Bioremediation of heavy metals polluted soil of active gold mines using bacteria biopolymers

AS AYANGBENRO

orcid.org/ 0000-0002-3220-1873

Thesis accepted in fulfilment of the requirements for the degree *Doctor of Philosophy in Biology* at the North-West University

Promoter: Prof OO Babalola

Graduation: April 2019

Student number: 28072693

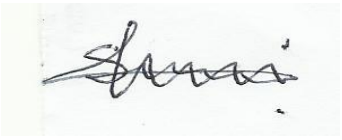
DECLARATION

I, the undersigned, declare that this thesis submitted to the North-West University for the degree of Doctor of Philosophy in Biology in the Faculty of Natural and Agricultural Sciences, School of Biological Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other University in part or entirety for the award of any degree.

STUDENT NAME

Ayansina Segun AYANGBENRO

SIGNATURE:



DATE: 20th February, 2019.

SUPERVISOR'S NAME

Professor Olubukola Oluranti BABALOLA

DEDICATION

This work is dedicated to the giver of life, who has directed my path this far and to all who have contributed to the success of this work.

ACKNOWLEDGMENTS

I have learnt many things through this journey and every little achievement has been through the help of people surrounding me. I will not pass on this opportunity without expressing my gratitude to people and organizations who have helped me thus far.

I would like to thank my supervisor, Prof. O.O. Babalola, for her support and constant encouragement during the course of this research work. I am indeed grateful. I equally want to thank all members and staff of the Department of Microbiology and staff of the Faculty of Natural and Agricultural Sciences for their support.

I also want to acknowledge the financial assistance of the National Research Foundation (NRF) of South Africa and The World Academy of Science (TWAS) for NRF-TWAS African Renaissance Doctoral Scholarship (Grant UID 99779). The financial support of North-West University postgraduate bursary is also gratefully appreciated.

I would also like to appreciate people who provided technical support: Mr. K. Mokalane, Mr. S. Loyilani and Dr. O.S. Aremu of the Department of Chemistry for the supply of chemicals and chemical analysis of my samples, Dr. B.R. Aremu for whole genome analysis and Mr. G. van Rensburg of the School of Chemical and Mineral Engineering, Potchefstroom Campus for ICP-OES analysis.

I would like to thank past and current members of Microbial Biotechnology Research Group and friends for all their support and for being who they are. Thank you for keeping me sane, especially during frustrating periods in the laboratory. Dr. M.F. Adegboye, Dr. C.F. Ajilogba, Dr. K.O. Afolabi, Dr. O.B. Ojuederie, Dr. M.O. Fashola, Dr. A.A. Adeniji, Dr. A.O. Yusuf, Dr. A.E. Amoo, Mrs. O.S. Abiodun-Salawu, Mrs. F.A. Chukwuneme, Mr. O.S. Olanrewaju, Mr. Simon Isaiah, Miss H.K. Mongadi and Miss. M. Khantsi. Thank you all for contributing to this success.

My gratitude also goes to my pastors, Revd. (Dr.) and Mrs. E.S. Ojo, Revd. O.I. Adegboyega, Prof. Abiodun Salawu and Prof. and Prof. (Mrs). Akpovire Oduaran. Thank you for your prayers and words of encouragement.

Lastly, to my parents, Mr. and Mrs. Theophilus A. Ayangbenro, thank you for your support and unconditional love. You have given me the best you could afford and I will always strive to make you proud. To my siblings, Ayanniyi, Ayanwale and Ayandele, thank you for giving me this opportunity to succeed. To my wife, Oluwadamilola, your unflinching support and love has been my motivation. You are a treasure indeed.

Thank you all for helping me succeed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

GENERAL ABSTRACT

Mining activities have increased environmental pollution which has consequently resulted in the release of large quantities of heavy metals into the environmental media. These metal species tend to accumulate in the environmental media because they are non-degradable causing several toxic effects on biological systems. Hence, there is a need for appropriate treatment techniques for effective removal of heavy metals from contaminated media. This study was designed to screen for prospective biopolymer producing heavy metal resistant bacterial isolates that can be used for metal removal from polluted media. Soil samples were collected from gold mining sites in Vryburg, South Africa. The soil physicochemical properties and metal concentrations were determined. Each soil sample was digested and the metal concentration determined through inducible conductivity plasma-optical emission spectroscopy. The pH values of the soil samples ranged from 5.9 to 7.5 which is slightly acidic to slightly alkaline. The properties of the soil revealed that the soil samples are sandy in nature and deficient in nutrients for microbial activities. Heavy metal concentrations for the soil samples ranged between 0.03-0.36 mg/kg for Cd, 0.22-0.41 mg/kg for Cr, 0.12-0.64 mg/kg for Cu, 127.9-1213.2 mg/kg for Fe, 0.2-1.9 mg/kg for Pb and 0.07-1.2 mg/kg for Zn. These values fall within the recommended limit for South African soil and sediments, but still have potential impacts on biological systems. Ninety-eight heavy metal (Cd, Cr and Pb) resistant bacterial isolates were isolated from the soil samples using Luria-Bertani agar supplemented with each of the heavy metals. These isolates were screened for biopolymer production and a total of 20 isolates (20.4%), that were positive for biopolymer production were selected for further studies. The metal tolerance of the biopolymer producing isolates were determined and 55% of the isolates grew on 1000 mg/L of Pb. Few isolates tolerated up to 300 mg/L of cadmium and chromium. All isolates were characterized biochemically and molecularly and the results showed that the phylum Firmicutes were the dominant organisms. The isolates were further characterized by 16S rRNA gene sequence analysis and identified as belonging to the genera *Acinetobacter*, *Agrobacterium*, *Bacillus*, *Citrobacter*, *Enterobacter*, *Pantoea* and

Pseudomonas. The isolates were screened for heavy metal resistance genes encoding for Cd (*cadA*, *CzcA*, *CzcB* and *CzcD*), Cr (*chrA* and *chrB*) and Pb (*PbrA* and *PbrT*). Multi- metal resistance was found in most of the isolates, notably, *Bacillus cereus* MH399230, *B. toyonensis* MH399231, *Pseudomonas korensis* MH399240 and *Pantoea* sp. MH399244. *B. cereus* MH399230 was positive for all the screening test for biosurfactant production and was selected for biosurfactant production and metal removal. *B. cereus* MH399230 has emulsification of 22%, 24%, 37% and 54% on hexadecane, vegetable oil, kerosene and engine oil respectively, and was able to reduce the surface tension of 39.5 mN/m. The biosurfactant produced by *B. cereus* MH399230 was identified as a lipopeptide with stability over a wide range of pH and temperature. The biosurfactant produced by *B. cereus* MH399230 was able to remove 78% of Pb, 56% of Cd and 35% of Cr from polluted soil after single washing with biosurfactant. The genome of *B. cereus* MH399230 was mined and revealed the presence of 44 gene clusters involved in antibiotic and secondary metabolite biosynthesis. These included non-ribosomal peptide synthetase (NRPS) gene clusters, lipoprotein, lipopolysaccharides, binding proteins, proteins related to degradation of toxic compounds and biofilm secretion genes. Genes responsible for resistance to toxic heavy metals such as arsenic, cadmium, chromium, cobalt, copper, lead, mercury and zinc were also detected in the genome of *B. cereus* MH399230. *P. korensis* MH399240 and *Pantoea* sp. MH399244 were further screened for bioflocculant production based on the high flocculating activity produced with kaolin clay. Maximum flocculating activity of 71.3% and 51.7% with glucose and yield of 2.98 g/L and 3.26 g/L was obatanied for *Pantoea* sp. and *P. korensis* respectively at optimum pH (7.5) and temperature (30˚C). Characterization of the partially purified bioflocculant using FTIR revealed the presence of carboxyl, hydroxyl and amino groups. These groups are responsible for metal binding. Metal sorption by the partially purified bioflocculants of *Pantoea* sp. removed 51.2%, 52.5%and 80.5% of Cd, Cr and Pb respectively while that of *P. korensis* removed 48.5%, 42.5% and 73.7% of Cd, Cr and Pb respectively from aqueous solution. This study shows the

potentials of these heavy metal resistant isolates for biopolymer production and removal of heavy metals from polluted media.

LIST OF PUBLICATIONS

Chapter Two: New strategy for heavy metal polluted environment: A review of microbial biosorbents. *Published in International Journal of Environmental Research and Public Health*. (2017) 14: 94 DOI: 10.3390/ijerph14010094

Authors: Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate‘s Contributions: managed the literature searches and wrote the first draft of the manuscript.

Chapter Three: Metal(loid) bioremediation: strategies employed by microbial polymers. *Published in Sustainability*. (2018) 10: 3028 DOI: 10.3390/su10093028

Authors: Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate’s Contributions: managed the literature searches and wrote the first draft of the manuscript.

Chapter Four: Identification and characterization of heavy metal resistant bacterial isolates from gold mining soil. *This chapter has been submitted for publication in Applied Microbiology and Biotechnology.*

Authors: Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate’s Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

Chapter Five: Heavy metal removal from contaminated soil by lipopeptide biosurfactant produced by *Bacillus cereus* NWUAB01. *This chapter has been submitted in this format for publication in Environmental Science and Pollution Research.*

Authors: Ayansina Segun Ayangbenro and Olubukola Oluranti Babalola

Candidate's Contributions: designed the study, managed the literature searches, wrote the protocol, carry out the laboratory work, performed all the analyses, interpretation of results and wrote the first draft of the manuscript.

Chapter Six: Draft genome sequence of heavy metal resistant *Bacillus cereus* strain NWUAB01. *Published in Micriobiology Resource Announcement.* (2019)8(7): e01706-18. DOI: 10.1128/mra.01706-18.

Authors: Olubukola Oluranti Babalola, Bukola Rhoda Aremu and Ayansina Segun Ayangbenro

Candidate's Contributions: managed the literature searches, wrote the protocol, performed part of the analyses and wrote the first draft of the manuscript.

Chapter Seven: Bioflocculant production and heavy metal sorption by metal resistant bacterial isolates from mining soil. *This chapter has been submitted in this format for publication in Chemosphere.*

Authors: Ayansina Segun Ayangbenro, Samuel Oluwole Aremu and Olubukola Oluranti Babalola

Candidate's Contributions: managed the literature searches, wrote the protocol, carry out the laboratory work, performed part of the analyses and wrote the first draft of the manuscript.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction to this chapter

Industrial activities have increased environmental pollution challenges and degradation of ecosystems with the buildup of many pollutants, such as toxic metals. Pollution with heavy metals is a consequnece of anthropogenic activities and thus becomes an important environmental and health problem (Siddiquee et al., 2015). Heavy metals are not degradable, unlike organic contaminants, and able to build up in the food web via bioaccumulation (Siddiquee et al., 2015). The toxic properties of metals are attributed to their ability to interfere with critical biological processes.

Heavy metals and metalloids are natural components of the earth's crust and are referred to as metallic elements. They have relatively high density and are toxic even at low concentration (Elekofehinti et al., 2012). They have been defined with a number of criteria which include cationic-hydroxide formation, specific gravity greater than 5 g/ml, complex formation, hard-soft acids and bases, and environmental toxicity (Rajendran et al., 2003). The stable oxidation states of these metals are the most toxic forms which react with body bio-molecules to form extremely stable bio-toxic compounds which are difficult to dissociate (Duruibe et al., 2007).

Natural occurence of heavy metals in the environment has been attributed to pedogenesis, a process of weathering of parent materials (Dixit et al., 2015). Examples of natural sources of metals includes volcanic activity, erosion and weathering of minerals. The anthropogenic sources are as a result of human activities such as biosolids, mining, atmospheric disposition, electroplating, smelting, pesticides and phosphate fertilizers (Fulekar et al., 2009).

Mining has been identified as one of the anthropogenic activities which have a destructive impact on environmental quality. The impacts range from physical destruction with accompanying loss

of biodiversity to accumulation of pollutants in different media of the environment (Getaneh and Alemayehu, 2006). Mining sites are usually contaminated with several kinds of heavy metals that come primarily from ore processing, disposal of tailings and wastewaters around the mines (Grimalt et al., 1999). These metals are then released into the environmental media, especially water, sediment and soil (Eisler, 2003; Getaneh and Alemayehu, 2006). Heavy metals in tailings can be transported to and accumulate in plants and animals, and can then be passed on to human beings in the food web, as a final consumer (Kim et al., 2001; Patel et al., 2005) and may also cause adverse effects on the ecosystem (Kim et al., 1998). Heavy metals associated with mining are of particular interest because they show a tendency to accumulate in sediments and soils and have a long persistence in the environment, as well as being ubiquitous in sediments and soils (Getaneh and Alemayehu, 2006).

The threat heavy metals pose to the health of living organisms is aggravated by their long-term persistence in the environment. The toxicity becomes more severe in acidic medium and nutrient-deficient ecosystems (Mukhopadhyay and Maiti, 2010). Most heavy metals are known to be carcinogenic, mutagenic, cause cardiovascular diseases, disrupt the endocrine system, and cause coordination problems (Wuana and Okieimen, 2011). They can accumulate in plant tissues to a level that affects growth and development, inhibits photosynthesis, interferes with metabolic functions and respiration, degenerates cell organelles and even leads to plant death (Liu et al., 2008).

Heavy metals cannot be degraded to harmless products and hence persist in the environment indefinitely (Rajkumar and Freitas, 2008). As a result, many different remediation methods have been employed to address the increase of heavy metal contaminated sites. Techniques for heavy metal removal from a polluted environment include chemical precipitation and sludge separation, chemical oxidation or reduction, and ion exchange and filtration. These conventional physicochemical methods are costly, non-specific, and become ineffective when metal

concentrations are less than 100 mg/L in such media (Ahluwalia and Goyal, 2007). The toxic sludge released from these methods of remediation can also pose adverse effects on biological activity; hence a biological approach for remediation of heavy metal contamination has been proposed (Congeevaram et al., 2007).

The biological approach for heavy metal removal is an attractive alternative to conventional techiques. The use of microorganisms for remediation purposes is a solution for heavy metal pollution because it is a sustainable technique to rectify and re-establish the natural soil condition (Kapoor and Viraraghavan, 1995). Due to high concentration of heavy metals in mining environments, resident microorganisms have developed resistance mechanisms that falicitate effective detoxification of toxic metals. They have developed strategies for their continued existence in heavy metal-polluted habitats and are known to develop and adopt different detoxifying mechanisms such as biosorption, bioaccumulation, biotransformation and biomineralization, which can be exploited for bioremediation processes (Lin and Lin, 2005). They secrete various kinds of metal-binding metabolites, biopolymers, capsules, extracellular polymeric substances, slimes and sheaths (Fomina and Gadd, 2014).

Microbial polymers are generating increasing attention in recent years due to their biodegradable nature, low toxicity, and diversity, which makes them superior to their chemical counterparts. These polymers provide defensive mechanisms and increase metal bioavailability in the soil (Valls and De Lorenzo, 2002; Vaishnav and Demain, 2011). One of the major impacts of microbial metabolic processes has been the exploitation of pathways for degradation and consequent remediation of pollutants in the environment. In contrast to organic pollutants, heavy metals presents a difficult challenge; metal ions can only be converted to base metal, precipitated, methlylated, volatilized or complexed with an organic ligand. One such approach to heavy metal removal involves the formation of stable complexes between metals and microbial biomass which

is a result of electrostatic interaction between metal ligands and negatively charged cellular biopolymers (Gutnick and Bach, 2000).

1.2 Problem statement

Mining activities generate different kinds of waste which are harmful to the environment. These wastes include mine waste, tailings, dump heap leach, and acid mine water (Yassir et al., 2015). A major source of the metal pollution is metal-rich acid mine drainage (AMD) from waste rock piles dumped in the surrounding soil. Heavy metals generated from mine wastes have been reported to travel several kilometers and impact downstream ecosystems. This is of great concern due to toxic effects on flora and fauna. The fate of metallic species after they enter the ecosystem becomes very difficult to track and they start to inflict damage as they move through from one ecological trophic level to another. Heavy metals such as chromium, cadmium and lead have always been present in the environment, but exploration and exploitation, and anthropogenic activities have increased their concentration in the biosphere (Prabhu et al., 2018). These metals have also been listed among toxic elements within the first twenty pollutants priority list that are of public health significance (Cáliz et al., 2013).

Microbial polymers are the first line of defense against metal toxicity and protect the interior of the cell (Li and Yu, 2014). The use of isolated biopolymers from microorganisms is desired due to their easy availability in heavy metal removal and circumvention of pathogenicity concerns of some producing organisms. Therefore, this research work becomes imperative to ascertain the removal of these toxic metals by biopolymers produced by heavy metal resistant bacterial isolates from polluted mine soil.

1.3 Research aims and objectives

The aims of this research are to isolate and characterize heavy metal resistant bacteria in soil samples from active mines, to screen these bacteria for biopolymer production and to use these microbial polymers for heavy metal removal.

The objectives of the study include:

1. To determine the concentrations of heavy metals in active mine soil samples.
2. To isolate and characterize heavy metal resistant bacteria isolates present in mine soil samples and to determine their phylogenetic relationship.
3. To screen heavy metal resistant bacterial isolates for biopolymer (biosurfactant and bioflocculant) production.
4. To remediate heavy metal (Cd, Cr, Pb) polluted soil with biopolymer produced.

CHAPTER TWO

NEW STRATEGY FOR HEAVY METAL POLLUTED ENVIRONMENT: A REVIEW OF MICROBIAL BIOSORBENTS

Abstract

Persistent heavy metal pollution poses a major threat to all life forms in the environment due to its toxic effects. These metals are very reactive at low concentrations and can accumulate in the food web, causing severe public health concerns. Remediation using conventional physical and chemical methods is uneconomical and generates large volumes of chemical waste. Bioremediation of hazardous metals has received considerable and growing interest over the years. The use of microbial biosorbents is eco-friendly and cost effective; hence, it is an efficient alternative for the remediation of heavy metal contaminated environments. Microbes have various mechanisms of metal sequestration that hold greater metal biosorption capacities. The goal of microbial biosorption is to remove and/or recover metals and metalloids from solutions, using living or dead biomass and their components. This review discusses the sources of toxic heavy metals and describes the groups of microorganisms with biosorbent potential for heavy metal removal.

Keywords: bioremediation; biosorbent; biosorption; heavy metals; microorganisms; remediation.

2.1 Introduction

Industrialization and technological advancement have put an increasing burden on the environment by releasing large quantities of hazardous waste, heavy metals (cadmium, chromium, and lead) and metalloids (elements with intermediate properties between those of typical metals and non-metals, such as arsenic and antimony), and organic contaminants that have inflicted serious damage on the ecosystem. The build-up of heavy metals and metalloids

in soils and waters continues to create serious global health concerns, as these metals and metalloids cannot be degraded into non-toxic forms, but persist in the ecosystem. Contamination of the environment with heavy metals has increased beyond the recommended limit and is detrimental to all life forms (Tak et al., 2013; Gaur et al., 2014; Dixit et al., 2015). The maximum permissible concentration of some heavy metals in water, as stated by the Comprehensive Environmental Response Compensation and Liability Act (CERCLA), USA, is 0.01, 0.05, 0.01, 0.015, 0.002, and 0.05 mg/L for Ar, Cd, Cr, Pb, Hg, and Ag respectively (Chaturvedi et al., 2015). The standard for soil, as established by the Indian standards for heavy metals, is 3–6, 135–270, 75–150, 250–500, and 300–600 mg/kg for Cd, Cu, Ni, Pb, and Zn respectively (Nagajyoti et al., 2010). The standard for soil as stipulated by the National Norms and Standards for Remediation of Contaminated Land and Soil Quality in South Africa is 5.8, 37, 80, 16, 1.0, 740, 91, 20, and 240 for As, Cd, Cr, Cu, Hg, Mn, Ni, Pb and Zn respectively (Olobatoke and Mathuthu, 2016).

Heavy metal pollution is currently a major environmental problem because metal ions persist in the environment due to their non-degradable nature. The toxicity and bioaccumulation tendency of heavy metals in the environment is a serious threat to the health of living organisms. Unlike organic contaminants, heavy metals cannot be broken down by chemical or biological processes. Hence, they can only be transformed into less toxic species.

The majority of heavy metals are toxic at low concentrations and are capable of entering the food chain, where they accumulate and inflict damage to living organisms. All metals have the potential to exhibit harmful effects at higher concentrations and the toxicity of each metal depends on the amount available to organisms, the absorbed dose, the route and the duration of exposure (Mani and Kumar, 2014). Due to the noxious effects of these metals, there are growing environmental and public health concerns, and a consequent need for increased awareness in order to remediate the heavy metal polluted environment. Thus, it is imperative to remove or

reduce heavy metal contamination in order to prevent or reduce contaminating the environment and the possibility of uptake in the food web. To achieve this, bioremediation is employed in order to increase metal stability (speciation), which in turn reduces the bioavailability of metal (Abbas et al., 2014; Akcil et al., 2015; Ndeddy Aka and Babalola, 2016). Speciation is defined as the identification and quantification of the different, defined species, forms, or phases, in which a metal occurs, while bioavailability is the portion of the total amount of a metal in an environment, within a time frame, that is available or made available for uptake by living organisms in their direct surroundings. Speciation of metal and its bioavailability determines the physiological and toxic effects of a metal on living organisms (Olaniran et al., 2013).

Bioremediation is a state-of-the-art technique used for heavy metal removal and/or recovery from polluted environments. The technique utilizes inherent biological mechanisms to eradicate hazardous contaminants using microorganisms and plants, or their products, to restore polluted environments to their original condition (Mani and Kumar, 2014; Akcil et al., 2015; Dixit et al., 2015). It is an environmentally friendly and cost-effective technique for heavy metal removal/recovery, when compared to the conventional chemical and physical techniques, which are often more expensive and ineffective, especially for low metal concentrations. In addition, these conventional methods generate significant amounts of toxic sludge.

Microbial remediation is described as the use of microorganisms to perform the absorption, precipitation, oxidation, and reduction of heavy metals in the soil (Su, 2014). Microorganisms possess astonishing metabolic pathways which utilize various toxic compounds as a source of energy for growth and development, through respiration, fermentation, and cometabolism. Due to their characteristic degradative enzymes for a particular contaminant, they have evolved diverse mechanisms for maintaining homeostasis and resistance to heavy metals, in order to adapt to toxic metals in the ecosystem (Brar et al., 2006; Wei et al., 2014). Strategies developed by microorganisms for continued existence in heavy metal polluted environments, include

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

This article presents insights into the use of microbial biosorbents for removing heavy metals from industrial waste and contaminated environments, as well as the sources and toxicity of these metals in the food web.

2.2 Sources of heavy metal pollution in the environment

Naturally occurring heavy metals are present in forms that are not readily available for uptake by plants. They are typically present in insoluble forms, like in mineral structures, or in precipitated or complex forms that are not readily available for plant uptake. Naturally occurring heavy metals have a great adsorption capacity in soil and are thus not readily available for living organisms. The bonding energy between naturally occurring heavy metals and soil is very high compared to that with anthropogenic sources. Examples of natural processes that bring about the occurrence of heavy metals in the environment are comets, erosion, volcanic eruptions, and the weathering of minerals. Heavy metals from anthropogenic sources typically have a high bioavailability due to their soluble and mobile reactive forms. These anthropogenic sources include alloy production, atmospheric deposition, battery production, biosolids, coating, explosive manufacturing, improper stacking of industrial solid waste, leather tanning, mining, pesticides, phosphate fertilizer, photographic materials, printing pigments, sewage irrigation, smelting, steel and electroplating industries, textiles, and dyes and wood preservation

(Fulekar et al., 2009; Dixit et al., 2015) (Table 2.1). Sources of heavy metals, concentrations in soil, soil properties, the degree and extent of uptake by plants, and the extent of absorption by animals, are the factors that influence the accumulation of metal ions in the food web (Bolan et al., 2014). According to D'amore et al. (2005), the geochemical cycle of heavy metals results in the buildup of heavy metals in the environment, which could cause risk to all life forms when they are above permitted levels. The routes of entry into the environment usually include the weathering of parent materials, the alteration of the geochemical cycle by man, soil ingestion (which is the primary exposure route to humans of soil-borne metals), the transfer from mines to other locations, and the discharge of high concentrations of metal waste by industries.

Mining has negatively impacted the environment, causing destruction and an alteration of the ecosystem, including a loss of biodiversity and an accumulation of pollutants in the environment. Mining and ore processing are major sources of heavy metal pollution in the soil, and the recovery of ecosystems from mining activities could take several decades. These activities produced large quantities of stockpiles and dumps, which are frequently abandoned without treatment. Abandoned mines contaminate water bodies through chemical run-off and particulates that accumulate in water sources (Adler et al., 2007), hence creating a need to treat wastewaters contaminated with heavy metals, before discharge into the environment occurs.

2.3 Toxicity of heavy metals to life forms

Although some heavy metals play important roles in the physiological, biochemical, and metabolic processes of living organisms, functioning as co-factors for some enzymes, micronutrients, regulators of osmotic pressure, and stabilization of molecules, the majority of them have no known biological function in living organisms and are toxic when generated in excess (Fashola et al., 2016). The toxicity of metals is the ability of a metal to cause undesirable effects on organisms. This depends on the heavy metal bioavailability and the absorbed dose (Rasmussen et al., 2000). The threat posed by heavy metals to the health of living organisms is

worsened by their continuously persistent nature in the environment. Toxicity increases when the medium becomes acidic and nutrient-deficient, and when the soil structure is poor, especially in mining environments (Mukhopadhyay and Maiti, 2010).

At acidic pH levels, heavy metals tend to form free ionic species, with more protons available to saturate metal binding sites. This means that at higher hydrogen ion concentrations, the adsorbent surface is further positively charged, thus reducing the attraction between adsorbent and metal cations. Therefore, the heavy metal becomes more bioavailable, thereby increasing its toxicity to microorganisms and plants. At basic conditions, metal ions replace protons to form other species, such as hydroxo-metal complexes. These complexes are soluble in some cases (Cd, Ni, Zn), while those of Cr and Fe are insoluble. The solubility and bioavailability of heavy metals can be influenced by a small change in the pH level. Variations in soil composition, such as the organic matter content of a soil, also affect the toxicity of heavy metals. In soil with relatively low organic matter content, high contamination by heavy metals is usually observed. Organic matter content has a strong influence on the cation exchange capacity, buffer capacity, as well as on the retention of heavy metals. Thus, metals present in organic soils contaminated with a combination of heavy metals are less mobile and less bioavailable to microorganisms and plants, than metals present in mineral soils (Olaniran et al., 2013).

Table 2.1: Toxicity of heavy metals to life forms

Metal	Source	Effects on human	Effects on plants	Effects on microorganisms	Reference
Antimony	Coal combustion, mining, smelting, soil erosion, volcanic eruption	Cancer, cardiovascular diseases, conjunctivitis, dermatitis, liver diseases, nasal ulceration, respiratory diseases	Decreases synthesis of some metabolites, growth inhibition, inhibit chlorophyll synthesis	Inhibit enzyme activities, reduced growth rate	Blais et al. (2008); An and Kim (2009)
Arsenic	Atmospheric deposition, mining, pesticides, rock sedimentation, smelting	Brain damage, cardiovascular and respiratory disorder, conjunctivitis, dermatitis, skin cancer,	Damage cell membrane, inhibition of growth, inhibits roots extension and proliferation, interferes with critical metabolic processes, loss of fertility, yield and fruit production, oxidative stress, physiological disorders	Deactivation of enzymes	Bissen and Frimmel (2003); Abdul-Wahab and Marikar (2012); Finnegan and Chen (2012)
Beryllium	Coal and oil combustion, volcanic dust	Allergic reactions, berylliosis, cancer, heart diseases, lung diseases	Inhibits seed germination	Chromosomal aberration, mutation	Gordon and Bowser (2003); Blais et al. (2008)
Cadmium	Fertilizer, mining, pesticide, plastic, refining, welding	Bone disease, coughing, emphysema, headache, hypertension, itai-itai, kidney diseases, lung and prostate cancer, lymphocytosis, microcytic hypochromic anemia, testicular atrophy, vomiting	Chlorosis, decrease in plant nutrient content, growth inhibition, reduced seed germination	Damage nucleic acid, denature protein, inhibit cell division and transcription, inhibits carbon and nitrogen mineralization	Nagajyoti et al. (2010); Sebogodi and Babalola (2011); Chibuike and Obiora (2014); (Sankarammal et al., 2014); Fashola et al. (2016)
Chromium	Dyeing, electroplating, paints production, steel fabrication, tanning, textile	Bronchopneumonia, chronic bronchitis, diarrhea, emphysema, headache, irritation of the skin, itching of respiratory tract, liver diseases, lung cancer, nausea, renal failure, reproductive toxicity, vomiting	Chlorosis, delayed, senescence, wilting, biochemical lesions, reduced biosynthesis germination, stunted growth, oxidative stress	Elongation of lag phase, growth inhibition, inhibition of oxygen uptake	Cervantes et al. (2001); Barakat (2011); Mohanty et al. (2012)

Copper	Copper polishing, mining, paint, plating, printing operations	Abdominal pain, anemia, diarrhea, headache, liver and kidney damage, metabolic disorders, nausea, vomiting	Chlorosis, oxidative stress, retard growth	Disrupt cellular function, inhibit enzyme activities	Salem et al. (2000); Nagajyoti et al. (2010); Dixit et al. (2015); Fashola et al. (2016)
Mercury	Batteries, coal combustion, geothermal activities, mining, paint industries, paper industry, volcanic eruption, weathering of rocks	Ataxia, attention deficit, blindness, deafness, decrease rate of fertility, dementia, dizziness, dysphasia, gastrointestinal irritation, gingivitis, kidney problem, loss of memory, pulmonary edema, reduced immunity, sclerosis	Affects antioxidative system, affects photosynthesis, enhance lipid peroxidation, induced genotoxic effect, inhibit plant growth, yield, nutrient uptake and homeostasis, oxidative stress	Decrease population size, denature protein, disrupt cell membrane, inhibits enzyme function	Wang et al. (2012); Ali et al. (2013); Fashola et al. (2016)
Lead	Coal combustion, electroplating, manufacturing of batteries, mining, paint, pigments	Anorexia, chronic nephropathy, damage to neurons, high blood pressure, hyperactivity, insomnia, learning deficits, reduced fertility, renal system damage, risk factor for Alzheimer's disease, shortened attention span	Affects photosynthesis and growth, chlorosis, inhibit enzyme activities and seed germination, oxidative stress	Denatures nucleic acid and protein, inhibits enzymes activities and transcription	Nagajyoti et al. (2010); Wuana and Okieimen (2011); Mupa (2013); Fashola et al. (2016)
Nickel	Electroplating, non-ferrous metal, paints, porcelain enameling	Cardiovascular diseases, chest pain, dermatitis, dizziness, dry cough and shortness of breath, headache, kidney diseases, lung and nasal cancer, nausea	Decrease chlorophyll content, inhibit enzyme activities and growth, reduced nutrient uptake	Disrupt cell membrane, inhibit enzyme activities, oxidative stress	Malik (2004); Chibuike and Obiora (2014); Fashola et al. (2016)
Selenium	Coal combustion, mining	Dysfunction of the endocrine system, gastrointestinal disturbances, impairment of natural killer cells activity, liver damage	Alteration of protein properties, reduction of plant biomass	Inhibits growth rate	Germ et al. (2007); Dixit et al. (2015)
Silver	Battery manufacture, mining, photographic processing, smelting	Argyria and argyrosis, bronchitis, cytopathological effects in fibroblast and keratinocytes, emphysema, knotting of cartilage, mental	Affects homeostasis, decrease chlorophyll content, inhibits growth	Cell lysis, inhibit cell transduction and growth	Prabhu and Poulose (2012); Qian et al. (2013)

		fatigue, nose, throat and chest irritation, rheumatism			
Thallium	Cement production, combustion of fossil fuels, metal smelting, oil refining	Alopecia, ataxia, burning feet syndrome, coma, convulsions, delirium, fatigue, gastroenteritis, hair fall, hallucinations, headache, hypotension, insomnia, nausea, tachycardia, vomiting	Inhibits enzyme activities, reduced growth	Damages DNA, inhibits enzyme activities and growth	Babula et al. (2008); Blais et al. (2008)
Zinc	Brass manufacturing, mining, oil refinery, plumbing	Ataxia, depression, gastrointestinal irritation, hematuria, icterus, impotence, kidney and liver failure, lethargy, macular degeneration, metal fume fever, prostate cancer, seizures, vomiting	Affects photosynthesis, inhibits growth rate, reduced chlorophyll content, germination rate and plant biomass	Death, decrease in biomass, inhibits growth	Chibuike and Obiora (2014); Gumpu et al. (2015)

Temperature also plays an important role in the adsorption of heavy metals. It has two major effects on the adsorption process. Increasing the temperature will also increase the rate of adsorbate diffusion across the external boundary layer and in the internal pores of the adsorbate particles, because liquid viscosity decreases as temperature increases. It also affects the equilibrium capacity of the adsorbate, depending on whether the process is exothermic or endothermic. Temperature changes affect the stability of the metal ion species initially placed in solution; stability of the microorganism–metal complex depends on the biosorption sites, microbial cell wall configuration, and ionization of chemical moieties on the cell wall. An increase in the sorption capacity of lead, from 0.596 to 0.728 mg/g, was obtained when the temperature was raised from 25 to 40 °C by olive stone (Arjoon et al., 2013).

Metal toxicity is also shown in their ability to disrupt enzyme structures and functions by binding with thiol and protein groups, or by replacing co-factors in prosthetic groups of enzymes. Exposure to lead and mercury can cause the development of autoimmunity, which can result in joint diseases, such as rheumatoid arthritis, kidney diseases, circulatory and nervous system disorders, and the damaging of the fetal brain in humans. Exposure to lead and mercury in children causes reduced intelligence, impaired development, and an increased risk of cardiovascular disease. Cadmium is known to be carcinogenic and mutagenic, and can disrupt the endocrine system, damage fragile bones and lungs, and affect the regulation of calcium in biological systems. Chromium causes hair loss, headaches, diarrhea, nausea, and vomiting in humans (Table 2.1).

Heavy metal contaminated soils limit plant habitats due to toxicity, resulting in ecological, evolutionary, and nutritional problems, as well as severe selection pressures (Abdul-Wahab and Marikar, 2012; Mani and Kumar, 2014). The toxicity of heavy metals in plants varies, depending on the plant species, specific metal involved, concentration of metal, chemical form of the metal, and soil composition and pH (Nagajyoti et al., 2010). There can be a build-up of

heavy metals in plant tissues that affects or inhibits nutrient uptake, homoeostasis, growth, and development. They disrupt metabolic functions, such as physiological and biochemical processes, biochemical lesions, cell organelles destruction, chlorosis, delayed germination, induced genotoxicity, inhibition of photosynthesis and respiration, loss of enzyme activities, oxidative stress, premature leaf fall, reduced biomass, reduced crop yield, senescence, stunted growth, wilting and can even cause the death of plants (Table 2.1).

Heavy metal toxicity affects microbial population size, diversity, and activity, as well as their genetic structure. It affects the morphology, metabolism, and growth of microorganisms by altering the nucleic acid structure, disrupting the cell membranes, causing functional disturbance, inhibiting enzyme activity and oxidative phosphorylation, and causing lipid peroxidation, osmotic balance alteration, and protein denaturation (Fashola et al., 2016; Xie et al., 2016) (Table 2.1).

2.4 Bioremediation of heavy metals by microorganisms

Several techniques have been used for the removal and/or recovery of heavy metals from polluted environments. Some established conventional procedures for heavy metal removal and/or recovery from solution, include adsorption processes, chemical oxidation or reduction reactions, chemical precipitation, electrochemical techniques, evaporative recovery, ion exchange, reverse osmosis, and sludge filtration (Siddiquee et al., 2015). However, these techniques are expensive, sometimes impracticable, and are not specific for metal-binding properties. Furthermore, the generation of toxic waste, the high reagent requirement, and the unpredictable nature of metal ion removal, highlights some of the disadvantages of these methods. The majority of these methods are ineffective when metal concentrations in solution are less than 100 mg/L (Ahluwalia and Goyal, 2007). Separation by physical and chemical techniques is also challenging due to the high solubility of most heavy metal salts in solution. Thus, there is a need to evaluate alternative techniques for a given procedure and such an

approach should be suitable, appropriate, and applicable to the local conditions, and must be able to meet the established permissible limits.

Bioremediation is an innovative technique for the removal and recovery of heavy metal ions from polluted areas, and involves using living organisms to reduce and/or recover heavy metal pollutants into less hazardous forms, using the activities of algae, bacteria, fungi, or plants. It has been employed for the removal of heavy metals from contaminated wastewaters and soils. This method is an appealing alternative to physical and chemical techniques, and the use of microorganisms plays a significant role in heavy metal remediation. Similarly, the use of microorganisms to remediate polluted environments is sustainable and helps to restore the natural state of the polluted environment with long term environmental benefits and cost effectiveness (Dixit et al., 2015). These organisms help to detoxify hazardous components in the environment. The process can function naturally or can be improved through the addition of electron acceptors, nutrients, or other factors.

Detoxification can occur through the valence transformation mechanism. This is particularly applicable in the case of metals whose different valence states vary in toxicity. In mercury-resistant bacteria, organomercurial lyase converts methyl mercury to Hg(II), which is one hundred-fold less toxic than methyl mercury (Wu et al., 2010a). The reduction of Cr(VI) to Cr(III) is widely studied, with Cr(III) having less mobility and toxicity. Other detoxification mechanisms of heavy metals are accomplished through metal binding, vacuole compartmentalization, and volatilization. Metal binding involves chelators, such as metallothein, glutathione-derived-peptides called phytochelatin, and metal binding peptides. These chelators bind to heavy metals and facilitate microbial absorption and the transportation of metal ions. Volatilization mechanisms involve turning metal ions into a volatile state. This is only possible with Se and Hg, which have volatile states. Mercury-resistant bacteria utilize the MerA enzyme to reduce Hg(II) to the volatile form Hg(0) (Wu et al., 2010a). The reduction

of Se(V) to elemental Se(0) has been employed to remediate contaminated waters and soils. The metabolic processes of these organisms help to transform pollutants in the environment (Siddiquee et al., 2015).

Biosorption, bioaccumulation, biotransformation, and biomineralization are the techniques employed by microorganisms for their continued existence in metal polluted environment. These strategies have been exploited for remediation procedures (Gadd, 2000; Lin and Lin, 2005). Heavy metal removal can be carried out by living organisms or dead biological materials. Large scale feasibility applications of biosorptive processes have shown that dead biomass is more applicable than the bioaccumulation approach, which involves the use of living organisms and thus requires nutrient supply and a complicated bioreactor system. Also, the toxicity of pollutants, as well as other unfavorable environmental conditions, can contribute to the inability to maintain a healthy microbial population. However, many characteristic attributes of living microorganisms have not been exploited in large scale applications (Park et al., 2010). The choice organism must develop resistance towards metal ions as it comes into contact with the heavy metal pollutant to achieve the goal of remediation. The organism of choice may be native to the polluted environment, or isolated from another environment and brought to the contaminated site (Sharma et al., 2000).

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

reported that a microbial cell develops resistance to heavy metals through the excretion of metal chelating substances, or through a problem in a particular transport system, which results in a reduced cell accumulation of the metal ion. Another resistance mechanism includes the binding of a metal ion to intracellular molecules, such as metallothionein, vacuole, or mitochondria, which results in changes in the distribution of metal ion (Siddiquee et al., 2015). Microorganisms interact with metal ions through cell wall associated metals, intracellular accumulation, metalsiderophore, extracellular polymeric reactions with transformation, extracellular mobilization or immobilization of metal ions, and volatilization of metals (Siddiquee et al., 2015).

Various factors influence the microbial remediation of metals. They include the bioavailability of the metal to the microbe, concentration of pollutants, electron acceptors, moisture content, nutrients, osmotic pressure, oxygen, pH, redox potential, soil structure, temperature, and water activity. The bioavailability of each metal in soil is influenced by factors such as the buffering capacity, cation exchange capacity, clay minerals content, metal oxide, and organic matter (Brar et al., 2006; Tak et al., 2013; Mani and Kumar, 2014). In general, remediation of heavy metal is achieved through the removal of the metal ion from substratum to reduce the risk posed by exposure to such heavy metals.

The environmental conditions, prehistory, and pretreatment required for the removal of heavy metals need to be established in order to select the most appropriate biosorbent for a specific situation, from the extremely large pool of organisms that are readily available. Sometimes, the interest may be to recover a specific metal regardless of equilibrium concentration attained, or on the other hand, the interest may be to curtail levels of pollution in the effluent, so that they fall within the acceptable containment limit. Also, priority may be given to the recovery of a large quantity of metal, while also achieving low equilibrium concentrations. Whatever the case, the biosorbent used should have a high sorption capacity (Romera et al., 2007).

2.5 Mechanisms of heavy metal uptake by microorganisms

The cellular structure of a microorganism can trap heavy metal ions and subsequently sorb them onto the binding sites of the cell wall (Malik, 2004). This process is called biosorption or passive uptake, and is independent of the metabolic cycle. The amount of metal sorbed depends on the kinetic equilibrium and composition of the metal at the cellular surface. The mechanism involves several processes, including electrostatic interaction, ion exchange, precipitation, the redox process, and surface complexation (Yang et al., 2015) (Figure 1). The process is fast and can reach equilibrium within a few minutes. Biosorption can be carried out by fragments of cells and tissues, or by dead biomass or living cells as passive uptake via surface complexation onto the cell wall and other outer layers (Fomina and Gadd, 2014). The other method is a process in which the heavy metal ions pass across the cell membrane into the cytoplasm, through the cell metabolic cycle. This is referred to as bioaccumulation or active uptake. Bioaccumulation is a process of a living cell that is dependent on a variety of physical, chemical, and biological mechanisms (Figure 1). These factors include intracellular and extracellular processes, where biosorption plays a limited and ill-defined role (Fomina and Gadd, 2014). The organism that will accumulate heavy metals should have a tolerance to one or more metals at higher concentrations, and must exhibit enhanced transformational abilities, changing toxic chemicals to harmless forms that allows the organism to lessen the toxic effect of the metal, and at the same time, keep the metal contained (Mosa et al., 2016).

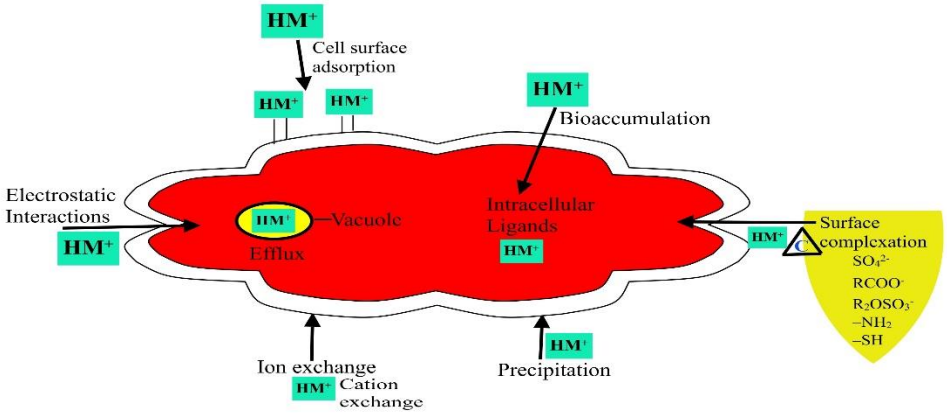


Figure 2.1: Mechanisms of heavy metal uptake by microorganisms

Metal uptake mechanisms by various biosorbents depend on the cellular surface of the microbes, as well as the exchange of metal ions and complex formations with the metal ions on the reactive chemical sites of the cell surface. These have been extensively studied with respect to various biosorption isotherms, derived from sorption experiments and the effect of various factors, such as pH, biomass pretreatment, and the biomass of the organisms. Precipitation of the excess metal ions, through nucleation reactions, then occurs at the cell surface. All microorganisms have a negative charge on their cell surface due to the presence of anionic structures, which enable them to bind to metal cations. The negatively charged groups that are involved in metal adsorption are the alcohol, amine, carboxyl, ester, hydroxyl, sulfhydryl, phosphoryl, sulfonate, thioether, and thiol groups (Gavrilescu, 2004).

An analysis of the cell wall components, which vary among the different microorganisms, helps in assessing metal uptake by different microorganisms. The peptidoglycan layer in Gram-positive bacteria, which contains alanine, glutamic acid, meso-di-aminopimelic acid, polymer of glycerol and teichoic acid, and that of the Gram-negative bacteria, which contains enzymes, glycoproteins, lipopolysaccharides, lipoproteins, and phospholipids, are the active sites

involved in metal binding processes (Lesmana et al., 2009; Fomina and Gadd, 2014; Gupta et al., 2015). Metals and metalloids are attached to these ligands on cell surfaces, which displace essential metals from their normal binding sites. Once the metal and metalloid are bound, microbial cells can transform them from one oxidation state to another, thus reducing their toxicity (Chaturvedi et al., 2015). Gavrilescu (2004) reported that the cell walls of bacteria are polyelectrolyte, which interacts with metal ions to maintain electro-neutrality by mechanisms of covalent bonding, extracellular precipitations, redox interactions, and van der Waals forces.

The rigid cell wall of fungi is made up of chitin, inorganic ions, lipids, nitrogen-containing polysaccharide, polyphosphates, and proteins. They can tolerate and detoxify metal ions by active uptake, extracellular and intracellular precipitation, and valence transformation, with many absorbing heavy metals into their mycelium and spores. The surface of their cell wall acts as a ligand for binding metal ions, resulting in the removal of metals (Gupta et al., 2015). The first barrier includes excreted substances like organic acids or/and proteins with an ability to immobilize heavy metals. The second barrier includes the (unspecific) binding of heavy metals by the cell wall and melanins located in the cell wall. Toxic heavy metals that could not be detained outside the cell must be detoxified inside the cell (Mishra and Malik, 2013).

The cell wall of all classes of algae is composed of cellulose with sulfonated polysaccharides present in the cell wall of brown and red algae. Other binding sites in algae are polysaccharides such as alginic acid, glycan, mannan, proteins, and xylans. The cell wall of cyanobacteria is composed of peptidoglycan, and some species also produce sheaths and extracellular polymeric substances, which are used for sorption. Characteristics of the biomass, chemical and physical properties of the metal of interest, and pH of the solution, influence the sorption capacity of algae (Lesmana et al., 2009).

Non-essential metal uptake usually consists of transporters which are committed to the acquisition of vital organic and inorganic ions. These transporters assist in either the co-

transport of these metals in complexes with low-molecular-mass ligands, or in the direct uptake of non-essential metals (Lemire et al., 2013). Microorganisms can also secrete many kinds of metal-binding metabolites, produce extracellular polymeric substances, which are made up of polysaccharide, capsules, slimes and sheaths, and biofilms, depending on the make-up of the polysaccharide and associated components. Biofilms bind substantial quantities of heavy metals under pristine conditions and serve as a medium for the precipitation of insoluble mineral phases (Fomina and Gadd, 2014).

2.6 Biosorption capacity of various microbial biosorbents

Various microbial biomasses have different biosorptive abilities, which also vary considerably within each group. However, the biosorption capacity of each biosorbent depends on its prehistory and pretreatment, as well as the experimental conditions. The biosorbent should be cheap, effective, and easy to grow and harvest. The organism should also lend itself to alteration of the bioreactor configuration, as well as physical and chemical conditions to enhance biosorption (Fomina and Gadd, 2014).

Bacteria have been used as biosorbents owing to their ubiquity, size, ability to grow under controlled conditions, and resilience to an extensive range of environmental conditions (Wang and Chen, 2009; Srivastava et al., 2015). Various heavy metals have been tested on bacteria species such as *Pseudomonas*, *Enterobacter*, *Bacillus*, and *Micrococcus* species (Table 2.2). Their excellent sorption capacity is due to their high surface-to-volume ratios and their numerous potential active chemosorption sites, such as the teichoic acid on the cell wall (Mosa et al., 2016).

Sinha et al. (2012) designed a laboratory scale sequential bioreactor for the removal of mercury from synthetic effluent (10 mg/L of Hg). The efficiency of mercury removal by *Bacillus cereus* (immobilized on alginate) was 104.1 mg/g on the third day. *Micrococcus luteus* was used to remove a large amount of Pb from a synthetic medium. Under optimal conditions, the

removal capacity was 1965 mg/g (Puyen et al., 2012). Kim et al. (2015) also designed a batch system using zeolite-immobilized *Desulfovibrio desulfuricans* for Cu, Cr, and Ni removal from contaminated seawater (Table 2.2). The removal efficiency was 98.2, 99.8, and 90.1 mg/g, respectively, after about seven days.

Yeasts and molds are easy to cultivate, can be genetically and morphologically manipulated, and can produce a high biomass yield. They are widely used in a variety of large-scale industrial fermentation processes, producing ferrichrome, gallic and kojic acid, and enzymes like lipases, glucose isomerase, pectinases, amylases, and glucanases (Wang and Chen, 2009). They are extensively used as biosorbents for the removal of toxic metals from polluted wastewaters, with excellent abilities for metal uptake and recovery (Dursun et al., 2003; Akar et al., 2005; Fu et al., 2012). They have developed a complex defense system to neutralize heavy metal toxicity. Akar et al. (2005) evaluated the Pb removal potential of *Botrytis cinerea* in a batch reactor. Lead (II) ions were found to be extracellularly accumulated on the cell surface and the rate of accumulation was affected by the pH, contact time, and initial metal concentration. The sorption capacity of Pb by *B. cinerea* was found to be 107.1 mg/g at an initial Pb concentration of 350 mg/L, after 180 min. Fu et al. (2012) recently reported the biosorption of Cu(II) ions by mycelial pellets of *Rhizopus oryzae*. The effects of pellet diameter, solution pH, contact time, initial metal concentration, and temperature were evaluated. Metal removal efficiency of Cu(II) ions using mycelial pellets was observed to be 34 mg/g after two hours (Table 2.2). Sharma and Adholeya (2011) reported that *Paecilomyces lilacinus* fungi accumulate only 24% of chromium from spent chrome effluent supplemented with cane sugar, while 100% removal was observed from a synthetic medium. Srivastava and Thakur (2006) also reported the efficiency of chromium removal by *Aspergillus* sp. from tannery effluent. Eighty-five percent of the chromium was removed at pH 6 in a bioreactor system from the synthetic medium, compared to a 65% removal from the tannery effluent. This is because of the presence of organic pollutants that inhibit the growth of the fungal species.

Algae have also been used as biosorbents for heavy metal removal. Brown algae have gained prominence as good biosorbents because of their high sorption capacity. Red, green, and brown algae have been used for adsorption studies and are all readily available in marine and fresh water environments (Srivastava et al., 2015). Algae are autotrophic, thus require a low number of nutrients and produce a large biomass compared to other microbial biosorbents. They have a high sorption capacity and are readily available in large quantities (Abbas et al., 2014). The sorption capacity of six different algae were evaluated for the recovery of Cd, Cu, Ni, Pb, and Zn from an aqueous solution by Romera et al. (2007). The maximum sorption of Cd (32.3 mg/g), Pb (63.7 mg/g), and Zn (21.6 mg/g), were recorded for *Asparagopsis armata*, while the maximum Cd (21.8 mg/g), Pb (63.3 mg/g), and Zn (23.8 mg/g) uptake, occurred in *Codium vermilara* (Table 2.2). Algae are effective biosorbents for the removal of Sb(III) from aqueous solutions (Ungureanu et al., 2015). The maximum adsorption capacity of Sb(III) by the algae *Sargassum muticum* was 5.5 mg/g, at pH 5. A slight effect of pH was observed in the removal efficiency of Sb by *S. muticum*.

Table 2.2: Metal biosorption by different microbial biosorbents

	Microbial biosorbent	Metal	pH	Temperature (◦C)	Time (h)	Initial metal ion conc. (mg/L)	Sorption capacity (mg/g)	Reference
Bacteria	*Bacillus cereus* (Immobilize on alginate)	Hg	7	30	72	30	140.1	Sinha et al. (2012)
	B. laterosporus	Cd	7	25	2	1000	159.5	Zouboulis et al. (2004)
		Cr (VI)	2.5				72.6	
	B. licheniformis	Cd	7	25	2	1000	142.7	
		Cr (VI)	2.5				62	
	Desulfovibrio desulfuricans (immobilize on zeolite)	Cu	7.8	37	168	100	98.2	Kim et al. (2015)
		Ni				100	90.1	
		Cr(VI)				100	99.8	
	Enterobacter cloacae	Pb	-	30	48	7.2	2.3	Kang et al. (2015)
	Kocuria rhizophila	Cd	8	35	1	150	9.07	Haq et al. (2015)
		Cr	4			150	14.4	
	Micrococcus luteus	Cu	7	27	12	80.24	408	Puyen et al. (2012)
		Pb				272.39	1965	
	Pseudomonas aeruginosa	Co	5.2	25	10	58.93	8.92	Kang et al. (2005)
		Ni	5.5			58.69	8.26	
		Cr(III)	3.4			52	6.42	

	P. jessenii	Ni	-	25	6	275	1.36	Rajkumar and
		Cu				300	10.22	Freitas (2008)
		Zn				400	4.39	
	Pseudomonas sp.	Ni		25	6	275	2.79	
		Cu				300	5.52	
		Zn				275	3.66	
	Sulphate-reducing bacteria	As (III)	6.9	-	24	1	0.07	Teclu et al. (2008)
		As(V)					0.11	
Fungi	*Aspergillus niger*	Cu	5	30	1	100	15.6	Dursun et al. (2003)
		Pb	4.5			100	34.4	
		Cr (VI)	3.5			50	6.6	
	Botrytis cinerea	Pb	4	25	1.5	350	107.1	Akar et al. (2005)
	Phanerochaete	Pb	6	20	1	100	88.16	Iqbal and Edyvean
	chrysosporium (immobilized	Cu				100	68.73	(2004)
	on loofa sponge)	Zn				100	39.62	
	Pleurotus platypus	Ag	6	20	2	200	46.7	Das et al. (2010)
	Rhizopus oryzae	Cu	4	35	2	100	34	Fu et al. (2012)
Algae	*Asparagopsis armata*	Cd	6	-	2	135	32.3	Romera et al. (2007)
		Ni	6			141	17.7	
		Zn	6			182	21.6	

	Cu	5			134.4	21.3	
	Pb	4			124	63.7	
Codium vermilara	Cd	6	-	2	135	21.8	Romera et al. (2007)
	Ni	6			147	13.2	
	Zn	6			182	23.8	
	Cu	5			140	16.9	
	Pb	5			83	63.3	
Cystoseira barbata	Cd	4	20	1	117.4	37.6	Yalçın et al. (2012)
	Ni				224.8	78.7	
	Pb				414	196.7	
Lessonia nigrescens	Ar(V)	2.5	20	5	200	45.2	Hansen et al. (2006)
Sargassum muticum	Sb	5	23	4	10	5.5	Ungureanu et al. (2015)
Spirogyra sp.	Pb	5	25	1.6	200	140	Gupta and Rastogi (2008)

Conclusions

This review revealed the contributions of the various biosorbents which are potentially effective and readily available for heavy metal removal. These biosorbents present attractive opportunities as low cost means of protecting the environment from pollution. Biosorbent selection and implementation for industrial wastewater management and soil remediation requires more effort, as most reported adsorption studies have been confined to laboratory investigations in a batch system. A sustainable approach needs to be developed in order to select the most appropriate biosorbent, operating conditions, and efficient mechanism of heavy metal removal in industrial effluent, to sufficiently address the major challenges involved. Also, in order to develop a reliable biosorption process, more research is needed in biosorbent characterization, in terms of surface morphology and area, zeta potential, functional groups, and particle size, as these are important in biosorption experiments, influenced by the pretreatment of the biosorbents. Equally, growing microbial biomass with the potential for metal uptake needs further investigation, with the aim of exploring the metabolic potential of these growing biomasses and their application in industrial wastewater management.

CHAPTER THREE

METAL(LOID) BIOREMEDIATION: STRATEGIES EMPLOYED BY MICROBIAL POLYMERS

Abstract

Environmental pollution arising from metal(loid)s is a result of industrialization, and has led to serious health issues. Conventional methods of metal(loid) removal often results in generation of secondary waste which is toxic to the environment. Bioremediation in combination with physicochemical techniques offers an excellent and effective means of removal. The use of secondary metabolites and extracellular polymers produced by microorganisms is an effective procedure employed in metal(loid) sequestration and reduction in toxicity of contaminated environments. These biopolymers have different chemical structures and have shown varied selectivity to different metal(loid)s. This review discusses various microbial polymers, their mechanism of metal(loid) removal and their potential application in remediation of contaminated environment.

Keywords: biodegradation; biofilms; bioflocculant; biopolymers; biosurfactant; secondary metabolites; pollution.

3.1 Introduction

Environmental pollution due to swift growth in chemical industries, battery production, electroplating and steel industries, fertilizer and pesticide production, and mining activities is one of the most important challenges that are of human health concern. Pollution caused by metal(loid)s poses a health risk to living organisms. The accumulation of metal(loid)s in the environment as a result of natural and anthropogenic activities has significantly altered the nature of the environment (Sarubbo et al., 2015; Luna et al., 2016). In the midst of diverse metal ions, cadmium, chromium, lead and mercury in wastewater and soil are hazardous to the environment

and have been linked to certain ailments such as birth defects, damage of internal organs such as kidney and liver, certain learning disabilities, and a host of other diseases. Due to their carcinogenic, genotoxic, mutagenic or toxic effects, their presence in the environment poses health risks and harmful effects on living organisms (Das et al., 2009; Sriram et al., 2011; Fashola et al., 2016; Ayangbenro and Babalola, 2017).

