IDENTIFICATION AND CHARACTERIZATION OF HEAVY METAL (Cd, Ni and Cr) TOLERANT BACTERIA ISOLATED FROM MINE TAILINGS AND THEIR POTENTIAL USE FOR PHYTOEXTRACTION

A Dissertation Submitted Under the Department Of Biological Sciences to the North-West University (Mafikeng Campus), In Partial Fulfillment of the Requirements for the Degree:

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DECLARATION

I declare that this dissertation entitled identification and characterization of heavy metal–tolerant bacteria isolated from mine tailings and their potential use for phytoextraction, is a true outcome of the research performed by me at the School of Biological Sciences, North West University (Mafikeng Campus) under the supervision of Prof Olubukola O Babalola. I declare that the work has not previously been submitted by me for a degree at this or any other University, and that all information derived from the literature has been duly acknowledged in the text and a list of references provided.

Signed: Akia Date: 26/10/2016

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Thanks and God bless.

STRUCTURE OF DISSERTATION

This study comprises of two main chapters submitted for publication in international journals. Chapters are not projected to be individual articles but describe the research work which was carried out to achieve the aim and objectives of this study:

Chapter 1 presents the general introduction, literature review, objectives, and outline of this project

Chapter 2 describes the isolation, characterization and identification of Ni, Cr and Cd tolerant bacteria from mine tailings

Chapter 3 studies the effect of bacterial inoculation on growth and heavy metal (Cd, Cr, and Ni) uptake by *B. juncea,* in a phytoextraction approach

Chapter 4 consists of the general conclusions from chapters 2 and 3 including future research that can be pursued.

ABSTRACT

The main objective of this study was to isolate and characterize metal tolerant bacteria from heavy metal-contaminated soils with the aim of selecting suitable bacterial isolates that can be used to improve growth and heavy metal accumulation in Indian mustard (*Brassica juncea*). Eleven bacterial isolates were recovered from soil samples collected from a mine tailings facility and checked for tolerance against heavy metals (Cr, Ni, and Cd). All isolates showed multiple tolerances against heavy metals but most promising results were shown by bacterial isolates BCr3, BCd33 and BNi11 that were tolerant to 15 mM of Cr^{6+}, 7.5 mM of Cd^{2+} and 10 mM of Ni^{2+} respectively. The effect of heavy metals on bacterial growth was tested together with their ability to grow at different concentrations of NaCl, pH, and temperature values. Bacterial isolates grew well between pH 7.5 and 8.5. The optimum temperature for maximum growth was between 35–37^{0}C and no significant change in bacterial growth was observed in the presence of 2% NaCl. In addition, the bioaccumulation potential of bacterial isolates was investigated. Bacterial isolates BCr3, BCd33 and BNi11 showed high bioaccumulation ability of Cr (68.7%), Cd (72.4%) and Ni (69.8%) respectively. All bacterial isolates were identified by 16S rRNA gene sequencing. Analysis of plasmid content revealed that all bacterial isolates contained a single plasmid. Further, polymerase chain reaction together with DNA sequence analysis was used to screen all bacterial isolates for the presence of metal tolerant genes (*CzcD, ChrA, ChrB, CzcB, CzcC, NccA* and *CadA*) on both plasmid and chromosomal genomes.

Among the metal tolerant bacterial isolates recovered from mine tailings, three bacterial isolates (BCr3, BCd33 and BNi11), identified as *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, were selected based on their ability to tolerate high concentrations of multiple heavy metals. Bacterial isolates also exhibited multiple plant growth beneficial characteristics including the production of indole-3-acetic acid, hydrogen cyanide, ammonia, insoluble phosphate solubilization together with the potential to protect plants against fungal pathogens. Bacterial inoculation improved seeds germination of *B. juncea* in the presence of 0.1 mM Cr, Cd and Ni, as compared to the control treatment. In addition, the ability of bacterial isolates to enhance metal solubilization was also analyzed. Compared with control treatment, soil inoculation with bacterial isolates, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 significantly increased the amount of soluble heavy metals in soil by 51% (Cr), 50% (Cd) and

44% (Ni) respectively. Subsequently, a pot experiment was performed to study the effects of bacterial inoculation on the growth and heavy metal accumulation by *B. juncea* grown in soil spiked with 100 mg kg^{-1} of $NiCl_2$, 100 mg kg^{-1} of $CdCl_2$, and 150 mg kg^{-1} of $K_2Cr_2O_7$. The results revealed that inoculation with metal tolerant bacteria not only protected plants against the toxic effects of heavy metals, but also increased growth and metal accumulation of plants significantly. These findings suggest that such metal–tolerant plant growth promoting bacteria are valuable tools which could be used to develop bio-inoculants for enhancing the efficiency of phytoextraction.

KEYWORDS: Heavy metals, heavy metal tolerant bacteria (HMTB), bioaccumulation, metal tolerant genes, phytoextraction, *B. juncea*, soil contamination.

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LIST OF ACRONYMS

bp	Base pairs
B. juncea	Brassica juncea
Cd	Cadmium
$CdCl_2$	Cadmium chloride
CDF	Cation diffusion facilitator
Cr	Chromium
EDTA	Ethylenediaminetetra acetic acid
$K_2Cr_2O_7$	Potassium dichromate
LB	Luria Bertani agar
Ni	Nickel
$NiCl_2$	Nickel (II) chloride
PCR	Polymerase chain reaction
PGPB	Plant growth promoting bacteria
RND	Resistance nodulation division
rpm	Revolutions per minute
sp	Species
TAE	Tris-Acetate-EDTA
Tris	Tris hydroxymethylaminoethane

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CHAPTER 1: LITERATURE REVIEW AND OBJECTIVES

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Consequent to global industrialization, heavy metal contamination has increased steadily over the years and has become one of the most significant environmental problems facing us today (Lalitagauri *et al.,* 2000). Heavy metals are not degraded biologically or chemically. This property together with high toxicity allows them to bioaccumulate in living systems via food webs, thus posing serious ecological and human health problems (Gerhardt *et al.,* 2009; Liu *et al.*, 2007). Due to the grave nature of this threat, immense scientific efforts are being made to develop effective remediation technologies that will help preserve the quality of soils by reducing heavy metal concentrations to safe and acceptable levels (Valls and Lorenzo 2002).

Biological systems have emerged as effective and less expensive tools for the remediation of heavy metal–contaminated sites, in contrast to physico–chemical methods (Wu *et al.*, 2006). The idea is to use metal tolerant bacteria and plants (identified as hyperaccumulators) to remove metals from contaminated environments.

1.2 HEAVY METALS: AN OVERVIEW

The term "heavy metal" has no standardized definition by a legitimate body such as the IUPAC (International Union of Pure and Applied Chemistry) although various definitions have been used in scientific publications over the last few years. According to some authors, the term "heavy metal" generally refers to a series of metals and metalloids whose atomic density is greater than 5.0 g/cm^3 (Nies, 1999). Among 90 elements found on the periodic table, 21 are non-metals, 16 are light metals and 53 are heavy metals. Common examples include chromium (Cr), copper (Cu), antimony (Sb), zinc (Zn), mercury (Hg), nickel (Ni), cadmium (Cd), arsenic (As), thallium (Tl), lead (Pb), selenium (Se), cobalt (Co), and iron (Fe). The heavy metals examined in this dissertation are Ni, Cr and Cd.

1.3 SOIL CONTAMINATION WITH HEAVY METALS: A GLOBAL PROBLEM

Generally, any heavy metal may be considered a contaminant if it is found in a place where it does not occur naturally or in a form or quantity that is unusually higher than normal background levels (McIntyre, 2003). Soils naturally contain low background concentrations of heavy metals such as those that originate from pedogenic/geogenic processes including volcanic eruptions, wind erosion, weathering of rocks and minerals. However, the primary source of heavy metal contamination derives from anthropogenic activities including mining and smelting, waste disposal, atmospheric deposition, industry, and the intensive use of agrochemicals (pesticides, herbicides, phosphate fertilizers), sewage and industrial effluents in farm lands (Carrillo-Chavez *et al.*, 2003; Duzgoren-Aydin *et al.*, 2006; Feng *et al.*, 2010; Herawati *et al.*, 2000; Jung 2008; Miclean *et al.*, 2009; Rutkowska *et al.*, 2009; Sienkiewicz *et al.*, 2009; Taylor *et al.*, 2010; Wang *et al.*, 2007; Zouboulis *et al.*, 2004). Various heavy metals produced by such activities are shown in Figure 1.

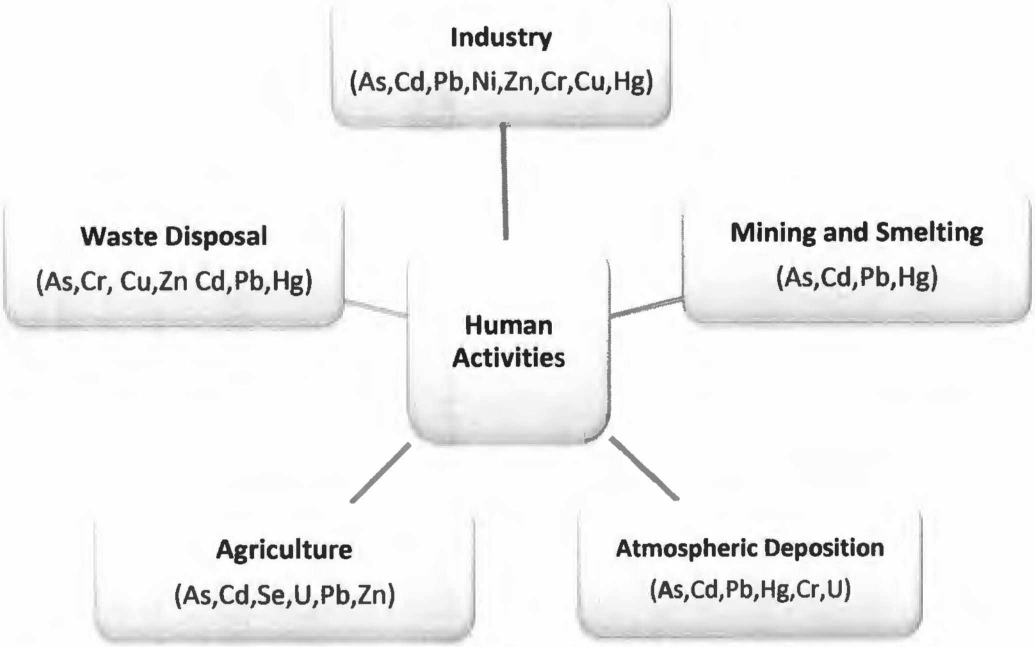

Figure 1: Human activities which contribute to soil contamination with heavy metals (Adapted from Ahemad, 2012)

All these sources cause accumulation of heavy metals in agricultural soils and pose significant threats to human health and food safety in the vicinity of mining areas (Mclaughlin *et al.*, 1999). Some common human health problems associated with the accumulation of toxic metals in humans are presented in Table 1.

Table 1: Heavy metals uses and health effects on humans

Metals	Uses	Health Effects
Cd	Electroplating, paints, plastics, alloys, batteries, pesticides, galvanized pipes, phosphate fertilizers	Cancer, cardiovascular disease, liver and kidney damage, damage to nervous tissue, emphysema, high blood pressure, anemia, arthritis, osteoporosis, obstructive lung disease, alopecia, growth impairment
Cr	Electroplating, galvanometry, pulp, leather tanning, mining, dyes, textile, nuclear weapons, petroleum refining	Ulcers, skin irritation, nausea cancer, vomiting, ulcers, asthma, liver and kidney damage
Ni	Ceramics, coins, fertilizers, batteries electroplating, , steel production	Liver necrosis, cancer, asthma, heart and liver damage, anemia, headache, fatigue, skin irritation, chronic damage to the nervous system, kidney and gastrointestinal tract

1.3.1 SITUATION IN SOUTH AFRICA

Currently, the mining industry in South Africa generates over R330 billion annually to the gross domestic product (GDP) of the country making it the fifth largest mining sector in the world. The country is famous for producing and supplying up to 56.7% of the world's platinum group metals (platinum, palladium, rhodium, ruthenium, osmium and iridium) and has a large reserve of other minerals including manganese (±80%), chromite ore (±72%), gold (±40%), vanadium, nickel and diamonds (Chamber of Mines South Africa, 2003). Despite these economic advantages, mining operations in South Africa have also led to widespread contamination of soils and water bodies with toxic heavy metals and other hazardous chemicals (McCarthy, 2011). The largest and presumably most heavily contaminated sites are found close to industrialized areas encompassing Johannesburg, Pretoria and Rustenburg (Gzik *et al.*, 2002). To address these concerns, several chemical and biological processes have been developed to remove toxic heavy metal from contaminated sites.

1.4 REMEDIATION TECHNOLOGIES FOR HEAVY METAL CONTAMINATED SOIL

The remediation of metal contaminated soil has focused primarily on physicochemical technologies including soil excavation and land fill, soil washing, and electrokinetic decontamination. However, these methods are unfeasible on large areas such as industrial or agrochemically contaminated soils because they are expensive and labour intensive (Green-Ruiz *et al.*, 2008; Pulford and Watson 2003; Tekerlekopoulou *et al.*, 2010). They also have the potential to adversely affect the physicochemical and biological properties of the treated soil which may lead to loss of soil fertility along with other negative impacts on the environment (Kumino *et al.*, 2001; Lasat, 2002; Quartacci *et al.*, 2006). These concerns have prompted the development of effective, low cost and eco-friendly technologies for soil remediation. Bioremediation is an example of such a technology.

1.4.1 BIOREMEDIATION

Bioremediation is a natural process that involves the use of biological agents (bacteria, algae, fungi, and plants) to neutralize or remove hazardous materials from the environment including heavy metals (Carrasco *et al.*, 2005; Delorme *et al.*, 2003).

1.4.2 BACTERIA–HEAVY METAL INTERACTIONS

The soil is an important ecological habitat that supports the growth of numerous types of bacteria whose survival depends on their ability to adapt to changes in environmental conditions caused by changes in temperature, pH, salinity, carbon, energy sources and water availability including high loads of metal contaminants. In low concentrations, some heavy metals such as Fe, Co, Cu, Zn, Cr, Ni, and Mn serve as essential micronutrients needed for bacterial growth and development. They serve as vital structural components in enzymes and nucleic acid, regulate gene expression, maintain osmotic balance and stabilize protein structures through electrostatic interactions (Bruins *et al.*, 2000).

On the other hand, heavy metals like Cd, Hg and Pb are non-essential. They have no known biological or physiological function in bacterial cells and thus are toxic even if present in small quantities (Pan and Wang 2012). Heavy metals can negatively influence soil bacterial communities by decreasing their growth, metabolic activity as well as functional diversity (Selvin *et al.*, 2009). Some toxic effects of heavy metals on bacteria are presented in Figure 2.

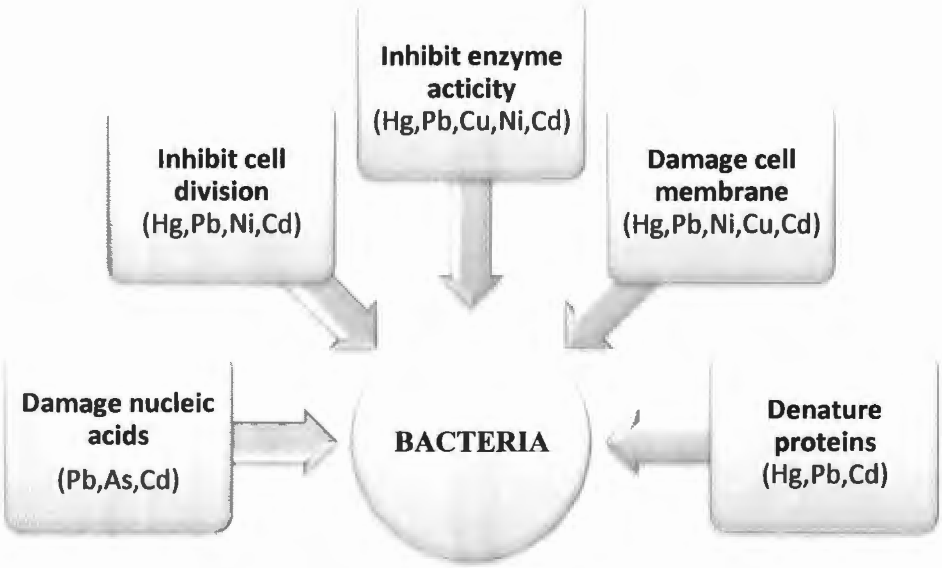

Figure 2: Effects of heavy metals on bacterial cells (Adapted from Khan *et al.*, 2009).

1.4.3 HEAVY METAL TOLERANCE IN BACTERIA

In order to survive in metal contaminated environments, bacteria have evolved a complex array of mechanisms to counteract the toxic effects of metal ions. Such adaptation mechanisms can be grouped into four main categories as follows:

1.4.3.1 EFFLUX SYSTEMS

Efflux systems are the most widely recognized and generally utilized mechanism for heavy metal tolerance in bacteria. They can be either P-type ATPases or chemiosmotic cation/H^+ antiporters e.g. Cation diffusion facilitator (CDF) exchangers and ATP-binding cassette (CBA) transporters. P-type ATPases are a superfamily of proteins which transport cations across the cell membrane using energy ATP hydrolysis. CBA transporters are large polypeptide complexes (proteins of the Resistance Nodulation Division) that transport metal ions across the cytoplasmic membrane into the exterior of the cell, while CDF exchangers are single-subunit systems that export metal ions from the cytoplasmic membrane into the periplasm (Arguello *et al.*, 2007; Franke *et al.*, 2003).

1.4.3.2 INTRACELLULAR BIOACCUMULATION

Some bacterial cells synthesize and release specific metal-binding proteins such as metallothioneins (MTs) and phytochelators. These proteins are low molecular weight, sulfhydryl-containing, cysteine-rich polypeptides that bind metal ions and reduce their bioavailability (Adamis *et al.,* 2004, Kao *et al.,* 2006; Umrania, 2006). For instance, SmtA, a metallothinein of *Synechococcus* PCC 7942, binds and detoxifies Zn^{2+} and Cd^{2+}. Another well-known metallothionein is BmtA that binds Zn^{2+} (Morby *et al.*, 1993).

1.4.3.3 EXTRACELLULAR METAL SEQUESTRATION AND COMPLEXATION

Researchers have demonstrated that bacterial surface structures (cell wall or envelope) are capable of adsorbing metal ions through a non-enzymatic process called biosorption (Pardo *et al.*, 2003). This process involves non-specific binding of metal cations to the cell envelope or extracellular polymeric substances (EPS) like proteins, polysaccharides, siderophores, teichoic and teichuronic acids (Henriques and Love 2007; Velasquez and Dussan 2009). These substances contain several functional groups like carboxyl, amide, phosphate, imidazole, sulhydryl, amino, carbonyl, phosphodiester and hydroxyl groups that give bacterial cell walls a strong negative charge (Gupta *et al.*, 2000; Vijayaraghavan and Yun 2008), allowing them to bind and regulate the interaction of metal cations with vital intracellular structures (Valls *et al.*, 2000).

1.4.3.4 ENZYMATIC DETOXIFICATION

Bacteria are able to reduce the toxicity of many heavy metals by converting them from a more toxic to a less toxic state through various processes such as oxidation, reduction, biomethylation, and demethylation (Elena *et al.*, 2005). An example is the reduction of Hg^{2+} to elemental Hg by mercuric reductase (MerA). Another is the reduction of Cr^{6+} to the less toxic Cr^{3+}.

These mechanisms are regulated by genes which have been found on bacterial chromosomes, transposons and plasmids. Examples of heavy metal ions with genetically-encoded mechanisms include Pb^{2+}, AsO^{2-}, AsO_4^{3-}, Bi^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+}, Ni^{2+}, TeO_3^{2-}, Tl^{+}, Zn^{2+} and CrO_4^{2-}.The tolerance mechanisms in bacteria to selected heavy metals are described in the section below.

1.4.4 GENETIC DETERMINANTS FOR CHROMIUM TOLERANCE IN BACTERIA

Bacterial tolerance against chromium is mainly achieved by a specific efflux system that pumps chromate ions out of the cell, subsequently reducing the intracellular concentration to sub-toxic levels (Branco *et al.*, 2008; Nies and Silver 1995; Ramırez-Dıaz *et al.*, 2008). This system is mediated by the *Chr* determinant which encodes a number of proteins including *ChrA, ChrB, ChrC, ChrE* and *ChrF* (Juhnke *et al.*, 2002). The *ChrA* is a membrane bound protein that transports chromate ions from the cytoplasm (Pimentel *et al.*, 2002), *ChrB* reduces chromate accumulation and also functions as a regulator of the *Chr* operon, *ChrC* is proposed to be responsible for the production of superoxide dismutase and *ChrF* encodes a protein with an unknown function (Morais *et al.*, 2011). These tolerance determinants have been identified in *Cupriavidus metallidurans* CH34 (Nies *et al.*, 1990), *Pseudomonas aeruginosa* PAO1 (Cervantes *et al.*, 1990), *Shewanella* sp. strain ANA (Aguilar-Barajas *et al.*, 2008), *Synechococcus elongatus* PCC 7942 (Aguilar-Barajas *et al.*, 2012), *Lysinibacillus fusiformis* ZC1 (He *et al.*, 2011), *Bacillus cereus* SJ1 (He *et al.*, 2010).

1.4.5 GENETIC DETERMINANTS FOR CADMIUM TOLERANCE IN BACTERIA

Cd tolerance in bacteria is based on two main mechanisms: *CadA* ATPase, a membrane-localized efflux pump that has been widely studied in Gram-positive bacteria (e.g. *Staphylococcus aureus*) and the plasmid-borne *Czc* operon in gram negative bacteria (e.g. *Cupriavidus metallidurans* CH34 (Mergeay *et al.*, 1985; Nies *et al.*, 1987). The *Czc* operon is an inducible, energy-dependent cation efflux system that exports Cd^{2+}, Zn^{2+} and Co^{2+} from bacterial cells (Figure 3). It is made up of three structural genes (*CzcC, CzcB* and *CzcA*) and two regulatory genes (*CzcD* and *CzcR*) (Nies, 2003). The *CzcA* is an inner membrane transport protein that functions as a chemiosmotic cation/H^+ antiporter driven by an electrochemical proton gradient (Leedjärv *et al.*, 2008). *CzcB* is a membrane bound protein that links the inner and outer cell membrane of gram-negative bacteria. It is responsible for removing metal ions from the cytoplasm (Choudhury and Srivastava 2001). The *CzcC* is an outer membrane protein, thought to be a modulator of substrate specificity (Nies, 1992).

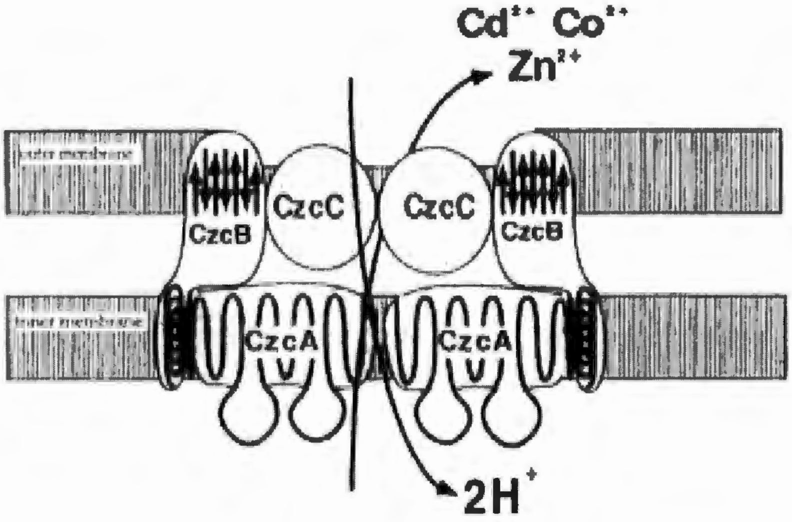

Figure 3: *Czc* model for Cd^{2+}, Zn^{2+} and Co^{2+} tolerance mechanism functioning as a chemiosmotic cation/H^+ antiporter consisting of an inner membrane transport protein (*CzcA*), is a membrane fusion protein (*CzcB*) and an outer membrane protein (*CzcC*) (Adapted from Silver (1996).

1.4.6 GENETIC DETERMINANTS FOR NICKEL TOLERANCE IN BACTERIA

Bacterial tolerance to nickel is encoded by two systems: the *Cnr* (nickel-cobalt) and *Ncc* (nickel-cobalt-cadmium) operons. The *Cnr* operon is usually located on the mega plasmid pMOL28 and consists of three structural genes (*CnrA, CnrB, CnrC*), that are homologous to those of *CzcABC* and three regulatory genes (*CnrY, CnrX, CnrH*), all of which mediate inducible tolerance to Co^{2+} and Ni^{2+} (Liesegang *et al.*, 1993). The *CnrA* protein, which is a member of the RND, is responsible for proton/cation antiporter efflux system whereas *CnrB* and *CnrC* are membrane fusion proteins which influence the degree and specificity of metal ion efflux. The Ncc operon consists of seven open reading frames (ORF's) i.e. *NccNYXHCBA*, in which *NccCBA* represent the structural genes. *NccXYH* is involved in the regulation of the structural genes while *NccA* forms a tunnel that allows transport across the membrane. *NccB* and *NccC* are proposed to be involved in cation binding and substrate specificity.

1.4.7 PHYTOREMEDIATION TECHNOLOGY: OVERVIEW

The term phytoremediation includes a set of technologies in which plants are used to remove organic or/and inorganic contaminants from the environment (Angle and Linacre 2005; Chehregani *et al.*, 2009; Ghosh and Singh 2005; Haque *et al.*, 2008; Kotrba *et al.*, 2009; Ma *et al.*, 2009; Peng *et al.*, 2009; Suresh and Ravishankar 2004; Weyens *et al.*, 2009). In recent years, this concept has received increasing interest because of certain beneficial features:

1. It is generally less expensive to manage and operate when compared to physiochemical remediation methods. This is mainly because the energy required for the process is acquired from sunlight.
2. It also has the potential to treat sites containing multiple contaminants and can be used to clean up large areas of soil with low to moderate levels of contamination.
3. It is an aesthetically pleasing technology in that it helps improve the natural properties and functioning of soil by promoting microbial activity and diversity. It also helps to prevent landscape destruction by water and wind erosion (Fischerova *et al.*, 2006; Miretzky, 2004; Sekhar *et al.*, 2005).

On the other hand, phytoremediation is a relatively slow process (i.e. between 10 and 20 years) because of its dependence on plant growth and tolerance to contaminants. Secondly, it cannot be used to treat sites with heavy contamination because the harsh conditions could inhibit plant growth. Effectiveness may be influenced by prevailing climatic conditions and the consumption of contaminated plants by animals with subsequent transfer into the food chain is also an issue of great concern. Depending on the nature of contaminant involved and the way plants are used, phytoremediation can be broadly categorized into several distinct processes summarized in Table 2.

Table 2: Various phytoremediation processes

Phytoremediation technique	**Mode of action**
Phytoextraction	Absorption and accumulation of heavy metals from the soil through the roots into harvestable tissues of plants
Phytostabilisation	Plants reduce the mobility of organic or inorganic contaminants in soil and water as a result of adsorption or accumulation within the root zone
Rhizofiltration	Plants are used to reduce or adsorb contaminants from aquatic environments (water and wastewater) through absorption or precipitation onto their roots
Phytovolatilization	Plants extract volatile organic compounds (TCE and MTBE) and metal contaminants from soil and water, translocate to the aerial parts and then release them into the atmosphere by evaporation through the leaf surface
Phytodegradation	Use of plants in association with microbes to break down organic compounds (herbicides, TNT, MTBE, and TCE) in soil and surface water through metaboilic processes with enzymes

The following section provides a summary of the use of plants in phytoremediation, with emphasis on phytoextraction.

1.4.7.1 PHYTOEXTRACTION OF METAL–CONTAMINATED SOILS

The primary objective of phytoextraction is the utilization of metal-tolerant plants to accumulate and store large amounts of metal ions in their shoots. Plants are grown on contaminated sites for a few months after which they are harvested and incinerated or composted for use as fertilizer (Whiting *et al*., 2004). Certain important metals can also be recycled through a process called phytomining. For viable and effective phytoextraction, plants must not only have the capacity to tolerate and accumulate high concentrations of one or more metals, but must also be able to grow very quickly and produce a high above-ground biomass (Dary *et al*., 2010; McGrath *et al.,* 2002; Nie *et al.,* 2002).

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1.4.7.2 HYPERACCUMULATORS

Hyperaccumulators are usually defined based on accumulating capability or bioconcentration factor (BCF): the ratio of metal concentration in plants relative to that in soil. Based on this fact, Baker *et al.,* 2000 described hyperaccumulators as herbaceous and woody plants that are capable of accumulating more than 1000 mg/kg of heavy metals (Cu, Co, Cr, Ni, or Pb) in their dry shoots and leaves. Currently, there are approximately 400 plant species from at least 45 plant families that have the ability to tolerate and accumulate large amounts of heavy metals without showing any symptoms of toxicity (McIntyre, 2003; Prasad and Freitas 2003). Common examples include; *Ricinus communis* (castor bean), *Brassica juncea* (Indian mustard), *Brassica napus* (turnip), *Thlaspi caerulescens* (alpine pennycress), *Zea mays* (maize), *Helianthus annuus* (sunflower*),* and *Salix spp* (willow) (Milner and Kochian 2008). Although these plants are capable of accumulating large amounts of metal ions in their shoot tissues, most of them are not suitable for cleaning up soils with high levels of metals due to their slow growth and low biomass production.

1.4.7.2.1 *Brassica juncea* – A HYPERACCUMULATOR OF HEAVY METALS

Brassica juncea or Indian mustard is an oil producing plant of the Brassicaceae family that has been described by some researchers as a 'model' plant species for phytoextraction because of its ability to grow in harsh environments together with a high capacity for metal ion accumulation (Gisbert *et al.*, 2006; Quartacci *et al.*, 2006). It is an annual plant that is capable of self-pollination and can grow up to 1 m or more. The lower leaves are deeply lobed with toothed, scalloped or frilled edges, while the upper leaves are narrow and entire and short petioled. This plant species has been found to be tolerant to a number of heavy metals, including Ni, Pb, Zn, Cd and Se, a physiological trait which can be exploited for the bioremediation of metal contaminated soils and waters.

1.4.8 MECHANISMS OF METAL TOLERANCE AND HYPERACCUMULATION IN PLANTS

Hyperaccumulators have adaptive mechanisms for tolerating high concentrations of heavy metals which also allow accumulation of large quantities of metals (Xiao *et al.*, 2008). Although such mechanisms are complex and often less understood, there is a consensus that four major processes are involved:

1. Active transport of metal ions through the plasma membrane transport proteins of root cells
2. Xylem loading and transport to shoots
3. Cytoplasmic chelation and compartmentalization within (cell walls, vacuoles)
4. Sequestration and detoxification (glutathione–phytochelatin) in specific cell compartments within the leaves (Jabeen *et al.*, 2009; Lombi *et al.*, 2002; Mendoza-Cozatl and Moreno-Sanchez 2006).

Despite these mechanisms, the application of most hyperaccumulators in phytoextraction is greatly restricted by their low uptake of metal ions, small annual biomass production and slow growth in heavily contaminated soils (Begonia *et al.,* 2005; Carrasco *et al.,* 2005; Dickinson *et al.*, 2008; Doty 2008; Luo *et al.,* 2005; Martha *et al.*, 2009; Weyens *et al.*, 2010).

1.4.9 FACTORS THAT INFLUENCE THE RATE OF PHYTOEXTRACTION

Metal accumulation by plants is mainly influenced by the existing state of the metal i.e. bioavailability (Chen *et al.*, 2004; Garbisu and Alkorta 2001; Sheng *et al.*, 2008). Metal bioavailability with regards to phytoextraction represents the fraction or percentage of metals in the soil that is available for plant root uptake. It is strongly governed by several physicochemical and biological properties of soils such as pH, redox reactions, cation exchange capacity (CEC), organic matter content, electrical conductivity of clay minerals, hydrous oxides, water content, temperature, soil particle size, and climate changes.

