours. Growth of colonies would confirm presence of bacteria. 3 colonies differing in morphological characteristics were selected and labeled for use in further studies.

3.4 Characterization of the Isolates

The bacterial isolates differing in morphological characteristics were grown on Eosin Methylene Blue (EMB) agar (Himedia, India). The shape and colors of the colonies were then examined under the microscope after Gram staining. This was followed by biochemical analysis for the activities of Oxidase, Catalase, MR-VP test, Citrate Utilization, Acid production from carbohydrates. These tests were used to identify the isolates according to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3.5 Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) of Cr^{6+} resistant isolates was determined by serial dilution method (Calomiris *et al*., 1984) in LB medium with Cr^{6+} concentrations ranging from 20 to 200 mg/L and the minimum concentration of metal in the medium inhibiting complete growth taken as the (MIC). Based on the evaluation MIC was determined at 37 ºC for 24 hours. The minimum Concentration of the chromium ($K_2Cr_2O_7$) at which no growth was observed was considered the MIC.

3.6 Reduction of Chromium by the Isolates

Chromate-resistant bacteria isolates were inoculated into nutrient broth (pH 7.0) containing different concentration of Cr^{6+} (from 20 to 200 mg/L) and incubated for 72 hours at 30 ºC under orbital shaking. The inoculum was 2% of the total volume of medium. Reduction of chromium was determined from extracted solution by using UV-Vis spectrophotometers at 540 nm with 1,5-diphenylcarbazide as a pink colored complex agent (APHA, 1992).

3.7 Molecular Identification of Isolates

Genomic DNA was isolated from the isolates using the CTAB protocol for molecular characterization and amplified by Polymerase Chain Reaction (PCR) using universal bacterial primers 1492R (5' -TACGGYTACCTTGTTACGACTT- 3') and Bac8f (5′-AGAGTTTGATCCTGGCTCAG-3′) for the rRNA gene (Weisburg, 1991). The amplified gene was then sequenced and the resulting 16S rRNA gene sequences were compared with sequences deposited in GenBank by performing a blast n search.

(Thompson *et al*., 1994). Sequence data was then aligned and analyzed to find the closest homology for the microbes. Sequences were aligned with the ClustaLW algorithm using default parameters (Thompson *et al*., 1994). Phylogenetic trees were generated with a Neighbour-Joining (NJ) algorithm. Confidence values for NJ trees were generated by bootstrapping, based on 1000 replicates.

3.8 Statistical Data Analysis

The different Cr^{6+} reducing capabilities of the isolated Cr^{6+} resistant bacteria were then analysed using One-way ANOVA with post-hoc Tukey HSD Test. The statistical formula applies two steps. The first step ANOVA examines whether there is a difference in the gradient of the slope and the second step finds out which among the three is different if at all there is a difference.

CHAPTER FOUR

RESULTS

4.1 Physicochemical properties of tannery waste

The results of the physicochemical properties of the effluent are shown in Table 4.1

Table 4.1: Physicochemical properties of tannery waste

SAMPLE	Temperature (ºC)	pH	Fat Content (%)	Total Cr(mg/L)	Cr^{6+}(mg/L)
KIRDI	28.1	3.55	0.441±0.03	35.077±0.07	**0.0080±0.0002**
DANDORA	27.9	3.23	0.628±0.07	37.565±0.5	**0.0056±0.0002**

± : Standard Deviation

The samples from both sites were found to have high concentrations of total chromium compared to the National Environmental Management Authority (NEMA) permissible limit of 1 mg/L. Concentrations of Cr^{6+} as well as those of fat were found to be lower than the limit set by the National Environmental Management Authority which is 0.05 mg/L for Cr^{6+}.

4.2 Isolation, Characterization and Identification of chrome resistant Bacteria

Three bacterial species were isolated from the chrome shavings sampled from the two sites in Dandora and KIRDI. Two from Dandora were labeled as CRB01 and CRB03 and the one from KIRDI was labeled as CRB02.