Metal(loid)s are natural components of the earth's crust and are also produced from anthropogenic sources. Some metal(loid)s act as essential micro nutrients for living organisms, but at higher concentrations they can cause severe poisoning. Metal(loid) ions are non-degradable and thus persist in the ecosystem. They become bioavailable for removal as there is decrease in the surface tension of water at polluted sites (Hashim et al., 2011).

Metal(loid) removal from contaminated environments usually involves three technologies namely: (1) Chemical, (2) physicochemical, and (3) biological techniques (Hashim et al., 2011; Sarubbo et al., 2015). The techniques can overlap in some cases as a result of multidisciplinary research and paradigms shift in technology. However, there can be limitations to a particular technique as the operation condition might not be effective in a particular environment (Sarubbo et al., 2015).

Conventional techniques for metal(loid) removal involve treatment of polluted soil with surfactants, organic and inorganic acids, water, reverse osmosis, adsorption processes, and metal(loid) chelating agents. However, these physicochemical techniques are expensive and do not guarantee effective removal of metal(loid) ions from the soil especially when metal(loid) concentrations are less than 100 mg/L. Other disadvantages include non-specificity of the techniques for metal(loid) binding, high energy demand, land space requirements, and sometimes the methods are impracticable and often exchange one problem for another (Das et al., 2009; Voica et al., 2016). Effective sequestration of metal(loid) from polluted environments demands a good metal(loid) complexing agent. Such a complexing agent must possess characteristics such as

environmental stability, high solubility and good metal(loid) complexation potentials (Das et al., 2009).

Bioremediation is a cost-effective alternative for metal(loid) decontamination of polluted environments. It offers a long term recovery and/or removal of metal(loid) ions from contaminated environments and can be used in combination with other techniques (Sarubbo et al., 2015). Various strategies have been developed by microbes for their continued existence in metal(loid) polluted environments. They adopt diverse detoxifying mechanisms that include bioaccumulation, biomineralization, biosorption and biotransformation for their survival (Lin and Lin, 2005) (Figure 1). These mechanisms are often triggered and activated when the concentration of metal(loid)s in their environment increases. They secrete many kinds of metal(loid)-binding metabolites, ranging from simple organic acids, alcohols to extracellular polymeric substances (EPS), slimes and sheaths, humic and fulvic acids, capsules, and biofilms which help in metal(loid) uptake from solution (Wu et al., 2010a; Fomina and Gadd, 2014). Metal sulfides and oxides are also trapped and absorbed by these extracellular polymers and proteins produced by microorganisms (Wu et al., 2010a).

Microbial products have generated increasing attention lately due to their biodegradable nature, diversity, and low toxicity. This makes them superior to their chemical counterparts. These metabolites provide defensive mechanisms, increase metal(loid) bioavailability and facilitate reproductive processes (Valls and De Lorenzo, 2002; Vaishnav and Demain, 2011). The metabolic pathways of these microorganisms have been exploited for degradation and removal of pollutants in the environment. In contrast to organic pollutants, metal(loid) contamination presents a difficult challenge; metal(loid) ions are not biodegradable but can be converted to base metal(loid), complexed with an organic ligand, methylated, precipitated or volatilized. An approach to metal(loid) removal involves stable complex formation between metal(loid) ion and microbial

polymers. This is as a consequence of the electrostatic interaction between metal(loid) ions and negatively charged microbial polymers (Gutnick and Bach, 2000).

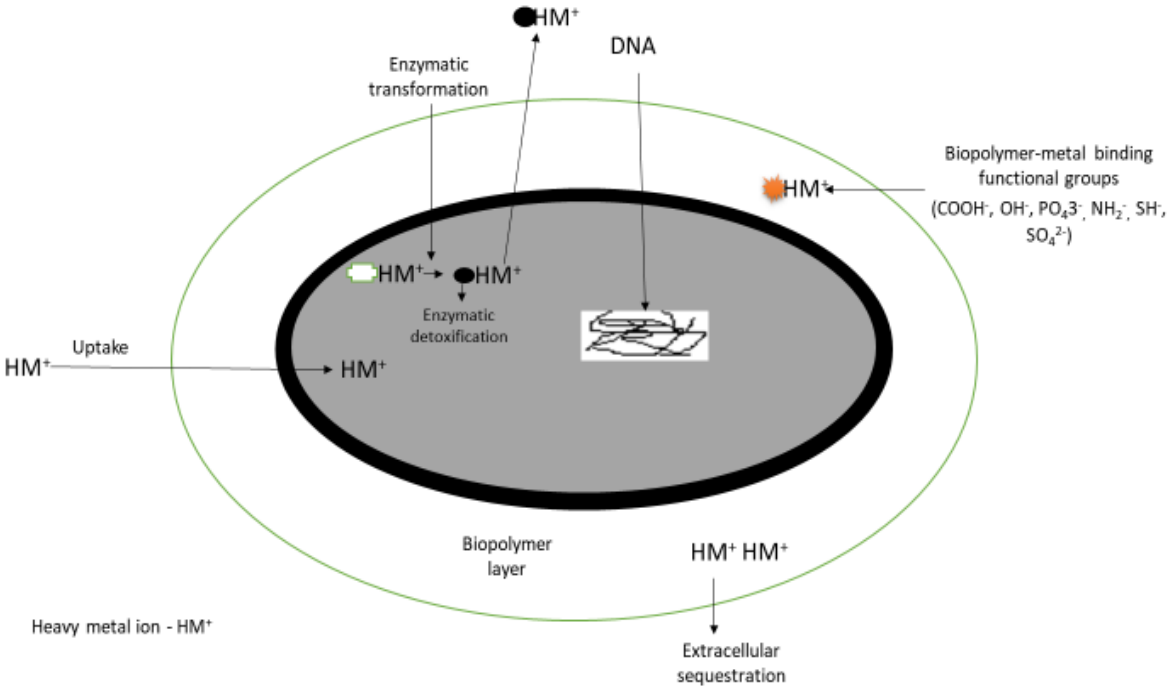


Figure 3.1: Mechanism of metal(loid) sequestration

From the beginning of cellular life, microorganisms are periodically exposed to metal(loid)s. This is evident in many of their enzymes that are composed of different essential divalent and transition metals at their active sites. These essential metal(loid)s are involved in maintaining protein structure and catalyzing key enzymatic reactions. They are thus needed in small quantity for cellular metabolism. The uptake of these metal(loid)s is regulated via the homeostatic mechanisms that ensure sufficient, but not excessive intake (Valls and De Lorenzo, 2002). However, other metal(loid)s have no known biological function, but instead, they damage, inactivate or block enzyme functions because of their affinity for the sulfhydryl groups of proteins. Resistance to metal(loid)s most likely evolved shortly after life started, in an early metal(loid)-polluted world (Valls and De Lorenzo, 2002). The synthesis of microbial polymers is driven by the need to survive in hostile environments and to assist in neutralization of toxic elements by sequestration (Van Hamme et al., 2006).

Microbial processes that facilitate the detoxification and metal(loid) mobility using resistant microbial strains can be used for removal of metal(loid)s from soil, effluents and wastewaters. The use of microbial polymers is evolving as a promising technique to improve and enhance the effectiveness of metal(loid) removal. These compounds are often economic, versatile, environmentally friendly and can reduce the concentration and toxicity of pollutants.

In addition, these biopolymers have the capability to survive a wide range of temperature and pH and can affect interfaces. pH affects the functional groups involved in metal(loid) binding; therefore providing varying binding sites on the surface of the organisms at different pH values. At high pH, there are less H^+ to compete with metal(loid)s for binding sites on the biopolymers. At neutral pH, they have functional groups that are negatively charged and are capable of forming stable organo-metallic complexes with metal cations by means of diverse interactions (Li and Yu, 2014). A high temperature favors biopolymer-metal adsorption (Kiran and Kaushik, 2008). As the temperature increases, the affinity for the binding sites increases which favors direct contact between the biopolymer functional groups and metal ions (Kiran and Kaushik, 2008). However a very high temperature can cause structural damage of the biopolymer and some functional groups, which can reduce the efficiency of the polymer (Li and Yu, 2014). The metal binding ability of these biopolymers depends on the nature of biomass, specificity, affinity, pH, temperature, hydrodynamics and functional groups present (Pal and Paul, 2008; Li and Yu, 2014).

The nature of the biopolymer varies depending on the origin, extraction procedure and adsorption characteristics (Li and Yu, 2014). Guibaud et al. (2009) made a comparison of the origin of some biopolymers. Their results showed that biopolymers from activated sludge have higher affinity for Cd^{2+} and Pb^{2+} than polymers from pure strains of bacteria. Polymers from anaerobic granular sludge were found to have lower affinity and proton exchange capability (Guibaud et al., 2012). The functional groups present on these biopolymers also vary in their affinity for metal(loid) ions (Li and Yu, 2014). The carboxyl and the phosphate groups are the main functional groups that are

involved in binding of metal ions and are effective at neutral pH. Other functional groups include the amides, hydroxyl groups and nucleic acids (Zhang et al., 2010). These groups showed weaker binding ability compared to carboxyl and phosphate groups due to their high pKa values (Guibaud et al., 2003).

Microbial polymers are usually the first line of defense against metal(loid)s, and are important in protecting the interior of the microbial cell (Li and Yu, 2014). The use of isolated biopolymers is desired for easy availability in metal(loid) removal and circumvention of pathogenicity concerns of some producing organisms. This review article discusses the activities of useful microbial polymers in metal(loid)-binding, their mechanism of action and applications in bioremediation processes.

3.2 Biosurfactant as a metal(loid)-complexing biopolymer

One of such microbial polymers is biosurfactant, which has been reported to be an effective metal(loid) complexing agent. They are surface-active metabolites with amphiphilic configuration. This amphiphilic metabolite has both hydrophilic and hydrophobic moieties, causing them to aggregate or form micelles at interfaces between fluids of different polarities, hence why it decreases interfacial surface tension between such fluids (Mazaheri and Tabatabaee, 2010). Typical hydrophilic groups of biosurfactants are amino acids, anions or cations, carbohydrates (mono-, di-, or polysaccharides) and peptides. The hydrophobic groups are the fatty acids which include branched, saturated, unsaturated, or hydroxylated fatty acids (Singh and Cameotra, 2004; Kitamoto et al., 2009).

Surfactants reduce the interfacial and surface tension, and increase the bioavailability, mobility, solubility and subsequent degradation of pollutants. There has been growing interest in this biopolymer due to several advantages it has over chemical surfactants. These characteristics include: (a) One or more chiral centers and functional groups; (b) complex structural diversity; (c) enhanced activity at extreme conditions of pH, salinity and temperature; (d) excellent ability to

form molecular assembly and liquid crystals; (e) gradual adsorption and continuing activity; (f) advanced surface activity and lower critical micelle concentration; (g) superior biodegradability and lower toxicity; (h) versatile biological activity; and (i) production from renewable feedstocks (Kitamoto et al., 2009; Sriram et al., 2011; Dadrasnia and Ismail, 2015; Bezza and Chirwa, 2016; Henkel et al., 2017).

Biosurfactants are extracted constituents of the bacterial or yeast cell membrane with structural variability. These secondary metabolites include high and low molecular-weight polymers that have the ability to effectively reduce interfacial and surface tension, and act as highly efficient emulsifiers. This stimulates the bioavailability of hydrophobic molecules by dissolving and emulsifying these hydrophobic compounds or by altering the cell's surface properties. Their production is considered as an important strategy to overcome pollutant-related stress by microorganisms (Bezza and Chirwa, 2016). They speed up the rate of mass transfer of hydrophobic compounds by making them readily available to microbes and thus acting as mediators. Furthermore, they induce changes in cell membrane properties which increases microbial adherence. This process is important when two immiscible fluids are present and direct substrate uptake is plausible (Dadrasnia and Ismail, 2015). Consequently, they help to surmount the diffusion-related mass transfer limitations (Bezza and Chirwa, 2016).

Biosurfactants are produced by different microorganisms and have unique metal(loid)binding capacities and selectivity (Arjoon et al., 2013). The selectivity in metal(loid)—binding is species specific (Miller, 1995) and it is important to remove the target metal(loid) as other metal(loid)s in solution compete with the target metal(loid). The selectivity depends on the ionic radius and the stability of the surfactant-metal(loid) complex (Yuan et al., 2008). It has been shown that cadmium and lead have higher affinities for rhamnolipids from *P. aeruginosa* than any other soil components to which they are bound. Rhamnolipids have special affinity for non-essential and essential

metallic cations, potentially leading to greater toxicity when in excess in exposure pathways, than with macronutrient cations (Torrens et al., 1998; Singh and Cameotra, 2004).

Biosurfactants with anionic, cationic, or neutral charge are employed as washing agents. They can solubilize, disperse and desorb metal(loid)s from excavated sediments or soils in a washing unit (Dahrazma and Mulligan, 2007). Anionic biosurfactants have been proposed to bind metal(loid) ions by complexation or electrostatic techniques. These complexations result in an increase in the apparent metal(loid) solubility (Rufino et al., 2012). Therefore, the bioavailability of metal(loid)s is affected through their reduction by this biopolymer, which leads to the formation of less soluble metal salts including phosphate and sulfide precipitates (Mosa et al., 2016). By adsorbing onto the surface of sediments, biosurfactants form complexes with metal(loid) ions, and thus detaching metal(loid) ions from the sediment into the pore water. Therefore, they are able to remove metal(loid)s adsorbed to the sediment in association with surfactant micelles (Mazaheri and Tabatabaee, 2010; Franzetti et al., 2014).

Biosurfactants are produced by a variety of actinobacteria, bacteria and fungi with different classes. The classes include glycolipids, fatty acids, lipopeptides, lipoproteins, phospholipids, particulate and polymeric surfactants (Singh and Cameotra, 2004; Sriram et al., 2011). They have achieved significant importance with their activities and structural characteristics. Several bacterial genera such as *Arthrobacter*, *Bacillus* and *Pseudomonas* have been reported to produce surfactants with metal(loid) binding characteristics (Table 3.1).

3.2.1 Mechanism of biosurfactant removal of metal(loid)s

Most microorganisms utilize the reductive pathway as an alternative to the oxidative pathway during transformation of metal(loid)s. This is due to the less toxic nature of the reduced form of metal(loid)s. The processes used include accumulation, complexation, efflux system and reduction mechanisms to tolerate metal(loid) exposure. Resistance to metal(loid) ions can also be conferred

by gene clustering on plasmid when exposed to stress conditions (Gnanamani et al., 2010). Biosurfactants act as a link between the fluid interfaces, due to their amphiphilic nature, and thus reduce the surface tension. Decrease in the surface tension of water increases mobilization of metal(loid) from unsaturated soils, consequently making removal of metal(loid)s possible.

Biosurfactants have been proposed to utilize two fundamental principles for metal(loid) removal from solution. Metal sorption mediated by biosurfactant is promoted through complexation of the free metal ion. This process employs Le Chatelier's principle and the solution phase activity of metal(loid) ion diminishes to promote desorption of metal(loid). Another mechanism is the direct contact with metal(loid) under reduced interfacial tension at solid-solution interface. This permits the biosurfactant to accumulate at solid-solution interface which aids metal(loid) binding (Singh and Cameotra, 2004; Chakraborty and Das, 2014).

Soils contaminated with metal(loid)s are treated by two basic techniques. The first technique involves metal(loid) immobilization on a strongly bound solid matrix to reduce migration. Nevertheless, the process does not permanently solve the problem as soil reuse is limited and also requires long-term monitoring. As a result, solidification is often limited to highly toxic or radioactive wastes. The other technique promotes mobility and migration of metal(loid) into liquid state by desorption and solubilization. This procedure offers a permanent solution to the pollution challenge. In addition, the treated soil can be recycled, which also improves further land-use alternative (Hong et al., 2002).

The mechanism of metal(loid) extraction by biosurfactant includes ion exchange, counter binding and precipitation. Ion exchange mechanism involves negatively charged anionic biosurfactants which bind with positively charged metal cations to form a bond that is stronger than the bond formed between metal ion and soil. The cell surface is conferred with a negative charge by biosurfactant. This process supports the electrostatic interaction with metal ion. The polar head moieties of surfactant micelles bind with the metal ions making them water soluble. The surfactant

micelles then move the metal ions into solution, thus making it easier for metal recovery by flushing, washing, or pumping (Mulligan, 2005; Aşçı et al., 2010). The metal(loid)-biosurfactant complex forms a strong bond and flushing with water removes the complex from soil matrix (Mulligan, 2005). Cationic biosurfactants replace the same charged ions by competing with negatively charged surfaces (Franzetti et al., 2014; Sarubbo et al., 2015). The metal(loid) ion can then be recovered from the complex and used for different applications. The carboxyl group of the biosurfactant mono-rhamnolipid produced by *P. aeruginosa* has preference for metals such as Cd^{2+}, Pb^{2+} and Zn^{2+} (Juwarkar et al., 2007; Franzetti et al., 2014). The complex formed between metal and biosurfactant is non-toxic to bacteria. In the precipitation process, biosurfactants are added to soil during washing. As a result of the foaming characteristics of biosurfactants, the biosurfactant-metal complex can be removed from soil by the addition of air to cause foaming. The biosurfactant can then be recovered and recycled through precipitation by reducing the pH (Mulligan, 2005).

Surfactant concentration at which aggregation of ions or molecules (micelles) first begins to form is called the critical micelle concentration (CMC) where the surfactants assemble into an organized molecular arrangement (Chakraborty and Das, 2014). Above the CMC, the surfactant molecules assemble together, forming aggregates and a range of micellar and vesicular configuration which depend on the pH of the solution (Aşçı et al., 2010). The surfactants exist as single molecules or monomers at low concentration in aqueous solutions. The CMC depends on the composition and structure of the surfactant, temperature, ionic strength, and the presence and nature of organic compounds in the solutions. At the CMC of surfactant solutions, there is a radical change that occurs in many physico-chemical properties of the surfactant (Bustamante et al., 2012).

Biosurfactant is applied to contaminated soil for metal removal in a small quantity by using a cement mixer. The complex formed between the biopolymer and metal ion is then flushed out and the soil deposited back on site. Biosurfactant is then precipitated out of the complex formed leaving

behind the metal ion. The bond formed by biosurfactant-metal ion is so strong that flushing with water eliminates the complex from soil matrix. Likewise, the process can be repeated for deep subsurface (in situ) contamination, but with increased flushing activities. Biosurfactants in cell free broth can be used directly or in dilute concentrations on polluted sites. They are stable and effective in the production medium. Biosurfactants also enhance the degradation of organic contaminant in the presence of metal(loid)s by lowering the toxicity of metal(loid) ions and can be simultaneously used to eliminate metal(loid)s and organic pollutants (Pacwa-Płociniczak et al., 2011).

Metal(loid)s form a strong bond with soil, thus making its bioavailability difficult for microbes. The activities of biosurfactant-metal(loid) ion complexes in soil are influenced by the soil's cation exchange capacity, soil composition and pH, particle size, type and time of contamination, and geological pattern (Singh and Cameotra, 2004; Marchant and Banat, 2012; Chakraborty and Das, 2014). Long duration of metal(loid) contamination provides sufficient time for metal(loid) ion to stabilize and attach to soil, thus making removal difficult (Singh and Cameotra, 2004; Chakraborty and Das, 2014).

Decontamination tests have been performed on metal contaminated soil and sludge with different biosurfactants (Table 3.1). Studies have revealed the potential of lipopeptides, rhamnolipids, sophorolipids and surfactin in metal removal (Table 3.1). Dahrazma and Mulligan (2007) examined the capability of rhamnolipids to remove metal ions from contaminated soil sediments. The rhamnolipid was able to remove up to 37% of Cu, 33% of Ni, and 7.5% of Zn when applied in a continuous flow configuration. Rhamnolipid from *Pseudomonas* was shown to remove 19% of Fe and 52% of Zn in a cyclic treatment (Table 3.1).

Other biosurfactants that have been effective soil washing agents for metal(loid) removal include lipopeptide from *Bacillus subtilis*. This surfactant has been demonstrated to remove metals like Cd, Cu, Co, Pb, Ni and Zn from metal and hydrocarbon polluted soil (Singh and Cameotra, 2013).

The efficiency ranged from 26% for Cu to 44% for Cd (Table 3.1). Sophorolipids produced by *Torulopsis bombicola* have also been employed successfully in the removal of metal ions (Mulligan et al., 2001b). It has demonstrated the capacity to remove 60% of Zn and 25% of Cu in a soil sediment washing experiment (Table 3.1).

Wang and Mulligan (2009) used a commercially available rhamnolipid biosurfactant JBR425 produced by *P. aeruginosa* to remove arsenic and other metals (copper, lead and zinc) from mine tailings. They observed that 0.1% of the surfactant removed 148, 74, 2379 and 259 mg/kg of As, Cu, Pb and Zn respectively under alkaline condition. They concluded that the mobilization of arsenic by the rhamnolipid surfactant is enhanced by the presence of other metals under alkaline condition. Consequently, allowing metal bridging through the formation of the biosurfactant-metal complex.

3.3 Metal(loid) removal by microbial flocculants

Substances used in the separation of suspended solid-liquid are called flocculants (Okaiyeto et al., 2016). Flocculation involves the floc formation through colloid aggregation. This is stimulated by a chemical substance called flocculant. There are three types of flocculating agents namely inorganic (alum, aluminum chloride and sulfate, ferrous sulfate, ferric chloride and polyaluminium chloride), natural (cellulose, chitosan, gum and mucilage, starch, tannin and microbial flocculant), and organic polymeric (polyacrylamide and polyethylene amine) flocculants (Lee et al., 2014; Salehizadeh and Yan, 2014; Sajayan et al., 2017). Chemically synthesized flocculants possess inherent drawbacks of being toxic, cause serious health issues, produce large quantities of sludge, and are not readily degradable (Zhai et al., 2012; Farag et al., 2014; Lee et al., 2014). Therefore, bioflocculants have gained increased attraction because they are environmentally friendly, non-toxic, free of secondary pollution risks, and they provide effective elimination of metal(loid)s and suspended solids from wastewaters (Guo, 2015).

Bioflocculants are polymers produced by bacteria during growth and contain complex mixtures of macromolecular polyelectrolytes produced by different organisms. They have various organic groups, such as aspartic and glutamic acid in the protein component, galacturonic and glucuronic acid in the polysaccharide constituent and uronic acids (containing a carbonyl and carboxylic acid component). These groups are responsible for metal binding (Lin and Harichund, 2011). The carboxyl groups on the molecular chain of this biopolymer allow the chain to stretch out due to electrostatic repulsion, and the extended molecular chains offer more effectual sites for attachment of particles. Metals form complexes with the carboxyl and amino functional groups of the bioflocculant by neutralizing and stabilizing the residual charge as the distance of binding is shortened (Pathak et al., 2014).

The flocculating efficiency depends on the contact area between the adsorbate and bioflocculant, suspended particle size, concentration and structure of adsorbate, contact time, ionic strength, pH, temperature and the type of producing organism (Okaiyeto et al., 2016). The flocculant choice has a significant effect on the flocculation performance, strength of particle aggregation, and the strength and number of bonds formed due to flocculation (Zhang et al., 2014).

3.3.1 Mechanism of metal(loid) removal by flocculation

The formation of interface with metal(loid) ions was attributed to be the mechanism of metal(loid) removal by bioflocculant. The interface formation depends on ionic groups such as amino, carboxyl and hydroxyl groups in the biopolymer which enable metal-floc interactions (Deng et al., 2005; Sathiyanarayanan et al., 2016). It is a metabolic-independent process in which communication between metal ions and the flocculant functional groups can be established by complexation, immobilization, ion exchange, physical adsorption and precipitation processes (Sathiyanarayanan et al., 2016).

Table 3.1: Biosurfactant washing of some metal polluted environment

Organism	Biosurfactant Type	Contaminated Environment	pH	Temperature (°C)	Metals	Efficiency (%)	Reference
Bacillus subtilis A21	Lipopeptide (50 × CMC)	Soil	9	25	Cd (989.8 mg/kg)	44.2	Singh and Cameotra (2013)
					Co (166.8 mg/kg)	35.4	
					Cu (173.6 mg/kg)	26.2	
					Ni (227.9 mg/kg)	32.2	
					Pb (143.7 mg/kg)	40.3	
					Zn (404.7 mg/kg)	32.07	
Bacillus subtilis	Surfactin (0.1%)	Soil	-	-	Cd (1 mM)	15	Mulligan et al. (1999)
					Cu (1 mM)	70	
					Zn (1 mM)	25	
Pseudomonas aeruginosa CVCM 411	Rhamnolipid (1 mg/mL)	Soil	8	25	Fe	19	Diaz et al. (2015)
					Zn	52	
Commercial	Rhamnolipid (5%)	Soil	6.5	25	Cu (140 mg/kg)	37	Dahrazma and Mulligan (2007)
					Ni (76 mg/kg)	33.2	
					Zn (4854 mg/kg)	7.5	
Bacillus circulans	Crude surfactant (5 × CMC)	Soil	-	-	Cd (100 ppm)	97.66	Das et al. (2009)
					Pb (100 ppm)	100	
Commercial	Saponin (30 g/dm3)	Sludge	3	25	Cr (1000 ppm)	56.1	Gao et al. (2012)
					Ni (1000 ppm)	64.2	
					Pb (1360 ppm)	73.2	
Candida sphaerica UCP 0995		Soil		27	Fe (1877 mg/kg)	95	Luna et al. (2016)
					Pb (3038 mg/kg)	79	

				Zn (1470 mg/kg)	90	
Torulopsis bombicola	Sophorolipid (4% and 0.7% HCl)	Soil	5.4 -	Cu	25	Mulligan et al. (2001b)
				Zn	60	
Candida lipolytica UCP 0988	Lipoprotein	Soil		Cd (0.9 mg/kg)	50	Rufino et al. (2011)
				Cu (13.3 mg/kg)	96	
				Fe (52.5 g/kg)	16.5	
				Pb (14 mg/kg)	15.4	
				Zn (19.1 mg/kg)	96	

Similarly, charge neutralization, electrostatic patch and polymer bridging are other mechanisms of flocculation. Metal ions are positively charged and most bioflocculants are negatively charged which makes charge neutralization applicable. The cation exchange ability makes the electrostatic forces between metal ions and bioflocculant possible (Lee et al., 2014). During the process of charge neutralization, the surface charge density of the particle is reduced by bioflocculant adsorption and the particles sufficiently aggregate for the effectiveness of the attractive forces (Salehizadeh and Shojaosadati, 2001).

In a bridging mechanism, the bioflocculant serves as a bridge in the complex formed by particle-polymer-particle. Some functional groups of the biopolymer adsorb to the metal ions when the biopolymer comes in contact with the metal ion, thus leaving the unabsorbed metal ions to extend out into the solution. The second biopolymer with unattached adsorption sites then comes in contact with the extended segments for attachment to occur. A metal-biopolymer-metal complex is thus formed with the biopolymer serving as a bridge (Lee et al., 2014). Before flocculation can occur, the biopolymer must not only come in contact with the particles, it must also conquer the electrostatic repulsive forces between particles (Wang et al., 2011; Okaiyeto et al., 2016). Efficient bridging mechanisms are attributed to the following characteristics: The ionic strength of the suspension, the molecular weight of the biopolymer, the net charge of the biopolymer, the suspended particles, and the kind of mixing (Okaiyeto et al., 2016).

The producing microorganisms determine the structural characteristics of the biopolymer produced. Metal(loid) ions demonstrate varying effects on different biopolymers. These effects depend on the concentration and valence and metal(loid) ions (Wu and Ye, 2007b; Okaiyeto et al., 2016). The metal(loid)-binding interactions are influenced by various factors such as bioflocculant dosage, physico-chemical characteristics of metal(loid) ions, initial metal(loid) concentration, ionic strength, charge density, appropriate positioning with the binding site, conformation of

polymer with adsorbed ions and tertiary structure of floc (Deng et al., 2005; Lin and Harichund, 2011; Gomaa, 2012; Salehizadeh and Yan, 2014).

Several bioflocculants have been reported in literature with strong metal(loid) adsorption capability (Table 3.2). Many of them have been isolated from soil and wastewaters and have various metal(loid) biosorption capacity.

3.4 Biofilms and metal(loid) removal

Bacteria exist in colonies in nature, accumulating at interfaces to form cell mass as seen in biofilms, mats, flocs and sludge. Singh et al. (2006), described biofilms as clusters of microbial cells attached to a surface. This usually occurs in moist environments where there is sufficient flow of nutrients. They can be formed by single or several species of organisms consisting of algae, bacteria, fungi and protozoa. Biofilm is a stable, highly structured ecosystem usually enclosed in a matrix adhering to a dormant or living surface (Farasin et al., 2017). Biofilms develop on different surfaces such as living tissues, soil and aquatic environments, medical devices, industrial or potable water piping systems (Vu et al., 2009). Microbial biofilms develop an increased preformed resistance to hard-surface disinfectants, antibiotics, extreme pH and oxygen levels, desiccation and metal(loid) ions (Morel et al., 2009).

Biofilm offers the advantage of availability of nutrient, persistence in different metabolic states, and communication and exchange of genetic materials. Biofilm-mediated removal of metal(loid)s is crucial as high concentration of metal(loid)s is detrimental to planktonic organisms. Planktonic communities usually have a problem when they come in contact with metal(loid)s because metal(loid) ions have unique chemistries, and function via varied biochemical routes of toxicity. Biofilm increases the tolerance of microorganisms to metal(loid)s and acidic environment, due to better adaptation and survival as they are protected within a matrix (Edwards and Kjellerup, 2013). The macromolecules within the biofilm matrix exhibit properties such as electrostatic interactions, hydrogen bonding and London dispersion forces. The extracellular polymeric substances (EPS)

plays an active role in metal(loid) sorption as many of them are anionic in nature (van Hullebusch et al., 2003). The close proximity within the biofilm and the physiological interactions among the organisms enhance metal(loid) tolerance and removal (Singh et al., 2006).

Metal(loid) sorption sites on biofilm include cell membrane and cell wall, extracellular polymeric substances and cytoplasm. Extracellular polymers consist mainly of polysaccharides, proteins, uronic acids (D-galacturonic, D-glucuronic and mannuronic acids), humic acids, ketal-linked pyruvates, lipids, nucleic acids, ionizable functional groups, and cell fragments (Quintelas et al., 2006; Pal and Paul, 2008; Vu et al., 2009). Removal of metal(loid) mediated by microbial biofilm offers an effective and innocuous alternative to sequestration by planktonic organisms. This is due to the better chance of adaptation that cells in biofilm offer and their subsequent survival in a protected matrix (Singh et al., 2006; Vu et al., 2009). The mineralization process is improved in biofilm mediated removal by maintaining optimum pH conditions, localized solute and redox potential (Vu et al., 2009). Acquired traits through horizontal gene transfer processes from the high cell density in the biofilm community also enhance their resistance abilities to metal(loid) ions (Farasin et al., 2017).

The factors that influenced biofilm EPS metal(loid) binding include the surrounding pH, ionic strength, metal(loid) concentration, EPS composition, physical gel state, organic matter component, and biomass. Furthermore, the ratio of carbohydrate to protein in the EPS also plays a major role in metal(loid) removal from wastewaters and in monitoring the state of the biofilm. A higher pH results in metal(loid) chelation with maximum binding affinity between pH 8–8.2, while a lower pH favors the release of meta(loid) ions from a bound state (van Hullebusch et al., 2003; Pal and Paul, 2008). At acidic pH, the binding anions such as COO^- groups are not readily available, thus a few metallic ions are bounded (Dominguez-Benetton et al., 2018). The encasing EPS protects microbial cells in biofilm from metal(loid) stress through metal(loid) ion binding or by preventing metal(loid) diffusion within the biofilm (Pal and Paul, 2008).

Table 3.2: Metal(loid) removal by bioflocculant produced by different bacterial species

Isolate	Source	Flocculating efficiency (%)	Metals removed	Metal removal (%)	pH	Reference
Pseudomonas aeruginosa		62.25	Cu (20 ppm)	87.39	7	Gomaa (2012)
			Hg (20 ppm)	89.09		
			Pb (40 ppm)	79.7		
			Cd (40 ppm)	79.73		
			As (60 ppm)	72.96		
			Zn (60 ppm)	80.59		
Rhodococcus erythropolis	Swine wastewater	44.7	Cu (100 mg/L)	96.9	6	Guo (2015)
Bacillus subtilis WD 90	Activated sludge	90.69	Cd (60 ppm)	90.9	8	Kaewchai and Prasertsan (2002)
			Ni (10 ppm)	90.7		
Enterobacter agglomerans	Activated sludge	87.84	Cd (60 ppm)	84.2	7	
			Ni (10 ppm)	92.8		

Paenibacillus elgii	Soil	87	Al (2 mM) Co (2 mM) Cu (2 mM) Pb (2 mM)	72 49 53 60	7.2	Li et al. (2013)
Bacillus cereus	Marine sponge (*Dendrilla nigra*)	94	Cu (1-3 mM) Hg (1-3 mM) Zn (1-3 mM)	Significant reduction	7	Sajayan et al. (2017)
Bacillus firmus	Soil		Cu (1000 mg/L) Pb (1000 mg/L) Zn (1000 mg/L)	74.9 98.3 61.8	4 4.5 6	Salehizadeh and Shojaosadati (2003)
Rothia sp. ZHT4-13	*Ruditapes philippinarum* conglutination mud	86.22	Cr (1 mg/L) Ni (20 mg/L)	69.3 19.2	9	Gao et al. (2009)

Colonization of microorganisms on metal(loid)s is mediated by biofilms which play a crucial role in their attachment. Metal sulfide dissolution is proposed to take place in the EPS layer of biofilm, and the method of occurrence is based on two postulates. It is first presumed that the complex formed by the metal ion and the EPS is decreased as a result of an electron tunneling effect. In addition, the glucuronic acid-metal ion complex is unstable, thus permitting metal ions to move inside the EPS space. If the metal ions migrate near the outer membrane, they will be re-oxidized by the cell's enzymatic system and can then reenter the oxidation/reduction cycle (Vu et al., 2009).

Pseudomonas aeruginosa biofilm was demonstrated to be more resistant to Cu, Pb and Zn ions than the free planktonic cells in a rotating-disk biofilm reactor (Teitzel and Parsek, 2003). The EPS encasing the biofilm was found to protect against metal(loid) by binding metal(loid) ions and preventing metal(loid) ion diffusion into the biofilm. In like manner, Au, Fe and La were demonstrated to bind to *P. aeruginosa* PAO1 biofilm in a more significant amount than the planktonic cells (Langley and Beveridge, 1999). The binding of metals by the biofilm of *P. aeruginosa* led to an increase in the electron density of the cells' surfaces when observed with a transmission electron micrograph. The shift in the production of lipopolysaccharide in *P. aeruginosa* PAO1 biofilm could be responsible for metal(loid) resistance. This showed that the mode of growth (biofilm or planktonic) affects the extent to which cells bind and precipitate metal(loid) ions (Langley and Beveridge, 1999).

The planktonic and biofilm cells of *Candida tropicalis* were exposed to a diverse range of metal(loid) ions (Ag, Al, Au, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Se, Te and Zn) in a time-dependent experiment (Harrison et al., 2006). The biofilm of *C. tropicalis* was found to be highly resistant to metal(loid) toxicity and was also found to be 65 times more tolerant than planktonic cells. The extracellular matrix of the biofilm was observed to be the contributing factor responsible for metal(loid) resistance. The EPS restricted the movement of metal(loid) ions into the biofilm of *C. tropicalis*. The densely packed cells formed by the biofilm, which later developed into dome-

shaped microcolonies, could also be responsible for the metal(loid) resistance observed in the study (Harrison et al., 2006).

The negatively charged carboxyl, phosphoryl and sulfhydryl groups existing in the EPS of biofilm produced *Escherichia coli* PHL628 was suggested to be responsible for sorption of copper (Hu et al., 2007). In addition, increased exopolysaccharide produced per cell within the biofilm and the tortuosity that possibly impede transport of Cu into biofilms were also reported to be responsible for Cu sorption. The study showed that Cu sorption by the biofilm is 6 times more than planktonic cells of *E. coli* PHL628.

Quintelas et al. (2006) investigated the removal of chromium by biofilm of *Arthrobacter viscosus* supported on granular activated carbon from wastewater. They observed a simultaneous increase in chromium uptake by *A. viscosus* biofilm in the presence of organic compounds (o-cresol, phenol and chlorophenol). The biofilm can be applied in wastewater removal of metal(loid)s.

Von Canstein et al. (2002) evaluated the diversity of species and the efficiency of mercury-reducing biofilm in a packed-bed reactor. They found that biofilms composed of multi-species organisms retained large quantities of mercury compared to mono-species biofilms in a changing environment. The diversity of the microbial species inhabiting the biofilm resulted in superior bioreactor performance in a rapidly changing environment, hence leading to efficient metal(loid) removal (Von Canstein et al., 2002). Environmental and metal(loid) stress related issues that affect planktonic organisms and monoculture biofilms were reduced using multi-species biofilms. Biofilms with multi-species offer a superior survival niche, and increase the metabolic abilities in the presence of metal(loid)s (Singh and Cameotra, 2004). There has been growing interest in metal(loid) removal by biofilm, and with genetic engineering, it could be manipulated and improved upon for efficient metal(loid) removal.

Conclusions

The exploration of the properties of microbial polymers for in situ bioremediation will enhance metal(loid) removal from contaminated environments as most experiments are still laboratory based. The metabolic properties of microorganisms can be exploited for this purpose since the polymers are specific, tolerant to extreme conditions and have low toxicity. Techniques to elucidate the mechanisms of action of these polymers are needed to further understand their role in metal(loid)-binding and metal(loid) decontamination. Molecular techniques that enable the construction and improvement of the production of these biopolymers with specific metal(loid)-binding characteristics from robust environmental strains hold future promise. This can be accomplished through alteration of metabolic pathways, and insertion of new genes and plasmids into the genome of robust organisms. The description and characterization of enzymes, genes, and proteins responsible for the synthesis and metal(loid)-binding mechanism of these biopolymers will also provide insight on the level of alteration needed under different environmental conditions.

CHAPTER FOUR

IDENTIFICATION AND CHARACTERIZATION OF HEAVY METAL RESISTANT BACTERIAL ISOLATES FROM GOLD MINING SOIL

Abstract

The selection of tolerant bacterial species for remediation of contaminated environments is based on their response and growth kinetics in polluted medium. Microbial population and diversity in heavy metal polluted environment are influenced by the bioavailable concentration of the heavy metals. This study investigated bacterial diversity in gold mining soil, their metal tolerance, growth kinetics and biopolymer production potential. Ninety-eight (98) heavy metal resistant bacteria were isolated and tested for their ability to tolerate cadmium, chromium and lead. Fifty-five percent of the isolates were resistant to Pb (1000 mg/L) and 10% and 20% of the isolates were able to resist Cd and Cr at level greater than 200 mg/L respectively. The tolerance pattern showed by the resistant isolates was the trend of Pb>Cr >Cd. Fifty percent of the isolates are multi-metal tolerant. *Bacillus* species were the dominant organisms with *Acinetobacter*, *Citrobacter*, *Enterobacter*, *Pantoea* and *Pseudomonas* species also isolated. Metal resistance genes: *cadA* and *czcD* (Cd), *chrA* (Cr), *PbrA* and *PbrT* (Pb) were shown to be present in some of the isolates using PCR. Fifty percent of the isolates showed α-hemolysis with emulsification of various hydrocarbons. The highest flocculating efficiency of 71.3% was shown by *Pantoea* sp. The multi-metal tolerance and growth kinetics assays revealed the potential of these isolates in heavy metal removal from contaminated environment.

Keywords: bacteria resistance, bioflocculant, bioremediation, biosurfactant, metal tolerance, pollution

4.1 Introduction

Incautious use of natural resources coupled with industrialization, mining, and urbanization have resulted in the buildup of pollutants in the environment. While mining activities have advanced the socio-economic development of national economies around the world, they have impacted negatively on the environment resulting in buildup of pollutants (Ayangbenro et al., 2018).

Amongst such pollutants are metal(loid)s which have generated wide interest because of their toxicity and the threat they pose to the health of biological systems (Ayangbenro and Babalola, 2018; Prabhu et al., 2018). Metal pollution arising from mining activities has negative impacts on the environment, and is of particular interest because of the persistent nature of these metals in the environment (Abdul-Wahab and Marikar, 2012), and are often irreversible resulting in difficulties in remediation (Deng et al., 2018). These metals are toxic at low concentration and could accumulate in the food web, causing damage to biological systems (Ayangbenro and Babalola, 2017).

The mobility, bioavailability, leaching and toxicity of metals and metalloids in soil is dependent on the soil properties. The mobility and toxicity of metals in soil is greatly influenced by pH, presence and amount of organic and inorganic components, redox reactions, sorbent nature, and nutrients (Violante et al., 2010). High concentration of metals in the soil are highly toxic to microorganisms and impact on the ecosystem functioning (Singh et al., 2014).

Metal toxicity affects microbial diversity and population size, morphology, activities, metabolism and growth, enzyme activities, and genetic and nucleic acid structure (Fashola et al., 2016; Xie et al., 2016; Ayangbenro and Babalola, 2017). This suppresses bacterial population and diversity in soil that plays a key role in cycling of nutrients in the environment, consequently, impacting negatively on the health and productivity of such environment (Zampieri et al., 2016).

Microorganisms have developed defense strategies for their continued existence in metal polluted environments and stress combating mechanisms against heavy metals. The coping mechanisms include complex formation, efflux of metals out of the cell, reduction of metals to less toxic forms, precipitation, and transformation (Bolan et al., 2014; Zampieri et al., 2016; Ayangbenro and Babalola, 2017). Due to the transformation of metal species to less toxic species by bacteria, they have potential application in removal of metals and metalloids from polluted environments. Studies have shown the potential application of metal resistant bacterial isolates from contaminated soil in the removal of metals (Shin et al., 2012; Chihomvu et al., 2014; Oladipo et al., 2018).

With increasing mining activities in the North West province of South Africa, there is the need to study indigenous resistant bacterial isolates associated with mining activities and their diversity. Thus, this study aimed to isolate and characterize heavy metal resistant bacteria from gold mining sites in Vryburg, South Africa and to screen the isolates for biopolymer production.

4.2 Materials and methods

4.2.1 Soil sampling and analysis

Soil samples were collected from active gold mines in Vryburg, North West Province, South Africa. Control soil samples were collected around the mines. Soil samples were collected in triplicate at depths of 10-30 cm with a soil auger in June 2016 and transported to the laboratory for analysis in sterile plastic bags.

Some of the samples were dried at 80˚C and sieved through a 2 mm sieve and used to determine the heavy metal composition (Freitas et al., 2004). The pH of the soil samples was determined in soil/water suspension (1:2.5) with a pH meter (Jenway, Bibby Scientific Ltd, Stone, Staffs, UK).

Soil samples were analyzed for heavy metals by digesting with acids (HCl-HNO_3) using a microwave reaction system (Multiwave 3000, Anton Paar GmbH, Graz, Austria). The digested samples were analyzed for total concentration of metals using Inducible Conductivity Plasma-Optical Emission Spectroscopy (725 ICP-OES, Agilent Technologies, Palo Alto, CA, USA) (Wei and Yang, 2010; Ali and Shakrani, 2014).

The ammonium acetate method was used in determining the Cation Exchange Capacity (CEC) and extractable ions of the soil samples. Soil samples (25 g each) were weighed into Erlenmeyer flasks (500 ml) and 125 ml 1 M ammonium acetate was added. The mixture was shaken and allowed to stand for 5 h. The solution was allowed to leach through a leaching tube and the filtrate was used for determination of Ca, K, Mg and Na in the samples. The ammonium saturated sample in the leaching tube was washed with 50% ethyl alcohol (150 ml) and then by 90% ethyl alcohol (30 ml) to remove the excess ammonium. The leachate was then transferred into a 250 ml volumetric flask and brought to volume with 1 M potassium chloride. The adsorbed ammonium was leached with 1 M potassium chloride (25 ml). The CEC was calculated as:

CEC (cmol (+)/Kg) = (NH_4^+ in extract - NH_4^+ in blank)/18.

The procedure of Filgueira et al. (2006) was used for the determination of the particle size. Forty gram of air dried and sieved soil sample was weighed into 500 ml beaker. One hundred millilitre (100 ml) of 50 g/L sodium-hexametaphosphate solution and 250 ml distilled water were added. The mixture was stirred with a glass rod, left overnight and transferred to a dispersing cup, and homogenized for 5 min with an electric mixer. The mixture was then transferred to a sedimentation cylinder and distilled water added to make up one litre. A plunger was used to mix the suspension thoroughly before inserting a hydrometer into the suspension. The reading was taken after 40 s. The suspension was homogenized again and the reading was taken after 4 min. The hydrometer was then removed and cleaned. The hydrometer reading was taken again after 6 h to determine the

clay particle density. The following equation was used to calculate the percentage of clay, sand and silt in each of the samples:

$$\%\ clay\ = \frac{corrected\ hydrometer\ reading\ after\ 6\ h}{mass\ of\ sample} \times 100$$

$$\%\ silt = \frac{corrected\ hydrometer\ reading\ after\ 40\ s}{mass\ of\ sample} \times 100\ - \%\ clay$$

$$\%\ Sand = 100\ - (\%\ silt - \%\ clay).$$

Organic carbon of the soil samples was determined using the Walkley-Black method (Abollino et al., 2002). The organic carbon was oxidized to CO_2 in the presence of $K_2Cr_2O_7$ in the presence of H_2SO_4. The unreacted $K_2Cr_2O_7$ was titrated with $FeSO_4.7H_2O$. The average organic carbon in soil organic matter is equal to 58%, then the conversion factor of 1.724 was used to calculate the organic carbon content.

The soil moisture content was determined by oven-drying the soil samples at 105 °C for 24 h while the soil nitrate was extracted using potassium chloride solution (Colombo et al., 2016). Five gram of the fresh soil sample and 50 ml of 2 M KCl solution were mixed and shaken for 60 min at 150 rpm and the mixture was filtered. The filtrate was analyzed for nitrate. The extractable phosphorus was determined using Bray-1 reagent. The phosphorus was extracted with 0.025 N HCl and 0.03 N NH_4F. One gram of the air-dried soil sample was extracted with 10 ml of Bray-1 reagent and shaken for 5 min. The phosphorus extracted was determined by measuring the intensity of blue color developed by the filtrate when treated with molybadate- ascorbic acid reagent using a Brinkman PC 900 probe colorimeter at 880 nm.

Sulfate was measured by weighing 10 g of each of the soil sample separately into a 100 ml Erlenmeyer flask and extracting with 25 ml of the extracting solution (39 g NH_4OAC and 1 litre of 0.25 M acetic acid). The mixture was mixed and shaken at 200 rpm for 30 min and 0.25 g activated charcoal was added. The resultant mixture was then shaken for 3 min and filtered using sulfate free filter paper and washed with the extracting solution. The filtrate (10 ml) was pipetted

into an Erlenmeyer flask (50 ml) and 1 ml of acid seed (6 M HCl + K_2SO_4 (20 mg) and 50 ml of 40 mg standard solution plus 50 ml of concentrated HCl) was added. The mixture was shaken and 0.5 g of $BaCl_2.H_20$ crystals was added and allowed to stand for 1 min and shaken regularly to dissolve the crystals. The sulfate content of the mixture was determined by measuring the optical density using a UV spectrophotometer at 420 nm.

4.2.2 Isolation and screening of heavy metal resistant bacteria isolates

Heavy metal (Cd, Cr, Pb) resistant bacteria were isolated as described by Rajkumar and Freitas (2008). One gram of each of the collected soil samples were serially diluted and 10^{-4} plated out on Luria-Bertani (LB) agar that had been supplemented with 50 mg/L of each metal solution and a mixture of all the metals. The metal solutions ($CdSO_4$, K_2CrO_4 and $Pb(NO_3)_2$) were filter sterilized through 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA). The plates were incubated at 30˚C for 48 h. Resistant isolates were then screened for ability to grow in different metal concentrations (100 to 1000 mg/L) on LB agar plates (Srinath et al., 2002). Growth of resistant isolates was monitored in the presence of heavy metals (100 mg/L of each metal) in LB broth and the optical density was measured at 24 h interval for 6 days.

4.2.3 Biochemical characterization of bacteria isolates

The following biochemical tests were carried out on the heavy metal resistant isolates: Gram reaction, catalase test, citrate utilization, gelatin liquefaction, hydrogen sulfide production, oxidase, nitrate reduction, starch hydrolysis, sugar fermentation test (glucose, arabinose, fructose, maltose, sucrose, lactose, mannose, melibiose, sorbitol, maltose), urease, and Voges-Proskauer test. Pure cultures of the isolates were identified according to the Bergey's manual of Determinative Bacteriology (Garrity et al., 2004).

4.2.4 Genomic DNA extraction

The DNA of the selected heavy metal resistant bacterial isolates were extracted from pure cultures grown on LB agar using a ZR soil microbe DNA mini prep ™ DNA extraction kit (Zymo Research, CA, USA) according to the kit's protocol. The DNA quality and quantity was determined using a NanoDrop Lite spectrophotometer (Thermo Fischer Scientific, CA, USA).

4.2.5 Amplification of 16S rDNA and detection of heavy metal resistance genes

The 16S rRNA gene was amplified for each isolate using universal primers F1 (forward, 5′-AGAGTTTGATCCTGGCTCAG-3′) and R2 (reverse, 5′-ACGGCTACCTTGTTACGACTT-3′) with approximately 1500 base pair amplicon (Marin et al., 2011). The PCR was performed with a total volume of 25 µl of a reaction mixture consisting of 12.5 µl 2X master mix (One Taq® Hot Start Quick Load, Biolabs, England), 1 µl of the DNA template, 0.5 µl (10 µM working solution) of each of the forward and reverse primer, and 11 µl nuclease free water. The PCR was performed in a C1000 thermal cycler (Bio-Rad, CA, USA) with preincubation step of 95˚C for 5 min and 35 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 30 s, and extension at 72˚C for 1 min, followed by a final extension step of 72˚C for 10 min. The PCR amplicons were analyzed by electrophoresis on agarose gel (1% w/v) containing ethidium bromide (1 µl) and the size of the band was determined using 1 kb molecular marker. The gel images were captured with gel documentation system (Gel Doc 2000, Bio-Rad, USA).

Analysis of heavy metal encoding genes were done with primers encoding for cadmium (*cadA*, *CzcA*, *CzcB* and *CzcD*), chromium (*chrA* and *chrB*) and lead (*PbrA* and *PbrT*) for each isolate. The sequences of each primer set are presented in Table 4.1 with their expected band size. All the primer sets used for PCR amplifications were synthesized by Whitehead Scientific, Integrated DNA Technologies, South Africa.

4.2.6 Sequencing reaction

Polymerase chain reaction amplicons were sequenced with PRISM TM Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Bio systems, USA) at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa. The forward and reverse primers were used in sequencing of the purified PCR products. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analyzed using CLC Main Workbench 7.

The 16S rRNA gene sequences were aligned and compared to other 16S rRNA genes in the NCBI GenBank database. The most similar sequences were aligned, analyzed and the phlogenetic analysis was done using MEGA 7.0 software (Kumar et al., 2016b).

4.2.7 Screening of biosurfactant production by heavy metal resistant isolates

Biosurfactant production was quantified in pure cultures of the isolates by different methods which include hemolytic activity, drop collapse test, and emulsification activity. All tests were carried out in duplicate. The hemolytic test on blood agar was carried out as described by Bicca et al. (1999).

The cultivation medium for biosurfactant production contains (g/L): yeast extract, 0.5; sucrose, 5.0; $Na_2HPO_4 \cdot 12H_2O$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.02; KH_2PO_4, 0.4 and peptone, 20.0 (Sriram et al., 2011). The medium was seeded with 3% inoculum prepared from LB broth into 50 ml cultivation medium in a 250 ml Erlenmeyer flask and incubated at 37˚C for 7 days at 160 rpm.
Hemolysis, drop collapse and emulsification tests were taken with supernatant obtained by centrifuging the cultures at 10,000 rpm for 20 min.

The drop collapse test was determined as described by Sriram et al. (2011). Mineral oil (2 µl) was added to each well of a 96-well microtiter plate and allowed to equilibrate for one hour at 30˚C. Thereafter, 5 µl of the culture supernatant was added to the center of each well over the oil layer.

After one minute, the shape of the oil drop was examined. A flattened drop was recorded as positive for biosurfactant production. Water was used as negative control.

Table 4.1: Primer sets used in PCR amplification of heavy metal resistance genes

Gene	Primer sequence (5′ →3′)	PCR conditions	PCR product size (bp)	Reference
CzcA	F- GTTTGAACGTATCATTAGTTTC R - GTAGCCATCCGAAATATTCG	Initial denaturation at 95°C for 5 min, 34 cycles at 94°C for 90 s, 57°C for 90 s, 72°C for 2 min and a final extension at 72°C for 7 min.	1885	Nies et al. (1989)
CzcD	F- CAGGTCACTGACACGACCAT R- CATGCTGATGAGATTGATGATC	Initial denaturation at 95°C for 5 min, 34 cycles at 94°C for 90 s, 52°C for 90 s, 72°C for 2 min and a final extension at 72°C for 7 min.	398	Nies et al. (1989)
CzcB	F- CTATTTCGAACAAACAAAAGG R- CTTCAGAACAAAACTGTTGG	Initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 90 s, 57°C for 1 min, 72°C for 90 s and a final extension at 72°C for 3 min.	1520	Abou-Shanab et al. (2007)
PbrT	F- ATGGTGATTGCTTTAGTT R- TTAGGCTTGCTTCTTTTT	Initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 30 s, 50°C for 90 s, 72°C for 2 min and a final extension at 72°C for 7 min.		Shin et al. (2012)
PbrA	F- ATGAGCGAATGTGGCTCGAAG R- TCATCGACGCAACAGCCTCAA	Initial denaturation at 95°C for 4 min, 35 cycles at 95°C for 30 s, 60°C for 90 s, 72°C for 2 min and a final extension at of 72°C for 7 min	766	Shin et al. (2012)
*chr*A	F- CTTATACGCTACGCCAACTG R- GTAATGGCATTCAGTCGCTTG	Initial denaturation at 95°C for 5 min, 35 cycles at 94°C for 30 s, 58°C for 90 s, 72°C for 2 min and a final extension step of 72°C for 5 min	1292	Nies et al. (1990)
chrB	F- GTCGTTAGCTTGCCAACATC R- CGAAAGCAAGATGTCGATCG	Initial denaturation at 95°C for 5 min, 30 cycles at 94°C for 30 s, 57°C for 60 s, 72°C for 2 min and a final extension at 72° C for 3 min	450	Nies et al. (1990)

cadA	F- GACAAGACYGGMACYMTCAC R- GCRTGGTTRATSCGTC	Initial denaturation at 95°C for 5 min, 34 cycles at 94°C for 90 s, 57°C for 60 s, 72°C for 2 min and a final extension at 72° C for 7 min	600	Dell'Amico et al. (2008)

The emulsification activity of resistant isolates was determined by measuring the emulsification index (E_{24}) after 24 h. Two millitre (2 ml) of the culture supernatant was mixed with kerosene (2 ml) in a test tube. The mixture was vortexed at high speed for 2 minutes. The E_{24} was calculated as the percentage of height of emulsified layer divided by total height of the liquid column (Rizzo et al., 2015). The test was also performed using engine oil and vegetable oil in place of kerosene.

4.2.8 Screening of metal resistant isolates for bioflocculating activity

Heavy metal resistant isolates were pre-cultured in 50 ml LB broth on a rotary shaker (200 rpm) at 30˚C for 24 hours. One mililitre (0.5 McFarland) of the culture broth was used to inoculate 100 ml fermentation medium after washing with sterile water. The fermentation medium contains (g/L): K_2HPO_4, 2; NH_4Cl, 1; $MgSO_4$, 0.5; $CaCO_3$ 0.2; KH_2PO_4, 5; glucose, 2; peptone, 5 and yeast extract, 5 (Subudhi et al., 2014). The medium was maintained at pH of 7.2 and incubated for 72 h at 30˚C on a rotary shaker (200 rpm). After incubation, the culture broth was centrifuged at 6000 *x*g for 30 min and the supernatant was evaluated for flocculating activity. The flocculating activity was measured using kaolin clay suspension (0.5 g of kaolin clay in 100 ml distilled water). Forty-five millilitre (45 ml) of the kaolin clay suspension was mixed with 0.5 ml of the supernatant and 4.5 ml of 1% $CaCl_2$ was added to the mixture. The mixture was vortexed for 2 minutes and left standing for 5 min at room temperature. The optical density of the supernatant and the blank (distilled water) was measured at 550 nm (Gomaa, 2012). The flocculating activity was defined as follows: flocculating activity = $\frac{A-B}{A} \times 100$

Where A and B are the optical densities of the control and sample respectively at 550 nm.