1.4.10 HOW TO MAKE METAL PHYTOEXTRACTION MORE EFFICIENT

To enhance metal ion mobility and solubility in phytoextraction studies, several synthetic chelating agents like EDTA (ethylene-diamine-tetra-acetic acid), DTPA (diethylene triaminepenta-acetic acid), EDGA (glycoletherdiamine tetra-acetic acid), HEDTA (hydroxyethyl-ethylenediaminetriacetic), NTA (nitrilotriacetic), and EDDS (ethylenediaminedisuccinic acid), have been used. These compounds function by absorbing heavy metals and forming soluble metal complexes which can either be taken up as free metal ions or entire complexes by plant roots (Chiu *et al.*, 2005; Evangelou *et al.*, 2007; Khan *et al.*, 2000; Luo *et al.*, 2005; Romken *et al.*, 2002). Unfortunately, the use of these complexing agents in field conditions may constitute a new source of soil pollution. For example, these chelators and the formed chelate–metal complexes generally show a low degree of biodegradability (Lombi *et al.*, 2001) and are highly toxic for plants and soil microbes (Bouwman *et al.*, 2005). Moreover, these synthetic compounds can leach into the subsoil thereby increasing the risk of surface and groundwater contamination as well as deleterious effects on soil fertility (White, 2001). All these limitations have prompted researchers to formulate alternative strategies to make phytoremediation a feasible technology.

1.4.11 PLANT–BACTERIA INTERACTIONS AND THEIR ROLE IN PHYTOEXTRATION OF HEAVY METALS

The inoculation of plant seeds with metal tolerant bacteria to promote plant growth and metal accumulation is currently an area of rapidly expanding research. Plant roots are known to produce and secrete a wide range of organic compounds (Table 3) into the soil that attract many different types of bacteria. Some bacteria are harmful and therefore inhibit plant development; some are neutral and so have no influence on plant growth while others are beneficial.

Such beneficial bacteria use plant root exudates as substrates to facilitate their growth and metabolism and in turn improve soil health and plant growth through several mechanisms (Gupta *et al.*, 2000; Vessey, 2003). For example, bacteria can enhance plant growth directly by providing the host plant with certain essential nutrients that are not easily accessible. They can: convert atmospheric nitrogen and supply plants with elemental nitrogen in the form of either ammonia or nitrate (Park *et al.*, 2005; Sahin *et al.*, 2004), convert unavailable forms of bound fixed and insoluble phosphorous into soluble forms ($H_2PO_4^-$ and HPO_4^{-2}) making it readily available for plant uptake (Jeon *et al.*, 2003), synthesize metal-chelating agents like siderophores which can solubilize Fe^{3+} reducing it to Fe^{2+} which is more easily absorbed by plant roots and other microorganisms (Belimov *et al.*, 2005; Burd *et al.*, 2000; Madhaiyan and Poonguzhali 2007; Patten and Glick 2002; Tripathi *et al.*, 2005).

Table 3: Organic Compounds Released by Plant Roots (Macario *et al*., 2003; Nicholas, 2007)

Compounds	**Single components**
Sugars and Polysaccharides	Arabinose, deoxyribose, fructose, galactose, glucose, maltose, mannose, mucilages of various composition, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose
Amino Acids	α-alanine, β-alanine, amino adipic, gamma amino butyric, arginine, asparagines, aspartic, citrulline, cystathionine, cysteine, cystine, deoxymugineic, 3-epihydroxymugineic, glutamic, glycine, homoserine, histidine, leucine, lysine, methionine, mugineic, ornithine, phenylalanine, proline, serine, threonine, tyroptophan, tyrosine, valine
Organic Acids	Acetic, aconitic, aldonic, ascorbic, benzoic, butyric, caffeic, citric, p-coumaric, erythonic, ferulic, formic, fumaric, glutaric, glycolic, glyoxilic, lactic, malic, malonic, oxalacetic, p-hydroxy benzoic, piscidic, propionic, pyruvic, succinic, syringic, tartaric, tetronic, valeric, vailli
Fatty Acids	Linoleic, linolenic, oleic, stearic
Sterols	Campesterol, cholesterol, sitosterol, stigmasterol
Growth Factors	p-amino benzoic acid, biotin, choline, n-methyl nicotinic acid, niacin, pantothenic, vitamins B_1 (thiamine), B_2 (riboflavin), and B_6 (pyridoxine)
Enzymes	Amylase, invertase, peroxidase, phenolase, phosphatases, polygalacturonase, protease
Flavonones and Nucloetides	P-amino benzoic acid, biotin, choline, n-methyl nicotinic acid, niacin, pantothenic, vitamins B_1 (thiamine), B_2 (riboflavin), and B_6 (pyridoxine).

Bacteria also synthesize several different phytohormones including auxins (IAA, indole-3-acetic acid), cytokinins and gibberelins, which all act to enhance the different phases of plant growth (Egamberdiyeva, 2005; Garcia de Salamone *et al.*, 2001; Gutierrez-Manero *et al.*, 2001). In addition, a number of bacteria are capable of decreasing metal toxicity to plants by inhibiting ethylene synthesis, thanks to the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme which hydrolyses ACC, the intermediate precursor of ethylene to yield ammonia and α- ketobutyrate (Dell'Amico *et al.*, 2005; Glick, 2005).

Bacteria can also enhance plant growth indirectly by providing protection against phytopathogenic microbes using a wide range of mechanisms. Such mechanisms include: competition for nutrients and colonization sites on roots, limiting the amount of iron available for the growth of pathogens through the production of siderophores, antibiotics (ammonia, butyrolactones, 2,4-diacetyl phloroglucinol, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, zwittermycin A), hydolytic enzymes (Chitinase, glucanases, pectinase cellulase, pectin lyase and cutinase) and other antimicrobial compounds like DAPG, phenazines, pyrrolnitrin, and Hydrocyanic acid (HCN) (Dobbelaere *et al.*, 2002; Dey *et al.*, 2004). Specific strains of non-pathogenic bacteria can also trigger plant defence mechanisms against a wide variety of pathogenic microbes by stimulating the production of multiple antimicrobial compounds. This phenomenon is called induced systemic resistance (ISR) (Glick *et al.*, 2007; Van Loon *et al.*, 1998). Several studies have shown that metal tolerant bacteria are able to enhance plant growth and increase metal uptake (Table 4).

Table 4: Examples of bacteria assisted phytoextraction

Experimental plant	**Bacterial strain**	**Mode of action**	**Effect on plant**	**Reference**
B. oxyrrhina	*Psychrobacter sp.*SRA1, SRA2, *Bacillus cereus* SRA10	Ni^{2+} mobilization, IAA, P solubilization, ACCD, siderophore production	Increased root and shoot length, vigour index of seedlings, fresh and dry weight, Ni^{2+} availability and uptake	Ma *et al.*, 2009
P. sativum	*Pseudomonas brassicacearum* Am3	ACCD production	Increased plant biomass and nutrient uptake	Safronova *et al.*, 2006
R. communis	*Pseudomonas sp.* PsM6, *Pseudomonas jessenii* PjM15	ACCD, siderophore, and IAA,[Ni^{2+}, Cu^{2+}, Zn^{2+}] biosorption , mobilization	Increased shoot length, root length, fresh and dry weight of plant Zn^{2+} uptake	Rajkumar *et al.*, 2008
Z. mays	*Burkholderia sp.* J62	ACCD, siderophore and IAA production , P solubilization	Increased shoot and root dry weight including Pb^{2+} accumulation	Jiang *et al.*, 2008
H. annuus	*B. weihenstephanensis* SM3	IAA, P solubilization, [Cu, Zn, Ni] biosorption and mobilization	Enhanced metal [Cu, Zn] accumulation Increased plant biomass	Rajkumar *et al.*, 2008
	Streptomyces tendae F4	siderophores	Increased iron content	Dimpka *et al.*, 2009
B. juncea	*Enterobacter aerogenes* and *Rahnella aquatilis*	P solubilization, IAA siderophore production	Increased plant biomass, protein, chlorophyll content and uptake of soil minerals	Kumar *et al.*, 2009
	Variovorax paradoxus, *Rhodoccus sp.*, *Flavobacterium sp.*	siderophores, ACCD	Increased root length; Increased growth	Belimov *et al.*, 2005
	Pseudomonas sp. A4, *Bacillus sp.* 32	IAA, siderophores, P solubilization	Increased root and shoot length	Rajkumar *et al.*, 2006
	Achromobacter xylosoxidans Ax10	ACCD, IAA, P solubilization	Enhanced Ni and Cr accumulation Increased plant biomass	Ma *et al.*, 2009

Experimental plant	**Bacterial strain**	**Mode of action**	**Effect on plant**	**Reference**
	Bacillus edaphicus NBT	IAA, siderophores, ACCD	Increased plant biomass	Sheng *et al.*, 2008
	B. subtilis SJ-101	IAA, P solubilization	Increased nickel uptake	Zaidi *et al.*, 2006
	Pseudomonas sp. 29C, Bacillus sp. 4C	IAA, siderophores, ACCD, P solubilization	Increased plant biomass	Rajkumar and Freitas 2008
B. napus	*Bacillus* sp. RJ16	IAA, Cd mobilization	Increased root and shoot length together with plant biomass Increased Cd uptake by plant	Sheng and Xia 2006
	Pseudomonas tolaasii ACC23, *P. fluorescens* ACC9, *Mycobacterium sp.* ACC14	ACCD, siderophore, IAA	Plant root elongation promotion activity, shoot and root dry biomass increased Cd uptake	Dell'Amico *et al.*, 2008
	Bacillus licheniformis BLMB1	unknown	Enhanced Cr uptake	Brunetti *et al.*, 2011
	Enterobacter cloacae CAL2		Increased plant biomass	Nie *et al.*, 2002
	Kluyvera ascorbata SUD165	ACCD	Increased plant biomass	Burd *et al.*, 1998
	Psuedomonas aspleni AC	IAA	Increased plant biomass	Reed and Glick 2005
A. murale	*Microbacterium oxydans* AY509223	Ni mobilization	Increased Ni uptake	Abou-Shanab *et al.*,2003
Orychophragmus violaceus	*Flavobacterium* sp.	unknown	Increased root length, biomass, metal uptake	He *et al.*, 2010
Cajanus cajan	*Proteus vulgaris* KNP3	unknown	Increased seed germination, plant biomass and chlorophyll	Rani *et al.*, 2008

Experimental plant	**Bacterial strain**	**Mode of action**	**Effect on plant**	**Reference**
Arabidopsis thaliana	*Pseudomonas putida* ARB86	unknown	Increased plant biomass and chlorophyll content	Someya *et al.*, 2007
Tomato	*Pseudomonas* sp. RJ10, *Bacillus* sp. RJ16	siderophores, IAA, ACCD	Increased root length and plant biomass	He *et al.*, 2009

1.5 HYPOTHESIS, AIM AND OBJECTIVES

Ni, Cr and Cd are toxic metals commonly found at contaminated sites. The use of bacteria and plants in bioremediation offers a less expensive option for the detoxification of metal contaminated sites. Research on this subject has led to the identification of various bacterial and plant species with the capacity to tolerate and immobilize metals in soil and water. However, additional information on metal tolerant organisms is required. This makes the identification and characterization of metal tolerant bacteria an important pathway to pursue.

We hypothesize that bacteria found in metal contaminated soils are prone to develop resistance and may eventually become much more tolerant to higher concentrations of metal ions. In addition, such bacteria could serve as growth–promoting inoculants for hyperaccumulators in metal contaminated soil. Based on these assumptions, this project was launched to obtain more information on how bacteria (bacteria-metal interactions, bacteria-plant interactions) can be used to develop new bioremediation processes for chromium, cadmium and nickel contaminated environments. Thus, our objectives were to:

1. Isolate and identify Ni, Cr and Cd tolerant bacteria from metal contaminated soil
2. Determine the maximum tolerable concentration (MTC) and bioaccumulation capacities of bacterial isolates
3. Screen bacterial isolates for the presence of plasmids and metal tolerance genes on both plasmid and chromosomal DNA
4. Screen metal tolerant bacteria for the production of plant growth promoting substances such as IAA, ammonia (NH_3) and hydrogen cyanide (HCN)
5. Evaluate the effects of bacterial inoculation on growth and heavy metal (Ni, Cr and Cd) accumulation by *B. juncea.*

REFERENCES

ABOU-SHANAB RA, ANGLE JS, DELORME TA, CHANEY RL, VERKUM P VAN, MOAWAD H, GHANEM K & GHOZLAN HA 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale. New Phytologist,* 158, 219-222.

ABOU-SHANAB RA, VAN BERKUM P & ANGLE JS 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale. Chemosphere,* 68, 360-367.

ADAMIS PD, GOMES DS, PEREIRA MD, FREIRE DE MESQUITA J, PINTO ML, PANEK AD & ELEUTHERIO EC 2004. The effect of superoxide dismutase deficiency on cadmium stress. *Journal of Biochemistry and Molecular Toxicology,* 18, 12-17.

AGUILAR-BARAJAS E, JERONIMO-RODRIGUEZ P, RAMIREZ-DIAZ MI, RENSING C & CERVANTES C 2012. The ChrA homologue from a sulfur-regulated gene cluster in *cyanobacterial* plasmid pANL confers chromate resistance. *World Journal of Microbiology and Biotechnology,* 28, 865-869.

AGUILAR-BARAJAS E, PALUSCIO E, CERVANTES C & RENSING C 2008. Expression of chromate resistance genes from *Shewanella* sp. strain ANA-3 in *Escherichia coli. FEMS Microbiology Letters,* 285, 97-100.

AHEMAD M 2012. Implications of bacterial resistance against heavy metals in bioremediation: a review. *The IIOAB Journal,* 3, 39-46.

ANGLE JS & LINACRE NA 2005. Metal Phytoextraction: A survey of potential risks. *International Journal of Phytoremediation,* 7, 241-254.

ARGUELLO JM, EREN E & GONZALEZ-GUERRERO M 2007. The structure and function of heavy metal transport P1B-ATPases. *Biometals,* 20, 233-248.

BAKER AJM, MCGRATH SP, REEVES R & SMITH JAC 2000. Metal hyperaccumulator plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils, *Boca Raton, Lewis Publishers*. 362-368.

BEGONIA MT, BEGONIA GB, IGHOAVODHA M & GILLIARD D 2005 Lead accumulation by tall fescue (*Festuca arundinacea Schreb*) grown on a lead-contaminated soil. *International Journal of Environmental Research and Public Health,* 2, 228-233.

BELIMOV A, HONTZEA AA SB, SAFRONOVAA VI, DEMCHINSKAYAA SV, PILUZZAC G, BULLITTAC S & GLICK BR 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern.*). *Soil Biology and Biochemistry,* 37, 241–250.

BOUWMAN LA, BLOEM J, ROMKENS PF & JAPENGA J 2005. EDGA amendment of slightly heavy metal loaded soil affects heavy metal solubility, crop growth and microbivorous nematodes but not bacteria and herbivorous nematodes. *Soil Biology and Biochemistry,* 37, 271-278.

BRANCO R, CHUNG AP, JOHNSTON T, GUREL V & MORAIS P 2008. The chromate-inducible *chrBACF* operon from the transposable element *TnOtChr* confers resistance to chromium (VI) and superoxide. *Journal of Bacteriology,* 190, 6996-7003.

BRUINS MR, KAPIL S & OEHME FW 2000. Microbial resistance to metals in the environment. *Ecotoxicology and Environmental safety,* 45, 198-207.

BRUNETTI G, FARRAG K, ROVIRA SP, NIGRO F & SENESI N 2011. Greenhouse and field studies on Cr, Cu, Pb and Zn phytoextraction by *brassica napus* from contaminated soils in the Apulia region, Southern Italy. *Geoderma* 160, 517-523.

BURD GI, DIXON DG & GLICK BR 1998. A plant growth promoting bacterium that decreases nickel toxicity in plant seedlings. *Applied Environmental Microbiology,* 64, 3663-3668.

BURD GI, DIXON DG & GLICK BR 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal of Microbiology,* 46, 237-245.

CARRASCO JA, ARMARIO P, PAJUELO E & BURGOS A 2005. Isolation and characterisation of symbiotically effective *Rhizobium* resistant to arsenic and heavy metals after the toxic spill at the Aznalcollar pyrite mine. *Soil Biology and Biochemistry,* 37, 1131-1140.

CARRILLO-CHAVEZ A, MORTON-BERMEA O, GONZALEZ-PARTIDA E, RIVAS-SOLORZANO H, OESLER G, GARCIA-MEZA V & HERNANDEZ E 2003. Environmental Geochemistry of the Guanajuato Mining District, Mexico, Ore. *Geoleogy Reviews,* 23, 277-297.

CERVANTES C, OHTAKE H, CHU L, MISRA TK & SILVER S 1990. Cloning, nucleotide sequence, and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid *pUM505*. *Journal of Bacteriology,* 172, 287-291.

CHAMBER OF MINES SOUTH AFRICA, 2003.The contribution of the mining and minerals industry to sustainable development in South Africa. */http://www.bullion.org.za.* Accessed (06.05.2014).

CHEHREGANI A, NOORI M & YAZDI HL 2009. Phytoremediation of heavy-metal-polluted soils: Screening for new accumulator plants in Angouran mine (Iran) and evaluation of removal ability *Ecotoxicology and Environmental safety,* 72, 1349-1353.

CHEN YH, SHEN ZG & LI XD 2004. The use of vetiver grass (*Vetiveria Zizanioides*) in the phytoremediation of soils contaminated with heavy metals. *Applied Geochemistry,* 19, 1553-1565.

CHIU KK, YE ZH & WONG MH 2005. Enhanced uptake of As, Zn and Cu by *Vetiveria zizanioides* and *Zea mays* using chelating agents. *Chemosphere,* 60, 1365-1375.

CHOUDHURY R & SRIVASTAVA S 2001. Zinc resistance mechanisms in bacteria. *Current Science,* 81, 768-775.

DARY AM, CHAMBER-PÉREZB MA, PALOMARESA AJ & PAJUELOA E 2010. In situ" phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. *Journal of Hazardous Materials,* 177, 323-330.

DELLAMICO E, CAVALCA L & ANDREONI V 2005 Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria *FEMS Microbiology and Ecology,* 52, 153-162.

DELLAMICO E, MAZZOCCHI M, CAVALCA L, ALLIEVI L & ANDREONI VA 2008. Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. *Microbiology Research* 163, 671-83.

DELORME TA, GAGLIARDI JV & ANGLE JS 2003. Phenotypic and genetic diversity of rhizobia isolated from nodules of clover grown in zinc and cadmium contaminated soil. *Soil Science Society of American Journal* 67, 1746-1752.

DEY R, PAL KK, BHATT DM & CHAUHAN SM 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea L.*) by application of plant growth-promoting rhizobacteria. *Microbiology Research,* 159, 371-394.

DICKINSON NM, BAKER AJM, DORONILA A, LAIDLAW S & REEVES RD 2008.

Phytoremediation of inorganics: realism and synergies. *International Journal of Phytoremediation,* 11, 97-114.

DOBBELAERE S, CROONENBORGHS A, THYS A, PTACEK D, OKON Y & VANDERLEYDEN J 2002. Effects of inoculation with wild type *Azospirillum brasilense* and *A. irakense* strains on development and nitrogen uptake of spring wheat and grain maize. *Biology and Fertility of Soils,* 36, 284-297.

DOTY SL 2008. Enhancing phytoremediation through the use of transgenics and endophytes. *New Phytologist,* 179, 318-333.

DUZGOREN-AYDIN NS, WONG CSC, AYDIN A, SONG Z, YOU M & LI XD 2006. Heavy metal contamination and distribution in the urban environment of Guangzhou, Se China. *Environmental Geochemistry and Health,* 28, 375-391.

EGAMBERDIYEVA D 2005. Plant-growth promoting rhizobacteria isolated from a Calcisol in a semi-arid region of Uzbekistan: biochemical characterization and effectiveness. *Journal of Plant Nutrition and Soil Science,* 168, 94-99.

ELENA DA, LUCIA C & VINCENZA A 2005. Analysis of rhizobacterial communities in perennial *Graminaceae* from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiology Ecology,* 52, 153-162.

EVANGELOU MWH, EBEL M & SCHAEFFER A 2007. Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. *Chemosphere,* 68, 989-1003.

FENG XB, FOUCHER D, HINTELMANN H, YAN HY, HE TR & QIU GL 2010. Tracing mercury contamination sources in sediments using mercury isotope compositions. *Environmental Science and Technology,* 44, 3363-3368.

FISCHEROVA Z, TLUSTOS P, SZAKOVA J & SICHOROVAK 2006. A comparison of phytoremediation capability of selected plant species for given trace elements *Environmental Pollution,* 144, 93-100.

FRANKE S, GRASS G, RENSING C, NIES DH, NIES A & SILVER S 2003. Molecular analysis of the copper transporting efflux system *CusCFBA* of *Escherichia coli. Journal of Bacteriology,* 185, 3804-3812.

GARBISU C & ALKORTA I 2001. Phytoextraction: a cost-effective plant based technology for the removal of metals from the environment. *Bioresource Technology,* 77, 229-236.

GARCIA DE SALAMONE IE, HYNES RK & NELSON LM 2001. Cytokinin production by plant growth promoting rhizobacteria and selected mutants. *Canadian Journal of Microbiology,* 47, 404-411.

GERHARDT KE, HUANG XD, GLICK BR & GREENBERG BM 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science,* 176, 20-30.

GHOSH M & SINGH SP 2005. A review on phytoremediation of heavy metals and utilization of its by-products. *Applied Ecology and Environmental Research,* 3, 1-18.

GISBERT C, CLEMENTE R, NAVARRO-AVINO J, BAIXAULI C, GINER A, SERRANO R, WALKER DJ & PILAR BERNAL M 2006. Tolerance and accumulation of heavy metals by Brassicaceae species grown in contaminated soils from Mediterranean regions of Spain. *Environmental and Experimental Botany*, 56, 19-27.

GLICK BR 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnology Advances,* 21, 383-393.

GLICK BR 2005. Modulation of plant ethylene levels by the bacterial enzyme *ACC* deaminase. *FEMS Microbiology Letters* 251, 1-7.

GLICK BR, CHENG Z, CZARNY J & DUAN J 2007. Promotion of plant growth by ACC deaminase containing soil bacteria. *European Journal of Plant Pathology,* 119, 329-339.

GREEN-RUIZ C, RODRIGUEZ-TIRADO V & GOMEZ-GIL B 2008. Cadmium and zinc removal from aqueous solutions by *Bacillus jeotgali*: pH, salinity and temperature effects. *Bioresource Technology,* 99, 3864-3870.

GUPTA R, AHUJA P, KHAN S, SAXENA RK & MOHAPATRA H 2000. Microbial biosorbents: meeting challenges of heavy metal pollution in aqueous solutions. *Current Science,* 78, 967-973.

GUTIERREZ-MANERO FJ, RAMOS-SOLANO B, PROBANZA A, MEHOUACHI J, TADEO FR & TALON M 2001. The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. *Plant Physiology,* 111, 206-211.

GZIK A, KUEHLING M, SCHNEIDER I & TSCHOCHNER B 2002. Heavy metal contamination of soils in a mining area in South Africa and its impact on some biotic systems. *DOI; http://dx.doi.org/10.1065/jss2002.08.054.*

HAQUE N, PERALTA-VIDEA JR, JONES GL, GILL TE & GARDEA-TORRESDEY JL 2008. Screening the phytoremediation potential of desert broom (*Baccharis sarothroides Gray*) growing on mine tailings in Arizona, USA. *Environmental Pollution,* 153

HE LY, CHEN ZJ, REN GD, ZHANG YF, QIAN M & SHENG XF 2009. Increased cadmium and lead uptake of a cadmium hyperaccumulator tomato by cadmium-resistant bacteria. *Exotoxicology and Environmental Safety,* 72, 1343-1348.

HE M, LI X, GUO L, MILLER SJ, RENSING C & WANG G 2010. Characterization and genomic analysis of chromate resistant and reducing *Bacillus cereus strain SJ1. BMC Microbiology,* 10, 221.

HE M, LI X, LIU H, MILLER SJ & WANG G 2011. Characterization and genomic analysis of a highly chromate resistant and reducing bacterial strain *Lysinibacillus fusiformis ZC1. Journal of Hazardous Materials,* 185, 682-688.

HENRIQUES IDS & LOVE NG 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. *Water Research,* 41, 4177-4185.

HERAWATI N, SUSUKI S, HAYASHI K, RIVAI IF & KOYAMA H 2000. Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia, and China by soil type. *Bulletin of Environmental Contamination and Toxicology,* 64, 33-39.

JABEEN R, AHMAD A & IQBAL M 2009. Phytoremediation of heavy metals: physiological and molecular mechanisms. *Botany Reviews,* 75, 339-364.

JEON JS, LEE SS, KIM HY, AHN TS & SONG HG 2003. Plant growth promotion in soil by some inoculated microorganisms. *Journal of Microbiology,* 41, 271-276.

JIANG CY, SHENG XF, QIAN M & WANG QY 2008. Isolation and characterization of a heavy metal resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal polluted soil. *Chemosphere,* 72, 157–164.

JUHNKE S, PEITZSCH N, HIIBENER N, GROBE C & NIES DH 2002. New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34. *Archives of Microbiology,* 179, 15-25.

JUNG MC 2008. Contamination by Cd, Cu, Pb, and Zn in mine wastes from abandoned metal mines classified as mineralization types in Korea. *Environmental Geochemistry and Health,* 30, 205-217.

KAO PH, HUANG CC & HSEU ZY 2006. Response of microbial activities to heavy metals in a neutral loamy soil treated with biosolid. *Chemosphere,* 64, 63-70.

KHAN AG, KUEK C, CHAUDHRY TM, KHOO CS & HAYES WJ 2000. Role of plants, mycorrhizal and phytochelators in heavy metal contaminated land remediation. *Chemosphere,* 41, 197-207.

KHAN MS, ZAIDI A, WANI PA, & OVES M 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. *Environmental Chemistry Letters*, 7:1-19.

KOTRBA P, NAJMANOVA J, MACEK T, RUML T & MACKOVA M 2009. Genetically modified plants in phytoremediation of heavy metal and metalloid soil and sediment pollution. *Biotechnology Advances,* 27, 799-810.

KUMAR KV, SRIVASTAVA S, SINGH N & BEHL HM 2009 Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea. Journal of hazardous materials,* 170, 51-57.

KUMINO T, SEAKI K, NAGAOKA K, OYAIZU H & MATSUMOTO S 2001. Characterization of copper resistant bacterial community in rhizosphere of highly copper-contaminated soil. *European Journal of Soil Biology,* 37, 95-102.

LALITAGAURI R, SUBHAM P, DEBABRATA B & PARIMAL C 2000. Bioaccumulation of Pb (II) from aqueous solutions by *Bacillus cereus* M16. *Journal of Hazardous Substances and Research,* 5, 516-529.

LASAT MM 2002. Phytoextraction of toxic Heavy metals. A review of biological mechanisms. *Journal of Environmental Quality,* 31, 109-120.

LEEDJÄRV A, IVASK A & VIRTA M 2008. Interplay of different transporters in the mediation of divalent heavy metal resistance in *Pseudomonas putida* KT2440. *Journal of Bacteriology,* 190, 2680-2689.

LIESEGANG H, LEMKE K, SIDDIQUI RA & SCHLEGEL HG 1993. Characterization of the inducible nickel and cobalt resistance determinant *Cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. *Journal of Bacteriology,* 175, 767-778.

LIU WX, SHEN LF, LIU JW, WANG YW & LI SR 2007. Uptake of toxic heavy metals by rice

(*Oryza Sativa L.*) cultivated in the agricultural soils near Zhengzhou city, People's Republic of China. *Bulletin of Environmental Contamination and Toxicology,* 79, 209-213.

LOMBI E, TEARALL KL, HOWARTH JR, ZHAO FJ, HAWKESFORD MJ & MCGRATH SP 2002. Influence of iron status on calcium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology,* 128, 1359-67.

LOMBI E, ZHAO FJ, DUNHAM SJ, MCGRATH SP 2001. Phytoremediation of Heavy Metal-Contaminated Soils: Natural Hyperaccumulation versus Chemically Enhanced Phytoextraction. *Journal of Environmental Quality*, 30, 1919-1926.

LUO C, SHEN Z & LI X 2005. Enhanced phytoextraction of Cu, Pb, Zn and Cd with EDTA and EDDS. *Chemosphere,* 59, 1-11.

MA Y, RAJKUMAR M & FREITAS H 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea Journal of environmental management,* 90, 831-837.

MACARIO BJ, SARA AF, ELSA VZ, STEPHANE B, EDUARDO Z & EDGAR PC 2003. Chemical characterization of root exudates from rice *(Oryza sativa)* and their effects on the chemotactic response of endophytic bacteria. *Plant Soil,* 249, 271-277.

MADHAIYAN M, POONGUZHALI S & SA T 2007. Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum L.*). *Chemosphere,* 69, 220-228.

MARTHA LL, JOSE RPV, JASON GP, JORGE LGT & MARIA DG 2009. Effect of indole-3-acetic acid, kinetin and ethylenediaminetetraacetic acid on plant growth and uptake and translocation of lead, micronutrients and macronutrients in alfalfa plants. *International Journal of Phytoremediation,* 11, 131-149.

MCCARTHY TS 2011. The impact of acid mine drainage in South Africa. *South African Journal of Science,* 107, Art. #712, 7 pages. doi:10.4102/sajs.v107i5/6.712.

MCGRATH SP, ZHAO FJ & LOMBI E 2002. Phytoremediation of metals, metalloids, and radionuclides. *Advances Agronomy,* 75, 1-56.

MCINTYRE T 2003. Phytoremediation of heavy metals from soils. *Advances in Biochemistry, Engineering and Biotechnology,* 78, 97-123.

MCLAUGHLIN MJ, PARKER DR & CLARKE JM 1999. Metals and micronutrients-food safety issues. *Field Crops Research,* 60, 143-163.

MENDOZA-COZATL DG & MORENO-SANCHEZ R 2006. Control of glutathione and phytochelatin synthesis under cadmium stress. Pathway modelling for plants. *Journal Theoretical Biology,* 238, 919-936.

MERGEAY M, NIES D, SCHLEGEL HG, GERITS J, P, C. & VAN GIJSEGEM F 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *Journal of Bacteriology,* 162, 328-334.

MICLEANM, LEVEI EA, SENILA M, ROMAN C & CORDOS EA 2009. Assessment of Cu, Pb, Zn And Cd availability to vegetable species grown in the vicinity of tailing deposits from Baia Mare Area. *Reveista de Chimie,* 60, 1-4.

MILNER MJ & KOCHIAN LV 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. *Annals of Botany,* 102, 3-13.

MIRETZKY P 2004. Aquatic macrophytes potential for the simultaneous removal of heavy metals (Buenos Aires, Argentina). *Chemosphere,* 57, 997-1005.

MORAIS PV, BRANCO R & FRANCISCO R 2011. Chromium resistance strategies and toxicity: what makes *Ochrobactrum tritici* 5bvl1 a strain highly resistant. *Biometals,* 24, 401-410.

MORBY AP, TURNER JS, HUCKLE JW & ROBINSON NJ 1993. SmtB is a metal-dependent repressor of the *cyanobacterial* metallothionein gene smtA: Identification of a Zn-inhibited DNA-protein complex. *Nucleic Acid Research,* 21, 921-925.

NICHOLAS CU 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-ground plants, 1: 1-20. In: Pinto R, Varanini Z & Nannipieri P.(Ed.), The rhizosphere, biochemistry and organic substances at the soil-plant interface.

NIE L, SHAH S, BURD GI, DIXON DG & GLICK BR 2002. Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium *Enterobacter cloacae* CAL2 *Plant Physiology and Biochemistry,* 40, 355-361.