4.2.1 Morphological Characterization of Chrome Resistant Bacteria

The colonies of CRB01 were observed as being white with a rough dull surface. The colonies were also observed to be transparent with a butyrous (butter like) with flat elevation. The white colonies were observed to turn light yellow in older colonies. Fresh colonies exist as dots on the surface of nutrient media but on the second and third day they spread over the whole surface of the media. Colonies of the second isolate CRB02

were observed to be slightly yellowish and mostly opaque. The texture of the colonies was smooth and were wrinkled and irregular. The colonies spread over the surface of the agar. The third isolate CRB03 was observed to have dull white colonies with irregular margins and were mostly opaque.

4.2.2 Biochemical Characterization of Chrome resistant Bacteria

Their morphological and biochemical characteristics are shown in Table 4.2.

Table 4.2: Biochemical characteristics of chrome resistant bacteria

Characteristic	CRB01 (Dandora)	CRB02 (KIRDI)	CRB03 (Dandora)
Shape	Rods	Short Rods	Rods
Gram Stain	Gram Positive	Gram positive	Gram Negative
EMB	+	+	-
Lactose	-	-	-
Methyl Red	-	+	+
VogesProskeur	-	+	-
Citrate Utilisation	+	+	-
Catalase	+	+	+
Oxidase	-	+	+

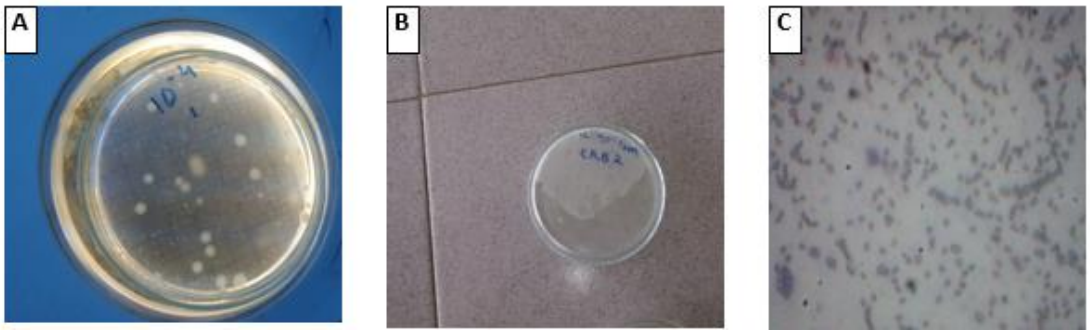

Figure 4. 1: A: Colonies of CRB01, B: Spreading colonies of CRB02, C: Gram negative CRB03 on safranin counterstain

4.3 Minimum Inhibitory Concentration

A stock solution of potassium dichromate was prepared and different concentrations of the potassium dichromate amended to nutrient agar from 20 mg/L to 200 mg/L. The minimum concentration that inhibited growth of bacteria was found to be 60 mg/L, 80 mg/L and 80 mg/L for CRB01, CRB02 and CRB03, respectively.

4.3.1 Reduction of Cr^{6+} by the Isolates

The bacterial isolates were found to be able to reduce the Cr^{6+} to Cr^{3+} in different concentrations of potassium dichromate amended to nutrient broth. The results are as shown in Table 4.3 and demonstrated in figure 4.2

Table 4.3: Table showing percentage reduction of Cr^{6+} by the isolates

Initial Cr^{6+} concentration	**Percentage Cr^{6+} reduction**		
	CRB01	**CRB02**	**CRB03**
20	100	100	100
40	97.5	100	100
60	77.7	86.7	81.7
80	75	79	76.3
100	70	76.3	73.3
120	68.5	60.6	70.6
140	67.5	59.2	65.7
160	65	55	65
180	64	54.5	55
200	63.8	54.3	45

