Role of Metallo-resistant Microorganisms in Remediation of Heavy Metals using Biofilter

By

Kalsoom

Department of Microbiology
Faculty of Biological Sciences
Quaid-I-Azam University
Islamabad
2022

Role of Metallo-resistant Microorganisms in Remediation of Heavy Metals using Biofilter

A thesis submitted in partial fulfillment of the requirements for the

Degree of

Doctor of Philosophy

In

Microbiology

By

Kalsoom

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad

2022

In the name of Allah, the Most Gracious, the Most Merciful

To

My beloved parents, Sisters and brother for their love, support and prayers

Author's Declaration

I **Ms. Kalsoom** hereby state that my Ph.D. thesis titled **"Role of Metallo-Resistant Microorganisms in Remediation of Heavy Metals using Biofilter"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Ms. Kalsoom
Date: 07-12-2022

Plagiarism Undertaking

"Role of Metallo-Resistant Microorganisms in Remediation of Heavy Metals using Biofilter" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: _____

Name: **Ms. Kalsoom**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Role of Metallo-Resistant Microorganisms in Remediation of Heavy Metals using Biofilter"** was conducted by **Ms. Kalsoom** under the supervision of **Prof. Dr. Aamer Ali Shah.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Ms. Kalsoom** Signature:

Examination Committee:

a) External Examiner 1: Signature:

Prof. Dr. Azra Khanum
University Institute of Biochemistry & Biotechnology
PMAS Arid Agriculture University, Murree Road
Rawalpindi

b) External Examiner 2: Signature:

Dr. Jehangir Arshad Khan
House No. 68, Street No. 51
F-11/3, Islamabad

Supervisor Name: **Prof. Dr. Aamer Ali Shah** Signature:

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:

Contents

List of Tables

List of Figures

List of Acronym/abbreviations

˚C	Degree Celsius
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium chloride
HM	Heavy metal
Cr	Chromium
Pb	Lead
Cu	Copper
$CuSO_4$	Copper sulfate
EDX	Energy Dispersive X-ray Spectroscopy
DNA	Deoxyribonucleic acid
EPA	Environmental Protection Agency
EDTA	Ethylene diaminetetraacetic acid
ChR	Chromate Reductase Gene
HCl	Hydrochloric acid
IUBM	International Union of Biochemistry and Molecular Biology
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
LH	Light harvesting complex
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
$MgSO_4$	Magnesium sulfate
Na_2CO_3	Sodium carbonate
$Na_3C_6H_5O_7$	Tri-sodium citrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
$(NH4)_2SO_4$	Ammonium sulfate
nm	Nanometer
OD	Optical density
PDA	Potato Dextrose Agar
PEG	Polyethylene glycol
pH	Paviour of hydrogen
PK	Proteinase K
Rpm	Revolutions per minutes
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
TEMED	Tetramethylethylenediamine

ACKNOWLEDGEMENTS

Starting with millions of thanks to **ALLAH**, the Omnipotent and the Omniscient, who is so kind to mankind and who enabled me for successfully completing this research and bestowed upon me the wealth of such nice, supportive, understanding and cooperative family. All the respect for the last and **Holy Prophet, Hazrat Muhammad** (peace be upon him) who enlightened the world with the essence of faith in Allah, and guiding the mankind true.

I deem it a great honor and privilege to record my profound gratitude and indebtedness to my research supervisor and chairman **Professor Dr. Aamer Ali Shah,** Department of Microbiology, Quaid-i-Azam University, Islamabad, who has supported and encouraged me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I have no words to express my thanks to him for all of his kindness, constructive critical suggestions and consistent advice throughout the process in the incitation and completion of this thesis.

I wish to thank to the entire faculty and non-teaching staff at the Department of Microbiology, Quaid-i-Azam University, Islamabad. Words do not suffice for the ineffable gratitude I would like to extend to **Prof. Dr. Fariha Hasan** for her ever kind attitude, encouragement and guidance, which have been pivotal throughout the course of my Ph.D.

I take this opportunity to express my deep sense of gratitude to my foreign advisor **Prof. Dr. Ali Osman Belduz** and his research team (Karadenez Technical University) for his kind cooperation, inspiration and guidance.

Words are insufficient to express thanks to my Ph.D fellow **Mr. Salah Ud Din** whose constant presence during my research work made it possible for me to complete this hard job quite smartly. It was because of his inspiring guidance and dynamic cooperation during entire research work that makes it possible.

I would like to express my appreciations and sincere thanks to M. Phil Scholars especially **Afshan Batool, Nazia Shah** and **Amna Hanif** for their immense help and equally vital role that helped me to complete this project in due time.

I am extremely grateful to my father (**M. Ibrahim Khan**), who facilitated and encouraged me at every single step of my education.

I express my great gratitude and my deepest affection for my parents, sisters and brother, and my caring friends (**Johar Jamil** and **Mehnaz**) for their love, good wishes, inspiration and all those, whose hands always rose in prayer for me. May I fulfill their expectations (Ameen).

I express my gratitude and thanks to **Higher Education Commission (HEC)** of Pakistan for providing indigenous scholarship to financially support my Doctorate studies.

Finally, I express my gratitude and apology to all those who provided me the opportunity to achieve my endeavors but I missed to mention them personally.

KALSOOM

ABSTRACT

Summary

Heavy metals are non-degradable and highly toxic in low concentration. Heavy metals are intermittently released from industries into ground water and soil, leading to human and environmental health problems. It is evident from recent studies that heavy metal i.e. Chromium (Cr) and Lead (Pb) toxicity and concentration is increasing day by day due to increase in their use in different industries. Before it goes beyond control, there is an emergent need to develop effective strategies for control and removal of heavy metals (Cr and Pb) contamination. The aim of this study was to evaluate the role of metallo-resistant microorganisms in remediation of heavy metals such as Cr and Pb. Total of 53 strains were isolated from soil and sludge samples Korangi and Lyari, Karachi (24˚52′46.0″N 66˚59′25.7″E and 24˚48′37.5″N 67˚06′52.6″E) respectively. Out of these 53 strains, two of the bacterial isolates designated as S48 and S54 from Korangi sludge were selected on the basis of high metal tolerance against $K_2Cr_2O_7$(1500 mg/L) and $Pb(NO_3)_2$ (1200mg/L).The optimum temperature and pH for both the strains S48 and S54 were found as 30˚C and 35˚C and while their optimum pH was observed 6.0 and 7.0, respectively. Both biochemical and molecular basis of identification indicated that strain S48 was related to genus *Bacillus* with 99% similarity with *Bacillus paramycoides* whereas strain S54 to genus *Escherichia* with 99% similarity with *Escherichia fergusonii*. *Bacillus* sp. strain S48 can effectively reduce toxic Cr(VI) to less toxic Cr(III) and maximum reduction achieved was up to 65% after 96 hours. Similarly, *Escherichia* sp. strain S54 has proved Pb biosorption potential and can affectively remove 60% of Pb. Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDX) and Fourier-transform infrared (FT-IR) spectroscopy result showed clear morphological and functional group shifts on bacterial strains S48 and S54 cell surfaces treated with metals.

The enzyme chromate reductase activity was found during Cr reduction experiment with strain S48. The physicochemical variables influencing chromate reductase production were identified by statistical tools, Plackett-Burman Design (PBD) and Central Composite Design (CCD). Maximum production of chromium reductase was achieved at temperature 35°C and pH 7.0. The chromate reductase was purified to homogeneity by

size exclusion column chromatography with specific activity of 1416.5 U/mg, 59.5% yield, and 6.6-fold increase in purity. The maximum activity of chromate reductase was observed at temperature 40˚C and pH 7.0. The K_m and V_{max} values of enzyme were 1.36 µM and 909.09 µM respectively, using $K_2Cr_2O_7$ as a substrate. The activity of chromate reductase was strongly inhibited by Mercury (Hg), sodium dodecyl sulphate (SDS) and Cetyl-tri-methyl-ammonium Bromide (CTAB). *Bacillus* sp. strain S48 reduced 69% of chromium at concentration upto100 mg/L after 96 hrs. Furthermore, effluent containing 100 mg/L of $K_2Cr_2O_7$ was treated with 1-10% of both crude and purified chromate reductase. The crude and purified chromate reductase (10% v/v) showed maximum reduction of 72% and 78% after 120 hours, respectively.