NIES A, NIES DH & SILVER S 1990. Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. *Journal of Biology and Chemistry,* 265, 5648-5653.

NIES D, MERGEAY M, FRIEDRICH B & SCHLEGEL HG 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus CH34.*

Journal of Bacteriology, 169, 4865-4868.

NIES D. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews,* 27, 313-39

NIES DH & SILVER S 1995. Ion efflux systems involved in bacterial metal resistances *Journal of Industrial Microbiology,* 14, 186-199.

NIES DH. 1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. *Plasmid,* 27, 17-28.

NIES DH. 1999. Microbial heavy metal resistance. *Applied Microbiology and Biotechnology,* 51, 730-750.

PAN K & WANG WX 2012. Trace metal contamination in estuarine and coastal environments in China. *Science of the Total Environment,* 16, 421-422.

PARDO R, HERGUEDAS M & BARRADO E 2003. Biosorption of cadmium, copper, lead and zinc by inactive biomass of *Pseudomonas putida. Analytical and Bioanalytical chemistry,* 376, 26-32.

PARK M, KIM C, YANG J, LEE H, SHIN W, KIM S & SA T 2005. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbiological Research,* 160, 127-133.

PATTEN CL & GLICK BR 2002. The role of bacterial indole acetic acid in the development of the host plant root system. *Applied Environmental Microbiology,* 68, 3795-3801.

PENG JF, SONG YH, YUAN P, CUI XY & QIU GL 2009. The remediation of heavy metals contaminated sediment. *Journal of Hazardous Materials,* 161, 633-640.

PIMENTEL BE, MORENO-SANCHEZ R & CERVANTES C 2002. Efflux of chromate by *Pseudomonas aeruginosa* cells expressing the ChrA protein. *FEMS Microbiology Letters,* 212, 249-254.

POLZ MF & CAVANAUGH CM 1998. Bias in template-to-product ratios in multi-template PCR. *Applied and Environmental Microbiology,* 64, 3724-3730.

PRASAD MNV & FREITAS HMD 2003. Metal hyperaccumulation in plants-biodiversity prospecting for phytoremediation technology. *Electronic Journal of Biotechnology,* 6, 285-321.

PULFORD ID & WATSON C 2003. Phytoremediation of heavy metal-contaminated land by trees—a review. *Environment International,* 29, 529-540.

QUARTACCI MF, ARGILLA A, BAKER AJM & NAVARI-IZZO F 2006. Phytoextraction of metals from a multiply contaminated soil by *Indian mustard. Chemosphere,* 63, 918-925.

RAJKUMAR M & FREITAS H 2008. Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. *Chemosphere,* 71, 834-842.

RAJKUMAR M, NAGENDRAN R, LEE KJ, LEE WH & KIM SZ 2006. Influence of plant growth promoting bacteria and Cr6+ on the growth of Indian mustard. *Chemosphere,* 62, 741-748.

RAMIREZ-DIAZ MI, DIAZ-PIREZ C, VARGAS E, RIVEROS-ROSAS H & CAMPOS-GARCIA J 2008. Mechanisms of bacterial resistance to chromium compounds. *Biometals,* 21, 321-332.

RANI A, SHOUCHE YS & GOEL R 2008. Declination of copper toxicity in pigeon pea and soil system by growth-promoting *Proteus vulgaris* KNP3 strain. *Current Microbiology,* 57, 78-82.

REED MLE & GLICK BR 2005. Growth of canola (*Brassica napus*) in the presence of plant growth promoting bacteria and either copper or polycyclic aromatic hydrocarbons. *Canadian Journal of Microbiology,* 51, 1061-1069.

REED MLE & GLICK BR 2005. Growth of canola (*Brassica napus*) in the presence of plant growth promoting bacteria and either copper or polycyclic aromatic hydrocarbons. *Canadian Journal of Microbiology,* 51, 1061-1069.

ROMKEN P, BOUWMAN L, JAPENGA J & DRAAISMA KP 2002. Potentials and drawbacks of chelate-enhanced phytoremediation of soils. *Environmental Pollution,* 116, 109-121.

RUTKOWSKA B, SZULC W & LABETOWICZ J 2009. Influence of soil fertilization on concentration of microelements in soil solution of sandy soil. *Journal Elemementology,* 14, 349-355.

SAFRONOVA VI, STEPANOK VV, ENGQVIST GL, ALEKSEYEV YV & BELIMOV AA 2006. Root associated bacteria containing 1-aminocyclopropane-1-1carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. *Biology and Fertility of Soils,* 42, 267-272.

SAHIN F, CAKMAKCI R & KANTAR F 2004. Sugar beet and barley yields in relation to inoculation with N2-fixing and phosphate solubilizing bacteria. *Plant Soil* 265, 123-129.

SEKHAR KC, KAMALA CT, CHARY NS, BALARAM V & GARCIA G 2005. Potential of

Hemidesmus indicus for phytoextraction of lead from industrially contaminated soils *Chemosphere*, 58, 507-514.

SELVIN J, SHANMUGHA PS, SEGHAL KG, THANGAVELU T & SAPNA BN 2009. Sponge-associated marine bacteria as indicators of heavy metal pollution. *Microbiological Research,* 164, 352-363.

SHENG XF & XIA JJ 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere,* 64, 1036–1042.

SHENG XF, XIA JJ, JIANG CY, HE LY & QIAN M 2008. Characterization of heavy-metal resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environmental Pollution* 156, 1164-1170.

SIENKIEWICZ S, WOJNOWSKA T, KRZEBIETK S, WIERZBOWSKA J & ZARCZYNSKI P 2009. Content of available forms of some micronutrients in soil after long-term differentiated fertilization. *Journal of Elementology,* 4, 787-794.

SILVER S 1996. Bacterial resistances to toxic metal ions *Reviews Gene,* 179, 9-19.

SOMEYA N, SATO Y, YAMAGUCHI I, HAMAMOTO H, ICHIMAN Y & AKUTSU K 2007. Alleviation of nickel toxicity in plants by a rhizobacterium strain is not dependent on its siderophore production. *Communications in Soil Science and Plant Analysis,* 38, 1155-1162.

SURESH B & RAVISHANKAR GA 2004. Phytoremediation – a novel and promising approach for environmental clean-up. *Critical Reviews in Biotechnology,* 24, 97-124.

TAYLOR MP, MACKAY AK, HUDSON-EDWARD KA & HOLZ E 2010 Soil Cd, Cu, Pb And Zn contaminants around mount Isa City, Queensland, Australia: Potential sources and risks to human health. *Applied Geochemistry,* 25, 841-855.

TEKERLEKOPOULOU AG, TSIAMIS G, DERMOU E, SIOZIOS S, BOURTZIS K & VAYENAS DV 2010. The effect of carbon source on microbial community structure and Cr(VI) reduction rate. *Biotechnology and Bioengineering,* 107, 478-487.

TRIPATHI M, MUNOT HP, SHOUCHE Y, MEYER JM & GOEL R 2005. Isolation and functional characterization of siderophore-producing lead- and cadmium-resistant *Pseudomonas putida* KNP9. *Current Microbiology,* 50, 233-237.

UMRANIA V 2006. Bioremediation of toxic heavy metals using acidothermophilic autotrophes.

Bioresource Technology, 97, 1237-1242.

VALLS M & LORENZO VD 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiological Reviews,* 26, 327-338.

VALLS M, DE LORENZO V, GONZALEZ-DUARTE R & ATRIAN S 2000. Engineering outer-membrane proteins in *Pseudomonas putida* for enhanced heavy-metal bioadsorption. *Journal of Inorganic Biochemistry,* 79, 219-223.

VAN LOON LC, BAKKER PA & PIETERSE CMJ 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Reviews of Phytopathology,* 36, 453-483.

VELASQUEZ L & DUSSAN J 2009. Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus. Journal of Hazardous Materials,* 167, 713-716.

VESSEY JK 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil,* 255, 571-586.

VIJAYARAGHAVAN K & YUN YS 2008. Bacterial biosorbents and biosorption. *Biotechnology Advances,* 126, 266-29.

WANG YP, SHI JY, WANG H, LIN Q, CHEN XC & CHEN YX 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter *Ecotoxicology and Environmental safety,* 67, 75-81.

WEYENS N, TRUYENS S, SAENEN E, BOULET J, DUPAE J, VAN DER LELIE D 2010 . Endophytes and their potential to deal with co-contamination of organic contaminants (toluene) and toxic metals (nickel) during phytoremediation. International *Journal of Phytoremediation*, 13, 244-255.

WEYENS N, VAN DER LELIE D, TAGHAVI S & VANGRONSVELD J 2009. Phytoremediation: plant–endophyte partnerships take the challenge. *Current Opinions in Biotechnology,* 20, 248-254.

WHITE PJ 2001. Phytoremediation assisted by microbes. *Trends in Plant Science*, 6, 502.

WHITING SN, REEVES RD, RICHARDS D, JOHNSON MS, COOKE JA, MALAISSE F, PATON A, SMITH JAC & ANGLE JS 2004. Research priorities for conservation of metallophyte biodiversity and their potential for restoration and site remediation. *Restoration Ecology,* 12, 106-116.

WU CH, WOOD TK, MULCHANDANI A & CHEN W 2006. Engineering plant-microbe symbiosis for rhizoremediation of heavy metals. *Applied Environmental Microbiology,* 72, 1129-1134.

XIAO X, CHEN T, AN Z, LEI M, HUANG Z & LIAO X 2008. Potential of *Pteris vittata L.* for phytoremediation of sites co-contaminated with cadmium and arsenic: the tolerance and accumulation. *Journal of Environmental Science,* 20, 62-67.

ZAIDI S, USMANI S, SINGH BR & MUSARRAT J 2006. Significance of *Bacillus subtilis* SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. *Chemosphere* 64, 991-997.

ZOUBOULIS AI, LOUKIDOU MX & MATIS KA 2004. Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. *Process Biochemistry,* 39, 909-916.

CHAPTER 2: IDENTIFICATION AND CHARACTERIZATION OF Cr, Cd and Ni TOLERANT BACTERIA ISOLATED FROM MINE TAILINGS

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2.1 INTRODUCTION

Imagine going to work on horseback! Yes. Human life can be miserable without gadgets such as, cars, jewellery and television, just to name a few things that humans cannot do without in their daily lives. These wonderful commodities are manufactured from minerals especially heavy metals. The term 'heavy metal' is often used to describe a group of elements (both metals and metalloids whose atomic density is greater than 5 gcm^{-3} (Duffus, 2002). These include the transitional elements from V to the half-metal As, from Zr to Sb, and from La to Po. The demand and production of these metals has increased exponentially over the past few decades, making them one of the most precious commodities on the global market. Currently, the mining industry in South Africa generates over R330 billion annually to the gross domestic product (GDP) of the country making it the fifth largest mining sector in the world. The country is famous for producing and supplying up to 56.7% of the world's platinum group metals (platinum, palladium, rhodium, ruthenium, osmium and iridium) and a large reserve of other minerals including manganese (±80%), chromite ore (±72%), gold (±40%), vanadium, nickel and diamonds (Chamber of Mines South Africa, 2003). However, mining operations have been recognized as a major contributor to soil contamination with heavy metals. Mining operations have frequently been reported to generate/discharge massive amounts of waste materials (tailings, and acid mine drainage); which often contain high concentrations of heavy metals (Cu, Zn, Fe, Mn, Ni, Pb and Cd) (Monica *et al.*, 2008), that can result in widespread contamination of soils and water bodies including the atmosphere. Nowadays, contamination of soils, groundwater, sediments, surface water and air with heavy metals represents a serious threat to the environment and health of all living organisms since most metals are highly toxic and cannot be degraded like carbon-based (organic) molecules and thus, persist in the environment indefinitely (Navarro *et al.*, 2008; Zulkali *et al.*, 2006). Therefore, cleanup of metal contaminated sites is necessary for environmental and human health preservation. In this regard, several physiochemical methods such as precipitation, ion exchange, reverse osmosis, electrodialysis, and ultrafiltration are commonly used to remove metal ions from aqueous media (Hashim *et al.*, 2011). However, most of these methods are very costly, ineffective and environmentally destructive (Wang and Chen, 2009), which is why alternative processing methods, such as those using bacterial biomass have received a great deal of attention over the past few years (King *et al.*, 2007; Ahluwalai and Goyal 2007). This is because bacterial biomass can be used *in situ*, are more efficient, offer possibility of metal recovery and are cost-effective; being easily integrated with many other remediation technologies. It has been found that metal contaminated environments usually contain bacteria that exhibit a complex array of biochemical and genetically encoded mechanisms to overcome the toxic effects of heavy metals in their surroundings. These may include efflux systems that remove metal ions from the cell by means of transport systems, intracellular sequestration of the metal by specific metal–ion binding proteins, extracellular precipitation into complex compounds and enzymatic transformation of metal ions to a less toxic species (Malik 2004; Yan and Virarghavan 2000; Lee *et al.,* 2006). Bacteria with such abilities can be isolated and used and/or manipulated in a number of ways for remediation of heavy metal contaminated sites (Umrania, 2006). Nonetheless, a better understanding of their ability to flourish in contaminated environments is a critical prerequisite for the development and optimization of such schemes. Present efforts to understand adaptive mechanisms of metal tolerance in bacteria are mainly centered on phenotypic changes that occur with very limited information at the molecular level. Analysis of bacterial genetic characteristics may help elucidate the mechanisms involved in bacteria-metal ion interaction, as well as information on heavy metal resistance genes in metal contaminated soil. Knowledge gained will serve as a guide in selecting beneficial bacterial isolates that can be used to develop or improve biotechnological systems that are currently being used to clean or detoxify metal contaminated soils. Hence, the objectives of this study were to:

1. Isolate and identify Ni, Cd and Cr tolerant bacteria from metal contaminated soil
2. Determine the maximum tolerable concentration (MTC) and bioaccumulation capacities of bacterial isolates
3. Study the effect of metals (Ni, Cd and Cr), temperature, pH and salinity on bacterial growth
4. Screen bacterial isolates for the presence of plasmids and metal tolerance genes on both plasmid and chromosomal genomes.

2.2 MATERIALS AND METHODS

2.2.1 SOIL COLLECTION AND ANALYSIS

Five soil samples were randomly collected from a platinum mine (Rustenburg, South Africa). Samples were taken from the surface layer (0-10 cm) placed in labeled polythene bags, and then transported to the laboratory (North-West University) for analysis. For physical and chemical analysis, a subsample of each collected sample was pulverized using a sterile mortar and pestle, dried and then passed through a 2 mm metal screen to remove large pieces of stones, wooden pieces, gravel, and organic debris (Lei *et al.*, 2008).

2.2.2 DETERMINATION OF SOIL pH, REDOX POTENTIAL, AND ELECTRICAL CONDUCTIVITY

For this, 5 g of each soil sample was suspended in 12.5 ml of distilled water. The mixture was allowed to stand for 30 mins and the pH and redox potential was recorded using a calibrated glass electrode pH meter. All assays were conducted in triplicate. The electrical conductivity of each sample was recorded using a conductivity meter (EC 215), after calibration with HI 7030 solutions.

2.2.3 TOTAL METAL (Cr, Ni, Cd) CONCENTRATION IN SOIL

The amount of heavy metals present in each soil sample was determined using a microwave digester (Anton Paar microwave 3000) according to the standard methods published by US environmental protection agency (USEPA, 2006). After digestion, the volume of each sample was adjusted to 50 ml using distilled water and the concentrations of Cr, Cd, and Ni in each sample was measured using a Perkin Elmer (Nexion 300 Q) inductively coupled plasma mass spectrophotometer (ICP-MS).

2.2.4 PREPARATION OF METAL SOLUTIONS

The metals Ni, Cr and Cd were used as $NiCl_2$, $K_2Cr_2O_7$, and $CdCl_2$ respectively. Stock solutions (1M) were prepared by dissolving metal salts in distilled water. Each stock solution was sterilized by filtration and stored at 4^0C. All plastic and glassware used were acid-washed in 2N HNO_3 and thoroughly rinsed several times with deionized water before use to avoid metal contamination.

2.2.5 ISOLATION AND SELECTION OF METAL–TOLERANT BACTERIA

For isolation and enumeration of metal tolerant bacteria, duplicate composite soil samples (1g) was suspended in 90 ml of sterile saline solution (0.9% NaCl) in a 250 mL conical flask and mixed thoroughly on a magnetic stirrer at 150 rpm for 5 min. Standard serial dilutions (10^{1} to 10^{-7}) were prepared by adding 1 ml of the suspension to test tubes containing 9 ml of sterile saline solution. Aliquots (100 µl) from each dilution were spread with a glass rod over triplicate Luria-Bartani (LB) medium supplemented (separately) with 0.5 mM of $NiCl_2$, $CdCl_2$ or $K_2Cr_2O_7$. The pH of the medium was adjusted to 7.0 using a 1 N HCl or 1 N NaOH solution to minimize complexation of heavy metal ions. Plates were placed in an incubator for 3 days at 37^0C. The number of colony forming units (CFUs) was determined and the percentages of bacteria tolerant to each metal were calculated as follows (Prescott and Harley 2002):

$$\text{Total CFU/ml} = \frac{\text{No. of colonies x dilution factor}}{\text{Inoculum size (ml)}}$$

$$\text{\% Metal tolerant bacteria} = \frac{\text{Metal tolerant bacteria}}{\text{Total Bacteria}} \text{ x } 100$$

Well isolated, metal tolerant bacterial colonies which varied in shape and color were selected at random and then purified by repeated streaking on fresh LB medium containing 0.1 mM of $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$ in order to maintain their ability to tolerate heavy metals (Rajkumar *et al.*, 2006). Strains were maintained on agar slants at 4^0C.

2.2.6 STUDY OF BACTERIAL COLONIAL AND CELL MORPHOLOGY

Isolated colonies of purified strains grown on LB plates were observed and data was recorded regarding the size, shape, color, elevation and margins (Prescott and Harley 2002). In order to determine the cellular morphology, bacterial colonies were gram stained using standard procedure. Slides were observed under a light microscope (oil immersion at 100 to X 400 magnification) to determine the shape and arrangement of cells along with Gram-reaction.

2.2.7 EVALUATION OF BACTERIAL TOLERANCE TO HEAVY METALS

Isolated bacterial strains were screened for tolerance to heavy metals on LB medium using the agar dilution method (Mergeay *et al.*, 1985) with slight modifications. For this, bacterial isolates were inoculated individually into 10 ml of sterile LB broth supplemented with 0.5 mM of Ni^{2+}, Cd^{2+} or Cr^{6+}. The cultures were incubated at 37^0C for 24 h (150 rpm) after which, the visual turbidity of each isolate was adjusted to 0.1 OD (600 nm) using a spectrophotometer. Experimental plates were prepared by supplementing LB agar with varying concentrations (0.01; 0.02; 0.05; 0.1; 0.2; 0.5; 1.0; 2.0; 5.0; 10; 20 and 40 mM) of $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$. Each plate was sub-divided into 8 sections and then spot inoculated with 10 µl of bacteria. Triplicate plates were prepared for each metal concentration to confirm the maximum tolerable concentrations (MTC) for each isolate. Similar plates without metal complements were set-up as controls. Plates were incubated at 37^0C for 48 h. The highest concentration of metal ion beyond which no visible growth occurred was recorded as the MTC (Piotrowska-Seget *et al.*, 2005). Tolerant strains were defined as those having MTC values greater than 1 mM (Nieto *et al.*, 1987).

2.2.8 EFFECT OF METALS ON BACTERIAL GROWTH

Growth profiles of bacterial isolates were determined in 250 ml Erlenmeyer flasks containing 100 ml LB medium supplemented with 1 mM of heavy metals (Cd, Cr, or Ni). Flasks were inoculated with 1 ml of freshly prepared bacterial cultures (optical density of 0.090 at 600 nm) and incubated at 37^0C in a shaker at 200 rpm. Bacterial growth was determined at 24 h intervals by measuring the optical density of every culture (1 ml) at 600 nm using UV spectrophotometer (Thermo Spectronic, Merck, SA) (Hassan *et al.*, 1999). Controls consisted of a metal-free medium inoculated with bacteria. The growth profile of each bacterial strain was represented graphically by plotting absorbance versus incubation time. Tests were performed in triplicate.

2.2.9 EFFECT OF pH ON BACTERIAL GROWTH

To study the effect of pH on growth of bacteria, test tubes containing 20 ml of LB broth amended with 1 mM of heavy metals (Cd, Cr, or Ni) were prepared by adjusting the pH to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 using conc HCl or 1N NaOH. After adjusting the pH, all test tubes were autoclaved followed by inoculation with 20 µl of overnight grown cultures (optical density of 0.090 at 600 nm). Tubes were placed on shaking incubator (150 rpm) at 37^0C for 48 h and OD of each culture was measured at 600 nm using a UV Spectrophotometer (Thermo Spectronic, Merck, SA).

2.2.10 EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

To study the effect of temperature on growth of bacterial isolates, 20 µl of each bacterial isolate was inoculated in 20 ml of sterilized LB broth containing 1 mM of metal salts at different temperatures (28^0C, 30^0C, 37^0C, and 40^0C). All test tubes were incubated for 24 h at 150 rpm. After incubation, the OD of each isolate was measured at 600 nm using an UV Spectrophotometer (Thermo Spectronic, Merck chemicals SA).

2.2.11 EFFECT OF NaCl ON BACTERIAL GROWTH

Bacterial tolerance to NaCl was evaluated in test tubes containing 20 ml of LB broth supplemented with increasing concentrations of NaCl (0.2, 0.4, 0.6, 0.8, and 1.0%). Flasks were inoculated with 100 µl (OD=0.5) of overnight bacterial cultures and then incubated at 37^0C with constant shaking for 24 h. The OD was measured at 600 nm using a UV Spectrophotometer (Thermo Spectronic, Merck chemicals SA).

2.2.12 ACCUMULATION OF Cd, Cr AND Ni BY BACTERIAL ISOLATES

For bioaccumulation studies, an aliquot (10 ml) of overnight bacterial cultures was inoculated into cotton plugged Erlenmeyer flask (250 ml) containing 100 ml of LB broth amended with 150 mg L^{-1} of the metal salt ($NiCl_2$, $K_2Cr_2O_7$, or $CdCl_2$). Flasks were incubated at 37^0C at optimum pH and temperature while shaking at 150 rpm for 7 days. After every 24 h, 10 ml aliquots were taken out of every sample and centrifuged at 10000 rpm for 15 min. The supernatant was digested in 5 ml of HNO_3 (65%) in glass tubes for 24 h in a microwave. Digested samples were cooled and diluted with 100 ml of distilled water and acidified with 100 µl of conc HNO_3 before analysis. The concentration of Cd, Ni, and Cr in the supernatant was determined using an ICP-MS. The amount of metal accumulated by each bacterial isolate was expressed in percentages using the following equation.

$$R = \frac{(C1 - C2)}{C1} X\ 100$$

Where R = bioaccumulation percentage, C1= initial concentration of heavy metal used (mg L^{-1}) and C2 = concentration of heavy metals ions in the treated solution (mg L^{-1}) (Qin *et al.,* 2006). Bacterial isolates grown without metals were used as blank for spectrophotometric analysis.

MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

2.2.13 GENOMIC DNA EXTRACTION

For genomic DNA extraction, the bacteria were grown in 10 ml LB Broth containing 0.1 mM of $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$. The cultures were grown for 24 h in a shaking incubator (100 rpm) at 37^0C after which 1.5 ml of each culture was transferred to sterile Eppendorf tubes and centrifuged at 10000 rpm for 5 min. The supernatants were discarded and cells were re-suspended in 650 µl of TE buffer. Total DNA was extracted from each bacterial suspension using a DNA extraction kit (ZR soil microbe DNA MiniPrep^{TM} kit (Zymo Research, USA)) according to the manufacturer's instructions.

2.2.14 PLASMID DNA EXTRACTION

Bacterial cells grown overnight in 10 ml of LB broth were harvested by centrifuging 1.5 ml of each culture in microfuge tubes for 5 min at 6,000 rpm (revolutions per min) and screened for the presence of plasmid DNA using high pure plasmid isolation kit (Roche Diagnostics, SA) according to the manufacturer's instruction. The resulting plasmid preps were stored at -80^0C for further use.

2.2.15 DNA QUANTIFICATION AND PCR AMPLIFICATION OF 16S rRNA GENE

DNA concentration (ng/µl) and purity of DNA was assessed by NanaDrop LITE. Amplification of a 1500 bp fragment of the 16S rRNA gene was performed by polymerase chain reaction (PCR) using universal bacterial 16S rRNA primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGTTAC CTT GTT ACG ACT T-3′) (Liu *et al.*, 2007) in order to confirm the identity of bacterial isolates. Amplifications were carried out in a final reaction volume of 25 µl containing 12.5 µl of 2x PCR Master Mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas), 1 µl of genomic DNA template (10 ng), 0.5 µl (10 µM) of each forward and reverse primer and 11 µl nuclease-free water. This reaction mixture was subjected to 30 cycles in a C1000 thermal cycler (BioRad) using the following program: an initial denaturation, 95^0C for 5 min; followed by denaturation at 95^0C for 1 min; annealing, 58^0C for 30s; extension, 72^0C for 1 min and final extension, 72^0C for 7 min.

2.2.16 PCR ANALYSIS TARGETING GENES ENCODING HEAVY METAL TOLERANCE IN BACTERIA

Genes associated with cadmium (*Cad*), nickel (*Ncc*), chromium (*Chr*) and multiple heavy metal tolerance (*Czc*) in each isolate were screened by PCR amplification. The oligonucleotide sequences used as primers for the partial amplification of the *CzcD, ChrA, ChrB, CzcB, CzcC*, *NccA* and *CadA* loci are given in Table 1.

Table 1: Oligonucleotide primers for PCR amplification of genes involved in metal tolerance

Gene	Forward sequence	Reverse sequence	Reference
NccA	ACGCCGGACATCACGAACAAG	CCAGCGCACCGAGACTCATCA	Abou-Shanab *et al.*, 2007
CzcC	ACATACCTTGGTGCAATTCGA	ATGTTTTATGAATCCCGTCTTACC	Dell'Amico 2008
CadA	GACAAGACYGGMACYMTCAC	GCRTGGTTRATSCGTC	Dell'Amico 2008
ChrB	GTCGTTAGCTTGCCAACATC	CGGAAAGCAAGATGTCGATCG	Nies *et al.*, 1990
CzcD	CAGGTCACTGACACGACCAT	CATGCTGATGAGATTGATGATC	Nies *et al.*, 1989
CzcB	CTATTTCGAACAAACAAAAGG	CTTCAGAACAAAACTGTTGG	Abou-Shanab *et al.*, 2007
ChrA	CTTATACGCTACGCCAACTG	GTAATGGCATTCAGTCGCTTG	Nies *et al.*, 1990

Amplification reactions were carried out in 25 µl volumes containing: 11 µl of nuclease-free water, 12.5 µl 1x PCR Buffer; 1 µl of genomic or plasmid DNA from the respective strain; 0.5 µl of each primer (100 mM); 200 µM of each dNTP and 0.5 µl 1.25 U *Taq* DNA polymerase (Fermentas) Master Amp *Taq* DNA polymerase and Epicentre failsafe PCR 2x premix buffers D, E, and F for amplification of *chrB, NccA, CzcD* respectively. The thermal cycler (C1000 Biorad) was programmed with the following five steps: initial denaturation at 95C for 5 min; followed by

35 cycles of denaturation (94^{0}C for 30 s), annealing (52^{0}C for 30 s), elongation (72^{0}C for 2 min); and a final elongation step at 72^{0}C for 5 min.

2.2.17 AGAROSE GEL ELECTROPHORESIS

DNA, plasmid and genomic including PCR products were checked in a 1% (w/v) agarose gel prepared by dissolving 1.0 g of agarose (Bio-Rad, SA) in 100 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE, pH 8). The mixture was heated for 3 mins in a microwave oven. After cooling, EtBr (1 µl/ml) was added to the molten gel which was then poured in a gel casting tray and then allowed to solidify. After solidification, combs were removed and the gel was carefully placed in the electrophoresis tank containing 1X TAE buffer (40 mM Tris, 20 mM Acetic acid, and 100 mM EDTA pH 8.0). DNA samples were prepared by mixing 10 µl genomic DNA/ plasmid DNA/ PCR product with 5 µl of 6x DNA loading dye (Fermentas) and then carefully loaded in pre-formed wells in the gel. A GeneRuler™ DNA Ladder (100 bp–10,000 bp) was used to estimate the sizes of genomic DNA and plasmid DNA bands. The electrophoresis was performed at 100V for 1.5 h. Gels were visualized and photographed using a gel documentation system (Gel Doc 2000, Bio-Rad).

2.2.18 DNA PURIFICATION AND SEQUENCING ANALYSIS

Before sequencing, PCR products were purified with a PCR purification kit (Roche diagnostics) according to the manufacturer's instructions. Sequencing was carried out at Inqaba Biotech (SA). Acquired 16S rDNA, *czcD, czcC, czcB, cadA*, nccA, *chrB* and *chrA* sequences were assembled within Bioedit (version 7.0.9.0) and matched with similar sequences in the National Centre for Biotechnology Information website (NCBI, www.ncbi.nih.gov) using the Basic Local Alignment Search Tool (BLAST).

2.3 DATA ANALYSIS

The physicochemical properties of soil samples, the number of metal tolerant bacteria, growth curve of each bacterial isolate, and their MTCs were statistically analyzed by calculating mean values and standard error (SE) using the SPSS statistical program (Version 22.0).

2.4 RESULTS AND DISCUSSION

2.4.1 SOIL CHARACTERISTICS

Some physico-chemical properties of the soils investigated in this study are presented in Table 2. The soils were sampled from a platinum mine tailings dam located on the outskirts of Rustenburg, South Africa. The mean pH of our samples ranged from 7.3 to 8.6 i.e. neutral to slightly alkaline. The redox potential varied from 265.3 to 457.8 Eh while the electrical conductivity (EC) ranged from 306.1 to 802.7 µs cm^{-1}. Soil pH, Eh and EC are important parameters which strongly influence the chemical behavior of metal ions present in terrestrial and aquatic environments. They have a direct/indirect effect on the solubility and mobility of metal ions, including their potential to form chelates with other soil constituents (Jan *et al.*, 2010). For instance, at neutral pH, the solubility of metal ions in any given soil solution is usually low. Wu *et al.* (2006) observed that a decrease in pH resulted in an increase in cation concentration in the soil solution. During their study, the solubility and mobility of metals such as zinc were affected by the decrease in pH.

Table 2: Physico–chemical characteristics of soil samples used for isolation of heavy metal tolerant bacteria

Soil sample	**pH (H_2O)**	**EC (µs cm^{-1})**	**RP (Eh)**	**Heavy metal concentration (mg/kg)**		
				Cd^{2+}	**Cr^{6+}**	**Ni^{2+}**
SS1	7.3	456.9	301.9	32.7	115.2	254.5
SS2	7.8	802.7	457.8	10.2	432.9	120.1
SS3	8.3	672.4	324.2	112.5	278.6	105.8
SS4	7.5	406.6	289.7	18.3	242.4	275.2
SS5	8.6	306.1	265.3	43.4	545.8	338.3

*EC= Electrical conductivity, RP= redox potential

ICP-MS analysis of soil samples revealed a reasonably high level of heavy metal ions in the following order of concentration: Cr>Ni>Cd with maximum concentrations of 545.8, 338.3 and 112.5 mg/kg respectively. The mining industry is one of the major contributors to soil contamination with heavy metals in South Africa. Mining activities generate huge volumes of chemical waste products that are deposited on tailings dams. These waste products usually contain high levels of heavy metals.

2.4.2 ISOLATION AND SELECTION OF Cr, Cd and Ni BACTERIA

Soils containing high levels of heavy metals are potential sources of metal-tolerant bacteria (Clausen, 2000). Data from numerous studies suggests that the presence of high concentrations of metal ions provides a selective medium favoring the evolution of bacterial communities which are more tolerant to heavy metals, but with lower diversity (Pal *et al.*, 2005; Tsai *et al.*, 2005; Rosewarne *et al.*, 2010). With this in mind, the present study was initiated to isolate and identify heavy metal (Cr, Ni and Cd) tolerant bacterial isolates followed by an investigation of common genetic mechanisms involved in bacterial tolerance to heavy metals including their potential to remove metals from an aqueous solution.