4.2.9 Statistical analysis

The principal component analysis was used to determine the relationship between physicochemical parameters using SPSS statistical package (v 25.0). Analysis of variance (ANOVA) with Least significant difference test (LSD) and Duncan multiple test ($p < 0.05$) were used to compare treatment means using SPSS.

4.3 Results

4.3.1 Physicochemical properties of the soil samples

The sampling location is shown in Table 4.2 and the geographical map of the sampling sites is presented in Figure 4.1. A total of seven gold mining soil samples (sampling sites 1-7) and a control soil sample (sampling site 8) were collected from Vryburg, South Africa in June 2016. The particle size distribution of the soil samples is presented in Table 4.2 The soil samples were generally sandy in nature which accounts for over 60% in each sample. The range of the particle size is between 12-26%, 62-86% and 0-26% for clay, sand and silt respectively for the soil samples. The soil sample from site 3 has the highest sand distribution (86%) while the sample from site 4 has the lowest (62%). The silt composition is very low in all samples. Site 5 (26%) has the highest clay composition while sites 3 and 4 have the lowest (12%). The mean moisture content of the soil samples is also shown in Table 4.2. The moisture content ranged between 0.153-3.89%. Sampling site 4 had the highest moisture content of 3.89% and also had the lowest sand composition (62%). The particle size of the samples were statistically significant ($P < 0.05$).

The average pH of the samples ranged between 5.9-7.5 (Figure 4.2). This indicates that the soil samples are slightly acidic to neutral. The soil sample from sampling site 3 has the highest pH of 7.5 which falls within the neutral pH range. Soils from sampling sites 1, 2, 5, 6 and 7 are slightly acidic in nature (Figure 4.2). Samples 3, 4 and 8 fall within the neutral pH range.

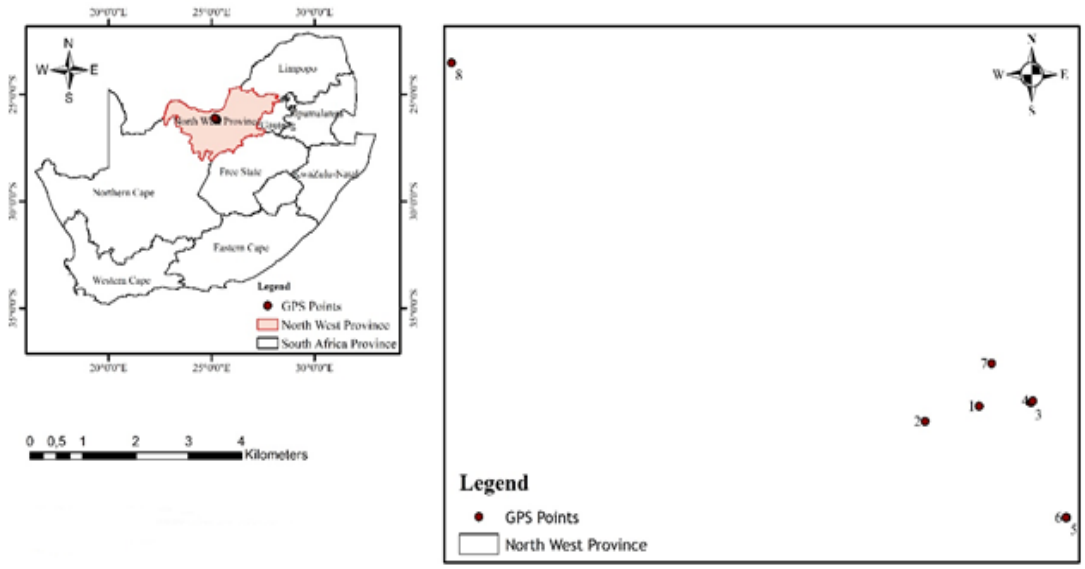


Figure 4.1: Map showing the soil sampling location

The cation exchange capacity (CEC) of the soil samples varied from each sampling location. It ranged between 2.71 cmol(+)/kg from sampling site 8 to 12.98 cmol(+)/kg from sampling site 2 (Figure 4.3). The CEC values were statistically significant ($P < 0.05$) with each samples.

The other chemical and physical properties of the soil samples are presented in Table 4.3. The organic carbon ranged between 0.09 to 1.07% (Table 4.3). This showed that the soil samples have low organic content as a result of mining activities in that environment which had impacted on the activities of plants and microorganisms in the environment. The control soil sample also had a very low amount of organic carbon (0.3%) which can be attributed to the parent material of the soil sample.

The soil samples are rich in sulfur (Table 4.3). The range of the sulfur value is between 1.973 mg/kg in the control (sample 8) to 30.78 mg/kg in sample 4. High sulfate content in the samples can be attributed to sulfur minerals in gold mining which has been exposed to atmosphere and water after excavation. Sulfate values ranged between 2.583 mg/kg (in sample 1) to 2312.858 kg/mg (in sample 4) (Table 4.3). In all samples, the clay content and organic carbon are low which makes the soil to be permeable.

Table 4.2: Sampling location and particle size distribution of the soil samples

Sampling site	Location	Sand (2.0-0.05 mm) (%)	Silt (0.05-0.002 mm) (%)	Clay (<0.002 mm) (%)	Moisture content (%)
1	S 26.1681354 E 25.246582	84.00 (0.57) ^{ab}	2.00 (0.58) ^{de}	14.00 (0.00) ^{cd}	0.153 (0.00) ^{h}
2	S 26.1706352 E 25.237386	84.00 (1.16) ^{ab}	0.00 (0.00) ^{e}	16.00 (1.16) ^{c}	0.325 (0.00) ^{e}
3	S 26.1675545 E 25.255411	86.00 (0.58) ^{a}	2.00 (0.00) ^{de}	12.00 (0.58) ^{d}	0.234 (0.00) ^{f}
4	S 26.1672729 E 25.255739	62.00 (0.58) ^{e}	26.00 (0.58) ^{a}	12.00 (1.56) ^{d}	3.890 (0.03) ^{a}
5	S 26.1863994 E 25.261444	68.00 (2.31) ^{de}	6.00 (1.73) ^{bc}	26.00 (0.58) ^{a}	2.317 (0.11) ^{b}
6	S 26.1863937 E 25.261355	78.00 (2.89) ^{bc}	8.00 (1.16) ^{b}	14.00 (0.57) ^{cd}	0.188 (0.00) ^{g}
7	S 26.1611553 E 25.248723	74.00 (3.46) ^{cd}	4.00 (1.16) ^{cd}	22.00 (1.73) ^{b}	1.469 (0.00) ^{d}
8	S 26.1118325 E 25.156812	84.00 (2.31) ^{ab}	2.00 (0.57) ^{de}	14.00 (0.57) ^{cd}	2.043 (0.01) ^{c}

Values are means of triplicate readings and values in parenthesis are ± standard error
Values with the same letter within a column are not significantly different ($P < 0.05$)

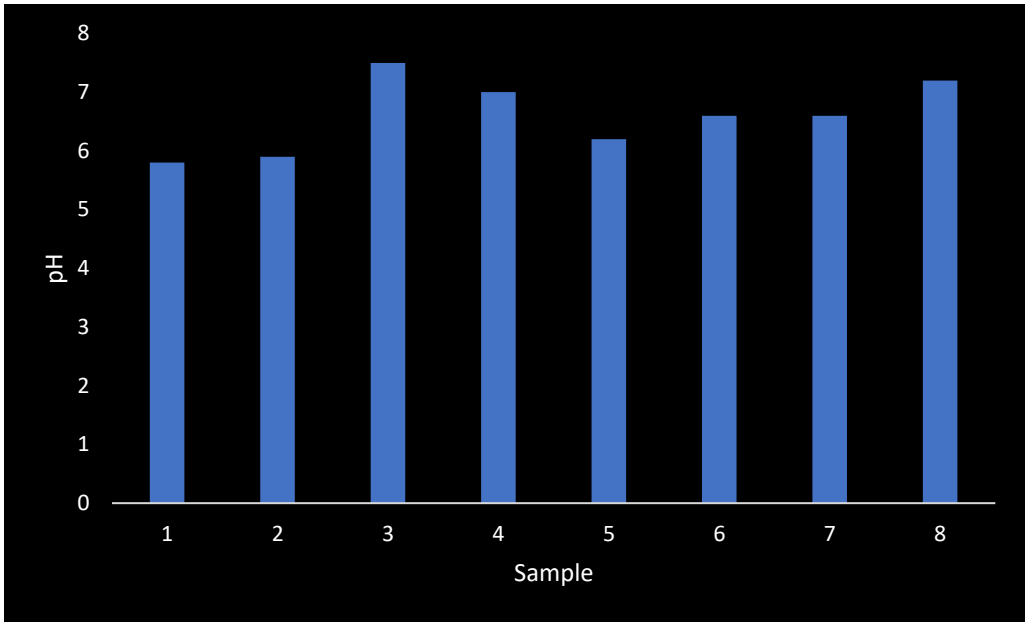


Figure 4.2: The pH of the soil samples from different sampling locations

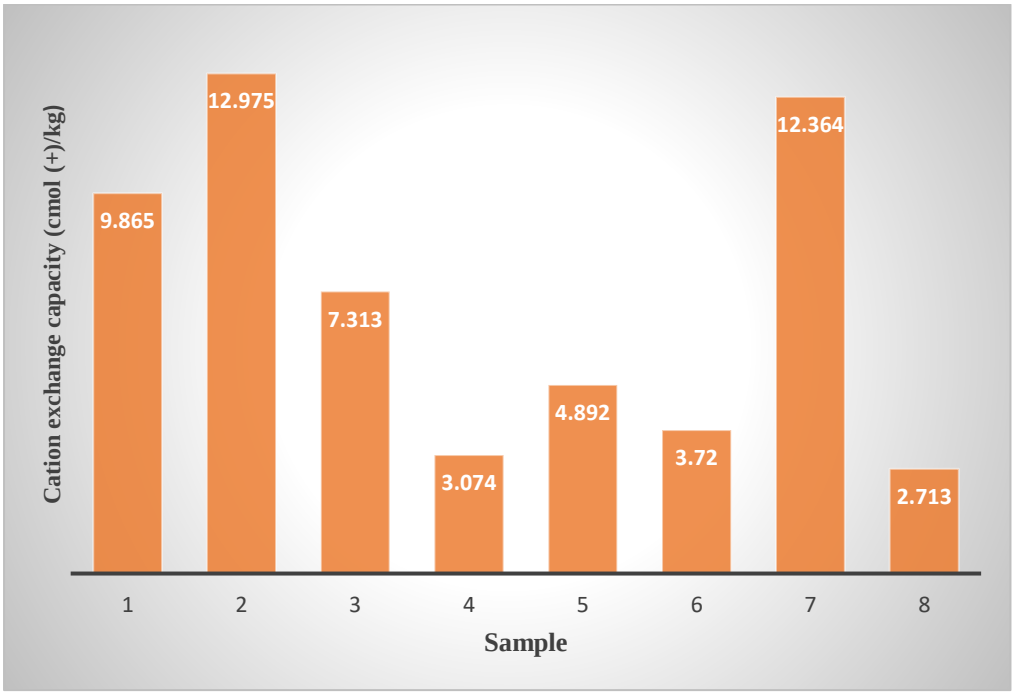


Figure 4.3: Cation exchange capacity of the soil samples

Low nitrogen contents were observed in all samples which is between 0.027 mg/kg of soil in sample 4 to 0.101 mg/kg of soil in sample 7 (Table 4.3). Nitrate concentration in the samples is within the range of 0.63 mg/kg of soil in sample 2 to 11.28 mg/kg of soil in sample 4.

Low concentrations of sodium, potassium and magnesium were observed in the collected soil samples (Table 4.3). The concentration of sodium ranged between 0.073 mg/kg in the control soil sample to 1.173 mg/kg of soil in sample 4. The values recorded for potassium is between 0.173 mg/kg in sample 2 to 0.73 mg/kg in sample 7. Magnesium is between 0.51 mg/kg in sample 8 to 8 mg/kg in sample 4. The calcium content is relatively high compared to sodium, potassium and magnesium. The concentration is between 0.983 mg/kg in sample 2 to 21.4 mg/kg in sample 4. These values are significantly different ($P < 0.05$) with each sampling location. Sample 4 generally had the highest concentration of SO_4, S, Mg, Ca, Na and NO_3, while sample 2 had the lowest concentration of organic carbon, NO_3, K and Ca (Table 4.3).

The correlation between all the physicochemical characteristics of the soil samples is presented in Table 4.4. The correlation matrix showed that there were positive correlations between organic carbon and P, N, NO_3, CEC, K, Ca, Mg, and S. Negative correlations were observed between organic carbon and pH, Na and SO_4, phosphorus and pH, Na, Ca, Mg, S and SO_4, and nitrogen and pH, Na, Na, Ca, Mg, S and SO_4. Strong correlations were observed between sodium and sulfate ($r = 0.999$), calcium and sulfur ($r = 0.999$), calcium and magnesium ($r = 0.989$) and, magnesium and sulfur ($r = 0.994$) (Table 4.4).

Table 4.3: Physical and chemical properties of the soil samples

Sample	Organic carbon (%)	Phosphorus (mg/kg)	Total Nitrogen (%)	Nitrate (mg/kg)	Sodium (mg/kg)	Potassium (mg/kg)	Calcium (mg/kg)	Magnesium (mg/kg)	Sulfur (mg/kg)	Sulfate (mg/kg)
1	0.240 (0.12) ^{d}	1.020 (0.01) ^{a}	0.045 (0.00) ^{d}	2.800 (0.04) ^{d}	0.084 (0.00) ^{de}	0.345 (0.00) ^{c}	1.491 (0.00) ^{e}	0.774 (0.00) ^{f}	2.693 (0.00) ^{f}	2.583 (0.06) ^{f}
2	0.090 (0.06) ^{e}	0.300 (0.00) ^{a}	0.032 (0.00) ^{f}	0.630 (0.01) ^{h}	0.079 (0.00) ^{ef}	0.173 (0.03) ^{e}	0.983 (0.00) ^{g}	0.769 (0.01) ^{f}	2.021 (0.01) ^{g}	5.150 (0.03) ^{e}
3	0.230 (0.06) ^{d}	0.663 (0.02) ^{a}	0.040 (0.02) ^{e}	2.260 (0.06) ^{g}	0.107 (0.00) ^{c}	0.198 (0.00) ^{e}	3.169 (0.00) ^{d}	1.249 (0.00) ^{e}	4.724 (0.01) ^{e}	5.820 (0.04) ^{e}
4	0.350 (0.23) ^{c}	0.450 (0.03) ^{a}	0.027 (0.00) ^{g}	11.283 (0.01) ^{a}	1.173 (0.00) ^{a}	0.258 (0.00) ^{d}	21.436 (0.03) ^{a}	7.957 (0.01) ^{a}	30.784 (0.00) ^{a}	2312.857 (1.19) ^{a}
5	0.750 (0.11) ^{b}	2.333 (0.02) ^{a}	0.061 (0.00) ^{b}	6.640 (0.23) ^{b}	0.089 (0.00) ^{d}	0.540 (0.00) ^{b}	8.132 (0.00) ^{b}	2.322 (0.00) ^{d}	11.085 (0.00) ^{d}	11.080 (0.02) ^{c}
6	0.340 (0.01) ^{c}	17.340 (16.83) ^{a}	0.054 (0.00) ^{c}	4.747 (0.01) ^{c}	0.312 (0.00) ^{b}	0.361 (0.00) ^{c}	8.035 (0.00) ^{c}	2.954 (0.00) ^{b}	11.677 (0.01) ^{b}	537.793 (0.50) ^{b}
7	1.070 (0.04) ^{a}	9.323 (0.01) ^{a}	0.101 (0.00) ^{a}	2.440 (0.04) ^{f}	0.078 (0.00) ^{fg}	0.732 (0.00) ^{a}	8.034 (0.00) ^{c}	2.387 (0.00) ^{c}	11.255 (0.03) ^{c}	8.403 (0.30) ^{d}
8	0.300 (0.01) ^{c}	7.707 (0.01) ^{a}	0.046 (0.00) ^{d}	2.570 (0.04) ^{e}	0.073 (0.00) ^{g}	0.363 (0.00) ^{c}	1.029 (0.00) ^{f}	0.506 (0.00) ^{g}	1.973 (0.00) ^{h}	3.097 (0.00) ^{f}

Values are means of triplicate readings and values in parenthesis are ± standard error
Values with the same letter within a column are not significantly different ($P < 0.05$)

Table 4.4: Correlation matrix between the physicochemical properties of the soil samples

	Organic carbon	Phosphorus	Total nitrogen	Nitrate	Cation exchange capacity	pH	Sodium	Potassium	Calcium	Magnesium	Sulfur	Sulfate
Organic carbon	1.000	0.656	0.888	0.144	0.133	-0.044	-0.115	0.949	0.281	0.146	0.252	-0.110
Phosphorus	0.656	1.000	0.728	-0.267	0.104	0.211	-0.331	0.727	-0.152	-0.231	-0.165	-0.322
Total nitrogen	0.888	0.728	1.000	-0.228	0.323	-0.094	-0.409	0.934	-0.055	-0.177	-0.081	-0.400
Nitrate	0.144	-0.267	-0.228	1.000	-0.635	0.182	0.870	0.015	0.924	0.916	0.923	0.869
Cation exchange capacity	0.133	0.104	0.323	-0.635	1.000	-0.502	-0.465	0.141	-0.419	-0.424	-0.420	-0.469
pH	-0.044	0.211	-0.094	0.182	-0.502	1.000	0.293	-0.163	0.241	0.253	0.244	0.283
Sodium	-0.115	-0.331	-0.409	0.870	-0.465	0.293	1.000	-0.260	0.908	0.958	0.922	0.999
Potassium	0.949	0.727	0.934	0.015	0.141	-0.163	-0.260	1.000	0.109	-0.022	0.082	-0.249
Calcium	0.281	-0.152	-0.055	0.924	-0.419	0.241	0.908	0.109	1.000	0.989	0.999	0.908
Magnesium	0.146	-0.231	-0.177	0.916	-0.424	0.253	0.958	-0.022	0.989	1.000	0.994	0.959
Sulfur	0.252	-0.165	-0.081	0.923	-0.420	0.244	0.922	0.082	0.999	0.994	1.000	0.923
Sulfate	-0.110	-0.322	-0.400	0.869	-0.469	0.283	0.999	-0.249	0.908	0.959	0.923	1.000

Positive correlation means that as one data set increases, the other data set increases as well. Negative correlation means that as one data set increases, the other decreases.

Table 4.5 showed the heavy metal concentration in each sampling site by total acid digestion. The concentration of cadmium in the samples ranged between 0.024 mg/kg to 0.36 mg/kg, chromium is between 0.215 mg/kg to 0.413 mg/kg in the digested samples. Lead concentration is between 0.2 mg/kg in sample 6 to 1.9 mg/kg in sample 1, copper is between 0.12 mg/kg in sample 5 to 0.64 mg/kg in sample 5. Iron had the highest concentration among the samples collected with values between 127.9 mg/kg in sample 5 to 1213.2 mg/kg in sample 4. Sample 4 generally has the highest concentration of heavy metals (Table 4.5).

The correlation between heavy metal concentration soil samples and pH of the soil samples is presented in Table 4.6. There is a positive correlation between all metals and pH. This indicates that the bioavailability of these metals is pH dependent and these metals are associated with one another.

4.3.2. Diversity of bacterial isolates from the study sites

A total of ninety-eight (98) heavy metal resistant bacteria were isolated from all the soil samples. Their distribution is shown in Figure 4.4. Twenty-six percent of the bacterial isolates were isolated from sample 3 while sample 1 accounted for 24% of the isolates. The lowest number of isolates were from sample 4 with 5% (Figure 4.4), which also has the highest concentration of the metals determined in the digested samples (Table 4.5). Figure 4.5 showed the pictures of some of the plates used for isolation and the appearance of some of the heavy metal resistant isolates on nutrient agar.

Table 4.5: Heavy metal concentration (mg/kg) of the digested soil samples

Sample	Cadmium	Chromium	Copper	Lead	Nickel	Iron	Zinc
1	0.2616 (0.001) [b]	0.3474 (0.003) [c]	0.5360 (0.001) [b]	1.8576 (0.003) [b]	0.6993 (0.003) [b]	980.0427 (1.592) [b]	0.9603 (0.001) [b]
2	0.0213 (0.003) [d]	0.2671 (0.013) [f]	0.2638 (0.025) [c]	0.2323 (0.014) [de]	0.1666 (0.013) [c]	96.6412 (3.814) [f]	0.3012 (0.021) [c]
3	0.0362 (0.000) [c]	0.3708 (0.001) [b]	0.2204 (0.001) [d]	0.2979 (0.010) [c]	0.1154 (0.002) [d]	178.5447 (0.813) [c]	0.1631 (0.002) [d]
4	0.3579 (0.002) [a]	0.4126 (0.004) [a]	0.6360 (0.001) [a]	2.4015 (0.040) [a]	0.7454 (0.002) [a]	1213.2267 (1.080) [a]	1.1965 (0.001) [a]
5	0.0245 (0.001) [d]	0.2872 (0.002) [e]	0.1209 (0.000) [f]	0.2093 (0.005) [de]	0.1247 (0.003) [d]	127.8600 (0.0352) [e]	0.0698 (0.001) [g]
6	0.0235 (0.001) [d]	0.2154 (0.001) [g]	0.1366 (0.000) [f]	0.1972 (0.002) [e]	0.0845 (0.001) [e]	130.5643 (0.360) [e]	0.0701 (0.001) [g]
7	0.0241 (0.001) [d]	0.2668 (0.001) [f]	0.1926 (0.000) [e]	0.2518 (0.006) [cd]	0.0937 (0.003) [e]	128.7373 (0.204) [e]	0.1356 (0.000) [e]
8	0.0343 (0.000) [c]	0.3262 (0.002) [d]	0.1459 (0.001) [f]	0.2842 (0.008) [c]	0.1281 (0.005) [d]	138.3280 (0.452) [d]	0.1086 (0.002) [f]
*Ref. value	7.50	Cr (IV) 6.50	16.00	20.00	91.00	N/A	240.00

Values are means of triplicate readings and values in parenthesis are ± standard error
Values with the same letter within a column are not significantly different ($P < 0.05$)
*National Norms and Standards for Remediation of Contaminated Land and Soil Quality in South Africa. N/A- Not available

Table 4.6: Correlation matrix between soil pH and heavy metals concentration of the acid digested soil samples

	Cd	Cr	Pb	Ni	Zn	Cu	Fe	pH
Cd	1.000	0.714	0.999	0.984	0.983	0.967	0.998	0.520
Cr	0.714	1.000	0.707	0.677	0.687	0.703	0.704	0.554
Pb	0.999	0.707	1.000	0.988	0.986	0.971	0.999	0.518
Ni	0.984	0.677	0.988	1.000	0.988	0.973	0.988	0.467
Zn	0.983	0.687	0.986	0.988	1.000	0.994	0.982	0.486
Cu	0.967	0.703	0.971	0.973	0.994	1.000	0.966	0.533
Fe	0.998	0.704	0.999	0.988	0.982	0.966	1.000	0.541
pH	0.520	0.554	0.518	0.467	0.486	0.533	0.541	1.000

Positive correlation means that as one data set increases, the other data set increases as well. Negative correlation means that as one data set increases, the other decreases.

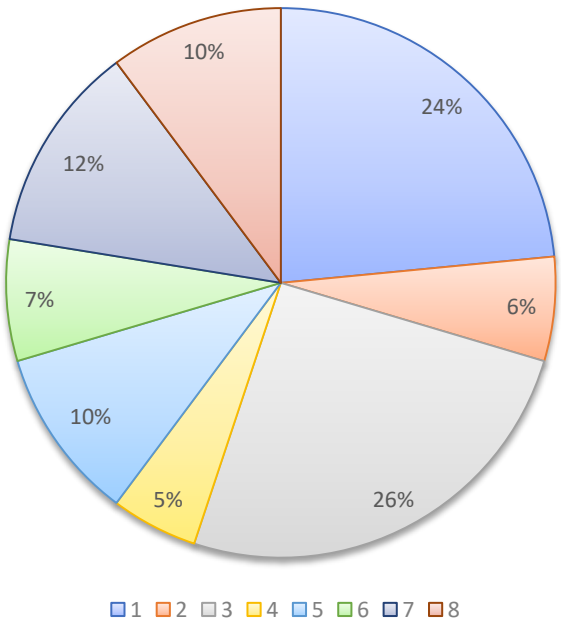


Figure 4.4: Distribution of bacterial isolates from each soil sample

The bacterial counts from each soil sample are presented in Table 4.7. The total bacterial counts for the samples varied between 1.25-6.3 × 10^6 CFU/g. The population of the heterotrophic bacteria from the soil samples were sensitive to heavy metals as the number decreases when heavy metals were added to the medium for isolation. The counts (in CFU/g) obtained for heavy metal resistant isolates are between 1.6-7.6 × 10^3, 0.15-2.37 × 10^3, and 1.28-6.95 × 10^3 for cadmium, chromium, and lead respectively. The total bacterial counts obtained in the presence of the combination of all metals (Cd, Cr and Pb) is between 0.13-2.08 × 10^2. The presence of heavy metals in the growth medium had a negative effect on the bacterial counts. The combination of all metals also had a negative effect on the bacterial counts (Table 4.7). The resistant isolates were screened for their ability to grow on increasing concentration of heavy metals. Twenty isolates that showed appreciable growth were selected for further studies. Table 4.8 showed metal resistance pattern of the twenty resistant isolates on different concentrations of heavy metals. Most of the bacterial isolates were able to grow on 400 mg/L of Pb and about 55% of the isolates were able to grow in the presence of 1 g/L of Pb. Forty percent of the isolates were able to grow on 200 mg/L of Cd with 10% grew on 300 mg/L of Cd. Limited growth was observed on medium containing chromium. Fifty percent of the isolates were able to grow on 100 mg/L of Cr and 20% on 300 mg/L of Cr. As the metal concentration increases, there was a decrease in the number of organisms that were able to grow on them (Table 4.8).

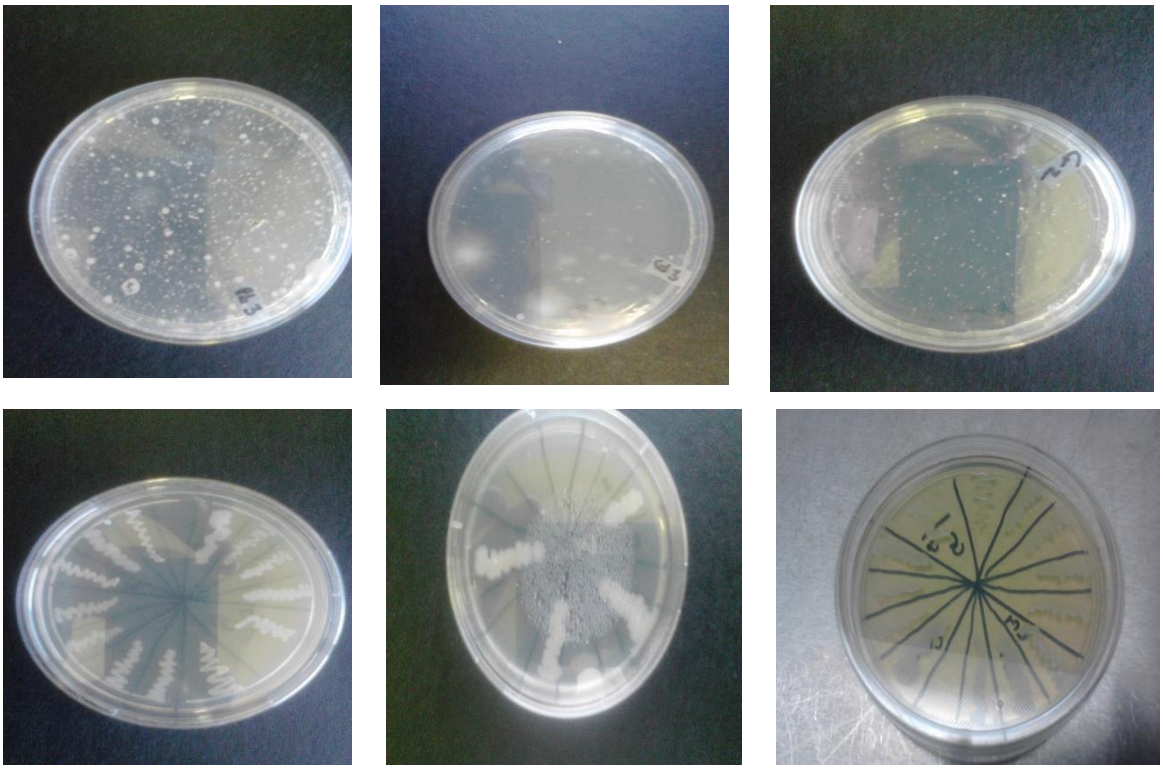


Figure 4.5: Plates showing bacterial counts and appearance of heavy metal resistant isolates

4.3.3. Biochemical profile of heavy metal resistant isolates

Most of the isolates are rod-like in shape with 70% Gram positive organisms (Table 4.9). All isolates were catalase positive, oxidase negative, and did not produce hydrogen sulfide and indole. Sixty-four percent of the isolates utilize citrate as a carbon source while 5% produce urease. Eighty-five percent of the isolates liquefy gelatin, 80% reduce nitrate to nitrite while 65% of the isolates hydrolyze starch. Five percent of the isolates ferment lactose, 10% ferment maltose, 65% ferment sucrose while 80% of the isolates ferment glucose (Table 4.9).

4.3.4. Molecular characterization of resistant isolates

The 16S rRNA gene amplification of the genomic DNA of the resistant isolates yielded the expected 1500 bp fragments using universal primers (F1, R2) as shown in Figure 4.6. The organisms were identified based on the 16S rRNA gene sequence using the BLAST search of the

NCBI database (Table 4.10). The organisms were grouped into *Acinetobacter* (1 isolate), *Agrobacterium* (1 isolate), *Bacillus* (13 isolates), *Citrobacter* (1 isolate), *Enterobacter* (1 isolate), *Pantoea* (1 isolate) and *Pseudomonas* (2 isolates) (Table 4.10). *Bacillus* species accounts for 65% of the metal resistant isolates while *Pseudomonas* is 10% of the isolated metal resistant organisms.

The phylogenetic relationship of the 16S rRNA gene of the identified heavy metal resistant bacteria with their aligned reference sequences of related taxa are presented in Figures 4.7- 4.12. The relationship between the *Bacillus* species, *Enterobacter asburiae*, *Citrobacter freundii*, *Pseudomonas* species and their related taxa in the NCBI GenBank database using maximum likelihood method based on Jukes-Cantor model (Jukes and Cantor, 1969) is presented in Figures 4.7, 4.9, 4.10, and 4.11 respectively. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). All positions containing gaps and missing data were eliminated.

Table 4.7: Enumeration of heavy metal resistant bacterial isolates from each sample

Sample	Total bacterial counts (10^6 CFU/g)	Cd (10^3 CFU/g)	Cr (10^3 CFU/g)	Pb (10^4 CFU/g)	MM (10^2 CFU/g)
1	6.30 (0.20) [a]	2.50 (0.20) [bc]	0.26 (0.06) [d]	2.53 (0.13) [c]	0.13 (0.03) [f]
2	6.20 (0.30) [a]	6.75 (0.75) [a]	0.78 (0.13) [c]	1.28 (0.75) [d]	0.38 (0.02) [e]
3	1.35 (0.25) [c]	1.65 (0.25) [c]	1.53 (0.13) [b]	1.75 (0.15) [cd]	0.23 (0.03) [f]
4	1.25 (0.05) [c]	7.60 (0.30) [a]	0.15 (0.05) [d]	6.95 (0.56) [a]	1.23 (0.07) [b]
5	2.15 (0.15) [b]	3.30 (0.10) [b]	2.37 (0.75) [a]	2.17 (0.17) [cd]	0.86 (0.04) [d]
6	1.40 (0.10) [c]	2.60 (0.20) [bc]	1.23 (0.23) [b]	5.25 (0.45) [b]	2.08 (0.02) [a]
7	1.80 (0.20) [bc]	1.60 (0.30) [c]	0.84 (0.04) [c]	6.51 (0.41) [a]	1.06 (0.05) [c]
8	1.65 (0.25) [bc]	1.95 (0.15) [c]	0.38 (0.02) [d]	1.28 (0.02) [d]	0.86 (0.15) [d]

MM is multi-metal (Cd, Cr, Pb) of mixture of 50 mg/kg of each metal
Values are means of duplicate readings and values in parenthesis are ± standard error
Values with the same letter within a column are not significantly different ($P < 0.05$)

Table 4.8: Growth pattern of heavy metal resistant isolates on different concentrations of heavy metals

Isolate code	Cd (mg/L)			Cr (mg/L)			Pb (mg/L)					
	100	200	300	100	200	300	100	200	300	400	500	1000
AB4	+	+	-	+	+	-	+	+	+	+	+	+
AB5	+	+	-	-	+	-	+	+	+	+	+	+
AB6	+	+	-	+	+	+	+	+	+	+	+	+
AB9	+	+	-	+	-	-	+	+	+	+	+	+
AB14	-	-	-	-	-	-	+	+	+	+	+	+
AB18	+	-	-	+	+	-	+	+	+	+	+	-
AB19	-	-	-	-	-	-	+	+	+	+	+	-
AB21	-	-	-	-	-	-	+	+	+	+	+	-
AB22	-	-	-	-	-	-	+	+	+	+	+	+
AB23	+	+	+	-	-	-	+	+	+	+	+	+
AB30	+	-	-	-	-	-	+	+	+	+	+	+
AB36	+	+	-	+	-	-	+	+	+	+	+	+
AB58	-	-	-	-	-	-	+	+	+	+	+	+
AB61	+	-	-	-	-	-	+	+	+	+	+	+
AB66	-	-	-	-	-	-	+	+	+	-	-	-
AB77	+	-	-	+	+	+	+	+	+	+	-	-
AB79	+	+	-	+	+	+	+	+	+	+	-	-
AB82	+	-	-	+	+	-	+	+	+	+	-	-
AB91	-	-	-	+	+	+	+	+	+	+	-	-
AB92	+	+	+	+	+	-	+	+	+	+	-	-

(+) = Growth in the presence of metals; (-) = No growth in the presence of metal

The relationship between *Acinetobacter calcoaceticus* and its related taxa in the NCBI GenBank database using neighbor-joining method (Saitou and Nei, 1987) is presented in Figure 4.8. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site.

The relationship between *Pantoea* sp. and its closely related taxa in the NCBI GenBank database was determined using the maximum parsimony method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The maximum parsimony tree was obtained using the subtree-pruning-regrafting algorithm (Nei and Kumar, 2000) with search level 2 in which the initial trees were obtained by the random addition of sequences.

Table 4.9: Biochemical profile of heavy metal resistant bacterial isolates

ISOLATE CODE	TESTS																				
	GRAM RXT	SHAPE	CAT	OXI	IN	VP	CIT	URE	SH	H_2S	GL	MAN	NIT	GLU	ARA	SUC	MAN	SOR	MEL	MAL	LAC
AB4	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+	+	+	-	-	-	-	-
AB5	+	Rod	+	-	-	+	+	-	+	-	+	-	+	+	+	+	-	-	-	-	-
AB6	+	Coccobacilli	+	-	-	+	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-
AB9	-	Rod	+	-	-	+	-	-	+	-	+	+G	+	+G	+	+G	+	+G	+	+G	-
AB14	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+G	-	+	-	-	-	-	-
AB18	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-
AB19	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-
AB21	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+G	-	+	-	-	-	-	-
AB22	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-
AB23	-	Rod	+	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-
AB30	-	Rod	+	-	-	+	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-
AB36	-	Rod	+	-	-	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-
AB58	-	Rod	+	-	-	-	-	-	-	-	-	+G	+	+G	+	+G	+	+G	+	+G	+G
AB61	+	Rod	+	-	-	-	+	-	+	-	+	-	-	-	-	+	-	-	-	-	-
AB66	+	Rod	+	-	-	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-
AB77	-	Rod	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-
AB79	+	Rod	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-
AB82	+	Rod	+	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
AB91	+	Rod	+	-	-	-	-	-	+	-	+	-	+	+	-	+	-	-	-	-	-
AB92	+	Rod	+	-	-	+	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-

Key: + (Positive), - (Negative), G (Gas production), CAT (Catalase), OXI (Oxidase), IN (Indole), VP (Voges Proskaeur), CIT (Citrate), URE (Urease), SH (Starch hydrolysis), GL (Gelatin liquefaction), MAN (Mannose), NIT(Nitrate), GLU (Glucose), ARA (Arabinose), SUC (Sucrose), MAN (Mannose), SOR (Sorbitol), MEL (Melibiose), MAL (Maltose), LAC (Lactose)

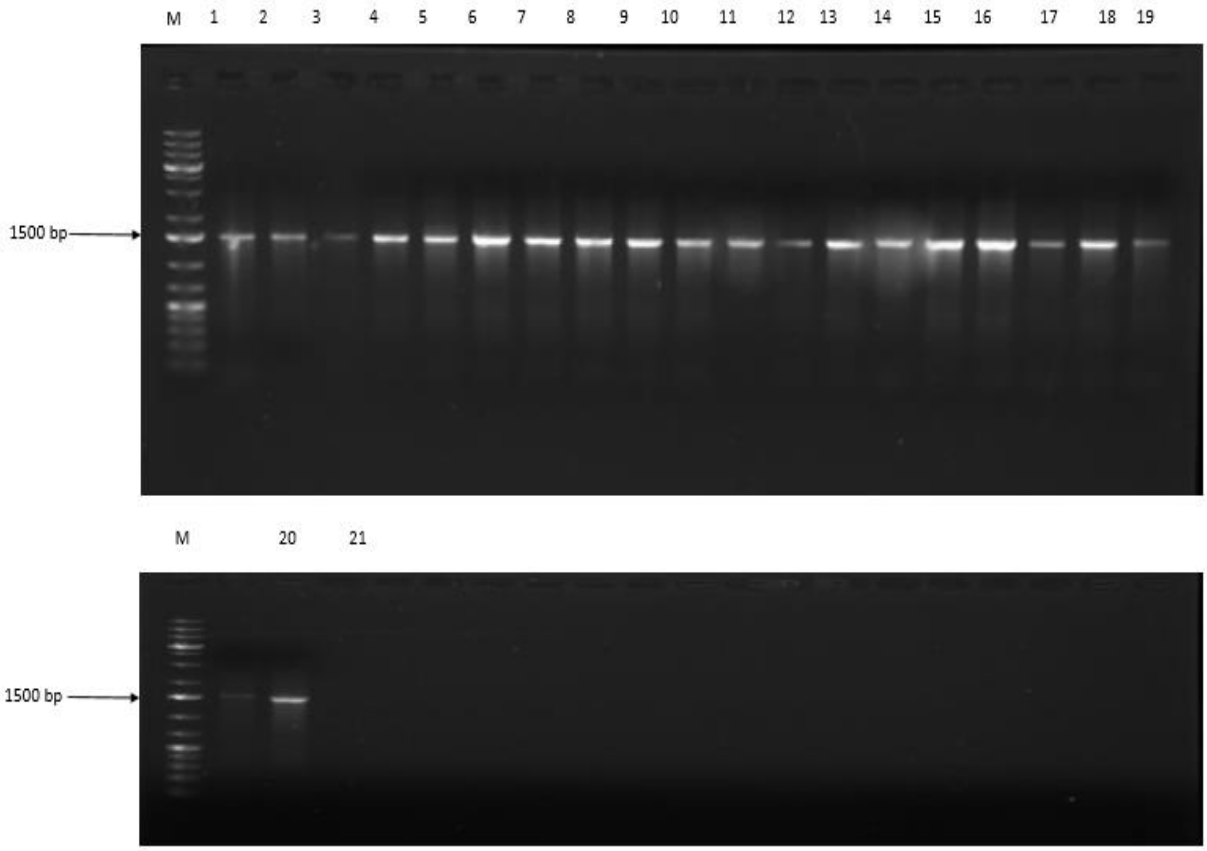


Figure 4.6: Photograph of the agarose gel showing 16S rRNA gene amplicons of 1500 bp of metal resistant bacteria. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92, Lane 21: Control

Table 4.10: 16S rRNA gene sequence-based identification of the isolates and their accession numbers

Isolate code	Strain code	Isolate name	Accession number	BLAST ID (closest cultured representative)	Percentage similarity (%)	E value
AB4	NWUAB01	*Bacillus cereus*	MH399230	*Bacillus cereus* (MH021873	100	0.0
AB5	NWUAB02	*Bacillus toyonensis*	MH399231	*Bacillus* sp. (KY064069)	99	0.0
AB6	NWUAB03	*Acinetobacter calcoaceticus*	MH399232	*Acinetobacter calcoaceticus* (KM114923)	100	0.0
AB9	NWUAB04	*Enterobacter asburiae*	MH399233	*Enterobacter* sp. (MG754444)	95	0.0
AB14	NWUAB05	*Bacillus wiedmannii*	MH399234	*Bacillus wiedmannii* (MF681995)	100	0.0
AB18	NWUAB06	*Bacillus cereus*	MH399235	*Bacillus cereus* (KJ612536)	99	0.0
AB19	NWUAB07	*Bacillus cereus*	MH399236	*Bacillus* sp. (KP992119)	99	0.0
AB21	NWUAB08	*Bacillus thuringiensis*	MH399237	*Bacillus thuringiensis* (MH260380)	100	0.0
AB22	NWUAB09	*Bacillus subtilis*	MH399238	*Bacillus subtilis* (KY495216)	100	0.0
AB23	NWUAB10	*Agrobacterium tumefaciens*		*Agrobacterium tumefaciens* (KP050793)	99	0.0
AB30	NWUAB11	*Pseudomonas fluorescens*	MH399239	*Pseudomonas fluorescens* (KU977136)	99	0.0
AB36	NWUAB12	*Pseudomonas korensis*	MH399240	*Pseudomonas korensis* (MH211310)	99	0.0
AB58	NWUAB13	*Citrobacter freundii*	MH399241	*Citrobacter freundii* (KM406465)	99	0.0
AB61	NWUAB14	*Bacillus cereus*	MH399242	*Bacillus cereus* (MH356588)	99	0.0
AB66	NWUAB15	*Bacillus wiedmannii*	MH399243	*Bacillus wiedmannii* (MF681995)	99	0.0
AB77	NWUAB16	*Pantoea* sp.	MH399244	*Pantoea* sp. (JN082730)	91	0.0
AB79	NWUAB17	*Bacillus megaterium*	MH399245	*Bacillus megaterium* (MH368091)	100	0.0
AB82	NWUAB18	*Bacillus aryabhattai*	MH399246	*Bacillus aryabhattai* (MF 681982)	99	0.0
AB91	NWUAB19	*Bacillus megaterium*		*Bacillus megaterium* (MF681883)	99	0.0
AB92	NWUAB20	*Bacillus* sp.	MH399247	*Bacillus* sp. (MH191109)	99	0.0

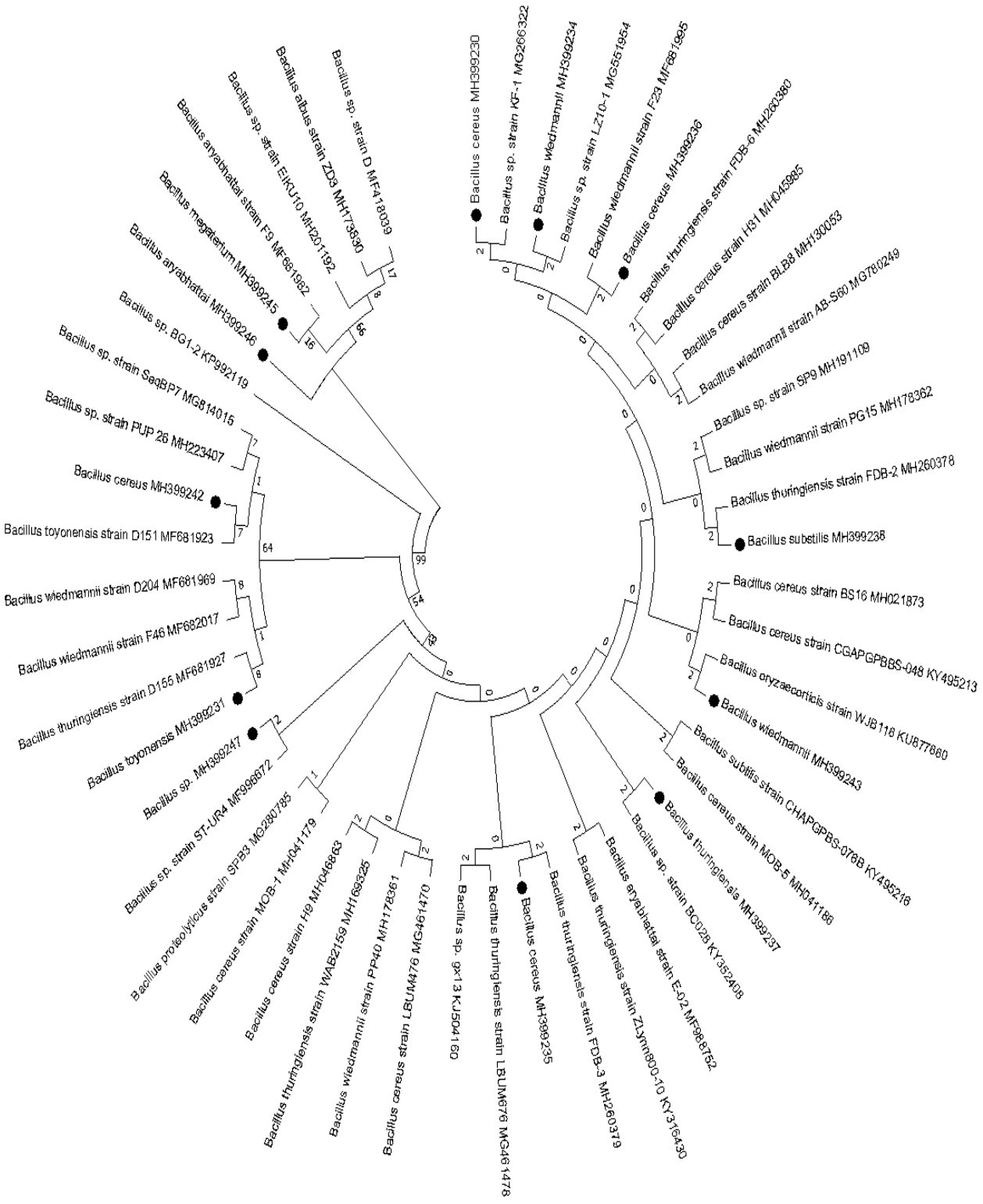


Figure 4.7: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using maximum likelihood method showing relationships between the resistant *Bacillus* species isolates and closely related strains from NCBI GenBank

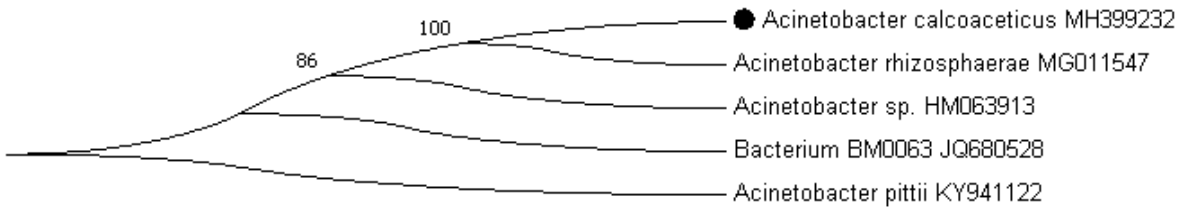


Figure 4.8: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using neighbor-joining method showing relationships between the resistant *Acinetobacter calcoaceticus* and its closely related strains from NCBI GenBank

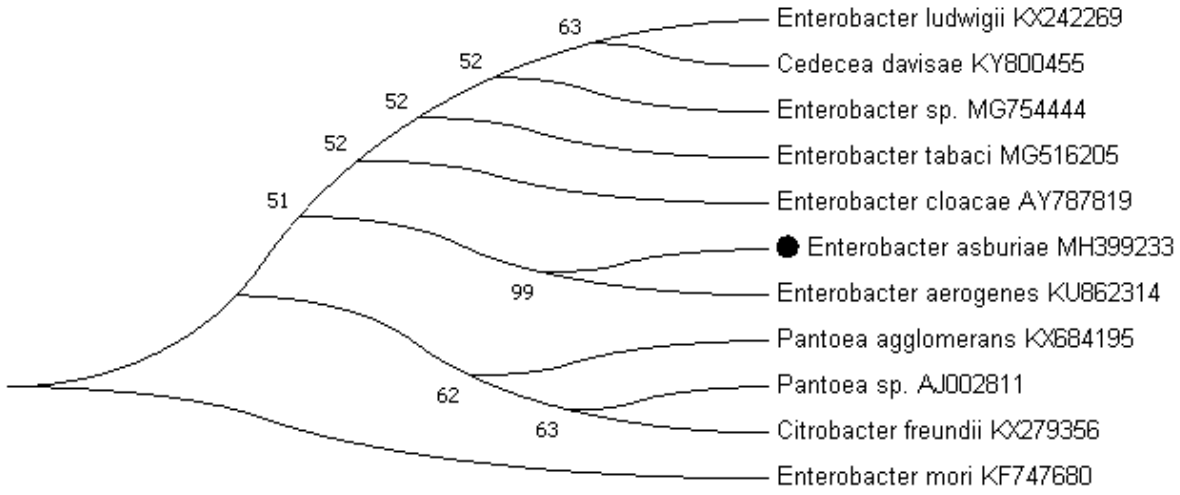


Figure 4.9: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using maximum likelihood based on the Jukes-Cantor model showing relationships between the resistant *Enterobacter asburiae* and its closely related strains from NCBI GenBank

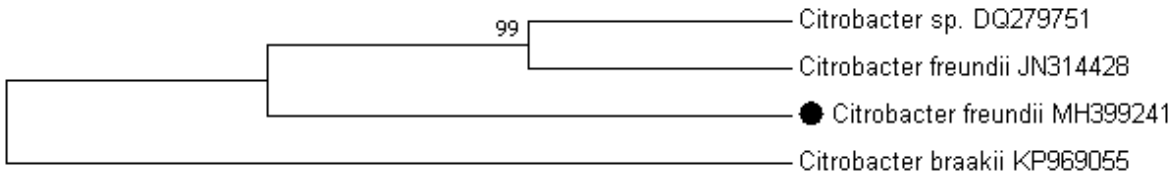


Figure 4.10: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using maximum likelihood based on the Jukes-Cantor model showing relationships between the resistant *Citrobacter freundii* and its closely related strains from NCBI GenBank

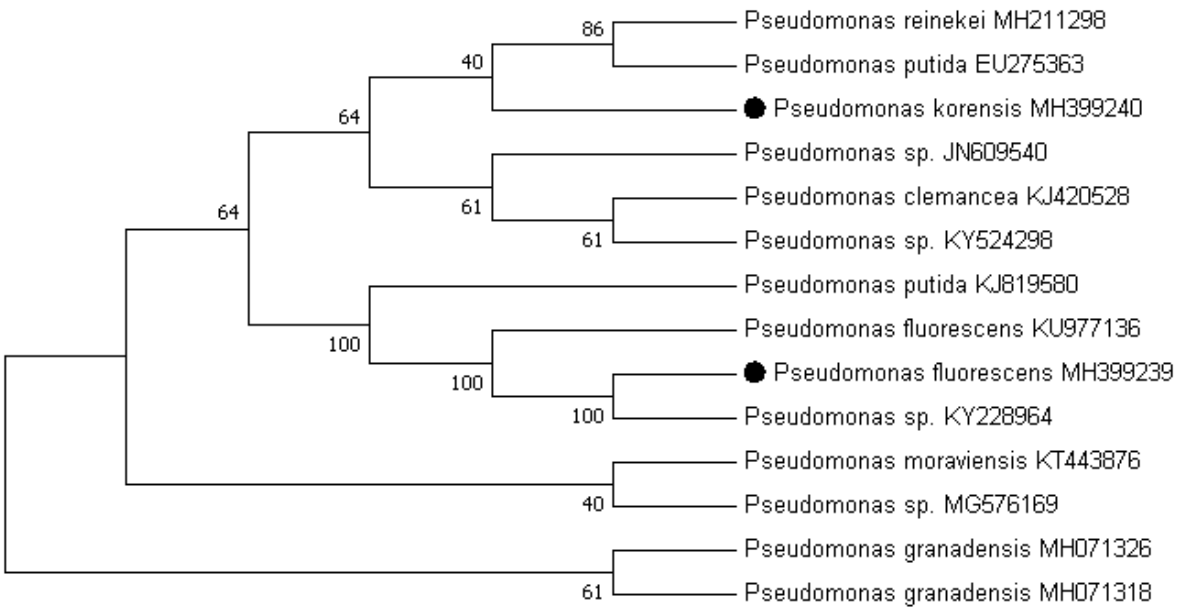


Figure 4.11: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using maximum likelihood based on the Jukes-Cantor model showing relationships between the resistant *Pseudomonas* species and its closely related strains from NCBI GenBank

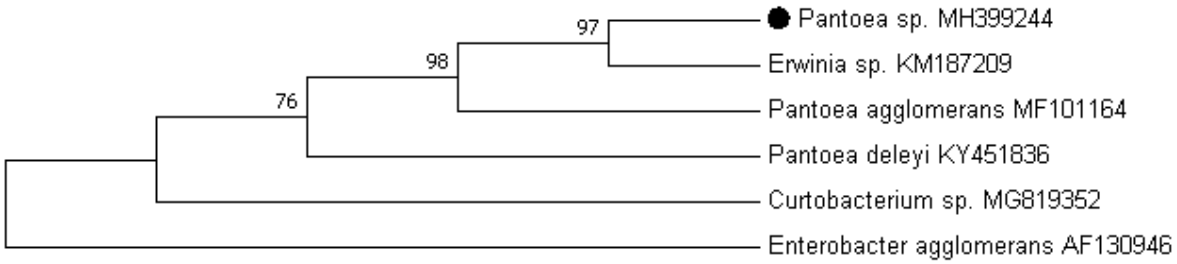


Figure 4.12: Evolutionary relationships of taxa tree based on partial 16S rDNA sequences using maximum parsimony method based on the Jukes-Cantor model showing relationships between the resistant *Pantoea* sp. and its closely related strains from NCBI GenBank

4.3.5. Disstribution of heavy metal resistance genes among the resistant isolates

The amplification of metal resistance genes responsible for Cd, Cr, and Pb were performed with specific primers for PCR amplification of the genomic DNA. No amplification products were obtained for primers representing *CzcA*, *CzcB* and *chrB*. The amplification of cadmium resistance genes (*cadA* and *CzcD*) with the expected band size are shown in Figures 4.13 and 4.14. Of the 20 isolates, 9 (45%) were positive for *cadA* and one unexpected band size was observed at Lanes 4, 5, and 12 (Figure 4.13). *cadA* primers produce fragments of 600 base pairs in *Bacillus cereus* (Lane

1, 6 and 14), *B. toyonensis* (Lane 2), *Citrobacter freundii* (Lane 13), *Pantoea* sp. (Lane 16), *Bacillus aryabhattai* (Lane 18), *B. megaterium* (Lane 17 and 19). Ninety percent of the isolates had the *CzcD* gene which is responsible for cadmium, cobalt and zinc resistance (Figure 4.14). There was no band in Lane 9 and an unexpected band size was observed at Lane 15. All the isolates produce *CzcD* fragments of 398 bp except *B. subtilis* (Lane 9) and *B. wiedmannii* (Lane 15) (Figure 4.14).