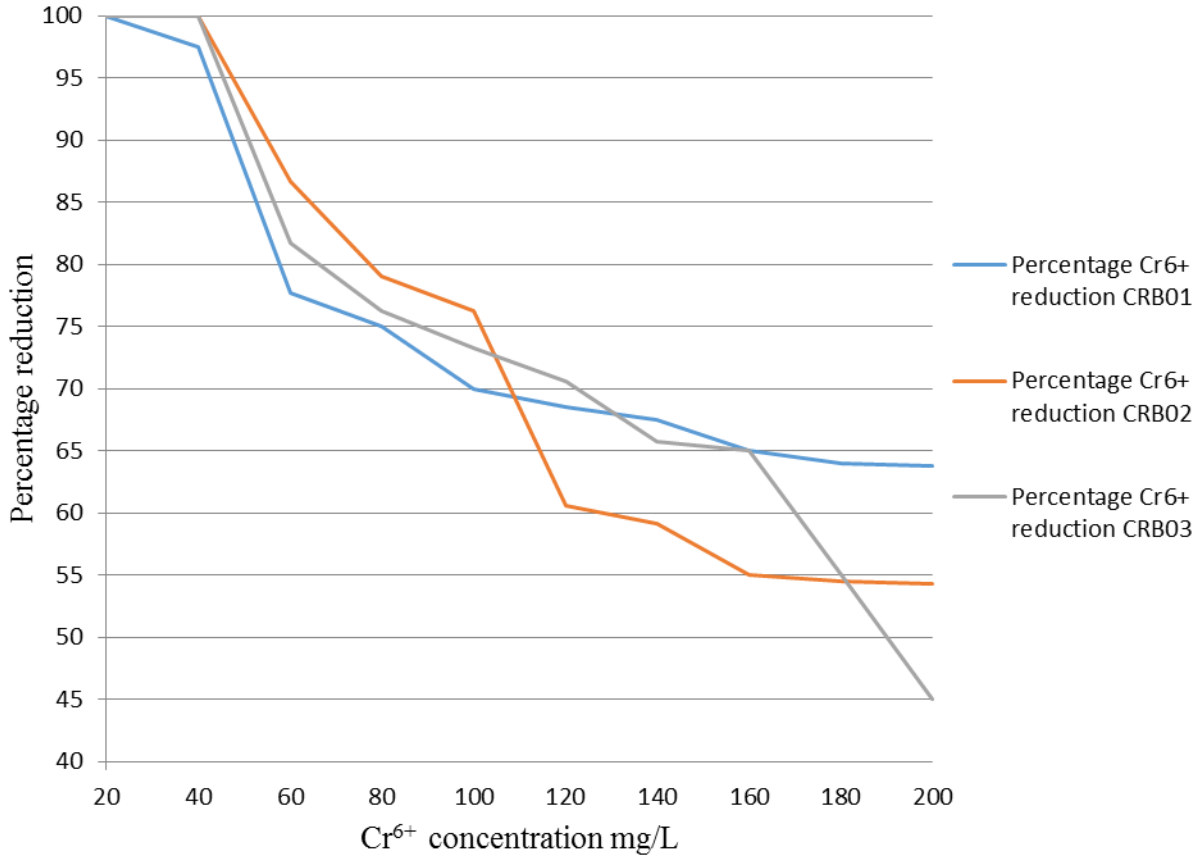

Figure 4.2: Graph showing different reduction capabilities of bacteria isolates

4.4 Molecular Identification

The DNA of the three bacteria species was extracted using CTAB protocol and amplified using PCR using two primers reverse and forward, 1492R and Bac_8F. The amplified 16S rDNA gene was then run on agarose gel electrophoresis using 1kbp ladder and the result observed under UV light. The amplicons were then sent to Macrogen for sequencing. The DNA sequences obtained were then used to identify the species that they closely matched based on the NCBI database. The Blast N results showed that the three chromium resistant bacteria belonged to the species *Lysinibacillus pakistanensis*