A relatively large number of potential metal tolerant bacteria were observed on LB medium amended with 0.5 mM of $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$. The number of viable colony forming units (CFUs) and the percentages of bacterial tolerance to each metal salt are presented in Table 3. The total plate count of the bacterial colonies grown on LB plates with no metal induction ranged from $14.8x10^4$ to $3.5x10^5$. The percentage of bacterial colonies (CFU/g soil) tolerant to Cd^{2+} fluctuated from 28.41% to 77.14%. Approximately 38.14% to 65.36% of all bacterial colonies where tolerant to Cr^{6+}, while 33.71% to 61.44% exhibited tolerance to Ni^{2+}. Several investigators have also reported isolation and characterization of Cr, Cd and Ni-tolerant bacteria from metal contaminated soils and wastewater. For instance, Belimov *et al.* (2005) isolated 42 Cd-tolerant bacterial strains from the rhizoplane of plants grown in the soils, sewage sludge and mining waste. Thacker *et al.* (2007) isolated 38 morphologically different bacterial isolates from soil samples which were heavily contaminated with chemical industrial wastes and found that all isolates were able to grow on LB agar plates containing 50–300 mg l^{-1} of $K_2Cr_2O_7$. Sevgi *et al.* (2010) recovered 433 bacterial strains from soils containing high levels of Ni and Cr and found that 73.9% of the bacterial strains were tolerant to Cr, whereas 26% and 11.5% were tolerant to Ni and Cd respectively. In another study, 70 bacterial strains were recovered from industrial and agricultural soils and tested for their ability to tolerate Cr, Ni Zn, and Cd. The investigators found that 88.8% of the strains exhibited tolerance to Ni, while 82.8%, 80%, and 71.4% were tolerant to Zn, Cd and Cr, respectively (Malik *et al.*, 2002). Abou-Shanab *et al.* (2007) isolated 46 bacterial strains from Ni-rich serpentine soil including the rhizospheric soil of

Alyssum murale, and found that all isolates were tolerant to Ni, Pb, and Zn whereas 53% and 42% were tolerant to Cr and Cd, respectively.

Table 3: Numbers of culturable bacteria (Cfu g^{-1}) and proportions of metal-tolerant populations in soil samples

Sample No.	**S1**	**S2**	**S3**	**S4**	**S5**
Total Cfu/g	$19.4x10^4$	$17.6x10^4$	$3.5x10^5$	$14.8x10^4$	$15.3x10^4$
Cd TB (Cfu/g)	$8.7x10^4$	$5.0x10^4$	$2.7x10^5$	$6.4x10^4$	$7.2x10^4$
% tolerance	44.5%	28.41%	77.14%	43.24%	47.1%
Cr TB (Cfu/g)	$7.4\ x10^4$	$8.9\ x10^4$	$16.4\ x10^4$	$6.6\ x10^4$	$10.7\ x10^4$
% tolerance	38.14%	50.57%	45.7%	44.59%	65.36%
Ni TB (Cfu/g)	$7.06\ x10^4$	$6.9\ x10^4$	$11.8\ x10^4$	$7.1\ x10^4$	$9.4\ x10^4$
% tolerance	39.18%	39.20%	33.71%	47.8%	61.44%

***TB= Tolerant Bacteria**

During the initial screening process, 50 morphologically distinct colonies were selected at random and tested for tolerance against increasingly higher metal concentrations. Finally, eleven bacterial isolates were selected for further characterization. Bacterial codes and phenotypic characteristics are listed in Tables 4 and 5.

2.4.3 COLONIAL AND CELLULAR MORPHOLOGY OF BACTERIAL ISOLATES

Morphological characterization of bacterial isolates showed that 73% of all bacterial cell shapes where circular while 27% where irregular (Table 4). Some elevations were flat (27%), convex (46%), and umbonate (27%). Bacterial margins where entire (73%), lobate (18%) and irregular (9%). The various colors exhibited by various bacteria were white (42%), yellow (8%), red (8%), orange (17%). brown (17%), and white cream (8%). All bacterial cells were rod shaped. Most bacterial isolates were gram-positive (75%) while the remaining 25% were gram-negative. Cellular arrangement was singles (16%) and scattered (84%).

Table 4: Colonial and Cellular morphology of bacterial isolates

Isolate Codes	Color	Shape	Margin	Elevation	Shape	Arrangement	Color	Gram Reaction
BCr27	White	Circular	Entire	Flat	Rods	Scattered	Purple	+ve
BCd16	White	Circular	Entire	Convex	Rods	Scattered	Purple	+ve
BNi6	White	Circular	Entire	Umbonate	Rods	Scattered	Pink	-ve
BCr7	Brown	Circular	Entire	Flat	Rods	Scattered	Purple	+ve
BCr3	White	Irregular	Lobate	Convex	Rods	Singles	Pink	-ve
BNi12	Yellow	Circular	Entire	Convex	Rods	Singles	Purple	+ve
BCd33	Orange	Circular	Entire	Umbonate	Rods	Scattered	Purple	+ve
BNi11	Yellow	Irregular	Lobate	Convex	Rods	Scattered	Purple	+ve
BCr32	CW	Circular	Entire	Umbonate	Rods	Scattered	Purple	+ve
BNi22	Orange	Circular	Entire	Convex	Rods	Scattered	Purple	+ve
BCd2	Brown	Irregular	Irregular	Flat	Rods	Scattered	Purple	+ve

CW=Cream White

2.4.4 BACTERIAL TOLERANCE TO HEAVY METALS

High bacterial metal tolerance is an important factor to be considered for remediation of heavy metals because it is directly related to the survival and growth of bacteria in metal contaminated soil or water. In this study, bacterial tolerance to Cr^{6+}, Cd^{2+} and Ni^{2+} was determined using the agar dilution method. A log phase culture of each bacterial isolate was aseptically inoculated on LB agar plates containing $NiCl_2$, $CdCl_2$ and $K_2Cr_2O_7$, at concentrations ranging from 0.5 to 40 mM. The MTCs of all bacterial isolates against the above metal salts are given in Table 6. Our data indicated that Cr^{6+} was less toxic, whereas Ni^{2+} and Cd^{2+} were highly toxic to most bacterial isolates. Metal toxicity to bacterial isolates was found in the following order Cd>Ni>Cr. The high tolerance to Cr^{6+} was probably due to the binding of Cr ions to the organic constituents of the medium, which reduced its toxicity (Laxman and More, 2002).

All bacterial isolates showed multiple tolerances to various heavy metal ions and were capable of growing at high concentrations of heavy metals. A large variety of bacteria with multiple metal tolerance to metal ion have also been reported by several researchers (Thacker *et al.*, 2007; Abou-Shanab *et al.*, 2007; Dell'amico *et al.*, 2008).

Table 5: Maximum tolerable concentration (MTC) of 3 metal ions tested against bacteria isolates

Isolate Code	**MTC (mM)**		
	Cr^{6+}	**Ni^{2+}**	**Cd^{2+}**
BCd33	1	2.5	7.5
BCr3	15	2	1.5
BNi11	1.5	10	2.5
BCd16	1	0.5	5
BNi6	1.5	5	2.5
BCr26	5	2.5	0.2
BCr32	5	2	2
BNi12	2	5	0.1
BCr7	10	2	0.5
BNi22	1	5	2.5
BCd2	2	0.5	5

Among all bacterial isolates examined in this study, BCd33 showed maximum tolerance to Cd^{2+} (7.5 mM) followed by BCd16 and BCd2 which showed the same level of tolerance to Cd^{2+} with MTC of 5 mM, while BCr7 was the least tolerant to Cd^{2+} with MTC of 0.5 mM.

Bacterial tolerance to Ni^{2+} was found to be in the range of 0.5 to 10 mM of $NiCl_2$. Isolate BNi11 showed highest tolerance to Ni^{2+} (10 mM) while BNi6, BNi12 and BNi22 showed the same level of tolerance to Ni^{2+} in LB medium with the MTC of 5 mM. BCd16 was the least tolerant with MTC of 0.5 mM.

Tolerance to Cr^{6+} was found to be in the range of 1.0 to 10 mM. Bacterial isolate BCr3 was the most tolerant to with MTC of 15 mM followed by BCr7 with MTC of 10 mM. While BCd33, BCd16, BNi22 were least tolerant with MTCs of 1 mM.

This high level of tolerance against multiple metal ions could be attributed to the fact that the bacteria used in this study were isolated from a soil containing relatively high levels of Ni^{2+}, Cd^{2+} and Cr^{6+}. Moreover, bacteria exhibit several physiological and genetic mechanisms to counteract the toxic effects of metal ions. For instance, many bacteria can mitigate the toxicity of heavy metal ions like Cr^{6+} by converting it to a less toxic form (Cr^{3+}) through enzymatic reduction. Bacterial tolerance to heavy metals is also attributed to a number of processes including bioaccumulation by cell biomass, efflux systems, complexation, precipitation and oxidation reactions. These mechanisms could be utilized for remediation of metal contaminated environments (Ahmed *et al.*, 2005; Malik, 2004; Canovas *et al.*, 2003).

The MTC values for Ni, Cr and Cd observed in our study are much higher than those obtained by Samanta *et al.* (2012), Banerjee *et al.* (2015) and Bhagat *et al.* (2016). However, the MTC values for Cr (15 mM) and Cd (7.5 mM) are much lower than those reported by Oyetibo *et al.*, 2010 where the MTC for Cd and Cr were 14 mM and 17 mM, respectively. Similar results were reported by Coral *et al.* (2005) who demonstrated that certain *Enterobacter* strains, which were isolated from water drainage near a landfill (Sofulu village, Adana), were tolerant to 16 mM of Ni^{2+}, 14 mM of Cd^{2+} and 16 mM of Cr^{6+}. In another study conducted by Stoppel and Schlegel (1995), *A. eutrophus* was tolerant to 25 mM of Ni^{2+} which is much higher than the MTCs for Ni reported in this study. These differences can be attributed to several factors including; the type of growth media used and incubation conditions.

2.4.5 EFFECTS OF Cr, Cd AND Ni ON BACTERIAL GROWTH

Growth inhibition curves of all bacterial isolates in LB medium containing 1 mM of Cr, Cd and Ni, in comparison to their respective growth patterns without heavy metal addition are presented in Figures 1, 2 and 3. Results obtained were in good agreement with bacterial tolerance (MTC) for each heavy metal. In a nutshell, we observed a decrease in optical density of all bacterial isolates in the presence of heavy metal compared to the metal free medium, which was similar to observations reported by Suresh *et al.* (1998), Pal *et al.* (2004) and Raja *et al.* (2006).

In the absence of Cr^{6+} (Figure 1b), the growth rates of all bacterial isolates increased steadily and reached a maximum after 72 h before declining sharply. Isolates BCr3 and BCr26 reached maximum growth in 48 h and then decreased sharply when Cr^{6+} was present in the growth medium (Figure 1a). BCr7 achieved maximum growth after 72 h, while the growth of BCr3 increased steadily and finally reached a plateau at about 1.84 after 72 h and then decreased after 96 h.

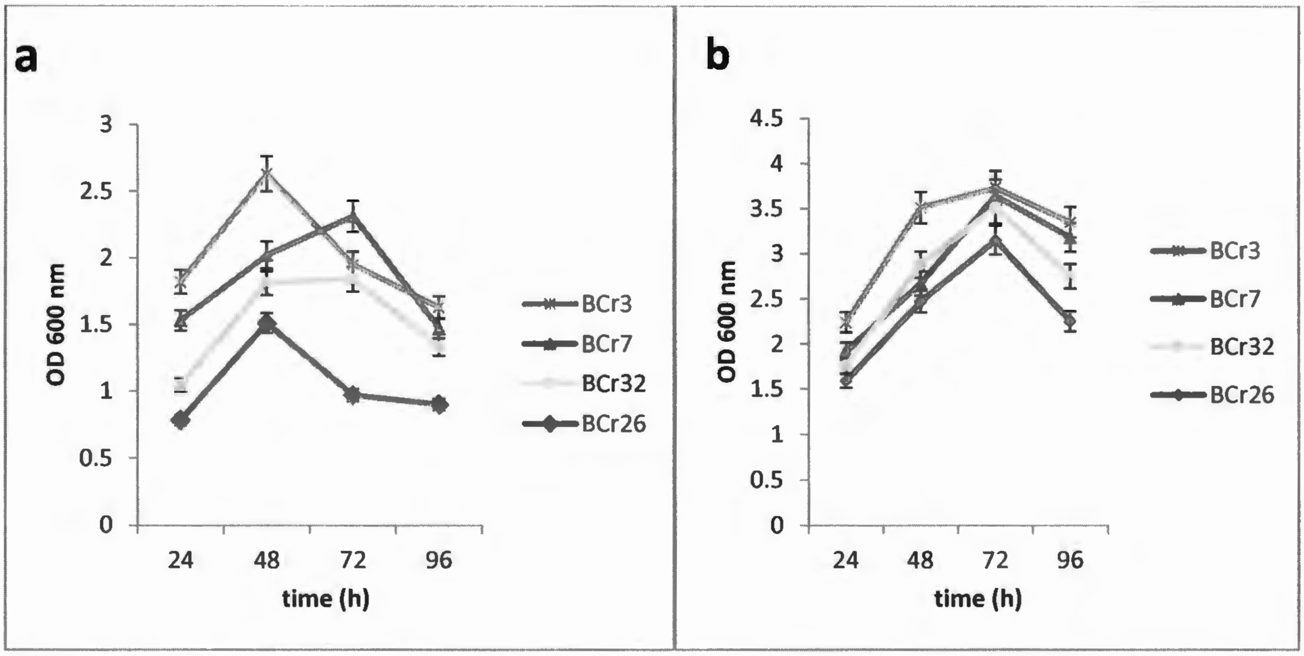

Figure 1: Growth curves of bacterial isolates, BCr3, BCr32, BCr7 and BCr26 in the presence (a) and absence of 1 mM Cr^{6+} (b), 96 h after incubation at 37^0C.

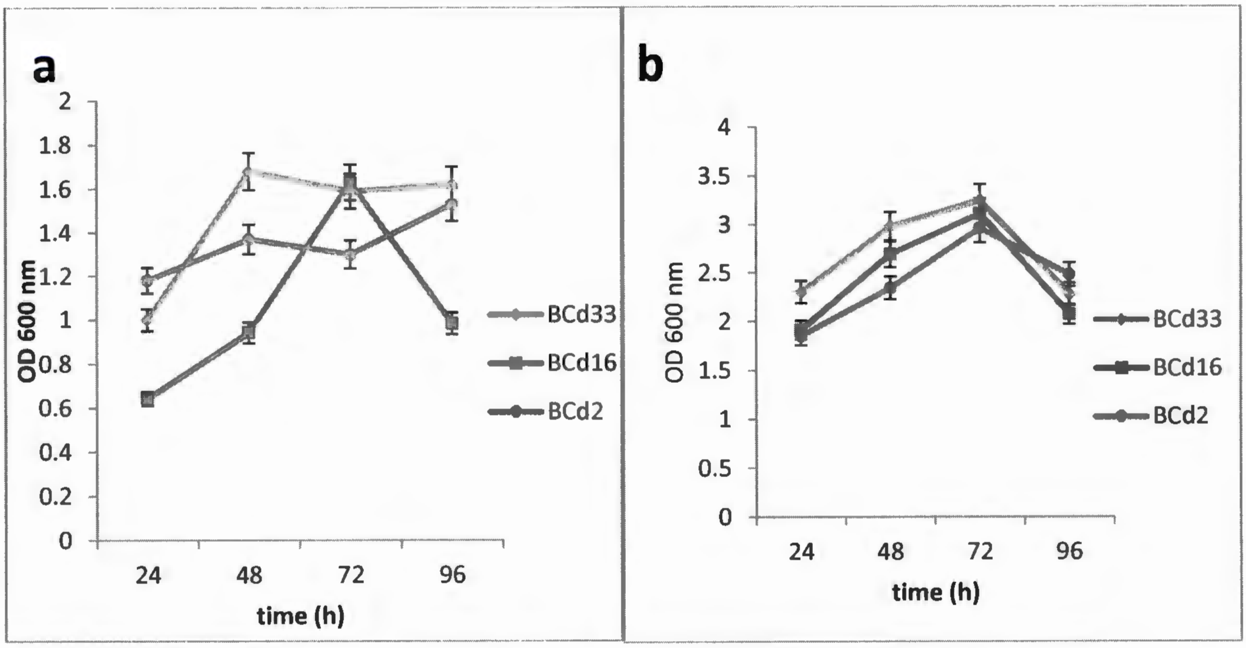

Figure 2: Growth of BCd33, BCd2 and BCd16 in the presence (a) and absence of 1 mM Cd^{2+} (b) 96 h after incubation at 37^0C.

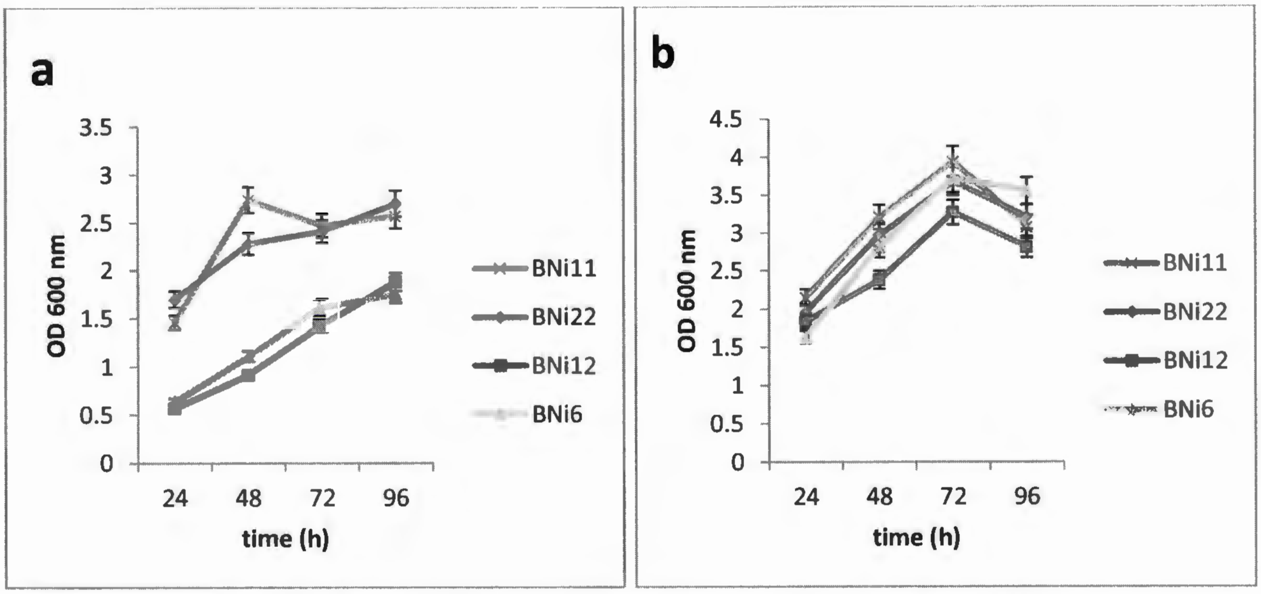

Figure 3: Growth curves of bacterial isolates, BNi11, BNi12, BNi6, and BNi22, in the presence (a) and absence of 1 mM of Ni^{2+} (b) 96 h after incubation at 37^0C.

The growth of bacterial isolates, BCd33, BCd2 and BCd16 was significantly reduced when LB medium contained Cd^{2+}, indicating that this metal ion had a high degree of toxicity towards the bacterial isolates (Figure 2a). BCd16 was the strain most affected by the presence of Cd^{2+} within the first 48 h compared to isolates BCd33 and BCd2. However, growth increased rapidly after 72 h. An unexpected phenomenon was observed in isolates BCd33 and BCd2. The growth of these isolates increased steadily after 48 h, decreased slightly after 72 h and then increasing again after 96 h.

Growth curves for isolates BNi6, BNi12, BNi22 and BNi11 in the presence of 1 mM Ni^{2+} are shown in Figure 3a. The results indicate that the growth of BNi12, BNi6 and BNi22 increased steadily over the entire 96 h testing period even though the growth rates of BNi12 and BNi6 were significantly lower than that of BNi6 and BNi11. However, maximum growth in Ni containing medium was observed in BNi11 after 48 h.

Metal ions are directly/indirectly involved in many cellular processes of microorganisms. Ni is an essential trace element, while Cr and Cd are non-essential elements for bacteria and are often toxic with no biological role (Nies, 1999). Several studies have proven that high levels of these metals can negatively influence soil bacterial communities by decreasing their growth, metabolic activity as well as functional diversity (Kamnev *et al.*, 2005; Wang *et al.*, 2010).

2.4.6 EFFECT OF TEMPERATURE ON BACTERIAL GROWTH

Temperature is an important factor that has a significant effect on bacterial growth. The effect of temperature on bacterial growth in the presence of Cd^{2+}, Ni^{2+} and Cr^{6+} is shown in Figures 4a, b and c. The results suggest that all the isolates preferred an optimum temperature of 35-37^{0}C for maximum growth. Growth of bacterial cultures decreased significantly when the temperature was raised to 40^{0}C. This can be attributed to a decrease in metabolic activity caused by the increase in temperature above optimum. High temperatures of metal solutions can inhibit or denature enzymes and also damage structural components of the plasma membrane, consequently slowing down bacterial growth. Likewise, at low temperatures, the rate of bacterial growth is also reduced because most enzymes are inactivated at low temperatures, and thus decrease the rate of metabolism (Prescott and Harley 2002; Whiteley and Lee 2006).

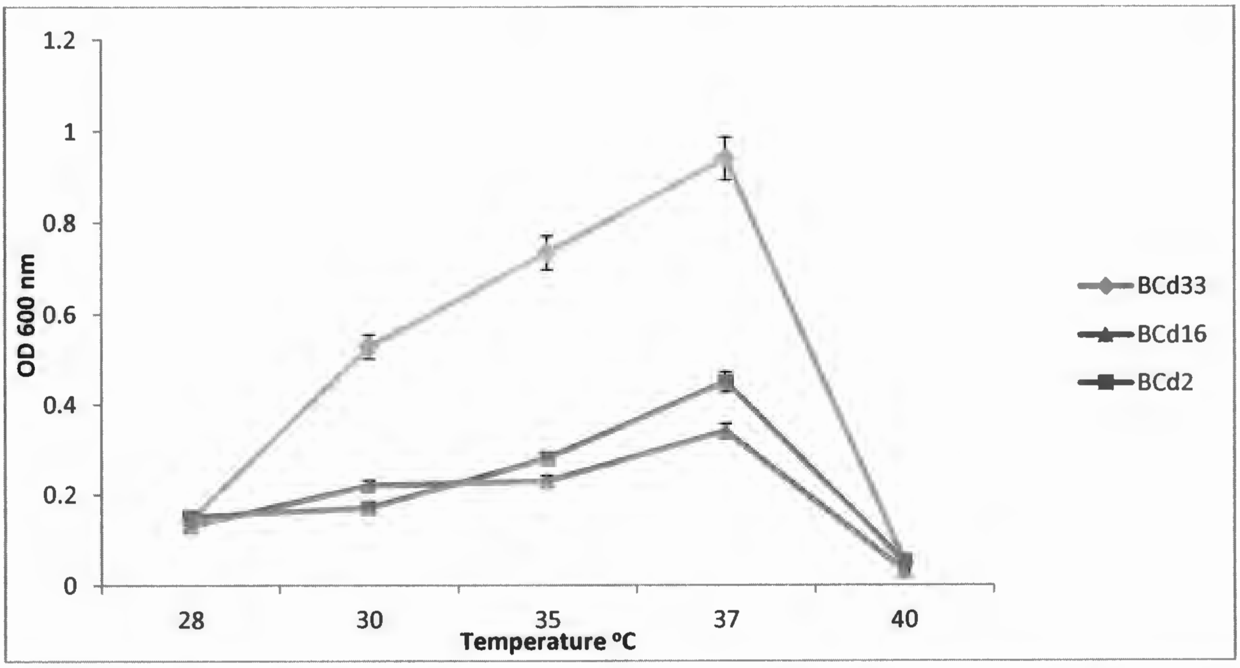

Figure 4a: Growth curves of BCd33, BCd2 and BCd16 at different temperatures in LB broth containing 1 mM Cd^{2+} after 24 h of incubation.

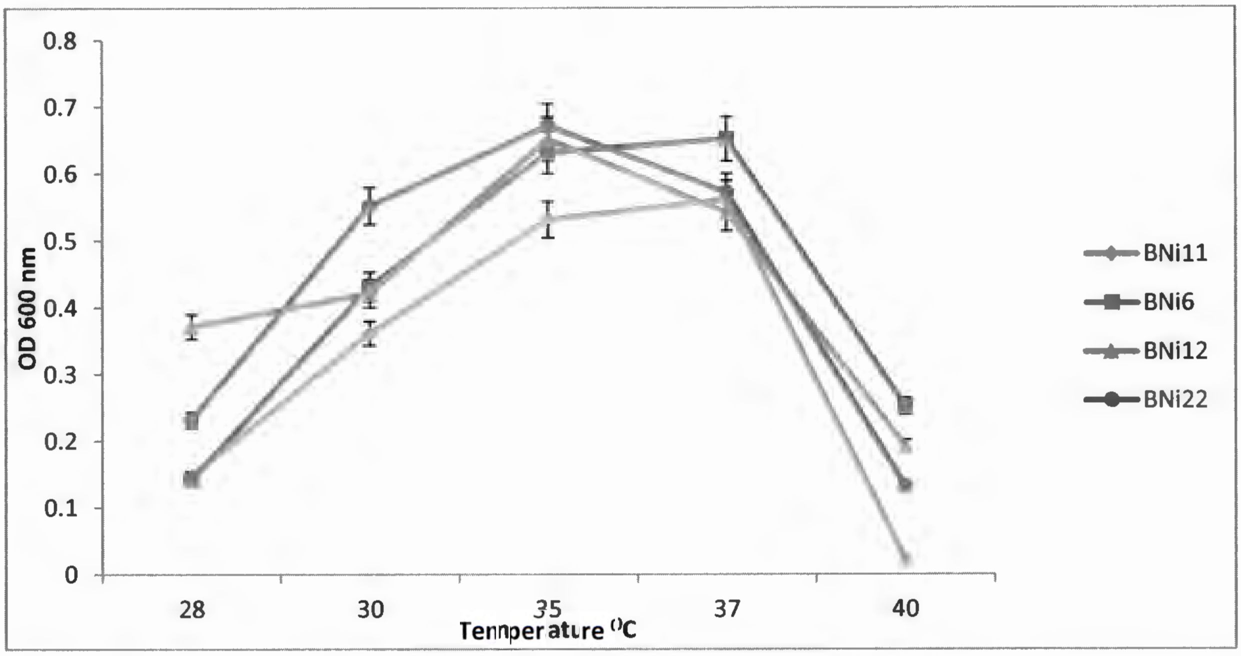

Figure 4b: Growth curves of BNi11, BNi12, BNi6, and BNi22 at different temperatures in LB broth containing 1 mM Ni^{2+} after 24 h of incubation.

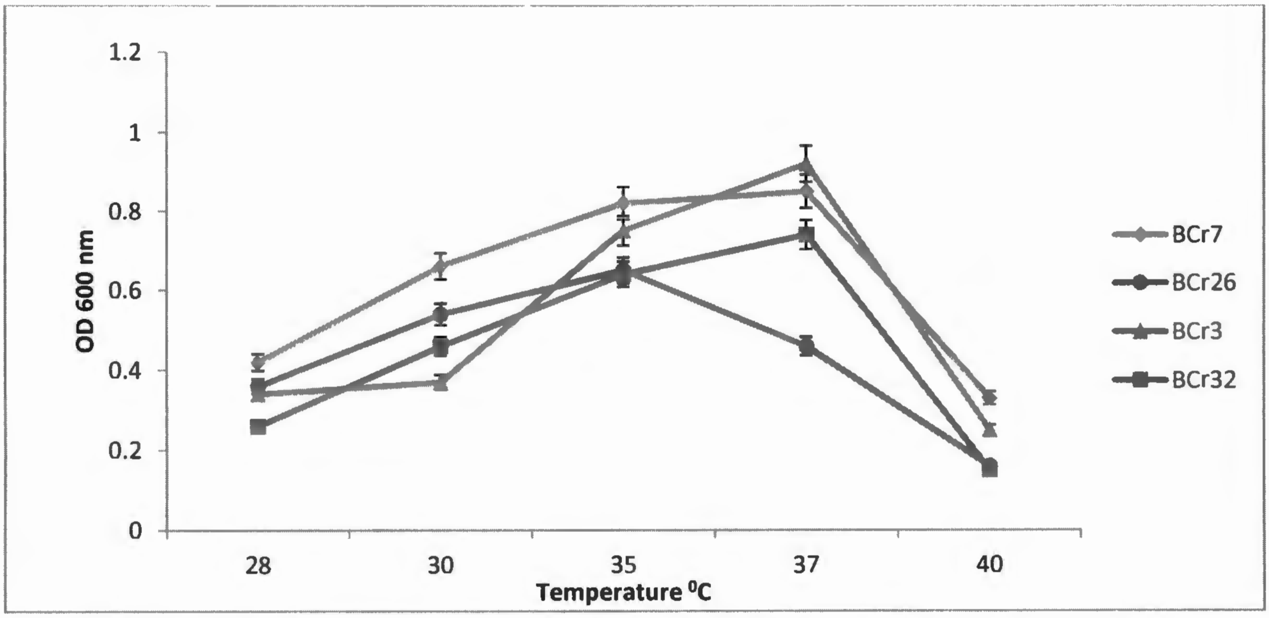

Figure 4c: Growth curves of BCr3, BCr32, BCr7 and BCr26 at different temperatures in LB broth containing 1 mM Cr^{6+} after 24 h of incubation.

2.4.7 EFFECT OF pH ON BACTERIAL GROWTH

The pH value is an important environmental factor that not only affects bacterial activity but also the chemical behavior of metal ions in solution (Exposito *et al.*, 2002). In this study, the effect of pH on bacterial growth in the presence of 1 mM Cd^{2+}, Ni^{2+} and Cr^{6+} is presented in Figures 5a, b and c. The data revealed lower optical densities of all bacterial isolates at acidic pH range (5.5-6.5). Whereas higher optical densities where observed at basic pH. Whereas, a slight decrease in growth was observed at pH 9. Above pH 9.0 growth studies were not performed since most metal ions tend to form hydroxyl – complexes which results in their precipitation. The optimum pH for the growth of BCr32, BCr3, BCr7, BNi11, BCd2, BCd16 and, BCd33 was 8.5. It was 8, BNi22, BNi6 and BCr26 while BNi12 showed optimum growth at 7.5.