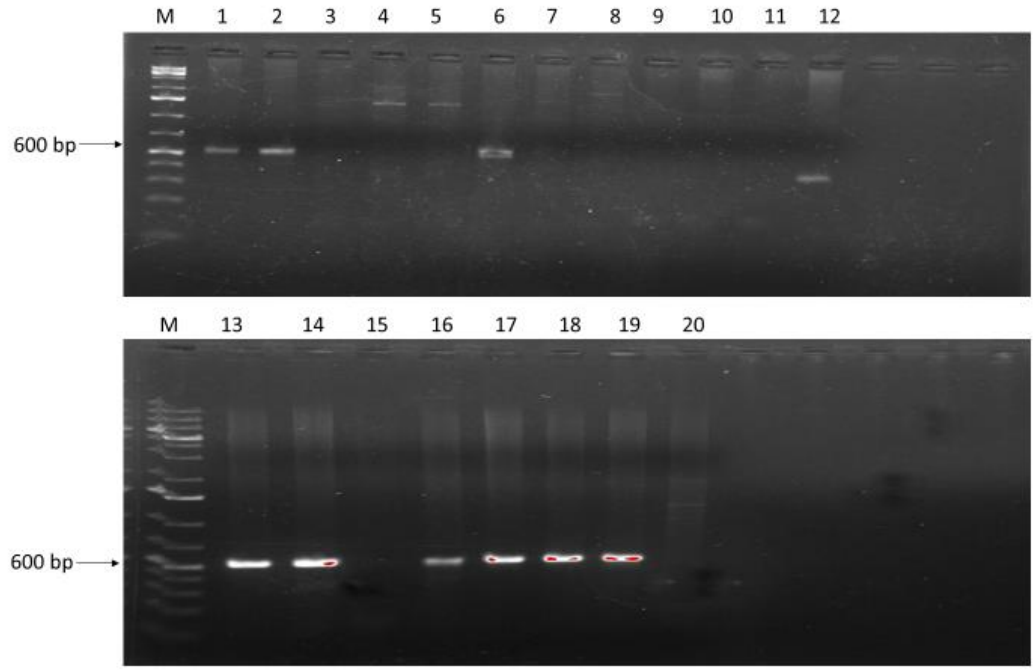


Figure 4.13: Photograph of the agarose gel showing *cadA* gene amplicons of 600 bp for cadmium resistance. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92

The amplification of lead resistance genes (*PbrA* and *PbrT*) are presented in Figures 4.15 and 4.16. Five of the isolates produce *PbrA* primer fragments at 766 bp in *B. cereus* (Lane 1), *B. toyonensis* (Lane 2), *A. calcoaceticus* (Lane 3), *Pantoea* sp. (Lane 16) and *B. megaterium* (Lane 17). There was unexpected band size in Lanes 9,10, 14,15, 19 and 20 (Figure 4.15). *PbrT* primers produce fragments of 740 bp in two isolates (Figure 4.16): *Bacillus cereus* (Lane 7) and *Bacillus aryabhattai* (Lane 18).

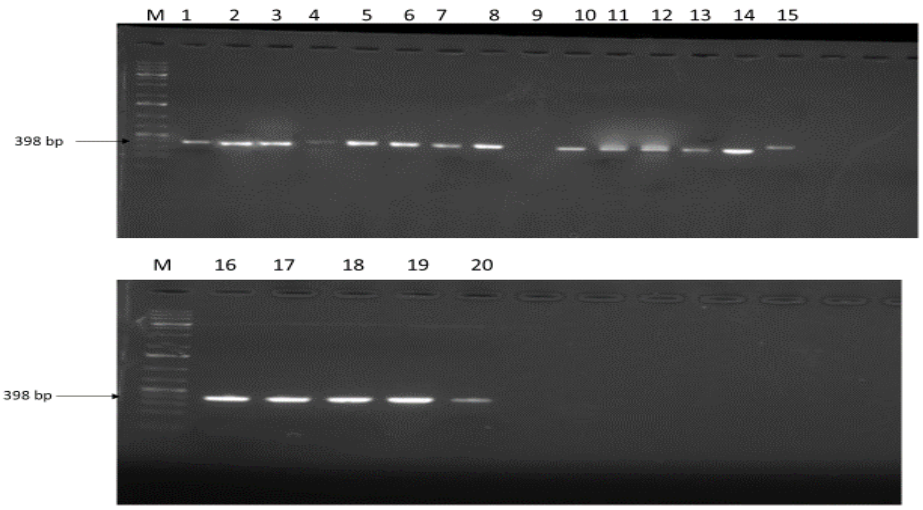


Figure 4.14: Photograph of the agarose gel showing *CzcD* gene amplicons of 398 bp for cadmium, cobalt and zinc resistance. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92

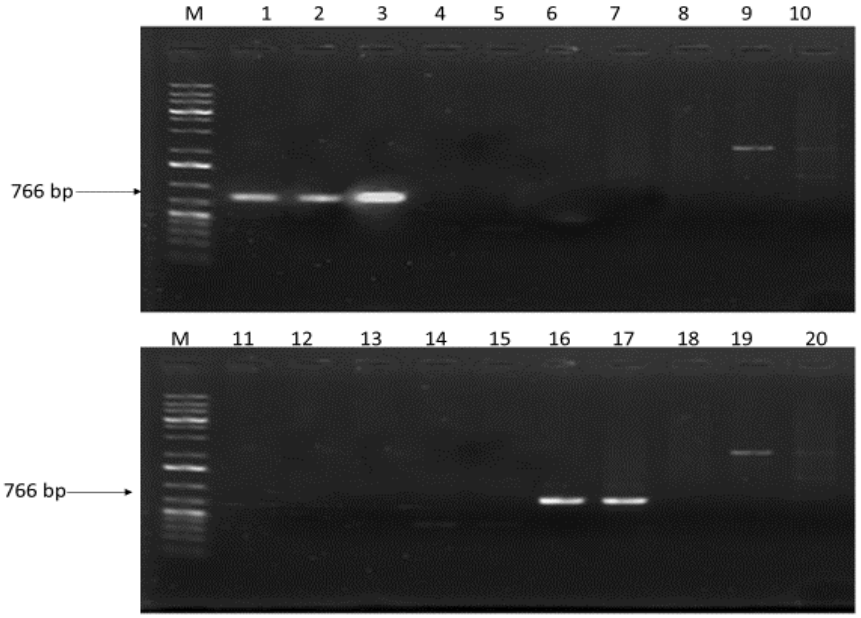


Figure 4.15: Photograph of the agarose gel showing *PbrA* gene amplicons of 766 bp for lead resistance. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92

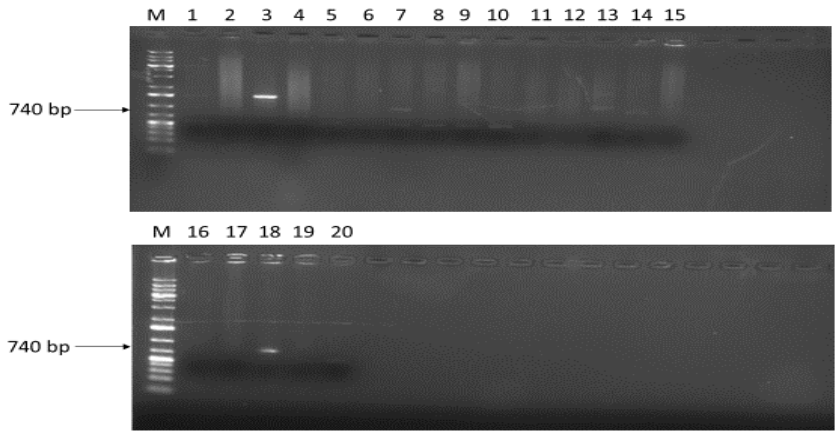


Figure 4.16: Photograph of the agarose gel showing *PbrT* gene amplicons of 740 bp for lead resistance. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92

Chromium resistance gene (*chrA*) produce a fragment of 1292 bp in *B. cereus* (Lane 6), *P. fluorescens* (Lane 11), *P. korensis* (Lane 12) and *Bacillus* sp. (Lane 20) (Figure 4.17). There were multiple fragments at Lanes 2 and 4, and unexpected band size at Lane 15 (Figure 4.17).

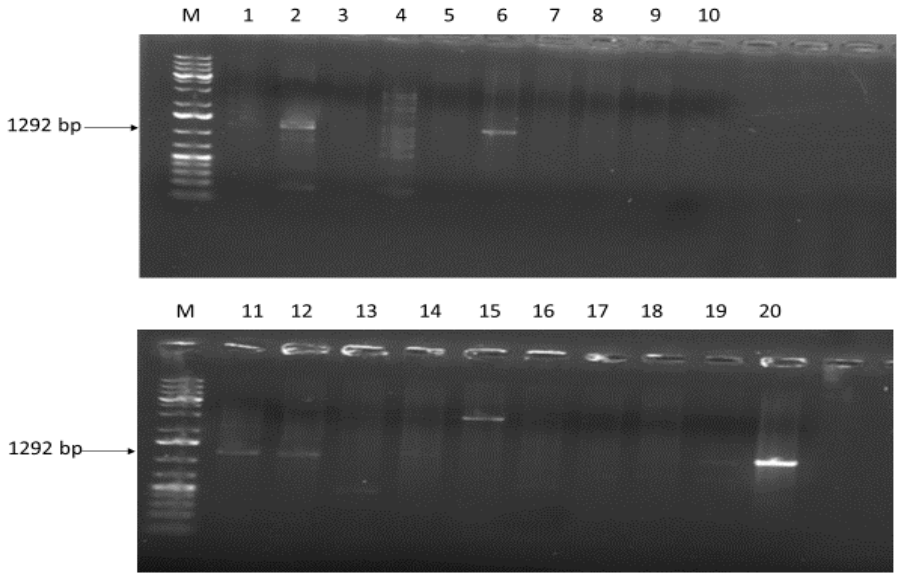


Figure 4.17: Photograph of the agarose gel showing *chrA* gene amplicons of 1292 bp for chromium resistance. M= 1Kb molecular weight marker. Lane 1: AB04, Lane 2: AB05, Lane 3: AB06, Lane 4: AB09, Lane 5: AB14, Lane 6: AB18, Lane 7: AB19, Lane 8: AB21, Lane 9: AB22, Lane 10: AB23, Lane 11: AB30, Lane 12: AB36, Lane 13: AB58, Lane 14: AB61, Lane 15: AB66, Lane 16: AB77, Lane 17: AB79, Lane 18: AB82, Lane 19: AB91, Lane 20: AB92

4.3.6. The effect of heavy metals on bacteria growth

The growth of metal resistant isolates was monitored in LB broth for six days in the presence of the metal tested (Cd, Cr, Pb; 100 mg/L of each metal) and the results are presented in Figures 4.18 to 4.22. In all, a decrease in optical density value was observed in the presence of heavy metals compared with the metal-free medium for each isolate (Figure 4.18-4.22). Also, most of the isolates were able to utilize Pb better than Cd and Cr. Generally, the growth of the bacterial isolates in the presence of the metals tested follows this pattern, Pb > Cr > Cd.

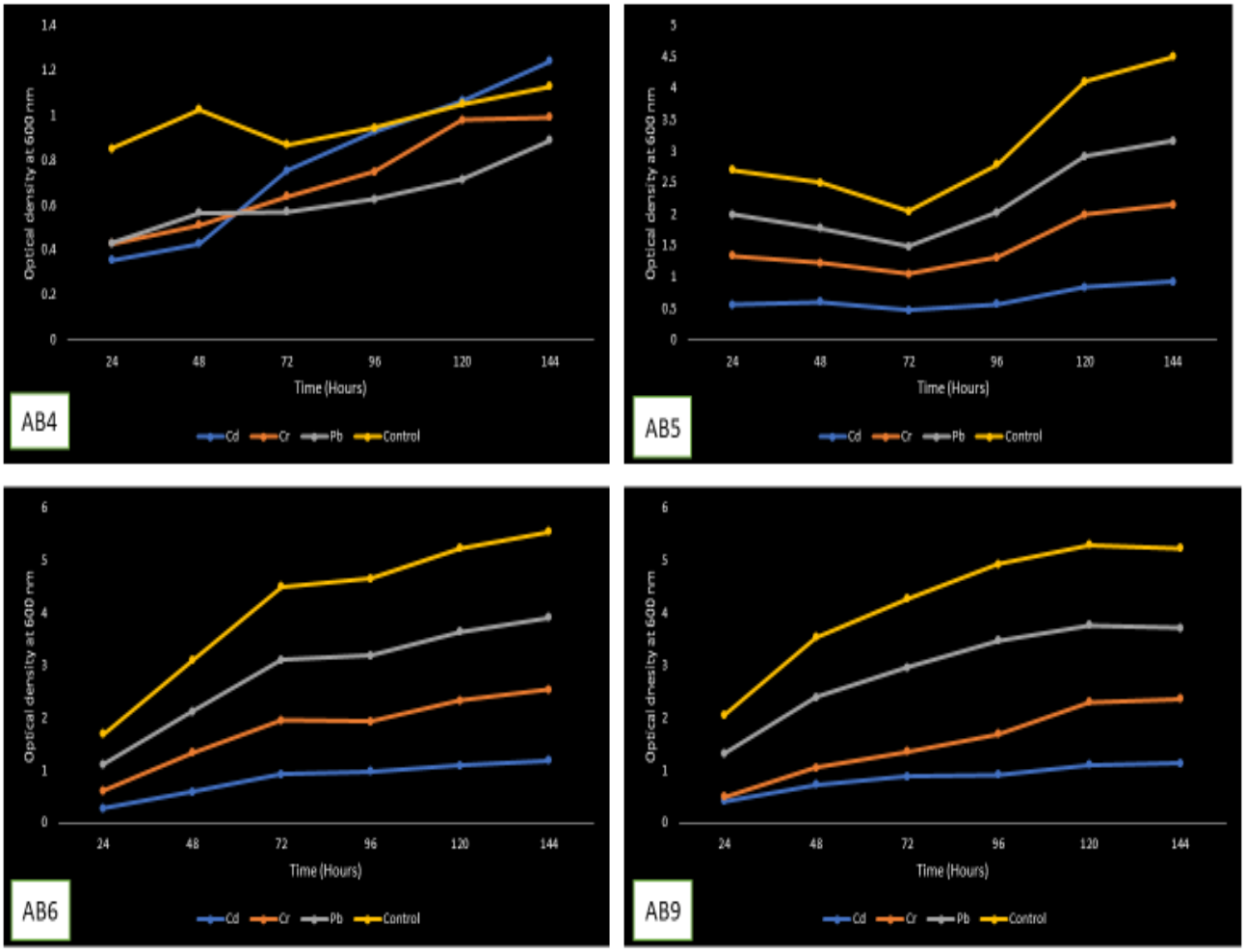


Figure 4.18: The growth patterns of *B. Cereus* (AB4), *B. Toyonensis* (AB5), *A. Calcoaceticus* (AB6), and *E. Asburiae* (AB9) in the presence of heavy metals

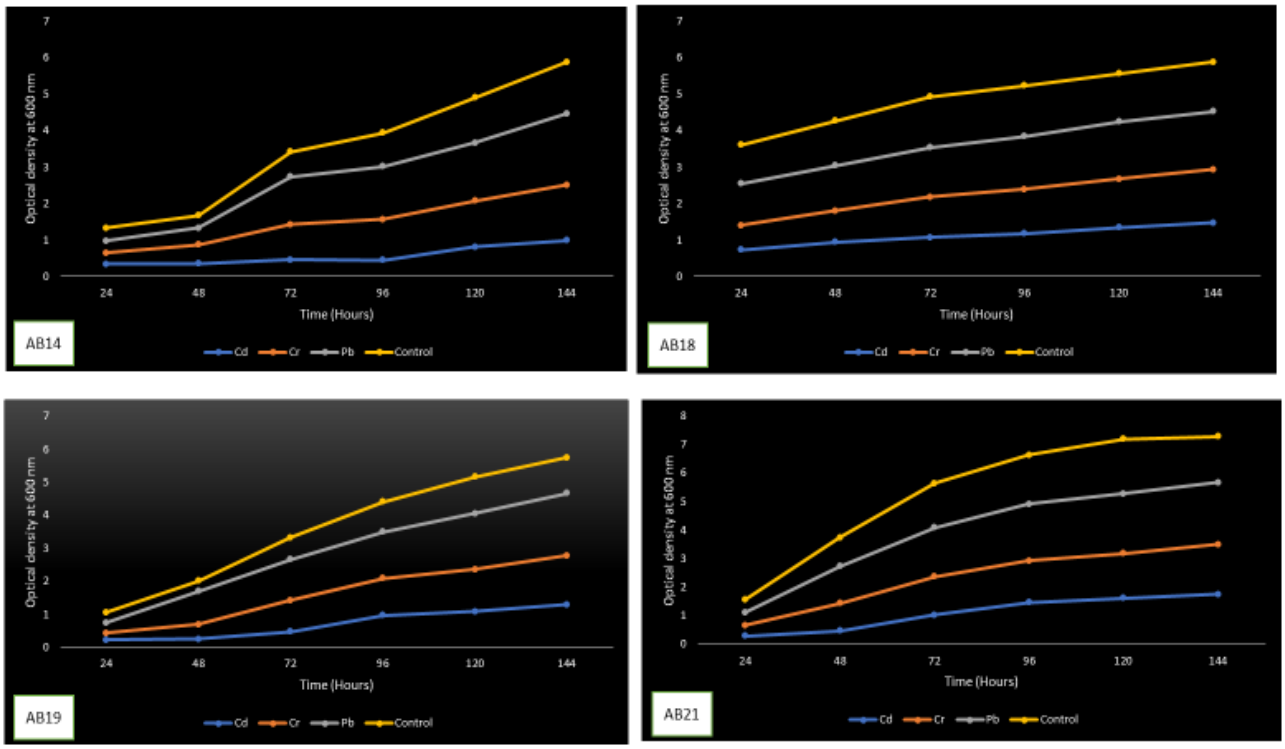


Figure 4.19: The growth patterns of *B. wiedmannii* (AB14), *B. cereus* (AB18), *B. cereus* (AB19), and *B. thuringiensis* (AB21) in the presence of Cd, Cr and Pb

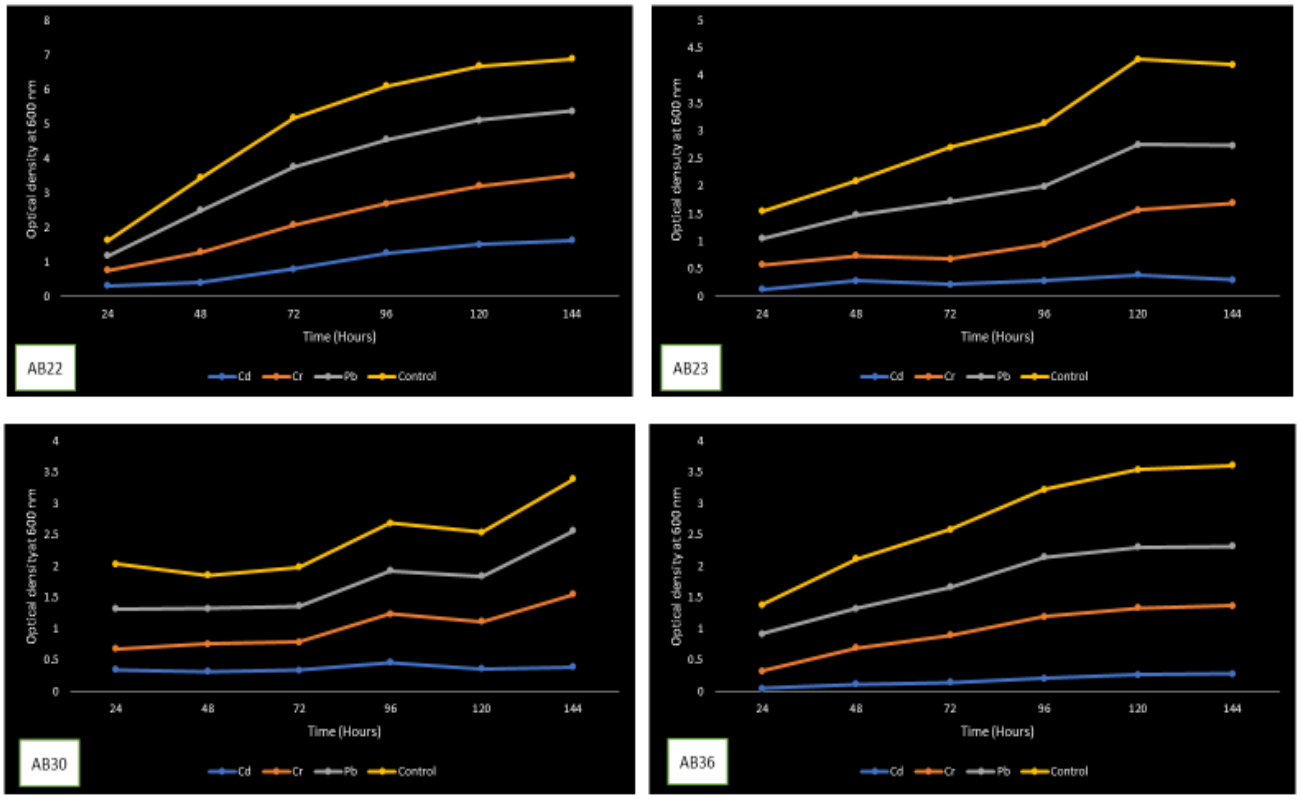


Figure 4.20: The growth patterns of *B. subtilis* (AB22), *A. tumefaciens* (AB23), *P. fluorescens* (AB30), and *P. korensis* (AB36) in the presence of Cd, Cr and Pb

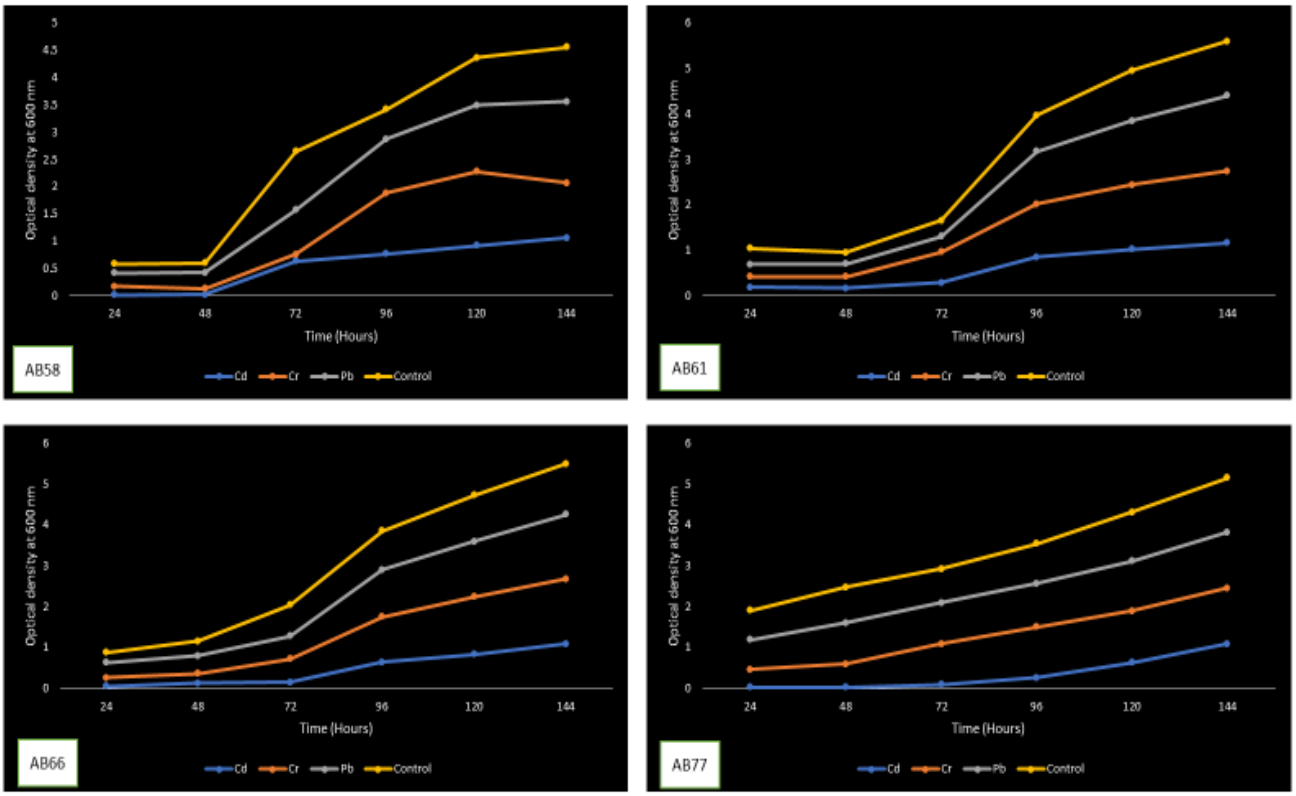


Figure 4.21: The pattern of growth of *C. freundii* (AB58), *B. cereus* (AB61), *B. wiedmannii* (AB66), and *Pantoea* sp. (AB77) in the presence of heavy metals

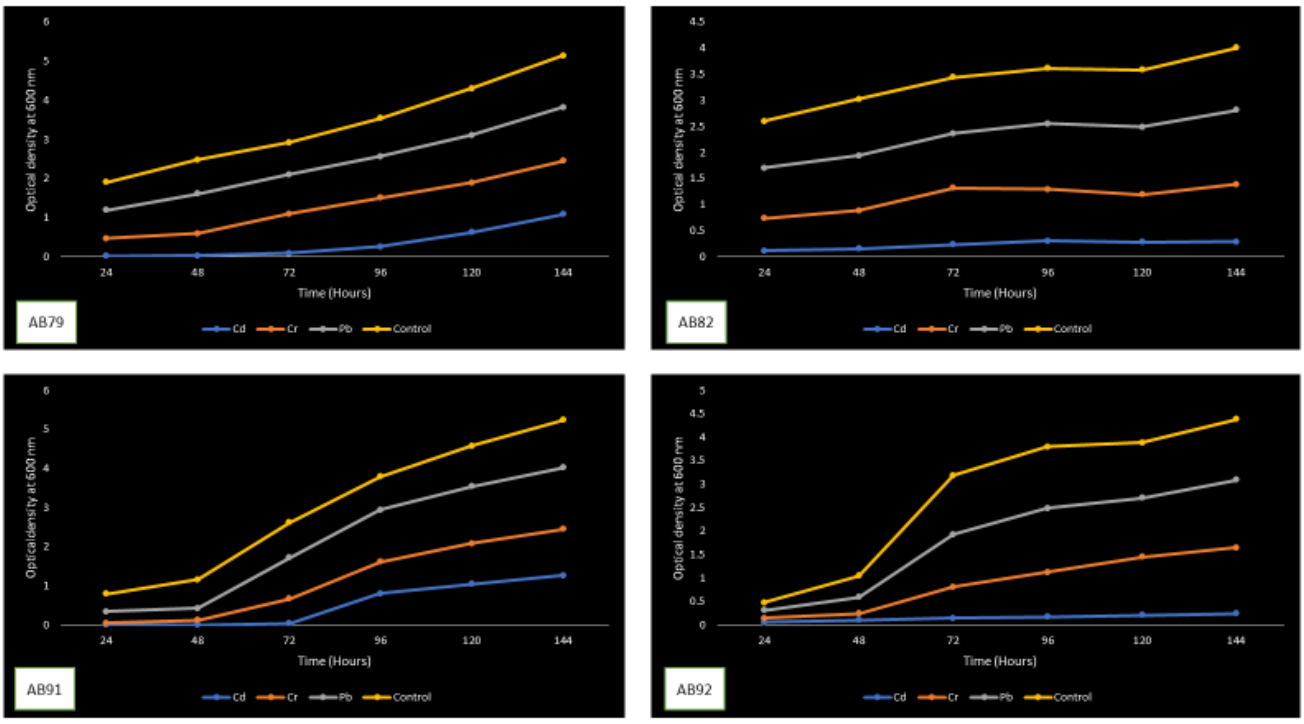


Figure 4.22: The growth patterns of *B. megaterium* (AB79), *B. aryabhattai* (AB82), *B. megaterium* (AB91), and *Bacillus* sp. (AB92) in the presence of Cd, Cr and Pb

4.3.7. Biosurfactant production by heavy metal resistant isolates

Biosurfactant production profiles by the heavy metal resistant isolates are presented in Table 4.11. Of the twenty isolates, 50% showed complete hemolysis of red blood cells on blood agar (Figure 4.23 and Table 4.11). *Bacillus* sp., *B. cereus* (2), *B. toyonensis, B. wiedimannii, B. thuringiensis, B. substilis, P. fluorescens A. calcoaceticus* and *Pantoea* sp. showed complete hemolysis of red blood cells. Forty-five percent of the isolates were positive for drop collapse with most hemolytic organisms also showing positive results for drop collapse.

The emulsification of different hydrocarbons and vegetable oil by different strains showed contrasting results as shown in Table 4.11. Most of the organisms were able to produce emulsion with engine oil at high percentage after 24 hours. The range of the emulsification of engine oil is between 0 – 93%. The emulsification of vegetable oil by different isolates is between 0 – 46% and that of kerosene is between 0 – 50.6%. Most of the isolates are able to form a stable emulsion with at least two of the oils used as the substrate.

Organisms such as *B. cereus* (AB4), *A. calcoaceticus* (AB6), *B. wiedmannii* (AB14), *B. cereus* (AB19), *B. thuringiensis* (AB21), *Pantoea* sp. (AB77), and *Bacillus* sp. (AB92) were positive for all the biosurfactant production tests. They are also able to completely hemolyse red blood cells, are positive for drop collapse and are able to emulsify engine oil, vegetable oil, and kerosene.

Table 4.11: Biosurfactant production profiles of the bacterial isolates

Isolate code	Organism	Hemolysis	Emulsification index (%)			Drop collapse
			Engine oil	Vegetable oil	Kerosene	
AB4	*Bacillus cereus*	+	53.40 (0.50)	23.50 (0.50)	37.25 (0.25)	+
AB5	*Bacillus toyonensis*	+	49.00 (1.00)	20.50 (0.50)	36.90 (0.90)	-
AB6	*Acinetobacter calcoaceticus*	+	44.00 (2.00)	14.00 (2.00)	40.85 (0.85)	+
AB9	*Enterobacter asburiae*	-	13.25 (0.75)	22.50 (1.50)	48.5 (1.50)	-
AB14	*Bacillus wiedmannii*	+	81.50 (1.50)	17.50 (1.50)	4.50 (0.50)	+
AB18	*Bacillus cereus*	-	87.25 (0.25)	25.00 (1.00)	14.00 (2.00)	-
AB19	*Bacillus cereus*	+	14.75 (0.75)	25.00 (1.50)	5.50 (1.50)	+
AB21	*Bacillus thuringiensis*	+	4.50 (0.50)	42.00 (2.00)	0.00 (0.00)	+
AB22	*Bacillus subtilis*	+	28.70 (0.70)	40.50 (0.50)	5.15 (1.15)	-
AB23	*Agrobacterium tumefaciens*	-	0.00 (0.00)	26.50 (0.50)	36.75 (0.75)	-
AB30	*Pseudomonas fluorescens*	+	76.75 (0.25)	46.00 (2.00)	45.50 (2.50)	-
AB36	*Pseudomonas korensis*	-	80.50 (0.50)	28.65 (0.65)	13.25 (0.75)	+
AB58	*Citrobacter freundii*	-	74.80 (0.20)	0.00 (0.0)	37.60 (1.60)	+
AB61	*Bacillus cereus*	-	87.25 (0.25)	28.50 (0.50)	0.00 (0.00)	-
AB66	*Bacillus wiedmannii*	-	93.00 (4.24)	14.00 (2.00)	0.00 (0.00)	-
AB77	*Pantoea* sp.	+	10.50 (0.50)	29.80 (0.60)	29.60 (0.40)	+
AB79	*Bacillus megaterium*	-	41.30 (0.60)	30.30 (0.70)	37.75 (1.35)	-
AB82	*Bacillus aryabhattai*	-	83.80 (0.50)	24.05 (0.95)	39.30 (4.20)	-
AB91	*Bacillus megaterium*	-	82.05 (0.95)	23.55 (0.45)	5.15 (0.95)	-
AB92	*Bacillus* sp.	+	23.75 (0.25)	39.7 (0.60)	50.60 (1.70)	+

Values are means of duplicate readings at $p < 0.05$
Values in parenthesis are ± standard error

4.3.8. Flocculating activity of heavy metal resistant bacterial isolates

The flocculating activities of heavy metal resistant isolates are presented in Table 4.12. The flocculating activity of the different isolates ranged between 0 – 71.3%. *Pantoea* sp. had the highest flocculating activity of 71.3% while some of the *Bacillus* species, *Acinetobacter calcoaceticus* and *Enterobacter asburiae* did not show any activity.

Table 4.12: Flocculating activity of the heavy metal resistant bacterial isolates

Isolate code	Organism	Flocculating activity (%)
AB4	*Bacillus cereus*	0.00 (0.00)
AB5	*Bacillus toyonensis*	0.00 (0.00)
AB6	*Acinetobacter calcoaceticus*	0.00 (0.00)
AB9	*Enterobacter asburiae*	0.00 (0.00)
AB14	*Bacillus wiedmannii*	17.5 (0.25)
AB18	*Bacillus cereus*	21.35 (0.65)
AB19	*Bacillus cereus*	32.70 (0.30)
AB21	*Bacillus thuringiensis*	23.10 (0.90)
AB22	*Bacillus subtilis*	25.75 (0.45)
AB23	*Agrobacterium tumefaciens*	21.90 (0.60)
AB30	*Pseudomonas fluorescens*	22.90 (0.60)
AB36	*Pseudomonas korensis*	51.70 (1.70)
AB58	*Citrobacter freundii*	27.85 (0.75)
AB61	*Bacillus cereus*	13.00 (0.50)
AB66	*Bacillus wiedmannii*	20.00 (1.00)
AB77	*Pantoea* sp.	71.25 (0.65)
AB79	*Bacillus megaterium*	3.80 (1.30)
AB82	*Bacillus aryabhattai*	0.00 (0.00)
AB91	*Bacillus megaterium*	42.05 (0.95)
AB92	*Bacillus* sp.	16.60 (1.40)

Values are means of duplicate readings ($P < 0.05$)
Values in parenthesis are ± standard error

4.4. Discussion

The bioavailability and mobility of metals in soil is controlled by the physical and chemical properties of soil that determine their biding ability to soil (Dube et al., 2001). Metal availability and behavior in mining environment is influenced by pH, cation exchange capacity (CEC), organic matter, particle size, moisture content, and sulfate composition of such soil (Dube et al., 2001; Finzgar et al., 2007). Soil type and pH, particle size, CEC and permeability of the soil affect metal removal efficiency from soil with high organic matter and clay content having a detrimental effect on metal removal (Mulligan et al., 2001a). The soil samples used in this study have a low organic matter and clay content that will make metal removal from the soil samples effective for remediation purposes.

The soil samples are sandy in nature which increases their permeability. These soil samples have numerous pore volumes that makes removal of metals easier and effective. Soil with high clay content and layered systems are difficult to remediate because they are less permeable (Mulligan et al., 2001a), but they accommodate more diverse and large populations of microorganisms than sandy soils (Deng et al., 2018). Organic carbon is often correlated with microbial biomass, with clay soils having higher number of biomass than sandy soils (Reeve et al., 2010). The low organic carbon causes disruption in the function of the ecosystem. Ashraf et al. (2012) reported that mining soils are low in organic carbon and organic matter which also corroborate the results of this study. The sandy nature of the soil samples indicates that these soils have poor nutrient retention capacity and do not support good microbial habitats. This impacts negatively on the diversity and function of microbial populations in the soil environment (Reeve et al., 2010). The sandy nature of the soils also makes metals readily bioavailable in the environment as a result of low binding capacity of sandy soils, consequently resulting in high metal mobility in such environments.

Low soil pH tends to increase metal solubility and toxicity and thus has a strong effect on the microbial population (Deng et al., 2018). The soil samples in this study are slightly acidic to neutral

in nature which accounts for the low diversity of bacteria obtained in this study. The dominant microbial population isolated in this study (*Bacillus* and *Pseudomonas* species) have been known to grow and survive at the pH range obtained for soil samples in this study (Das and Mukherjee, 2007). The results showed that slightly acidic to slightly alkaline environment is suitable for isolation of these dominant organisms. This correlates with similar findings reported by Ndeddy Aka and Babalola (2017) who reported slightly alkaline soil from mining soil with *Bacillus* and *Pseudomonas* species as the dominant organisms, and Wahl (2014), who reported slightly acidic to slightly neutral pH for mining soils from North West province of South Africa in their study.

Maiti (2006) reported that moisture content of mining soil is a fluctuating parameter that depends on sampling period, organic carbon content, stone content, dump height, texture, and thickness of litter layers. It is an important parameter for biogeochemical, biological and hydrological processes (Sadhu et al., 2012). Low moisture content from this study can be attributed to low organic carbon content and the sandy nature of the soil samples. Low moisture content of between 2-3% during summer and 5% during winter was reported by Maiti et al. (2002). The moisture content of soil samples in this study are lower than those reported by Sadhu et al. (2012) except for sampling site 4 with moisture content of 3.890%.

The cation exchange capacity of a soil is the capacity of the soil to absorb or release cations (Ashraf et al., 2012), and a measure of soil fertility and nutrient retention (Mukhopadhyay et al., 2014). Clay content and organic matter of the soil are responsible for the CEC. The CEC of soil decreases with increasing soil size distribution (Dube et al., 2001) with clay soils having high CEC values. This suggest that negatively charged humic acids serve as binding sites for organic matter that helps in the retention of cations (Abollino et al., 2002). The CEC of metal species depends on the ionic strength of the soil colloid surfaces and relative charges of metal ions in solution (Finzgar et al., 2007). Sandy soils typically have low CEC as reflected in this study and indicates the poor ability of the soil to retain cations and hold water, consequently increasing metal availability

potential in the soil. The sandy nature of the soil samples is due to high leaching caused by porous soil samples. Similar results were reported by Sadhu et al. (2012) for sandy soils with low CEC values.

Organic carbon, phosphorus and nitrogen have been reported as growth limiting factors in mining soils (Mukhopadhyay et al., 2014). The total nitrogen and phosphorus in the soil samples are very low except for sample 6 which has high phosphorus content. The low nitrogen content of mining soil is as a result of inadequate organic input, nitrogen fixation and lack of mineralizable organic nitrogen (Saviour and Stalin, 2012; Mukhopadhyay et al., 2014). The low nitrogen content of the soil can be attributed to lack of microbial activity and removal of natural vegetation in mining soil. Phosphorus content of soil is influenced by pH, organic matter and weathering processes (Mukhopadhyay et al., 2014) and has an important effect on metal bioavailability as a result of sparingly soluble salts (Finzgar et al., 2007).

The microbial activities are reduced in soil affected by high sulfur content which ultimately affects biogeochemical cycles and mineral nutrition (Menz and Seip, 2004).

The iron content of the soil samples showed that they are adequate for mine soil samples as they contain concentrations greater than 4.5 mg/kg of soil samples (Maiti, 2006). The copper content of the soil samples is also low and within the toxic limits of the National Norms and Standards for Remediation of Contaminated Land and Soil Quality in South Africa of 16 mg/kg of soil for all land uses. The low copper content can be attributed to the low organic matter content of the soils that possibly immobilize metals (Maiti, 2006). The copper content of the soil samples were lower than those reported by Wahl (2014) from gold mining soils in North West province of South Africa.

Mining activities have resulted in destruction and alteration of the ecosystem through metal pollution of the surrounding soil (Ayangbenro and Babalola, 2017). Although the concentration of

heavy metals in the soil samples falls within the permissible limits of National Norms and Standards for Remediation of Contaminated Land and Soil Quality in South Africa, these metals still have potential significant impacts on biological systems (Guo et al., 2009). This was observed in the lower microbial diversity that were found in the polluted sites compared with the control soil. The findings of this study are similar to the concentrations of heavy metals reported by Wahl (2014) from gold mining soil from North West province of South Africa which also falls below the permissible limits. The heavy metal concentration recorded in this study is lower than those in the study conducted by Olobatoke and Mathuthu (2016) from gold mining sites in Johannesburg, South Africa. Metal concentrations were found to exceed the recommended limits set by the South African regulatory body. Soil particle size influences the heavy metal content of such soil (Dube et al., 2001). Hence, metal content is higher in clay soil than in sandy soil because clay minerals have large surface area and weak pH dependence of CEC (Dube et al., 2001). The low metal contents in the soil samples in this study can be attributed its sandy nature. This implies that high amounts of metals will be in soil solution, thereby increasing metal bioavailability in the environment. The binding forces between soil particles and metal ions are dependent on the pH and the ionic properties of the metal ions (Dube et al., 2001). Increase in pH of the environment decreases the binding forces between soil particles and metal ions. The slightly acidic pH to slightly alkaline pH of the soil samples can also be attributed to the low heavy metal concentrations obtained from the soil samples. Likewise, the affinity of each metal varies between different soil mineral components and organic matter.

Bacteria in polluted environments have been exposed to high concentrations of heavy metals from nearby environments which have occurred steadily over a long period (Oliveira et al., 2010; Ayangbenro and Babalola, 2018). Mining soils contaminated with metals and metalloids are sources of metal resistant bacteria isolates belonging to *Acinetobacter*, *Bacillus*, *Pseudomonas*, and *Enterobacter* species (Fan et al., 2011; Ndeddy Aka and Babalola, 2017), with *Bacillus* species reported to be the most abundant in metal contaminated soil (Ellis et al., 2003). These soils are

considered an interesting model for the development of metal resistant bacteria isolates (Rajkumar and Freitas, 2008). The isolated organisms have been previously isolated from mining and metal contaminated environments (Sharma and Fulekar, 2009; Zhang et al., 2011; Mohite et al., 2018; Oladipo et al., 2018). The ability of these isolates to survive in metal polluted environment can be attributed to their cell wall composition that binds and interacts with metal ions and their genetic makeup (Abou-Shanab et al., 2007; Shin et al., 2012).

The total bacterial counts from each sample were higher than the counts obtained in the presence of heavy metals from each sample. This can be attributed to growth inhibition in the presence of heavy metals which suppresses the diversity and population of bacteria in the growth medium (Chihomvu et al., 2014; Zampieri et al., 2016). Screening of metal resistant bacterial isolates is a continuous process in the search for isolates that have the potential to remediate polluted environments. Hence, there is a need to screen for resistant isolates from mining soils to harness the potential benefits of these organisms. The metal tolerances reported in this study are higher than those reported by Rajesh and Rajesh (2016), Mohite et al. (2018), Oladipo et al. (2018) and Marzan et al. (2017). However, they are lower than those reported by Zhang et al. (2011). The different responses of each isolate to different concentrations of heavy metals suggests different action mechanisms, unique chemistry and toxicity of each metal ion (Edwards and Kjellerup, 2013; Oladipo et al., 2018).

Multi-metal resistance by microbial strains gives mutual benefits to the single components and are suitable for metal removal (Alisi et al., 2009). Multi-metal resistance showed the presence of various genetic determinants for metal resistance, which could have evolved in the natural environment of the organism. The genetic determinants encode for specific metal transport proteins involved in sequestration of metal ions and regulating active efflux (Pal et al., 2005). Multi-metal resistance could also be attributed to existence of concomitant regulating factors for co-selection of metals that is widespread in the environment (Rahman and Singh, 2016).

Bacteria have adapted to environmental changes by physiological and genetic mechanisms that confer new traits, such as metal resistance and xenobiotic degradation, to survive and colonize their environment (Anjum et al., 2011). Metal resistance is known to be achieved through efflux systems as found in Gram-negative organisms (Blair et al., 2014) that pump metals out of the cytoplasm into the periplasmic space, and other mechanisms such as complexation, accumulation, transformation, sorption, oxido-reduction reactions and mineralization (Govarthanan et al., 2013; Ayangbenro and Babalola, 2017). Bacterial isolates were screened for metal resistance genes by PCR amplification of genomic DNA for genes responsible for Cd, Cr, and Pb resistance. These genes have the potential to reduce or eliminate metal toxicity (Wei et al., 2009).

Most of the isolates harbor the *CzcD* operon that is responsible for multi-metal resistance (Cd, Co and Zn), which is widespread in most isolates. It is necessary for the activation of *Czc* determinant known to encode for Cd, Co and Zn resistance together (Nies et al., 1989). It is a protein involved in detoxification of these metals, by exporting these metal ions from the periplasm and/or from the cytoplasm directly into the extracellular medium and regulation of *CzcCBA* pump (Legatzki et al., 2003). *cadA* is a cadmium efflux and that is involved in soft-metal-transporting ATPases. This protein binds the inducing cations with high affinity to cysteine residues and interacts directly with the promoter or operator region of the ATPase-encoding gene (Legatzki et al., 2003). The expression of *cadA* by bacteria isolates in this study is controlled by the cytoplasmic concentration of zinc and cadmium in their cells. The *cadA* and *Czc* are energy dependent efflux systems, *cadA* is constitutively expressed while *Czc* is inducible (Nies et al., 1989). *cadA* appears to be the main detoxification mechanism of Cd in this study as most of the isolates that have *CzcD* operon do not possess the *cadA* gene.

The *PbrA* is an efflux transporter (P-type ATPases) protein and *PbrT* is an uptake protein that is involved in lead resistance (Shin et al., 2012).The organisms were screened for efflux transporter *PbrA* and lead uptake protein *PbrT* and some of the isolates harbor these genes. *ChrA* confers

chromate resistance on the organisms which results from the accumulation of CrO_4^{2-} (Nies et al., 1990). It is an efflux pump responsible for chromate extrusion (Pimentel et al., 2002). Therefore, organisms possessing metal resistance genes are ideal isolates for metal removal in contaminated environments and potential sources of novel metal resistant genes. The organisms that did not harbor any of the metal resistance genes could be explained by the lack of genetic systems responsible for metal resistance or the presence of other efflux mechanisms for metal resistance (Shin et al., 2012). Furthermore, the primers used for resistant gene amplification may be inappropriate.

The low tolerance for Cd and Cr which also follows the decrease in growth rate of the bacterial isolates in the presence of these metals can be explained by the slow utilization efficiency as a result of high energy cost of the organisms in the presence of metal stress (Giller et al., 2009). This is also a common phenomenon as isolates from metal polluted environment only proliferate in the presence of small amounts or the absence of metal in the growth medium under laboratory conditions (Gadd, 1990). Other factors that could be responsible for this are media composition, dosage and incubation conditions. Whatever the case, heavy metals usually suppress microbial population and species diversity in a particular environment, thus selecting for resistant isolates (Zampieri et al., 2016). The growth rate of all the isolates in the presence of heavy metals was generally lower than that of the control. This is similar to the pattern observed by Raja et al. (2006) and Pal et al. (2004).

Microbial metabolites are generating increasing attention and are usually the first barrier against metal toxicity that is essential in protecting the internal cell (Li and Yu, 2014; Ayangbenro and Babalola, 2018). These biopolymers are desirable to circumvent the pathogenicity concerns of some of the producing isolates. Metal resistant isolates were screened for biopolymer production that are useful in metal(loid) removal from contaminated environment. The presence of

extracellular substances which serve as barrier in Gram-positive bacteria enhances resistance to metal compared to Gram-negative organisms (Ianeva, 2009).

The screening for biosurfactant production was based on the concept that contaminated environment serves as a source of potential biosurfactant-producing isolates and the isolates were evaluated under laboratory conditions (Eddouaouda et al., 2012). Hemolysis of red blood cells on nutrient agar has been used to screen for biosurfactant production by bacteria (Bicca et al., 1999; Kumar et al., 2016a). Carrillo et al. (1996) and Kumar et al. (2016a) found a correlation between hemolysis of red blood cell and surfactant production, and suggested its use as a screening procedure for biosurfactant production. However, not all biosurfactant producing isolates have the ability to lyse red blood cells. It could exclude some potential isolates, but has helped in the initial screening of surfactant producing strains (Youssef et al., 2004).

Drop collapse method was suggested as an easy and sensitive method for biosurfactant production by Jain et al. (1991). The organisms were therefore screened for drop collapse and it was observed that some isolates that were able to hemolyze red blood cells were negative for drop collapse. This can be attributed to the fact that some isolates act as biosurfactants themselves and do not produce extracellular polymer (Thavasi et al., 2011). The drop collapse is a criterion that excludes hemolytic organisms that do not produce biosurfactant.

Emulsification index is another criterion used in the selection of surface active producing bacterial isolates. Satpute et al. (2008) suggested that more than one screening method should be used in the primary screening of potential surface-active agents. Emulsification enhances the bioavailability of metals and thus increases the removal rate. Emulsification test is a reliable test for surfactant production which makes an organism a suitable surface-active agent. It was however observed that some bacterial isolates were able to emulsify some of the oils tested but did not produce hemolysis or are positive for drop collapse.

Biosurfactant production by bacteria isolated from mining environment was reported by Toribio-Jim et al. (2014) and Wang and Mulligan (2009) and were found to be useful in metal removal from contaminated environment. Production of biosurfactant could be a mechanism of metal uptake and metal resistance by the isolated organisms.

Bioflocculant is another biopolymer produced by bacterial isolates during growth. The flocculating properties of the metal resistant isolates were determined to access the aggregation of particles and cells (Yim et al., 2007). Bacterial isolates that were able to flocculate suspended kaolin clay can adsorb to other particles to form flocs (Tang et al., 2014). The flocs are capable of effective metal removal from solution.

Conclusion

This study has identified many heavy metal resistant bacterial isolates with high tolerance and capable of producing biopolymers which are metal complexing agents for future biotechnological use. The study also showed that mining soils are deficient in nutrients which affects the distribution, population and diversity of microorganisms in the environment. Moreover, *Bacillus* species were the dominant organisms in the areas and they showed high metal resistance.

CHAPTER FIVE

HEAVY METAL REMOVAL FROM CONTAMINATED SOIL BY LIPOPEPTIDE BIOSURFACTANT PRODUCED BY *BACILLUS CEREUS* NWUAB01

Abstract

Microbial polymers, such as biosurfactants, are good metal(loid) complexing agents and have been used for the removal of metal(loids) from polluted environmental media. Biosurfactant from *Bacillus cereus* NWUAB01 isolated from a gold mining soil was evaluated for heavy metal (Cd, Cr and Pb) removal from contaminated soil. Heavy metal resistance of the isolate was determined on Luria Bertani agar. Biosurfactant production was determined by conventional screening methods that include hemolysis, drop collapse, emulsification and surface tension measurement. Strain NWUAB01 was able to grow on 200 mg/L of Cd and Cr, and was resistant to 1 g/L of Pb. The surfactant produced is a lipopeptide with a metal complexing property. It has emulsification of 22%, 24%, 37% and 54% on hexadecane, vegetable oil, kerosene and engine oil respectively. The surfactant had a surface tension of 39.5 mN/m. The crude biosurfactant was able to remove 35%, 56% and 78% of Cr, Cd and Pb respectively from contaminated soil. The biosurfactant produced by strain NWUAB01 is a promising metabolite for remediation of heavy metal contaminated soil.

Keywords: bioremediation, biosurfactant, metal(loid)s, mining, pollution, secondary metabolite

5.1 Introduction

Industrialization and mining activities have continued to put an increasing burden on the environment as a result of metal(loid) pollution (Das et al., 2009; Ayangbenro et al., 2018). The unrestrained release of metal(loid)s into the environment from these activities poses a serious threat to the ecosystem and the health of living organisms. Heavy metals have been known to cause various diseases and ailments in humans, such as allergic reactions, birth defects, damage to

internal organs, cardiovascular and respiratory disorder, learning disabilities and cancer (Ayangbenro and Babalola, 2017).

Conventional methods of heavy metal removal involve treatment with chelating agents, organic and inorganic acids, reverse osmosis, surfactants and water. However, these techniques are often expensive and ineffective for low heavy metal concentration removal. Other challenges often encountered with the use of these conventional techniques include non-specificity of these methods, high energy demand, space requirements and the impractical nature of some of the techniques (Das et al., 2009; Voica et al., 2016).

Bioremediation of heavy metals offers an alternative and effective means of decontaminating metal polluted environments. Heavy metal remediation of contaminated environment mediated by microorganisms is efficient and cost effective (Voica et al., 2016). Microorganisms have developed various mechanisms for detoxifying heavy metals. These mechanisms include bioaccumulation, biomineralization, biosorption and biotransformation (Lin and Lin, 2005). These organisms also secrete a range of metal sequestering biopolymers that are employed in metal uptake (Wu et al., 2010a; Fomina and Gadd, 2014). These biopolymers also trap and absorb metal sulfides and oxides (Wu et al., 2010a).

Biopolymers of microbial origin are emerging as a promising technique to enhance the effectiveness of metal(loid) removal. Similarly, these polymers can survive in a wide range of pH and temperature. Their metal binding ability depends on the producing organism, metal affinity and specificity, functional groups on the biopolymer, temperature and pH (Pal and Paul, 2008; Li et al., 2014; Ayangbenro and Babalola, 2018). They are environmentally friendly, versatile and economic compared to chemical polymers.

One of the numerous polymers of microbial origin is biosurfactant, with numerous applications in detergents, cosmetics, medicine, food industries, petroleum and bioremediation (Valls and De

Lorenzo, 2002). Biosurfactants have been reported as a metal complexing agent for removal of metal(loid)s from polluted soil and wastewaters (Singh and Cameotra, 2004; Mulligan, 2005; Dahrazma and Mulligan, 2007; Ayangbenro and Babalola, 2018). They solubilize metal ions through surface tension reduction and increase in wettability, thereby bringing metal ions out of the soil matrix. Biosurfactants of microbial origin are good metal complexing agents due to their stability, degradability, low toxicity and environmental compatibility (Rizzo et al., 2015). They form stable complexes with metal ions as a result of electrostatic interaction between charged polymers.

Cadmium, chromium and lead have been listed among toxic elements within the first twenty pollutants priority list that are of significance to public health (Cáliz et al., 2013). The use of isolated biopolymers from microorganisms is desired to circumvent the pathogenicity concerns of some producing organisms. Therefore, this research work investigate the removal of these toxic metals (Cd, Cr, and Pb) by biosurfactants produced by heavy metal resistant *B. cereus* NWUAB01 isolated from gold mining polluted soil in Vryburg, South Africa.

5.2 Materials and method

5.2.1 Isolation and screening of heavy metal resistant bacterial isolates

Soil samples were collected from active gold mine in Vryburg (26˚10’5.28” S, 25˚14’47.69” E), North West Province, South Africa. Control soil samples were also collected around the mines. Soil samples were collected in triplicates at depth of 10-30 cm with a soil auger in June 2016 and transported to the laboratory for analysis in sterile plastic bags. Isolation of heavy metal (Cd, Cr, Pb) resistant isolates was done as described by Rajkumar and Freitas (2008). One gram of each of the collected soil samples was serially diluted and plated out on Luria-Bertani agar that has been supplemented with 50 mg/L of each metal solution and a mixture of metals. The metal solutions: $CdSO_4$ (Sigma-Aldrich, India), K_2CrO_4 and $Pb(NO_3)_2$) (Sigma-Aldrich, USA) were filter-

sterilized through 0.22 µm membrane filter (Millipore Corporation, Bedford, MA, USA). The plates were incubated at 37˚C for 48 h. Resistant isolates were then screened for ability to grow on different concentrations (100 to 1000 mg/L) of each metal salt on LB agar plates (Srinath et al., 2002). The plates were incubated at 37˚C for 48 h and observed for growth. The lowest concentration of metal that completely inhibits the growth of bacterial isolates was considered the minimum inhibitory concentration (MIC). Metal sorption by strain NWUAB01 was performed with 100 mg/L of each metal and optical density measured at 24 h interval.

5.2.2 Identification of strain NWUAB01

The following biochemical tests were carried out on strain NWUAB01: Gram reaction, catalase test, citrate utilization, indole production, hydrogen sulfide production, oxidase, nitrate reduction, methyl red, sugar fermentation test (fructose, glucose, galactose, lactose, starch, sorbitol, sucrose, maltose, mannitol), and Voges-Proskauer test. The organism was identified according to the Bergey's manual of determinative bacteriology (Garrity et al., 2004).

The DNA of the strain NWUAB01 was extracted from pure culture grown on LB agar using a ZR soil microbe DNA mini prep ™ DNA extraction kit (Zymo Research, CA, USA) according to the kit's protocol. The quality and quantity of the DNA was determined using a NanoDrop Lite spectrophotometer (Thermo Fischer Scientific, CA, USA).

5.2.3 Amplification of 16S rDNA and detection of heavy metal resistance genes

Universal primers F1 (forward, 5′- AGAGTTTGATCCTGGCTCAG -3′) and R2 (reverse, 5′- ACGGCTACCTTGTTACGACTT -3′), with approximately 1500 base pair amplicon, were used to amplify the 16S rRNA gene of strain NWUAB01 (Marin et al., 2011). The PCR was performed with a total volume of 25 µl of a reaction mixture consisting of 12.5 µl 2X master mix (One Taq® Hot Start Quick Load, Biolabs, England), 0.5 µl (10 µM working solution) of each of the forward and reverse primer, 1 µl of the DNA template, and 11 µl nuclease free water. The PCR was

performed with a thermal cycler (Bio-Rad, CA, USA) with preincubation step of 95˚C for 5 min and 35 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 30 s, and extension at 72˚C for 1 min, followed by a final extension step of 72˚C for 10 min. Polymerase chain reaction amplicons were analyzed by electrophoresis on agarose gel (1% w/v) containing ethidium bromide (1%) and the band size was determined using 1 kb molecular marker.

The sequencing method was as described in section 4.2.6. The nucleotide BLAST of the 16S rRNA gene sequence homology was searched on the NCBI GenBank database. The sequence was aligned and analyzed by Molecular Evolutionary Genetics Analysis (MEGA 7.0) software (Kumar et al., 2016b). (Kumar et al., 2016b)(Kumar et al., 2016b)(Kumar et al., 2016b)(Kumar et al., 2016b)(Kumar et al., 2016b)(Kumar et al., 2016b)The phylogenetic tree was constructed based on the 16S rDNA using the maximum parsimony method. The sequence was deposited in the NCBI GenBank database.

Analysis of heavy metals encoding genes was done with primers encoding for cadmium (*cadA*, *CzcA*, *CzcB* and *CzcD*), chromium (*chrA* and *chrB*) and lead (*PbrA* and *PbrT*) for the isolate. The sequences of each primer set and their PCR conditions are presented in Table 4.1. All the primer set used for PCR amplifications were synthesized by Whitehead Scientific, Integrated DNA Technologies, South Africa.