NCCP 54, *Bacillus pumilus* SAFR-032*and Bacillus safensis* strain NBRC 100820. The blastN results are shown in Table 4.2.1

Table 4.4: BlastN results of the bacterial isolates

Isolate	Description	Max score	Total score	Query cover	E value	Ident	Accession
CRB01	*Lysinibacillus pakistanensis* NCCP 54	627	627	96%	6e-179	72%	NR 113166.1
CRB02	*Bacillus pumilus* SAFR-032	294	294	19%	9e-79	85%	NR 074977.1
CRB03	*Bacillus safensis* strain NBRC 100820	1659	1659	73%	0.0	94%	NR 113945.1

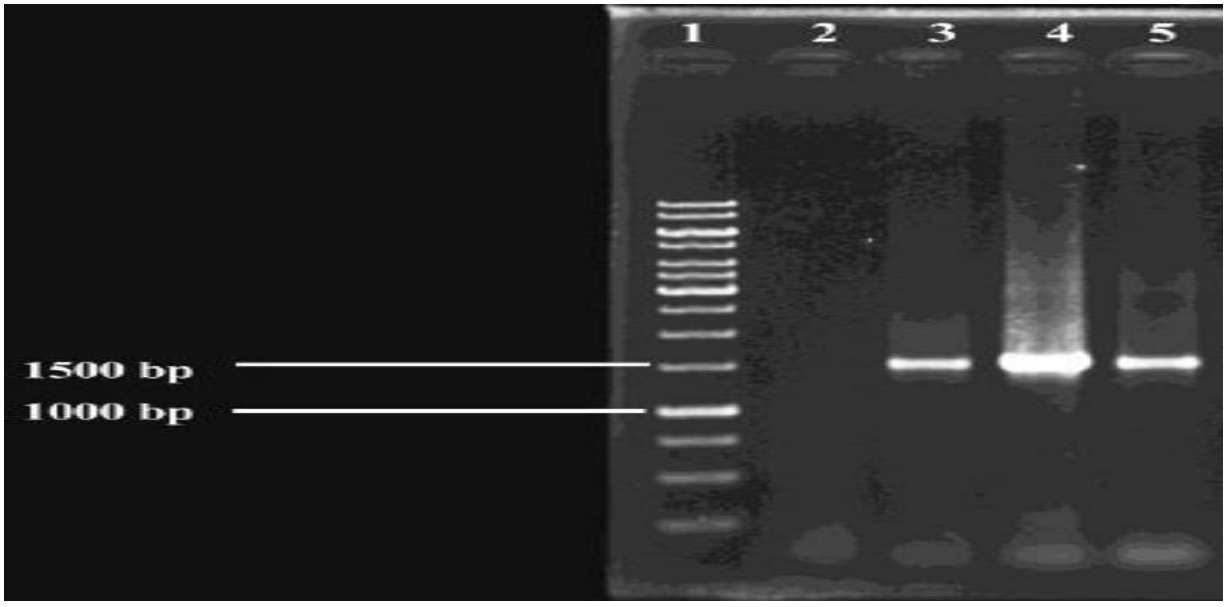

Figure 4.3 Agarose gel electrophoresis after 16S rDNA amplification. Lane 1: GeneRuler 1 kb DNA ladder Lanes 3 to 5: 16S PCR products of DNA isolated from chrome resistant bacteria CRB01 to CRB03 respectively.

4.4.1 Phylogenetic Analysis

The sequences of the isolates *L. pakistanensis, B. pumilus and B. safensi*, and that of a reference organism which a *Picropilus* species were taken through multiple sequence alignment and the result used to create a phylogenetic tree. The result is shown in figure 4.4.