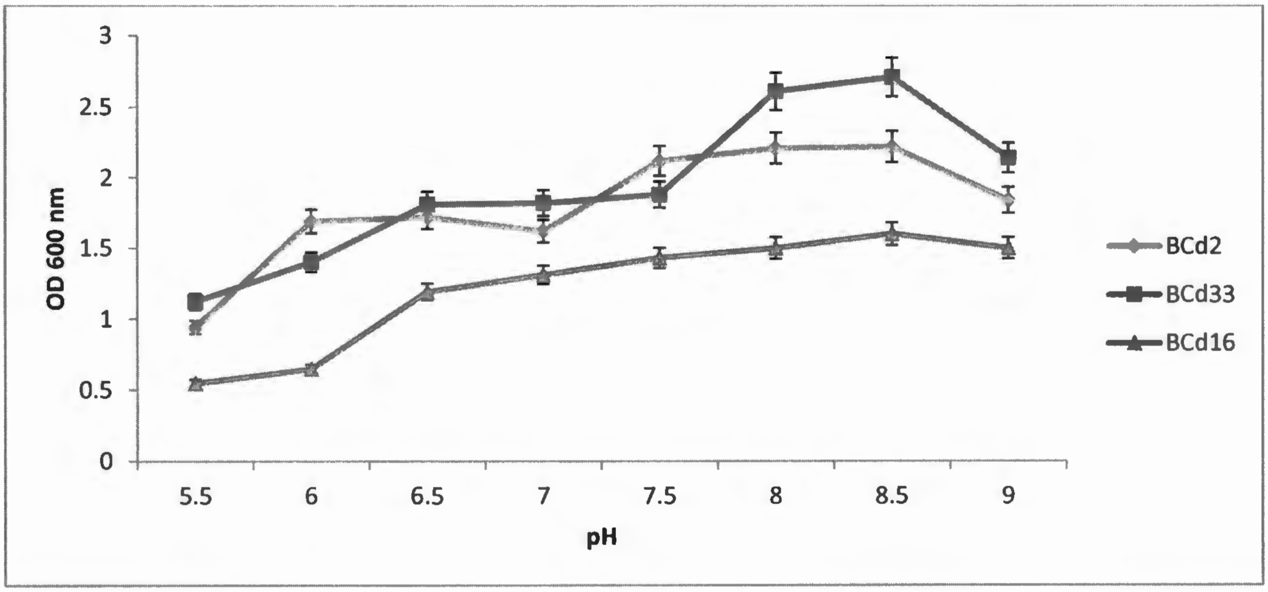

Figure 5a: Growth curves of BCd33, BCd2 and BCd16 at different pHs in LB broth containing 1 mM Cd^{2+} after 48 h of incubation.

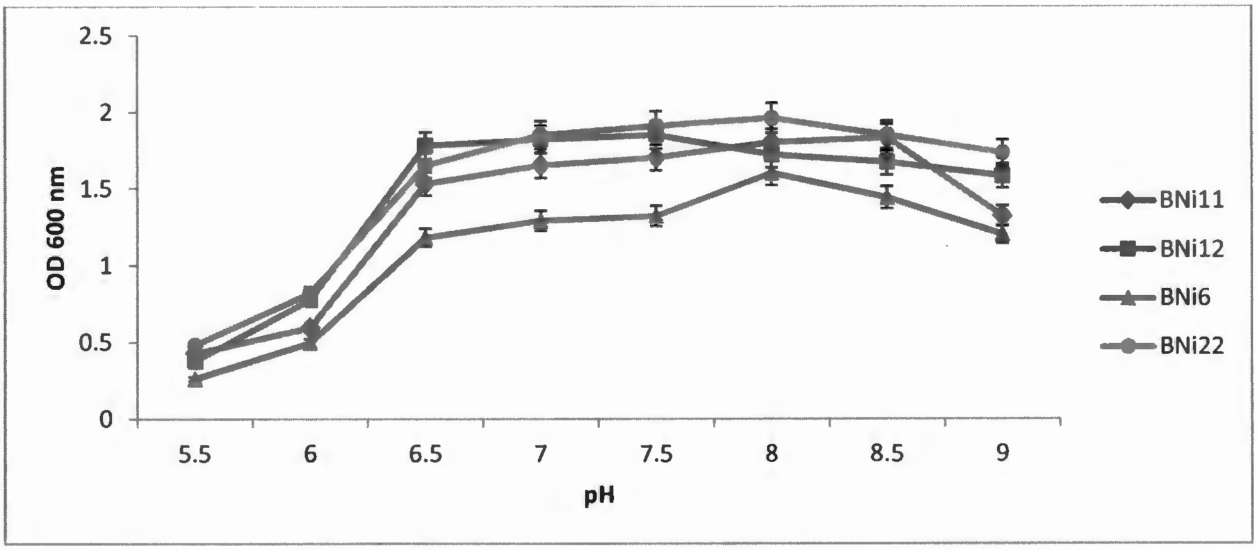

Figure 5b: Growth curves of BNi11, BNi12, BNi6, and BNi22 at different pHs in LB broth containing 1 mM Ni^{2+} after 48 h of incubation.

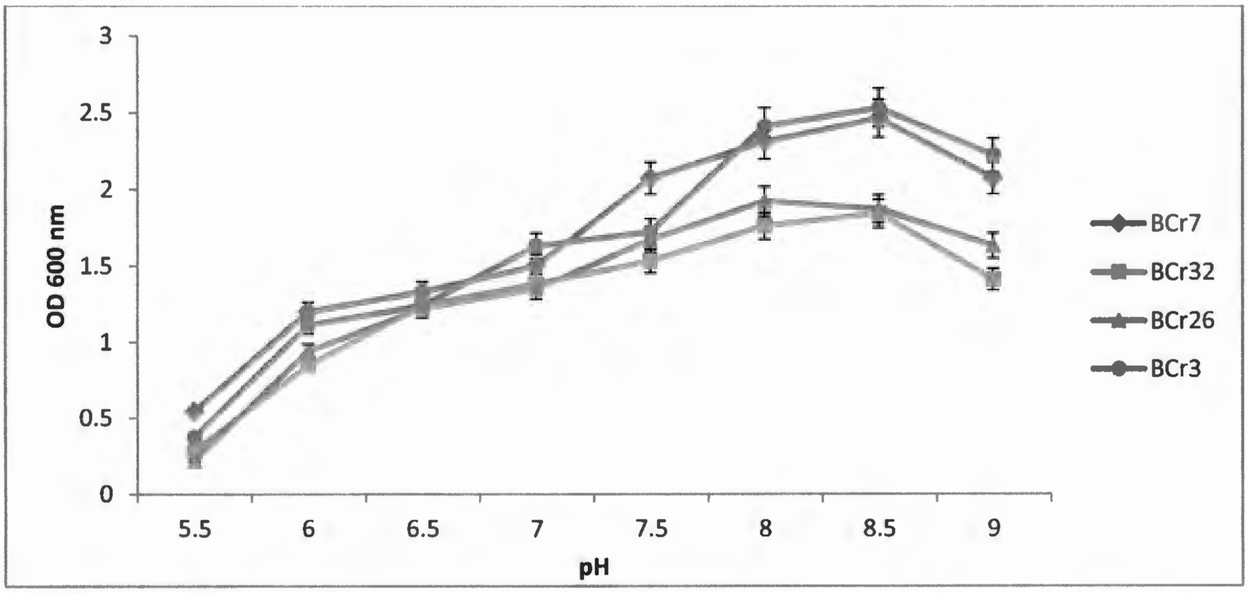

Figure 5c: Growth curves of BCr3, BCr32, BCr7 and BCr26 at different pHs in LB broth containing 1 mM Cr^{6+} after 48 h of incubation.

2.4.8 EFFECT OF SALINITY ON BACTERIAL GROWTH

Data presented in Figures 6a, b and c indicates that bacterial growth decreased progressively along with increase in NaCl concentration. Bacteria have the capacity to produce osmolytes such as sugars and amino acids which provide protection against a hypertonic environment (Bacilio *et al.*, 2004). No significant increase in bacterial growth was observed in LB medium containing 2% NaCl and beyond. This can be attributed to the osmotic gradient generated due to a high concentration of Na^+ in LB medium. The high concentration of Na^+ in the surrounding medium causes water molecules to move out of the cell resulting in a decrease in total internal pressure. This causes the cell to shrink and die (Chowdhury *et al.*, 2011; Ibekwe *et al.*, 2010).

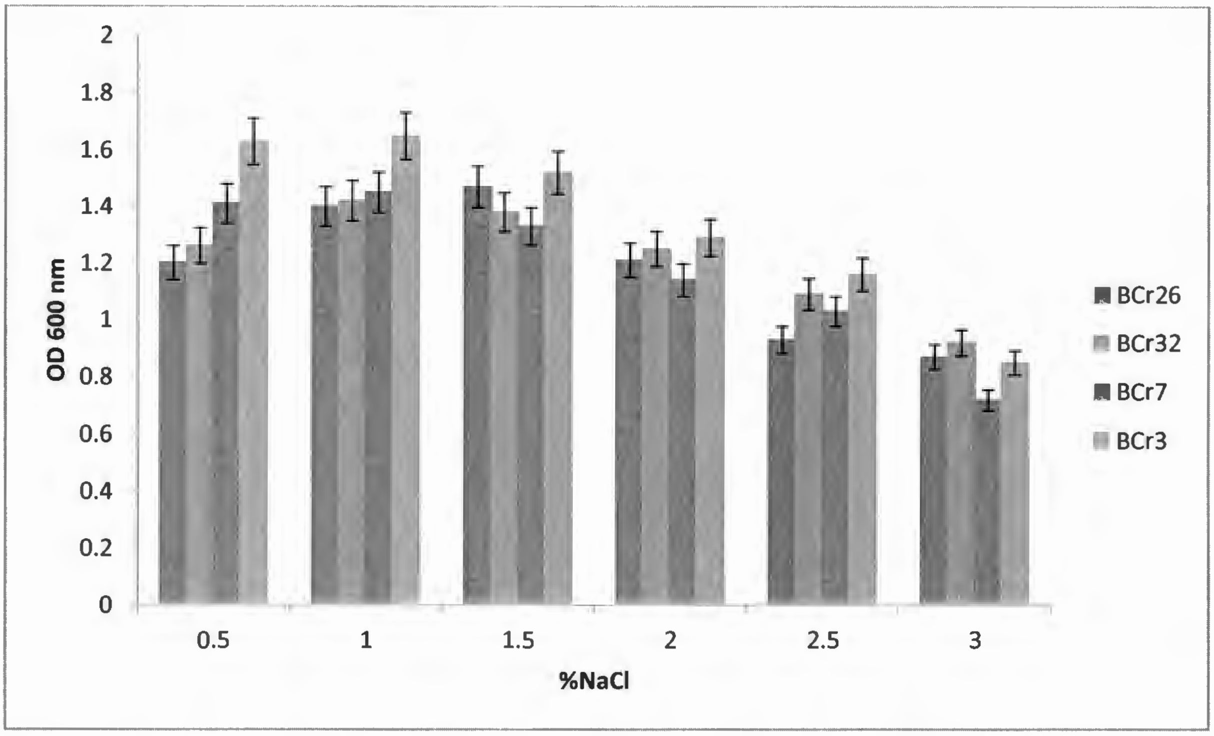

Figure 6a: Effect of NaCl on BCr3, BCr32, BCr7 and BCr26 in LB medium containing 1 mM Cr^{6+} after 24 h of incubation.

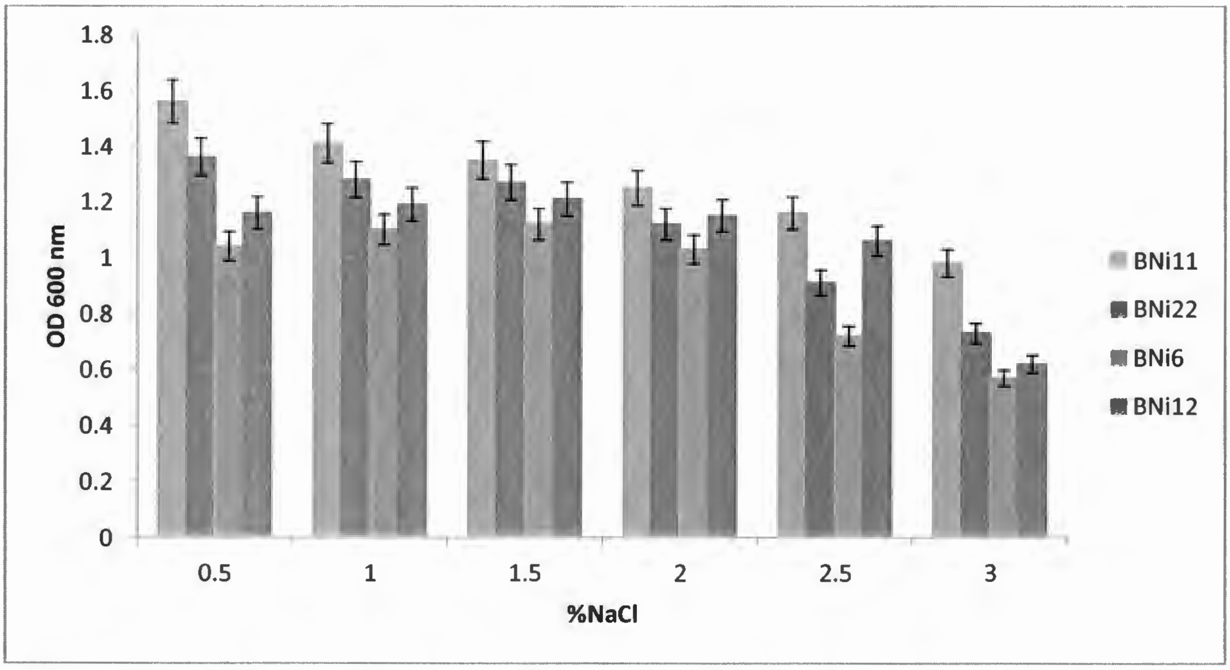

Figure 6b: Effect of NaCl on BNi11, BNi12, BNi6, and BNi22 in LB medium containing 1 mM Ni^{2+} after 24 h of incubation.

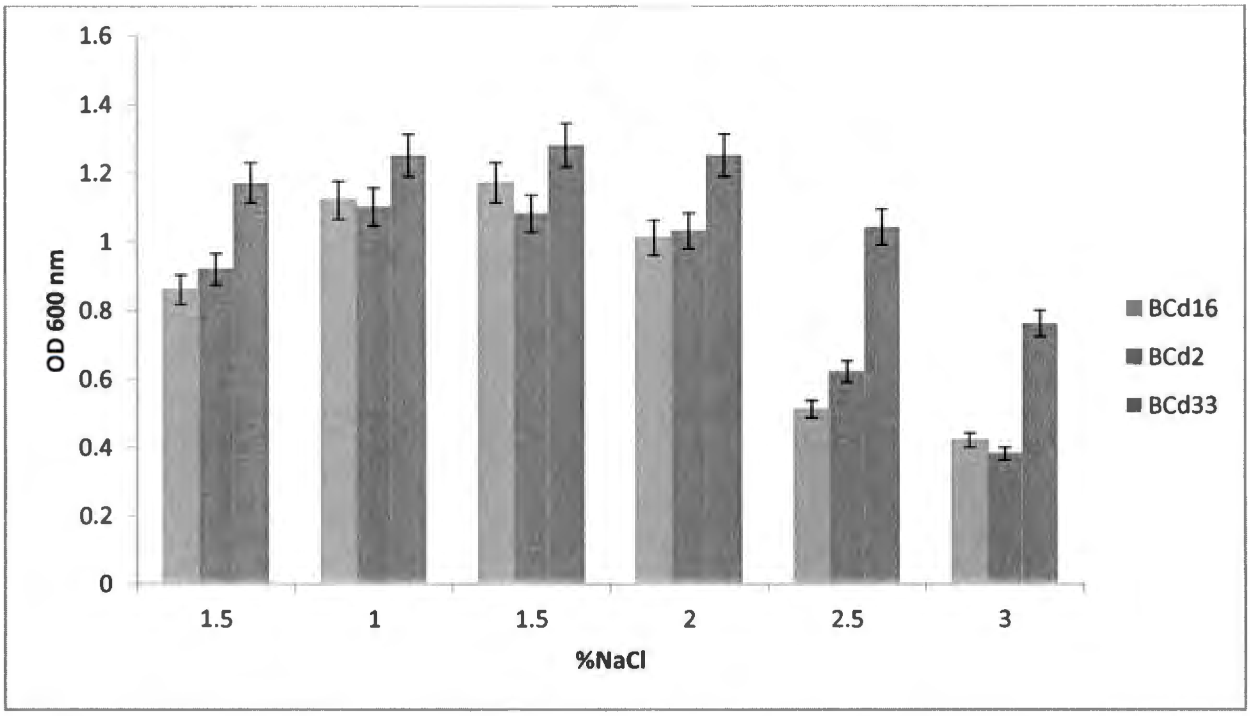

Figure 6c: Effect of NaCl on BCd33, BCd2 and BCd16 in LB medium containing 1 mM Cd^{2+} after 24 h of incubation.

2.4.9 ACCUMULATION OF Cr, Cd and Ni BY BACTERIAL ISOLATES

The accumulation of Cd, Cr, and Ni by the bacterial isolates was characterized to evaluate their potential to remove heavy metals from a solution. The amount (%) of metal uptake by each bacterial isolate was examined as a function of time, after having added the biomass to metal solution. Bacterial isolates also exhibited different accumulation capacities towards the various metal ions. Most bacterial isolates exhibited high accumulation of Cr and Ni, while the accumulation of Cd was relatively low (Table 7). The maximum percentage of metal accumulation in all bacterial isolates was observed within 72 h. After this time, further incubation up to 7 days did not improve the extent of accumulation. This can be attributed to intense saturation of bacterial cells with heavy metal ions, metal toxicity or a decrease in viable cell count (Al-Garni 2005).

Among Cr tolerant isolates, BCr3 showed maximum accumulation (68.7%) after 72 h, whereas minimum accumulation (16.7%) was observed in BCr26. BCr32 showed 44.8% accumulation while BCr7 absorbed 33.9% of Cr.

For Ni tolerant strains, the highest quantity of Ni was absorbed by BNi11 (69.8%) while the maximum accumulation percentage for BNi22, BNi12 and BNi6 was 32.3%, 38.6% and 49.2% respectively.

Among the Cd tolerant isolates, the maximum percentage of metal removal was observed in BCd33 (72.4%) while the maximum accumulation percentage for BCd2 and BCd16 was 40.5% and 29.7% respectively.

Table 6: Accumulation of heavy metals (Cr, Ni and Cd) by bacterial isolates

Isolate code	**Metal salt**	**Accumulation percentage (%) (days)**						
		1	**2**	**3**	**4**	**5**	**6**	**7**
BCd33	$CdCl_2$	35.5	48.7	72.4	61.8	52.5	31.6	28.9
BCd2	$CdCl_2$	22.1	38.9	40.5	39.1	38.3	35.8	34.5
BCd16	$CdCl_2$	16.3	29.7	22.8	21.6	21.2	20.2	16.8
BNi6	$NiCl_2$	48.0	47.6	49.2	45.5	45.1	24.8	24.8
BNi11	$NiCl_2$	21.4	51.3	69.8	46.7	45.9	40.2	40.1
BNi12	$NiCl_2$	25.7	26.4	38.6	37.1	32.4	32.9	31.8
BNi22	$NiCl_2$	15.8	23.6	32.3	24.4	16.1	12.7	11.5
BCr26	$K_2Cr_2O_7$	14.2	15.3	16.7	9.3	7.5	7.3	6.9
BCr7	$K_2Cr_2O_7$	28.3	29.7	33.9	16.7	14.3	13.6	9.4
BCr32	$K_2Cr_2O_7$	36.4	37.3	44.8	39.6	38.1	25.2	22.5
BCr3	$K_2Cr_2O_7$	56.8	64.3	68.7	45.4	38.7	38.4	37.9

Many investigators have also demonstrated Cr, Ni and Cd removal by several types of bacterial species. For example, Paul and Pal (2004) carried out a study to evaluate the biosorption capacity of several chromium tolerant bacteria obtained from serpentine soil and reported that the percentage removal of Cr by *Bacillus sphaericus* from a culture medium containing 100 mg/L Cr ions was approximately 65% in 8 h. Mishra and Doble (2008) reported 90% of 200 mg/L of Cr in 5 h. In a study by Pardo *et al.* (2003), approximately 80% of Cd^{+2} was removed by *Pseudomonas putida* from aqueous medium.

Metal ion accumulation by bacterial cells is generally achieved via two main mechanisms. These include binding of metal ions to cell surfaces (biosorption), and/or intracellular sequestration/ translocation (bioaccumulation) (Vieira and Volesky 2000; Wu *et al.*, 2006). Bacterial surface structures (cell wall or envelope) are also capable of adsorbing metal ions through electrostatic interactions. This process involves non-specific binding of metal cations to the cell envelope or extracellular polymeric substances (EPS) like proteins, polysaccharides, siderophores, teichoic and teichuronic acids (Henriques and Love 2007; Velasquez and Dussan 2009). These substances contain several functional groups like carboxyl, amide, phosphate, imidazole, sulhydryl, amino, carbonyl, phosphodiester and hydroxyl groups that give bacterial cell walls a strong negative charge (Vijayaraghavan and Yun 2008). Metal cations may be attracted to the cell surface structures due to opposite charges of chemical functional groups. They can also be transported across the cell membrane into the cell via ion pumps, carrier mediated transport, endocytosis, complex permeation, and lipid permeation. Most of these mechanisms have been studied and documented in numerous scientific reports. For instance Zolgharnein *et al.* (2010) studied the biosorption of heavy metals (Cu, Zn, Cd and Pb) by *Pseudomonas aeruginosa* MCCB 102 and reported that accumulation of metal ions involves both surface phenomena and diffusion. In another study, transmission electron microscopic (TEM) analysis revealed intracellular and periplasmic accumulation of cadmium in *Pseudomonas putida* 62 BN that was grown in the presence of 0.5 mM of cadmium (Rani *et al.*, 2009). This was attributed to the presence of metal-binding proteins and/or efflux systems within the cells. Subcellular fractionation studies performed by Guo *et al.*, 2010 to determine the distribution of Cd ions in a multi-metal resistant bacterium, *Bacillus* sp. L14, revealed that almost 80.8% of the Cd^{2+} taken up by the cell was found on the membrane fraction. Yoshida *et al.* (2002) and Naz *et al.* (2005) demonstrated that intracellular and periplasmic accumulation of heavy-metal ions in *Escherichia coli* was as a result of metallothionein expression. Our results indicate that bacterial isolates BCd33, BNi11 and BCr3 are excellent candidates for bioremediation of Cd, Ni and Cr respectively.

2.4.10 16S rRNA BASED IDENTIFICATION

DNA extraction using the Zymo ZR soil microbe DNA MiniPrep kit was fast and yielded high quality DNA. The amplification of the 16S rRNA genes was performed successfully using a set of universal primers (Figure 7). The molecular marker (A GeneRuler™ DNA Ladder) can be clearly seen in Lane 1 and fragments of 1500 bp (Lanes 2-11). No non-specific amplification was observed.

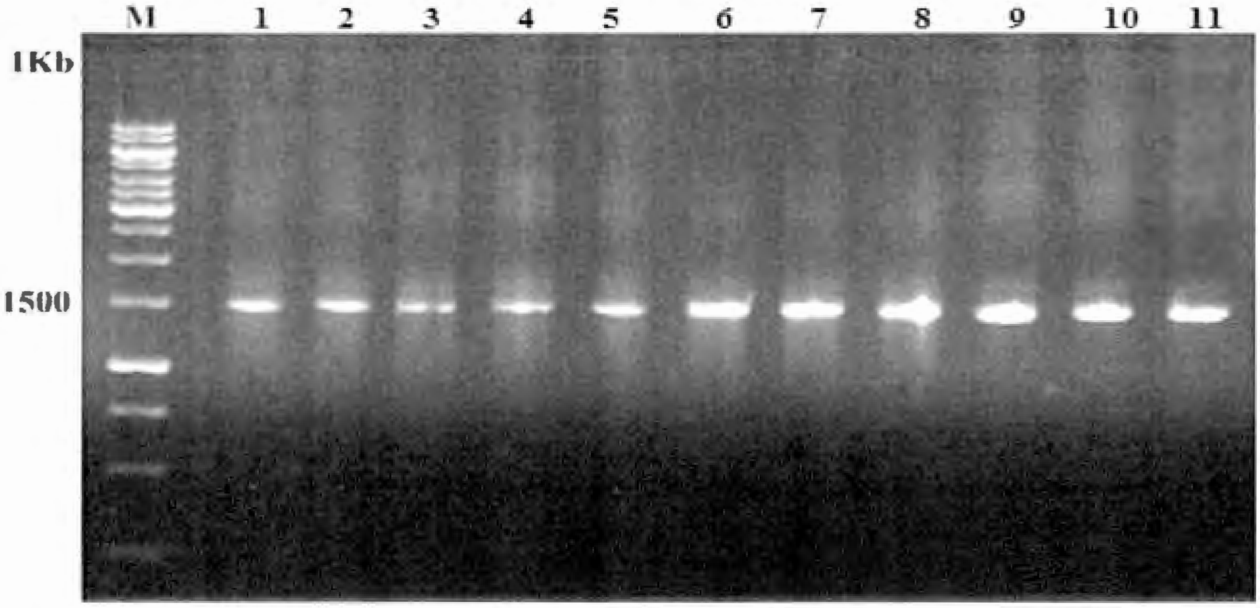

Figure 7: Agarose gel showing amplified DNA sequences of 1500 bp. Lane M= 1Kb molecular weight marker. Lane M= 1Kb molecular weight marker, Line 1: BCd16, Line 2: BCr7, Line 3: BCr3, Line 4: BCr32, Line 5: BCd2, Line 6: BCd33, Line 7: BCr26, Line 8: BNi6, Line 9: BNi22, Line 10: BNi11, Line 11: BNi12

Partial 16S rDNA nucleotide sequences from all bacterial strains were matched with similar sequences in the NCBI website using BLAST. The blast query revealed that the strains were close to the genus *Proteus*, *Bacillus, Alcaligenes* and *Pseudomonas* species. The identities of all bacterial isolates and their Genbank accession numbers are presented in Table 8. Several studies have reported that Gram-negative bacteria are more tolerant to heavy metals than Gram-positive (Bennisse *et al.*, 2004; Madigan *et al.*, 2003; Wenderoth and Reber 1999). Benyehuda *et al.* (2003) argued that the cell wall in Gram-negative bacteria is a much more effective barrier against toxic metals. He also stated that the surface structures that make up the cell wall interact with metal ions resulting in their detoxification. In the study done by Mounaouer *et al.* (2014), *Pseudomonas aeruginosa* and *Staphylococcus aureus* appeared as the most dominant genera. They also discovered that Gram-negative bacterial isolates were generally more tolerant than their Gram positive counterparts. The opposite was found in our study. Most metal tolerant strains were Gram positive and belonged to the genus *Bacillus*. This corresponds to the findings of Piotrowksa-Seget *et al.* (2005) who reported that metal tolerance is often associated with members of the Gram positive genera such as *Bacillus, Arthrobacter* and *Corynebacterium.* Moreover, they indicated that Gram negative organisms such as *Pseudomonas*, *Alcaligenes* and *Ralstonia* can also display tolerance to heavy metals. Ellis *et al.* (2003) also reported that *Bacillus* sp. had a higher relative abundance in the most heavy metal contaminated soil.

Table 8: Identity of the bacterial isolates based on 16S rRNA gene sequence analysis

Isolate code	Closest match (%)	Genbank accession No.
BCr3	*Pseudomonas aeruginosa* (98%)	KP717554
BCr7	*Bacillus safensis* (97%)	KP717556
BCr32	*Alcaligenes feacalis* (98%)	KP717562
BCr27	*Bacillus cereus* (99%)	KP717558
BCd2	*Bacillus pumulis* (97%)	KP717553
BCd33	*Bacillus cereus* (95%)	KP717555
BCd16	*Bacillus sp* (67%)	KP717563
BNi11	*Bacillus subtilis* (99%)	KP717559
BNi12	*Alcaligenes feacalis* (98%)	KP717561
BNi6	*Proteus mirabilis* (98%)	KP717560
BNi22	*Bacillus cereus* (98%)	KP717557

2.4.11 DETECTION OF PLASMIDS

In the present study, a single band of plasmid DNA was observed in all bacterial strains. According to the electrophoretic separation, isolated plasmids were approximately 10 000 bp each in size (Figure 8). In a study conducted by Piotrowska-Seget (2005), it was found that tolerance to Zn and Cd was related to plasmids. The heavy metal tolerant genes in bacteria have often been reported to be located on plasmids and transposons (Lee *et al.*, 2006). Hence they have generated a high degree of interest in the area of environmental bioremediation. However, this study did not conclusively demonstrate that the metal tolerant traits are associated with plasmids. If tolerance mechanisms are expressed through plasmids, manipulation and enhancement of these strains will be useful.

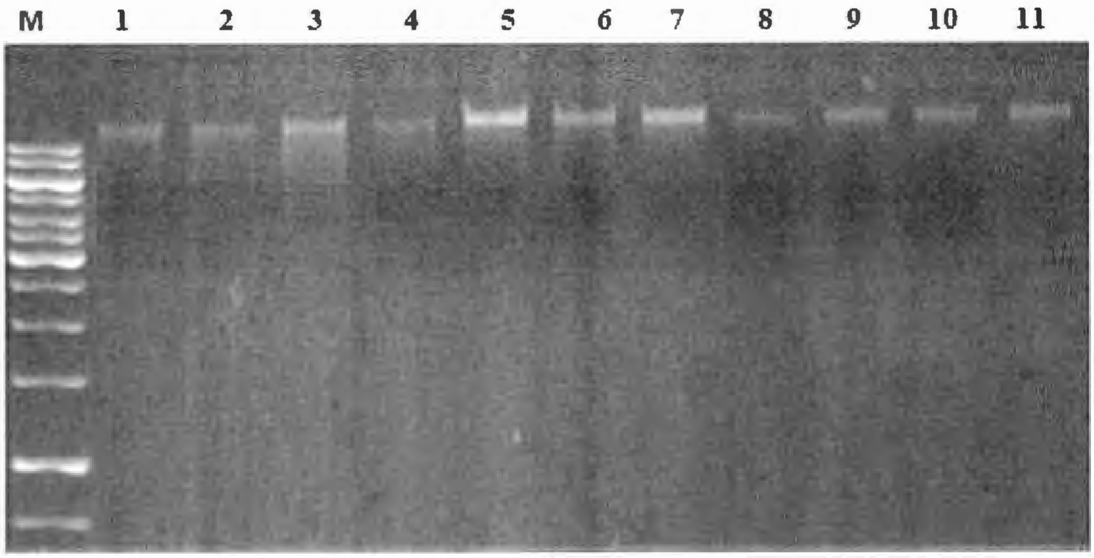

Figure 8: Electrophoretic separation profile of plasmid DNAs isolated from bacterial isolates. Lane M= 1Kb molecular weight marker, Line 1: BCd2, Line 2: BNi22, Line 3: BNi6, Line 4: BNi11, Line 5: BCd16, Line 6: BNi12, Line 7: BCr26, Line 8: BCr3, Line 9: BCr7, Line 10: BCr32, Line 11: BCd33

2.4.12 AMPLIFICATION OF HEAVY METAL TOLERANCE GENES

Through the evolutionary process, bacteria have developed heavy metal tolerance mechanisms to survive in hostile environments. This study was performed using primer-specific PCR amplifications on total genomic and plasmid DNA fractions, targeting some genetic systems responsible for Cr, Cd and Ni tolerance in bacteria. PCR amplification of the *chrA* gene yielded the expected ~ 1292 bp products for isolate BNi22 (Figure 9). Similar results were obtained with primer *chrB*, where expected fragment of 450 bp could be produced from BNi6 (Figure 10), while the rest of the isolates yielded no amplification. In the case of cadmium tolerance, the primer *cadA* yielded the expected 600 bp product with the BCr3, BCd2, BNi6, BCr7, BNi11, and

BNi12, but only multiple smaller bands in some isolates (figure 11). Amplification of *nccA* yielded the expected 1141 bp product in BNi6, BNi11, BCd33, BCd16 and BCr32 (Figure 12). Due to the poor amplification and reproducibility of results of most test isolates with the *CadA, NccA, ChrA* and *ChrB* primers, sequencing and nucleotide translation for these genes were not pursued further. Most isolates yielded no amplification products with primers *CzcC, CzcB* and *CzcD*. The absence of amplicons in bacterial isolates suggests that the tolerance to heavy metals could be due to other heavy metal resistance systems.