The isolate was sequenced on the Illumina Miseq platform. Sample DNA was fragmented by ultrasonication procedure (Covaris), and the fragments selected by size with AMPure XP beads and the end was repaired. Illumina specific adapter sequences were ligated to each fragment. The fluorometric method was used for quantification of the fragment and then diluted to a standard concentration (4 nM). A MiSeq v3 kit was used for sequencing of the fragment on the Illumina Miseq platform. The obtained sequences were processed and the quality of the reads were checked using FastQC v.1.0.1 of the Kbase platform (Arkin et al., 2016). The reads were trimmed to remove low quality sequences and adapter sequences using Trimmomatic v0.36. The CLC *de novo*

assembly was used to assemble the genome using the CLC platform. The Rapid Annotations using Subsystems Technology (RAST v2.0) server (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP v4.5) (Haft et al., 2017) were used for genome annotation.

5.2.4 Screening and characterization of biosurfactant produced by strain NWUAB01

Pure strain of NWUAB01 was used to quantify biosurfactant production by different methods which include hemolytic activity, drop collapse test, oil displacement test, and emulsification activity. All tests were carried out in triplicates. The hemolytic test on blood agar was carried out as described by Bicca et al. (1999).

The cultivation medium for biosurfactant production contains (g/l): yeast extract, 0.5; sucrose, 5.0; $Na_2HPO_4 \cdot 12H_2O$, 1.4; $MgSO_4 \cdot 7H_2O$, 0.02; KH_2PO_4, 0.4 and peptone, 20.0 (Sriram et al., 2011). The medium was seeded with 3% inoculum prepared from LB broth into 50 ml cultivation medium in a 250 ml Erlenmeyer flask and incubated at 37˚C for 7 days at 160 rpm.
All readings were taken with supernatant obtained by centrifuging the cultures at 10,000 rpm for 20 min.

The drop collapse test was determined as described by Sriram et al. (2011). Mineral oil (2 µl) was added to each well of a 96-well microtiter plate and allowed to equilibrate for one hr at 37˚C. Thereafter, 5 µl of the culture supernatant was added to the center of each well over the oil layer. After one min, the shape of the oil drop was examined. A flattened drop was recorded as positive for biosurfactant production. Water was used as negative control.

The oil displacement test was carried out as described by Sriram et al. (2011). A petri dish (150 mm diameter) was filled with 40 ml sterile distilled water and engine oil (15 µl) was added. Thereafter, 10 µl of the supernatant was added to the center of the oil film and the halo zone was measured after 30 s of incubation.

The emulsification activity of strain NWUAB01 was determined by measuring the emulsification index (E_{24}) after 24 h. A 2 ml volume of the culture supernatant was added to 2 ml of kerosene in a test tube and the mixture was vortexed at high speed for 2 min. The E_{24} was calculated as the percentage of height of emulsified layer divided by total height of the liquid column (Rizzo et al., 2015). The test was also performed using engine oil, hexadecane and vegetable oil in place of kerosene. The stability of the emulsion produced was determined at different temperature and pH. Surface tension of cell free supernatant was determined in a force tensiometer (Sigma 702, Biolin Scientific, Sweden) using the du Nouy ring method at room temperature.

The biosurfactant produced was extracted and purified as described by Gond et al. (2015). Strain NWUAB01 was grown in 1 L cultivation medium for 7 days at 37˚C at 200 rpm. The cell free supernatant obtained by centrifuging at 5000 rpm for 15 min at 4˚C was precipitated by adding conc. HCl to reduce the pH to 2 and incubated overnight at 4˚C. The precipitate was collected by centrifugation at 10000 rpm for 15 min at 4˚C and dissolved in methanol and then filtered using a membrane filter (0.22 µm PTFE) to remove cell components and larger particles. The resulting mixture was then concentrated using a vacuum evaporator at 30˚C and lyophilized.

The lyophilized biosurfactant was characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra, with a resolution of 4 cm^{-1}, were collected from 400 to 4000 wavenumbers (cm^{-1}) with an average of 32 scans using an Alpha II Platinum-ATR IR spectrophotometer (Brucker, USA).

The molecular mass of the surfactant was determined using Micromass ToFSpec matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MICRO-TOF-MS). Applied Biosystems 4800 Plus MICRO-TOF/TOF analyzer (AB Sciex, USA) was used to obtain a purified sample. The analyzer was operated in the positive ion mode with 337 nm nitrogen laser for ionization, accelerating voltage of 20 kV and a-cyano-4-hydroxycinnamic acid for matrix. The

molecular weight was determined by mass spectrum smart formula tools and the mass spectrometry was determined using the Bruker compass data analysis.

5.2.5 Remediation of heavy metal contaminated soil with biosurfactant

Biosurfactant washing of heavy metal polluted soil was performed in a batch experiment as described by Singh and Cameotra (2013). A gram of heavy metal (Cd, Cr, Pb: 100 mg/L of each metal salt) spiked soil sample was placed in a series of 50 ml centrifuge tubes with 25 ml of the biosurfactant solution. The suspensions were centrifuged at 5000 rpm for 12 min and the supernatant was filtered. The metal composition of the filtered supernatant was analyzed by Inducible Conductivity Plasma-Optical Emission Spectroscopy (725 ICP-OES, Agilent Technologies, Palo Alto, CA, USA). The positive control used is strain NWUAB01 while sterile distilled water served as the negative control. The metal removal efficiency was calculated using the formula:

$$Removal\ rate\ (\%) = \frac{Ci - Ce}{Ci}\ X\ 100$$

Where C_i and C_e are the initial and final concentrations of metal respectively.

5.3 Results and Discussion

Soils contaminated with heavy metals are usually sources of metal resistant bacteria isolates (Xie et al., 2016; Ndeddy Aka and Babalola, 2017). Mining soils are rich sources of potential bacterial populations that are resistant to heavy metals but with reduced bacteria diversity, population size and metabolic activities (Choudhary and Sar, 2009; Zampieri et al., 2016). Metal resistance might have developed as a result of the presence of metals in their growth medium (Çolak et al., 2011). In this study, soil samples from a gold mining environment, with natural occurrence of heavy metals, were used for isolation of metal resistant isolates. Thus, the metal-containing environment might have led to the evolvement of mechanisms of resistance to heavy metals in the isolated organisms. Ninety-eight heavy metal resistant bacteria isolates were isolated from the collected

soil samples. Elevated levels of tolerance to metals is an important criterion for metal removal by bacterial strains (Ndeddy Aka and Babalola, 2017). Strain NWUAB01 showed multiple resistance to the metals tested and good preliminary metal removal properties, with the ability to grow on all the concentrations of Pb tested while it showed growth on 100 and 200 mg/L of Cd and Cr (Table 5.1). The organisms showed the ability to withstand varying metal concentrations as reported in different studies (Gnanamani et al., 2010; Shim et al., 2014) from different polluted sites and higher tolerance compared to those observed by Oladipo et al. (2018). Varied responses of strain NWUAB01 to different metal ions observed in this study could be attributed to different mode of action, unique chemistry and level of toxicity of each metal (Edwards and Kjellerup, 2013; Oladipo et al., 2018).

Multi-metal resistance by microbial strains gives mutual benefits to the single components and are suitable for metal removal (Alisi et al., 2009). Multi-metal resistance showed the presence of various combinations of genetic determinants for metal resistance. This could probably have evolved in the natural environment of the organism. The genetic determinants encode for specific metal transport proteins involved in sequestration of metal ions and regulating active efflux (Pal et al., 2005). The resistance pattern to the tested heavy metals follows the order Pb > Cd = Cr. Our results showed that Cd and Cr are more toxic to strain NWUAB01 than Pb due to the toxicity of the metals on the organism. Many reports had also reported many bacteria with multi-metal resistance abilities (Abou-Shanab et al., 2007; Dell'Amico et al., 2008; Ndeddy Aka and Babalola, 2017). Multi-metal tolerance in *Bacillus* species has been well documented.

The presence of extracellular substances which serve as a barrier in Gram-positive bacteria enhances metal resistance compared to Gram-negative organisms (Ianeva, 2009). A direct comparison of metal resistance by strain NWUAB01 with other studies is difficult due to the composition and strength of medium, nature of the medium that influences metal bioavailability,

complexation, organic constituents, diffusion rate and incubation period which causes variations in inhibitory concentrations (Govarthanan et al., 2013; Shim et al., 2014).

Various mechanisms are employed by microbial cells for metal removal (Lin and Lin, 2005). These include bioaccumulation, biomineralization, biosorption and biotransformation. The growth inhibition curve of strain NWUAB01 on 100 mg/L of each metal at pH 7, agitation of 150 rpm and 25 ˚C is presented in Figure 5.1. The highest optical density was obtained on the sixth day of growth for each of the metals tested and the control. The optical density generally increases with time for all the metals and control (Figure 5.1). In summary, a decrease in the optical density of strain NWUAB01 was observed in the presence of heavy metals compared with the metal-free medium. This is similar to the pattern observed by Shim et al. (2014) and Raja et al. (2006). The presence of heavy metal in the growth medium is consistently less than that observed in the control. The decrease in growth of *B. cereus* in the presence of heavy metals might be due to metal ion interaction with cell membrane and thus increases metal binding sites which makes it less effective for transport of materials essential for growth (Achal et al., 2012). To understand the mechanism of resistance to metals, the growth kinetics is used as an index of adaptation (Oladipo et al., 2018). The inverse growth rate relationship observed between metal concentrations and growth rate in tolerant bacteria are characteristic of bacteria growth in response to external stress (Bloem and Breure, 2003). The high inhibitory values obtained for Cd and Cr along with decrease in growth rate in the presence of these metals could be attributed to decline in efficiency of substrate utilization as a result of high energy cost of the organism subjected to metal stress (Giller et al., 2009).

The biochemical profile of strain NWUAB01 is presented in Table 5.2. The organism was able to ferment glucose, fructose, sucrose, and starch; utilize citrate and is catalase positive (Table 5.2).

Table 5.1: Growth of *B. cereus* NWUAB01 on different concentrations of heavy metals

Metal concentration (mg/L)	100	200	300	400	500	600	700	800	900	1000
Cd	+	+	-	-	-	-	-	-	-	-
Cr	+	+	-	-	-	-	-	-	-	-
Pb	+	+	+	+	+	+	+	+	+	+

(+) Positive reactions; (-) negative reactions

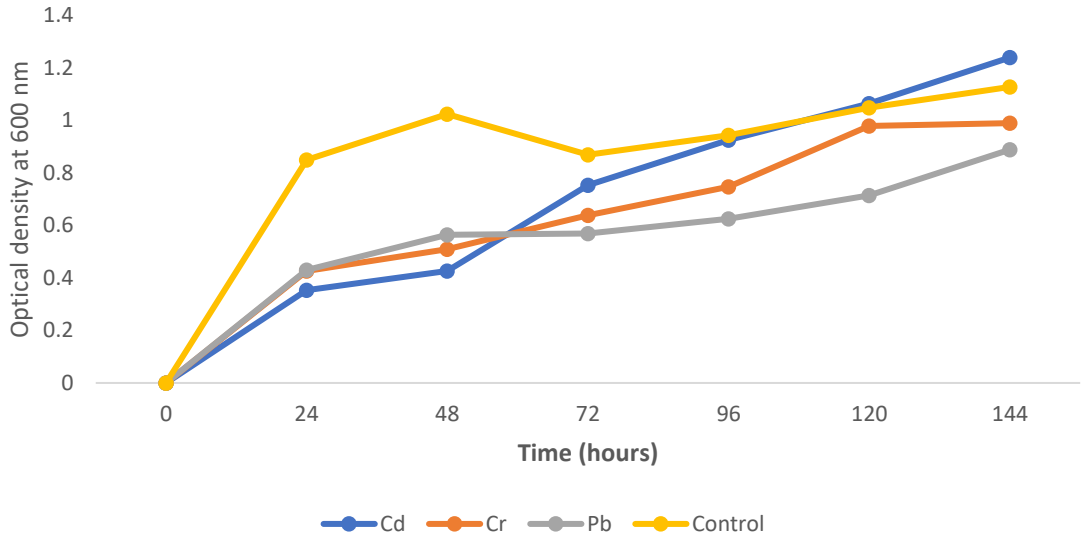


Figure 5.1: Time course growth of *B. cereus* NWUAB01 on different heavy metals

Table 5.2: The biochemical properties of *B. cereus* NWUAB01

Test	Result	Test	Result
Gram reaction	+	Sugar fermentation	
Shape	Rod	Glucose	+
Catalase	+	Galactose	-
Citrate utilization	+	Lactose	-
H_2S production	-	Starch	+
Indole	-	Sucrose	+
Methyl red	-	Sorbitol	-
Nitrate reduction	+	Maltose	-
Oxidase	-	Mannitol	-

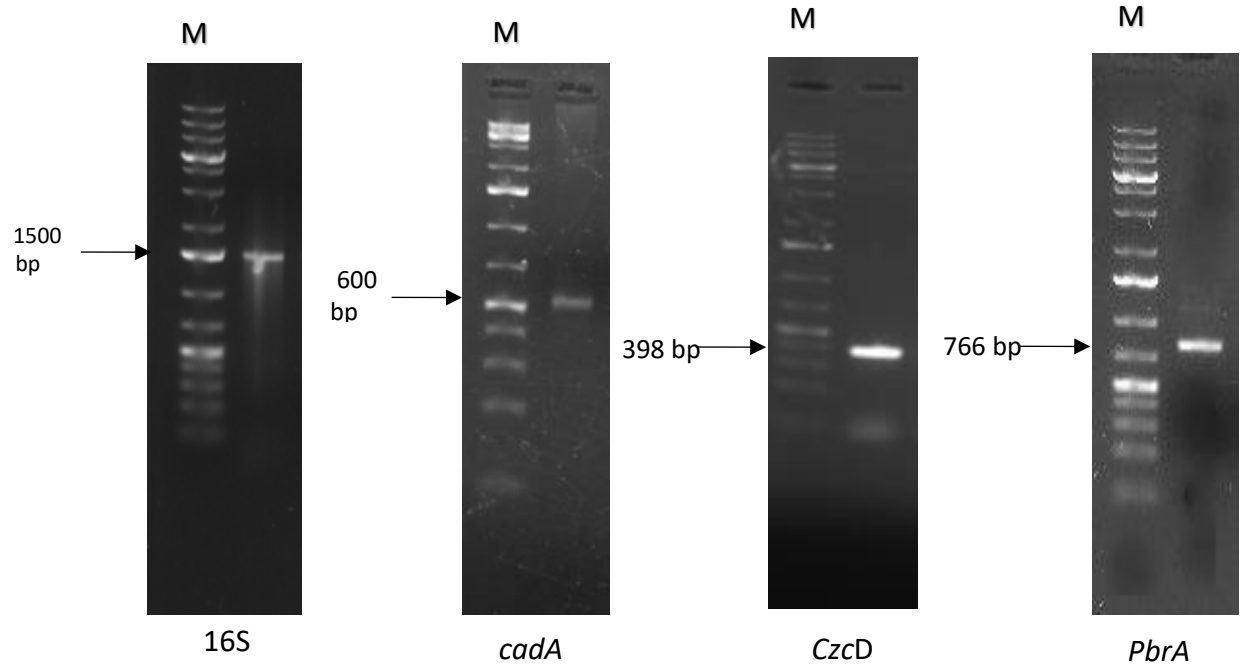
(+) Positive reactions; (-) negative reactions

The amplification of the 16S rRNA gene of strain NWUAB01 yielded the expected 1500 bp amplicon (Figure 5.2). The amplicon sequence was compared with the 16S rRNA gene sequences in NCBI database and it showed that strain NWUAB01 had 100% similarity with *Bacillus cereus* strain BS16 (MH021873), *B. wiedmanni* strain F23 (MF681995), *B. thuringiensis* strain FDB-6 (MH260380), *Bacillus* sp strain SP9 (MH191109) and 99% similarity with *B. proteolticus* strain SPB3 (MG280785) with E-value of 0.0. The organism has a 94.6% relationship with *B. cereus* 172560W and *B. cereus* BAG30-2 genomes (Figure 5.4). *B. cereus* NWUAB01 has been deposited at the NCBI database under the 16S rRNA gene accession number MH399230 and whole genome accession number QNGD00000000. *Bacillus cereus* has been reported to have a strong relationship with gold mines (Reith et al., 2005) which is evident in this study.

Genes encoding metal resistance can eliminate or reduce metal toxicity (Shin et al., 2012). Hence, strain NWUAB01 was screened for metal resistance genes. The amplification of primer-specific heavy metal resistance genes of chromosomal DNA of strain NWUAB01 yielded amplicons of the expected band size of 600 bp for *cadA*, 398 bp for *CzcD*, and 766 bp for *PbrA* (Figure 5.2). *PbrA* is the gene responsible for lead uptake and down-regulation of the metal concentration which occur in response to high levels of lead (Wu et al., 2016). It thus revealed that isolate NWUAB01 has a functional gene that is key in lead resistance. *PbrA* is an active efflux pump gene that transports Pb ions against concentration gradient using energy provided by ATP hydrolysis (Shin et al., 2012). *cadA* which is a P-type ATPase, was also found to be present on the organism. *cadA* is cadmium specific ATPase used for Cd efflux and confers metal resistance to strain NWUAB01. *CzcD* is responsible for efflux of cobalt, zinc and cadmium. Both *CzcD* and *cadA* operons are energy dependent efflux and confer cadmium resistance (Nies et al., 1989). The efflux systems are actively involved in the pumping out of toxic metal ions that enter the cell through ATPase diffusion. Metal transport proteins are involved in transporting metal ions outside the cell membrane (Naik and Dubey, 2013). These metal transporting proteins are a group of PIB-type ATPases which governs metal resistance. *cadA, CzcD* and *PbrA* belong to these groups of proteins

present in strain NWUAB01 which are involved in metal resistance. These proteins prevent metal accumulation of highly reactive and toxic metals within the cell membrane and also play a key role in metal resistance by strain NWUAB01 (Naik and Dubey, 2013).

No amplification was observed for *CzcA, CzcB, PbrT, chrA*, and *chrB*. This might be as a result of the lack of mechanisms responsible for metal resistance in the genetic system of the organism. The organism may also use other mechanisms different from the efflux system for metal tolerance. It is likely that amplification of the genes was done with inappropriate primers or the genes were not present in the organism.



Lane M-1kb molecular marker

Figure 5.2: The 16S rRNA and heavy metal resistance genes amplification of DNA sequence of strain NWUAB01

The evolutionary relationship of strain NWUAB01 was deduced by the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The phylogenetic relationship of strain NWUAB01 is presented in Figure 5.3, which shows the relationship of the organisms with closely related strains from the GenBank. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree for the heuristic search was

obtained automatically by applying the maximum parsimony method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 6 nucleotide sequences. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016b).

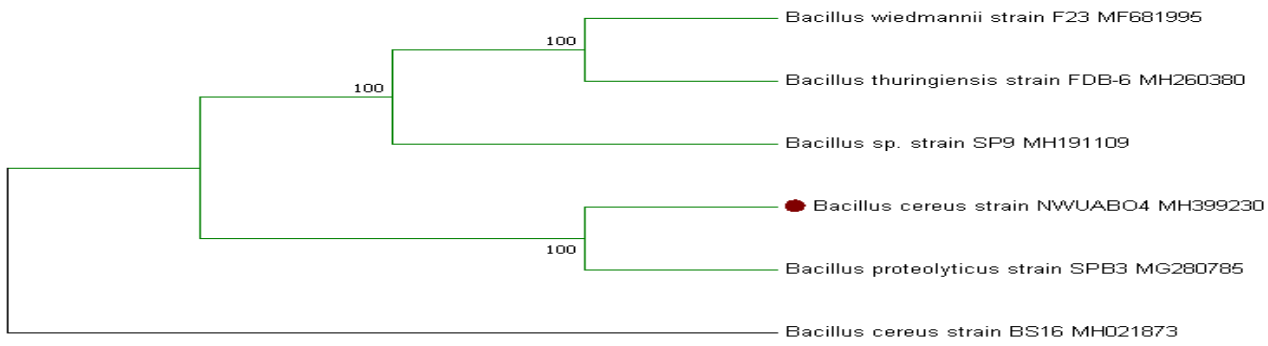


Figure 5.3: Phylogenetic tree using maximum likelihood method of strain NWUAB01 based on 16S rRNA gene sequence

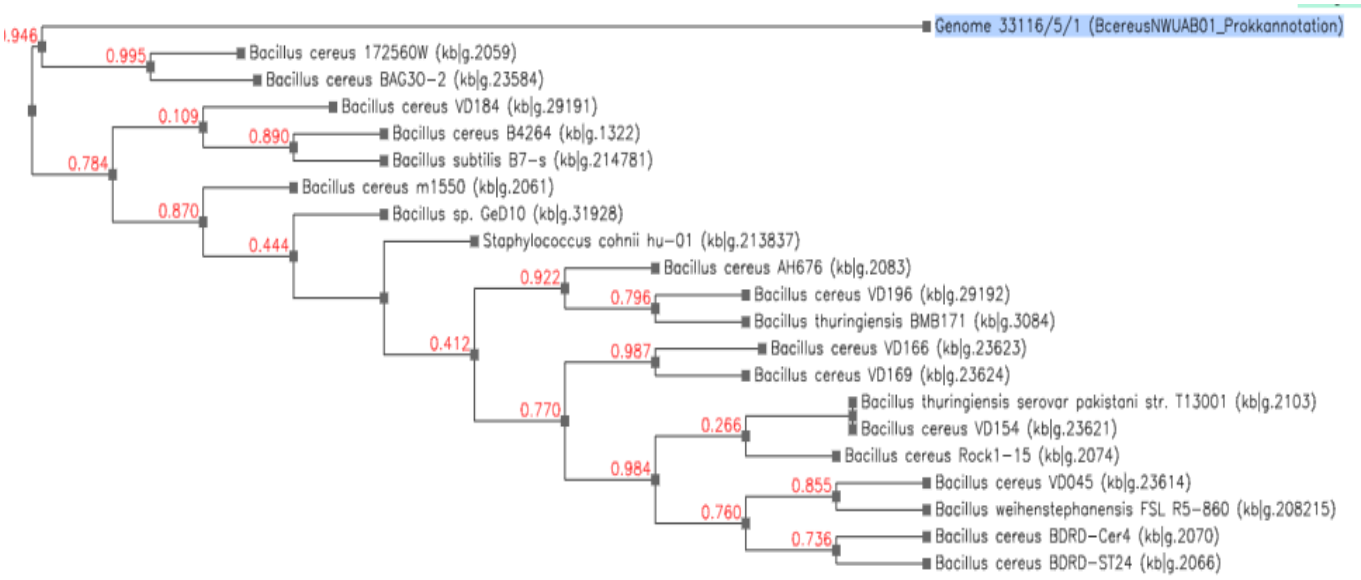


Figure 5.4: The phylogenetic relationship of the pangenome sequence of strain NWUAB01 with other closely related organisms generated from KBase database

The emulsification of different hydrocarbons and vegetable oil by strain NWUAB01 is presented in Figure 5.5 and the biosurfactant properties of the organism are presented in Table 5.3. The organism was able to completely hemolyze red blood cells (α-hemolysis), was positive for drop collapse test and reduced the surface tension of the medium to 39.48 mN/m (Table 5.3). Blood agar has been used to quantify and screen for biosurfactant production by bacteria (Bicca et al.,

1999; Kumar et al., 2016a). Carrillo et al. (1996) and Kumar et al. (2016a) found an association between surfactant production and hemolytic activity, and recommended the use of lysis of blood agar as screening method for biosurfactant production. Although lysis of red blood cells could exclude some biosurfactant producing organisms, it has helped in initial screening of biosurfactant producing organisms. Strain NWUAB01 showed complete hemolysis on red blood cells and was used as the initial screening test for its selection. The reduction in surface tension of water has been reported by several studies (Tuleva et al., 2005; Sriram et al., 2011; Kumar et al., 2016a) for various biosurfactant producing *Bacillus* species. The reduction in surface tension confirmed the production of biosurfactant by strain NWUAB01. The ability to reduce the surface tension of water from 72 to 35 mN/m has been considered as a characteristic of a good surfactant (Mulligan, 2005). Strain NWUAB01 has a surface tension that is similar to that produced by *B. cereus* NK1 which has a value of 38 mN/m (Sriram et al., 2011). It has a better surface tension compared to *B. cereus*, *B. sphaericus* and *B. fusiformis*, which has surface tension of 50, 55.2 and 56.4 mN/m respectively (Bento et al., 2005), and *B. amyloliquefaciens* and *B. thuringiensis* with surface tension values of 57.7 m/Nm each (Barakat et al., 2017). Strain NWUAB01 has lower surface tension potential compared to *Bacillus* sp reported by Heryani and Putra (2017), that had a value of 27.1 mN/m. The differences in the surface tension values can be attributed to different production medium, condition of growth and uniqueness of individual organisms.

Emulsification index is another criterion used in the selection of surface active producing bacterial isolates. Satpute et al. (2008) suggested that more than one screening method should be used in the primary screening of potential surface-active agents. Strain NWUAB01 produced stable emulsions with various hydrocarbons and vegetable oil with the highest E_{24} of 54% obtained with engine oil and lowest E_{24} of 22% with hexadecane (Table 5.3). This appreciable emulsifying property makes the organism a suitable surface-active agent. The ability of biosurfactant to emulsify different hydrocarbons and vegetable oil had been reported for *Bacillus* species with different results. Sriram et al. (2011), reported *B. cereus* NK1 to emulsify motor oil, diesel oil,

crude oil, petrol and vegetable oil with E_{24} of 80.36, 55.5, 70, 44 and 50.47% respectively. Strain NWUAB01 had lower emulsification index compared to what was reported for *B. cereus* NK1. Likewise, Barakat et al. (2017) reported emulsification index of 60 and 69% with paraffin oil for *B. amyloliquefaciens* and *B. thuringiensis* respectively. This might be as a result of the different components of the production medium and different carbon sources used for producing biosurfactant (Pereira et al., 2013).

The production of bioactive metabolites that confer resistance to microorganisms growing in polluted environments is an important defense mechanism against environmental stress and for survival (Rizzo et al., 2015). Biosurfactants are applied in several fields and the applicability depends on their stability at different temperatures and pH values (Khopade et al., 2012). The stability of the emulsion produced by strain NWUAB01 was tested at different temperatures and pH (Figure 5.6). Gradual increase in emulsion stability was observed and optimum pH for emulsion stability of 37.5% was obtained at pH 7 at 30˚C. At 40˚C, the organism gave a stable emulsion with E_{24} of 30% (Figure 5.6). Reduction in the stability of the emulsion was observed at extreme temperature and pH. As the pH increases, there was an increase in the stability of the emulsion until pH 7, after which, the stability begins to reduce. The result indicated that increase in pH had a positive effect on stability of emulsion. This could be as a result of the precipitation of biosurfactant at high pH values (Khopade et al., 2012). Lower stability at reduced pH (<4) can be attributed to distortion of the biosurfactant structure and precipitation (Zou et al., 2014).

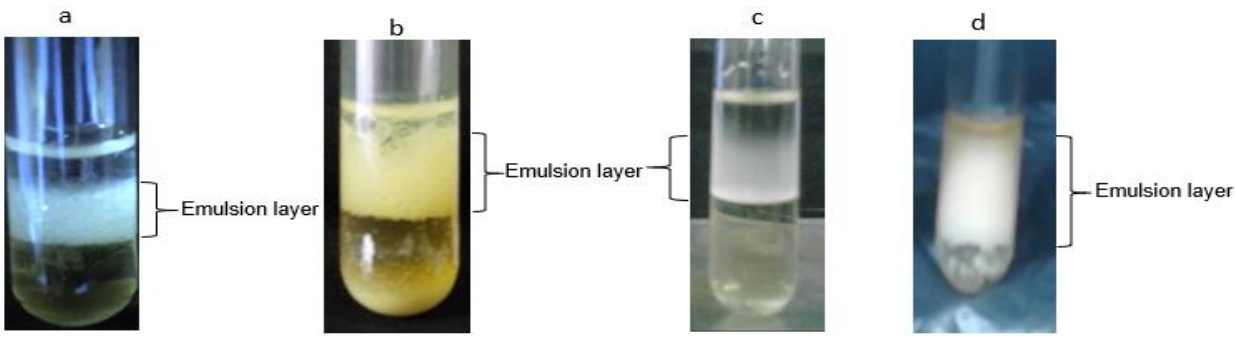


Figure 5.5: Emulsification of (a) kerosene (b) kerosene in the presence of heavy metals (c) vegetable oil (d) engine oil by strain NWUAB01

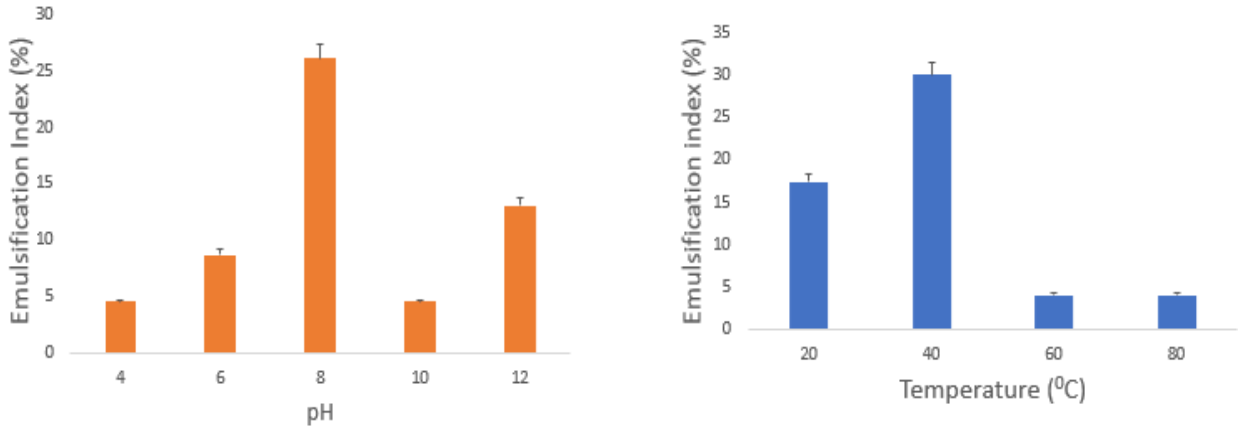


Figure 5.6: Stability of the emulsion produced from kerosene at different pH and temperature

The scanning electron micrograph (SEM) of the surfactant produced by strain NWUAB01 is presented in Figure 5.7. This shows the morphology of the lipopeptide surfactant. The FTIR characterization of biosurfactant produced by strain NWUAB01 is presented in Figure 5.8. The spectra had characteristic absorbance band of peptides at 3273 cm^{-1} (stretching mode N-H), 1624 cm^{-1} (stretching mode CO-N) and 1526 cm^{-1} (N-H deformation and C-N stretching mode). The bands obtained at 2961-2878 cm^{-1} and 1624-1447 cm^{-1} represent the presence of aliphatic chains. This suggest that the surfactant produced by strain NWUAB01 contained peptide-like moieties which is typical of lipopeptides surfactants produced by *Bacillus* species described in literature (Rivardo et al., 2009; Sriram et al., 2011; Pereira et al., 2013).

The MALDI-TOF spectra of the surfactant produced by strain NWUAB01 is shown in Figure 5.9. There are only well resolved groups of peaks at m/z values between 182.76 and 696.37. The detected groups could be attributed to the iturin variants as described by Jasim et al. (2016) and Cho et al. (2003). The lack of some specific iturin homologs can be attributed to loss of some of the amino acids such as asparagine, glycine and tyrosine in the structure of iturin, which makes many homologs of the lipopeptide difficult to identify (Chen et al., 2014). The composition of the medium of production of lipopeptides can be attributed to some of the variations in the structure of the lipopeptide produced (Akpa et al., 2001). This showed that different compounds could be expressed by *Bacillus* species during changes in growth condition (Chen et al., 2014).

Table 5.3: Evaluation of *B. cereus* NWUAB01 for biosurfactant production

Test		Result
Hemolysis test		Positive
Oil displacement		Negative
Drop collapse		Positive
Surface tension		39.48 ± 0.25 mN/m
Emulsification index	Engine oil	54.0 ± 0.58
(E_{24}) (%)	Hexadecane	22.4 ± 0.6
	Kerosene	37.5 ± 0.29
	Vegetable oil	24.0 ± 0.58
Biosurfactant yield		0.38 g/L

Values are means of triplicate readings ± standard error

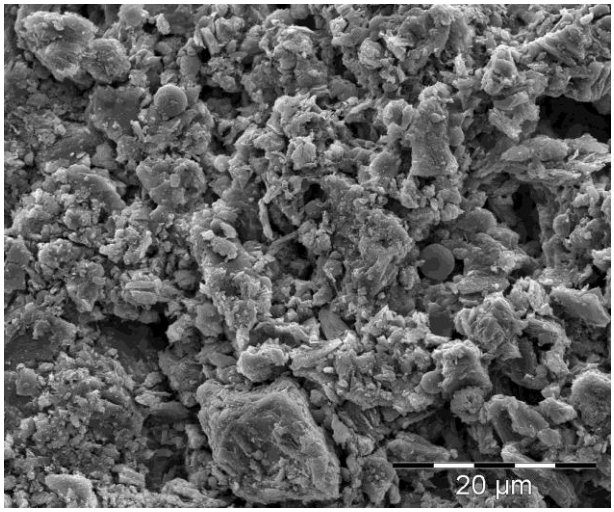


Figure 5.7: The SEM image of the biosurfactant produced be strain NWUAB01

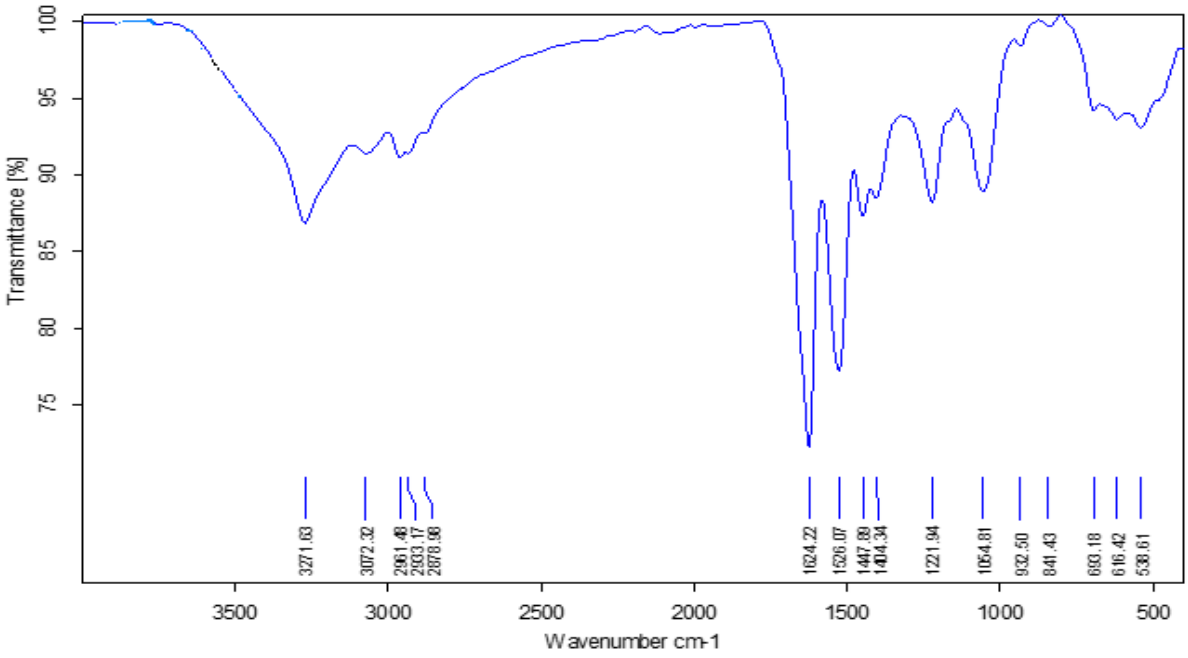


Figure 5.8: The FTIR spectra of biosurfactant produced by strain NWUAB01

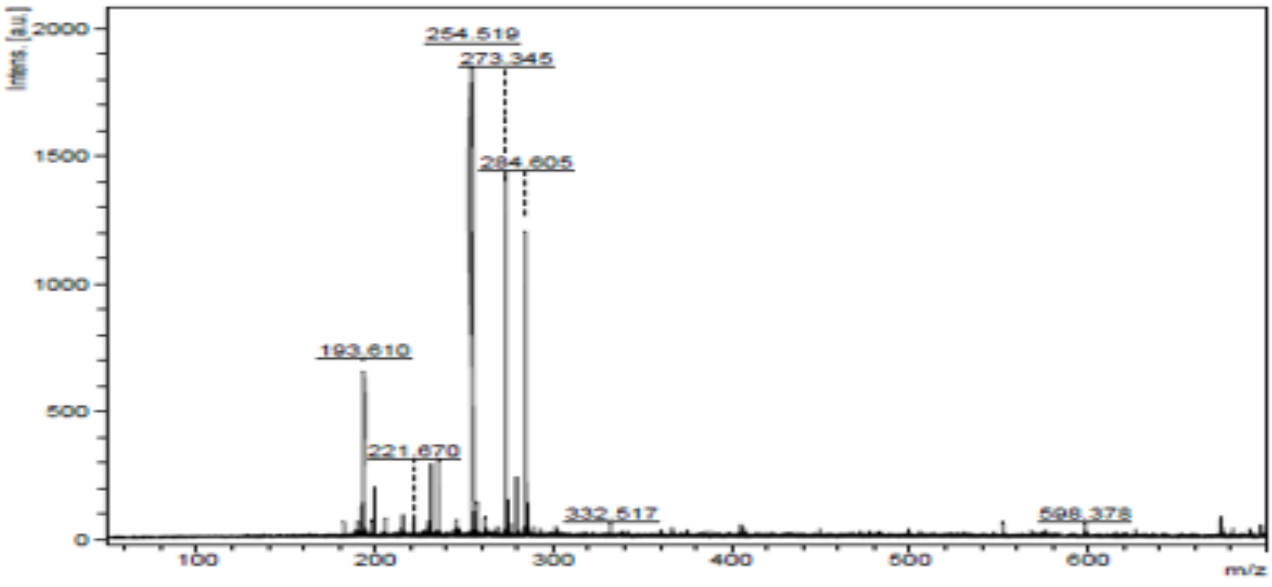


Figure 5.9: The mass spectrum of the purified biosurfactant produced by strain NWUAB01

Table 5.4: Heavy metal removal by strain NWUAB01 and its biosurfactant

	Biosurfactant	Strain NWUAB01
Metal	Percentage removal (%)	Percentage removal (%)
Cd	56.06	60.90
Cr	35.00	30.85
Pb	78.73	83.83
	Multi-metal	
Cd	35.34	45.80
Cr	32.23	33.72
Pb	73.84	72.39

Biosurfactant soil washing has been used for the removal of metals from contaminated soils and sediments due to their biodegradability environmental compatibility and low toxicity (Mulligan, 2005; Singh and Cameotra, 2013). In this study, we evaluated the metal removal capability of biosurfactant produced by strain NWUAB01. This study showed that the crude biosurfactant was able to remove 78% of Pb, 56% of Cd and 35% of Cr from the batch experiment (Table 5.4). We found that biosurfactant produced by strain NWUAB01 is efficient in removing metal from contaminated soil. In a multi-metal system, the percentage removal of each metal decreases compared to a single metal system. The ability of the biosurfactant to remove metals from contaminated soil was also examined in comparison with strain NWUAB01. It was observed that the percentage metal removal was higher for the organisms than the surfactant with significant removal of cadmium. The results of this study showed highest removal of Pb overall followed by Cd and Cr. This could be attributed to the affinity of the biosurfactant to different metals (Ochoa-Loza et al., 2007; da Rocha Junior et al., 2018). The efficiency of metal removal of biosurfactants also depends on the type of biosurfactant and its concentration, soil characteristics and other additives such as acids and bases that may be added (da Rocha Junior et al., 2018). Metal removal efficiency of strain NWUAB01 biosurfactant is higher than those reported by Singh and Cameotra (2013). However, the metal removal efficiency of lipopeptides of marine origin reported by Das

et al. (2009) was higher than those of strain NWUAB01. The metal removal potential of strain NWUAB01 corroborated the work of Mulligan et al. (2001b), who reported the use of lipopeptide from *B. subtilis* for the removal of Cd, Cu and Zn. Lipopeptides which are anionic in nature have better metal sequestration properties and are more effective in metal removal (Mulligan, 2005). Removal of metals by biosurfactant has been proposed to occur by surfactant sorption to soil surface which is followed by complexation with metals, thus leading to metal detachment from soil surface by the reduction in interfacial tension (Mulligan, 2005).

Conclusion

Biosurfactant is a promising technique for enhanced remediation of polluted environments. The present study shows the feasibility of using biosurfactant produced by strain NWUAB01 in remediation of heavy metal contaminated soil. Findings of this study demonstrate the potential of the biosurfactant produced for effective removal and recovery of heavy metals for environmental applications.

CHAPTER SIX

DRAFT GENOME SEQUENCE OF HEAVY METAL RESISTANT *BACILLUS CEREUS* STRAIN NWUAB01

Abstract

Bacillus cereus NWUAB01 was isolated from a gold mining site from Vryburg, South Africa for its multiple heavy metal resistance properties. Here we report the draft genome sequence of *B. cereus* NWUAB01, obtained by Illumina sequencing.

6.1 Introduction

Bacteria from polluted environments have evolved resistance and adaptive genes required for their survival in hostile environments. The search for novel molecular mechanisms from polluted environments provides strong informational resources that lead to new technologies (Astolfi et al., 2018). As a result, the draft genome of *B. cereus* NWUAB01 isolated from gold mining soil in Vryburg, South Africa is hereby reported.

Bacillus cereus is a spore forming Gram positive bacterium that is ubiquitous in nature and always detected in hostile environments (Rasko et al., 2005). The spore forming ability helps them to survive in different habitats (Singh et al., 2010). *B. cereus* NWUAB01 is a metal resistant spore forming isolate. Microbial degradation is a major route of recovery of pollutants from polluted environments by means of transformation and detoxification mechanisms (Lin and Lin, 2005).

6.2 Materials and Method

B. cereus NWUAB01 was identified from 98 bacterial isolates from soil samples obtained from a gold mining site (Section 4.3.2). The isolate was screened for heavy metal (Cd, Cr, Pb) resistance, and antibiotic and secondary metabolites production.

The draft genome of *B. cereus* NWUAB01 was obtained by whole genome shotgun sequencing using one Illumina paired-end library with an average insert size of ~300 bp. The sample was fragmented using an ultrasonication approach (Covaris). The resulting DNA fragments were size selected (300 – 800bp), using AMPure XP beads. The fragments were end repaired and Illumina specific adapter sequences were ligated to each fragment. The sample was indexed, and a second size selection step was performed. The sample was then quantified, using a fluorometric method, diluted to a standard concentration (4 nM) and then sequenced on Illumina's MiSeq platform, using a MiSeq v3 (600 cycle) kit as described in the kit protocol.

6.3 Results and Discussion

The raw sequence was processed to obtain high-quality reads using the Kbase (Arkin et al., 2016) platform to check quality of reads (FastQC v.1.0.1), trim reads and to remove adapter and low-quality sequences, and ambiguous reads (Trimmomatic v0.36). The CLC *de novo* assembly was used to assemble the genome using the CLC platform. The Rapid Annotations using Subsystems Technology (RAST v 2.0) server (Aziz et al., 2008) and the NCBI Prokaryotic Genome Annotation Pipeline (PGAP v4.5) (Haft et al., 2017) was used for genome annotation.

The genome of *B. cereus* NWUAB01 consists of a linear chromosome (5,859,863 bp) with GC content of 35.7%, 849 contigs, 6094 protein coding genes, 2 rRNA operons, 12 tRNA genes and 286 pseudogenes. The subsystem information of *B. cereus* NWUAB01 was predicted with SEED viewer (Overbeek et al., 2014) (Figure 6.1).

Analysis of the secondary metabolite biosynthesis gene clusters with antiSMASH v3.0 (Weber et al., 2015) revealed that *B. cereus* NWUAB01 had 44 gene clusters involved in antibiotic and secondary metabolite biosynthesis. These include non-ribosomal peptide synthetase (NRPS) gene clusters, lipoprotein, lipopolysaccharides, petrobactin, siderophore, binding proteins, bacillibactin, proteins related to degradation of toxic compounds and biofilm secretion genes. Genes responsible

for resistance to toxic heavy metals such as arsenic, cadmium, chromium, cobalt, copper, lead, mercury and zinc were also detected. Five questionable clustered regularly interspaced short palindromic repeats (CRISPR) sequences were identified by CRISPRFinder (Grissa et al., 2007). Further investigation will provide additional information into the mechanism involved in multi-metal resistance role of *B. cereus* NWUAB01 that facilitates environmental applications.

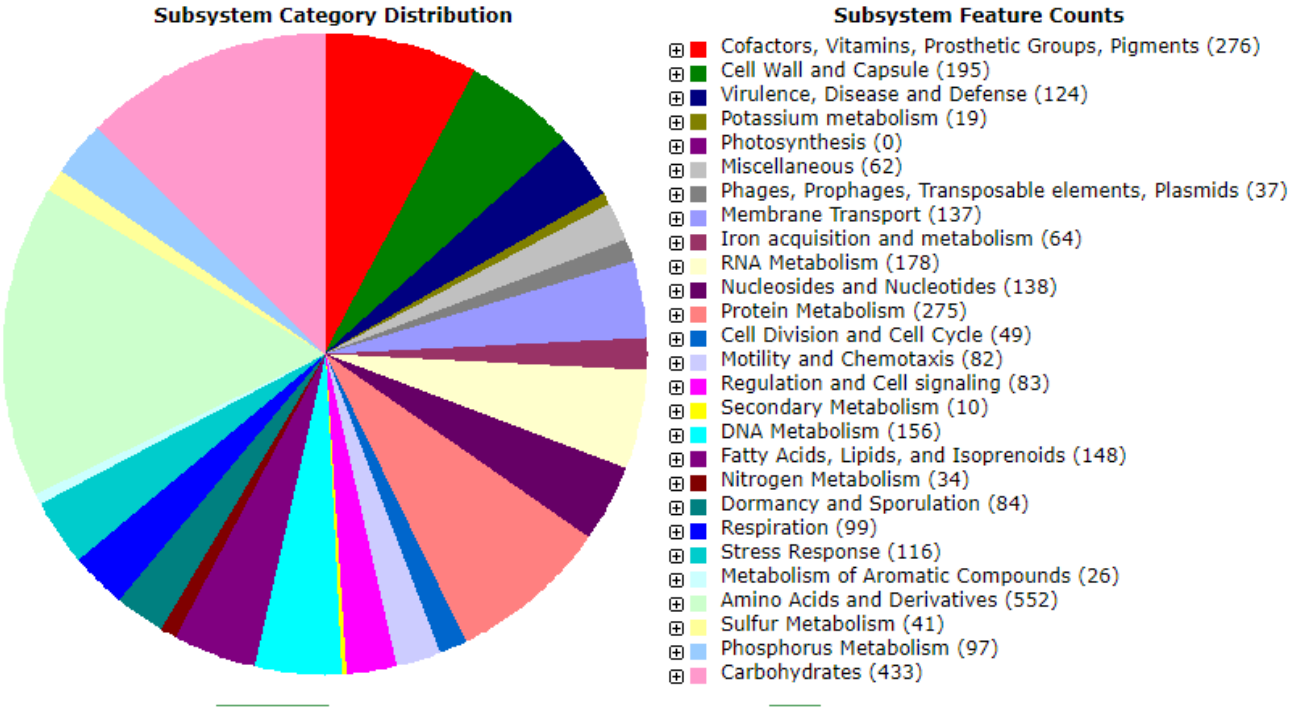


Figure 6.1: The subsystem information of the genome of *Bacillus cereus* NWUAB01 as predicted by SEED Viewer v2.0. Genomic features are colored according to their functional classification types

Accession number(s). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QNGD00000000 and BioProject number PRJNA476495. The version described in this paper is version QNGD01000000.

CHAPTER SEVEN

BIOFLOCCULANT PRODUCTION AND HEAVY METAL SORPTION BY METAL RESISTANT BACTERIAL ISOLATES FROM MINING SOIL

Abstract

Two bioflocculant producing bacterial isolates from mining soil samples were investigated for their application in heavy metal removal. The bacterial isolates were identified as *Pseudomonas korensis* and *Pantoea* sp. Cadmium resistance genes *cadA* and *CzcD* were detected in *Pantoea* sp. while *P. korensis* harbor *CzcD* and *chrA* responsible for Cd and Cr resistance respectively. The isolates showed maximum flocculating activity of 71.3% and 51.7% with glucose and yield of 2.98 g/L and 3.26 g/L for *Pantoea* sp. and *P. korensis* respectively. The optimum flocculating activity was achieved at pH 7.5 and temperature of 30˚C. Fourier transform infrared analysis of the bioflocculants produced by the two isolates showed the presence of carboxyl, hydroxyl and amino groups characteristic of polysaccharide and protein. Heavy metal sorption by bioflocculant of *Pantoea* sp. removed 51.2%, 52.5% and 80.5% of Cd, Cr and Pb respectively while that of *P. korensis* removed 48.5%, 42.5% and 73.7% of Cd, Cr and Pb respectively from aqueous solution. The bioflocculants produced have potential application in metal removal from industrial wastes.

Keyword: Biopolymer, biosorption, flocculation, pollution, remediation

7.1 Introduction

Heavy metal pollution as a result of industrialization and urbanization has put an increasing burden on the ecosystem due to the deleterious effect on living organisms (Sajayan et al., 2017). Industries such as mining and metallurgy release large tons of heavy metal containing waste into the surrounding environment. These metals are of serious global health concern because of their non-degradable and persistent nature (Subudhi et al., 2016; Ayangbenro and Babalola, 2017). These

metals exert toxic effects on living organisms even at low concentrations, thus there is a need for removal of these metals from the environment (Subudhi et al., 2016; Sajayan et al., 2017).

Flocculation involves floc formation through aggregation of colloids and suspended solids with the help of a stimulant (Sajayan et al., 2017; Ayangbenro and Babalola, 2018). Flocculants are materials used in the separation of suspended substances and have several industrial applications. Flocculants are of two types: chemically synthesized organic and inorganic flocculants, and natural flocculants (Lee et al., 2014; Subudhi et al., 2014; Okaiyeto et al., 2016). Chemically synthesized organic and inorganic flocculants are toxic, cause health complications, produce a large quantity of waste and are difficult to degrade (Zhai et al., 2012; Farag et al., 2014). Thus, bioflocculants are receiving increasing attention due to their biodegradable nature, and because they are environmentally benign, non-toxic and lead to efficient removal of suspended particles, dye and heavy metals from wastewaters (Sajayan et al., 2017).

In the last few decades, much attention has been given to environmental pollution management of hazardous pollutants (Umrania, 2006). Heavy metal removal from water and soil has been a challenge in/and around mining environments. Current chemical and physical approaches of metal removal from polluted environments are not environmentally friendly, they are expensive, some of the methods are non-specific, have high energy demands, and impracticable nature (Das et al., 2009; Voica et al., 2016). Biosorption is one of the promising techniques for heavy metal removal from wastewater. The technique is simple and economically feasible (Subudhi et al., 2016).

Secondary metabolites of microbial origin are generating increasing attention lately as a result of their diversity, low toxicity and biodegradable nature, which makes them superior to chemical products. Microbial products provide defensive mechanisms and increase bioavailability of metals (Vaishnav and Demain, 2011; Ayangbenro and Babalola, 2018). One of these bioproducts is bioflocculant, an extracellular polymer that is made up of exopolysaccharides, proteins, glycoproteins, lipids and glycolipids (More et al., 2014; Subudhi et al., 2016). Bioflocculant is a

promising alternative to synthetic flocculant and is generally recognized as being a non-toxic, environmentally-friendly and highly biodegradable substitute with no secondary pollution (Bukhari et al., 2018). Different studies have reported on the various applications of bioflocculants in remediation processes to treat wastewater, removal of heavy metals and toxic materials, potential emulsifier, synthesis of nanoparticles and biomass recovery (Buthelezi et al., 2009; Gomaa, 2012; Sathiyanarayanan et al., 2013; Drakou et al., 2017; Bukhari et al., 2018).

Mining industries discharge waste that contains heavy metals into the surrounding environment. High concentrations of these metals are hazardous and tend to form toxic complex compounds which affects biological functions (Subudhi et al., 2014). Thus, it becomes imperative to explore effective processes of metal removal from contaminated soil and wastewater. This study investigates the isolation and bioflocculant production of heavy metal resistant bacterial isolates from mining soil and their potential in heavy metal (Cd, Cr and Pb) biosorption.

7.2 Materials and methods

7.2.1 Soil sampling and isolation of heavy metal resistant bacteria isolates

Soil samples were collected from active gold mines in Vryburg, North West Province, South Africa. Control soil samples were also collected around the mines. Soil samples were collected in triplicate at depths of 10-30 cm with a soil auger in sterile plastic bags.

Heavy metal (Cd, Cr, Pb) resistant bacterial isolates were isolated as described by Rajkumar and Freitas (2008). One gram of each of the collected soil samples were serially diluted and plated out on Luria-Bertani (LB) agar that has been supplemented with 50 mg/L of each metal solution and a mixture of all the metals. The metal solutions ($CdSO_4$, K_2CrO_4 and $Pb(NO_3)_2$) were filter sterilized through 0.22 µm membrane filters (Millipore Corporation, Bedford, MA, USA). The plates were incubated at 30˚C for 48 h. Resistant isolates were then screened for ability to grow on different metal concentrations (100 to 1000 mg/L) on LB agar plates (Srinath et al., 2002).

Growth of resistant isolates was monitored in the presence of heavy metals (100 mg/L of each metal) in LB broth and the optical density measured at 24 h intervals for 6 days.

7.2.2 Biochemical characterization of bacteria isolates

The following biochemical tests were carried out on heavy metal resistant isolates: Gram reaction, catalase test, citrate utilization, gelatin liquefaction, hydrogen sulfide production, oxidase, nitrate reduction, starch hydrolysis, sugar fermentation test (glucose, arabinose, fructose, maltose, sucrose, lactose, mannose, melibiose, sorbitol, maltose), urease, and Voges-Proskauer test. Pure cultures of the isolates were identified according to the Bergey's manual of Determinative Bacteriology (Garrity et al., 2004).

7.2.3 Genomic DNA extraction

The DNA of the selected heavy metal resistant bacterial isolates were extracted from pure cultures grown on LB agar using a ZR soil microbe DNA mini prep ^{TM} DNA extraction kit (Zymo Research, CA, USA) according to the kit's protocol. The DNA quality and quantity of the bacterial isolates wee determined using NanoDrop Lite spectrophotometer (Thermo Fischer Scientific, CA, USA).

7.2.4 Molecular characterization and detection of heavy metal resistance genes

The 16S rRNA gene was amplified for each isolate using universal primers F1 (forward, 5′-AGAGTTTGATCCTGGCTCAG-3′) and R2 (reverse, 5′-ACGGCTACCTTGTTACGACTT-3′) with approximately 1500 base pair amplicon (Marin et al., 2011). The PCR was performed with a total volume of 25 µl of a reaction mixture consisting of 12.5 µl 2X master mix (One Taq® Hot Start Quick Load, Biolabs, England), 1 µl of the DNA template, 0.5 µl (10 µM working solution) of each of the forward and reverse primer, and 11 µl nuclease free water. The PCR was performed in a C1000 thermal cycler (Bio-Rad, CA, USA) with a preincubation step of 95˚C for 5 min and 35 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 30 s, and extension at 72˚C for

1 min, followed by a final extension step of 72˚C for 10 min. Polymerase chain reaction amplicons were analyzed by electrophoresis on agarose gel (1% w/v) containing ethidium bromide and the band size was determined using 1 kb molecular marker. Gel Doc 2000 (Bio-Rad, USA) was used for gel visualization and photograph.

Analysis of heavy metal encoding genes were done with primers encoding for cadmium (*cadA*, *CzcA*, *CzcB* and *CzcD*), chromium (*chrA* and *chrB*) and lead (*PbrA* and *PbrT*) for each isolate. The sequences of each primer set are presented in Table 4.1 with their expected band size. All the primer sets used for PCR amplifications were synthesized by Whitehead Scientific, Integrated DNA Technologies, South Africa.

7.2.5 Sequencing reaction

Sequencing of the PCR amplicons was done at Inqaba Biotechnical industrial (Pty) Ltd, Pretoria, South Africa with PRISM TM Ready Reaction Dye Terminator Cycle Sequencing Kit using dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Bio systems, USA) by following the manufacturer's instructions. The forward and reverse primers were used in sequencing of the purified PCR products. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™) were analyzed using CLC Main Workbench 7.

The 16S rRNA gene sequences were blast searched on the NCBI GenBank database. Aligned sequences were analyzed using MEGA 7.0 software (Kumar et al., 2016b).