Furthermore, the activity of Chromate reductase and Lead binding protein were enhanced by cloning and expression of gene for chromate reductase (ChrR) and Lead binding protein (PbR) into expression host. Both chromate reductase gene (*BparChR)* from *Bacillus* sp. strain S48 and Lead binding protein (*EferPbR*) from *Escherichia* sp. strain S54were successfully cloned into intracellular PET-28a and expressed into BL21 (DE3). Ni-Affinity ion exchange column chromatography was used for the purification of *BparChR* and *EferPbR* and fractions were collected for measuring enzyme activity. The purity and molecular size of protein was checked through SDS-PAGE and approximately 35 kDa size of chromate reductase (ChR) and 20 kDa of lead binding protein (PbR) were observed. Chromate reductase (ChR) exhibited specific activity of 1680 U/mg, 34.19% yield, and 5.73-fold increase in purity under optimum physicochemical conditions. The maximum chromate reductase activity was observed at temperature 35˚C and pH 7.0, while the activity was strongly inhibited by Hg^+. A Total of 90% chromium reduction was achieved after 96 hours by treating effluent with recombinant *BparChR.*

Biofilter was designed and operated with recombinant *PbREcoliBL21* strain carrying PbR from *Escherichia* sp. strain S54.Both the experiments, batch and continuous column results showed that recombinant *PbREcoliBL21*significantly improved the adsorption capacity of the ceramic balls. SEM results showed clear morphological changes on recombinant *PbREcoliBL21* cell surfaces treated with metals. FTIR spectra showed that *PbREcoliBL21* provides binding sites for the retention of the metal cations. A total of 95% biosorption in lead were achieved by *PbREcoliBL21* in fix bed biofilter. Finally, it is

concluded from the results that both chromium and lead-resistant bacteria can be effectively utilized in chromium and lead removal from contaminated industrial effluents. Biological based techniques using bacteria will help to provide a cheaper and environmentally friendly method for heavy metals removal, recovery and detoxification.

CHAPTER 1
INTRODUCTION

Introduction

Heavy metals (HMs) are toxic environmental pollutants and are present as natural constituents of the earth's surface. Heavy metal ions have densities greater than 5gm/cm^3 and they are five times denser than water which is highly lethal at very low concentrations. Metals such as lead, mercury, chromium, cadmium and arsenic are major environmental pollutants (Vardhan et al., 2019). Heavy metals i.e. Copper (Cu), Iron (Fe) and Zinc (Zn) exhibit a significant effect on plants and animals and are essential for their growth and metabolism such as chromium at low concentration enhances plant growth(Fu & Xi, 2020). Similarly, copper is important for the photosynthesis of higher plants and algae. It serves as an important co-factor for different oxidative stress-associated enzymes (Chrysargyris et al., 2019). Whereas some toxic heavy metals such as As, Hg and Pb have a detrimental effect on the kidney and central nervous system (CNS) in humans resulting in mental disorders, headache, weakness, anemia, abdominal cramps, and diarrhea (Efome et al., 2018).

Heavy metals enter the environment as a result of various natural and anthropogenic activities. Natural sources include rock weathering and abrasion, volcanic eruptions, the formation of aerosols, and forest fire. Sedimentary rocks and igneous rocks are minor sources of heavy metals. Whereas anthropogenic activities include burning of fossil fuel, smelting, mining, digging, utilization of fertilizers, manure of animals, pesticides, leatherworking, sewage sludge, and compost (Li et al., 2019).

Heavy metals have physical properties portraying them distinguishable from other metals. Mostly HMs are hard and cannot be easily bent or cut. Nevertheless, their melting point varies from low to high depending upon their atomic weight and number. Their tensile strength is mostly high but thermal expansion is usually low (Popovych et al., 2019). The heavy metals also constitute certain unique chemical characteristics; very less reactive, highly insoluble in both hydroxides and sulfides, give colored solutions with salts and form colored complexes (Li et al., 2019).

Heavy metals are generally classified as non-essential metals and essential. Essential heavy metals are micronutrients i.e. Molybdenum (Mo), Manganese (Mn), Cobalt (Co),

Copper (Cu), Nickel (Ni) and Iron (Fe) required in very low concentrations for cellular activities. Such as Zinc plays important role in the formation of carbohydrates and many oxidation processes in plants are catalyzed by zinc (Asati et al., 2016).Whereas high concentrations of essential HMs are as toxic as nonessential heavy metals. The enzymatic activities in soil are influenced by HMs by altering the microbial community which is responsible for enzyme synthesis (Gauthier et al., 2014).Nonessential HMs are highly lethal in trace amount, and induces oxidative stress in organisms. Contamination of HMs in aquatic bodies limited the diversity of aquatic organisms (Javed & Usmani, 2019). Accumulation of HMs in the body causes permanent damage to human health. Many ailments, for example, Menkes disease, Alzheimer's disease, and malignancies can be persuaded by the unnecessary consumption of HM ions (Bornhorst et al., 2017).

Heavy metals have both significant and detrimental impacts on the life of humans, plants, aquatic creature, and microorganisms depending on the concentration of heavy metals. High HMs concentration uptake by the plants and subsequent accumulation of HM in human tissue followed by the biomagnification via the food chain, pose a threat to both human health and the environment. For example, chromium enters the body through ingestion, breathing, or direct dermal contact. After entering the body, hexavalent chromium (Cr-VI) binds to double-stranded ribonucleotide, inhibiting normal physiological functions such as DNA repair. Hexavalent chromium cause Cr-DNA complex and DNA protein cross-links, which can block replication and transcription(Wang et al., 2017; Bhat, 2019), causing kidney dysfunction, lungs cancer, bronchitis, dermatitis, and asthma (Prasad et al., 2021). Similarly, lead (Pb) poisoning interferes and destroys enzymes of the body, hence disrupting its normal functions. Pb is a toxicant and accumulates over time to cause Edema, the leakage of the plasma membrane into interstitial fluids of the body from the blood-brain barrier. Acute levels of Pb metal cause headache, hallucination, loss of appetite, abdominal pain, hypertension, renal dysfunction, fatigue, sleeplessness, and in some cases arthritis (Boey & Ho, 2020).

The untreated effluents released from numerous industries like electroplating, electrolysis, smelting, and mining are increasing gradually and polluting the

environment. Therefore, there is an urgent need to remove and recover toxic metals from industrial wastes for the safety of the environment and ecosystem. HMs has been eliminated using a variety of conventional physicochemical methods from contaminated sites such as chemical precipitation, reverse osmosis, ion exchange, ultrafiltration, membrane filtration, sorption, and evaporation but, these approaches are incompetent as a result of high energy and power supplies, produce unwanted by-products, and unsuccessful to eradicate all-metal pollution (Singh et al., 2015). In comparison to other physiochemical methods, biological treatments are more economical, inexpensive, and eco-friendly due to their cost (Trellu et al., 2016). Microorganisms can live in heavy metal contaminated soil due to their ubiquitous existence and can convert heavy metals into non-toxic forms. CO_2 and H_2O are the end products of the bioremediation process of organic pollutants by microbes or organic pollutants are mineralized into metabolic intermediates, which are used as essential nutrients for bacterial cell growth (Verma & Kuila, 2019). Generally, heavy metals pollution is one of the highly considerable environmental problems. The level of chromium and lead in drinking water is increasing and it's above the permissible limit of 0.015 mg/L (EPA-US). Therefore, it is urgently required to reduce, remove and recover chromium and lead from industrial effluents for safety of human health and environmental protection. Many studies have shown that different strains isolated from the contaminated environment have greater efficacy towards metals and are highly considerable in bioremediation processes i.e. Biosorption, Bioaccumulation, Biotransformation, and enzymatic bioreduction can be effectively immobilized or convert the toxic form of metal into nontoxic form (Jacob et al., 2018).

Biosorption is a passive uptake, executed through both living cells and dead biomass as passive uptake via surface complexation (Fomina & Gadd, 2014). Bioaccumulation is active uptake using intracellular HMs accumulation including a range of physicochemical and biological methods. It incorporates a mixture of exterior reactions, intracellular as well as extracellular precipitation and intracellular-complexation and extracellular complexation reactions (Fomina & Gadd, 2014). The intracellular mechanistic pathway inside the cytoplasm will sequester metals that cross the cell surface and prevent to acquire a lethal stage. Many microbes use cytosolic

polyphosphates, proteins rich in cysteine and sulfides to transform heavy metals (Sharma et al., 2018). Microorganisms have the ability of chemical modification or biotransformation which is used as a defense mechanism against stresses, the same mechanism microorganisms can use for the transformation of metals from lethal form to less destructive form (Liu et al., 2017). Bioreduction is carried out through different types of oxidoreductase enzymes such as Iron reductase, quin one reductases nitro reductase, Flavin reductases, and hydrogenases are involved in the tolerance mechanism of heavy metals chromium (Patra et al., 2010). These oxidoreductase enzymes are present either in the cell membrane or in the cytosol portion (Jobby et al., 2018).

The removal of heavy metals can be enhanced by molecular approaches, and thus the genetically modified microbes can be applied in sites with severe heavy metal contamination (Naik et al., 2013). Moreover, the bacteria can produce more metal-binding proteins and enzymes involved in heavy metal removal and increase heavy metal removal by 33% using genetically modified methods (Yin et al.,2019). Various studies have been repoted for removal of toxic metals by microbes using recombinant technology. Modification in the metal-binding proteins on the bacterial surfaces has proven to be best biological method for heavy metals removal.