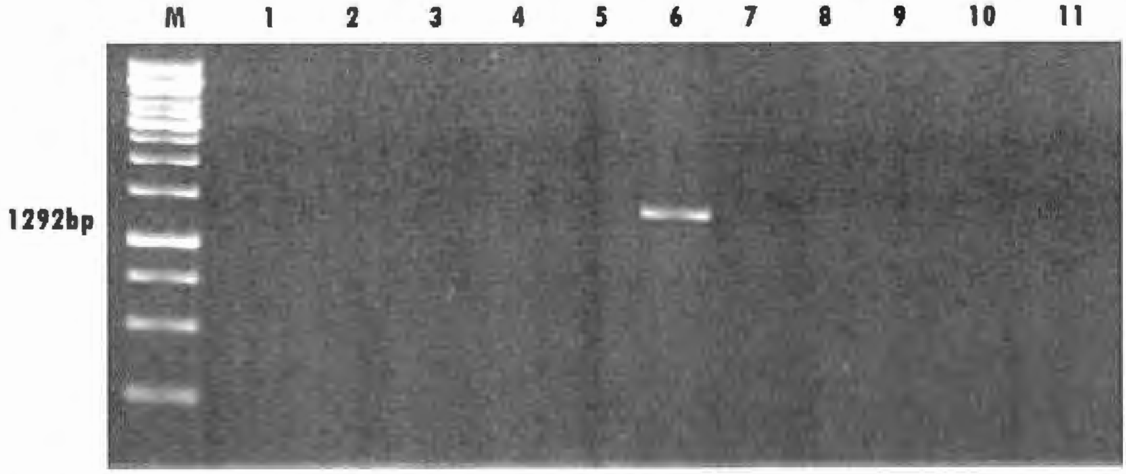

Figure 9: Agarose gel electrophoresis of *ChrA* PCR products. Lane M= 1Kb molecular weight marker, Line 1: BNi12, Line 2: BCd2, Line 3: BCr7, Line 4: BNi11, Line 5: BCr32, Line 6: BCd16, Line 7: BCd33, Line 8: BCr3, Line 9: BNi6, Line 10: BNi22, Line 11: BCr26.

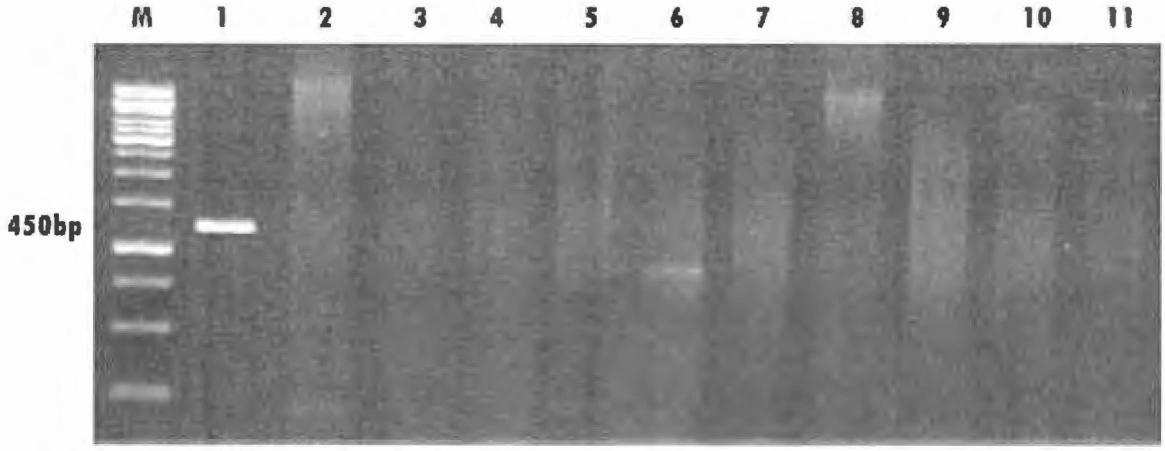

Figure 10: Agarose gel electrophoresis of *ChrB* PCR products. Lane M= 100 bp molecular weight marker, Line 1: BNi6, Line 2: BCr32, Line 3: BCd2, Line 4: BCr7, Line 5: BNi11, Line 6: BNi12, Line 7: BNi22, Line 8: BCd33, Line 9: BCr26, Line 10: BCd16, Line 11: BCr3.

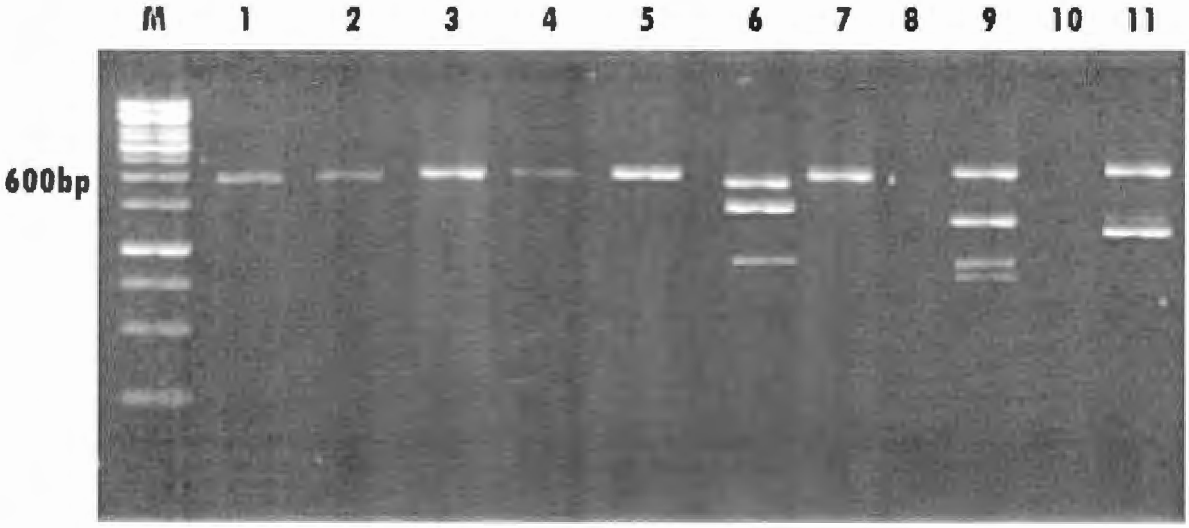

Figure 11: Agarose gel electrophoresis of *CadA* PCR products. Lane M= 100bp molecular weight marker, Line 1: BCr3, Line 2: BNi12, Line 3: BCd2, Line 4: BNi6, Line 5: BCr7, Line 6: BCd33, Line 7: BNi11, Line 8: BCr32, Line 9: BCd16, Line 10: BCr26, Line 11: BNi22.

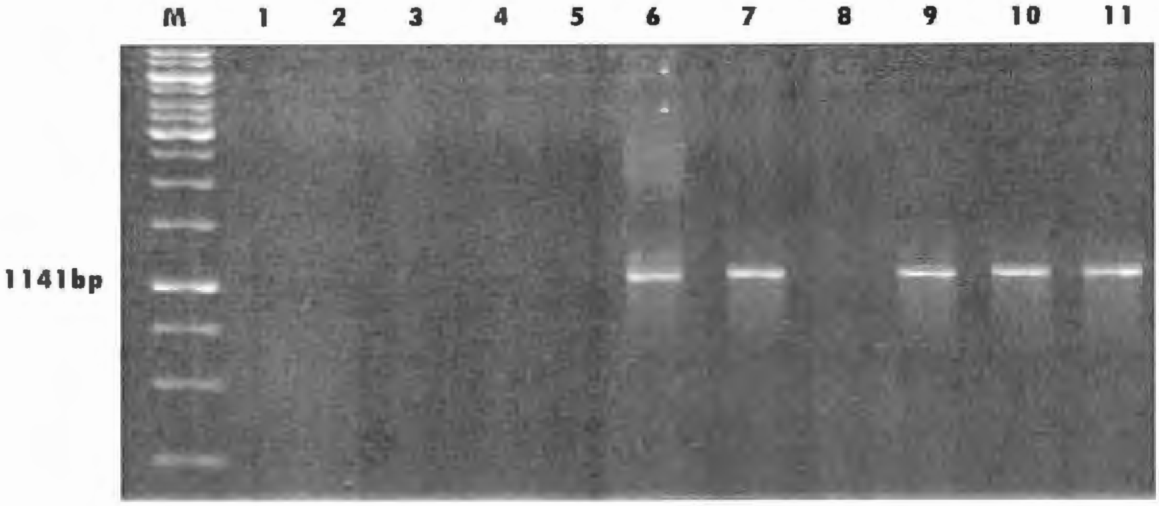

Figure 12: Agarose gel electrophoresis of *NccA* PCR products. Lane M= 1Kb molecular weight marker, Line 1: BCr7, Line 2: BCr3, Line 3: BCr26, Line 4: BCd2, Line 5: BNi12, Line 6: BCd16, Line 7: BNi6, Line 8: BNi22, Line 9: BCd33, Line 10: BCr32, Line 11: BNi11.

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REFERENCES

ABOU-SHANAB RAI, VAN BERKUM P & ANGLE JS 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. *Chemosphere,* 68, 360–367.

AHLUWALAI SS & GOYAL D 2007. Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource Technology*, 98, 2243-2257.

AHMED N, NAWAZ A & BADAR U 2005. Screening of copper tolerant bacterial species and their potential to remove copper from the environment *Bulletin of Environmental Contamination and Toxicology,* 74, 219-226.

AL-GARNI S 2005. Biosorption of Lead by Gram-ve capsulated and non-capsulated bacteria. *Water SA*, 31,345-350.

BACILIO M, RODRIGUEZ H, MORENO M, HERNANDEZ JP & BASHAN Y 2004. Mitigation of salt stress in wheat seedlings by a gfp-tagged *Azospirillum lipoferum*. *Biology and Fertility of Soil,* 40, 188-193.

BANERJEE S, GOTHALWAL R, SAHU PK & SAO S 2015. Microbial Observation in Bioaccumulation of Heavy Metals from the Ash Dyke of Thermal Power Plants of Chhattisgarh, India. *Advances in Bioscience and Biotechnology*, 6, 131-138.

BELIMOV A, HONTZEA AA SB, SAFRONOVAA VI, DEMCHINSKAYAA SV, PILUZZAC G, BULLITTAC S & GLICK BR 2005. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea L. Czern.*). *Soil Biology and Biochemistry,* 37, 241-250.

BENNISSE R, LABAT M, ELASLI A, BRHADA F, CHANDAD F, LIEGBOTT PP, HIBTI M & QATIBI AI 2004. Rhizosphere bacterial populations of metallophyte plants in heavy metal-contaminated soils from mining areas in semiarid climate. *World Journal of Microbiology and Biotechnology,* 20, 759-766.

BENYEHUDA G, COOMBS J, WARD PL, BALKWILL D & BARKEY T 2003. Metal resistance among aerobic chemoheterotrophic bacteria from the deep terrestrial subsurface. *Canadian Journal of Microbiology*, 49, 151-156.

BHAGAT N, VERMANI M & BAJWA HS 2016. Characterization of heavy metal (cadmium and nickle) tolerant Gram negative enteric bacteria from polluted Yamuna River, Delhi. *African Journal of Microbiology Research*, 10, 127-137

CANOVAS D, CASES I & DE LORENZO V 2003. Heavy metal tolerance and metal homeostasis in *Pseudomonas putida* as revealed by complete genome analysis. *Environmental Microbiology,* 5, 1242-1256.

CHAMBER OF MINES SOUTH AFRICA, 2003.The contribution of the mining and minerals industry to sustainable development in South Africa. */http://www.bullion.org.za.* Accessed (06.05.2014).

CHOWDHURY N, MARSCHNER P & BURNS RG 2011. Soil microbial activity and community composition: impact of changes in matric and osmotic potential. *Soil Biology and Biochemistry,* 43, 1229-1236.

CLAUSEN CA 2000. Isolating metal tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood. *Waste Management Research*, 18, 264–268.

CORAL MNU, KORKMAZ H, ARIKAN B & CORAL G 2005.Plasmid mediated heavy metal resistances in *Enterobacter* spp. Isolated from Sofulu landfill, in Adana, Turkey. *Annals of Microbiology*, 55, 175-179.

DELL'AMICO E, CAVALCA L & ANDREONI V 2008. Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biology and Biochemistry,* 40, 74-78.

DUFFUS JH 2002. Heavy Metals-A Meaningless Term? (IUPAC Technical Report) *Pure and Applied. Chemistry,* 74**,** 793-807.

ELLIS RJ, MORGAN P, WEIGHTMAN AJ & FRY JC 2003. Cultivation-Dependent and -Independent Approaches for Determining Bacterial Diversity in Heavy-Metal-Contaminated Soil. *Applied and Environmental Microbiology,* 69, 3223-3230.

ESPOSITO A, PAGNANELLI F & VEGLIO F 2002. pH-related equilibria models for biosorption in single metal systems. *Chemical Engineering Science*, 57, 307-313.

GUO H , LUO S, CHEN L, XIAO X, XI Q, WEI W, ZENG G, LIU C, WANA Y, CHEN J, & HE Y 2010. Bioremediation of heavy metals by growing hyperaccumulator endophytic bacterium *Bacillus sp.* L14. *Bioresource Technology,* 101, 8599-8605.

HASHIM MA, MUKHOPADHYAY S, SAHU JN & SENGUPTA B 2011. Remediation technologies for heavy metal contaminated groundwater. *Journal of Environmental Management,* 92, 2355-2388.

HASSAN MT, VAN DER LELIE D, SPRINGAEL D, ROMLING U, AHMED N & MERGEAY M 1999. Identification of a gene cluster, czr, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa. Gene,* 238, 417-425.

HENRIQUES IDS & LOVE NG 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. *Water Research,* 41, 4177–4185.

IBEKWE AM, POSS JA, GRATTAN SR, GRIEVE CM & SUAREZ D 2010. Bacterial diversity in cucumber (*Cucumis sativus*) rhizosphere in response to salinity, soil pH, and boron. *Soil Biology and Biochemistry*, 42, 567-575.

JAN FA, ISHAQ M, KHAN S, IHSANULLAH I, AHMAD I & SHAKIRULLAH M 2010. A comparative study of human health risks via consumption of food crops grown on wastewater irrigated soil (Peshawar) and relatively clean water irrigated soil (Lower Dir). *Journal of Hazardous Materials*, 179, 612–621.

KAMNEV AA, TUGAROVA AV, ANTONYUK LP, TARANTILIS PA, POLISSIOU MG & GARDINER PH 2005. Effects of heavy metals on plant-associated rhizobacteria: comparison of endophytic and non-endophytic strains of *Azospirillum brasilense. Journal of Trace Elements and Medical Biology,* 19, 91–95.

KING P, RAKESH N, BEENALAHARI S, KUMAR YP & PRASAD VSR 2007. Removal of lead from aqueous solution using *Syzygium cumini L.*: equilibrium and kinetic studies. *Journal of Hazardous Materials,* 142, 340–347.

LAXMAN SR & MORE S 2002. Reduction of hexavalent chromium by *Streptomyces griseus. Mineralogy and Engineering*, 15, 831–837.

LEE YK, CHANG HH, LEE HJ, PARK H, LEE KH & JOE MH 2006. Isolation of a novel plasmid, pNi15, from *Enterobacter sp.* Ni15 containing a nickel resistance gene. *FEMS Microbiology Letters,* 257, 177–181.

LEI JQ, LI SY, JIN ZZ 2008. Comprehensive eco-environmental effects of the shelter-forest ecological engineering along the Tarim Desert Highway. *Chinese Science Bulletin*, 53, 190-202.

LIU WX, SHEN LF, LIU JW, WANG YW & LI SR 2007. Uptake of toxic heavy metals by rice (*Oryza sativa L.*) cultivated in the agricultural soils near Zhengzhou City, People's Republic of China. *Bulletin of Environmental Contamination and Toxicology,* 79, 209–213.

MADIGAN MT, MARTINKO JM & PARKER J 2003. Brock Biology of Microorganisms 10th Ed.. Upper Sadle River, USA. *Pearson Education, Inc.*

MALIK A 2004. Metal bioremediation through growing cells. *Environment International*, 30, 261–278.

MALIK A, KHAN IF & ALEM A 2002. Plasmid incidence in bacteria from agricultural and industrial soils. *World Journal of Microbiology and Biotechnology*, 18, 827–833.

MERGEAY M, NIES D, SCHLEGEL HG, GERTIS J, CHARLES P & VAN GUSEGEM F 1985. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *Journal of Bacteriology,* 162, 328–334.

MISHRA S & DOBLE M 2008. Novel chromium tolerant microorganisms: Isolation, characterization and their biosorption capacity. *Ecotoxicology and Environmental Safety,* 71, 874–879.

MONICA OM, JULIA WN & RAINA MM 2008. Characterization of a bacterial community in an abandoned semiarid lead-zinc mine tailing site. *Applied and Environmental Microbiology,* 74, 3899–3907.

MOUNAOUER B, NESRINE A & ABDENNACEUR H. 2014. Identification and characterization of heavy metal-resistant bacteria selected from different polluted sources. *Desalination and Water Treatment,* 52, 7037–7052.

NAVARRO MC, PÉREZ-SIRVENT C, MARTÍNEZ-SÁNCHEZ MJ, VIDAL J, TOVAR PJ & BECH J 2008. Abandoned mine site as a source of contamination by heavy metals: a case study in a semi-arid zone. *Journal of Geochemical Exploration,* 96, 183-193.

NAZ N, YOUNG HK, AHMED N & GADD GM 2005. Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. *Applied and Environmental Microbiology,* 71, 4610-4618.

NIES A, NIES DH & SILVER S 1990. Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus. Journal of Biology and Chemistry* 265, 5648-5653.

NIES DH 1999. Microbial heavy metal resistance. *Applied Microbiology and Biotechnology,* 51, 730–750.

NIES DH, NIES A & SILVER S 1989. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proceedings of the National Academy of Sciences USA,* 86, 7351–7355.

NIETO JJ, VENTOSA A & RUIZ-BERRAQUERO F 1987. Susceptibility of halobacteria to heavy metals. *Applied and Environmental Microbiology,* 53, 1199.

OYETIBO GO, ILORI MO, ADEBUSOYE SA, OBAYORI OS & AMUND OO 2010. Bacteria with dual resistance to elevated concentrations of heavy metals and antibiotics in Nigeria in contaminated systems. *Environmental Monitoring Assessment*, 168, 305-314.

PAL A, CHOUDHURIP, DUTTAS, MUKHERJEE PK & PAUL AK 2004. Isolation and characterization of nickel-resistant microflora from serpentine soils of Andaman. *World Journal of Microbiology and Biotechnology,* 20, 881-886.

PAL A, DUTTA S, MUKHERJEE PK & PAUL AK 2005. Occurrence of heavy metal-resistance in microflora from serpentine soil of Andaman. *Journal of Basic Microbiology,* 45, 207-218.

PARDO R, HERGUEDAS M & BARRADO E 2003. Biosorption of cadmium, copper, lead and zinc by inactive biomass of *Pseudomonas putida*. *Analytical and Bioanalytical chemistry,* 376, 26–33.

PAUL AK & PAL A 2004. Aerobic chromate reduction by chromium resistant bacteria isolated from serpentine soil. *Microbiology Research*, 159, 347–354.

PIOTROWSKA-SEGET Z, CYCON M & KOZDRO J 2005. Metal-tolerant bacteria occurring in heavily polluted soil and mine spoil. *Applied Soil Ecology,* 28, 237–246.

PRESCOTT LM & HARLEY JP 2002. Laboratory Exercises in Microbiology. Fifth Edition. The McGraw–Hill Companies.

QIN Y, SHI B & LIU J 2006. Application of chitosan and alginate in treating waste water containing heavy metal ions. *Indian Journal of Chemical Technology,* 13, 464-469.

RAJA E, ANBAZHAGAN K & SELVAM GS 2006. Isolation and characterization of a metal resistant *Pseudomonas aeruginosa* strain. *World Journal of Microbiology and Biotechnology,* 22, 577–586.

RAJKUMAR M, NAGENDRAN R, LEE KJ, LEE WH & KIM SZ 2006. Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. *Chemosphere,* 62, 741-748.

RANI A, YOGESH S, SOUCHE B & REETA GOEL 2009. Comparative assessment of in situ bioremediation potential of cadmium resistant acidophilic *Pseudomonas putida* 62BN and alkalophilic *Pseudomonas monteilli* 97AN strains on soybean. *International Biodeterioration and Biodegradation,* 63, 62–66.

ROSEWARNE CP, PETTIGROVE V, STOKES HW PARSONS YM 2010. Class 1 integrons in benthic bacterial communities: abundance, association with Tn_4O_2-like trans-position modules and evidence for co-selection with heavy-metal resistance. *FEMS Microbiology and Ecology,* 72, 35-46.

SAMANTA A, BERA P, KHATUN M, SINHA C, PAL P, LALEE A & MANDAL A 2012. An investigation on heavy metal tolerance and antibiotic resistance properties of bacterial strain *Bacillus sp.* isolated from municipal waste. *Journal of Microbiology and Biotechnology Research*, 2, 178-189.

SEVGI E, CORAL G, GİZİR MA &SANGÜN MK 2010. Investigation of heavy metal resistance in some bacterial strains isolated from industrial soils. *Turkish Journal of Biology*, 34, 423-431.

STOPPEL R & SCHLEGEL HG 1995 Nickel-Resistant Bacteria from Anthropogenically Nickel-Polluted and Naturally Nickel-Percolated Ecosystems. *Applied and Environmental Microbiology*, 2276-2285.

SURESH KMK, SELVAM GS & JASMINE PS 1998. Cadmium tolerance and metal accumulation by bacterial strain from industrial effluents. *Journal of Scientific and Industrial Research*, 57, 817-820.

THACKER U, PARIKH R, SHOUCHE Y & MADAMWAR D 2007. Reduction of chromate by cell-free extract of *Brucella sp.* Isolated from Cr(VI) contaminated sites. *Bioresource Technology,* 98, 1541-1547.

TSAI YP, YOU SJ, PAI TY & CHEN KW 2005. Effect of cadmium on composition and diversity of bacterial communities in activated sludges. *International Biodeterioration and Biodegredation,* 55, 285–291.

UMRANIA VV. 2006. Bioremediation of toxic heavy metals using acidothermophilic autotrophes. *Bioresource Technology,* 97, 1237-1242.

US ENVIRONMENTAL PROTECTION AGENCY (USEPA) 2006. SW-846 test methods for evaluating solid wastes. *http://www.epa.gov/sw-846/main.htm.*

VELASQUEZ L & DUSSAN J 2009. Biosorption and bioaccumulation of heavy metals on dead and living biomass of *Bacillus sphaericus*. *Journal of Hazardous Materials,* 167, 713–716.

VIEIRA RH & VOLESKY B 2000. Biosorption; A solution to pollution. *International Microbiology,* 3, 17-24.

VIJAYARAGHAVAN K & YUN YS 2008. Bacterial biosorbents and biosorption. *Biotechnology Advances,* 126, 266–269.

WANG F, YAO J, SI Y, CHEN H, RUSSEL M, CHEN K, QIAN Y, ZARAY G & BRAMANTI E 2010. Short-time effect of heavy metals upon microbial community activity. *Journal of Hazardous Materials*, 173, 510-516.

WANG J & CHEN C 2009. Biosorbents for heavy metals removal and their future. *Biotechnology Advances,* 27, 195–226.

WENDEROTH DF & REBER HH 1999. Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. *Soil Biology and Biochemistry*, 31, 345-352.

WHITELEY CG & LEE DJ 2006. Enzyme technology and biological remediation. *Enzyme Microbiology and technology*, 38, 291–316.

WU SC, LUO YM, CHEUNG KC & WONG MH. 2006. Influence of bacteria on Pb and Zn speciation, mobility and bioavailability in soil: a laboratory study. *Environmental Pollution,* 144, 765–773.

YAN G & VIRARGHAVAN T 2000. Effect of pretreatment on the bioadsorption of heavy metals on *Mucor rouxii*. *Water SA*, 26, 119–123.

YOSHIDA N, KATO T, YOSHIDA T, OGAWA K, YAMASHITA M & MUROOKA Y 2002. Bacterium-based heavy metal biosorbents: enhanced uptake of cadmium by *Escherichia coli* expressing a metallothionein fused to b-galactosidase. *Biotechniques,* 32, 551–558.

ZOLGHARNEIN H, AZMI MLM, SAAD MZ, MUTALIB AR &B MOHAMED CA 2007. Detection of plasmids in heavy metal resistance bacteria isolated from the Persian Gulf and enclosed industrial areas. *Iranian Journal of Biotechnology*, 5, 232-239.

ZULKALI MM, AHMAD AL & NORULAKMAL NH 2006. *Oryza sativa L. husk* as heavy metal adsorbent: optimization with lead as model solution. *Bioresource. Technology*, 97, 21–25.

CHAPTER 3: EFFECT OF BACTERIAL INOCULATION of STRAINS of *Pseudomonas aeruginosa, Alcaligenes feacalis* AND *Bacillus subtilis* ON GERMINATION, GROWTH AND HEAVY METAL (Cd, Cr, and Ni) UPTAKE OF *Brassica juncea*

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3.1 INTRODUCTION

Heavy metals (Cr, Cd and Ni) are major ecological contaminants that negatively affect soil quality, crop production and public health in many countries around the globe today. Therefore, reducing the amount of metal contaminants in soil is necessary for human health and ecosystem preservation (Abou-Shanab *et al.*, 2006). However, this is a challenging task because unlike organic contaminants, heavy metals cannot be degraded and the dangers they pose are aggravated by their high toxicity and persistence in the environment. At present, a range of physicochemical processes including excavation, land-fill, solidification, stabilization, soil washing, and electrokinesis are used for remediation of heavy metal contaminated soils (Mulligan *et al.*, 2001; Susarla *et al.*, 2002). However, these processes have certain limitations. Not only are they too expensive to manage and operate, they lack specificity and may generate large amount of waste that are potentially harmful to the environment (Quartacci *et al.*, 2006). This makes them unsuitable for cleaning up large areas such as industrial and agrochemically contaminated soils.

Recently, phytoextraction has gained much attention within the scientific community because it is cheap and environmentally compatible. This relatively new technology uses hyperaccumulator plants to extract large amounts of metals and accumulate them in their above-ground tissues (Lasat, 2002). Unfortunately, most of the identified metal hyperaccumulators are not suitable for phytoextraction due to their slow growth and low biomass production in soils containing high concentrations of heavy metal ions (Khan *et al.*, 2000). To solve this problem, the use of metal tolerant bacteria with plant growth promoting properties has been proposed (Sheng and Xia 2006). Bacteria are a key constituent of the soil and are quite capable of forming mutual beneficial relationships with most terrestrial plants. They can enhance metal bioavailability and provide protection to plants against the toxic effects of heavy metals using a variety of processes including biosorption, bioaccumulation and biotransformation (Yang *et al.*, 2005). In addition, bacteria also have the capacity to fix atmospheric nitrogen, solubilize inorganic phosphate, synthesize antibiotic substances as well as plant growth regulators like siderophores, 1-aminocyclopropane-1-carboxylic acid, indole acetic acid (IAA) gibberellin acid (GA) and cytokinin, which stimulate growth and allow greater metal accumulation by host plants (Dell'Amico *et al.*, 2008; Rajkumar *et al.*, 2009; Sheng and Xia 2006). Therefore, the present study was conducted to:

1. Screen metal tolerant bacteria for the production of plant growth promoting substances such as IAA, ammonia (NH_3) and hydrogen cyanide (HCN)
2. Evaluate the effects of bacterial inoculation on growth and heavy metal (Ni, Cr and Cd) accumulation by *B. juncea.*

3.2 MATERIALS AND METHODS

3.2.1 ISOLATION AND SELECTION OF METAL TOLERANT BACTERIA

In a previous study, eleven metal tolerant bacteria were isolated from mine tailings and three strains: *Pseudomonas aeruginosa* KP717554 (BCr3), *Alcaligenes feacalis* KP717561 (BCd33) and *Bacillus subtilis* KP717559 (BNi11) were selected based on their high tolerance to heavy metals Cd, Cr and Ni respectively. Selected bacterial isolates were screened for plant growth promoting traits and their influence on plant growth and heavy metal accumulation by *B. juncea* was evaluated.

QUALITATIVE DETERMINATION OF PLANT-GROWTH PROMOTING CHARACTERISTICS OF BACTERIAL ISOLATES

3.2.2 CATALASE ACTIVITY

Bacterial cultures were grown on Luria Bertani (LB) medium for 24 h. The cultures were mixed with a drop of 3% hydrogen peroxide on a clean glass slide and observed for effervescence (Chacko *et al.*, 2009).

3.2.3 INDOLE-3-ACETIC ACID (IAA) PRODUCTION

Bacterial isolates were inoculated in LB medium amended with 50 mg ml^{-1} of tryptophan and then incubated at 37^0C for 24 h. After incubation, 1 ml of cell suspension was centrifuged at 10000 rpm for 10 min and 2–3 drops of orthophosphoric acid was added to the supernatant along with 4 ml of Solawaski's reagent (50 ml 35% perchloric acid; 1 ml of 0.5 M $FeCl_3$). The tubes were allowed to stand at room temperature for 20 min. IAA production was indicated by the development of pink cooler (Brick *et al.*, 1991). IAA production assays were repeated twice for each isolate.

3.2.4 MINERAL PHOSPHATE SOLUBILIZATION

Bacterial isolates were evaluated for their ability to solubilize inorganic phosphate. A loop full of each isolate was streaked on Pikovskya's medium (Pikovskaya, 1948) modified with tri-calcium phosphate ($Ca_3\ (PO_4)_5$) and placed in an incubator at 37^0C for 7 days. The formation of a clear zone around the bacterial colony was considered as positive result (Husen, 2003). The experiment was performed twice.

3.2.5 AMMONIA (NH_3) PRODUCTION

For the detection of NH_3 production, 100 µl of freshly grown bacterial cultures were inoculated in separate test tubes containing 10 ml peptone water and incubated at 37^0C for 72 h. After incubation, 1 ml Nessler's reagent was added to each tube and the color change was noted. The development of a yellow to brown color was considered a positive result for NH_3 production (Bakker and Schippers, 1987). NH_3 production assays were performed twice per isolate.

3.2.6 HYDROGEN CYANIDE (HCN) PRODUCTION

HCN production was determined by a modified method of Bakker and Shippers (1987). Bacterial cultures (24 h) were streaked on LB medium supplemented with 4.4 gl^{-1} of glycine. The plates were covered with sterile filter paper saturated with 0.5% picric acid in 2% sodium carbonate (Na_2CO_3), after which they were sealed with parafilm and incubated at 37^0C for 4 days. Bacterial isolates that produced an orange color on the filter paper were scored positive for HCN production. The experiment was conducted twice.

3.2.7 ANTIFUNGAL ACTIVITY

Antifungal activity of all bacterial isolates was evaluated on plant pathogen, *Fusarium solani* ATCC 36031 (Davies Diagnostic, South Africa) using plate diffusion method on potato dextrose agar (PDA) as described by Mehnaz and Lazarovits (2006). The antifungal activity of each bacterial isolate was evaluated by measuring the diameter (mm) of fungal growth towards and away from the bacterial colony. Percentage growth inhibition was calculated using the following formula:

$$\%\ \text{inhibition} = \frac{(R-r)}{R} \text{X } 100$$

Where, r = growth of pathogen in control (mm) and, R= colony growth of pathogen in dual culture (mm).

GREENHOUSE EXPERIMENTS

Greenhouse pot culture experiments were conducted to evaluate the effect of bacterial inoculation on the growth and removal of heavy metals (Cd, Ni and Cr) by the hyperaccumulator *B. juncea*. The experimental plant was selected based on its ability to accumulate substantial amounts of metals in its shoots and rapid growth rate (Quartacci *et al.*, 2006).