7.2.6 Screening of metal resistant isolates for bioflocculant production

Heavy metal resistant isolates were pre-cultured in 50 ml LB broth on a rotary shaker (200 rpm) at 30˚C for 24 hours. The culture medium was used to inoculate 100 ml fermentation medium. The fermentation medium contains (g/L): K_2HPO_4, 2; NH_4Cl, 1; $MgSO_4$, 0.5; $CaCO_3$ 0.2; KH_2PO_4, 5; glucose, 2; peptone, 5 and yeast extract, 5 at pH 7.2 (Subudhi et al., 2014). The medium was

incubated for 72 hours at 30˚C on a rotary shaker at 200 rpm. After incubation, the medium was centrifuged at 6000 g for 30 minutes and the supernatant was evaluated for flocculating activity.

The flocculating activity was measured using kaolin clay suspension (0.5 g of kaolin clay in 100 ml distilled water). Kaolin suspension (45 ml) was mixed with 0.5 ml of the supernatant and 4.5 ml of $CaCl_2$ (1%) was added to the mixture. The mixture was vortexed for 2 minutes and left standing for 5 minutes at room temperature. The optical density of the supernatant and the blank (distilled water) was measured at 550 nm (Gomaa, 2012). The flocculating activity was defined as follows: flocculating activity = $\frac{A-B}{A} \times 100$

Where A and B are the optical densities of the control and sample respectively at 550 nm.

7.2.7 Bioflocculant purification

The purification of the crude bioflocculant was done as described by Nwodo et al. (2012). The fermentation broth was centrifuged at 3000 rpm for 30 minutes at 15˚C. The supernatant was mixed with cold ethanol at a ratio of 1:4 (v/v) respectively in order to precipitate the bioflocculant. The mixture was kept for 16 hours overnight at 4˚C. The mixture was then centrifuged at 1000 rpm for 30 minutes at 15˚C to collect the resulting precipitate. The precipitate was dissolved in distilled water at a ratio of 1:4 (v/v) and the bioflocculant produced was lyophilized.

7.2.8 Characterization of the bioflocculant produced

The lyophilized bioflocculant was characterized by Fourier transform infrared spectroscopy (FTIR). The FTIR spectra, with a resolution of 4 cm^{-1}, were collected from 400 to 4000 wavenumbers (cm^{-1}) with an average of 32 scans under ambient conditions using Alpha II Platinum-ATR IR spectrophotometer (Brucker, USA).

The lyophilized bioflocculant was subjected to scanning electron microscope (JSM-6390LV, JEOL, Tokyo, Japan).

7.2.9 Effect of culture conditions on flocculation activity

The flocculating activities of the isolates were determined as described by Subudhi et al. (2014) at different pH (5-9), temperature (25, 30, 37 and 50˚C) and carbon source (glucose, fructose, lactose and sucrose).

7.2.10 Heavy metal sorption by bioflocculant produced by bacterial isolates

The purified bioflocculant produced by each isolate was used for heavy metal (Cd, Cr and Pb) removal as described by Subudhi et al. (2016) with little modifications. A 0.1 g of the lyophilized partially purified bioflocculant was added to 100 ml of solution of 100 mg/L of each of the metal salt ($CdSO_4$, K_2CrO_4 and $Pb(NO_3)_2$). The mixture was incubated at 30˚C for 24 hours at 150 rpm. Afterwards, the mixture was centrifuged at 10,000 rpm for 30 mins to remove insoluble bioflocculants. The metal concentrations in the supernatant were measured using Inducible Conductivity Plasma-Optical Emission Spectroscopy (725 ICP-OES, Agilent Technologies, Palo Alto, CA, USA). The metal removal efficiency was calculated using the formula:

$$Removal\ rate\ (\%) = \frac{Ci - Ce}{Ci}\ X\ 100$$

Where C_i and C_e are the initial and final concentrations of metal respectively.

7.2.11 Statistical analysis

Data were means values of replicate readings and were subjected to analysis of variance (ANOVA) at significant level of $p < 0.05$ using SPSS statistical package (v 25.0).

7.3 Results and Discussion

The heavy metal tolerance of the two bioflocculant producing strains is presented in Table 7.1. The two isolates showed appreciable growth on Pb, with *Pseudomonas korensis* growing on 1000 mg/L of Pb and *Pantoea* sp. was able to tolerate 400 mg/L of Pb. However, the two organisms showed limited growth on Cd and Cr. *P. korensis* was able to tolerate 200 mg/L of Cd and 100

mg/L of Cr while *Pantoea* sp. was able to tolerate 100 mg/L of Cd and 200 mg/L of Cr (Table 7.1).

The biochemical characteristics of the two isolates are presented in Table 7.2. Both isolates are Gram negative, rod-like in shape, catalase positive and have the ability to utilize citrate, glucose and sucrose. *Pantoea* sp. was able to produce nitrate, ferment arabinose and mannose while *P. korensis* has the ability to liquefy gelatin (Table 7.2).

Bacterial isolates from heavy metal contaminated environments are usually more resistant to high concentrations of heavy metals than those isolated from pristine environments (Umrania, 2006). Soils from mining environments are potential sources of heavy metal resistant isolates (Choudhary and Sar, 2009; Zampieri et al., 2016) and these organisms may have developed resistance as a result of the presence of metals in their medium of growth (Çolak et al., 2011).

The 16S rRNA gene amplification of the genomic DNA of the resistant isolates yielded 1500 bp fragments using universal primers (F1, R2) as shown in Figure 4.6. (Lane 12: *Pseudomonas korensis* and Lane 16: *Pantoea* sp.). The organisms were identified based on the 16S rRNA gene sequence using the BLAST search of the NCBI database that identified the organisms as *Pseudomonas korensis* and *Pantoea* sp. The results showed that *Pantoea* sp. had maximum homology of 91% with *Pantoea* sp. (JN082730) and *Pseudomonas korensis* showed maximum homology of 99% with *Pseudomonas korensis* (MH211310).

Bacteria have adapted to environmental changes by physiological and genetic mechanisms that confer new traits, such as metal resistance and xenobiotic degradation, to survive and colonize their environment (Anjum et al., 2011). Metal resistance is known to be achieved through an efflux system as found in Gram-negative organisms (Blair et al., 2014) that pumps metals out of the cytoplasm into the periplasmic space and other mechanisms such as complexation, accumulation, transformation, sorption, oxido-reduction reactions and mineralization (Govarthanan et al., 2013;

Ayangbenro and Babalola, 2017). The isolates were screened for metal resistance genes by PCR amplification of genomic DNA for genes responsible Cd, Cr, and Pb. These genes have the potential to reduce or eliminate metal toxicity (Wei et al., 2009). The amplification of genes responsible for Cd, Cr, and Pb resistance were performed with specific primers. No amplification products were obtained for primers representing *CzcA, CzcB* and *chrB*, *PbrA* and *PbrT* for each isolate. *Pseudomonas korensis* possess resistance genes for cadmium (*CzcD*) (Figure 4.14; Lane 12) and chromium (*chrA*) (Figure 4.17; Lane 12) while *Pantoea* sp. possess genes responsible for cadmium resistance (*cadA*) (Figure 4.13; Lane 16) and (*CzcD*) (Figure 4.14; Lane 16).

The two isolates possess the *CzcD* operon that is responsible for Cd, Co and Zn resistance. The *CzcD* operon is used in the detoxification of Cd, Co and Zn by exporting them from the cytoplasm and/or periplasm into the extracellular medium (Legatzki et al., 2003). The *cadA* found in *Pantoea* sp. is responsible for Cd efflux which is energy dependent (Nies et al., 1989).

ChrA confers chromate resistance on *P. korensis* which results from the accumulation of Cr (Nies et al., 1990). It is an efflux pump responsible for chromate extrusion (Pimentel et al., 2002). Therefore, these bacterial isolates are ideal for heavy metal removal from polluted environments.

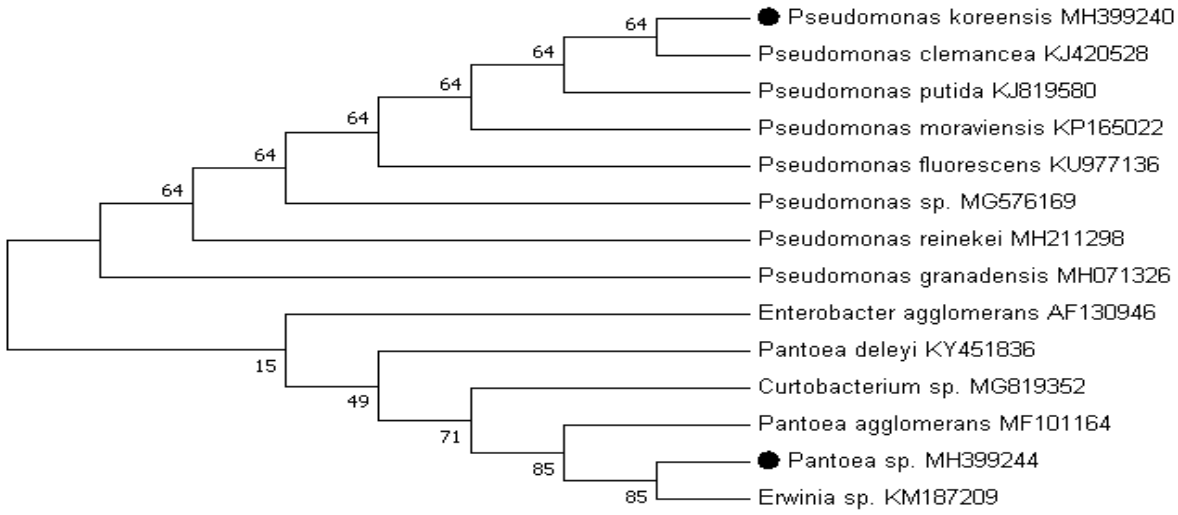


Figure 7.1: Maximum likelihood tree showing the phylogenetic relationship of the two bioflocculant producing isolates based on 16S rRNA gene sequence

The evolutionary history was inferred by using the maximum likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

The growth pattern of the isolates in the presence of heavy metals is presented in Figures 7.2 and 7.3. Both isolates showed a decrease in optical density while growing in heavy metal containing medium compared to a metal-free medium (Figure 7.2 and 7.3). The two isolates have similar growth patterns in the presence of heavy metals and follow this order Cr > Pb > Cd. The highest optical density of 0.283, 0.947 and 1.087 were observed on the sixth day of growth for *P. korensis* for Cd, Pb and Cr respectively (Figure 7.2). Similar results were also observed for *Pantoea* sp. on the sixth day with highest optical density of 1.089, 1.364 and 1.366 for Cd, Pb and Cr respectively (Figure 7.3). The optical densities observed for *Pantoea* sp. in the presence of Cr and Pb on the sixth day were higher than that of the control.

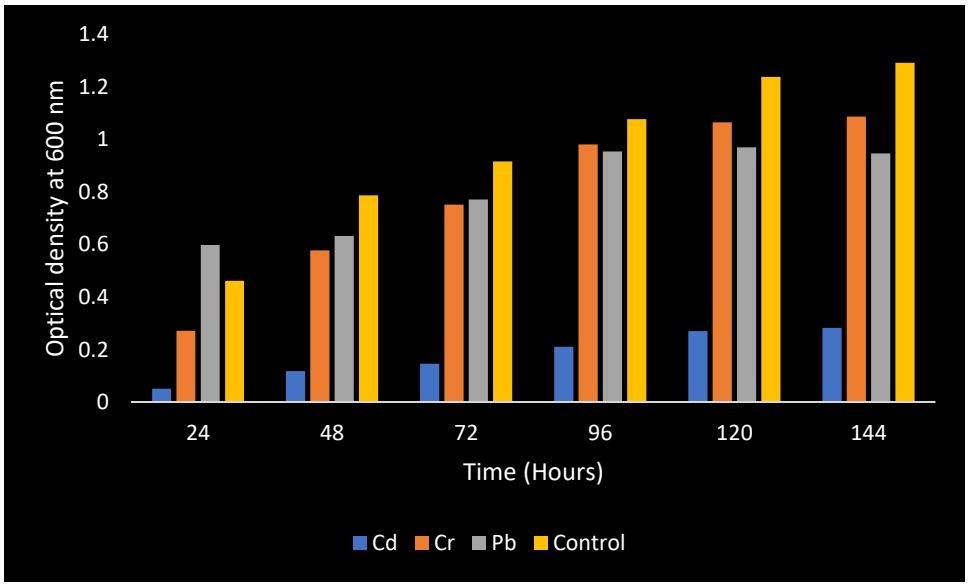


Figure 7.2: The growth pattern of *P. korensis* in the presence of heavy metals

Table 7.1: Heavy metal tolerance of the two bioflocculant producing isolates

Isolate	Cd (mg/L)			Cr (mg/L)			Pb (mg/L)					
	100	200	300	100	200	300	100	200	300	400	500	1000
Pseudomonas korensis	+	+	-	+	-	-	+	+	+	+	+	+
Pantoea sp.	+	-	-	+	+	+	+	+	+	+	-	-

(+) Positive reactions; (-) negative reactions

Table 7.2: Biochemical profile of heavy metal resistant bacterial isolates

ISOLATE	TESTS																				
	GRAM RXT	SHAPE	CAT	OXI	IN	VP	CIT	URE	SH	H_2S	GL	MAN	NIT	GLU	ARA	SUC	MAN	SOR	MEL	MAL	LAC
Pseudomonas korensis	-	Rod	+	-	-	+	+	-	-	-	+	-	+	+	-	+	-	-	-	-	-
Pantoea sp.	-	Rod	+	-	-	+	+	-	-	-	-	+	+	+	+	+	+	-	-	-	-

Key: + (Positive), - (Negative), G (Gas production), CAT (Catalase), OXI (Oxidase), IN (Indole), VP (Voges Proskaeur), CIT (Citrate), URE (Urease), SH (Starch hydrolysis), GL (Gelatin liquefaction), MAN (Mannose), NIT(Nitrate), GLU (Glucose), ARA (Arabinose), SUC (Sucrose), MAN (Mannose), SOR (Sorbitol), MEL (Melibiose), MAL (Maltose), LAC (Lactose)

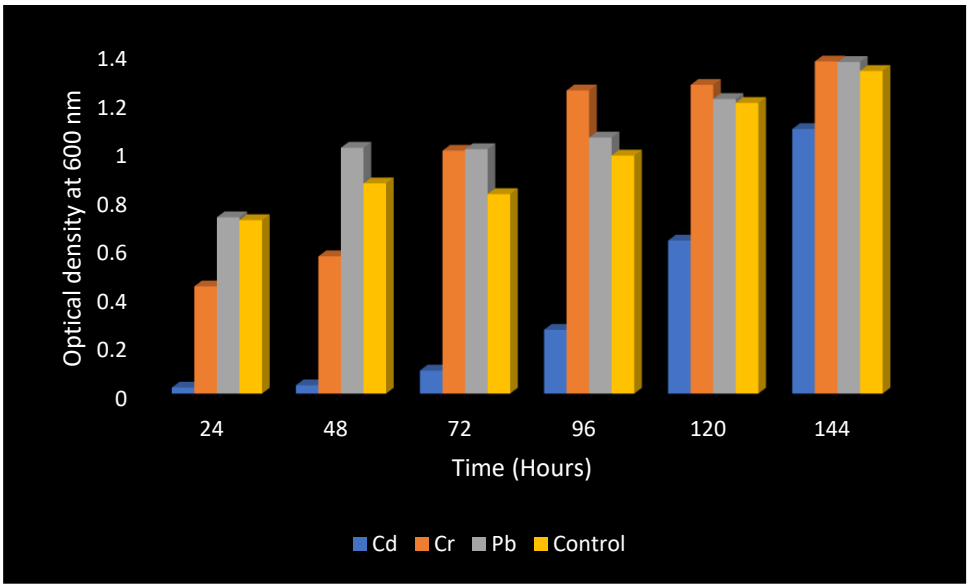


Figure 7.3: The growth pattern of *Pantoea* sp. in the presence of heavy metals

Bioflocculant is another biopolymer produced by bacterial isolates during growth. The flocculating properties of the metal resistant isolates were determined to access the aggregation of particles and cells (Yim et al., 2007). Bacterial isolates that were able to flocculate suspended kaolin clay can adsorb to other particles to form flocs (Tang et al., 2014). The flocs are capable of effective metal removal from solution.

Flocculation is an effective technique used in the treatment of wastewater for removal of suspended particles and heavy metals (Aljuboori et al., 2013). The metal resistant isolates were screened for bioflocculant production. Synthesis of bioflocculant is influenced by the composition of culture medium and other parameters. The carbon source for synthesis of bioflocculant was investigated. Glucose gave the highest flocculating activity of 71.3% for *Pantoea* sp. while sucrose served as the best carbon source for *P. korensis* resulting in flocculating activity of 56% (Table 7.3). Conversely, lactose and maltose showed the lowest flocculating properties. The least flocculating activity was observed when lactose was used as a carbon source for each isolate. The flocculating activity were 30.5% and 32% for *P. korensis* and *Pantoea* sp. respectively (Table 7.3).

Gomaa (2012), reported that changing carbon and nitrogen sources of the production medium influences flocculating activities of bacteria. Sugars, such as glucose, fructose and sucrose have been reported to be favorable for bioflocculant production by bacterial isolates (Nwodo et al., 2012; Nwodo and Okoh, 2013). The two organisms in this study preferred sucrose, glucose and fructose and the flocculating activity was higher compared to what was observed for lactose and maltose. Glucose was used as carbon source for further experiments. Sucrose was reported to be the most favorable carbon source for bioflocculant production by *Achromobacter xylosoxidans* (Subudhi et al., 2016) which is similar to what was observed for *Pseudomonas korensis* in this study. Nwodo and Okoh (2013), and Xia et al. (2008) reported glucose to be the best carbon source for bioflocculant production and yield by *Cellulomonas* sp. (Okoh) and *Proteus mirabilis* TJ-1 respectively. There is variation in carbon requirement by different organisms. Ethanol was reported to be preferred by *Pseudomonas aeruginosa* (Gomaa, 2012) while lactose was preferred by *Serratia ficaria* (Gong et al., 2008).

Table 7.3: Flocculating activity of the bacterial isolates using different carbon sources

Organism	Flocculating activity (%)				
	Glucose	Fructose	Sucrose	Maltose	Lactose
Pseudomonas korensis	51.70 (1.70)	49.38 (1.39)	56.02 (0.22)	40.15 (1.15)	30.51 (0.84)
Pantoea sp.	71.25 (0.65)	68.99 (0.02)	67.19 (0.82)	41.12 (0.88)	32.00 (1.00)

Values are means of duplicate readings ($P < 0.05$)
Values in parenthesis are ± standard error

The kinetics of flocculating activity was monitored for 5 days and presented in Figure 7.4. A continuous increase in flocculating activity was observed from 39.9% for *Pantoea* sp. and 22.5% for *P. korensis* after 24 hours of fermentation to a peak of 71.3% and 51.7% for *Pantoea* sp. and *P. korensis* respectively after 120 hours of fermentation. The bioflocculant synthesis increases during of growth. This can be attributed to synthesis during growth and not cell lysis (Gao et al., 2006). This is consistent with the report of Gong et al. (2008), Wu et al. (2010b) and Okaiyeto et al. (2013) that shows a link between bioflocculant synthesis and bacterial growth. There was a

decrease in pH with increase in flocculating activity which can be attributed to the production of organic acids from glucose during the growth phase (Gong et al., 2008; Xiong et al., 2010). The maximum flocculating activity was obtained at pH 6.9 and 7.1 for *P. korensis* and *Pantoea* sp. respectively (Table 7.4). In general, the flocculating activity increases with growth and peaked at the stationary phase.

The effect of initial pH on flocculating activities of the two isolates are presented in Figures 7.4 and 7.5. Increase in flocculating activity was observed as pH increases for each isolate with the optimum around neutral pH and then decreases with further increase in pH. This implied that the bioflocculant production occurred in the range between neutral to slightly alkaline pH. The optimum flocculating activity of 74.4% and 60.3% was observed at pH 7.5 for *Pantoea* sp. and *P. korensis* respectively and then decreases with increase in initial pH (Figures 7.4 and 7.5). It has been reported that the initial pH of the fermentation medium determines bioflocculant production, electric charge on the cell surface and the redox potential that consequently affects nutrient absorption, enzymatic reactions, growth and flocculating activity (Zhang et al., 2007; Xia et al., 2008). Similar results were observed for *Bacillus licheniformis* and *A. xylosoxidans* that has optimum flocculating activity at pH 7.5 (Xiong et al., 2010; Subudhi et al., 2016), while *P. aeruginosa*, *Cellulomonas* sp. and *S. ficaria* were reported to have optimum flocculating activity at pH 7 (Gong et al., 2008; Gomaa, 2012; Nwodo and Okoh, 2013). On the contrary, optimum flocculating pH of 4 was reported for *Gyrodinium impudicum* (Yim et al., 2007). Thus, different strains have different optimum pH for flocculating activity.

Table 7.4: Kinetics of bioflocculant producing isolates and their flocculating activity

Time (Hours)	24	48	72	96	120
			pH		
Pseudomonas korensis	6.2	6.2	6.5	6.7	6.9
Pantoea sp.	6.4	6.3	6.5	6.8	7.1
			Optical density		
Pseudomonas korensis	1.01 (0.75)	1.45 (1.17)	2.12 (1.56)	1.85 (0.59)	2.05 (1.78)
Pantoea sp.	1.73 (0.89)	1.90 (1.63)	2.04 (1.50)	1.99 (2.01)	2.36 (0.51)
			Flocculating activity		
Pseudomonas korensis	22.54 (2.35)	32.33 (2.01)	50.5 (1.50)	50.81 (1.49)	51.70 (1.70)
Pantoea sp.	39.89 (0.57)	47.90 (1.11)	68.40 (1.61)	69.15 (1.15)	71.25 (0.65)

Values are means of duplicate readings ($P < 0.05$)
Values in parenthesis are ± standard error

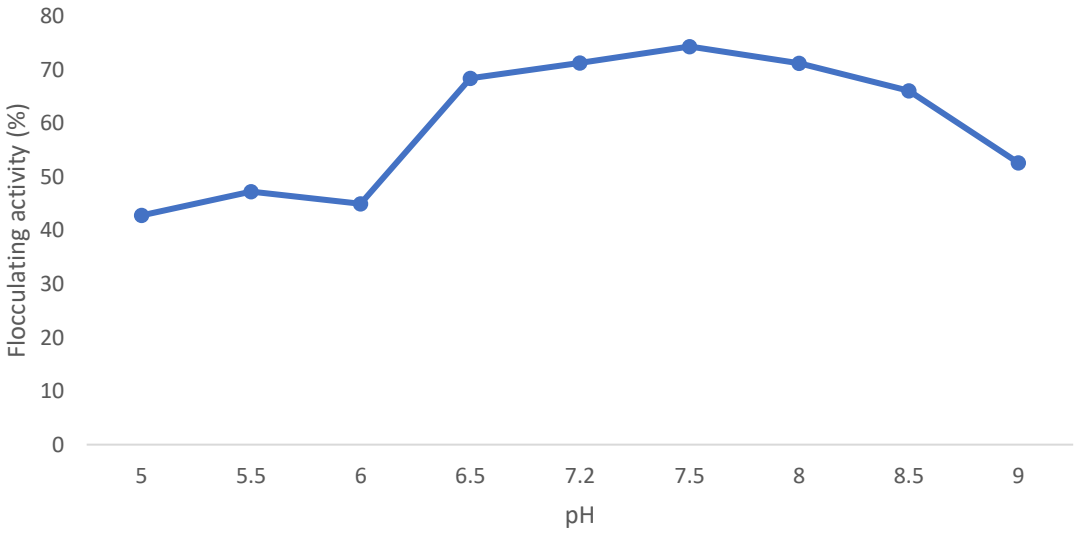


Figure 7.4: The effect of pH on flocculating potential of *Pantoea* sp.

The exponential condition is between pH 5.5-7.5

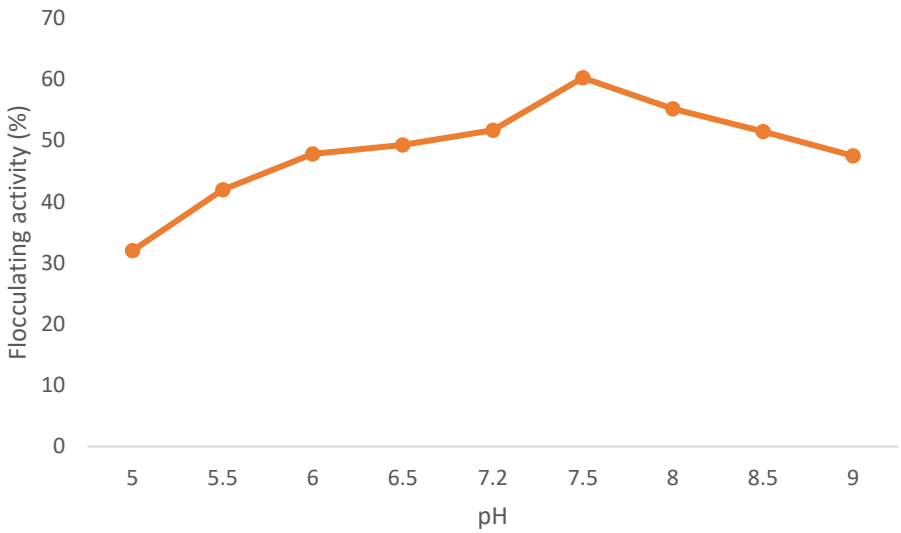


Figure 7.5: The effect of pH on flocculating activity of *Pseudomonas korensis*

Temperature affects enzymatic reactions and bacteria growth, and consequently the flocculating activity (Subudhi et al., 2016). The effect of culture temperature on flocculating activities of the two bacterial strains is presented in Figure 7.6. The maximum flocculating activity of 71.3% and 51.7% for *Pantoea* sp. and *P. korensis* respectively was observed at 30˚C and slightly above what was observed at 37˚C. Increase in initial culture temperature increases flocculating activity until the optimum was reached at 30˚C. Afterwards, flocculating activities were observed to steadily decrease as temperature increases due to decrease in enzymatic activities. This suggests that the synthesis of bioflocculant is strongly influenced by cultivation temperature and optimum enzymatic activation can only be obtained at optimum temperature (Zhang et al., 2007). This result corroborates the work of Nwodo and Okoh (2013) that reported optimum flocculating activity at 30˚C for *Cellulomonas* sp. However, Wu and Ye (2007a) and Gomaa (2012) reported optimum flocculating activity at 35˚C for *Bacillus subtilis* DYU1 and *P. aeruginosa*, while optimum flocculating activity at 37˚C was reported for *A. xylosoxidans* by Subudhi et al. (2016).

.

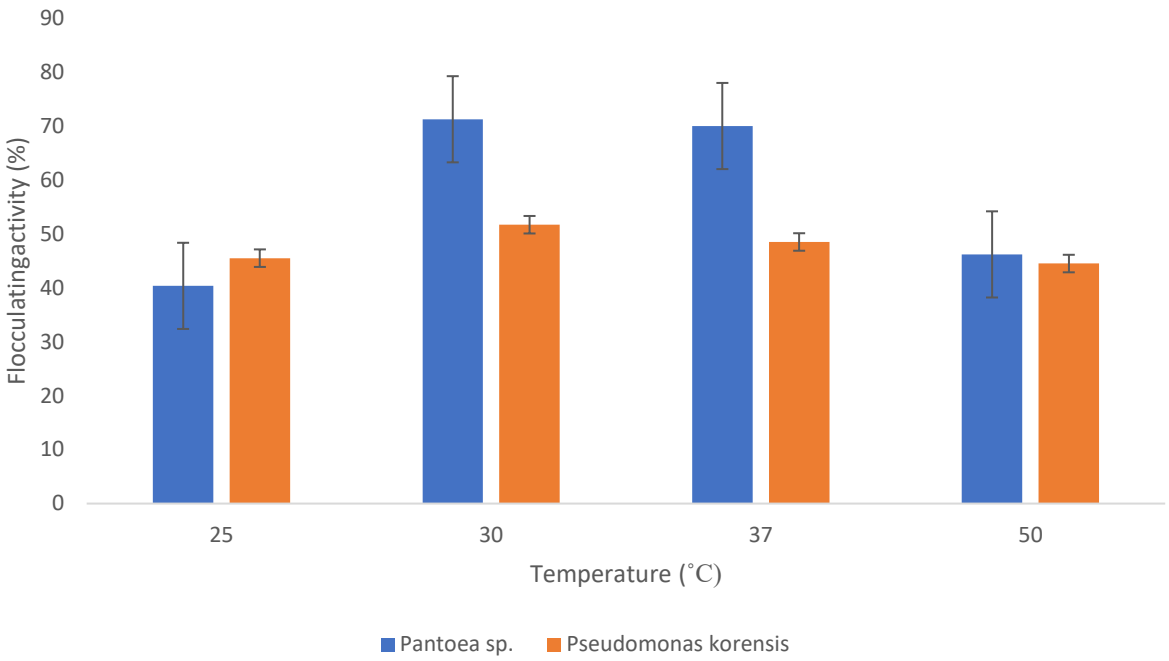


Figure 7.6: The effect of culture temperature on flocculating activity

The bioflocculant yield for each isolate is presented in Figure 7.7. The optimum yield of 3.26 g/L for *P. korensis* and 2.98 g/L for *Pantoea* sp. was obtained. The yield was lower than 5 g/L reported for *A. xylosoxidans* by Subudhi et al. (2016) using the same medium and culture conditions. This could be attributed to the different producing strains. The bioflocculant yield was also lower than those reported for *Paenibacillus elgii* B69 (25.63 g/L) (Sun et al., 2012), *Cellulomonas* sp. (4.04 g/L) (Nwodo and Okoh, 2013) and a consortium of *Streptomyces* and *Cellulomonas* species with a yield of 4.45 g/L (Nwodo et al., 2014). However, the yield produced by *P. korensis* and *Pantoea* sp. in this study was higher than those reported for *Halomonas* sp. (1.213 g/L) and *Micrococcus* sp. (0.738 g/L) (Okaiyeto et al., 2013). *Pseudomonas korensis* from this study also has a higher yield compared to *Bacillus megaterium* which was reported to have a yield of 3.11 g/L (Guo and Chen, 2017).

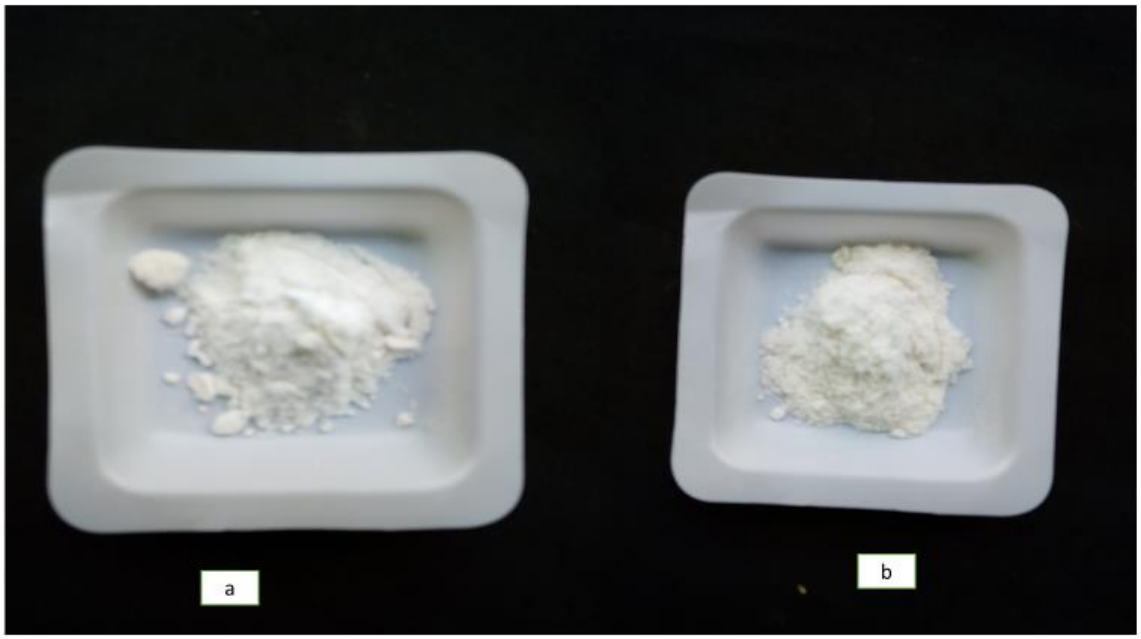


Figure 7.7: Bioflocculant yield from each isolate at optimum condition (a) *P. korensis* and (b) *Pantoea* sp.

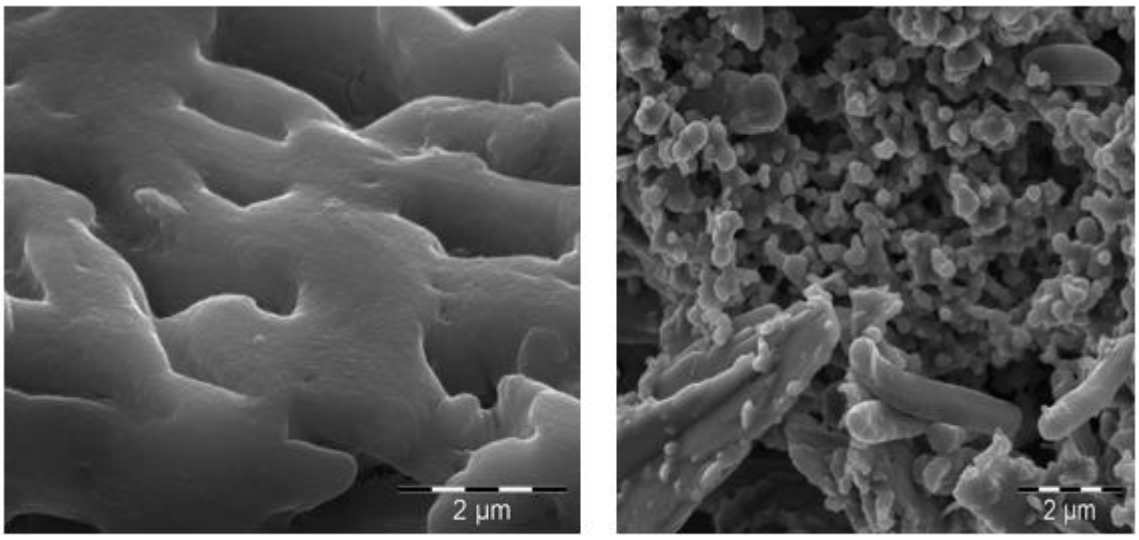


Figure 7.8: The scanning electron micrograph of the purified bioflocculant produced by (a) *P. korensis* and (b) *Pantoea* sp.

The scanning electron micrograph of the purified bioflocculant produced by the two isolates is shown in Figure 7.8. The micrograph of *P. korensis* revealed the irregular shaped amorphous

biopolymer (Figure 7.8a) while the *Pantoea* sp. showed particles with random size and shape with a large degree of aggregation.

The functional groups of bioflocculant produced differs for each organism. The flocculating activity depends on the functional groups and chemical structure of the bioflocculant (Okaiyeto et al., 2013). The partially purified bioflocculant produced by the two bacterial isolates were characterized by FTIR and are presented in Figures 7.9 and 7.10. The FTIR spectrum showed the presence of various functional groups. The spectrum in Figure 7.9 showed a weak absorption peak at 3471 cm^{-1} characteristic of hydroxyl group that could be as a result of vibration of -OH or -NH in the sugar ring of polysaccharide (He et al., 2010; Kavita et al., 2013). The broad weak peak at 2879 cm^{-1} is characteristic of C-H stretching vibration. In addition, the stretching peak at 1640 cm^{-1} showed the presence of carbonyl group vibration in the peptide (Okaiyeto et al., 2013; Okaiyeto et al., 2015). A strong carboxylic group vibration band was observed at 1432 cm^{-1} (Kavita et al., 2013).

The FTIR spectrum of the purified bioflocculant produced by *Pantoea* sp. (Figure 7.10) showed a broad, intense medium peak at 3231 cm^{-1} that is typical of either -OH or -NH stretching vibration. This indicates the presence of hydroxyl group (Subudhi et al., 2014). The band observed at 2335 cm^{-1} is attributed to C-H stretching. The asymmetric stretching band at 1637 cm^{-1} and peak at 1431 cm^{-1} revealed the presence of carboxyl groups (Yin et al., 2014).

Furthermore, the peaks between 1200 and 800 cm^{-1} for the two isolates were attributed to different polysaccharide derivatives (Pathak et al., 2017). In addition, the presence of strong absorption bands obtained at 983 cm^{-1} (Figure 7.8) and 979 cm^{-1} (Figure 7.9) showed stretching of =C-H bends of alkanes. The peaks between 690–515 cm^{-1} correspond to N-H vibrations of primary amine and C-X stretch of alkyl halides (Kavita et al., 2013). The FTIR spectrum of the bioflocculant produced by *P. korensis* and *Pantoea* sp. revealed the presence of carboxyl, hydroxyl groups (the main groups used for flocculation), amino acid and sugar derivative groups. The carboxyl

functional groups in the bioflocculant structure provides adequate binding sites for attachment of particles which makes bridging mechanisms between particles and flocculants effective (Pathak et al., 2017). Thus, it can be inferred that the two bioflocculants are polysaccharides containing proteins.

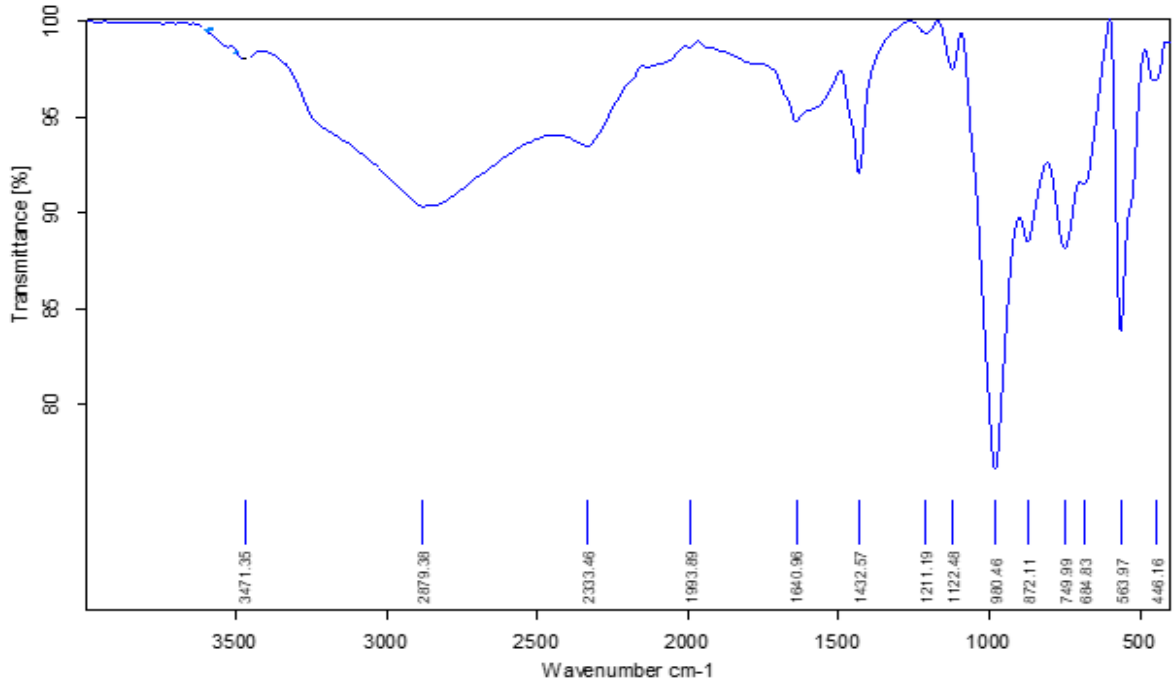


Figure 7.9: The FTIR spectroscopy of the purified bioflocculant produced by *P. korensis*

Microorganism develop resistance mechanisms, such as producing secondary metabolites, to thrive and survive in harsh environments. These metabolites have important ecological function and application in industrial waste treatment (Okaiyeto et al., 2016). Thus, the heavy metal removal potential of the bioflocculants produced by the two isolates were investigated and results is presented in Table 7.5. Lead had the highest percentage removal by the partially purified bioflocculants (Table 7.5). The bioflocculant from *Pantoea* sp. was able to remove 80.5% of Pb, 52.5% of Cr and 51.2% of Cd from aqueous solution of each metal. *Pseudomonas korensis* had removal efficiency of 42.5%, 48.5% and 73.7% for Cr, Cd and Pb respectively.

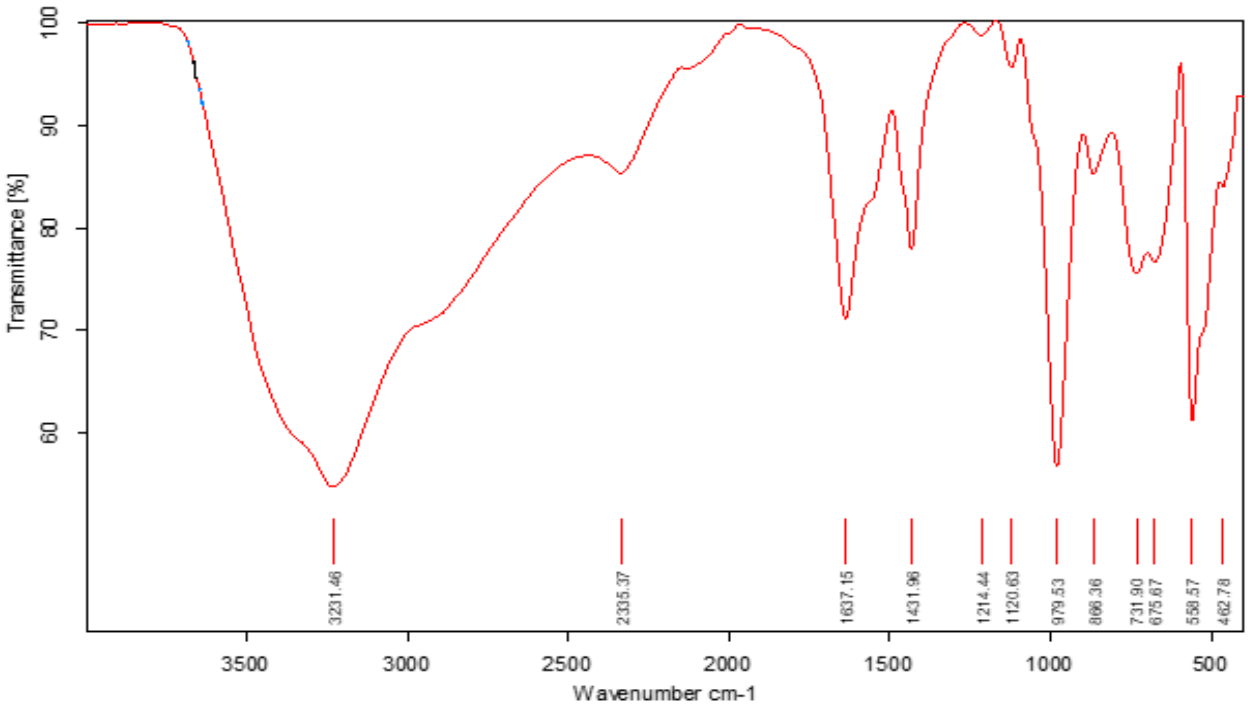


Figure 7.10: The FTIR spectroscopy of the purified bioflocculant from *Pantoea* sp.

Table 7.5: Metal removal efficiency by bioflocculant produced by the two isolates

	Heavy metal removal (%)		
	Cd	Cr	Pb
Pseudomonas korensis	48.50 (0.50)	42.50 (2.50)	73.70 (0.50)
Pantoea sp.	51.20 (1.20)	52.50 (1.50)	80.50 (1.50)

Values are means of duplicate readings ($P < 0.05$)
Values in parenthesis are ± standard error

The metal removal efficiencies observed in this study were lower than those reported for *Achromobacter xylosoxidans* for the removal of Pb from aqueous solution (Subudhi et al., 2016) and Cd removal by *Bacillus subtilis* WD 90 and *B. subtilis* SM 29 (Kaewchai and Prasertsan, 2002). *Achromobacter xylosoxidans* achieved 95% removal efficiency from 1500 mg/L of Pb while *B. subtilis* SM 29 and *B. subtilis* WD 90 were reported to remove 91.4% and 90.9% of Cd

(60 mg/L) respectively. However, the removal efficiency of bioflocculant produced by the two isolates were higher that those reported for *Bacillus* sp., *Halomonas* sp., and *Herbaspirillium* sp. for removal of Cd, Cr and Pb from different dyes and chemical industry effluents (Lin and Harichund, 2011). *Pantoea* sp. was observed to remove a slightly higher percentage of Pb compared to that reported for *Pseudomonas aeruginosa* that had 79.7% Pb removal efficiency (Gomaa, 2012).

The differences in metal removal efficiency by each bioflocculant can be attributed to their binding specificity, charge density, attractive interaction, types of conformation of the bioflocculant with the adsorbed metal ion (Gomaa, 2012) and bioflocculant concentration (Lin and Harichund, 2011).

Conclusion

Bioflocculants are produced by different bacterial species and have received increasing attention lately as a result of their applications in metal removal. The bioflocculants produced by the two isolates demostrate high metal removal potential from aqueous solution. The structure of the bioflocculants revealed the presence of polysaccharides and proteins which are responsible for metal binding. This study demonstrates possible evironmental and biotechnological applications of this biopolymer in heavy metal removal.

CHAPTER EIGHT

8.0 SUMMARY, CONCLUSION AND RECOMMENDATIONS

Mining activities have led to the release of large quantities of heavy metals into the environmental media, which pose significant health hazards to the ecosystem. These metals exert their toxic effect on microorganisms by disrupting cell membranes, causing functional disturbance, inhibiting enzyme activity, causing protein denaturation, loss of diversity and blocking essential functional groups and essential ions. However, polluted media harbor microorganisms that have the potential to remove these heavy metals from those environments. The surviving organisms have developed intrinsic or induced resistance mechanisms that counter the effect of high concentrations of heavy metals in their environment. These organisms secrete various kinds of metal-binding polymers that are important in metal removal.

This study showed that mining activities constitute a threat to the ecosystem. The soil samples collected had low moisture content, organic matter and nutrients needed for survival of microbial communities. Consequently, this affects microbial diversity, nutrient cycling and soil fertility. The soil samples are generally sandy in nature which also has effects on metal mobility and availability, thus leading to lower microbial population and diversity. Furthermore, the soil samples have low cation exchange capacity (CEC) and have high sulfur and sulfate content. The low CEC values also contribute to low water holding capacity of the soil samples and its attendant leaching of cations and nutrients in the soil samples. High sulfur content affects calcium and magnesium ions from sorption complexes, thereby increasing acidification of the soil. This impacts negatively on the microbial population in the soil samples.

Despite the low heavy metal concentrations in the digested mine soil samples, the microbial diversity was low as *Bacillus* species were the dominant organisms. These metals still have a potential significant impact on biological systems. A total of 98 heavy metal resistant bacterial isolates were recovered from the sampling sites and were clustered within a few genera which

corroborates the concept that bacterial diversity is affected by pollutants in contaminated environment when compared to pristine environment. The resistant organisms showed active growth, suggesting that they have been able to adapt to the metal polluted environment. A significant number of the isolates were able to grow at high concentration of heavy metals, but a few were susceptible to high metal concentration, perhaps owing to changes in the laboratory conditions they were subjected to. However, a few of the resistant organisms were able to secrete secondary metabolites that are involved in metal binding. The metabolites are usually the first line of defense against toxic metals.

As a result of the secretion of metal binding polymers and the multi-metal resistance of some of the isolates, a few of them were selected for further studies. *Bacillus cereus* was investigated for biosurfactant production and its metal removal potential. The organism was able to produce biosurfactant using different hydrocarbons as carbon source and was able to emulsify different hydrocarbons. The biosurfactant produced by the isolate had a good stability over a wide range of temperature and pH and was able to reduce the surface tension of water from 72 to 39.5 mN/m. Spectrometric analysis of the biosurfactant revealed that it was a lipopeptide with the potential to remove 35%, 56% and 78% of Cr, Cd and Pb respectively from contaminated soil. The genomic richness of the organism can be tapped into for biotechnological applications.

Similarly, two strains that showed potential flocculating activity were investigated for their bioflocculant production. The spectrometric analysis of the bioflocculant produced by the *Pantoea* sp. and *P. korensis* revealed the presence of carboxyl, hydroxyl and amino groups typical of polysaccharides and proteins involved in metal binding. The bioflocculant of *Pantoea* sp. was able to remove 51.2%, 52.5% and 80.5% of Cd, Cr and Pb respectively while that of *P. korensis* removed 48.5%, 42.5% and 73.7% of Cd, Cr and Pb respectively from aqueous solution.

The use of these biopolymers has provided valuable insight to their potential metal removal abilities. These polymers produced by frequently designated opportunistic pathogens bear a great

role for removal of heavy metals from polluted media. These polymers have also helped in circumventing the pathogenicity concerns that may arise from the use of these organisms as microbial inoculants for metal removal and have immense possibilities in treatment of soil and wastewaters.

Further investigation needs to be carried out in detail on the promising industrial application, large scale production through optimization and their ability for reuse. The economics of their production should also be evaluated. The exploitation of the metabolic pathways of the producing organisms and the roles of the biosynthetic genes would also give insight about other obscure benefits of these polymers.

REFERENCES

Abbas, S. H., Ismail, I. M., Mostafa, T. M. Sulaymon, A. H., 2014 Biosorption of heavy metals: a review. Journal of Chemical Science and Technology 3**,** 74-102.

Abdul-Wahab, S. Marikar, F., 2012 The environmental impact of gold mines: pollution by heavy metals. Open Engineering 2**,** 304-313.

Abollino, O., Aceto, M., Malandrino, M., Mentasti, E., Sarzanini, C. Petrella, F., 2002 Heavy metals in agricultural soils from Piedmont, Italy. Distribution, speciation and chemometric data treatment. Chemosphere 49**,** 545-557.

Abou-Shanab, R., Van Berkum, P. Angle, J., 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.

Achal, V., Pan, X., Fu, Q. Zhang, D., 2012 Biomineralization based remediation of As (III) contaminated soil by *Sporosarcina ginsengisoli*. Journal of Hazardous Materials 201**,** 178-184.

Adler, R. A., Claassen, M., Godfrey, L. Turton, A. R., 2007 Water, mining and waste: an historical and economic perspective on conflict management in South Africa. The Economics of Peace and Security Journal 2**,** 32-41.

Ahluwalia, S. S. Goyal, D., 2007 Microbial and plant derived biomass for removal of heavy metals from wastewater. Bioresource Technology 98**,** 2243-2257.

Akar, T., Tunali, S. Kiran, I., 2005 *Botrytis cinerea* as a new fungal biosorbent for removal of Pb (II) from aqueous solutions. Biochemical Engineering Journal 25**,** 227-235.

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

Akpa, E., Jacques, P., Wathelet, B., Paquot, M., Fuchs, R., Budzikiewicz, H. Thonart, P., 2001 Influence of culture conditions on lipopeptide production by *Bacillus* subtilis. Applied Biochemistry and Biotechnology 91**,** 551-561.

Ali, H., Khan, E. Sajad, M. A., 2013 Phytoremediation of heavy metals—concepts and applications. Chemosphere 91**,** 869-881.

Ali, M. Shakrani, S., 2014 A comparison of ICP-OES and UV-Vis spectrophotometer for heavy metals determination in soil irrigated with secondary treated wastewater. International Journal of Civil & Environmental Engineering 14**,** 8-15.

Alisi, C., Musella, R., Tasso, F., Ubaldi, C., Manzo, S., Cremisini, C. Sprocati, A. R., 2009 Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance. Science of The Total Environment 407**,** 3024-3032.

Aljuboori, A. H. R., Idris, A., Abdullah, N. Mohamad, R., 2013 Production and characterization of a bioflocculant produced by *Aspergillus flavus*. Bioresource Technology 127**,** 489-493.

An, Y.-J. Kim, M., 2009 Effect of antimony on the microbial growth and the activities of soil enzymes. Chemosphere 74**,** 654-659.

Anjum, R., Grohmann, E. Malik, A., 2011 Molecular characterization of conjugative plasmids in pesticide tolerant and multi-resistant bacterial isolates from contaminated alluvial soil. Chemosphere 84**,** 175-181.

Arjoon, A., Olaniran, A. Pillay, B., 2013 Co-contamination of water with chlorinated hydrocarbons and heavy metals: challenges and current bioremediation strategies. International Journal of Environmental Science and Technology 10**,** 395-412.

Arkin, A. P., Stevens, R. L., Cottingham, R. W., Maslov, S., Henry, C. S., Dehal, P., Ware, D., Perez, F., Harris, N. L. Canon, S., 2016 The DOE systems biology knowledgebase (KBase). bioRxiv096354.

Aşçı, Y., Nurbaş, M. Açıkel, Y. S., 2010 Investigation of sorption/desorption equilibria of heavy metal ions on/from quartz using rhamnolipid biosurfactant. Journal of Environmental Management 91**,** 724-731.

Ashraf, M., Maah, M. Yusoff, I., 2012 Chemical speciation and potential mobility of heavy metals in the soil of former tin mining catchment. The Scientific World Journal 2012**,** 1-11.

Astolfi, M. C. T., De Souza Carvalho, E. B., De Barros, A. M., Pinto, M. V., De Lacerda, L. B., Nogueira, V. B., Lopes, E. F. Astolfi-Filho, S., 2018 Draft Genome Sequence of the Novel Enterobacter cloacae Strain amazonensis, a Highly Heavy Metal-Resistant Bacterium from a Contaminated Stream in Amazonas, Brazil. Genome announcements 6**,** e00450-18.

Ayangbenro, A. S. Babalola, O. O., 2017 A new strategy for heavy metal polluted environments: a review of microbial biosorbents. International Journal of Environmental Research and Public Health 14**,** 94.

Ayangbenro, A. S. Babalola, O. O., 2018 Metal(loid) bioremediation: strategies employed by microbial polymers. Sustainability 10**,** 3028.

Ayangbenro, A. S., Olanrewaju, O. S. Babalola, O. O., 2018 Sulfate-reducing bacteria as an effective tool for sustainable acid mine bioremediation. Frontiers in Microbiology 9**,** 1986.

Aziz, R. K., Bartels, D., Best, A. A., Dejongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M. Kubal, M., 2008 The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9**,** 75.

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L. Kizek, R., 2008 Uncommon heavy metals, metalloids and their plant toxicity: a review. Environmental Chemistry Letters 6**,** 189-213.

Barakat, K. M., Hassan, S. W. Darwesh, O. M., 2017 Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea, Egypt. The Egyptian Journal of Aquatic Research 43**,** 205-211.

Barakat, M., 2011 New trends in removing heavy metals from industrial wastewater. Arabian Journal of Chemistry 4**,** 361-377.

Bento, F. M., De Oliveira Camargo, F. A., Okeke, B. C. Frankenberger Jr, W. T., 2005 Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiological Research 160**,** 249-255.

Bezza, F. A. Chirwa, E. M. N., 2016 Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil. Chemosphere 144**,** 635-644.

Bicca, F. C., Fleck, L. C. Ayub, M. a. Z., 1999 Production of biosurfactant by hydrocarbon degrading *Rhodococcus ruber* and *Rhodococcus erythropolis*. Revista de Microbiologia 30**,** 231-236.

Bissen, M. Frimmel, F. H., 2003 Arsenic—a review. Part I: occurrence, toxicity, speciation, mobility. Acta Hydrochimica et Hydrobiologica 31**,** 9-18.

Blair, J. M., Richmond, G. E. Piddock, L. J., 2014 Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiology 9**,** 1165-1177.

Blais, J., Djedidi, Z., Cheikh, R. B., Tyagi, R. Mercier, G., 2008 Metals precipitation from effluents: Review. Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management 12**,** 135-149.

Bloem, J. Breure, A. M. 2003. Microbial indicators. *(In* Markert, B. A., Breure, A. M. & Zechmeister, H. G., *eds*. Trace Metals and other Contaminants in the Environment. Elsevier. p. 259-282).

Bolan, N., Kunhikrishnan, A., Thangarajan, R., Kumpiene, J., Park, J., Makino, T., Kirkham, M. B. Scheckel, K., 2014 Remediation of heavy metal (loid) s contaminated soils–To mobilize or to immobilize? Journal of Hazardous Materials 266**,** 141-166.

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

Bukhari, N. A., Loh, S. K., Nasrin, A. B. Jahim, J. M., 2018 Enzymatic hydrolysate of palm oil mill effluent as potential substrate for bioflocculant BM-8 production. Waste and Biomass Valorization1-13.

Bustamante, M., Duran, N. Diez, M., 2012 Biosurfactants are useful tools for the bioremediation of contaminated soil: a review. Journal of Soil Science and Plant Nutrition 12**,** 667-687.

Buthelezi, S., Olaniran, A. Pillay, B., 2009 Turbidity and microbial load removal from river water using bioflocculants from indigenous bacteria isolated from wastewater in South Africa. African Journal of Biotechnology 8**,** 3261-3266.