In biofiltration technology, microbial biomass is fixed to the bedding material, whereas the treated fluid is mobile and passes through the filter. In biofilteration process microbes are immobilized onto membrane and contaminants containing effluents passed through it. The contaminants will bind to microbial surfaces and clean effluents passed to collection tank (Chen and Hoff, 2012). On biofilter application very few studies have been reported for the elimination of organic volatile compounds and certain heavy metals. Although, this method is not well documented in the field of toxic metal removal (Raghuvanshi and Babu, 2009; Majumder et al., 2016).

Research on the removal of different heavy metals by using microbes is currently limited to only screening and investigating their role in heavy metals removal i.e. *Serratia*sp.S2, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus*sp. SFC 500-1E

(Chihomvu et al., 2015; Zhou et al., 2017; Huang et al., 2016; Gan et al., 2018; Ontanon et al., 2018) and so on, but the molecular mechanisms behind the microbial potential of heavy metal resistance and reduction are less studied (Dong et al., 2018; Zhou et al., 2017; He et al., 2018). Furthermore, certain microorganisms cannot be directly used in the environment because they are pathogenic to humans. Bioengineering technology makes solutions for the treatment of heavy metal by using genetically modified bacteria.

Widespread use of heavy metals in industries has lead to increase the concentration of heavy metal in water, soil and atmosphere which further lead to severe health consequences. Less developed countries such as Pakistan, India and Bangladesh mostly have industrial set-up on sea sites and river banks in large cities, which are directly releasing metals into water resources. Many scientists are working on biological based technologies to find out cheaper and eco-friendly solution. However, major challenge faced by researchers is to incorporate most technical and economical processes of bioremediation into environmental technology. Previously more work was performed on the characterization of pure cultures of microorganisms and rarely focused on an efficient technology for complete reduction and removal of heavy metals.

Considering the above shortcomings, in this study we isolated metallo-resistant microorganisms from metal contaminated zones and focused on two bacterial strains genetically modified for bioremediation and biofiltration of chromium and lead.A total of 53 bacterial strains have been isolated and checked for different heavy metals resistance (Cr, Pb, Cu. Ni and Mn). Ten strains were selected on high metal tolerance ability, five strains were found resistant against chromium and five strains against lead. These highly resistant strains were further explored for different bioremediation studies. We investigated the role of chromium-resistant bacteria in bioreduction through chromate reductase enzyme and optimize fermentation conditions for high yield of reductase enzyme and applied this enzyme in bioreduction of chromium from effluents. We also setup experiments for biosorption and bioaccumulation of chromium and lead by metal resistant strains and results were analyzed through Atomic absorption spectroscopy, FTIR, SEM and TEM. We also cloned and expressed chromium and lead resistant genes from metallo-resistant strains into a host cell for acquiring high

efficiency and speedy removal of heavy metals. Biofilters based on genetically modified bacterial strains were developed and tested for their efficacy. It is evident from literature and recent studies that heavy metal toxicity and concentration is increasing day by day due increase in their use in different industries. Before its goes beyond control, there is an emergent need to develop effective strategies for control and removal of heavy metals contamination. This research design and outcome is a small step toward big problems caused by a release of heavy metals into the environment. This research will provide insights to the role of metallo-resistant microorganisms in the removal and reduction of heavy metals pollution. The operation of the continuous biofilter is a promising feasible technological solution to a serious environmental problem.

Hypothesis

Heavy metals are intermittently released from industries in form of effluents into water bodies and soil, posing threat to life. The industries need to be asked to develop regulations and ensure practical implementation of efficient and eco-friendly strategies for heavy metal removal. Heavy metals can be effectively removed using Metallo-resistant microorganisms isolated from the heavy metal contaminated site through different bioremediation mechanisms.

Research Questions

1. Can I isolate metallo-resistant bacteria from heavy metal contaminated sites?
2. Can these isolates be employed for biosorption and bioreduction of heavy metals?
3. Can chromate reductase enzyme prove to be an efficient tool in the reduction of heavy metals?
4. Can I improve the efficiency of heavy metal removal through cloning and expression of resistant genes into host cell?
5. Can these genetically modified bacteria be effectively employed in biofilters for heavy metals removal?

Aim and objectives

Aim

To evaluate the role of metallo-resistant microorganisms in remediation of heavy metals.

Objectives

1. Isolation and screening of metallo-resistant microorganisms from industrial waste.
2. Evaluation of selected bacteria for their bioremediation potential by optimization of process parameters.
3. Purification and functional characterization of chromate reductase for reduction of chromium.
4. Cloning and expression of ChR and PbR gene in microbial host.
5. Designing a lab scale biofilter and its performance assessment for lead removal using recombinant cell.

CHAPTER 2

REVIEW OF LITERATURE

Literature review

2.1 Heavy metals

Heavy metals (HMs) exist naturally in the earth's crust and have a high atomic density. Heavy metal ions have densities greater than 5gm/cm^{3}and are five times denser than water (Osman et al., 2019). Heavy metals are significant environmental contaminants and due to their high toxicity, they are becoming a serious problem for human life as well as for the environment. Heavy metals are categorized into two group i.e., non-essential and essential heavy metals. Essential heavy metals are recommended in minute concentration and are a part of metabolic activities. These include copper, zinc, manganese and iron (Fe). While other heavy metals like lead chromium, and cadmium are non-essential with no known biological significance and are lethal in trace amounts. Heavy metal toxicity can impair the functions of the lungs, brain, liver, kidneys, blood composition, and other organs, as well as reduce energy levels. Because of their long-term exposure, several metals and their compounds can cause cancer (Verma & Kuila, 2019). Heavy metals persist in soil and their continuous release can lead to an increase in HMs concentration in the environment (Lwin et al., 2018).

2.2 Heay metals sources

2.2.1 Natural sources

Various processes like weathering of rock and abrasion, volcanic eruptions, formation of aerosols, and fire forest contribute toward increasing the concentration of HMs in the soil. Drift deposits or rock outcroppings are the major natural source of HMs. The concentration and composition of HMs are affected by the type of rock and conditions of the environment, triggering the weathering process. The formation of soil takes place from muddy rocks and is considered a small source of HMs. Via various igneous rocks named augite, hornblende, and olivine add a considerable amount of Co, Mn, Cu, Ni, and Zn to the soil. High level of HMs such as Zn, Al, Pb, Mn, and Cu have been confirmed to be emitted by volcanoes. Forest fires and marine aerosols also have a maximum concentration of HM (Pandey & Tiwari, 2021)

2.2.2 Anthropogenic sources

Heavy metals have been introduced as a result of various anthropogenic activities like emission of wastes from metallurgical industries, metallurgy, electrolysis,

electroplating, metal mine tailings, utilization of organic and inorganic fertilizers, manure of animals, pesticides, leathern working, sewage sludge, compost, coal, corrosion, atmospheric deposition and combustion of fossil fuel residues (Edelstein & Ben-Hur, 2018).

2.2.2.1 Agricultural source

The utilization of fertilizers introduces high content of heavy metals in agricultural soils. The use of fungicides, pesticides, and irrigation from deep wells and canals are the major contributor to HMs contamination. Depending on the source, Cd, Ni, Cr, Zn, and Pb concentration varies in inorganic fertilizers, phosphate fertilizers, and fungicides. Due to the regular utilization of fertilizers, their accumulation in soil increases by a large amount (Qian et al., 2020). The main source of phosphate fertilizers is phosphate rocks. The ores of phosphate rocks contain HMs of varying concentrations. Superphosphate fertilizers are chemical fertilizers that contain heavy metals like mercury, chromium, lead, and cadmium. These heavy metals along with the fertilizers can be incorporated into the soil, where they are absorbed by plant tissue, causing serious health problems. For example, the toxicity of lead causes mental retardation, and inhibition of hemoglobin formation (Yang & Massey, 2019).

2.2.2.2 Waste water

Lead, cadmium, arsenic, chromium, mercury, zinc, nickel, and silver are the most common HMs present in wastewater. The discharge of these metals into water bodies causes a threat to environmental problems and also increases the cost of waste water treatment. The toxic and non-degradable nature of metals causes their persistence in wastewater. Effluents of waste water used for irrigation purposes have less concentration of heavy metals but expanded irrigation increases the concentration of metals in soil (Abd-Elwahed, 2018).

2.2.2.3 Mining of metals and industrial wastes

The metal ores are extracted and milled which results in large amount of metals in soil and also increases their concentration in soil. The intensive use of zinc and lead ores contaminates the soil, posing threat to the environment and human beings. Different techniques used in those areas are expensive and time-consuming and do not assess the soil's yield capacity. Industries manufacture various products like fertilizers, pesticides, utilization of oil-based stuff, tanning, and pharmaceutical facilities that are highly

inconsistent. Though some of these materials have agricultural benefits, most of them are hazardous due to the presence of HM contents (Punia, 2020).