3.2.8 PREPARATION OF BACTERIAL INOCULUM

Bacterial inocula were prepared by growing bacterial strains, *Pseudomonas aeruginosa* KP717554 (BCr3), *Alcaligenes feacalis* KP717561 (BCd33) and *Bacillus subtilis* KP717559 (BNi11) in separate 250 ml Erlenmeyer flasks containing 100 ml of sterilized LB broth amended separately with 1 mM of Cd, Cr or Ni. Flasks were placed in a shaking incubator at 120 rpm at 37^0C for 48 h. Bacterial cells were harvested by centrifugation at 10000 rpm for 15 min and washed twice with sterile distilled water. Cell pellets were re-suspended in phosphate buffer (0.01M, pH 7.0) and then adjusted to an absorbance of 1.5 at 600 nm (approximately = 5.7×10^7 cfu ml^{-1}) using a spectrophotometer (Thermo Spectronic, Merck, SA) (Kumar *et al.*, 2009).

3.2.9 PREPARATION OF POT EXPERIMENTS

Prior to sowing, a mixture of vermiculite, sphagnum peat moss and perlite (3:4:1) was oven dried at 70^0C for 72 h, passed through a 2 mm sieve, then sterilized by autoclaving at 121^0C for 15 min. After sterilization, 400 g of the medium was transferred into plastic pots (24 cm diameter) and spiked separately with 100 mg kg^{-1} $NiCl_2$, 100 mg kg^{-1} of $CdCl_2$, 150 mg kg^{-1} of $K_2Cr_2O_7$. Another set of pots were spiked with a combination of all three metals. Pots were placed in a greenhouse for two weeks (for metal stabilization) until inoculation.

3.2.10 EFFECTS OF BACTERIA ON THE MOBILITY OF SOIL METALS

Bacterial strains were grown in cotton plugged Erlenmeyer flasks (50 ml) containing 30 ml of LB broth. Inoculated flasks were placed inside a shaking incubator at 37^0C and 200 rpm. After 24 h, bacterial cells were harvested from cultures by centrifugation (6000 rpm for 15 min), washed twice with phosphate buffer (pH 7.0) and then resuspended in 5 ml sterile water. The optical density of each suspension was adjusted to 1.5 at 600 nm using an UV spectrophotometer (Thermo Spectronic, Merck SA). Bacterial cells (1 ml aliquots) were added to 1 g of metal (Cr, Cd, and Ni) contaminated soil in 50 ml Falcon tubes and placed on an orbital shaker at 200 rpm at room temperature. Control soils (axenic) received 1 ml of sterile water. After 7 days, 10 ml of sterile water were added to each tube to extract the water soluble heavy metals (Ni, Cd and Cr). Soil particles were removed from each tube by centrifugation at 7000 rpm for 10 min and the resulting solution was filtered. The concentrations of Ni, Cd and Cr in the filtrate were determined using an inductively coupled plasma mass spectrometer (Perkin Elmer Nexion 300 Q) (Rajkumar *et al.*, 2008).

3.2.11 SEED GERMINATION TEST IN PETRI DISH

Seed germination tests were carried out to investigate the effect of bacterial inoculation on seed germination in the presence of metal contaminants using a modified procedure of Huang *et al.*, (2013). For this, four clean glass Petri dishes were prepared by placing two filter papers on the bottom of each Petri dish followed by 10 ml bacterial suspensions or sterile tap water (control). Bacterial suspensions and water were supplemented or not with 0.1 mM of Cr, Ni or Cd. Before testing, *B. juncea* seeds were sterilized by immersion in 70% ethanol for 30 min and then washed three times with sterile distilled water. The seeds were then soaked in a 2% sodium hypochlorite ($NaClO_2$) solution for 10 min and then rinsed thoroughly with distilled water to remove any remains of disinfectant solution (Madhaiyan *et al.*, 2007). Sterile seeds were inoculated by immersion in 10 ml of bacterial suspension for 2 h on a rotary shaker (150 rpm) then placed in each Petri dish (25 seeds per plate) and incubated at room temperature. Each treatment was replicated three times. After 7 days, the number of germinated seeds in each Petri dish was counted. Five seedlings were randomly taken from each plate to determine growth parameters (shoot length, root length and dry weight) of seedlings. The germination percentage and vigor index were determined by the following formulas (Karnataka 2009; Ghorbanpour Hatami 2014).

$$\text{Germination rate}(\%) = \frac{n}{\mathrm{N}} \text{x } 100$$

Where *n* is the number of germinated seeds after 7 days; N is the total of number seeds

Vigor index = % germination x total length of seedling (shoot length+root length)

3.2.12 PLANT GROWTH EXPERIMENT

B. juncea seeds were sterilized by immersion in 70% ethanol for 30 min and then washed three times with sterile distilled water. The seeds were then soaked in a 2% $NaClO_2$ solution for 10 mins and then washed thoroughly with distilled water. Sterilized seeds of *B. juncea* were inoculated by immersion in bacterial cultures or sterile water (control) for 4 h, and then allowed to germinate on wet filter paper at room temperature before planting. Four seeds (inoculated and non-inoculated) were sown at a depth of 5 cm into each plastic pot containing 400 g of soil. Twelve days after germination, the pots were thinned by leaving 1 seedling per pot. Bacterial suspensions (50 ml per pot) were added to the soil near root zone during transplantation and then applied twice at 10 day intervals. Following planting, pots were placed in a glasshouse (North-West University, Mafikeng) and watered twice daily with either 50 ml of water (control) or a solution of 0.1 mM $NiCl_2/K_2Cr_2O_7/CdCl_2$. The Experiment general consisted of four treatments with three replicates:

1. *B. juncea* + non-contaminated soil (control)
2. *B. juncea* + metal contaminated soil
3. *B. juncea* + non-contaminated soil + bacteria
4. *B. juncea* + metal contaminated soil + bacteria

Thus, the total number of pots was 48. After 45 days, all plants were carefully removed from their respective pots and washed with distilled water to remove all soil material. The height and fresh weight of each plant were measured and then plants were separated into roots and shoots. Plant tissues (roots, stems and leaves) were placed in individual foil packages and dried at 70^0C for 72 h. Oven dried plant tissues were ground using a mortar and pestle before recording the dry weight of each plant using a digital balance (Radwag PS 750/C2). Plant samples were stored in polyethylene bags for further analysis.

3.2.13 ANALYSIS OF HEAVY METALS (Cr, Ni and Cr) IN PLANTS

For heavy metal analysis, 0.2 g of dried plant sample (root and shoot) from each sample was accurately weighed and placed inside a 100 ml Erlenmeyer flask containing 9 ml of HCL, 3 ml conc HNO_3 (69%) and 1 ml of H_2O_2. The mixture was heated in a microwave reaction system (Anton Paar microwave 3000) at 160^0C for 15 min. After digestion, the mixture was allowed to cool and then diluted to 10 ml with distilled de-ionized water. The resulting solution was filtered twice through filter paper after which the various concentrations of heavy metals (Ni, Cd and Cr) in the samples were measured using a Perkin Elmer (Nexion 300 Q) ICP-MS.

3.2.14 DETERMINATION OF BIOCONCENTRATION FACTOR (BCF) AND TRANSLOCATION FACTOR (TF)

The TF or total amount of heavy metal absorbed by the upper parts of the plants from soil was calculated using the formula:

$$\mathrm{TF} = \frac{\text{Metal in shoot}}{\text{Metal in root}}$$

BCF represents the ratio of metal concentration in the roots to that in soil (Yoon et al., 2006). It is usually expressed as:

$$\mathrm{BCF} = \frac{\text{Metal in root}}{\text{Metal in soil}}$$

3.3 DATA ANALYSIS

The experimental data obtained in this study were analyzed separately according to Li and Ramakrishna (2011). The data obtained for each treatment was presented as arithmetic mean with standard error. One-way analysis of variance (ANOVA) was used to determine the significant differences among the means based on Fisher's least significant difference (LSD) procedure. Statistical significance was defined at the level of $p<0.05$. All statistical analyses were performed by using SPSS 22.

3.4 RESULTS AND DISCUSSION

3.4.1 HEAVY METAL TOLERANCE AND PLANT–GROWTH PROMOTING CHARACTERISTICS OF BACTERIAL ISOLATES

Despite the toxic effects of heavy metals on microbes, bacteria possess adaptive mechanisms that enable them to survive in environments containing heavy metals. Such metal tolerant bacteria can be isolated and selected for their potential to promote plant growth and heavy metal accumulation by plants (Chen *et al.*, 2008; Dell'Amico *et al.*, 2008; Kumar *et al.*, 2008; Sheng *et al.*, 2008; Ma *et al.*, 2009; Rajkumar *et al.*, 2009). In this investigation, three bacterial isolates (BCr3, BNi11 and BCd33) were selected from mine tailings with an objective to assess their effects on growth and heavy metal accumulation on *B. juncea.* These strains were identified as *Pseudomonas aeruginosa* KP717554, *Bacillus subtilis* KP717559, and *Bacillus cereus* KP717555 respectively. *Pseudomonas aeruginosa* KP717554 showed an extremely high level of tolerance (15 mM) to Cr whereas; the strains *Bacillus subtilis* KP717559 and *Alcaligenes feacalis* KP717561 were able to tolerate up to 10 mM and 7.5 mM concentrations of Ni and Cd respectively. The order of the toxicity of the metals to all bacterial strains tested was found to be Cd > Ni > Cr (Table 1).

All bacterial isolates exhibited multiple plant growth promoting properties. All three strains were positive for catalase activity, inorganic phosphate solubilization, NH_3 and IAA production. Inorganic phosphate solubilization has been reported in a variety of bacteria sp including *Bacillus*, and *Pseudomonas*. It has been repeatedly stressed that phosphate solubilization is a key plant growth-promoting mechanism (Richardson, 2001). The various mechanisms through which bacteria help to increase the supply of phosphorus to plants have been described (Chen *et al.*, 2006; Rashid *et al.*, 2004). These include; production of organic acids/chelating substances which reduce the pH in the rhizosphere, and secretion of phosphatases to free phosphorous bound in organic matter. Chabot *et al.* (1993) demonstrated growth enhancement of maize and lettuce by a number of microorganisms capable of mineral phosphate solubilization. Besides supplying plants with essential nutrients, a number of bacteria have been reported to possess the ability to produce plant hormones, mainly indole-3-acetic acid that stimulate and facilitate plant growth (Patten and Glick, 2002). Bacterial synthesis of IAA has been reported in a number of genera including *Azospirillum, Burkholderia*, *Enterobacteria,* and

Pseudomonas (Patten and Glick, 2002; Kuklinski-Sobral *et al.*, 2004). Antoun *et al.* (1998) reported that 58% of 266 *Rhizobium* and *Bradyrhizobium* isolates produced IAA. Patten and Glick (2002) demonstrated the benefits of IAA synthesis using wild-type and mutant strains of *Pseudomonas putida* as PGPB on canola plants. The wild-type strain produced an average of 35-50% longer roots compared to plants inoculated with an IAA-deficient mutant.

Table 1: Plant growth promoting and antagonistic properties of metal tolerant *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559.

Bacterial isolates	NH_3 production	Catalase activity	Phosphorus solubilization	IAA production	HCN production	MTC (mM)		
						Ni	Cr	Cd
Alcaligenes feacalis KP717561	+	+	+	+	-	1	2.5	7.5
Bacillus subtilis KP717559	+	+	+	+	+	10	5	2.5
Pseudomonas aeruginosa KP717554	+	+	+	+	+	2	15	0.5

Bio-control of phytopathogens is another area which has gained much attention in recent years. Certain species of bacteria have been known to increase plant growth indirectly by suppressing the growth of pathogens (Dobbelaere *et al.*, 2003). Pathogen suppression may be as a result of competitive exclusion, secretion of volatile compounds such as NH_3, including other anti-fungal enzymes and production of HCN (Brimecombe *et al.*, 2001). Friedlander *et al.* (1993) identified strains of *Pseudomonas cepacia* that possessed the enzyme $\beta - 1,3$ glucanse, which has the ability to break-down fungal cell wall components. In this study, all bacterial isolates and fungal strains grew rapidly on PDA. The spectrum of antifungal activity of the three bacterial strains is presented in Figure 1. *Pseudomonas aeruginosa* KP717554 exhibited significant growth inhibitory activity against *F.solani,* followed by *Bacillus subtilis* KP717559. Production of HCN was detected in two isolates i.e. *Bacillus subtilis* KP717559 and *Pseudomonas aeruginosa* KP717554. The results of this study indicate that these isolates have potential to be used as biological control agents.

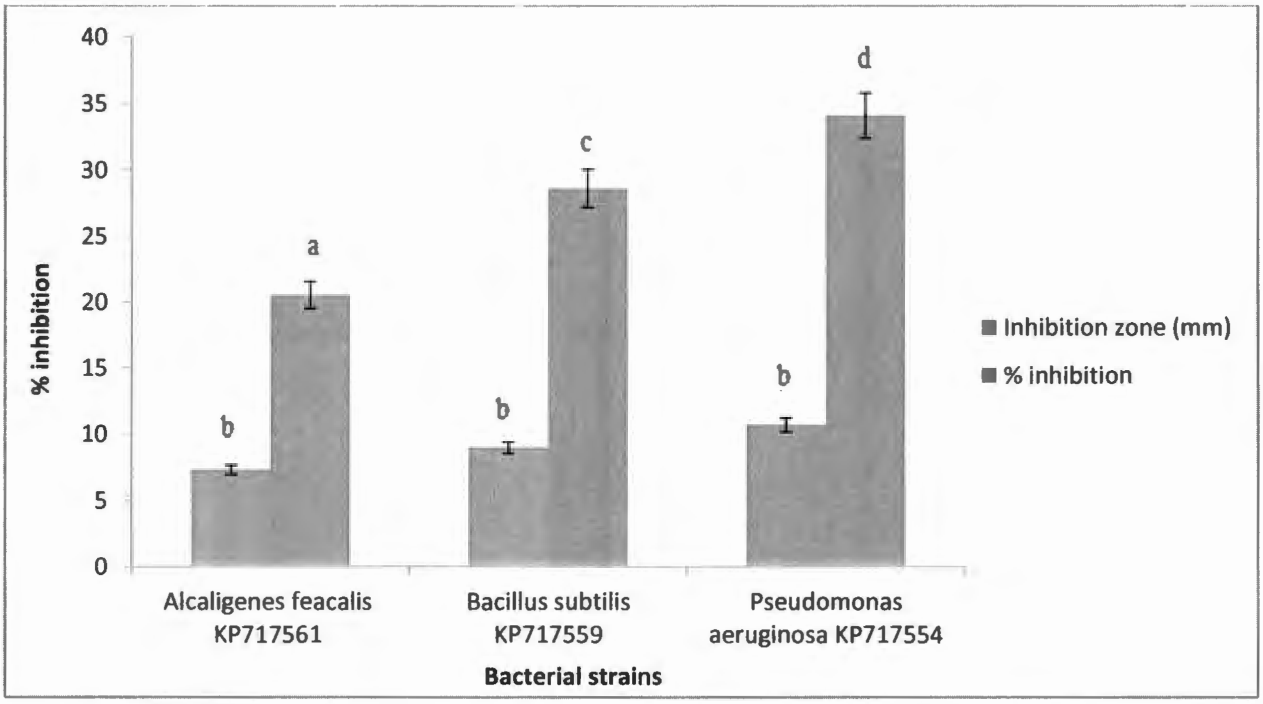

Figure 1: Inhibitory effect of *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 against mycelial growth of *F. solani.* Different letters indicate significant differences between means at $p<0.05$ according to Fisher's protected LSD test.

3.4.2 SEED GERMINATION TEST

Germination parameters were investigated to gather information on seed quality, plant tolerance to heavy metals and the ability of bacterial isolates to promote plant growth. Seed inoculation with bacterial strains significantly increased plant height, root length, seed germination and vigor index (Table 2). Maximum germination percentage (92%) was observed in seeds treated with a mixed culture of bacterial strains (*Pseudomonas aeruginosa*, *Alcaligenes feacalis* and *Bacillus subtilis*). The lowest germination percentage (44%) was recorded in un-inoculated seeds grown in Petri dishes containing all three metals (Cr, Ni and Cd).

The Vigour index reflects the health of the seedlings produced including the ability of seeds to withstand a variety of different stress factors. In the present study the highest seedling vigour index (1472) was reported in seeds treated with a mixed culture containing all three bacterial strains. The lowest value (324) was recorded in un-inoculated seeds grown in Petri dishes containing a mixture of metals. A high value of vigor index indicates better seedling health. The results presented in Table 2 indicate that the inoculation of metal tolerant, plant growth promoting strains significantly enhanced seed germination and seedling vigor in *B. juncea*. For instance, inoculation of *B. juncea* seeds with *Pseudomonas aeruginosa* KP717554 increased the root length, shoot length, seed germination and vigor index by 50%, 47.6%, 23.5% and 83.7% respectively; with *Bacillus subtilis* KP717559, root length (35.7%), shoot length (26.5%), seed germination (17.6%) vigour index (54.8%); with *Alcaligenes feacalis* KP717561, root length (31%), shoot length (64.7%), seed germination (11.8%), and vigor index (63.2%) respectively. Qualitative analysis for IAA production showed that all three bacterial strains (BCr3, BCd33 and BNi11) were able to use L-tryptophan as a precursor for growth and IAA biosynthesis. It is well known that bacterial IAA can help to break seed dormancy by initiating the emergence of roots as well as shoot development by stimulating cell division and expansion of the plant cells (Spaepen *et al*., 2007). Similar enhancement of seed germination parameters by inoculation with *Bacillus sp*. RM-2, and *bacilli* formulations (LS256 and LS257) has been observed in food crops such as cowpea (Minaxi, *et al*., 2012) and pearl millet (Niranjan *et al*., 2003). This effect to the production of different metabolites like IAA, GA, cytokinins and enzymes such as alpha-amylase. Bharathi *et al.* (2004) suggested that an increase in seedling vigour could be due to better synthesis of auxins.

Table 2: Effects of *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 on root length, shoot length, vigour index and germination of *B. juncea* seedlings

Treatments	**Shoot length (cm)**	**Root length (cm)**	**Vigour index**	**Germination (%)**
Control (H_2O)	3.4 ± 2.2^{b}	4.2 ± 1.3^{d}	516.8 ± 21.2^{bc}	68
Pseudomonas aeruginosa KP717554	5.1 ± 1.2^{a}	6.2 ± 2.4^{ab}	949.2 ± 27.5^{c}	84
$K_2Cr_2O_7$ (0.1 mM)	2.3 ± 1.6^{c}	3.2 ± 1.5^{b}	330.0 ± 32.1^{a}	60
Bacillus subtilis KP717559	4.3 ± 1.4^{d}	5.7 ± 1.2^{a}	800.0 ± 22.5^{d}	80
$NiCl_2$ (0.1 mM)	2.8 ± 2.3^{c}	2.6 ± 1.4^{c}	345.6 ± 22.7^{a}	64
Alcaligenes feacalis KP717561	5.6 ± 1.2^{a}	5.5 ± 1.3^{a}	843.6 ± 41.9^{b}	76
$CdCl_2$ (0.1 mM)	2.5 ± 1.1^{c}	3.1 ± 1.8^{b}	313.6 ± 23.9^{e}	56
T1	7.8 ± 2.6^{e}	8.2 ± 2.3^{e}	1472.0 ± 32.5^{ab}	92
T2	1.4 ± 1.8^{ab}	2.4 ± 1.6^{c}	167.2 ± 20.7^{f}	44

*Values represent Means ± Standard error from three replicates. Control refers to plants without heavy metal additions and bacterial inoculations. T1= Three bacterial strains applied together, and treated with water with no metal induction. T2 = brassica seeds grown in 0.1mM of $K_2Cr_2O_7$+ $CdCl_2$+ $NiCl_2$ without bacterial inoculum. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

3.4.3 EFFECTS OF METAL TOLERANT *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 ON PLANT GROWTH

Optimal plant growth is an important factor that influences the successful application of phytoextraction (Yu and Zhou 2009). In this study, pot culture experiments were used to assess the effectiveness of mixed bacterial cultures and single inoculant preparations on the growth of *B. juncea.* Generally, brassica plants inoculated with bacterial isolates exhibited a significant increase in shoot length, root length, plant fresh and dry weight in the presence and absence of metals, compared to the un-inoculated soil.

A mixed culture of three bacterial strains performed better than plant inoculation with individual strains. In the absence of heavy metals, co-inoculation of *B. juncea* with bacterial strains, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, increased root length, shoot length, fresh and dry weight of plants by 82.8%, 88.5%, 139.5% and 69.5% respectively (Table 4).

It is conceivable that all three bacterial strains interacted synergistically with each other, to supply their host plants with key nutrients, remove inhibitory substances and stimulate each other through physical and biochemical activities. For individual inoculations, maximum plant growth-promoting effect was observed in isolate *Pseudomonas aeruginosa* KP717554, which increased root length, shoot length, fresh and dry weight of *B. juncea* plants by 40.5%, 60.1%, 116.2% and 58.2% respectively (Table 3a), followed by *Bacillus subtilis* KP717559 which increased root length, shoot length, fresh and dry weight of plant by 40.5%, 68.2%, 97.3% and 43.2% respectively (Table 3b). Similarly, *Alcaligenes feacalis* KP717561, enhanced the root length, shoot length, fresh and dry weight by 31%, 46%, 66.6% and 32.6% respectively (Table 4). A similar observation was reported by several authors (Dell'Amico *et al*., 2008; Kumar *et al*., 2008; Ma *et al*., 2009), who stated that the increase in plant growth as a result of bacterial inoculation was due to the solubilization of minerals such as phosphorus, synthesis of plant growth substances such as IAA that promote plant root elongation and shoot growth resulting in improved mineral and nutrient uptake (Gadagi *et al.,* 2004; Lambrecht *et al*., 2000; Zhuang *et al*., 2007). Another important trait that may indirectly influence root and plant growth is the production of ammonia (Hardoim *et al*., 2008; Marques *et al*., 2010). Ammonia production by bacteria has also been reported to play an important role in biocontrol or suppression of phytopathogens (Brimecombe *et al.*, 2001; Minaxi, *et al.*, 2012). HCN is another secondary metabolite produced by bacteria that has the potential to protect plants against fungal pathogens (Voisard *et al.*, 1989).

In the presence of heavy metals, non-inoculated plants exposed to different heavy metals showed a significant decrease in growth, especially when plants were grown in soil containing a mixture of all three metals. Growth of plant roots, shoots, dry and fresh weights were reduced by 32.8%, 29.7%, 34.7%, and 38.7% respectively (Table 4). The percentage decrease in 100 mg kg^{-1} of $CdCl_2$ was 32.8% (roots), 29.7% (shoots), 34.7% (dry weight) and 38.7% (fresh weight) (Table 3c); for 150 mg kg^{-1} of $K_2Cr_2O_7$, 23.3% (roots), 44.6% (shoots), 39.3% (dry weight) and 37.6% (fresh weight) (Table 3a); and for 100 mg kg^{-1} $NiCl_2$, 38% (roots), 35.8% (shoots), 28.4% (dry weight) and 46.8% (fresh weight) (Table 3b). The toxic effects of heavy metals in plants are well-documented (Van Assche and Clijsters 1990). Cd, Ni and Cr are elements of unknown biological function in plants. They are known to cause chlorosis, decrease in root growth affect nutrient uptake, CO_2 fixation, electron transport and biosynthesis of chlorophyll including plant death (Padmaja *et al.*, 1990; Sandalio *et al.*, 2001; Sen and Bhattacharyya 2004). High amounts of heavy metals in soil can also interfere with the uptake of nutrients such as iron and phosphorus leading to chlorosis, stunting and even plant death (Halstead *et al.*, 1969; Zayad *et al.*, 2003).

In the presence of heavy metals, co-inoculation of *B. juncea* plants with *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, significantly enhanced root length, shoot length, fresh and dry weight by 40.4%, 35%, 30.7%, and 16% respectively (Table 4). Plants grown in 100 mg kg^{-1} of $CdCl_2$ and then inoculated with *Alcaligenes feacalis* KP717561, showed increase in root length (47.4%), shoot length (37.5%), fresh and dry weight (36.2 and 42.5%) respectively (Table 3c). Similarly, *Pseudomonas aeruginosa* KP717554 enhanced root length (25.8%), shoot length (40.2%), fresh and dry weight (34%, and 30.6%) respectively, of plants grown in 150 mg kg of $K_2Cr_2O_7$ (Table 3a). Likewise, in soils containing 100 mg kg^{-1} $NiCl_2$, *Bacillus subtilis* KP717559 increased root length (38%), shoot length (35.8%), fresh and dry weight (46.8% and 28.4%) respectively (Table 3b). These observations clearly indicated that the inoculation of *B. juncea* with bacterial strains, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, protected plants against growth inhibition caused Cr, Ni and Cd. Presumably, the bacteria strains were able to reduce the phytotoxic effects of the metals though bioaccumulation and biotransformation involving redox reactions. All bacterial strains were able to produce catalase. Singh *et al.* (2013) stated that bacterial strains showing catalase activity are highly tolerant to environmental, mechanical, and chemical stress. Burd *et al.*, (2000) mentioned that *Kluyvera ascorbata* SUD 165/26 protected plants against inhibitory effects of high levels of Ni, Pb and Zn. Rajkumar *et al.* (2006) found that *Pseudomonas* Psa4 and *Bacillus sp.* Ba32 protected *B. juncea* against negative effects of chromium. Our results also suggest that the plant growth promoting activity of all bacterial strains was affected by the presence of heavy metals. Several studies have demonstrated that the environmental stress caused by heavy metals may influence soil bacterial populations by reducing their growth and metabolic activity (Pérez-de-Mora *et al.*, 2006; Tsai *et al.*, 2005; Wang *et al.*, 2010).

Table 3a: Effects of *Pseudomonas aeruginosa* KP717554 inoculation on the growth of *B. juncea*

Treatments	**Root lengths (cm)**	**Shoot lengths (cm)**	**Dry weight (mg plant^{-1})**	**Fresh weight (mg plant^{-1})**
Control (H_2O)	11.6 ± 0.3^{b}	14.8 ± 0.2^{b}	28.5 ± 1.9^{d}	437.3 ± 1.4^{b}
Pseudomonas aeruginosa KP717554	16.8 ± 0.2^{a}	23.7 ± 1.4^{a}	45.1 ± 1.5^{b}	945.6 ± 2.1^{a}
$K_2Cr_2O_7$ (150 mg kg^{-1})	8.9 ± 0.2^{c}	8.2 ± 2.1^{c}	17.3 ± 2.3^{a}	279.9 ± 1.8^{d}
Pseudomonas aeruginosa KP717554 + $K_2Cr_2O_7$	11.2 ± 0.6^{b}	11.5 ± 2.2^{d}	22.6 ± 1.4^{b}	385.1 ± 1.6^{c}

*All the values are mean of three replicates ± SE. Control refers to plants without heavy metal additions and bacterial inoculations. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 3b: Effects of *Bacillus subtilis* KP717559 inoculation on the growth of *B. juncea*

Treatments	**Root lengths (cm)**	**Shoot lengths (cm)**	**Dry weight (mg plant^{-1})**	**Fresh weight (mg plant^{-1})**
Control (H_2O)	11.6±0.3^b	14.8±0.2^b	28.5±1.9^b	437.3±0.4^b
Bacillus subtilis KP717559	16.3±0.4^a	22.4±0.6^a	40.8 ±1.3^a	862.8±1.8^a
$NiCl_2$ (100 mg kg^{-1})	7.2±0.1^c	9.5±0.5^d	20.4±2.8^b	232.6±1.7^d
Bacillus subtilis KP717559 + $NiCl_2$	10.4±0.7^b	13.2±0.3^c	24.6±1.4^b	352.7±2.2^c

*All the values are mean of three replicates ± SE. Control refers to plants without heavy metal additions and bacterial inoculations. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 3c: Effects of *Alcaligenes feacalis* KP717561 inoculation on the growth of *B. juncea*

Treatments	**Root lengths (cm)**	**Shoot lengths (cm)**	**Dry weight (mg plant^{-1})**	**Fresh weight (mg plant^{-1})**
Control (H_2O)	11.6 ± 0.5^b	14.8± 0.8^b	28.5 ±1.0^a	437.4± 1.1^c
Alcaligenes feacalis KP717561	15.2 ± 0.6^a	21.6± 0.5^a	37.8 ± 1.1^c	728.4± 1.5^a
$CdCl_2$ (100 mg kg^{-1})	7.8 ± 0.1^c	10.4 ± 0.3^c	18.6 ± 0.4^b	268.1± 1.6^b
Alcaligenes feacalis KP717561 + $CdCl_2$	11.5 ± 0.2^b	14.3 ± 0.2^b	26.5 ± 0.9^a	365.2± 2.2^b

*All the values are mean of three replicates ± SE. Control refers to plants without heavy metal additions and bacterial inoculations Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

Table 4: Effect of mixed bacterial culture inoculation on the growth of *B. juncea*

Treatments	**Root length (cm)**	**Shoot length (cm)**	**Dry weight (mg plant^{-1})**	**Fresh weight (mg plant^{-1})**
Control (H_2O)	11.6±0.3^b	14.8± 0.2	28.5±1.9^b	437.3±1.4^b
T1	21.2±0.5^a	27.9±0.8^a	48.3±2.7^a	1047.3±2.3^a
T2	5.2±0.8^d	5.4±0.4^d	13.1±2.3^d	146.4±2.8^d
T1+T2	7.3±0.6^c	7.1±0.3^c	15.2±1.9^b	191.3±2.5^c

*All the values are mean of three replicates ± SE. Control refers to plants without heavy metal additions and bacterial inoculations. T1= *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 applied together, and treated with water with no metal induction. T2 = brassica plants grown in $K_2Cr_2O_7$+ $CdCl_2$+ $NiCl_2$ without bacterial inoculum. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

3.4.4 EFFECTS OF BACTERIA ON THE MOBILITY OF SOIL METALS

In addition to low plant biomass, the low availability of heavy metals in soils is another limiting factor of phytoextraction (Li *et al.*, 2007). The bioavailability of a metal determines its toxicity and their extraction by plant. It has been defined as the fraction of metal in soil or water, available to the utilizing organism (Dong *et al.*, 2007). Bacteria are well known for their involvement in the biogeochemical cycling of toxic heavy metals using mechanisms affecting transformations which enhance the availability of metals for plant uptake (Sheng *et al.*, 2008; He *et al.*, 2013). In this study, the concentrations of water soluble Ni, Cd and Cr in soil were examined to assess the efficiency of bacterial strains in enhancing metal solubilization from the soil. Compared with control treatment, soil inoculation with bacterial strains, *Pseudomonas* *aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, significantly increased the amount of soluble heavy metals in soil by 51%, 50% and 44% respectively (Figure 2). The increase in concentration of water-soluble metals caused by *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 may be attributed to the production of organic acids such as indole-3-acetic acid, phosphate solubilization and oxidation–reduction reactions (Abou-Shanab *et al.*, 2006; Jiang *et al.*, 2008; Rajkumar *et al.*, 2008; Zaidi *et al.*, 2006).

Metal availability in soils containing no bacterial inoculum (control) appeared to be low as shown by the water soluble contents and in the order: Ni>Cr>Cd. These results indicate that all bacterial strains used for this study have the potential of metal tolerance and insoluble metal solubilization.