Cáliz, J., Montserrat, G., Martí, E., Sierra, J., Chung, A. P., Morais, P. V. Vila, X., 2013 Emerging resistant microbiota from an acidic soil exposed to toxicity of Cr, Cd and Pb is mainly influenced by the bioavailability of these metals. Journal of Soils and Sediments 13**,** 413-428.

Carrillo, P., Mardaraz, C., Pitta-Alvarez, S. Giulietti, A., 1996 Isolation and selection of biosurfactant-producing bacteria. World Journal of Microbiology and Biotechnology 12**,** 82-84.

Cervantes, C., Campos-García, J., Devars, S., Gutiérrez-Corona, F., Loza-Tavera, H., Torres-Guzmán, J. C. Moreno-Sánchez, R., 2001 Interactions of chromium with microorganisms and plants. FEMS Microbiology Reviews 25**,** 335-347.

Chakraborty, J. Das, S. 2014. Biosurfactant-based bioremediation of toxic metals. *(In* Das, S., *ed.* Microbial. Biodegradation and Bioremediation Waltham, USA: Elsevier Inc. p. 167-201).

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

Chen, H., Chen, Z., Zhang, Y. Zhu, W., 2014 Identification of antifungal peptides from *Bacillus subtilis* Bs-918. Analytical Letters 47**,** 2891-2899.

Chibuike, G. Obiora, S., 2014 Heavy metal polluted soils: effect on plants and bioremediation methods. Applied and Environmental Soil Science 2014**,** 1-12.

Chihomvu, P., Stegmann, P. Pillay, M. Identification and characterization of heavy metal resistant bacteria from the Klip River. Proceedings of the International Conference on Ecological, Environmental and Biological Sciences, WASET, Cape Town, South African, 2014. 1107-1117.

Cho, S.-J., Lee, S. K., Cha, B. J., Kim, Y. H. Shin, K.-S., 2003 Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. FEMS Microbiology Letters 223**,** 47-51.

Choudhary, S. Sar, P., 2009 Characterization of a metal resistant *Pseudomonas* sp. isolated from uranium mine for its potential in heavy metal (Ni2+, Co2+, Cu2+, and Cd2+) sequestration. Bioresource Technology 100**,** 2482-2492.

Çolak, F., Atar, N., Yazıcıoğlu, D. Olgun, A., 2011 Biosorption of lead from aqueous solutions by *Bacillus* strains possessing heavy-metal resistance. Chemical Engineering Journal 173**,** 422-428.

Colombo, F., Macdonald, C. A., Jeffries, T. C., Powell, J. R. Singh, B. K., 2016 Impact of forest management practices on soil bacterial diversity and consequences for soil processes. Soil Biology and Biochemistry 94**,** 200-210.

Congeevaram, S., Dhanarani, S., Park, J., Dexilin, M. Thamaraiselvi, K., 2007 Biosorption of chromium and nickel by heavy metal resistant fungal and bacterial isolates. Journal of Hazardous Materials 146**,** 270-277.

D'amore, J., Al-Abed, S., Scheckel, K. Ryan, J., 2005 Methods for speciation of metals in soils. Journal of Environmental Quality 34**,** 1707-1745.

Da Rocha Junior, R. B., Meira, H. M., Almeida, D. G., Rufino, R. D., Luna, J. M., Santos, V. A. Sarubbo, L. A., 2018 Application of a low-cost biosurfactant in heavy metal remediation processes. Biodegradation 1-19.

Dadrasnia, A. Ismail, S., 2015 Biosurfactant production by *Bacillus salmalaya* for lubricating oil solubilization and biodegradation. International Journal of Environmental Research and Public Health 12**,** 9848-9863.

Dahrazma, B. Mulligan, C. N., 2007 Investigation of the removal of heavy metals from sediments using rhamnolipid in a continuous flow configuration. Chemosphere 69**,** 705-711.

Das, D., Das, N. Mathew, L., 2010 Kinetics, equilibrium and thermodynamic studies on biosorption of Ag (I) from aqueous solution by macrofungus *Pleurotus platypus*. Journal of Hazardous Materials 1**,** 765-774.

Das, K. Mukherjee, A. K., 2007 Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresource Technology 98**,** 1339-1345.

Das, P., Mukherjee, S. Sen, R., 2009 Biosurfactant of marine origin exhibiting heavy metal remediation properties. Bioresource Technology 100**,** 4887-4890.

Dell'amico, E., Mazzocchi, M., Cavalca, L., Allievi, L. Andreoni, V., 2008 Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. Microbiological Research 163**,** 671-683.

Deng, S., Ke, T., Li, L., Cai, S., Zhou, Y., Liu, Y., Guo, L., Chen, L. Zhang, D., 2018 Impacts of environmental factors on the whole microbial communities in the rhizosphere of a metal-tolerant plant: *Elsholtzia haichowensis* Sun. Environmental Pollution 237**,** 1088-1097.

Deng, S., Yu, G. Ting, Y. P., 2005 Production of a bioflocculant by *Aspergillus parasiticus* and its application in dye removal. Colloids and surfaces B: Biointerfaces 44**,** 179-186.

Diaz, M. A., De Ranson, I. U., Dorta, B., Banat, I. M., Blazquez, M. L., Gonzalez, F., Muñoz, J. A. Ballester, A., 2015 Metal removal from contaminated soils through bioleaching with oxidizing bacteria and rhamnolipid biosurfactants. Soil and Sediment Contamination: An International Journal 24**,** 16-29.

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

Dominguez-Benetton, X., Varia, J. C., Pozo, G., Modin, O., Ter-Heijne, A., Fransaer, J. Rabaey, K., 2018 Metal recovery by microbial electro-metallurgy. Progress in Materials Science 94**,** 435-461.

Drakou, E.-M., Amorim, C. L., Castro, P. M., Panagiotou, F. Vyrides, I., 2017 Wastewater valorization by pure bacterial cultures to extracellular polymeric substances (EPS) with high emulsifying potential and flocculation activities. Waste and Biomass Valorization 9**,** 2557–2564.

Dube, A., Zbytniewski, R., Kowalkowski, T., Cukrowska, E. Buszewski, B., 2001 Adsorption and migration of heavy metals in soil. Polish Journal of Environmental Studies 10**,** 1-10.

Dursun, A., Uslu, G., Cuci, Y. Aksu, Z., 2003 Bioaccumulation of copper (II), lead (II) and chromium (VI) by growing *Aspergillus niger*. Process Biochemistry 38**,** 1647-1651.

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

Eddouaouda, K., Mnif, S., Badis, A., Younes, S. B., Cherif, S., Ferhat, S., Mhiri, N., Chamkha, M. Sayadi, S., 2012 Characterization of a novel biosurfactant produced by *Staphylococcus* sp. strain 1E with potential application on hydrocarbon bioremediation. Journal of Basic Microbiology 52**,** 408-418.

Edwards, S. J. Kjellerup, B. V., 2013 Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. Applied Microbiology and Biotechnology 97**,** 9909-9921.

Eisler, R., 2003 Health risks of gold miners: a synoptic review. Environmental Geochemistry and Health 25**,** 325-345.

Elekofehinti, O., Omotuyi, I., Olaremu, A. Abayomi, T., 2012 Heavy metals distribution and lipid profile in the stomach of cow grazed in Akungba-Akoko, Ondo State, Nigeria. African Journal of Biochemistry Research 6**,** 146-149.

Ellis, R. J., Morgan, P., Weightman, A. J. Fry, J. C., 2003 Cultivation-dependent and-independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Applied and Environmental Microbiology 69**,** 3223-3230.

Fan, L.-M., Ma, Z.-Q., Liang, J.-Q., Li, H.-F., Wang, E.-T. Wei, G.-H., 2011 Characterization of a copper-resistant symbiotic bacterium isolated from Medicago lupulina growing in mine tailings. Bioresource technology 102**,** 703-709.

Farag, S., Zaki, S., Elkady, M. Abd-El-Haleem, D., 2014 Production and characteristics of a bioflocculant produced by *Pseudomonas* sp. strain 38A. Journal of Advances in Biology 4**,** 286-295.

Farasin, J., Koechler, S., Varet, H., Deschamps, J., Dillies, M. A., Proux, C., Erhardt, M., Huber, A., Jagla, B. Briandet, R., 2017 Comparison of biofilm formation and motility processes in arsenic-resistant *Thiomonas* spp. strains revealed divergent response to arsenite. Microbial Biotechnology 10**,** 789-803.

Fashola, M., Ngole-Jeme, V. Babalola, O., 2016 Heavy metal pollution from gold mines: environmental effects and bacterial strategies for resistance. International Journal of Environmental Research and Public Health 13**,** 1047.

Felsenstein, J., 1985 Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39**,** 783-791.

Filgueira, R. R., Fournier, L. L., Cerisola, C. I., Gelati, P. García, M. G., 2006 Particle-size distribution in soils: A critical study of the fractal model validation. Geoderma 134**,** 327-334.

Finnegan, P. Chen, W., 2012 Arsenic toxicity: the effects on plant metabolism. Frontiers in Physiology 3**,** 182.

Finzgar, N., Tlustos, P. Lestan, D., 2007 Relationship of soil properties to fractionation, bioavailability and mobility of lead and zinc in soil. Plant Soil and Environment 53**,** 225-238.

Fomina, M. Gadd, G. M., 2014 Biosorption: current perspectives on concept, definition and application. Bioresource Technology 160**,** 3-14.

Franzetti, A., Gandolfi, I., Fracchia, L., Van Hamme, J., Gkorezis, P., Marchant, R. Banat, I. M. 2014. Biosurfactant use in heavy metal removal from industrial effluents and contaminated sites. *(In* Kosaric N & Fv, S., *eds*. Biosurfactants: production and utilization—processes, technologies, and economics. Boca Raton, FL: CRC Press. p. 361-369).

Freitas, H., Prasad, M. Pratas, J., 2004 Analysis of serpentinophytes from north–east of Portugal for trace metal accumulation—relevance to the management of mine environment. Chemosphere 54**,** 1625-1642.

Fu, Y. Q., Li, S., Zhu, H. Y., Jiang, R. Yin, L. F., 2012 Biosorption of copper (II) from aqueous solution by mycelial pellets of *Rhizopus oryzae*. African Journal of Biotechnology 11**,** 1403-1411.

Fulekar, M., Singh, A. Bhaduri, A. M., 2009 Genetic engineering strategies for enhancing phytoremediation of heavy metals. African Journal of Biotechnology 8**,** 529-535.

Gadd, G. 1990. Metal tolerance. Microbiology of extreme environments. Milton Keynes UK: Open Universty Press. p. 178-210).

Gadd, G. M., 2000 Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Current Opinion in Biotechnology 11**,** 271-279.

Gadd, G. M., 2009 Biosorption: critical review of scientific rationale, environmental importance and significance for pollution treatment. Journal of Chemical Technology and Biotechnology 84**,** 13-28.

Gao, J., Bao, H.-Y., Xin, M.-X., Liu, Y.-X., Li, Q. Zhang, Y.-F., 2006 Characterization of a bioflocculant from a newly isolated *Vagococcus* sp. W31. Journal of Zhejiang University Science B 7**,** 186-192.

Gao, L., Kano, N., Sato, Y., Li, C., Zhang, S. Imaizumi, H., 2012 Behavior and distribution of heavy metals including rare earth elements, thorium, and uranium in sludge from industry water treatment plant and recovery method of metals by biosurfactants application. Bioinorganic chemistry and applications 2012**,** 173819.

Gao, Q., Zhu, X.-H., Mu, J., Zhang, Y. Dong, X.-W., 2009 Using Ruditapes philippinarum conglutination mud to produce bioflocculant and its applications in wastewater treatment. Bioresource Technology 100**,** 4996-5001.

Garrity, G. M., Bell, J. A. Lilburn, T. G., 2004 Taxonomic outline of the prokaryotes. Bergey's manual of systematic bacteriology. Springer, New York, Berlin, Heidelberg.

Gaur, N., Flora, G., Yadav, M. Tiwari, A., 2014 A review with recent advancements on bioremediation-based abolition of heavy metals. Environmental Science: Processes & Impacts 16**,** 180-193.

Gavrilescu, M., 2004 Removal of heavy metals from the environment by biosorption. Engineering in Life Sciences 4**,** 219-232.

Germ, M., Kreft, I., Stibilj, V. Urbanc-Berčič, O., 2007 Combined effects of selenium and drought on photosynthesis and mitochondrial respiration in potato. Plant Physiology and Biochemistry 45**,** 162-167.

Getaneh, W. Alemayehu, T., 2006 Metal contamination of the environment by placer and primary gold mining in the Adola region of southern Ethiopia. Environmental Geology 50**,** 339-352.

Giller, K. E., Witter, E. Mcgrath, S. P., 2009 Heavy metals and soil microbes. Soil Biology and Biochemistry 41**,** 2031-2037.

Gnanamani, A., Kavitha, V., Radhakrishnan, N., Rajakumar, G. S., Sekaran, G. Mandal, A., 2010 Microbial products (biosurfactant and extracellular chromate reductase) of marine microorganism are the potential agents reduce the oxidative stress induced by toxic heavy metals. Colloids and Surfaces B: Biointerfaces 79**,** 334-339.

Gomaa, E. Z., 2012 Production and characteristics of a heavy metals removing bioflocculant produced by *Pseudomonas aeruginosa*. Polish Journal of Microbiology 61**,** 281-289.

Gond, S. K., Bergen, M. S., Torres, M. S. White Jr, J. F., 2015 Endophytic *Bacillus* spp. produce antifungal lipopeptides and induce host defence gene expression in maize. Microbiological Research 172**,** 79-87.

Gong, W.-X., Wang, S.-G., Sun, X.-F., Liu, X.-W., Yue, Q.-Y. Gao, B.-Y., 2008 Bioflocculant production by culture of *Serratia ficaria* and its application in wastewater treatment. Bioresource Technology 99**,** 4668-4674.

Gordon, T. Bowser, D., 2003 Beryllium: genotoxicity and carcinogenicity. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 533**,** 99-105.

Govarthanan, M., Lee, K.-J., Cho, M., Kim, J. S., Kamala-Kannan, S. Oh, B.-T., 2013 Significance of autochthonous *Bacillus* sp. KK1 on biomineralization of lead in mine tailings. Chemosphere 90**,** 2267-2272.

Grimalt, J. O., Ferrer, M. Macpherson, E., 1999 The mine tailing accident in Aznalcollar. Science of the Total Environment 242**,** 3-11.

Grissa, I., Vergnaud, G. Pourcel, C., 2007 CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Research 35**,** W52-W57.

Guibaud, G., Bhatia, D., D'abzac, P., Bourven, I., Bordas, F., Van Hullebusch, E. D. Lens, P. N., 2012 Cd (II) and Pb (II) sorption by extracellular polymeric substances (EPS) extracted from anaerobic granular biofilms: evidence of a pH sorption-edge. Journal of the Taiwan Institute of Chemical Engineers 43**,** 444-449.

Guibaud, G., Tixier, N., Bouju, A. Baudu, M., 2003 Relation between extracellular polymers' composition and its ability to complex Cd, Cu and Pb. Chemosphere 52**,** 1701-1710.

Guibaud, G., Van Hullebusch, E., Bordas, F., D'abzac, P. Joussein, E., 2009 Sorption of Cd (II) and Pb (II) by exopolymeric substances (EPS) extracted from activated sludges and

pure bacterial strains: modeling of the metal/ligand ratio effect and role of the mineral fraction. Bioresource Technology 100**,** 2959-2968.

Gumpu, M. B., Sethuraman, S., Krishnan, U. M. Rayappan, J. B. B., 2015 A review on detection of heavy metal ions in water–An electrochemical approach. Sensors and Actuators B: Chemical 213**,** 515-533.

Guo, J., 2015 Characteristics and mechanisms of Cu (II) sorption from aqueous solution by using bioflocculant MBFR10543. Applied Microbiology and Biotechnology 99**,** 229-240.

Guo, J. Chen, C., 2017 Removal of arsenite by a microbial bioflocculant produced from swine wastewater. Chemosphere 181**,** 759-766.

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

Gupta, V. Rastogi, A., 2008 Biosorption of lead from aqueous solutions by green algae *Spirogyra* species: kinetics and equilibrium studies. Journal of Hazardous Materials 152**,** 407-414.

Gupta, V. K., Nayak, A. Agarwal, S., 2015 Bioadsorbents for remediation of heavy metals: Current status and their future prospects. Environmental Engineering Research 20**,** 1-18.

Gutnick, D. Bach, H., 2000 Engineering bacterial biopolymers for the biosorption of heavy metals; new products and novel formulations. Applied Microbiology and Biotechnology 54**,** 451-460.

Haft, D. H., Dicuccio, M., Badretdin, A., Brover, V., Chetvernin, V., O'neill, K., Li, W., Chitsaz, F., Derbyshire, M. K. Gonzales, N. R., 2017 RefSeq: an update on prokaryotic genome annotation and curation. Nucleic Acids Research 46**,** D851-D860.

Hansen, H. K., Ribeiro, A. Mateus, E., 2006 Biosorption of arsenic(V) with *Lessonia nigrescens*. Minerals Engineering 19**,** 486-490.

Haq, F., Butt, M., Ali, H. Chaudhary, H. J., 2015 Biosorption of cadmium and chromium from water by endophytic *Kocuria rhizophila*: equilibrium and kinetic studies. Desalination and Water Treatment 2015**,** 1-13.

Harrison, J. J., Rabiei, M., Turner, R. J., Badry, E. A., Sproule, K. M. Ceri, H., 2006 Metal resistance in *Candida* biofilms. FEMS Microbiology Ecology 55**,** 479-491.

Hasegawa, M., Kishino, H. Yano, T.-A., 1985 Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution 22**,** 160-174.

Hashim, M., Mukhopadhyay, S., Sahu, J. N. Sengupta, B., 2011 Remediation technologies for heavy metal contaminated groundwater. Journal of Environmental Management 92**,** 2355-2388.

He, J., Zou, J., Shao, Z., Zhang, J., Liu, Z. Yu, Z., 2010 Characteristics and flocculating mechanism of a novel bioflocculant HBF-3 produced by deep-sea bacterium mutant *Halomonas* sp. V3a'. World Journal of Microbiology and Biotechnology 26**,** 1135-1141.

Henkel, M., Geissler, M., Weggenmann, F. Hausmann, R., 2017 Production of microbial biosurfactants: Status quo of rhamnolipid and surfactin towards large-scale production. Biotechnology Journal 12**,** 1600561.

Heryani, H. Putra, M. D., 2017 Kinetic study and modeling of biosurfactant production using *Bacillus* sp. Electronic Journal of Biotechnology 27**,** 49-54.

Hong, K.-J., Tokunaga, S. Kajiuchi, T., 2002 Evaluation of remediation process with plant-derived biosurfactant for recovery of heavy metals from contaminated soils. Chemosphere 49**,** 379-387.

Hu, Z., Jin, J., Abruña, H. D., Houston, P. L., Hay, A. G., Ghiorse, W. C., Shuler, M. L., Hidalgo, G. Lion, L. W., 2007 Spatial distributions of copper in microbial biofilms by scanning electrochemical microscopy. Environmental Science & Technology 41**,** 936-941.

Ianeva, O. D., 2009 Mechanisms of bacteria resistance to heavy metals. Mikrobiolohichnyi Zhurnal 71**,** 54-65.

Iqbal, M. Edyvean, R., 2004 Biosorption of lead, copper and zinc ions on loofa sponge immobilized biomass of *Phanerochaete chrysosporium*. Minerals Engineering 17**,** 217-223.

Jain, D., Collins-Thompson, D., Lee, H. Trevors, J., 1991 A drop-collapsing test for screening surfactant-producing microorganisms. Journal of Microbiological Methods 13**,** 271-279.

Jasim, B., Sreelakshmi, K., Mathew, J. Radhakrishnan, E., 2016 Surfactin, iturin, and fengycin biosynthesis by endophytic *Bacillus* sp. from *Bacopa monnieri*. Microbial Ecology 72**,** 106-119.

Jukes, T. H. Cantor, C. R., 1969 Evolution of protein molecules. Mammalian Protein Metabolism 3**,** 132.

Juwarkar, A. A., Nair, A., Dubey, K. V., Singh, S. Devotta, S., 2007 Biosurfactant technology for remediation of cadmium and lead contaminated soils. Chemosphere 68**,** 1996-2002.

Kaewchai, S. Prasertsan, P., 2002 Biosorption of heavy metal by thermotolerant polymer-producing bacterial cells and the bioflocculant. Songklanakarin Journal of Science and Technology 24**,** 421-430.

Kang, C.-H., Oh, S. J., Shin, Y., Han, S.-H., Nam, I.-H. So, J.-S., 2015 Bioremediation of lead by ureolytic bacteria isolated from soil at abandoned metal mines in South Korea. Ecological Engineering 74**,** 402-407.

Kang, S., Lee, J. Kim, K., 2005 Metal removal from wastewater by bacterial sorption: Kinetics and competition studies. Environmental Technology 26**,** 615-624.

Kapoor, A. Viraraghavan, T., 1995 Fungal biosorption—an alternative treatment option for heavy metal bearing wastewaters: a review. Bioresource Technology 53**,** 195-206.

Kavita, K., Mishra, A. Jha, B., 2013 Extracellular polymeric substances from two biofilm forming *Vibrio* species: characterization and applications. Carbohydrate Polymers 94**,** 882-888.

Khopade, A., Biao, R., Liu, X., Mahadik, K., Zhang, L. Kokare, C., 2012 Production and stability studies of the biosurfactant isolated from marine *Nocardiopsis* sp. B4. Desalination 285**,** 198-204.

Kim, I. H., Choi, J.-H., Joo, J. O., Kim, Y.-K., Choi, J.-W. Oh, B.-K., 2015 Development of a microbe-zeolite carrier for the effective elimination of heavy metals from seawater. Journal of Microbiology and Biotechnology 25**,** 1542-1546.

Kim, K.-K., Kim, K.-W., Kim, J.-Y., Kim, I. S., Cheong, Y.-W. Min, J.-S., 2001 Characteristics of tailings from the closed metal mines as potential contamination source in South Korea. Environmental Geology 41**,** 358-364.

Kim, K., Lee, H. Yoo, B., 1998 The environmental impact of gold mines in the Yugu-Kwangcheon Au-Ag metallogenic province, Republic of Korea. Environmental Technology 19**,** 291-298.

Kiran, B. Kaushik, A., 2008 Chromium binding capacity of *Lyngbya putealis* exopolysaccharides. Biochemical Engineering Journal 38**,** 47-54.

Kitamoto, D., Morita, T., Fukuoka, T., Konishi, M.-A. Imura, T., 2009 Self-assembling properties of glycolipid biosurfactants and their potential applications. Current Opinion in Colloid & Interface Science 14**,** 315-328.

Kumar, A. P., Janardhan, A., Viswanath, B., Monika, K., Jung, J.-Y. Narasimha, G., 2016a Evaluation of orange peel for biosurfactant production by *Bacillus licheniformis* and their ability to degrade naphthalene and crude oil. 3 Biotech 6**,** 1-10.

Kumar, S., Stecher, G. Tamura, K., 2016b MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33**,** 1870-1874.

Langley, S. Beveridge, T., 1999 Metal binding by *Pseudomonas aeruginosa* PAO1 is influenced by growth of the cells as a biofilm. Canadian Journal of Microbiology 45**,** 616-622.

Lee, C. S., Chong, M. F., Robinson, J. Binner, E., 2014 A review on development and application of plant-based bioflocculants and grafted bioflocculants. Industrial & Engineering Chemistry Research 53**,** 18357-18369.

Legatzki, A., Grass, G., Anton, A., Rensing, C. Nies, D. H., 2003 Interplay of the Czc system and two P-type ATPases in conferring metal resistance to Ralstonia metallidurans. Journal of Bacteriology 185**,** 4354-4361.

Lemire, J. A., Harrison, J. J. Turner, R. J., 2013 Antimicrobial activity of metals: mechanisms, molecular targets and applications. Nature Reviews Microbiology 11**,** 371-384.

Lesmana, S. O., Febriana, N., Soetaredjo, F. E., Sunarso, J. Ismadji, S., 2009 Studies on potential applications of biomass for the separation of heavy metals from water and wastewater. Biochemical Engineering Journal 44**,** 19-41.

Li, K., Pidatala, V. R., Shaik, R., Datta, R. Ramakrishna, W., 2014 Integrated metabolomic and proteomic approaches dissect the effect of metal-resistant bacteria on maize biomass and copper uptake. Environmental Science & Technology 48**,** 1184-1193.

Li, O., Lu, C., Liu, A., Zhu, L., Wang, P.-M., Qian, C.-D., Jiang, X.-H. Wu, X.-C., 2013 Optimization and characterization of polysaccharide-based bioflocculant produced by *Paenibacillus elgii* B69 and its application in wastewater treatment. Bioresource Technology 134**,** 87-93.

Li, W.-W. Yu, H.-Q., 2014 Insight into the roles of microbial extracellular polymer substances in metal biosorption. Bioresource Technology 160**,** 15-23.

Lin, C. C. Lin, H. L., 2005 Remediation of soil contaminated with the heavy metal (Cd^{2+}). Journal of Hazardous Materials 122**,** 7-15.

Lin, J. Harichund, C., 2011 Isolation and characterization of heavy metal removing bacterial bioflocculants. African Journal of Microbiology Research 5**,** 599-607.

Liu, D., Zou, J., Wang, M. Jiang, W., 2008 Hexavalent chromium uptake and its effects on mineral uptake, antioxidant defence system and photosynthesis in *Amaranthus viridis* L. Bioresource Technology 99**,** 2628-2636.

Luna, J. M., Rufino, R. D. Sarubbo, L. A., 2016 Biosurfactant from *Candida sphaerica* UCP0995 exhibiting heavy metal remediation properties. Process Safety and Environmental Protection 102**,** 558-566.

Maiti, S., Karmakar, N. Sinha, I., 2002 Studies into some physical parameters aiding biological reclamation of mine spoil dump–a case study from Jharia coalfield. Int. Mining Environ. Journal 41**,** 20-23.

Maiti, S. K., 2006 Properties of mine soil and its affects on bioaccumulation of metals in tree species: case study from a large opencast coalmining project. International Journal of Surface Mining, Reclamation and Environment 20**,** 96-110.

Malik, A., 2004 Metal bioremediation through growing cells. Environment International 30**,** 261-278.

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

Marchant, R. Banat, I. M., 2012 Biosurfactants: a sustainable replacement for chemical surfactants? Biotechnology Letters 34**,** 1597-1605.

Marin, M., Garcia-Lechuz, J., Alonso, P., Villanueva, M., Alcala, L., Gimeno, M., Cercenado, E., Sanchez-Somolinos, M., Radice, C. Bouza, E., 2011 The role of universal 16S rRNA gene PCR and sequencing in the diagnosis of prosthetic joint infection. Journal of Clinical Microbiology 50**,** 583-589.

Marzan, L. W., Hossain, M., Mina, S. A., Akter, Y. Chowdhury, A. M. A., 2017 Isolation and biochemical characterization of heavy-metal resistant bacteria from tannery effluent in Chittagong city, Bangladesh: Bioremediation viewpoint. The Egyptian Journal of Aquatic Research 43**,** 65-74.

Mazaheri, A. M. Tabatabaee, M., 2010 Biosurfactants and their use in upgrading petroleum vacuum distillation residue: A review. International Journal of Environmental Research 4**,** 549-572.

Menz, F. C. Seip, H. M., 2004 Acid rain in Europe and the United States: an update. Environmental Science & Policy 7**,** 253-265.

Miller, R. M., 1995 Biosurfactant-facilitated remediation of metal-contaminated soils. Environmental Health Perspectives 103**,** 59-62.

Mishra, A. Malik, A., 2013 Recent advances in microbial metal bioaccumulation. Critical Reviews in Environmental Science and Technology 43**,** 1162-1222.

Mohanty, M., Pattnaik, M. M., Mishra, A. K. Patra, H. K., 2012 Bio-concentration of chromium—an in situ phytoremediation study at South Kaliapani chromite mining area of Orissa, India. Environmental Monitoring and Assessment 184**,** 1015-1024.

Mohite, B. V., Koli, S. H. Patil, S. V., 2018 Heavy Metal Stress and Its Consequences on Exopolysaccharide (EPS)-Producing *Pantoea agglomerans*. Applied Biochemistry and Biotechnology 186**,** 199-216.

More, T. T., Yadav, J. S. S., Yan, S., Tyagi, R. D. Surampalli, R. Y., 2014 Extracellular polymeric substances of bacteria and their potential environmental applications. Journal of Environmental Management 144**,** 1-25.

Morel, M. A., Ubalde, M. C., Olivera-Bravo, S., Callejas, C., Gill, P. R. Castro-Sowinski, S., 2009 Cellular and biochemical response to Cr (VI) in *Stenotrophomonas* sp. FEMS Microbiology Letters 291**,** 162-168.

Mosa, K. A., Saadoun, I., Kumar, K., Helmy, M. Dhankher, O. P., 2016 Potential biotechnological strategies for the cleanup of heavy metals and metalloids. Frontiers in Plant Science 7**,** 1-14.

Mukhopadhyay, S., Maiti, S. Masto, R., 2014 Development of mine soil quality index (MSQI) for evaluation of reclamation success: a chronosequence study. Ecological Engineering 71**,** 10-20.

Mukhopadhyay, S. Maiti, S. K., 2010 Phytoremediation of metal mine waste. Applied Ecology and Environmental Research 8**,** 207-222.

Mulligan, C., Yong, R. Gibbs, B., 2001a Surfactant-enhanced remediation of contaminated soil: a review. Engineering Geology 60**,** 371-380.

Mulligan, C. N., 2005 Environmental applications for biosurfactants. Environmental Pollution 133**,** 183-198.

Mulligan, C. N., Yong, R. N. Gibbs, B. F., 1999 Removal of heavy metals from contaminated soil and sediments using the biosurfactant surfactin. Journal of Soil Contamination 8**,** 231-254.

Mulligan, C. N., Yong, R. N. Gibbs, B. F., 2001b Heavy metal removal from sediments by biosurfactants. Journal of Hazardous Materials 85**,** 111-125.

Mupa, M., 2013 Lead content of lichens in metropolitan Harare, Zimbabwe: air quality and health risk implications. Greener Journal of Environmental Management and Public Safety 2**,** 75-82.

Nagajyoti, P., Lee, K. Sreekanth, T., 2010 Heavy metals, occurrence and toxicity for plants: a review. Environmental Chemistry Letters 8**,** 199-216.

Naik, M. M. Dubey, S. K., 2013 Lead resistant bacteria: Lead resistance mechanisms, their applications in lead bioremediation and biomonitoring. Ecotoxicology and Environmental Safety 98**,** 1-7.

Ndeddy Aka, R. J. Babalola, O. O., 2016 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*. International Journal of Phytoremediation 18**,** 200-209.

Ndeddy Aka, R. J. Babalola, O. O., 2017 Identification and characterization of Cr-, Cd-, and Ni-tolerant bacteria isolated from mine tailings. Bioremediation Journal 21**,** 1-19.

Nei, M. Kumar, S. 2000. Molecular evolution and phylogenetics: Oxford University Press.

Nies, A., Nies, D. H. Silver, S., 1990 Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. Journal of Biological Chemistry 265**,** 5648-5653.

Nies, D. H., Nies, A., Chu, L. 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 86**,** 7351-7355.

Nwodo, U. Okoh, A., 2013 Characterization and flocculation properties of biopolymeric flocculant (glycosaminoglycan) produced by *Cellulomonas* sp. Okoh. Journal of Applied Microbiology 114**,** 1325-1337.

Nwodo, U. U., Agunbiade, M. O., Green, E., Mabinya, L. V. Okoh, A. I., 2012 A Freshwater Streptomyces, isolated from Tyume River, produces a predominantly extracellular glycoprotein bioflocculant. International Journal of Molecular Sciences 13**,** 8679-8695.

Nwodo, U. U., Green, E., Mabinya, L. V., Okaiyeto, K., Rumbold, K., Obi, L. C. Okoh, A. I., 2014 Bioflocculant production by a consortium of *Streptomyces* and *Cellulomonas* species and media optimization via surface response model. Colloids and Surfaces B: Biointerfaces 116**,** 257-264.

Ochoa-Loza, F. J., Noordman, W. H., Jannsen, D. B., Brusseau, M. L. Maier, R. M., 2007 Effect of clays, metal oxides, and organic matter on rhamnolipid biosurfactant sorption by soil. Chemosphere 66**,** 1634-1642.

Okaiyeto, K., Nwodo, U. U., Mabinya, L. V. Okoh, A. I., 2013 Characterization of a bioflocculant produced by a consortium of *Halomonas* sp. Okoh and *Micrococcus* sp. Leo. International Journal of Environmental Research and Public Health 10**,** 5097-5110.

Okaiyeto, K., Nwodo, U. U., Mabinya, L. V., Okoli, A. S. Okoh, A. I., 2015 Characterization of a bioflocculant (MBF-UFH) produced by *Bacillus* sp. AEMREG7. International Journal of Molecular Sciences 16**,** 12986-13003.

Okaiyeto, K., Nwodo, U. U., Okoli, S. A., Mabinya, L. V. Okoh, A. I., 2016 Implications for public health demands alternatives to inorganic and synthetic flocculants: bioflocculants as important candidates. MicrobiologyOpen 5**,** 177-211.

Oladipo, O. G., Ezeokoli, O. T., Maboeta, M. S., Bezuidenhout, J. J., Tiedt, L. R., Jordaan, A. Bezuidenhout, C. C., 2018 Tolerance and growth kinetics of bacteria isolated from gold and gemstone mining sites in response to heavy metal concentrations. Journal of Environmental Management 212**,** 357-366.

Olaniran, A. O., Balgobind, A. Pillay, B., 2013 Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. International Journal of Molecular Sciences 14**,** 10197-10228.

Oliveira, A., Pampulha, M., Neto, M. Almeida, A., 2010 Mercury tolerant diazotrophic bacteria in a long-term contaminated soil. Geoderma 154**,** 359-363.

Olobatoke, R. Mathuthu, M., 2016 Heavy metal concentration in soil in the tailing dam vicinity of an old gold mine in Johannesburg, South Africa. Canadian Journal of Soil Science 96**,** 299-304.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J. Disz, T., 2014 The SEED and the rapid annotation of microbial genomes using subsystems technology (RAST). Nucleic Acids Res 42.

Pacwa-Płociniczak, M., Płaza, G. A., Piotrowska-Seget, Z. Cameotra, S. S., 2011 Environmental applications of biosurfactants: recent advances. International Journal of Molecular Sciences 12**,** 633-654.

Pal, A., Choudhuri, P., Dutta, S., Mukherjee, P. Paul, A., 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, P. Paul, A., 2005 Occurrence of heavy metal-resistance in microflora from serpentine soil of Andaman. Journal of Basic Microbiology 45**,** 207-218.

Pal, A. Paul, A., 2008 Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. Indian Journal of Microbiology 48**,** 49-64.

Park, D., Yun, Y.-S. Park, J. M., 2010 The past, present, and future trends of biosorption. Biotechnology and Bioprocess Engineering 15**,** 86-102.

Patel, K., Shrivas, K., Brandt, R., Jakubowski, N., Corns, W. Hoffmann, P., 2005 Arsenic contamination in water, soil, sediment and rice of central India. Environmental Geochemistry and Health 27**,** 131-145.

Pathak, M., Devi, A., Sarma, H. K. Lal, B., 2014 Application of bioflocculating property of *Pseudomonas aeruginosa* strain IASST201 in treatment of oil-field formation water. Journal of Basic Microbiology 54**,** 658-669.

Pathak, M., Sarma, H. K., Bhattacharyya, K. G., Subudhi, S., Bisht, V., Lal, B. Devi, A., 2017 Characterization of a novel polymeric bioflocculant produced from bacterial utilization of n-hexadecane and its application in removal of heavy metals. Frontiers in Microbiology 8**,** 170.

Pereira, J. F., Gudiña, E. J., Costa, R., Vitorino, R., Teixeira, J. A., Coutinho, J. A. Rodrigues, L. R., 2013 Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel 111**,** 259-268.

Pimentel, B. E., Moreno-Sánchez, R. Cervantes, C., 2002 Efflux of chromate by *Pseudomonas aeruginosa* cells expressing the ChrA protein. FEMS Microbiology Letters 212**,** 249-254.

Prabhu, S. Poulose, E. K., 2012 Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. International Nano Letters 2**,** 1-10.

Prabhu, S. G., Srinikethan, G. Hegde, S., 2018 Surface treated *Pteris vittata* L. pinnae powder used as an efficient biosorbent of Pb (II), Cd (II), and Cr (VI) from aqueous solution. International Journal of Phytoremediation 20**,** 947-956.

Puyen, Z. M., Villagrasa, E., Maldonado, J., Diestra, E., Esteve, I. Solé, A., 2012 Biosorption of lead and copper by heavy-metal tolerant *Micrococcus luteus* DE2008. Bioresource Technology 126**,** 233-237.

Qian, H., Peng, X., Han, X., Ren, J., Sun, L. Fu, Z., 2013 Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. Journal of Environmental Sciences 25**,** 1947-1956.

Quintelas, C., Sousa, E., Silva, F., Neto, S. Tavares, T., 2006 Competitive biosorption of ortho-cresol, phenol, chlorophenol and chromium (VI) from aqueous solution by a bacterial biofilm supported on granular activated carbon. Process Biochemistry 41**,** 2087-2091.

Rahman, Z. Singh, V. P., 2016 Assessment of heavy metal contamination and Hg-resistant bacteria in surface water from different regions of Delhi, India. Saudi Journal of Biological Sciences.

Raja, C. E., Anbazhagan, K. Selvam, G. S., 2006 Isolation and characterization of a metal-resistant *Pseudomonas aeruginosa* strain. World Journal of Microbiology and Biotechnology 22**,** 577-585.

Rajendran, P., Muthukrishnan, J. Gunasekaran, P., 2003 Microbes in heavy metal remediation. Indian Journal of Experimental Biology 41**,** 935-944.

Rajesh, N. Rajesh, V., 2016 Evaluation of the genetic basis of heavy metal resistance in an isolate from electronic industry effluent. Journal of Genetic Engineering and Biotechnology 14**,** 177-180.

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.

Rasko, D. A., Altherr, M. R., Han, C. S. Ravel, J., 2005 Genomics of the *Bacillus cereus* group of organisms. FEMS Microbiology Reviews 29**,** 303-329.

Rasmussen, L. D., Sørensen, S. J., Turner, R. R. Barkay, T., 2000 Application of a mer-lux biosensor for estimating bioavailable mercury in soil. Soil Biology and Biochemistry 32**,** 639-646.

Reeve, J. R., Schadt, C. W., Carpenter-Boggs, L., Kang, S., Zhou, J. Reganold, J. P., 2010 Effects of soil type and farm management on soil ecological functional genes and microbial activities. The ISME Journal 4**,** 1099.

Reith, F., Mcphail, D. Christy, A., 2005 *Bacillus cereus*, gold and associated elements in soil and other regolith samples from Tomakin Park Gold Mine in southeastern New South Wales, Australia. Journal of Geochemical Exploration 85**,** 81-98.

Rivardo, F., Turner, R., Allegrone, G., Ceri, H. Martinotti, M., 2009 Anti-adhesion activity of two biosurfactants produced by *Bacillus* spp. prevents biofilm formation of human bacterial pathogens. Applied Microbiology and Biotechnology 83**,** 541-553.

Rizzo, C., Michaud, L., Graziano, M., De Domenico, E., Syldatk, C., Hausmann, R. Giudice, A. L., 2015 Biosurfactant activity, heavy metal tolerance and characterization of *Joostella* strain A8 from the Mediterranean polychaete *Megalomma claparedei* (Gravier, 1906). Ecotoxicology 24**,** 1294-1304.

Romera, E., González, F., Ballester, A., Blázquez, M. Munoz, J., 2007 Comparative study of biosorption of heavy metals using different types of algae. Bioresource Technology 98**,** 3344-3353.

Rufino, R., Rodrigues, G., Campos-Takaki, G., Sarubbo, L. Ferreira, S., 2011 Application of a yeast biosurfactant in the removal of heavy metals and hydrophobic contaminant in a soil used as slurry barrier. Applied and Environmental Soil Science 2011**,** 939648.

Rufino, R. D., Luna, J. M., Campos-Takaki, G. M., Ferreira, S. R. Sarubbo, L. A., 2012 Application of the biosurfactant produced by *Candida lipolytica* in the remediation of heavy metals. Chemical Engineering 27**,** 61-66.

Sadhu, K., Adhikari, K. Gangopadhyay, A., 2012 Effect of mine spoil on native soil of Lower Gondwana coal fields: Raniganj coal mines areas, India. International Journal of Environmental Sciences 2**,** 1675-1687.

Saitou, N. Nei, M., 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4**,** 406-425.

Sajayan, A., Seghal Kiran, G., Priyadharshini, S., Poulose, N. Selvin, J., 2017 Revealing the ability of a novel polysaccharide bioflocculant in bioremediation of heavy metals sensed in a *Vibrio bioluminescence* reporter assay. Environmental Pollution 228**,** 118-127.

Salehizadeh, H. Shojaosadati, S. A., 2001 Extracellular biopolymeric flocculants: Recent trends and biotechnological importance. Biotechnology Advances 19**,** 371-385.

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

Salehizadeh, H. Yan, N., 2014 Recent advances in extracellular biopolymer flocculants. Biotechnology advances 32**,** 1506-1522.

Salem, H., E A, E. Farag, A. 2000 Heavy metals in drinking water & their environment impact on human health. *ICEHM 2000.* Cairo University, Egypt.

Sankarammal, M., Thatheyus, A. Ramya, D., 2014 Bioremoval of cadmium using *Pseudomonas fluorescens*. Open Journal of Water Pollution and Treatment 1**,** 92-100.

Sarubbo, L., Rocha Jr, R., Luna, J., Rufino, R., Santos, V. Banat, I., 2015 Some aspects of heavy metals contamination remediation and role of biosurfactants. Chemistry and Ecology 31**,** 707-723.

Sathiyanarayanan, G., Bhatia, S. K., Kim, H. J., Kim, J.-H., Jeon, J.-M., Kim, Y.-G., Park, S.-H., Lee, S. H., Lee, Y. K. Yang, Y.-H., 2016 Metal removal and reduction potential of an exopolysaccharide produced by Arctic psychrotrophic bacterium *Pseudomonas* sp. PAMC 28620. RSC Advances 6**,** 96870-96881.

Sathiyanarayanan, G., Kiran, G. S. Selvin, J., 2013 Synthesis of silver nanoparticles by polysaccharide bioflocculant produced from marine *Bacillus subtilis* MSBN17. Colloids and Surfaces B: Biointerfaces 102**,** 13-20.

Satpute, S., Bhawsar, B., Dhakephalkar, P. Chopade, B., 2008 Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian Journal of Marine Sciences 37**,** 243-250.

Saviour, M. N. Stalin, P., 2012 Soil and sand mining: causes, consequences and management. IOSR Journal of Pharmacy 2**,** 01-06.

Sebogodi, K. M. Babalola, O. O., 2011 Identification of soil bacteria from mining environments in Rustenburg, South Africa. Life Science Journal 8**,** 25-32.

Sharma, J. Fulekar, M., 2009 Potential of *Citrobacter freundii* for bioaccumulation of heavy metal–copper. Biology and Medicine 1**,** 7-14.

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

Sharma, S. Adholeya, A., 2011 Detoxification and accumulation of chromium from tannery effluent and spent chrome effluent by *Paecilomyces lilacinus* fungi. International Biodeterioration and Biodegradation 65**,** 309-317.

Shim, J., Babu, A. G., Velmurugan, P., Shea, P. J. Oh, B.-T., 2014 *Pseudomonas fluorescens* JH 70-4 promotes Pb stabilization and early seedling growth of Sudan grass in contaminated mining site soil. Environmental Technology 35**,** 2589-2596.

Shin, M.-N., Shim, J., You, Y., Myung, H., Bang, K.-S., Cho, M., Kamala-Kannan, S. Oh, B.-T., 2012 Characterization of lead resistant endophytic *Bacillus* sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator *Alnus firma*. Journal of Hazardous Materials 199**,** 314-320.

Siddiquee, S., Rovina, K., Azad, S., Naher, L. Suryani, S., 2015 Heavy metal contaminants removal from wastewater using the potential filamentous fungi bomass: a review. Journal of Microbial and Biochemical Technology 7**,** 384-393.

Singh, A. K. Cameotra, S. S., 2013 Efficiency of lipopeptide biosurfactants in removal of petroleum hydrocarbons and heavy metals from contaminated soil. Environmental Science and Pollution Research 20**,** 7367-7376.

Singh, B. K., Quince, C., Macdonald, C. A., Khachane, A., Thomas, N., Al-Soud, W. A., Sørensen, S. J., He, Z., White, D. Sinclair, A., 2014 Loss of microbial diversity in soils is coincident with reductions in some specialized functions. Environmental Microbiology 16**,** 2408-2420.

Singh, P. Cameotra, S. S., 2004 Enhancement of metal bioremediation by use of microbial surfactants. Biochemical and Biophysical Research Communications 319**,** 291-297.

Singh, R., Paul, D. Jain, R. K., 2006 Biofilms: implications in bioremediation. Trends in microbiology 14**,** 389-397.

Singh, S. K., Tripathi, V. R., Jain, R. K., Vikram, S. Garg, S. K., 2010 An antibiotic, heavy metal resistant and halotolerant *Bacillus cereus* SIU1 and its thermoalkaline protease. Microbial Cell Factories 9**,** 59.

Sinha, A., Pant, K. K. Khare, S. K., 2012 Studies on mercury bioremediation by alginate immobilized mercury tolerant *Bacillus cereus* cells. International Biodeterioration and Biodegradation 71**,** 1-8.

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

Sriram, M. I., Kalishwaralal, K., Deepak, V., Gracerosepat, R., Srisakthi, K. Gurunathan, S., 2011 Biofilm inhibition and antimicrobial action of lipopeptide biosurfactant produced by heavy metal tolerant strain *Bacillus cereus* NK1. Colloids and Surfaces B: Biointerfaces 85**,** 174-181.

Srivastava, S., Agrawal, S. Mondal, M., 2015 A review on progress of heavy metal removal using adsorbents of microbial and plant origin. Environmental Science and Pollution Research 22**,** 15386-15415.

Srivastava, S. Thakur, I. S., 2006 Isolation and process parameter optimization of *Aspergillus* sp. for removal of chromium from tannery effluent. Bioresource Technology 97**,** 1167-1173.

Su, C., 2014 A review on heavy metal contamination in the soil worldwide: situation, impact and remediation techniques. Environmental Skeptics and Critics 3**,** 24-38.

Subudhi, S., Batta, N., Pathak, M., Bisht, V., Devi, A., Lal, B. Al Khulifah, B., 2014 Bioflocculant production and biosorption of zinc and lead by a novel bacterial species, *Achromobacter* sp. TERI-IASST N, isolated from oil refinery waste. Chemosphere 113**,** 116-124.

Subudhi, S., Bisht, V., Batta, N., Pathak, M., Devi, A. Lal, B., 2016 Purification and characterization of exopolysaccharide bioflocculant produced by heavy metal resistant *Achromobacter xylosoxidans*. Carbohydrate Polymers 137**,** 441-451.

Sun, J., Zhang, X., Miao, X. Zhou, J., 2012 Preparation and characteristics of bioflocculants from excess biological sludge. Bioresource Technology 126**,** 362-366.

Tak, H. I., Ahmad, F. Babalola, O. O. 2013. Advances in the application of plant growth-promoting rhizobacteria in phytoremediation of heavy metals. *(In* Whitacre, D. M., *ed.* Reviews of environmental contamination and toxicology. Springer. p. 33-52).

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. Kumar, S., 2011 MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28.

Tang, W., Song, L., Li, D., Qiao, J., Zhao, T. Zhao, H., 2014 Production, characterization, and flocculation mechanism of cation independent, pH tolerant, and thermally stable bioflocculant from *Enterobacter* sp. ETH-2. PloS one 9**,** e114591.

Teclu, D., Tivchev, G., Laing, M. Wallis, M., 2008 Bioremoval of arsenic species from contaminated waters by sulphate-reducing bacteria. Water Research 42**,** 4885-4893.

Teitzel, G. M. Parsek, M. R., 2003 Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. Applied and Environmental Microbiology 69**,** 2313-2320.

Thavasi, R., Sharma, S. Jayalakshmi, S., 2011 Evaluation of screening methods for the isolation of biosurfactant producing marine bacteria. Journal of Petroleum & Environmental Biotechnology 1**,** 1-6.

Toribio-Jim, Enez, J., Rodr, A., Iguez-Barrera, M., Lucena, M. V., Flores, A. B., Segura, D., Wilson-Corral, V., Ictor, Alfaro, E. F. Romero, Y., 2014 Production of biosurfactants by bacteria isolated from a mine tailing zone in Southern Mexico and their resistance to heavy metals. African Journal of Bacteriology Research 6**,** 23-31.

Torrens, J. L., Herman, D. C. Miller-Maier, R. M., 1998 Biosurfactant (rhamnolipid) sorption and the impact on rhamnolipid-facilitated removal of cadmium from various soils under saturated flow conditions. Environmental science & technology 32**,** 776-781.

Tuleva, B., Christova, N., Jordanov, B., Nikolova-Damyanova, B. Petrov, P., 2005 Naphthalene degradation and biosurfactant activity by *Bacillus cereus* 28BN. Zeitschrift für Naturforschung C 60**,** 577-582.

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

Ungureanu, G., Santos, S., Boaventura, R. Botelho, C., 2015 Biosorption of antimony by brown algae *S. muticum* and *A. nodosum*. Environmental Engineering and Management Journal 14**,** 455-463.

Vaishnav, P. Demain, A. L., 2011 Unexpected applications of secondary metabolites. Biotechnology Advances 29**,** 223-229.

Valls, M. De Lorenzo, V., 2002 Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. FEMS Microbiology Reviews 26**,** 327-338.

Van Hamme, J. D., Singh, A. Ward, O. P., 2006 Physiological aspects: Part 1 in a series of papers devoted to surfactants in microbiology and biotechnology. Biotechnology Advances 24**,** 604-620.

Van Hullebusch, E. D., Zandvoort, M. H. Lens, P. N., 2003 Metal immobilisation by biofilms: mechanisms and analytical tools. Reviews in Environmental Science and Biotechnology 2**,** 9-33.

Violante, A., Cozzolino, V., Perelomov, L., Caporale, A. Pigna, M., 2010 Mobility and bioavailability of heavy metals and metalloids in soil environments. Journal of Soil Science and Plant Nutrition 10**,** 268-292.

Voica, D. M., Bartha, L., Banciu, H. L. Oren, A., 2016 Heavy metal resistance in halophilic bacteria and archaea. FEMS Microbiology Letters 363**,** fnw146.

Von Canstein, H., Kelly, S., Li, Y. Wagner-Döbler, I., 2002 Species diversity improves the efficiency of mercury-reducing biofilms under changing environmental conditions. Applied and Environmental Microbiology 68**,** 2829-2837.

Vu, B., Chen, M., Crawford, R. J. Ivanova, E. P., 2009 Bacterial extracellular polysaccharides involved in biofilm formation. Molecules 14**,** 2535-2554.

Wahl, J. J. 2014 *Soil ecological risk assessments of selected South African soils.* PhD, North-West University.

Wang, J. Chen, C., 2009 Biosorbents for heavy metals removal and their future. Biotechnology Advances 27**,** 195-226.

Wang, J., Feng, X., Anderson, C. W., Xing, Y. Shang, L., 2012 Remediation of mercury contaminated sites–a review. Journal of Hazardous Materials 221**,** 1-18.

Wang, L., Ma, F., Qu, Y., Sun, D., Li, A., Guo, J. Yu, B., 2011 Characterization of a compound bioflocculant produced by mixed culture of *Rhizobium radiobacter* F2 and *Bacillus sphaeicus* F6. World Journal of Microbiology and Biotechnology 27**,** 2559-2565.

Wang, S. Mulligan, C. N., 2009 Rhamnolipid biosurfactant-enhanced soil flushing for the removal of arsenic and heavy metals from mine tailings. Process Biochemistry 44**,** 296-301.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., Lee, S. Y., Fischbach, M. A., Müller, R. Wohlleben, W., 2015 antiSMASH 3.0—a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Research 43**,** W237-W243.

Wei, B. Yang, L., 2010 A review of heavy metal contaminations in urban soils, urban road dusts and agricultural soils from China. Microchemical Journal 94**,** 99-107.

Wei, G., Fan, L., Zhu, W., Fu, Y., Yu, J. Tang, M., 2009 Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of Lespedeza cuneata in gold mine tailings in China. Journal of Hazardous Materials 162**,** 50-56.

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

Wu, G., Kang, H., Zhang, X., Shao, H., Chu, L. Ruan, C., 2010a A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities. Journal of Hazardous Materials 174**,** 1-8.

Wu, H., Li, Q., Lu, R., Wang, Y., Zhuang, X. He, N., 2010b Fed-batch production of a bioflocculant from *Corynebacterium glutamicum*. Journal of Industrial Microbiology and Biotechnology 37**,** 1203-1209.

Wu, J.-Y. Ye, H.-F., 2007a Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtilis* DYU1 isolate. Process Biochemistry 42**,** 1114-1123.

Wu, J. Y. Ye, H. F., 2007b Characterization and flocculating properties of an extracellular biopolymer produced from a *Bacillus subtilis* DYU1 isolate. Process Biochemistry 42**,** 1114-1123.

Wu, W., Huang, H., Ling, Z., Yu, Z., Jiang, Y., Liu, P. Li, X., 2016 Genome sequencing reveals mechanisms for heavy metal resistance and polycyclic aromatic hydrocarbon degradation in *Delftia lacustris* strain LZ-C. Ecotoxicology 25**,** 234-247.

Wuana, R. A. Okieimen, F. E., 2011 Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. ISRN Ecology 2011**,** 1-20.

Xia, S., Zhang, Z., Wang, X., Yang, A., Chen, L., Zhao, J., Leonard, D. Jaffrezic-Renault, N., 2008 Production and characterization of a bioflocculant by *Proteus mirabilis* TJ-1. Bioresource Technology 99**,** 6520-6527.

Xie, Y., Fan, J., Zhu, W., Amombo, E., Lou, Y., Chen, L. Fu, J., 2016 Effect of heavy metals pollution on soil microbial diversity and bermudagrass genetic variation. Frontiers in Plant Science 7**,** 775.

Xiong, Y., Wang, Y., Yu, Y., Li, Q., Wang, H., Chen, R. He, N., 2010 Production and characterization of a novel bioflocculant from *Bacillus licheniformis*. Applied and Environmental Microbiology 76**,** 2778-2782.

Yalçın, S., Sezer, S. Apak, R., 2012 Characterization and lead (II), cadmium (II), nickel (II) biosorption of dried marine brown macro algae *Cystoseira barbata*. Environmental Science and Pollution Research 19**,** 3118-3125.

Yang, T., Chen, M.-L. Wang, J.-H., 2015 Genetic and chemical modification of cells for selective separation and analysis of heavy metals of biological or environmental significance. TrAC Trends in Analytical Chemistry 66**,** 90-102.

Yassir, B., Edine, K. M. Alain, P., 2015 Study of potential environmental risk of trace metallic elements in mine tailings: Case of Draa Lasfar functional mine in Marrakech-Morocco. African Journal of Agricultural Research 10**,** 3246-3252.

Yim, J. H., Kim, S. J., Ahn, S. H. Lee, H. K., 2007 Characterization of a novel bioflocculant, p-KG03, from a marine dinoflagellate, *Gyrodinium impudicum* KG03. Bioresource Technology 98**,** 361-367.

Yin, Y.-J., Tian, Z.-M., Tang, W., Li, L., Song, L.-Y. Mcelmurry, S. P., 2014 Production and characterization of high efficiency bioflocculant isolated from *Klebsiella* sp. ZZ-3. Bioresource Technology 171**,** 336-342.

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M. Mcinerney, M. J., 2004 Comparison of methods to detect biosurfactant production by diverse microorganisms. Journal of Microbiological Methods 56**,** 339-347.

Yuan, X., Meng, Y., Zeng, G., Fang, Y. Shi, J., 2008 Evaluation of tea-derived biosurfactant on removing heavy metal ions from dilute wastewater by ion flotation. Colloids and Surfaces A: Physicochemical and Engineering Aspects 317**,** 256-261.

Zampieri, B. D. B., Pinto, A. B., Schultz, L., De Oliveira, M. A. De Oliveira, A. J. F. C., 2016 Diversity and distribution of heavy metal-resistant bacteria in polluted sediments of the Araça Bay, São Sebastião (SP), and the relationship between heavy metals and organic matter concentrations. Microbial Ecology 72**,** 582-594.

Zhai, L.-F., Sun, M., Song, W. Wang, G., 2012 An integrated approach to optimize the conditioning chemicals for enhanced sludge conditioning in a pilot-scale sludge dewatering process. Bioresource Technology 121**,** 161-168.

Zhang, D., Pan, X., Mostofa, K. M., Chen, X., Mu, G., Wu, F., Liu, J., Song, W., Yang, J. Liu, Y., 2010 Complexation between Hg (II) and biofilm extracellular polymeric substances: an application of fluorescence spectroscopy. Journal of Hazardous Materials 175**,** 359-365.