2.2.2.4 Biosolids and manures

Biosolids are mainly organic matter that is recovered and recycled beneficially from sewage through the treatment of wastewater. Different types of biosolids e.g. livestock manure, compost, and sewage sludge in the soil increase the concentration of heavy metals like chromium, cadmium, copper, or lead in soil (Wang et al., 2011). The high content of Zn, Cd, As, and Cu is present in animal manure. As a consequence of long-term agricultural use, residues of HMs in manure may accumulate on the soil surface. Accumulation of HM could have a hazardous effect on soil fertility as well as product quality (Li et al., 2019).

2.2.2.5 Industrial source

Smelting, mining, recycling, and finishing metals are the industrial sources of heavy metals. The use of mercury in gold mining has been a major source of pollutants in the environment. The high-temperature processes in industries like casting and smelting release metals in the form of vapors. These vapor forms of HMs such as Cd, Zn, Sn, and Cu then react with water and form aerosols. These aerosols are then dispersed through wind and contaminate the environment (Nagajyoti et al., 2010).

2.3 Heavy Metals Toxicity

Different heavy metals i.e. chromium, lead, zinc, copper, etc. are abundantly found in polluted sites (Engwa et al., 2019). The chemical form of metals determines the accumulation and transference of metals in the surrounding environment. Initially, the metals are adsorbed by quick reaction but later on, the reaction becomes slow. As a result, various chemical forms of metals with varying bioavailability, toxicity, and mobility are redistributed in the environment (Rai, et al., 2019). Chromium, mercury, lead, cadmium, and arsenic are classified as priority metals due to their high toxicity; which has become a significant public health issue. Heavy metals that do not metabolize become toxic and accumulate after entering the body through water, air, or food. These metals are essential in a small amount for maintaining good health but become destructive when the concentration exceeds. The toxicity of these heavy metals can

cause damage to the kidney, lungs, brain, and other significant organs (Fig 2.1) (Bhat, 2019).

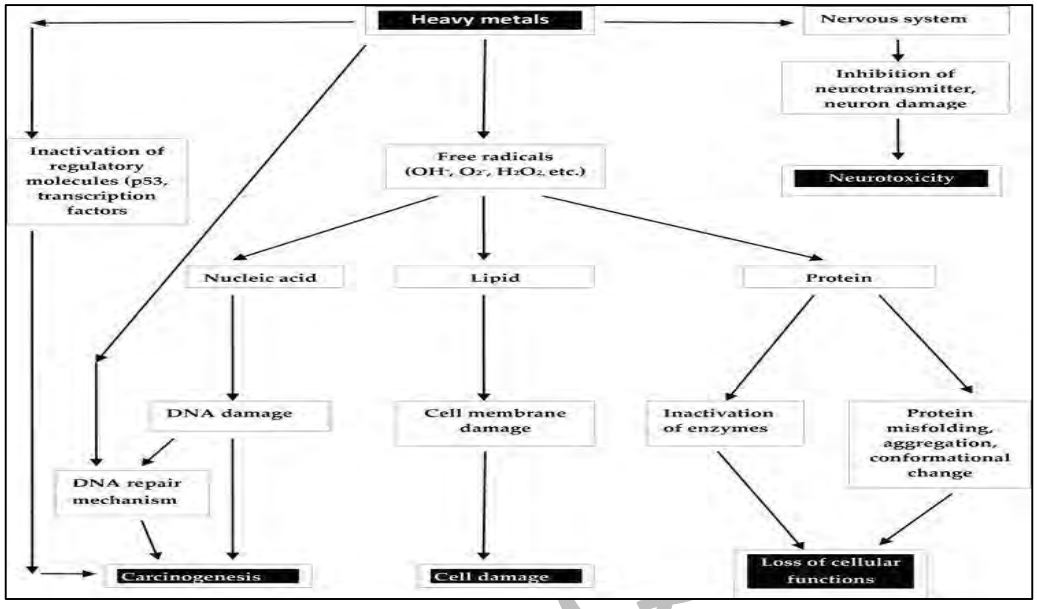


Fig 2.1 Diagrammatic representation of heavy metals toxicity

2.4 The Hazardous Effect of Heavy Metals

2.4.1 Effects on human health

Heavy metal ions can influence cellular organelles, and constituents, and stimulate oxidative pressure. Such as, arsenic can persuade DNA hypo-methylation and promote abnormal gene articulation (Phookphan et al., 2017). Chromium can cause DNA damage. Sister chromatid exchanges, alteration in transcription, and replication of DNA (Bhat, 2019). Human health is affected by mercury in a variety of ways. Excessive mercury exposure induces a variety of sicknesses such as renal system-related disease, allergies, nerves, and inability to speak (Azimi & Moghaddam, 2013). Cadmium exposure can cause damage to the liver, kidney, cardiovascular system, and skeleton system. Hearing and sight are also affected by exposure to cadmium (Genchi et al., 2020). Another highly toxic heavy metal is Pb, which is lethal even in trace amounts especially in children because exposure to Pb can adversely affect the mental development and behavior in children. It is also a cause of anemia in children. So, HM pollution has now turned out to be major ecological trouble and the treatment of dynamic HM ions is of prodigious significance (Fang et al., 2017).

2.4.2 Effects on plants

Plants require HMs for proper growth and development; these metals become toxic in excessive amounts. Chromium at low concentration enhances plant growth (Shanker et al., 2009), symptoms of chromium toxicity in plants comprise inhibition of seedling growth and root growth. High concentrations of mercury cause disarrangement of bio membrane lipids as well as cellular metabolism in a plant cell (Cargnelutti et al., 2006).The development of the plants in these heavy metal-polluted sites causes alteration in their metabolism, physiological and biochemical processes, which lead to the metal collection, reduce biomass production, and decline biomass development (Enniya et al., 2018).

2.4.3 Effects on aquatic life

Increased level of heavy metals in living organisms can have a detrimental impact on the health of aquatic animals and causes their population reduction (Luo et al., 2014). In fish, heavy metals are potent neurotoxins. Heavy metals interact with chemical stimuli in fish and may disrupt the fish's ability to communicate with their surroundings (Baatrup, 1991). Heavy metals deform fish in both natural and laboratory settings (Sfakianakis et al., 2015).

2.4.4 Effects on microorganisms

Heavy Metals uptake from soil influences microbial composition, differentiation, and metabolism. Several factors such as metal species, habitat, compositions, structure, and microbial functions influence the interaction of metals and their related compounds with soil microorganisms. Heavy metals like Cu, Zn, Co, and Fe are necessary for microbial growth. However, these metals become lethal, when concentration is exceeded (Oves et al.,2016). Heavy metals can cause a toxic impact on microbes by numerous significant methods that are responding as redox catalysts in the creation of ROS, enzymatic operations, breaking down ion regulation, and promptly influencing the creation of protein and DNA complex (Gauthier et al., 2014). Heavy metals halt important enzymatic activities by non-competitive or competitive relations with substrates, which affects enzyme configuration variations, modifies enzyme genetic factor articulation, and produces reactive oxygen species which might precede enzyme oxidation via producing carbonyl (Gauthier et al., 2014).

2.5 Biological Significance of Heavy Metals

Some heavy metals (Cu, Co, Fe, Ni, Mg, Zn and Cr) are important for growth, production and function of organic molecules i.e. Poly peptides, carbohydrates, chlorophyll and nucleic acids (Osman et al., 2019). Zinc is part of enzymes such as carbonic anhydrase and carboxypeptidase, which plays an important role in carbon dioxide regulation and protein digestion. In a trace amount, copper is a vital dietary nutrient. Copper is also a cofactor in the enzyme Cytochrome c oxidase and plays an important role in aerobic respiration (Osredkar, 2011). Many organs require an optimal balance of magnesium and vitamin D for proper physiological processes. Magnesium is required for the proper functioning of skeletal muscles, the heart, bones, teeth and other organs. Magnesium is also important for vitamin D activation. Vitamin D aids in the regulation of phosphate and calcium balance, which is necessary for bone health. Abnormally high quantities of either of these nutrients can cause organ damage (Uwitonze & Razzaque, 2018).

2.6 Heavy Metals treatment method

Different conventional physicochemical methods and biological methods have been used for heavy metals treatment from polluted sites so far; some of these methods are given below.

2.6.1 Chemical Methods

2.6.1.1 Chemical precipitation

Chemical precipitation method can significantly eliminate HMs from wastewater. In this method, chemicals bind to HMs dissolved in wastewater and developed insoluble deposits of HMs such as carbonates, sulfides, hydroxides, and phosphates. These precipitates are eliminated using the sedimentation process and the clean water is poured (Fu et al., 2011). There are many chemicals employed for chemical precipitation however the most well-known are calcium oxide and calcium hydroxide. Calcium oxide is more efficient but an extensively large quantity is needed for very low amount of heavy metal (Azimi et al., 2017).

2.6.1.2 Ion Exchange

Ion exchange is an efficient method for the elimination of heavy metals. In this method, reversible exchange of ions occurs between the liquid and solid phase in which the insoluble resin substance extracts ions from the electrolytic mixture and liberates other ions of analogous charge in an equal quantity without causing any structural change to the insoluble resin substance. This is an inexpensive method, it usually involves cost-effective constituents and appropriate processes, and it has proved to be very effective for eradicating HMs from aqueous mixtures, specific for handling water with little absorption of HMs (Dizge et al., 2009; Hamdaoui et al., 2009). This technique is extremely delicate with the pH of the aqueous stage. Certain disadvantages of the ion exchange process include a pretreatment system for the removal of suspended solids, and high operational cost (Grzegorzek et al., 2020).