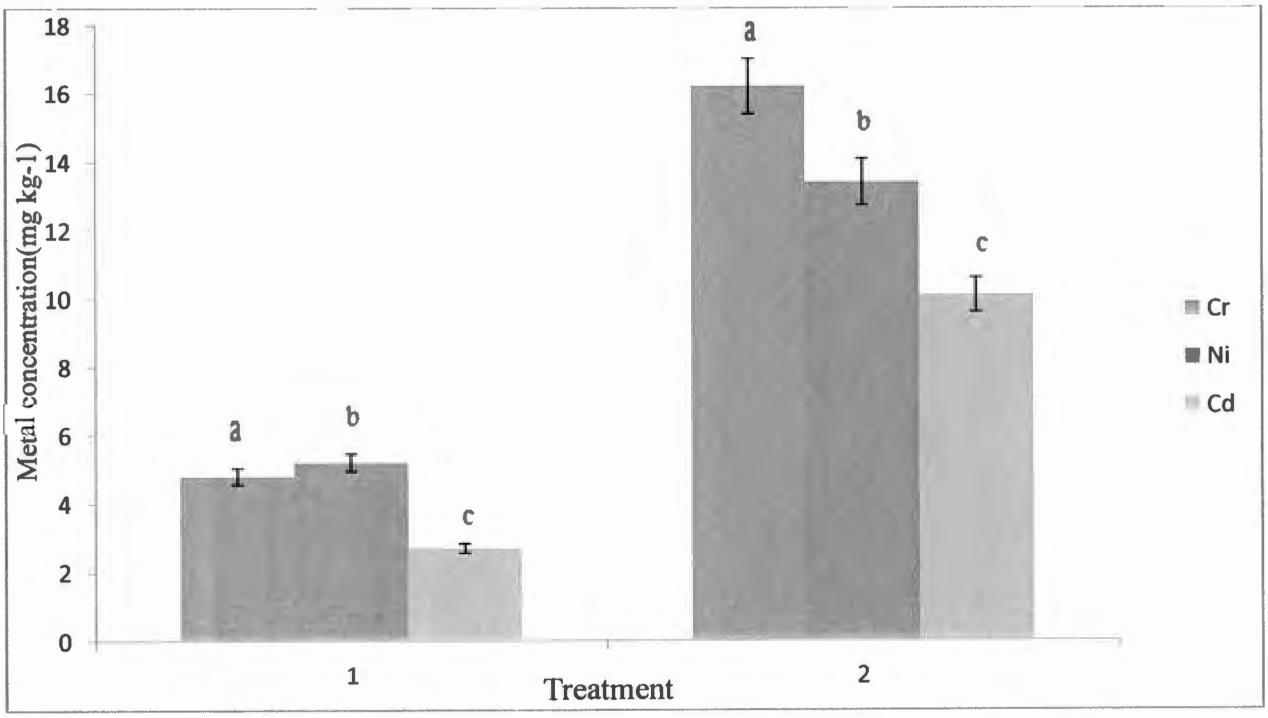

Figure 2: Effect of *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 on the concentration of water-soluble metals (100 mg kg^{-1} $NiCl_2$, 100 mg kg^{-1} $CdCl_2$, and 150 mg kg^{-1} of $K_2Cr_2O_7$) in soil. Treatment 1= Control (metal contaminated soils with no bacteria inoculation); Treatment 2 = metal contaminated soils + bacterial

inoculation. Each value is the mean of triplicates with SE. Data of columns indexed by the same letter are significantly different according to Fisher's protected LSD test ($p < 0.05$).

3.4.5 HEAVY METAL ACCUMULATION IN *Brassica juncea* TISSUES

Since the process of metal accumulation by plants depends on various internal and external factors (Gupta and Sinha 2006), we assessed whether inoculation with metal tolerant bacteria affected the accumulation of heavy metals (Ni, Cr and Cd) by *B. juncea.* The increase in plant biomass and metal availability induced by inoculation with metal tolerant strains, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, resulted in a corresponding increase in heavy metal accumulation in the root and shoot tissues of *B. juncea,* compared with non-inoculated controls (Table 5). For instance, strain *Pseudomonas aeruginosa* KP717554 increased the concentration of Cr in the root and shoot tissues of *B. juncea* by 56% and 73%, respectively. *Bacillus subtilis* KP717559 increased the Ni concentration in the root and shoot tissues by 55.9 % and 32% respectively, while *Alcaligenes feacalis* KP717561 increased the Cd concentration in the root and shoot tissues by 73% and 14% respectively. Halstead *et al.* (1969) suggested that the process of inorganic phosphate solubilization facilitates the uptake of the metals from soil by plants. Kumar *et al.* (2009) reported that *Enterobacter aerogenes* NBRI K24 and *Rahnella aquatilis* NBRI K3 increased plant growth and heavy metal (Ni and Cr) accumulation. In our study, BNi11 increased the Ni concentration in the root and shoot tissues by 55.9 % and 32%, respectively. Similar observations were also reported by Rajkumar and Freitas (2008) who observed that inoculation with metal tolerant *Pseudomonas jessenii* PjM15 increased Ni, Cu and Zn uptake by *Ricinus communis* compared with non-inoculated controls. Abou-Shanab *et al.* (2006) demonstrated that inoculation of *Alyssum murale* with *Microbacterium oxydans* AY509223, significantly increased Ni accumulation of the plant by 36.1%, 39.3%, and 27.7% in soils containing low, medium, and high levels of Ni respectively. Sinha *et al.* (2008) observed that inoculation of *B. juncea* with metal tolerant bacterium, *Pseudomonas aeruginosa* KUCd1 significantly increased plant growth and Cd uptake by 36.89%. Likewise Dell'Amico *et al.* (2008) found that the inoculation of *Brassica napus* with Cd tolerant rhizobacteria, *Pseudomonas fluorescens* ACC9 and *Pseudomonas tolaasii* ACC23, enhanced plant growth and total Cd uptake of plants by 72% and 107% respectively. In contrast to this observation, Madhaiyan *et al.* (2007) reported that inoculation with PGPB strains *Methylobacterium oryzae* CBMB20 and *Burkholderia* sp.

CBMB40 enhanced plant growth but decreased Ni and Cd uptake in tomato plants. Soil microbes and organic matter can immobilize heavy metals such as Cd; however root exudates including organic acids can enhance the mobility of heavy metals by forming soluble complexes (Janouskova *et al.*, 2006). Production of H^+ by plant roots can influence rhizosphere pH affecting the solubility and hence bioavailability of metals in soil (Hinsinger, 1998).

In the absence of bacterial inoculant, the quantity of metal accumulated by plants was significantly less. It is possible that low available heavy metals in soils limited the amount of heavy metals accumulated by plants. Heavy metals such as Cd can adversely affect the activity of proton pump and hence production of H^+ by roots (Tu *et al.*, 1989). Proton pump supplies energy for the uptake and movement of nutrients across the plasma membrane. Accordingly, as heavy metals alter the rhizosphere pH their solubility and hence their bioavailability is decreased with reducing soil pH.

Furthermore, inoculation with a mixed culture of all three bacterial strains significantly enhanced metal accumulation by *B. juncea*, compared to the single strain inoculants (Table 6). The mobility of metals from the soil into the root tissues of *B. juncea* plants and the capability to transfer the metals from roots to shoots were evaluated by means of the BCF and TF respectively. The results (Tables 5 and 6) showed that *B. juncea* contained high amounts of heavy metals in their roots as compared to shoots, as revealed by the TF of each metal that was less than one. This poor translocation of metals from root to shoot system can be attributed to the strong metal binding proteins in the roots which may be a natural toxicity response of the plant (Burd *et al.*, 2000; Garbisu and Alkorta 2001; Shanker *et al.*, 2004). The results also confirmed that *B. juncea* is an accumulator as revealed by the BCF values that were all greater than 1. Furthermore, the BCF and TF of each metal in plants were increased significantly as a result of bacterial inoculation. This indicated that plant inoculation with suitable metal tolerant-plant growth promoting bacteria can be used to enhance the efficiency of phytoextraction.

Table 5: Cd, Ni and Cr concentrations in *B. juncea* tissues after 45 days of sowing

Treatment	**Root** (mg kg^{-1} DW)	**Shoot** (mg kg^{-1} DW)	**BCF**	**TF**
$CdCl_2$	369.3± 8.4^{a}	118.6±20.4^{e}	3.7±0.01	0.32 ± 0.01
Alcaligenes feacalis KP717561+$CdCl_2$	689.5± 11.6^{b}	135.4±16.9^{c}	6.9±0.02	0.20 ±0.04
$NiCl_2$	289.2± 6.7^{c}	146.5±31.1^{b}	2.9 ± 0.09	0.16 ±0.12
Bacillus subtilis KP717559 +$NiCl_2$	451.1± 41.2^{d}	227.2±10.9^{d}	4.5±0.02	0.19 ±0.02
$K_2Cr_2O_7$	272.6±24.9^{e}	155.7±6.2^{f}	2.7±0.3	0.57 ±0.31
Pseudomonas aeruginosa KP717554 + $K_2Cr_2O_7$	556.3±26.5^{f}	339.2±5.4^{a}	3.7 ±0.02	0.61±0.02

*All the values are mean of three replicates ± SE. BCF= Bioconcentration factor, TF=Translocation factor. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$). DW = dry weight.

Table 6: Effect of mixed bacterial cultures on uptake of Cd, Cr and Ni into the shoots and roots of *B. juncea* in soils containing a mixture of heavy metals ($CdCl_2$+$NiCl_2$+$K_2Cr_2O_7$)

Treatment	**Metal ions (mg kg^{-1})**		
	Ni^{2+}	**Cr^{6+}**	**Cd^{2+}**
$CdCl_2$+$NiCl_2$+$K_2Cr_2O_7$ (control)			
Root	318.6±0.7^{b}	294.7±0.6^{b}	382.5±0.3^{b}
Shoot	172.5±0.2^{d}	192.3±0.4^{d}	161.9±0.8^{d}
BCF	3.2±1.3	2.0±2.4	3.8±1.5
TF	0.2±1.6	0.7±1.8	0.4±2.8
Mixed bacterial culture			
Root	508.4±1.9^{a}	624.3±2.1^{a}	419.1±2.5^{c}
Shoot	311.2±1.3^{c}	426.7±1.4^{c}	283.4±2.7^{a}
BCF	5.1±2.6	4.2±2.3	4.2±1.7
TF	0.3±1.5	0.7±1.6	0.5±2.1

*All the values are mean of three replicates ± SE. Data of columns indexed by the same letter are not significantly different according to Fisher's protected LSD test ($p < 0.05$).

REFERENCES

ABOU-SHANAB RAI, ANGLE JS & CHANEY RL 2006. Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils *Soil Biology and Biochemistry,* 38, 2882–2889.

ANTOUN H, BEAUCHAMP CJ, GOUSSARD N, CHABOT R & LALANDE R 1998. Potential of *Rhizobium* and *Bradyrhizobium* species as plant growth promoting rhizobacteria on non-legumes: effects of radishes (*Raphanus sativus L.*). *Plant Soil*, 204, 57-67.

BAKKER AW & SHIPPERS B 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas spp.-mediated plant growth-stimulation. *Soil Biology and Biochemistry*, 19, 451–57.

BHARATHI R, VIVEKANANTHAN R, HARISH S, RAMANATHAN A, & SAMIYAPPAN R. 2004. Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies. *Crop Protection*, 23, 835-843.

BRICK JM, BOSTOCK RM & SILVERSTONE SE 1991. Rapid *in situ assay* for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. *Applied and Environmental Microbiology*, 57, 535-538.

BRIMECOMBE MJ, DE LELJ FA & LYNCH JM 2001. The Rhizosphere. The effect of root exudates on rhizosphere microbial populations. In: Pinton R, Varanini Z, Nannipieri P, eds. The rhizosphere. Biochemistry and organic substances at the soil-plant interface. *Marcel Dekker, New York.* p. 95-140.

BURD GI, DIXON DG & GLICK BR 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal of Microbiology,* 46, 237–245.

CERVENTES C, GARCIA JC, DEVERS S, CORONA FG, TAVERA HL, GUZMAN JCT & SANCHEZ RM 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiology Reviews,* 25, 335-347.

CHABOT R, ANTOUN H & CESCAS MP 1993. Stimulation of growth of maize and lettuce by inorganic phosphorus solubilizing microorganisms. *Canadian Journal of Microbiology,* 39, 941-947.

CHACKO S, PRAMOD WR & SUCHIT AJ 2009. Amidase from plant growth promoting rhizobacterium. *Journal of Bacteriology Research*, 1, 46-50.

CHEN CZ, MA S & LIU L 2008. Studies on phosphorus solubilizing activity of a strain of phosphobacteria isolated from chestnut type soil in China. *Bioresource Technology*, 99, 6702-6707.

CHEN YP, REKHA PD, ARUN AB, SHEN FT, LAI WA &YOUNG CC 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. *Applied Soil Ecology*, 34, 33-41.

DELL'AMICO, CAVALCA L & ANDREONI V 2008. Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biology and Biochemistry*, 40, 74–84.

DOBBELAERE S, VANDERLEYDEN J & OKON Y 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *CRC Critical Reviews in Plant Science*, 22, 107-149.

DONG J, MAO W, ZHANG G, WU F, & CAI Y 2007. Root excretion and plant tolerance to cadmium toxicity. *Plant Soil and Environment*, 53, 193–200.

GADAGI RS, KRISHNARAJ PU, KULKARNI JH & SA T 2004. The effect of combined *Azospirillum* inoculation and nitrogen fertilizer on plant growth promotion and yield response of the blanket flower *Gaillardia pulchella*. *Scientific Horticulture,* 100, 323–332.

GARBISU C & ALKORTA I 2001. Phytoextraction: a cost-effective plant based technology for the removal of metals from the environment. *Bioresource Technology*, 77, 229-236.

GHORBANPOUR M & HATAMI M 2014. Biopriming of *Salvia officinalis* Seed with Growth Promoting Rhizobacteria Affects Invigoration and Germination Indices. *Journal Of Biological and Environmental Sciences*, 8, 29–36.

GUPTA AK & SINHA S 2006. Chemical fractionation and heavy metals accumulation in the plants of *Sesamum indicum (L.)* var. T55 grown on soil amended with tannery sludge: selection of single extractants. *Chemosphere,* 64, 161–173.

HALSTEAD RL, FINN BJ & MACLEAN AJ 1969. Extractability of nickel added to soils and its concentration in plants. *Canadian Journal of Soil Science,* 49, 335–342.

HARDOIM PR, VAN OVERBEEK LS & VAN ELSAS JD 2008. Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology,* 16, 467-471.

HE H, YE Z, YANG D ,YAN J, XIAO L & ZHONGB T 2013. Characterization of endophytic *Rahnella* sp.JN6 from *Polygonumpubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. *Chemosphere,* 90, 1960-1965.

HENRIQUES IDS & LOVE NG 2007. The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. *Water Research,* 41, 4177-4185.

HINSINGER P 1998. How do plant roots acquire mineral nutrients? Chemical processes involved in the rhizosphere. *Advances in Agronomy*, 64, 225-65.

HUANG G, TIAN H, LIU H, FAN X, LIANG YU & LI Y 2013. Characterization of Plant-Growth-Promoting Effects and Concurrent Promotion of Heavy Metal Accumulation in the Tissues of the Plants Grown in Polluted Soil by *Burkholderia* Strain LD-11. *International Journal of Phytoremediation,* 15, 991–1009.

HUSEN E 2003. Screening of soil bacteria for plant growth promotion activities in vitro. *Indian Journal of Agricultural Sciences,* 4, 27–31.

JANOUSKOVA M, PAVLIKOVA D & VOSATKA M 2006. Potential contribution of arbuscular mycorrhiza to cadmium immobilisation in soil. *Chemosphere*, 65, 1959-1965.

JIANG CY, SHENG XF, QIAN M & WANG QY 2008. Isolation and characterization of a heavy metal-resistant *Burkholderia sp.* From heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil *Chemosphere,* 72, 157–164.

KARNATAKA 2009. Seed germinability, root and shoot length and vigour index of soybean as influenced by rhizosphere fungi. *Journal of Agricultural Sciences,* 22, 1120–1122.

KHAN AG, KUEK C, CHAUDHRY TM, KHOO CS & HAYES WJ 2000. Role of plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. *Chemosphere,* 41, 197–207.

KUKLINSKI-SOBRAL J, ARAÚJO W, MENDES R, GERALDI I, PIZZIRANI KLEINER A & AZEVEDO J 2004. Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology*, 6, 1244-1251.

KUMAR KV, SINGH N, BEHL HM & SRIVASTAVA S 2008. Influence of plant growth promoting bacteria and its mutant on heavy metal toxicity in *Brassica juncea* grown in fly ash amended soil. *Chemosphere,* 72, 678–683.

KUMAR KV, SRIVASTAVA S, SINGH N & BEHL HM. 2009. Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of *Brassica juncea*. *Journal of Hazardous Materials*, 170, 51-57.

LAMBRECHT M, OKON Y, VANDE BROEK A & VANDERLEYDEN J 2000. Indole-3-acetic acid: a reciprocal signaling molecule in bacteria-plant interactions. *Trends in Microbiology*, 8, 298-300.

LASAT MM 2002. Phytoextraction of toxic metals: a review of biological mechanisms. *Journal of Environmental Quality*, 31, 109–120.

LI MS, LUO YP & SU ZY 2007. Heavy metal concentrations in soils and plant accumulation in a restored manganese mineland in Guangxi, South China. *Environmental Pollution, 147*, 168–175.

MA Y, RAJKUMAR M & FREITAS H 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans strain* Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *Journal of Environmental Management*, 90, 831–837.

MADHAIYAN, M, POONGUZHALI, S & SA T 2007. Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum L.*). *Chemosphere*, 69, 220–228.

MARQUES AP, PIRES C, MOREIRA H, RANGEL AO & CASTRO PM 2010. Assessment of the plant growth promotion abilities of six bacterial isolates using *Zea mays* as indicator plant. *Soil Biology and Biochemistry*, 42, 1229–1235.

MEHNAZ S & LAZAROVITS G 2006. Inocuation effect of *Pseudomonas putida*, *Gluconacetobacter diazotrophicus* and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. *Microbiology Ecology*, 51, 326–335.

MINAXI, NAIN L, YADAV RC & SAXENA J 2012. Characterization of multifaceted *Bacillus sp*. RM-2 for its use as plant growth promoting bioinoculant for crops grown in semi-arid deserts. *Applied soil ecology*, 59, 124–135.

MULLIGAN CN, YONG RN & GIBBS BF 2001. Remediation technologies for metal contaminated soils and groundwater: an evaluation. *Engineering Geology 60*, 193–207.

NIRANJAN SR, DEEPAK SA, BASAVARAJU P, SHETTY HS, REDDYAND MS, & KLOEPPER JW 2003. Comparative performance of formulations of plant growth promoting rhizobacteria in growth promotion and suppression of downy mildew in pearl millet. *Crop Protection*, 22, 579–588.

PADMAJA K, PRASAD DDK & PRASAD ARK 1990. Inhibition of chlorophyll synthesis in *Phaseolus vulgaris* seedlings by cadmium acetate. *Photosynthetica,* 24, 399–405.

PATTEN CL & GLICK BR 2002. The role of bacterial indole acetic acid in the development of the host plant root system. *Applied and Environmental Microbiology,* 68, 3795-3801.

PÉREZ-DE-MORA A, BURGOS P, MADEJÓN E, CABRERA F, JAECKEL P & 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.

PIKOVSKAYA RI 1948. Mobilization of phosphorus in soil connection with the vital activity of some microbial species. *Microbiologiya*, 17, 362–370.

QUARTACCI MF, ARGILLA A, BAKER AJM & NAVARI-IZZO F 2006. Phytoextraction of metals from a multiply contaminated soil by *Indian mustard. Chemosphere,* 63, 918-925.

RAJKUMAR M & FREITAS H 2008. Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. *Chemosphere,* 71, 834–842.

RAJKUMAR M, NAGENDRAN R, LEE KJ, LEE WH & KIM SZ 2006. Influence of plant growth promoting bacteria and Cr6+ on the growth of Indian mustard. *Chemosphere* 62,741-748.

RAJKUMAR M, PRASAD MNV & FREITAS H 2009. Biotechnological applications of serpentine soil bacteria for phytoremediation of trace metals. *Critical Reviews in Biotechnology,* 29, 120-130.

RASHID M, KHALIL S, AYUB N, ALAM S, LATIF F 2004. Oragnic acid production and phosphate solubilization by phosphate solubilizing microorganisms (PSMs) under conditions. Pakistan Journal of Biological Sciences, 7, 187–196.

RICHARDSON AE 2001. Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants. *Australian Journal of Plant Physiology*, 28, 897-90.

SANDALIO LM, DALURZO HC, GOMEZ M, ROMERO-PUERTAS M & DEL RIO LA 2001. Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany*, 52, 2115-2126.

SEN AK & BHATTACHARYYA M 2004. Studies of uptake and toxic effects of Ni (II) on *Salvinia natans*. *Water Air and Soil Pollution*, 78, 141–152.

SHANKER AK, DJANAGUIRAMAN M, SUDHAGAR R, JAYARAM R, & PATHMANABHAN G 2004. Expression of metallothionein 3 (MT3) like protein mRNA in Sorghum cultivars under chromium (VI) stress. *Current Science*, 86, 901- 902.

SHENG XF & XIA JJ 2006. Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. *Chemosphere*, 64, 1036-1042.

SHENG XF, XIA JJ, JIANG CY, HE LY & QIAN M 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. *Environmental Pollution,* 156, 1164–1170.

SINGH Y, RAMTEKE PW & SHUKLA PK 2013. Isolation and characterization of heavy metal Resistant *Pseudomonas spp.* and their plant growth promoting activities. *Advances in Applied Science Research*, 4, 269–272.

SPAEPEN S, VANDERLEYDEN J & REMANS R 2007 Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiology Reviews,* 31, 425–448.

SUSARLA S, MEDINA VF & MCCUTCHEON SC 2002. Phytoremediation: an ecological solution to organic chemical contamination. *Ecological Engineering,* 18, 647–658.

TSAI YP, YOU SJ, PAI TY & CHEN KW 2005. Effect of cadmium on composition and diversity of bacterial communities in activated sludges. *International Biodeterioration and Biodegredation,* 55, 285–291.

TU SI, NUNGESSER E, & BRAUER D 1989. Characterization of the effects of divalent cations on the coupled activities of the H^+ ATPase in tonoplast vesicles. *Plant Physiology*, 10, 1636–43.

VAN ASSCHE F & CLIJSTERS H 1990. Effect of metals on enzyme activity in plants. *Plant Cell and Environment,* 13, 195–206.

VOISARD C, KEEL C, HAAS D & DEFAGO G 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. *EMBO Journal,* 8, 351–358.

WANG F, YAO J, SI Y, CHEN H, RUSSEL M, CHEN K, QIAN Y, ZARAY G & BRAMANTI E 2010. Short-time effect of heavy metals upon microbial community activity. *Journal of Hazardous Materials*, 173, 510-516.

WANG F, YAO J, SI Y, CHEN H, RUSSEL M, CHEN K, QIAN Y, ZARAY G &BRAMANTI E 2010. Short-time effect of heavy metals upon microbial community activity. *Journal of Hazardous Materials*, 173, 510–516.

YANG XO, FENG Y, HE ZL & STOFFELLA PJ 2005. Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation. *Journal of Trace Elements in Medicine and Biology*, 18, 339–353.

YOON J, CAO X, ZHOU Q & MA QL 2006. Accumulation of Pb, Cu and Zn in native plants growing on a contaminated Florida site. *Science of the Total Environment* 368, 456-464.

YU Z & ZHOU Q 2009. Growth responses and cadmium accumulation of *Mirabilis jalapa L.* under interaction between cadmium and phosphorus. *Journal of Hazardous Materials,* 167, 38–43.

ZAIDI S, USMANI S, SINGH BR & MUSARRAT J. 2006. Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. *Chemosphere,* 64, 991–997.

ZHUANG XL, CHEN J, SHIM H & BAI Z 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. *Environment International*, 33, 406–413.

CHAPTER 4: GENERAL CONCLUSION AND FUTURE RESEARCH TRENDS

Mining activities have led to soil contamination with hazardous substances such as heavy metals, which pose danger to environmental and human health. The soil is an important habitat that supports the growth of numerous types of bacteria that have a great potential for agricultural use and environmental preservation. Heavy metals can generally exert their toxic effects on soil bacterial communities by inhibiting their growth and metabolic activities (Bruins *et al.*, 2000). However, long term exposure to heavy metals may lead to the selection of metal tolerant bacterial communities which have intrinsic biochemical, physiological and/or genetically acquired mechanisms that enable them to survive and flourish in metal contaminated environments (Abou-Shanab *et al.*, 2007; DellAmico *et al.*, 2005). This warranted us to characterize and identify metal tolerant bacteria from mine tailings with the aim of determining their potential for environmental cleanup activities.

The results obtained from this study provided evidence that a large number of metal tolerant bacteria are present in soils contaminated with heavy metals. The total plate count of the bacterial colonies grown on LB plates with no metal induction ranged from 14.8×10^4 to 3.5×10^5. The percentage of bacterial colonies (CFU/g soil) tolerant to Cd^{2+} fluctuated from 28.41% to 77.14%. Approximately 38.14% to 65.36% of all bacterial colonies where tolerant to Cr^{6+}, while 33.71% to 61.44% exhibited tolerance to Ni^{2+}.

All isolates showed multiple tolerances against heavy metals but most promising results were shown by bacterial isolates BCr3, BCd33 and BNi11 that were tolerant to 15 mM of Cr^{6+}, 7.5 mM of Cd^{2+} and 10 mM of Ni^{2+} respectively. The effect of heavy metals on bacterial growth was tested together with their ability to grow at different concentrations of NaCl, pH, and temperature values. Bacterial isolates grew well between pH 7.5 and 8.5. The optimum temperature for maximum growth was between $35–37^0$C and no significant change in bacterial growth was observed in the presence of 2% NaCl. In addition, the bioaccumulation potential of bacterial isolates was investigated. Bacterial isolates BCr3, BCd33 and BNi11 showed high bioaccumulation ability of Cr (68.7%), Cd (72.4%) and Ni (69.8%) respectively

According to Malik *et al.* (2008) only 1% of soil microorganisms are culturable and since only culture-dependent techniques were used, it is possible that higher numbers of different bacteria would be found using culture-independent techniques such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and *fluorescence in situ hybridization* (FISH). These methods could also be used in future investigations to quantify the expression of genes responsible for heavy metal tolerance in bacteria. The products encoded by heavy metal tolerant genes could be used to engineer new metal tolerant bacteria, construct biosensors or used as sources for probes.

All bacterial isolates displayed multiple tolerances to high levels of Cd, Ni and Cr. BCr3, BCd33 and BNi11 that were tolerant to 15 mM of Cr^{6+}, 7.5 mM of Cd^{2+} and 10 mM of Ni^{2+} respectively. However, further investigations are needed to determine their tolerance patterns in the presence of different or multiple combinations of heavy metals. Elucidation of the precise tolerance mechanisms needs further investigation.

Most tolerant bacterial isolates were able to grow at different pH, temperature and NaCl concentrations. Bacterial isolates grew well between pH 7.5 and 8.5. The optimum temperature for maximum growth was between 35–37^{0}C and no significant change in bacterial growth was observed in the presence of 2% NaCl. Bacterial strains (specifically BCr3, BCd33 and BNi11) also had the ability to remove large amounts of Cr, Ni and Cd from an aqueous medium, highlighting their potential for bioremediation of Cr, Ni and Cd-contaminated soil and water. Nonetheless, their rates of metal uptake should be determined in detail and the kinetics modeled. Molecular techniques such energy dispersive X-ray analysis (EDX), fourier transform infrared (FTIR) spectroscopy, scanning and transmission electron microscopy (SEM and TEM) should be used to further characterize the bacterial isolates. The effects of other parameters pH, temperature, combination of bacterial strains, and combination of metals should also be investigated through a series of experiments. Furthermore, bioaccumulation of metals using mixed bacterial cultures may prove pivotal in acquiring more information on the subject.

The screening of bacterial isolates for extra-chromosomal DNA revealed the presence of plasmids but they were not classified. Further characterization of the isolated plasmids is necessary to establish a connection between plasmid presence and heavy metal resistance tolerance in bacteria. Further characterization may also provide new genetic tools for future analysis of heavy metal tolerance mechanisms in bacteria.

Amplification of metal tolerant genes (*CzcD, ChrA, ChrB, CzcB, CzcC, NccA* and *CadA*) was attempted on two template fractions (genomic DNA and plasmid DNA) from all bacterial strains. However, this proved challenging since very few amplification products were consistently produced from DNA fractions in all bacterial strains tested. In addition, plasmid DNA fractions failed to produce amplification products for all genetic systems encoding resistance. To further investigate the presence of metal tolerant genes in bacteria, we may need to design alternative primers for the various genes to be used in PCR analysis and attempt amplification of these genes on both large and small-plasmid-isolation preparation from all bacterial strains.

Most metal tolerant bacterial isolates displayed multiple PGP traits. Three Bacterial strains (*Pseudomonas aeruginosa* KP717554, *Bacillus subtilis* KP717559, and *Bacillus cereus* KP717555) were positive for catalase activity, inorganic phosphate solubilization, NH_3 and IAA production. The effects of these bacterial strains on growth and heavy metal accumulation in *B. juncea* were examined in a greenhouse experiment. Bacterial inoculation improved seeds germination of *B. juncea* in the presence of 0.1 mM Cr, Cd and Ni, as compared to the control treatment. Maximum germination percentage was observed in seeds treated with a mixed culture of bacterial strains (*Pseudomonas aeruginosa*, *Alcaligenes feacalis* and *Bacillus subtilis*). The lowest germination percentage was recorded in un-inoculated seeds grown in Petri dishes containing all three metals (Cr, Ni and Cd). The bioavailability of a metal determines its toxicity and their extraction by plant. Bacteria are well known for their involvement in the biogeochemical cycling of toxic heavy metals using mechanisms affecting transformations which enhance the availability of metals for plant uptake thus; the ability of bacterial isolates to enhance metal solubilization was also analyzed. Compared with control treatment, soil inoculation with bacterial isolates, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 significantly increased the amount of soluble heavy metals in soil. A pot experiment was set-up to study the effects of bacterial inoculation on the growth and heavy metal accumulation by *B. juncea* grown in soil spiked with 100 mg kg^{-1} of $NiCl_2$, 100 mg kg^{-1} of $CdCl_2$, and 150 mg kg^{-1} of $K_2Cr_2O_7$. A mixed culture of three bacterial strains performed better than plant inoculation with individual strains. In the presence and absence the of heavy metals, co-inoculation of *B. juncea* with bacterial strains, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 significantly enhanced root length, shoot length, fresh and dry weight of plants. For individual inoculations, maximum plant growth-promoting effect was observed in isolate *Pseudomonas aeruginosa* KP717554, followed by *Bacillus subtilis* KP717559. In the presence of heavy metals, non-inoculated plants exposed to different heavy metals showed a significant decrease in growth, especially when plants were grown in soil containing a mixture of all three metals. Similarly, inoculation of *B. juncea* with *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559, resulted in a corresponding increase in heavy metal accumulation in the root and shoot tissues of the plant, compared with non-inoculated controls. Inoculation with a mixed culture of all three bacterial strains was more effective than single strain inoculants.

Although phytoextraction is an emerging cost-effective solution for the remediation of heavy metal-contaminated soils, slow growth and low bioavailability of heavy metals in soil are factors that greatly decrease the success of this green technology. In this study, we demonstrated that inoculation with metal tolerant bacterial strains, *Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561 and *Bacillus subtilis* KP717559 not only protects plants against growth inhibition caused by heavy metals (Cr, Cd, and Ni), but also increases plant growth, bioavailability of metals in the soil with a concurrent increase in metal accumulation by the host plant. In addition, all bacterial strains enhanced the translocation of heavy metals from roots to shoots. However, further investigations to understand molecular mechanisms of plant-bacteria interactions for plant growth, metal solubilization, uptake and translocation are necessary for efficient phytoextraction of heavy metals. Our long term objectives are to:

1. Test the inoculation effects of metal tolerant bacterial strains (BCr3, BCd33 and BNi11) under natural environmental conditions in different agro-climatic zones of the world
2. Study their effects or interactions with different hyperaccumulators
3. Investigate bacterial induced changes in the rhizosphere of hyperaccumulator plants in relation to mobilization and metal accumulation by plant tissues.

These are some of the challenges which require a thorough understanding by research scientists in order to make phytoextraction a highly reproducible and reliable process.

REFERENCES

ABOU-SHANAB RAI, VAN BERKUM P & ANGLE JS 2007. Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of *Alyssum murale*. *Chemosphere,* 68, 360–367.

DELL'AMICO E, CAVALCA L & ANDREONI V 2005. Analysis of rhizobacterial communities in perennial Graminaceae from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. *FEMS Microbiology Ecology*, 52, 153-162

MALIK S, BEER M, MEGHARAJ M & NAIDU R 2008. The use of molecular techniques to characterize the microbial communities in contaminated soil and water. *Environment International*, 34, 265-276.