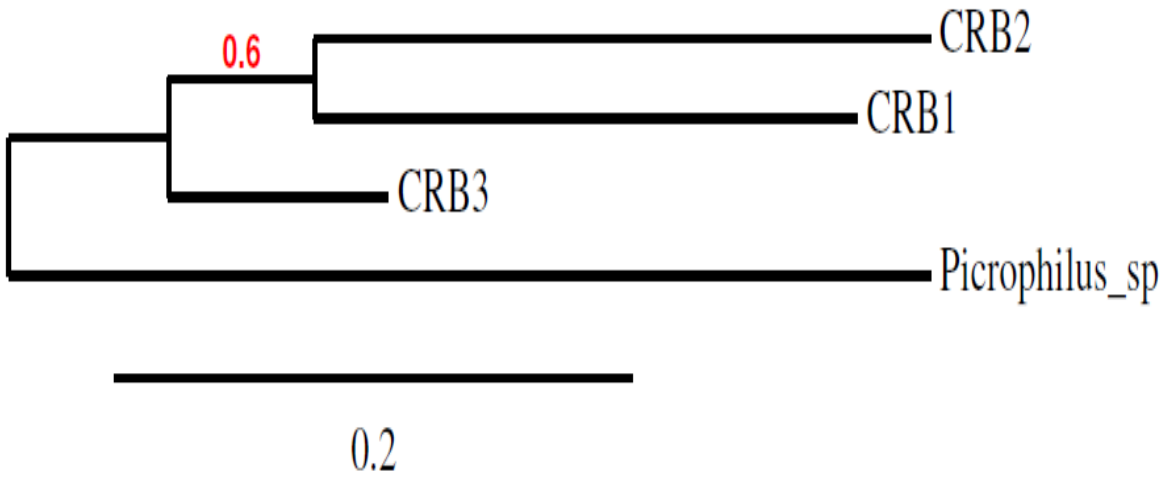

Figure 4.4: Neighbor-joining (NJ) tree of the three isolated bacteria based on the 16S rRNA gene sequences comparison, showing the relationship of *Bacillus. pumilus*, *Bacillus safensis* and *Lysinibacillus pakistanensis* using *Picropilus* as an out-group

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Chromium Sulphate is the tanning agent that is used for tanning in chemical tanning. The Chromium component is the trivalent form of Chromium. Total chromium in the tannery waste was found to be in high amounts compared to the National Environmental Management Authority (NEMA) permissible limit of 1 mg/L. This was expected because a very small percentage of the tanning agent is used during tanning while the rest is washed away with large quantities of water. In addition, after leather has been tanned, it has to be trimmed to the right shape and thickness through trimming and mechanical process called shaving. Through shaving, pieces of wet blue which contain high quantities of Cr^{3+} are produced to form part of the tannery waste and contribute to the levels of Cr^{3+} containing tannery waste. This finding is in line with a study undertaken by (Leghouchi *et al.,* 2009) who found out that the concentrations of total chromium upstream of a tannery were lower compared with high concentration, up to 860 times downstream of the tannery.

Hexavalent chromium on the other hand was found to be in low amounts of 0.008 mg/L and 0.0056 mg/L, lower than the limit set by the National Environmental Management Authority which is 0.05 mg/L. In normal situations, Cr^{6+} is not expected to be detected in tannery waste but certain factors and environmental condition may lead to the oxidation of Cr^{3+} to Cr^{6+}. Some of these factors such as amount of fat and pH were determined in this study. The pH was found to be 3.55 and 3.23 and the fat content was found to be less than 1 percent. As mentioned in chapter 2, high pH or high fat content leads to the oxidation of Cr^{3+} to Cr^{6+} and since pH was found to be low then it was expected that there would be low concentrations of Cr^{6+} if any. These findings are similar to the findings of a study carried out by Palop *et al.,* (2008) who found out that the type of fat-liquoring agent was vital in the formation of Cr^{6+} post tanning. Fat-

liquoring products with single or multiple unsaturated fatty acids either free or esterified are especially responsible for formation of Cr^{6+}.