2.6.1.3 Adsorption

Adsorption is a well-known procedure for the cleaning of water because it is an efficient, inexpensive, and biodegradable process. This method is strong enough to achieve water recycling requirements and high runoff norms in the industries. It is mainly a mass transfer method in which the metal ion is transported from the solution to the sorbent surface where it is physically and chemically bounded (Gupta et al., 2012). All adsorption methods are based on solid-liquid equilibrium and mass transfer rates. The advantage of adsorption includes low cost, high efficiency, and simple operation. Along with advantages, the drawbacks include high capital cost, and the spent absorbent is potentially hazardous waste (Tripathi et al., 2015).

2.6.2 Physical Methods

2.6.2.1 Membrane filtration

The membrane filtration process exhibits tremendous effects on the elimination of HM from the wastewater. Membrane filtration can remove organic compounds, suspended particles, and inorganic pollutants like heavy metals. The several categories of membranes which comprise reverse osmosis, ultra-filtration, and Nano-filtration were effectively used for the exclusion of poisonous metallic ions from the effluents based on the particle size that can be retained. The disadvantage of membrane filtration is

high equipment cost, reduction in permeate flux due to the membrane fouling effect, and high energy consumption (Gunatilake, 2015).

2.6.2.2 Ultrafiltration

The ultrafiltration (UF) method uses a porous membrane to detach HMs, high molecules, and suspended solids from a mineral mixture according to the size of the hole (5–20 nm) and molecular mass of detaching mixtures (1000-100,000Da) (Vigneswaran et al., 2004). This method can reach above 90% of exclusion proficiency with a metal absorption extending from 10 to 112 mg/L at pH varying from 5-9.5 and at 2–5 bar of pressure depending on the membrane features. This method has specific benefits for instance low energy consumption and a smaller area prerequisite because of its elevated compacting denseness. The disadvantage of ultrafiltration is membrane fouling, costly membranes, cannot remove dissolved solids from water (Crini & Lichtfouse, 2019).

2.6.2.3 Reverse Osmosis

It is a separation method, in which pressure is employed to make the polluted effluent endure the sheath that eliminates the pollutants to one end while allowing the solvent to pass through the other end. Their sheaths are semi-permeable allowing solvents to pass through but not metals. The sheaths frequently hold a dense barrier layer in the polymer medium where the majority of the separation procedures take place. This method can be utilized to eliminate many types of ions and molecules from several kinds of polluted H_2O, including manufacturing level uses. Though, this method involves diffusive processes; so, the effectiveness of the split-up is dependent greatly on water flux rate, pressure, and solute concentration (Gunatilake, 2015). Reverse Osmosis brine typically has huge quantities of extremely poisonous HMs (i.e. Mo, Cu, and Ni) and other less poisonous HMs (i.e. Zn and Fe) (Tran et al., 2012). Many scientists studied the effectiveness of the RO method for Copper elimination and noticed that extraordinary separation competence was accomplished in an assortment b/w 70–99.9% (Dialynas et al., 2009). The drawbacks of reverse osmosis are high energy consumption, the need for experienced personnel to run the process of reverse osmosis, flow rate decrease due to membrane fouling, and high operational cost (Crini & Lichtfouse, 2019).

2.6.3 Biological Methods

2.6.3.1 Bioremediation

The conversion of harmful and lethal substances to non-lethal substances by microbes is stated as bioremediation. In bioremediation, HMs polluted sites can be restored to their original state without harming the environment (Adhikari et al., 2004). Biological species like bacteria, yeast, fungi, and algae have shown adequate remediation capacities, but fungi and bacteria have mainly led to more effective remediation of heavy metals. Some of the merits of these techniques include the low requirement of energy, low operational expenses, high efficiency or performance, no health and environmental risk, and metal recovery (Garbisu et al., 2003). Microorganisms can live in heavy metal contaminated soil due to their ubiquitous existence and can convert heavy metals into non-toxic forms but can not be degraded. Whereas CO_2 and H_2O are the end products of the bioremediation process of organic pollutants by microbes or organic pollutants are mineralized to metabolic intermediates, which are used as essential nutrients for bacterial cell growth (Verma & Kuila, 2019). The chemical nature of the contaminant, nutritional state, moisture content of the pollutant, impurity structure, hydrogeology and pH of the soil, microbial consortium, and temperature are the essential parameters that affect the biological effectiveness of remediation. Microorganisms adopt various methods such as biosorption, bioleaching biotransformation, bioaccumulation, and bioreduction to convert the toxic form into a nontoxic form and survive in an HM-contaminated environment. Bioremediation was accepted as superior technology over traditional approaches to removing hexavalent Cr (VI) from the polluted environmental site (Jobby et al., 2016). Heavy metals like Cu, Zn, Co, and Fe are necessary for microbial growth and plays a vital role in microbes' metabolic processes and function as components of different enzymes (Yin et al., 2015; Oves et al.,2016)). The high concentration of these metals offers an adverse threat to microbes by hindering enzyme activity, damaging nucleic acid, and cell membranes, and distracting many cellular functions. Microbes adopted different strategies to interrelate and persist in the environment in the presence of metals like extrusion, exopolysaccharide production, enzyme detoxification, biotransformation, and production of metallothioneins (Dixit et al., 2015).

2.6.3.1.1 Biosorption

Biosorption of metals by microbes can be distinguished into metabolism-independent and dependent mechanism. Metabolism-independent biosorption occurs outside the cell and the mechanisms of this type of biosorption include physical adsorption, ion exchange, or precipitation, while metabolism-dependent biosorption includes chelation, oxidation-reduction reaction, or physical adsorption (Igiri et al., 2018). Living cells and dead biomass may carry out biosorption as passive uptake via surface complexation (Fomina & Gadd, 2014). Microbes' cell wall is charged negatively due to many anionic structures present on the cell's surface. The metal has been determined positively that allow microorganisms to form a chemical bond with metals. Phosphoryl, alcohol, hydroxyl, carboxyl, sulfonate, and thiol groups are the negatively charges sites in microbes that cause metal adsorption (Oves et al., 2016).

2.6.3.1.2 Intracellular Sequestration

The intracellular traps inside the cytoplasm will sequester metals that cross the cell surface and enter microbes, preventing them from gaining a lethal stage. As a result, the cellular parts that are sensitive can be shielded from heavy metal exposure. Many microbes use cytosolic polyphosphates, proteins rich in cysteine and sulfides, to transform heavy metals (Sharma et al., 2018).

2.6.3.1.3 Extracellular Sequestration

Extracellular Sequestration is the widely used process for removing heavy metals, several biological structures, such as siderophores, EPS, bio-surfactants, and glutathione, may immobilize heavy metals on the outer surfaces of the cell (Deschatre et al., 2013). EPS are important bio-sorbent for various metal ions, including chromium, silver, and copper, and can affect the metal distribution in microbes. As a result, EPS can shield microbes from heavy metals' toxic effects and be a helpful tool for bioremediation. Microbes that release glutathione have an excellent capability to bind metal ions. These metals form a complex with glutathione and cannot move into the bacterial cell. Furthermore, the microbial cell wall is also essential protection against the toxicity of metals. Cell walls consist of several anionic and cationic functional groups like carboxyl, amine, phosphate groups, or hydroxyl that could prevent metal ions from entering the cell via extracellular sequestration of heavy metals (Yin et al., 2016; Naik et al., 2013).

2.6.3.1.4 Bioaccumulation by Active Export

The efflux system can effectively control the accumulation of heavy metals inside the cell (Remenar et al., 2018). These efflux systems have been found in of several microbes, primarily those isolated from contaminated sites. The metal ion transporters' expression in the microbe's efflux system is regulated by resistance genes present in the plasmid or chromosome and is affected by bacterial species and metal ions. These transporters encoded by genes regulate metal uptake as well as its elimination. The cell membrane contains various metal ion exporting proteins like proton-cation antiporters, ABC transporters, and P-type efflux ATPase to facilitate metal ions' efflux (Yin et al., 2019).

2.6.3.1.5 Bioreduction by microbial enzyme

The ability of microbes to resist heavy metals is primarily due to chemical modification or biotransformation of metals from lethal form to less destructive form by enzymes (Liu et al., 2017). Metal toxicity can be reduced by changing the redox state of metal ions. Detoxifying enzymes are used as a defense mechanism, which is also regulated by microbial-resistant genes. During bacterial anaerobic respiration, metals in oxidized form could serve as the final electron acceptor. The reduction of heavy metals may contribute to the formation of a less-lethal form (Viti et al., 2003). Microbes that reduce metal ions are also referred to as dissimilarity metal ions reducing bacteria (Lonergan et al., 1996). Chromium reduction was studied in most bacterial strains among the various types of microbes. Microbial reduction of hexavalent chromium is divided into two processes. In aerobic conditions, removal of chromium is co-metabolic, while in the anaerobic situation; it is mainly dissimilatory (Giovanella et al., 2016).