Zhang, Y.-F., He, L.-Y., Chen, Z.-J., Zhang, W.-H., Wang, Q.-Y., Qian, M. Sheng, X.-F., 2011 Characterization of lead-resistant and ACC deaminase-producing endophytic bacteria and their potential in promoting lead accumulation of rape. Journal of Hazardous Materials 186**,** 1720-1725.

Zhang, Z.-Q., Bo, L., Xia, S.-Q., Wang, X.-J. Yang, A.-M., 2007 Production and application of a novel bioflocculant by multiple-microorganism consortia using brewery wastewater as carbon source. Journal of Environmental Sciences 19**,** 667-673.

Zhang, Z., Wu, C., Wu, Y. Hu, C., 2014 Comparison of coagulation performance and floc properties of a novel zirconium-glycine complex coagulant with traditional coagulants. Environmental Science and Pollution Research 21**,** 6632-6639.

Zou, C., Wang, M., Xing, Y., Lan, G., Ge, T., Yan, X. Gu, T., 2014 Characterization and optimization of biosurfactants produced by *Acinetobacter baylyi* ZJ2 isolated from crude oil-contaminated soil sample toward microbial enhanced oil recovery applications. Biochemical Engineering Journal 90**,** 49-58.

Zouboulis, A., Loukidou, M. Matis, K., 2004 Biosorption of toxic metals from aqueous solutions by bacteria strains isolated from metal-polluted soils. Process Biochemistry 39**,** 909-916.

APPENDICES

Appendix 1: Excerpt of research work presented at the ICIEM conference 2018

Biosurfactant production and heavy metal resistance of *Bacillus cereus* isolated from gold mining soil

Ayansina S. Ayangbenro and Olubukola O. Babalola
Food Security and Safety Niche, Faculty of Natural and Agricultural Sciences, North-West University, Mmabatho 2735, South Africa.
corresponding author e-mail (Olubukola.Babalola@nwu.ac.za)

ICIEM 2018

Poster ID : 128

Abstract

The effect of heavy metals on the growth and activity of biosurfactants produced by *Bacillus cereus* isolated from gold mining soil was investigated. The organism was screened for its ability to grow on different concentrations of heavy metals. Biosurfactant activity was first improved by evaluating the influence of abiotic parameters. The activity of biosurfactants was then evaluated in the presence of heavy metals Higher emulsification indices were achieved at 40 ˚C and pH 8 in mineral salt medium supplemented with glucose. Considerable surface tension reduction was recorded. The production of stable emulsion was observed in the presence of metals. These findings suggest that biosurfactant produced by *B. cereus* represent its adaptive strategy to defend its cells from a stress condition derived from heavy metals.

Introduction

The uncontrolled release of heavy metals into the environment from mining activities poses serious threat to the health of living organisms and the ecosystem. Conventional methods of heavy metal removal are often expensive and ineffective for low concentration removal [1]. Bioremediation offers an alternative and effective means of removal and recovery of heavy metals from polluted environment. Biosurfactant of microbial origin are good metal complexing agents and have been reported to be effective for metal remediation due to their stability, degradability, low toxicity and environmental compatibility [2]. They form stable complexes with metal ion as a result of electrostatic interaction between charged polymers. In this study, a biosurfactant producing *Bacillus cereus* was isolated from gold mining site in Vryburg, South Africa and its heavy metal resistant properties were determined.

Materials and Methods

Bacillus cereus isolation from soil samples collected from gold mine [3] and characterized using 16S rRNA gene. The isolate was screened for biosurfactant production (hemolysis, drop collapse, emulsification and surface tension). The resistance of the isolate to different concentration (100-1000 mg/L) of heavy metals (Cd, Cr, Pb). Metal sorption study was performed with 100 mg/L of each metal and optical density measured at 24 h interval for 144 h. The isolate was also screened for the presence of heavy metal resistant genes (*CzcA, CzcD, ChrA, ChrB, PbrA* and *PbrT*). The stability of biosurfactant produced was determined at different temperature, pH, and in the presence of metal ions by measuring the emulsification index [4].

Results

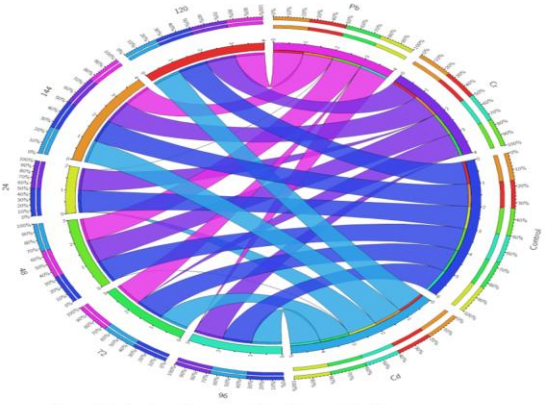


Figure 1: Evaluation of heavy metal resistance of *Bacillus cereus*

Results

Bacillus cereus is a Gram-positive, rod shaped, aerobic organism. The organism can grow in the presence of kerosene, hexadecane, engine oil and vegetable oil. The organism showed beta-hemolysis on blood agar and formed a stable emulsion with kerosene, engine oil and vegetable oil after 24 h (Table 1). *B. cereus* tolerated heavy metal in the order Cd>Cr>Pb. A lack of growth was observed at 200 mg/L and above for Cd and Cr while it tolerated all concentrations of Pb tested. Resistant genes *CzcD* and *PbrA* were present in the isolate.

Table 1: Evaluation of *B. cereus* for biosurfactant production

Hemolysis test	Drop collapse	Kerosene	Hexadecane	Engine oil	Vegetable oil	Surface tension
Positive	Positive	37.5%	22.4%	54%	24%	39.45 mN/m

B. cereus showed an OD of 1.24 after 144 h of incubation in the presence of 100 mg/L of Cd and 0.99 and 0.889 in the presence of Cr and Pb respectively (Figure 1). The optimum temperature for biosurfactant production was achieved at 40 ˚C with emulsification index (EI) of 30% (Figure 2) and the optimum pH was achieved at pH 8 with EI of 26.1% (Figure 5)

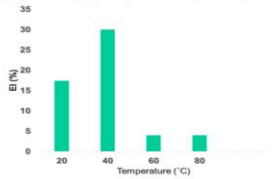


Figure 2: Effect of temperature on biosurfactant production by *B. cereus*

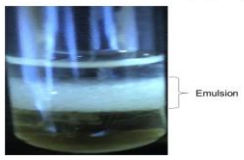


Figure 3: Emulsification of kerosene by *B. cereus*

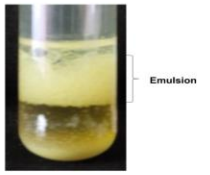


Figure 4: Emulsification of kerosene by *B. cereus* in the presence of heavy metals

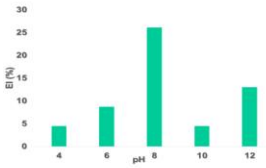


Figure 5: Effect of pH on biosurfactant production by *B. cereus*

The emulsification of kerosene by biosurfactant produced by *B. cereus* is presented in Figure 3. The EI showed 37.5% emulsification at 40 ˚C and pH 7.2. The EI of *B. cereus* biosurfactant in the presence of heavy metals (Cd, Cr, Pb) is 36% (Figure 4).

Discussion and Conclusions

- The result clearly showed the versatility of the isolate and its ability to tolerate higher concentration of heavy metals.
- Several parameters such as carbon source, pH and temperature influences biosurfactant production
- The biosurfactant produced can be utilized for effective removal and recovery of heavy metals from polluted environment.
- The sorption mechanism can be investigated to improve its potential application in metal removal and recovery.

References

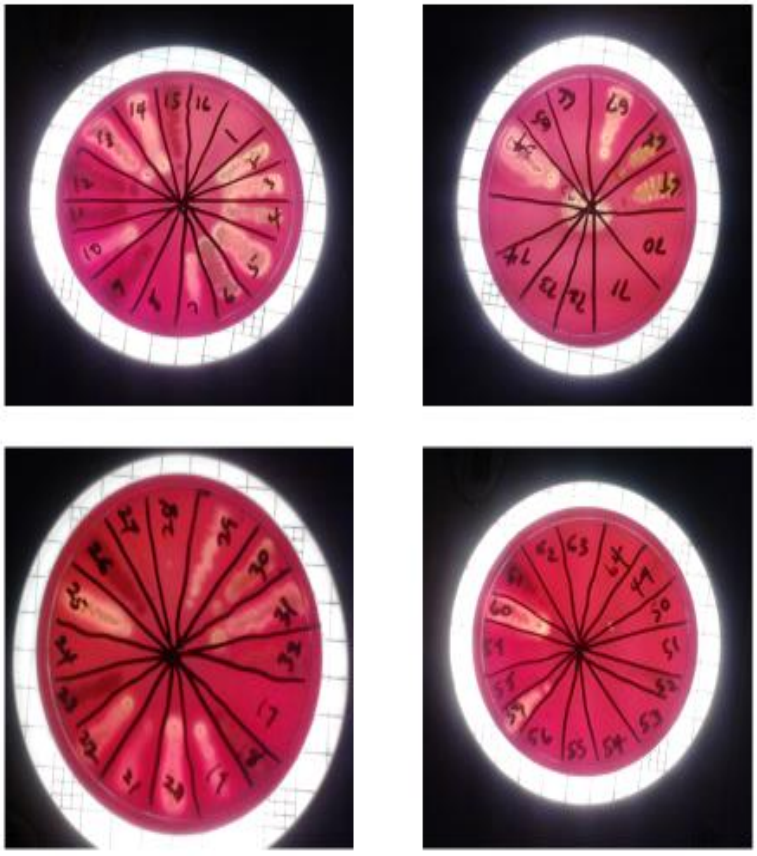
[1] Ayangbenro AS and Babalola OO (2017). Int. J. Environ. Res. Public Health 14, 94; doi:10.3390/ijerph14010094.
[2] Rizzo C et al. (2015). Ecotoxicology. 24, 1294-1304.
[3] Ndeddy Aka RJ and Babalola OO (2017). Bioremediat J. 21 doi: 10.1080/10889868.2017.1282933
[4] Mouafi FE et al. (2016). Biotechnol Rep. 9, 31-37.

Acknowledgements

National Research Foundation of South Africa (Grants 99779 and UID81192)

ICIEM 2018 - International Conference on Integrated Environmental Management for Sustainable Development www.iciem-conference.com

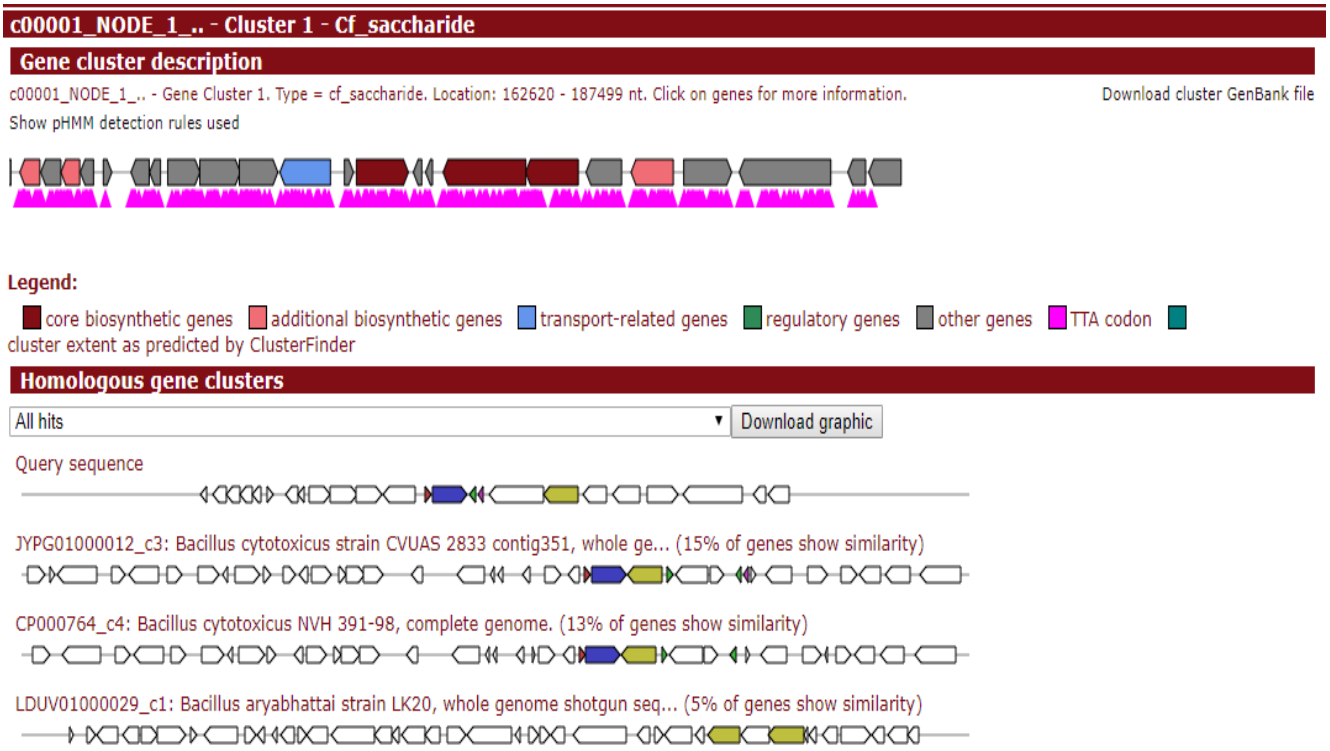
Appendix 2: Hemolysis of red blood cells by resistant bacteria isolates



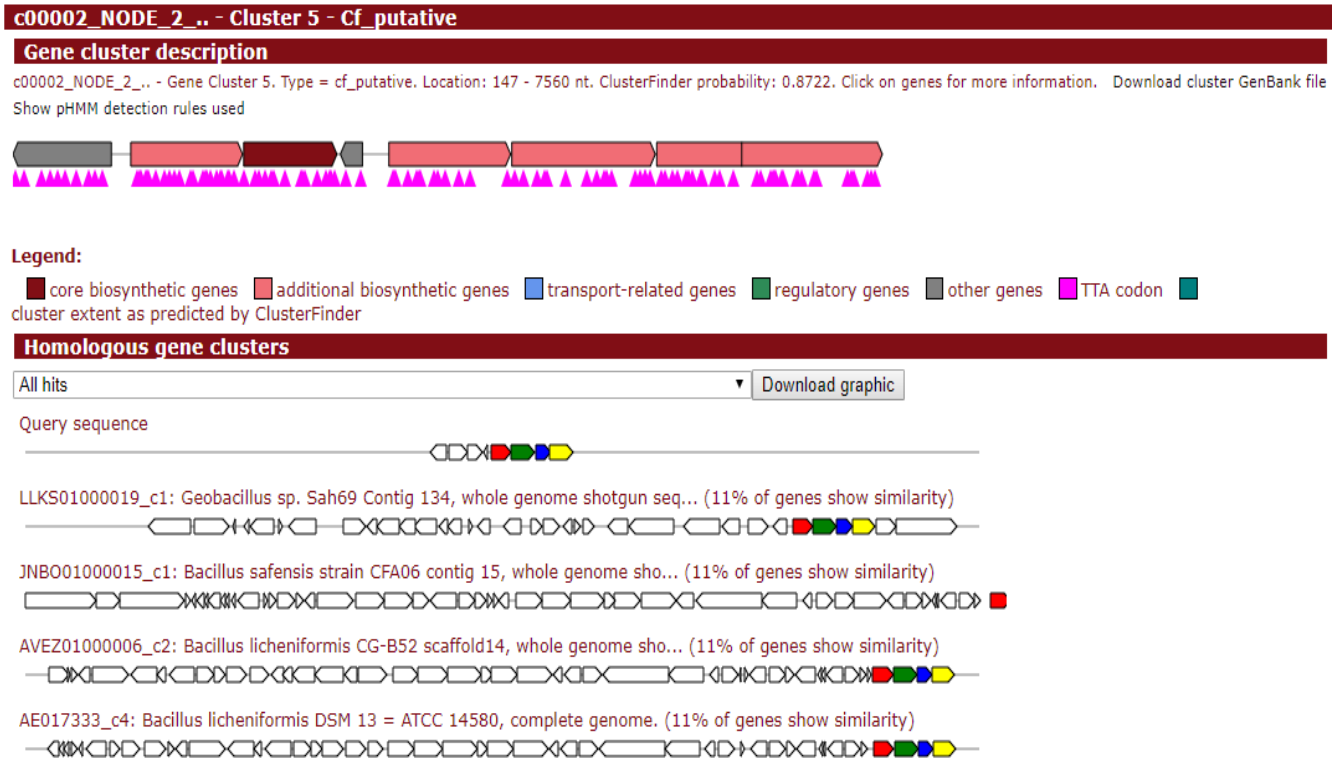
Appendix 3: Description and location of major BGC in *B. cereus* NWUAB01 identified *in silico*

Cluster ID	Type	Position/Region	Most Similar BGC predicted	Percentage similarity (%)
Cluster 1	Saccharide	162620-187499	Undefined	-
Cluster 5	Putative	145-7560	Citrulline	18
Cluster 7	Putative	91503-105724	Burkholderic acid	6
Cluster 11	Saccharide	221494-253173	S-layer glycan	9
Cluster 15	Siderophore	120072-133779	Petrobactin	100
Cluster 18	Lassopeptide	35492-57743	Undefined	-
Cluster 19	Fatty acid	175528-197730	Undefined	-
Cluster 20	Saccharide	208427-233412	S-layer glycan	19
Cluster 21	Bacteriocin	253699-267564	Undefined	-
Cluster 22	Putative	310537-317336	Polyhydroxyalkaoates	100
Cluster 25	Nrps	101717-148733	Undefined	
Cluster 26	Bacteriocin	166579-176845	Undefined	-
Cluster 28	Nrps	72712-122437	Bacillibactin	46
Cluster 36	Other	76321-119902	Undefined	-
Cluster 40	Terpene	139251-161104	Molybdenum cofactor	17
Cluster 41	Lantipeptide	1-20956	Undefined	-
Cluster 43	Saccharide-Nrps	3687-62177	Polyoxypeptin	5

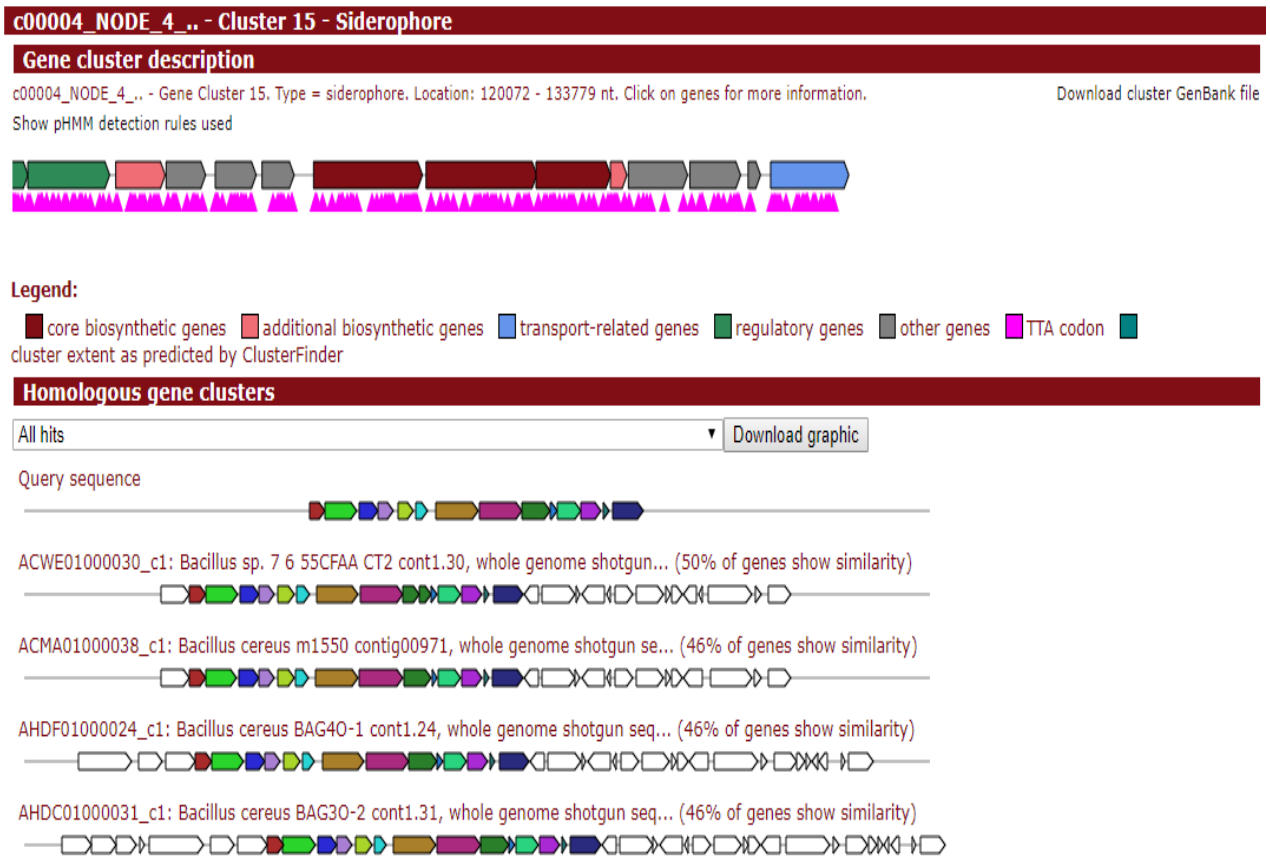
Appendix 4: Molecular annotation of the predicted clusters identified in the *Bacillus cereus* NWUAB01 genome



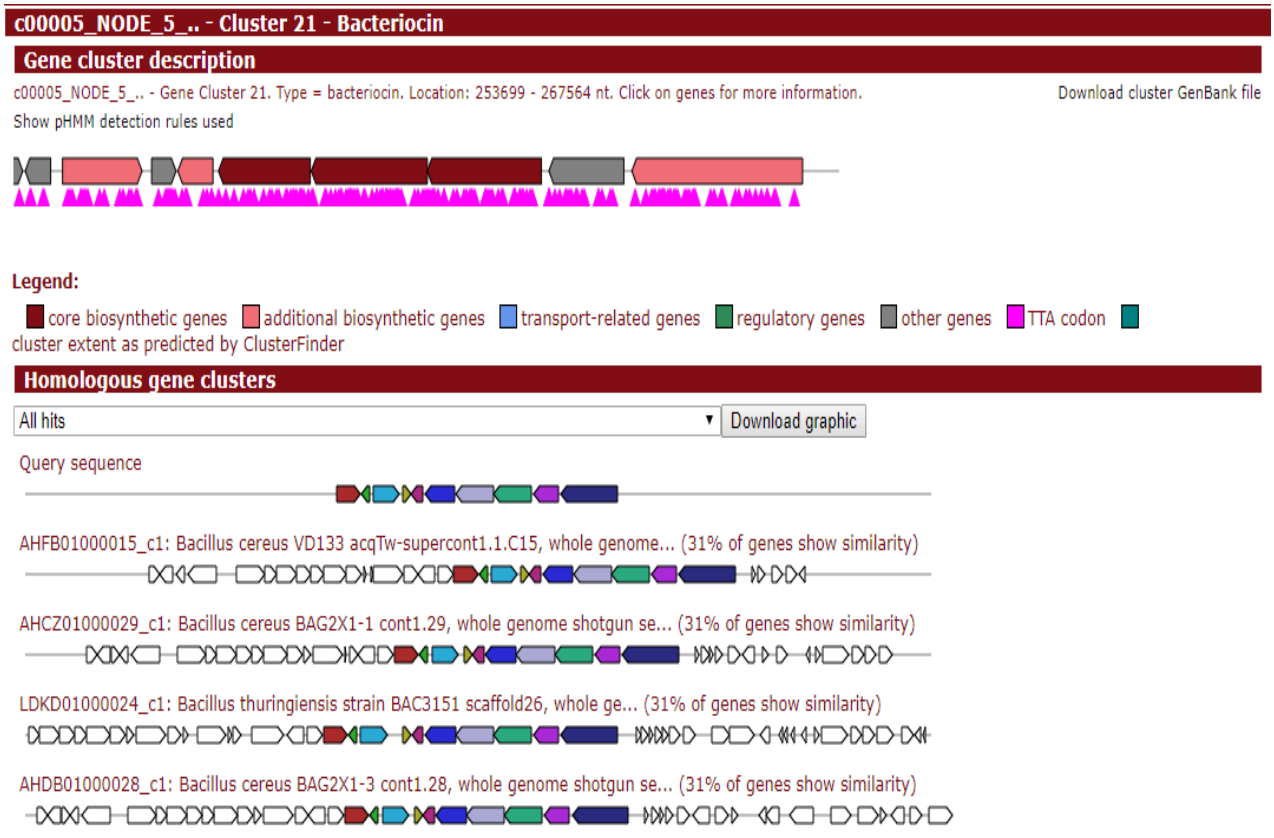
Cluster 1: saccharide annotation



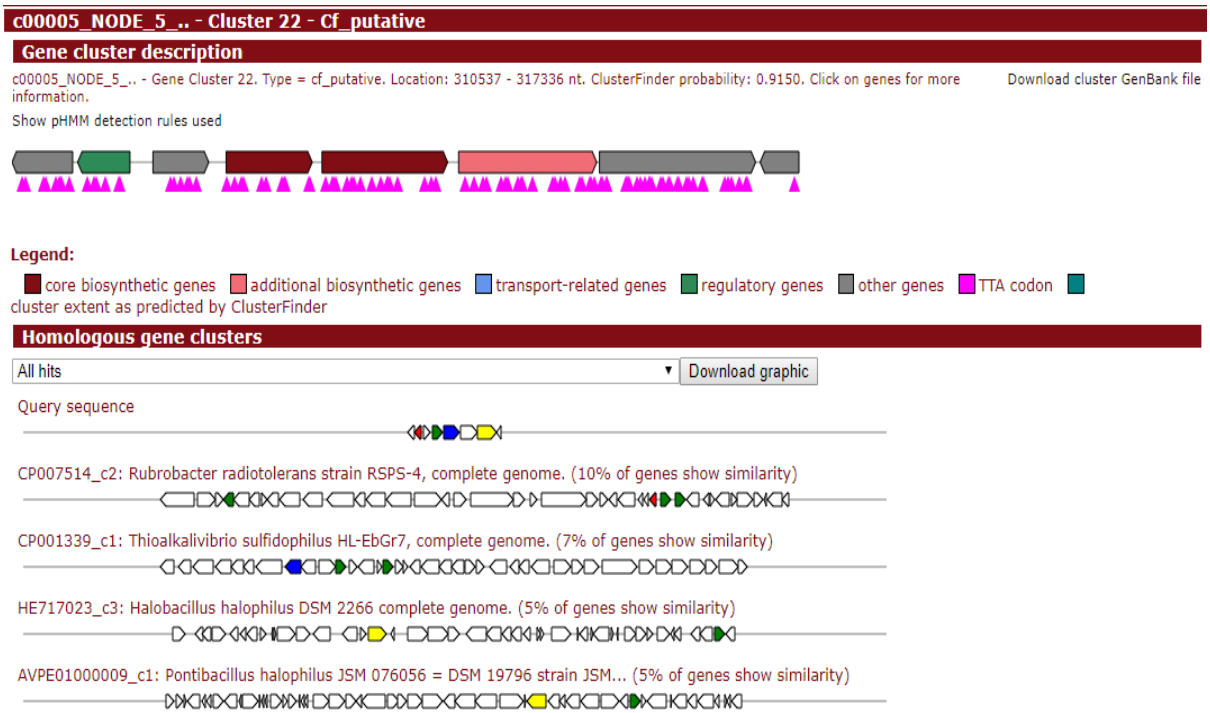
Cluster 2: putative annotation for predicted citrulline synthesis



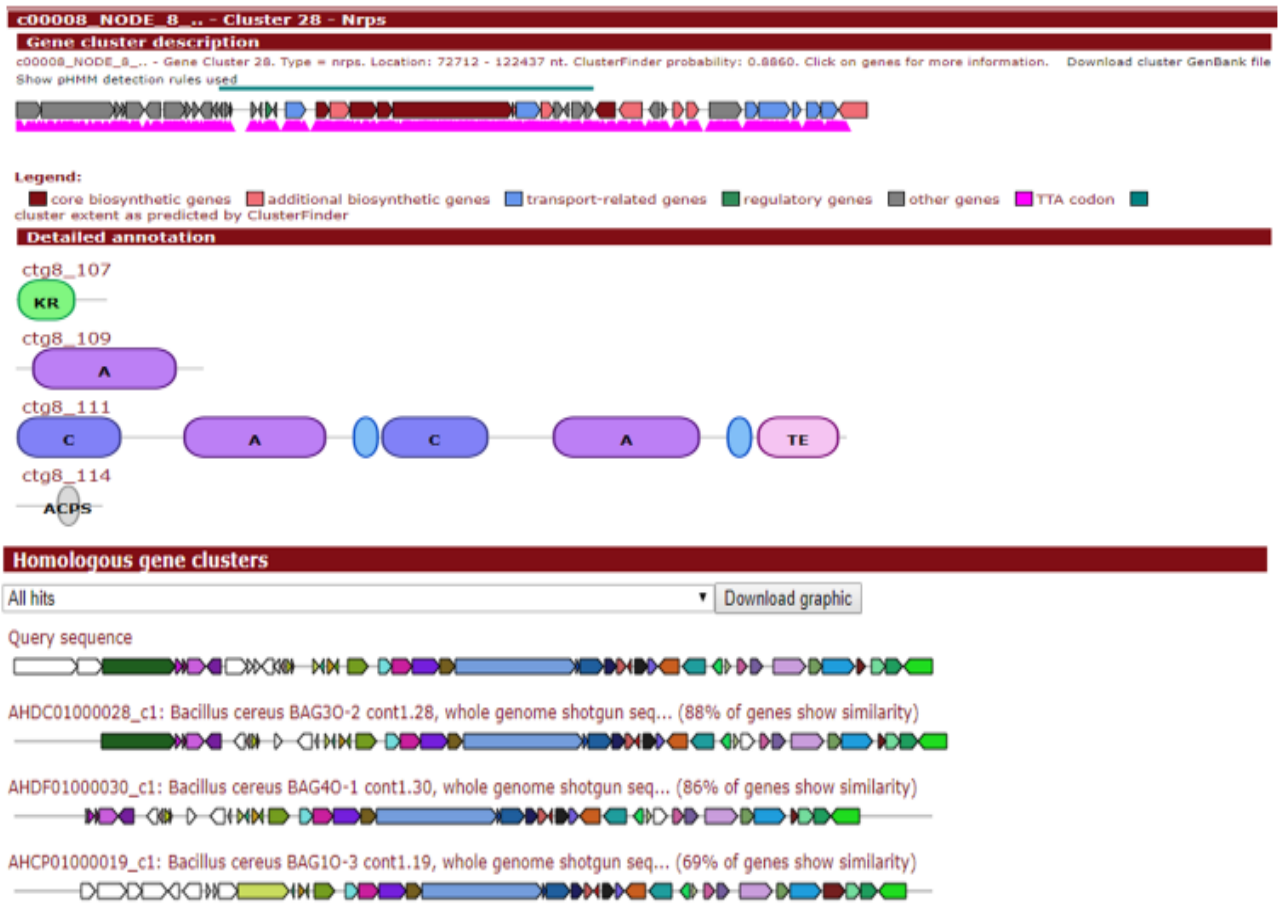
Cluster 15: siderophore annotation for petrobactin synthesis



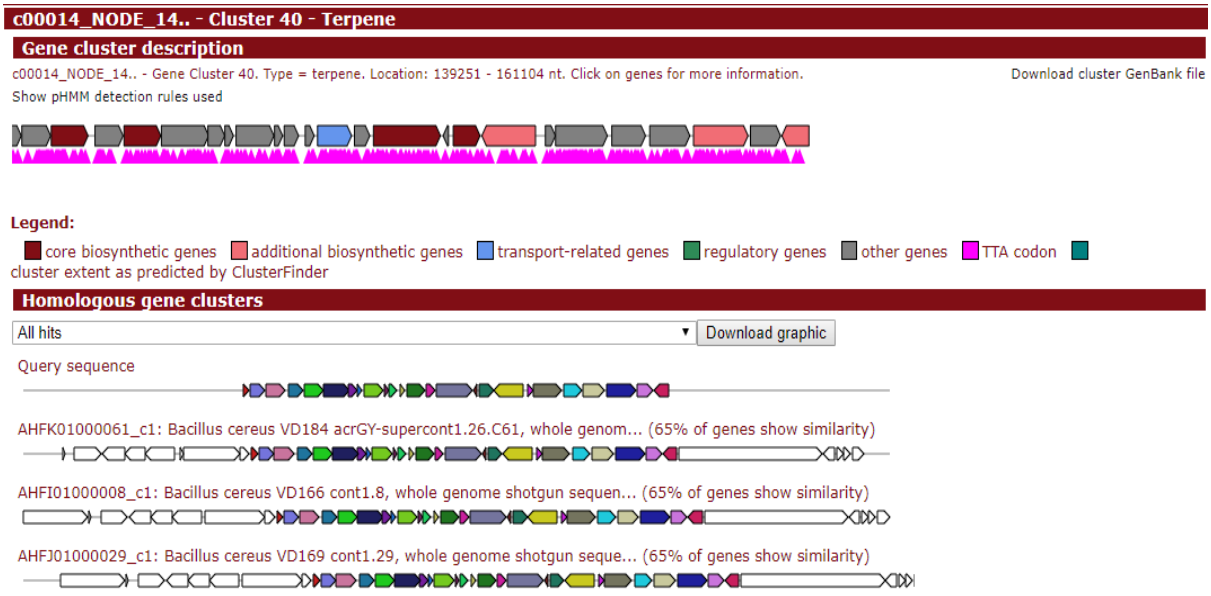
Cluster 21: bacteriocin annotation



Cluster 22: putative annotation for polyhdroxyalkanoates synthesis

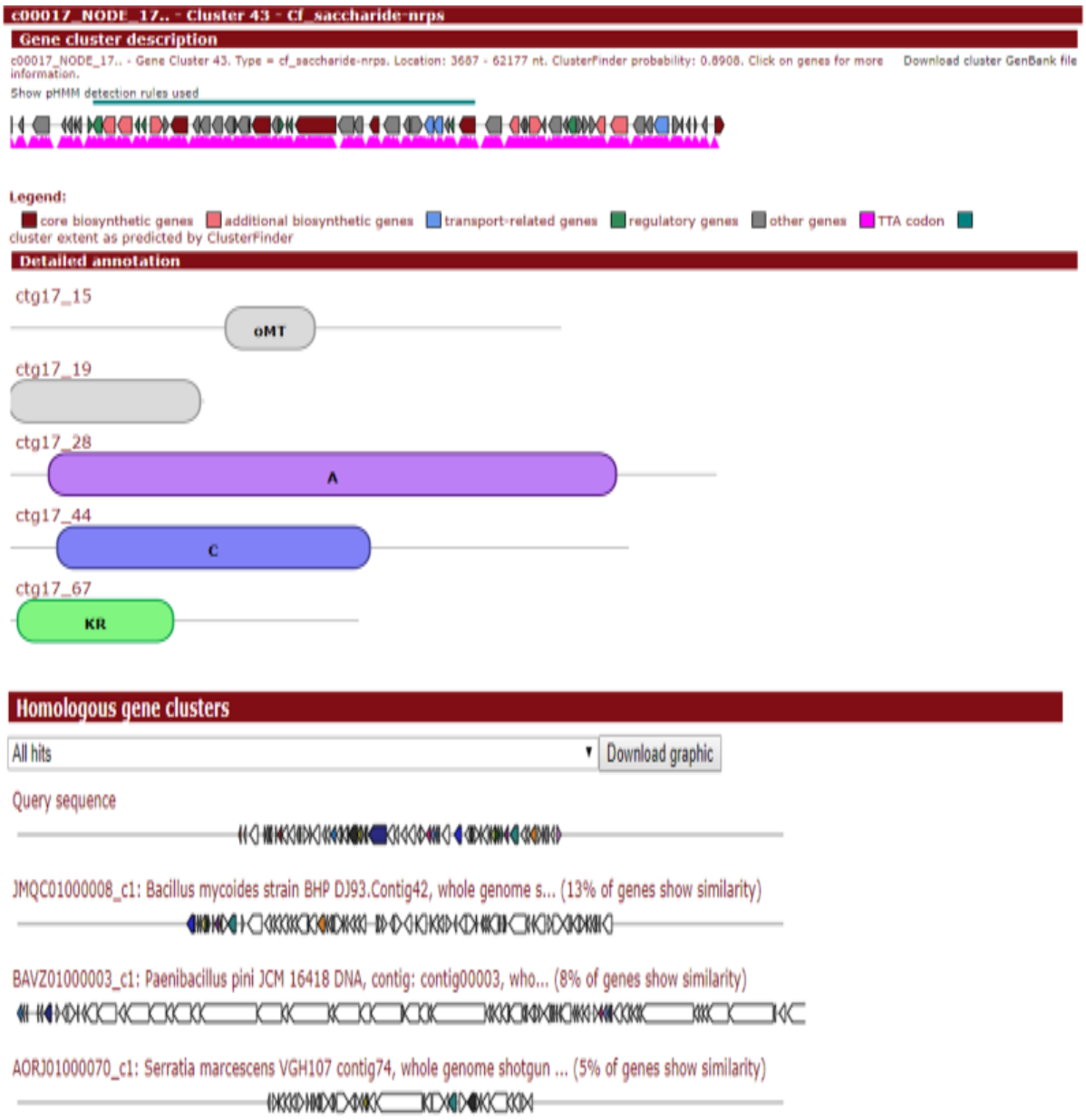


Cluster 28: Nrps annotation for bacillibactin synthesis



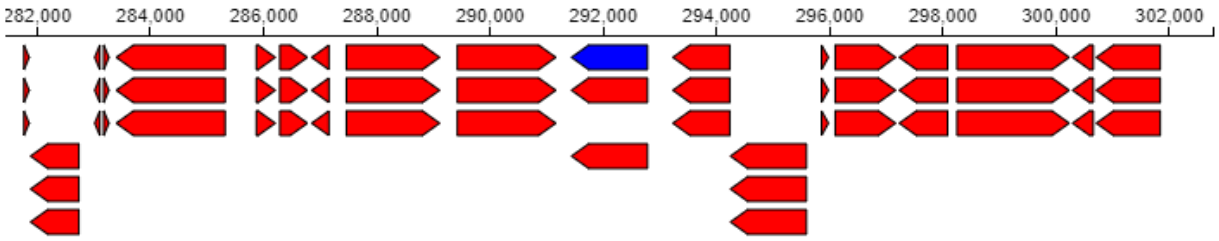
Cluster 40: terpene annotation for the synthesis of molybdenum cofactor

Cluster 41: lantipeptide annotation



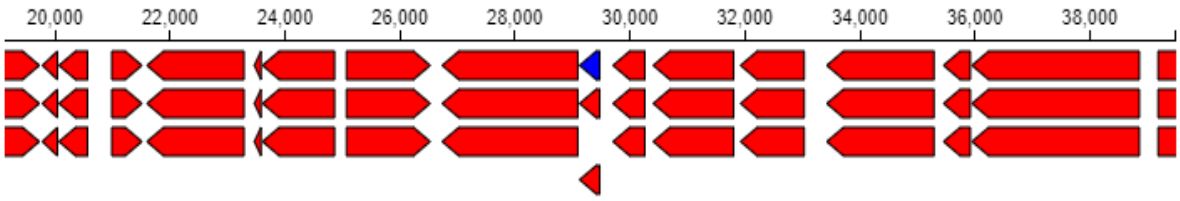
Cluster 43: saccharide-Nrps annotation for the synthesis of polyoxpeptin

Appendix 5: In silico annotation of heavy metals present in the genome of B. cereus NWUAB01 with their protein translations




```
  1:  MSIEIWITIIVFFLTMIVIFWRPRGLNEAWPAAIGAGIILITGLVSKPDV
 51:  IDIVSKIGGASITILATIVMAVILESFGFFHWSAAKLANLAKGSGRRLYW
101:  YIQLLCFLMTLLFNNDGSILITTPILILLLKNLQLKPHQQIPYLLSGALI
151:  ATASSAPIGVSNIVNLIALNIVNMTLYMHTAMMFVPATLGLLFMSWLMYT
201:  VLKKKLPKKLPVSSYDIEEIFFTKNFHPLKGNNSVDTKQKRTRFMLKVLG
251:  FVFLMRCLLFVASFLSIPIEIVAVLGSLVLLIWRWYYLRTNPVDILKKTP
301:  WHILIFAFSMYVIIYGLHNAGLTAVLVQWLEPIVNQHLLYASFAMGGLVS
351:  ILSNVFNNHPALMIGTITLTEMGLDPVTLKTIYLANIIGSDIGSLLLPIG
401:  TLASLIWMYILKQNKIKVKWKDYLSVSLIVIPLTTVVTLFLLFYWVHLFF
451:  AL
```

Arsenic efflux pump protein

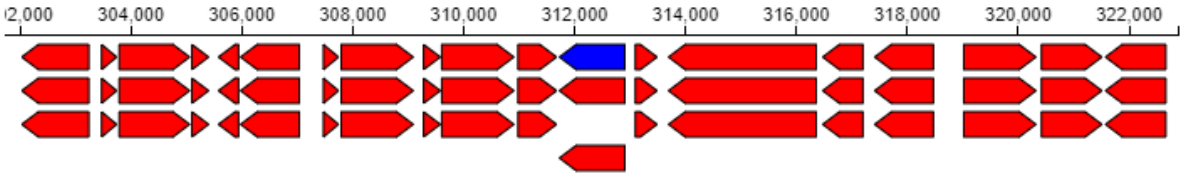



```
  1:  MAGNKVETPQETCSQTIIHEEVVEQVKQTIPTDESLSKVAELFKVLGDRT
 51:  RTRILHALFEAEMCVCDLAYLLGMTQSSISHQLRVLKQAKLVKNRKEGKV
101:  VYYSLADQHVIHIFEQAFEHVNEEE
```

Cadmium efflux system accessory protein

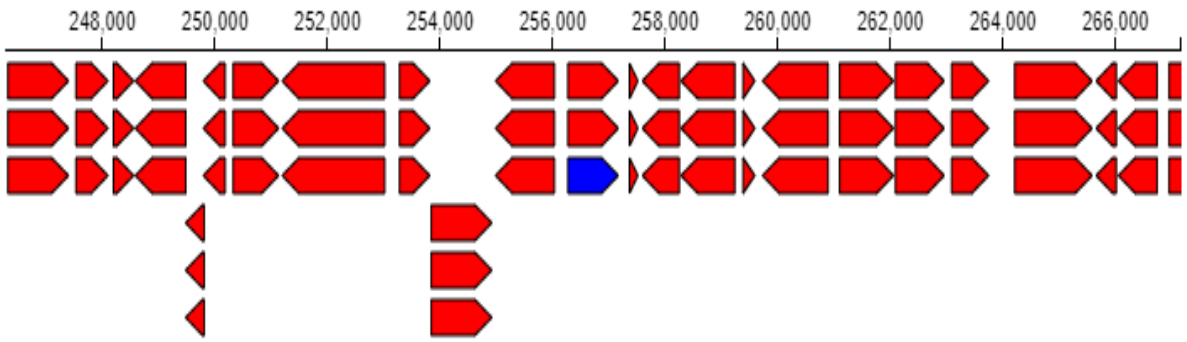

```
  1:  MNEKKEANLQISGMTCAACANRIEKGLKKVEGVHEANVNFALEKTKIMYD
 51:  PTKTNPQQFKEKVESLGYGIVSDKAEFTVSGMTCAACANRVEKRLNKLDG
101:  VNKATVNFALESATVDFNPNEVNVNEMKSAITKLGYKLEVKPDDQDASTD
151:  HRLQEIERQKKKFIISFILSFPLLWAMVSHFSFTSFIYLPDMLMNPWVQL
201:  ALATPVQFIIGGQFYVGAYKALRNKSANMDVLVALGTSAAYFYSVYLSIQ
251:  SIGSSKHMTDLYFETSAVLITLIILGKLFEAKAKGRSSEAIKKLMGLQAK
301:  TATVVRDGTEIKILIEEVVAGDIVYVKPGEKIPVDGEIVEGKSAIDESML
351:  TGESIPVDKSIGDVVIGSTINKNGFLKVKATKVGRDTALAQIIKVVEEAQ
401:  GSKAPIQRVADQISGIFVPVVVVIAIITFAVWMIFVTPGDFGGALEKMIA
451:  VLVIACPCALGLATPTSIMAGSGRSAEYGILFKGGEHLEATHRLDTVILD
501:  KTGTVTNGKPVLTDVIVADRFNENELLRLVGAAERNSEHPLAEAIVEGIK
551:  EKKIDIPSSETFEAIPGFGIESVVEGKHLLIGTRRLMKKFNIDIEEVSKS
601:  MEALEREGKTAMLIAIDKEYAGIVAVADTVKDTSKAAIARLKKMGLDVVM
651:  ITGDNTQTAQAIAKQVGIDHVIAEVLPEGKAEEVKKLQANGKKVAMVGDG
701:  INDAPALATANIGMAIGTGTDVAMEAADITLIRGDLNSIADAIFMSKMTI
751:  RNIKQNLFWALAYNALGIPIAALGFLAPWVAGAAMAFSSVSVVLNALRLQ
801:  RVKLKP
```

Lead, cadmium, zinc and mercury transporting ATPase



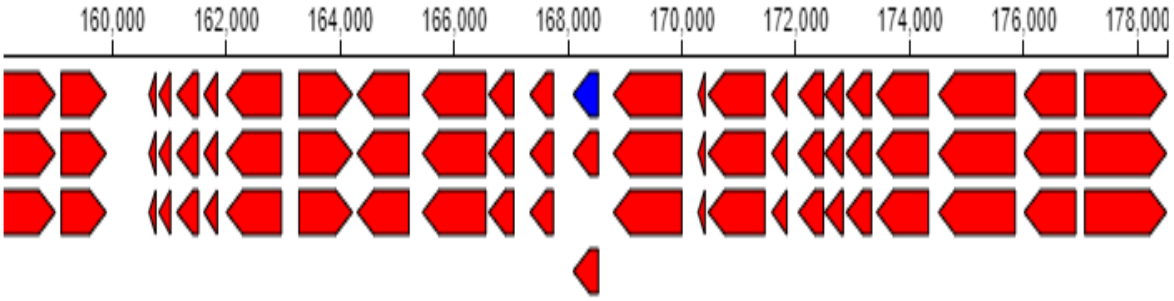

```
  1:  MKKNKHIFHTLLEIFLISFKLGLTSFGGPVAHLGYFHHEYVQKRKWMDER
 51:  SYGDLVALCQFLPGPASSQVGMGVGLLRGGLLGAIISWIGFTLPSVLVLV
101:  FFASFLNQFDLGSAGWIHGLKLVAVAIVAHAIWGMARKLTPDRNRATIAI
151:  VTAAIALLWPSSWTQVILIILCGFIGWFLYRNQPISQSQNIKVPISKKIA
201:  VSCLVLFFGLLLLLPILRPFSYYIALFDSFYRSGALVFGGGHVVLPLLEG
251:  EFVQNGMMTKEQFLAGYGLTQAVPGPLFTFASYIGAVLNGTLGATLATIA
301:  IFLPAFLLVIGVLPFWNSVRKISFIQGALLGVNAAVVGILIAAFYDPIWT
351:  STIINASDFVFASLLFCLLAFWKTPPWVIVILGAFGGYILSIL
```

Chromate transport protein



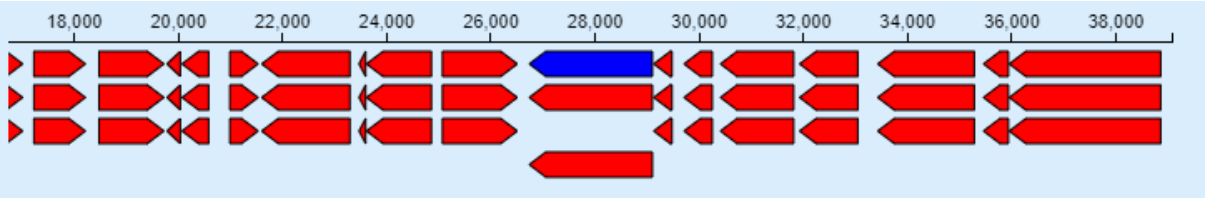

```
  1:  MGHSHDHGHSKNKKALLIAFLLTTSFMIAEVVGGFVTNSLALLSDAGHML
 51:  SDAVSLALSLLAFKLGEKTATTAKTYGYKRVEMLAALCNGVVLIVISVYI
101:  FIEAIRRFKEPVEIASNGMLIIAVLGLLINILSAWILMRGGDVKGNLNLR
151:  SAFLHVLGDLLGSVGAIIAALLIKFFGWTAADAIASILVSILVIISGWRV
201:  TRDTVHILMEGAPQHINVEEVKSTLLNIPIVKEVHDLHIWSVTSDFQVLT
251:  CHLIIKGNETQSVLKEATYVLKEKFHVEHVTIQVEIDGEFHHDETTCKV
```

Cobalt-zinc-cadmium resistance protein CzcD



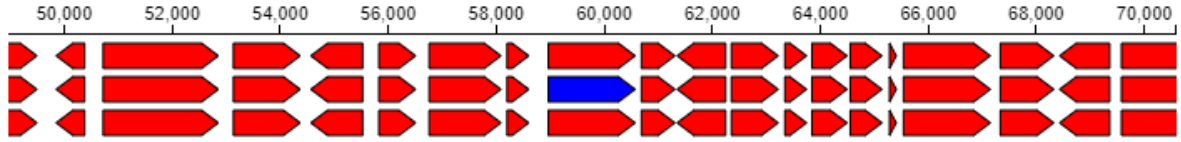

```
  1:  MITTIISSVVAFATTNIDDIFILLVLFSQVRTEGRAVREKAMRKKVYIVI
 51:  GQYVGFSMIIFLSIVGSLSSFFIPVSWIGLLGFVPIYMGIKGLFSIRSNK
101:  SNEVIDNTSESLFKVAAITLANGADNISIYIPMFASQSLEANIVTLMIFF
151:  P
```

Cadmium resistance transporter




```
  1: MAEALVKKKLMLEGLDCANCAMKIEKGVGNIEGVNSCSVNFATKTMILET
 51: AQNKENEVVTEAKQLVTKLEPHIKVQEEQKNKIAKEVFILEGLDCANCAM
101: KIENKVKEMPAVSEATVDFVSKKLRVEVANKRELEATVANITNVVQKLEP
151: DVKVVREEKNGHDHGHSHDHGEANVKKMVGRLVVGGILTAIAALAGLPQM
201: VTIPLFVLAYLLIGGDIVWRAVRNITRGQVFDENFLMAIATVGAFAIQQY
251: SEAVAVMLFYQVGELFQSIAVNRSRKSITSLMDIRPDYANVKVGNETKQV
301: SPEDVQIGDYIIVKPGEKVPLDGKVIEGTSMVDTSALTGESVPREVEVGN
351: DVLSGFVNQNGVLTIEVTKEFGESTVSKILDLVQNASSKKAPTENFITKF
401: ARYYTPVVVITAAIMAFIPPLILEGATFSEWIYRALVFLVISCPCALVVS
451: IPLGFFGGIGGASKSGVLVKGSNYLEALNDVKYIVFDKTGTLTKGVFKVT
501: KMEPSEGTTSEELLEYAAFAEVYSNHPIAQSIRKAYGKSIDEKIIDDYNE
551: ISGHGTVVKVQGKEIFAGNAKLMRKENIEFKQPETVGTLVHVAVDGRYAG
601: YIVISDEVKEDSKQAIQKLKELGIKKTVMLTGDAKPVGEAVGKELGLDEV
651: HAELLPQQKVEEIEKIDAAKHGKEKIAFVGDGINDTPVLARADVGIAMGG
701: LGSDAAIEAADIVIMTDEPSKIATAVKIAKRTRSIVWQNIIFALGVKGIV
751:             LLLGAFGIATMWEAVFSDVGVTLLAVLNAMRVLRVKDL
```

Cadmium-transporting ATPase




```
  1:   MRRLGTWLLLACVLIILIPKSASAHAYVVKSNPMENETLKRAPSVVKIEF
 51:   DEDIQVSRFNTLYVRDTSGKRVDLKDAHIDKKNKKLLEAGLKENLKNGLY
101:   SIQWKAISADGHPIQGVIPFRIGLAEAGTDDVQVEEMGYVPQIDMIMERG
151:   ILYASFSLFLGVLFFNLIMYKGNATSVRSRSKNIIWISLFGIFNSLLFNL
201:   PLQAKINADVSWLEAFNPLLLKETLQLSVFGYVWITQMVLISTLIIVTYF
251:   AVKHEKFSSFKVWSIPILLFIGLLVMKAFNSHAYGLKFKDIAVVMDFLHL
301:   FAASLWVGGLSSIILLLRKEDNKWHMYWDMIKRFSPWATGAVIVILITGL
351:   FNSTFFIPTIHSLFDTKYGLALLTKILLFIFMGILGIIHYMKGRMRAKQG
401:   LGATVKVEFIIGIIIFVIVAFMTNVQTPPMPPTGPFTESKQLDNGYEMTL
451:   NVSPNRVGQNIFHITLKDENGQPVTDMEQIILTTQSLDMNMGKGLFKVSA
501:   VSPGEYEAEGMYINMTGNWNIQVHGLTKSLDSFDTDYKFIVGGR
```

Copper resistance protein CopC / Copper resistance protein CopD

Appendix 6: Published review article from this study

frontiers in Microbiology

REVIEW
published: 22 August 2018
doi: 10.3389/fmicb.2018.01986

Sulfate-Reducing Bacteria as an Effective Tool for Sustainable Acid Mine Bioremediation

*Ayansina S. Ayangbenro, Oluwaseyi S. Olanrewaju and Olubukola O. Babalola**

Food Security and Safety Niche, Faculty of Natural and Agricultural Sciences, North-West University, Mmabatho, South Africa

Mining industries produce vast waste streams that pose severe environmental pollution challenge. Conventional techniques of treatment are usually inefficient and unsustainable. Biological technique employing the use of microorganisms is a competitive alternative to treat mine wastes and recover toxic heavy metals. Microorganisms are used to detoxify, extract or sequester pollutants from mine waste. Sulfate-reducing microorganisms play a vital role in the control and treatment of mine waste, generating alkalinity and neutralizing the acidic waste. The design of engineered sulfate-reducing bacteria (SRB) consortia will be an effective tool in optimizing degradation of acid mine tailings waste in industrial processes. The understanding of the complex functions of SRB consortia vis-à-vis the metabolic and physiological properties in industrial applications and their roles in interspecies interactions are discussed.

Keywords: bioleaching, heavy metals, microorganism, mine wastes, mining, tailings

OPEN ACCESS

Edited by:
Rajesh K. Sani, South Dakota School of Mines and Technology, United States

Reviewed by:
Sema Sevinc Sengor, Southern Methodist University, United States
Venkataramana Gadhamshetty, South Dakota School of Mines and Technology, United States

****Correspondence:***
Olubukola O. Babalola
Olubukola.Babalola@nwu.ac.za

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *12 March 2018*
Accepted: *07 August 2018*
Published: *22 August 2018*

Citation:
Ayangbenro AS, Olanrewaju OS and Babalola OO (2018) Sulfate-Reducing Bacteria as an Effective Tool for Sustainable Acid Mine Bioremediation. Front. Microbiol. 9:1986. doi: 10.3389/fmicb.2018.01986

INTRODUCTION

Urbanization and increase in the world's population, with an attendant rise in energy and mineral demands, have continued to drive mining activities. The goal of the mining industry is to meet the requirements for energy and mineral resources, enhance infrastructural development and quality of life of the populace. While mining and mineral extraction have contributed significantly to the advancement of human civilization and national economies, they have also caused serious environmental degradation. Mineral extraction and its resultant need for the disposal of wastes, slurry and water can have major environmental implications (Jain et al., 2016).

Mining and mineral processing activities generate massive amounts of toxic, corrosive, or flammable materials. Mining sites are surrounded by stockpiles of waste dumps, tailings ponds and processing chemicals. Mine wastes are by-products of mining operations which have no economic value. They consist of ash, flue dust, gangue, industrial minerals, loose sediment, metals, metallurgical slag and wastes, mill tailings, mineral fuels, ore, particulate emissions, processing chemicals and fluids, roasted ore and rock. It has been established that at least a ton of mining waste is generated for every ton of metal ore extracted. These waste streams demand informed planning and decision-making in matters of waste reduction, resource recovery, waste disposal and environmental protection (Hudson-Edwards et al., 2011). The release of these wastes into the environment can have significant impact on surface water, groundwater, air and land resources (**Figure 1**).

Striking features of mining industries are the incidents of poor waste management. Dam failures, seepages, tailings spills, unrehabilitated sites and instances of direct disposal into