The colonies of the isolated bacteria were observed to be white with rod shapes on nutrient agar amended with potassium dichromate. This is a characteristic that is common with bacteria of the genus *Bacillus. B. subtilis,* for instance, is a good example of bacteria that has white colonies with wrinkled form and with rod shapes. The most commonly known *Bacilli* are those that are pathogenic. However, it is important to note that most *Bacilli* are saprophytes that feed on dead decaying matter for instant decaying organic matter from industrial wastes. The colonies isolated were observed to spread throughout the media, which again is associated with members of the *bacilli* genus. Members of the genus *Bacilli* are often the major population found in sites with a high concentration of toxic substances such as heavy metals including chromium and because of their ability to produce spores they survive harsh environments.

The Minimum Inhibitory Concentration (MIC) of the three bacteria towards Cr^{6+} was determined to find out which among the three bacteria could tolerate the highest concentration of Cr^{6+}. This is because the best candidate for bioremediation is that microorganism that can tolerate the highest concentration of the pollutant (Spain and Alm, 2003). If those bacteria that cannot tolerate high concentrations of the pollutant are used then they would die and bioremediation would not be achieved. Of the three bacterial species isolated, CRB02 and CRB03 had a higher MIC of 80 mg/L while the MIC of CRB01 was found to be 60 mg/L.

The three bacterial species each showed different reduction capabilities with different concentrations of initial Cr^{6+} concentration as shown in figure 4.3.1. However, upon statistical analysis using One-way ANOVA with post-hoc Tukey HSD Test, it was shown that the difference in the reducing potential among the three bacteria is not statistically significant. Therefore none of the bacterial species can be said to be better than the other as a potential candidate for application in bioremediation. Total reduction of Cr^{6+} was observed only at low concentrations of 20 mg/L and 40 mg/L.

The three Chromium resistant bacteria species, CRB01 and CRB03 from a tannery in Dandora and CRB02 from a tannery at KIRDI were characterized in this study. Upon extraction of their DNA following the CTAB protocol and sequencing followed by comparison in the database at NCBI Gene bank, the bacterial isolates CRB01, CRB02 and CRB03 were identified as *Lysinibacillus pakistanensis* NCCP 54, *Bacillus pumilus* SAFR-032 *and Bacillus safensis* strain NBRC 100820 respectively. These three were the indigenous species of bacteria that were isolated from tanneries in Dandora and KIRDI that were capable of reducing Cr^{6+} to Cr^{3+}.

This is the first time *L. pakistanensis* is being implicated in the biotransformation of Cr^{6+}. A different species, *Lysinibacillus fusiformis* ZC1 was found to contain quite a number of genes that confer metal resistance such as ChrA gene, yieF gene and several others that are known to encode for reductases (He *et al*., 2011). *Lysinibacillus sphaericus* has also been reported for its great larvicidal activity against mosquito larvae and toxic metal resistance. This implies that this specific genus should be studied more to identify the probability of having more species that are chrome resistant.

The revelation in this study that *Bacillus pumilus* can reduce Cr^{6+} is in agreement with a study carried out by Ejaz *et al.,* (2013), who were able to isolate a different strain of *B pumilus* capable of reducing Cr^{6+}. They isolated *Bacillus pumilus* S-4 in Pakistan from a tannery effluent. In another study, Bacillus pumilus was found to be able to completely degrade protocatechuic and caffeic acids and reduce achieve a 50% reduction in the phenolic content of an oil mill waste water (McNamara *et al*., 2008). In our study, *Bacillus pumilus* SAFR-032 was isolated in Kenya from tannery waste.

Bacillus safensis was originally isolated from a National Aeronautics and Space Administration (NASA) assembly plant (Satomi *et al.,* 2006). Strains of this species have been reported to be resistant to Boron and Arsenic (Raja, 2014). It has also been isolated in Brazil from biodegraded petroleum (Laborda *et al.,* 2014). Here we report isolation of *Bacillus safensis* strain NBRC 100820 from a Tannery site in Dandora

capable of reducing Cr^{6+}. There have been very few reports implicating *B safensis* in the reduction of Cr^{6+}.