2.6.3.2 Biofiltration

Human activities and industrialization release harmful pollutants into the environment that pose serious threats to human health and the ecosystem. Several technologies for pollutants and their reservoirs were implemented to overcome this problem. Considering this, biofiltration has been known as a sustainable solution for pollutants removal (Vikrant et al., 2018). When contaminants passed through the medium containing microbes, they are degraded eventually (Jeong et al., 2017). Microorganisms formed biofilm on supporting medium which provides large amount of surface area for biofilm binding (Rene et al., 2018), homogenous distribution of the pollutants, and moisture is needed for active biofilm (Mačaitis et al., 2014; Ibanga et al., 2018). In

biofilteration process microbes are immobilized onto membrane and contaminants containing effluents passed through it. The contaminants will bind to microbial surfaces and clean effluents passed to collection tank (Cohen, 2001; Chen and Hoff, 2012).

Table 2.1 Microorganisms applied for heavy metals removal

Microorganisms	Technology	Metals
Eichhorniacrassipes	Biofiltration	Pb, Cr and Cu
Pseudomonas taiwanensis,	Biofiltration	Cr (VI) and Zn (II)
Thaueraselenatis	Biofiltration	Cd, Ni, Pb and Cr
Enterobacter cloacae	Biofiltration	Cr (VI), Pb and Hg
Bacillus sp., Pseudomonas	Biofiltration	Cd, Cu and Pb
Thiobacillus thiooxidans	Biofiltration	Zn and Cu
Rhodospirillum sp.	Biofiltration	Hg, Cd and Pb

2.7 Bio-filters

Biological filters were used in the biofiltration process, which were cheaper, easy to design and operate. Bio-filter consists of a supporting medium that provides additional nutrients for microbial growth (Fig. 2.2). Supporting media in the biofilter includes compost, soil, bamboo, bio balls, or fish tank stones (Kumar et al.,2019). Microorganisms gradually develop biofilm on the supporting biofilter medium. Porous clay or polystyrene spheres can also be used as biofilter supporting material. The supporting medium provides large surface area for biofilm development, also decrease backpressure and increase biofilter material operational time, thus providing favorable conditions for microbial growth (Barupal et al., 2019). Microorganisms have a vital role in the degradation of contaminants in the internal environment (Srivastava et al., 2008). In biofilter, contaminants are degraded to less or non-toxic form within short time without producing any other secondary contaminants (Gopinath et al, 2018). The efficiency of biofilters in removing pollutants is determined by the following factors:

1. The quality and nature of its filter media;
2. The biofilter media must be less compact and porous to avoid its replacement.
3. Provision of appropriate internal environments i.e. temperature, pH and moisture contents.
4. Large surface area for microorganism's attachment.
5. Potential to keep rate of degradation high (Srivastava et al., 2008; Majumder, 2015).

These factors are important for maintenance of internal environment of biofilter for active biofilm development. Microbes use movement, initial attachment followed by hard attachment, development of biofilm and finally colonization onto support (Kumar et al., 2013). For growth of microorganisms in the biofilter, dissolved oxygen is delivered by incoming natural air (Chandranet al., 2020).

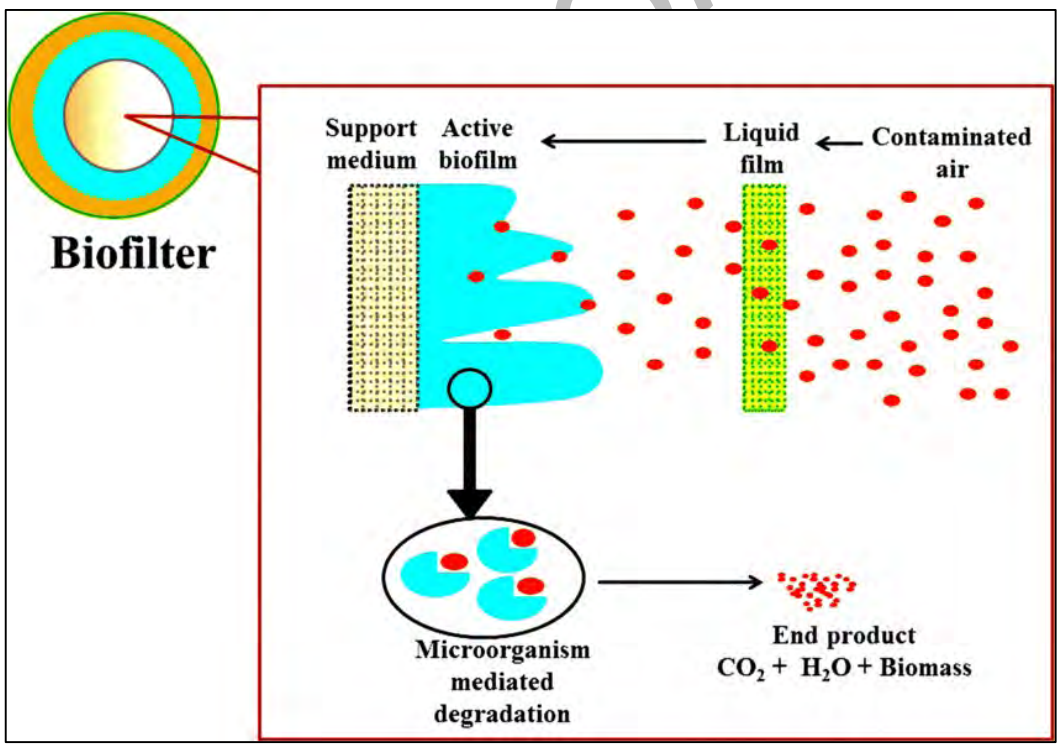


Fig 2.2 Biofilm development and colonization onto biofilter media

2.7.1 Types of biofilters

Different types of filters have been used in past for waste water treatments such as fixed bed filter, fluidized bed reactor, trickling filter and membrane bioreactor. Some of biofilters and their designing and uses are mentioned below;

2.7.1.1 Trickling Filter

Highly used filters for wastewater treatment was trickling filter (TF) in which the filter media is present on the surface and wastewater is sprinkled onto the media (Fig 2.3). Different packing material such as rocks and synthetic plastics can be used as a fixed film (Metcalf & Eddy, 2014). In this filteration system, the microorganisms in the wastewater use the organic matter as a nutrients released from the biofilm layer developed on the supporting medium (Alves et al., 2002). Each TF consists of a dose supply system, filter media Collection system for treated water, and a settling system to settle the sludge and separate it from the effluent (Shahot et al., 2014).

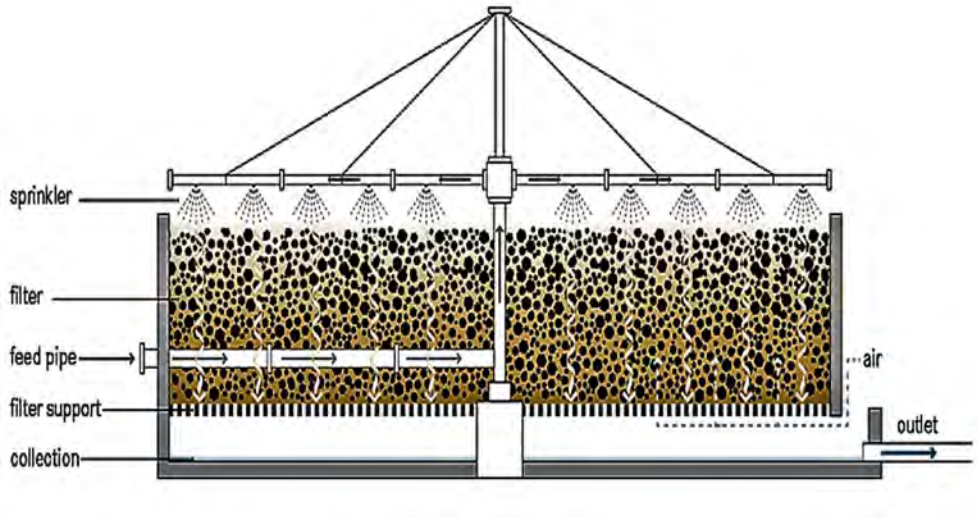


Fig 2.3 Typical trickling filter

2.7.1.2 Rotating Biological Contactor (RBC)

RBC is now widely used in wastewater treatment. It consists of a series of closely spaced circular discs of polystyrene or polyvinylchloride that are partially submerged in wastewater and rotated through it (Fig 2.4). In RBC, aeration is achieved by exposing

discs to the air and microorganisms attached on the discs can effectively degrade the contaminants present in wastewater (Metcalf & Eddy, 2014).

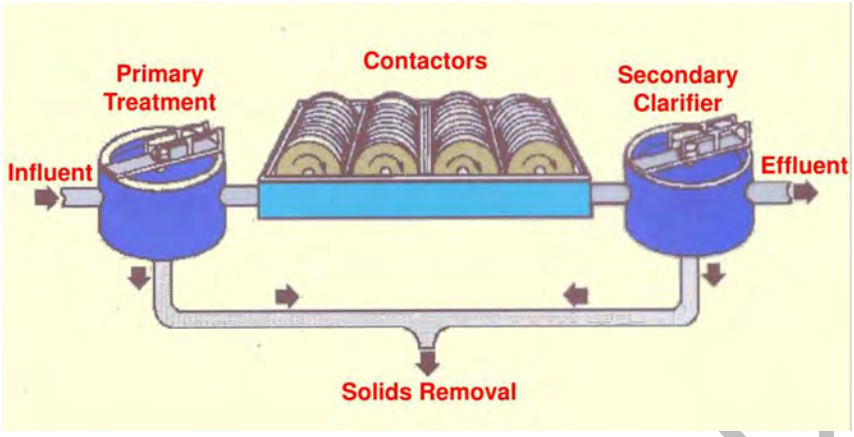


Fig 2.4 Rotating Biological Contactor

2.7.1.3 Fixed Bed Filters

Fixed bed biofilters commonly applied in wastewater treatment required less volume and area with the high loading rate (Fig 2.5) as compared to other filters (Galluciet al, 2009). In fixed bed biofilters, Microbes are fixed on the solid surface to develop a biofilm layer in aerobic/ anaerobic systems (Shahot et al, 2014). Figure 2.5 shows the configuration of this system. Researchers are now working on the anaerobic application of this system.

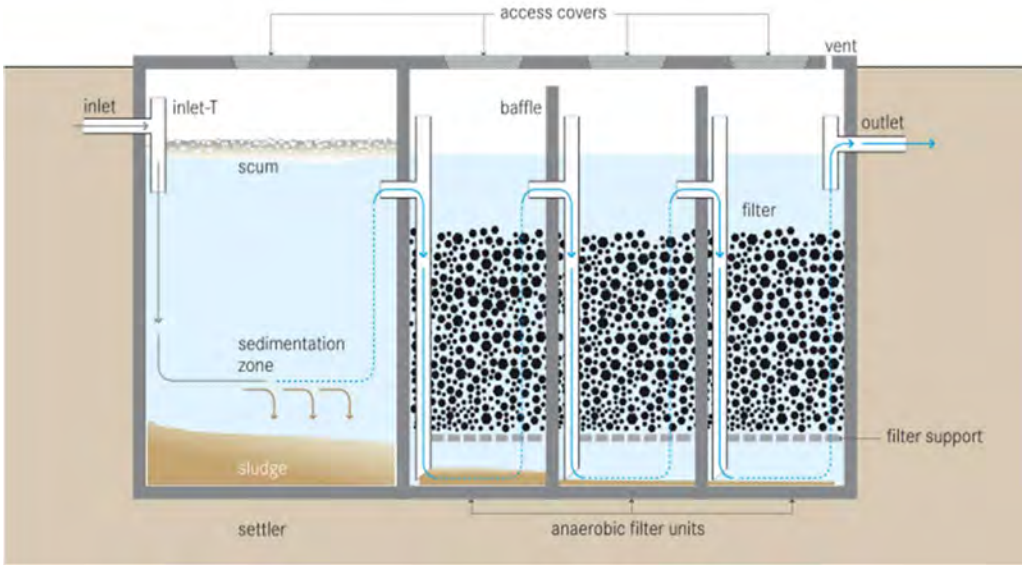


Fig 2.5 Fixed Bed Filters

2.7.1.4 Fluidized Bed Filters (FBR)

Fluidized bed filters are the latest technology in the wastewater treatment system operated in both aerobic and anaerobic systems with floated or submerged supporting media (Fig 2.6). When the wastewater is mixed with the filter media, microorganisms develop a biofilm layer on the media to consume organic contaminants present in wastewater (Burghate et al., 2013). These filters consist of a reactor with the supporting media denser than water and small in diameter. Supporting media that can be used are sand, slag, plastic balls, etc. This system has various advantages as compared to other biological filtration systems. The uniqueness of this system for wastewater treatment is that FBR combine both activated sludge and trickling filter in one process. The FBR removal efficiency is ten times more than activated sludge process. The main drawback of this system is the excessive cost of packing material and requires power for pumping wastewater.

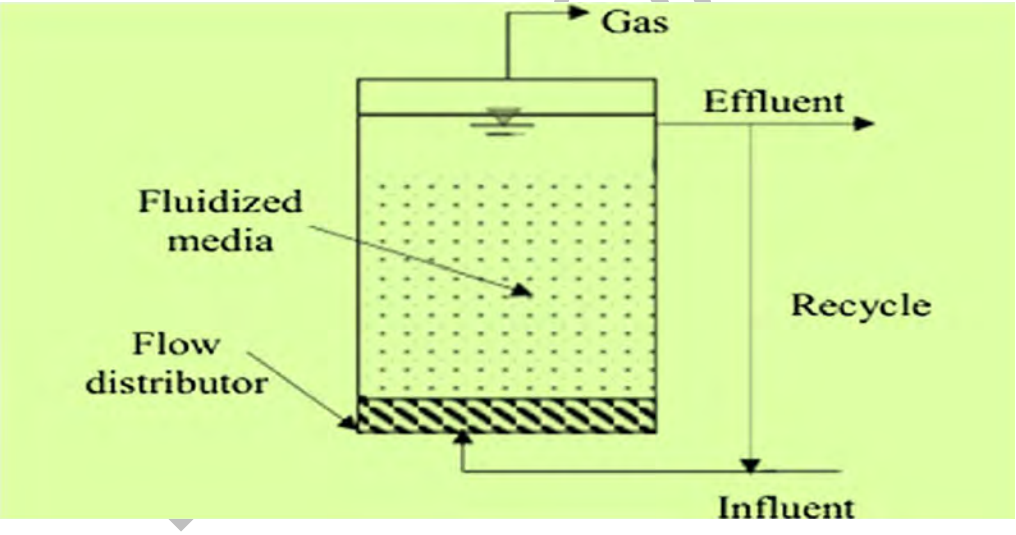


Fig 2.6 Fluidized Bed Filters

2.7.1.5 Moving Bed Biofilm Reactors (MBBR)

The operating mechanism of moving bed biofilm reactors is like an activated sludge system coupled with a small cylindrical shaped filter for biofilm growth. The typical size of these filters is about 10mm in diameter and 7mm in height. Inside the MBBR, these small cylindrical-shaped filters continuously mix with wastewater in terms of suspension either in aerobic or anaerobic basins (Metcalf & Eddy, 2014). The MBBR has various advantages as compared to other biological filtration systems in removing

organic compounds such as BOD5 and COD with high organic loading rates. Studies have been conducted on the characteristics of biofilm carrier elements of moving bed biofilm reactors. The studies indicate that carrier biofilm elements with high specific surface area permit higher biomass concentration in small reactors and good control for system efficiency on which the biofilm concentration is between 3000 and 4000g $TSSm^{3-}$ which is the same rate as in the activated sludge system (Shahot et al, 2014). The main disadvantage of this system is the high operational cost and the settling of biosolids in the reactor.

2.8 Chromium Occurrence and Chemistry

Chromium is a firm, steel-gray metal present in the form of chromite ore in the earth's mantle. It mainly belongs to the transition elements and is the first member of group VI of the periodic table. Chromium occurs in different oxidation states (from Cr^{+2} to Cr^{+6}). trivalent and hexavalent forms of the chromium are the most stable. Chromium is present naturally in trace amounts in all environmental components like water, air, and soil. (Coetzee et al., 2020).

2.8.1 Toxicity of chromium

The uptake of hexavalent chromium by eukaryotic and prokaryotic becomes more accessible than trivalent chromium because of its high solubility and mobility. Chromium is considered an essential micronutrient in human beings' and animals' diets because it plays a vital role in the metabolism of proteins, sugar, and lipids. The oxidation state of Chromium decides its level of toxicity, e.g., $Cr^{+6}$is more destructive than $Cr^{+3}$and causes adverse health effects on animals and humans like ulcers, diarrhea, kidney dysfunction, irritation of the skin and eye, and carcinoma of the lungs (Qian et al., 2017). The toxicity of chromium in eukaryotes and prokaryotes is linked to its fast diffusion of chromium through the bacterial membrane and produce free radicals through the reduction of chromium. These free radicals cause alteration of DNA and other lethal effects (Jobby et al., 2018). Trivalent chromium shows a positive effect in adjusting the glucose level in blood by facilitating the binding of receptors of cells with insulin (Pechova & Pavlata, 2007). Cr^{+3}also play a vital role in decreasing fats, level of cholesterol, and triglycerides. Hexavalent chromium causes lung carcinoma, and its mechanism of carcinogenesis was discovered by Browning and Wise (2017).