Bacteria are organisms that can adapt to environmental stresses such as high concentration of heavy metals such as Cr^{6+}. In a study carried out by Megharaj *et al.,* (2003), an *Arthrobacter* sp. and a *Bacillus* sp. were isolated from a long-term tannery waste contaminated soil. It is probable that in their study, Megharaj *et al.,* (2003) isolated indigenous bacteria because the waste was long term meaning it had been in that for a period long enough to be colonized by native bacteria. The findings of this study that found native bacteria capable of biotransorming Cr^{6+} are also concur with findings of a study carried out by Alisi *et al*., (2009) where a formula of native bacteria were employed with success in bioremediation of wastes contaminated with heavy metals and diesel oil. It is important to note that the three species isolated in this study were Bacilli similar to the results obtained by a study carried out by Camargo *et al*., (2003) where it was observed that most of the isolates capable of bio transforming Cr^{6+} were those of the genus *Bacillus*.

From the phylogenetic tree, it was observed that there is a close relationship between *L. pakistanensis, B. pumilus and B. safensis.* Since all the three isolated species demonstrated ability to survive and biotransform Cr^{6+}, it could be that Mobile genetic elements (MGE) could be involved in the spreading of Cr^{6+} resistance determinants, facilitating the adaptation of bacterial communities to Cr^{6+}. Bacteria exposed to Cr^{6+} for a long period of time may acquire MGE such as plasmids carrying Cr^{6+} determinants and, therefore, they become Cr^{6+} resistant bacteria. In agreement with this hypothesis, is a study carried out by De *et al.,* (2008) which found out that showed the presence of the copA gene in metagenomic DNA from the three Cu-polluted soils and the absence of copA gene in metagenomic DNA from the non-polluted soil.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- Concentration of hexavalent Chromium was determined and was found to be below the maximum limit set by NEMA. Total Chromium however, was found to be in higher concentrations which increase the overall heavy metal concentration in sewage.
- Three bacterial species were isolated from tannery waste which implies that communities of microorganisms exist in the tannery wastes that are tolerant to high concentrations of heavy metals. The three species of bacteria isolated each had the capability of reducing Cr^{6+} to Cr^{3+} which suggests that they can be used to improvise bioremediation techniques for the cleaning of industrial wastes from industries associated with hexavalent Chromium.
- Phylogenetic studies indicated that these three species *Lysinibacillus pakistanensis* NCCP 54, *Bacillus pumilus* SAFR-032 *and Bacillus safensis* strain NBRC 100820 are closely related which suggests that maybe their close relatives also possess the genes that enable these bacteria to bio transform Cr^{6+}. According to the available information, this is the first time Chromium reducing bacteria have been isolated in these locations KIRDI and Dandora.

6.2 Recommendations

- The three chrome resistant bacterial species may be exploited commercially for the bioremediation of Cr^{6+} from industrial wastes in industries that are associated with Cr^{6+} especially the leather industry in Kenya. However, before this can be done successfully, the relationships between these native chrome reducing bacteria and other microorganisms in the tannery waste has to be established. Microbial communities often have symbiotic relationships with each other which make them very good at breaking down wastes naturally. Understanding these relationships will go a long way in ensuring that we take full advantage in their

capacity to bio remediate Cr^{6+}. Different optimum conditions for the reduction of hexavalent Chromium were not investigated in this study.

- Conditions such as temperature be investigated because temperature is one of the factors that determine the efficiency of bacteria in bioremediation. It is important to provide the bacteria with the best substrate for it to carry out bioremediation optimally.
- It is therefore imperative to determine the mechanism of Cr^{6+} reduction that each of the three bacteria uses.

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