Chromium inhibits plants' growth by interrupting the process of photosynthesis and decreasing the uptake of nutrients. It has a detrimental effect on different morphological, biochemical and physiological processes in plant cells. Plants show chromium toxicity in the form of necrosis and chlorosis (Shahid et al., 2017)

2.8.2 Chromium induced contamination

Because of the corrosion resistance and hardness quality of chromium, it has a wide variety of industrial applications. It is commonly utilized in several industries like electroplating, metallurgical, preservation of wood, tanning, stainless steel production, pigments, paints, paper, and pulp production (Grace et al., 2019). Fly ash, slag, and sludge are the primary sources of waste that contain a large concentration of chromium in the environment. The utilization of chromium substances to tan hides in tanneries is the primary source of its contamination. The leathers cannot absorb the entire chromium, and a considerable amount of chromium is released into effluents that lead to contamination. About forty million tons of effluents are produced in tanneries every year worldwide, having chromium, a significant contaminant (Pushkar et al., 2021). The effluent from these tanning industries is directly disposed of in rivers and on lands without being treated. In Australia a significant contamination of chromium was observed in water bodies and agricultural land because of the direct disposal of waste from tanneries (Megharaj et al., 2003).

2.8.3 Bacterial resistance mechanism to chromium

Bacteria use different strategies the reduction of chromium in a polluted environment. Some of the strategies are extracellular chromium reduction, intracellular chromium reduction, reduced uptake of chromium, ROS scavenging, and DNA repair enzyme mechanisms (Fig 2.7) (Nanda et al., 2019).

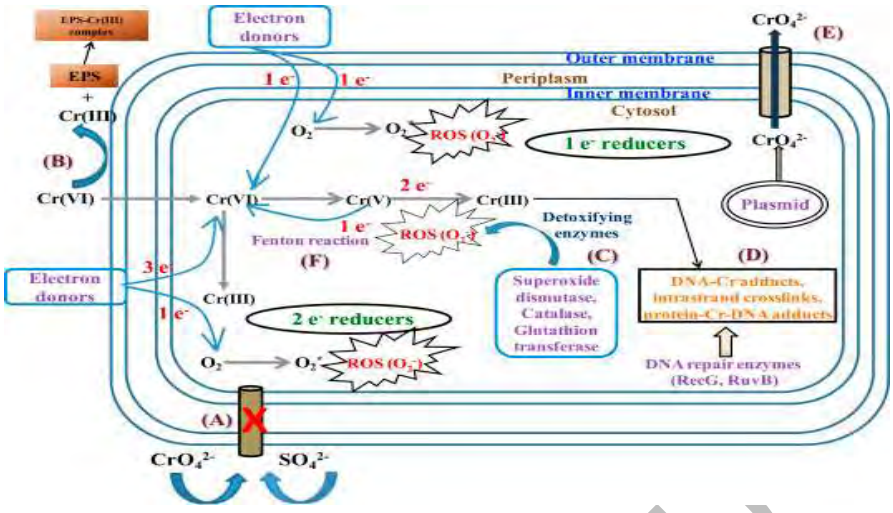


Fig 2.7. Bacterial resistance to hexavalent chromium (Thatoi et al., 2014)

2.8.4 Uptake of reduced chromium

Reduced uptake of chromium similar to the sulfate uptake pathway is one of the significant defense strategies used by bacteria against the toxic effect of hexavalent chromium. The structure of sulfate ion is similar to that of chromate ion. Through nonspecific anionic carriers or sulfate transport pathways, the chromate ion easily passes into the cell by crossing the cell membrane (Speer et al., 2018). When the chromosome-encoded sulfate uptake pathway in the organism undergoes mutation, the transfer of chromate is declined. Microbes found in metal-polluted sites evolve chromium tolerance by causing mutation which results in uptake of reduced chromium via the sulfate transport pathway (Gang et al., 2019)

2.8.5 Enzymatic reduction of chromium

The reduction of chromium is carried out by enzymes from several organisms, ranging from prokaryotic bacteria to eukaryotic organisms. Iron reductase, quinone reductases, nitro reductase, Flavin reductases, and hydrogenases are the different types of oxidoreductase enzymes that are involved in the tolerance mechanism of chromate (Patra et al., 2010). The nature of the bacteria performing the reduction mechanism determines the type of chromate reductases. Oxidoreductase enzymes are present either in the cell membrane or in the cytosol portion (Cheung et al., 2006). In bacteria, enzymatic chromium reduction mechanisms are produced in a variety of ways. Soluble

reductases reduce hexavalent chromium intracellularly or extracellularly, whereas, membrane bond oxidoreductase can only reduce Cr(VI) extracellularly (Das et al., 2014).

2.8.5.1 Intracellular reduction of chromium

In the intracellular reduction mechanism, reactive and short-lived intermediate Cr(V) is produced during the conversion of Cr(VI) into Cr(III). Cr(V) is highly unstable so it oxidizes back to Cr(VI) by providing electrons to di-oxygen and forming reactive oxygen species. In bacteria, these reactive oxygen species produce oxidative stress. Chromate also induces some proteins for defense purposes, resulting in an additional chromate tolerance mechanism (Ramírez-Díaz et al., 2008).

2.8.5.2 Extracellular reduction of chromium

In this protective mechanism, hexavalent chromium through a chemical bond attaches to the functional groups found on the surface of the bacterial membrane(Ngwenya et al., 2011). The potential of reduced chromium Cr(III) to bind to the surface of the cell membrane promotes its removal from polluted sites. The peptidoglycan components are the efficient binders of trivalent chromium present in the bacterial cell wall. Some of the bacterial species have adsorptive characteristics for the removal of metals. This characteristic of metal removal is determined by the distribution of functional groups present on the cell surface like hydroxyl, sulfhydryl, or amine (Das et al., 2014)

2.8.5.3 DNA repair mechanism of chromate enzymes

A defense mechanism in which DNA repair enzymes provide a defense to bacterial cells by repairing the DNA damaged by chromium. After entering the bacterial cell, via different non-enzymatic and enzymatic mechanism Cr(VI) transform into Cr(III). As a result, ROS are produced that exert negative consequences on DNA or proteins in the bacterial cell. DNA is damaged by ROS such as double-strand breaks and single-strand breaks or base modification of DNA. Bacteria use DNA repair enzymes such as SOS repair enzyme (RecG, RuvB, RecA) to repair the damaged DNA(Hu et al., 2005)e.g. because of chromate, *E. coli* induces SOS response enzyme against oxidative stress to protect DNA (Zhitkovich, 2011).

2.8.5.4 ROS scavenging of chromium

After entering the bacterial cell, Cr^{+6} accepts an electron from NADH or NADPH or other few organic substances such as glucose and converts it into highly active and

unstable intermediate Cr^{+5}.By chromate reductase enzyme, this reactive intermediate is converted into Cr^{+3} via two-electron transfer. This reaction is not always rapid due to which some part of Cr^{+5} is re-oxidized into Cr^{+6}. ROS were generated as a result of this process (Das et al., 2014). In this reduction process, O_2 radicals are formed when molecular oxygen is reduced and dis-mutation produced H_2O_2. Hydroxyl radicals (OH) are produced as a result of the reaction between Cr(VI) and H_2O_2 by Fenton-like reaction (Shao et al., 2019).

2.8.5.5 Application of microbial enzymes in bioremediation of chromium

The use of microbes to degrade the pollutant is a slow practice and decreases the viability of remediation in the actual situation (Ghosh et al., 2017).To overcome the above hindrances, rather than using whole microbes, enzymes extracted from microbial cells were used for remediation (Thatoi et al., 2014). Enzymes are complex biomolecules that catalyze a variety of biochemical reactions (Kalogerakis et al., 2017). To enhance the reaction rate, enzymes lower the activation energy of molecules. Partially purified and purified enzyme-based bioremediation is not dependent on the microbial growth in contaminated sites, but, on the enzyme catalytic activity extracted by microorganisms (Ruggaber et al., 2006). The purified enzyme can be used for bioremediation in soil with fewer nutrients. Microbial biotransformation produces lethal side products that are harmful to the environment while enzymatic biotransformation is safe to the environment because no lethal products are formed.

2.8.6 Bioreduction of Chromium Via Chromate reductase

By forming a chemical bond between the metal ions and functional groups, chromium is adsorbed on the cell surface. After adsorption, either chromium precipitates on the microbial cell surface or is converted into a more diminutive toxic form of chromium. Chromate reductase enzyme catalyzed this transformation of sulfate transporter present on the surface of the biological membrane of both eukaryotes and prokaryotes are involved in chromate diffusion across the membrane. It is translocated within the microbial cell via specific metal-binding protein and reduced into trivalent chromium. The capability to transform hexavalent chromium to trivalent chromium is present in aerobic and anaerobic microbes. In aerobic conditions, NADPH or NADH acts as an

electron donor while in anaerobic conditions, chromate itself acts as a final electron acceptor as depicted in fig 2.8 (Thatheyus & Ramya, 2016).

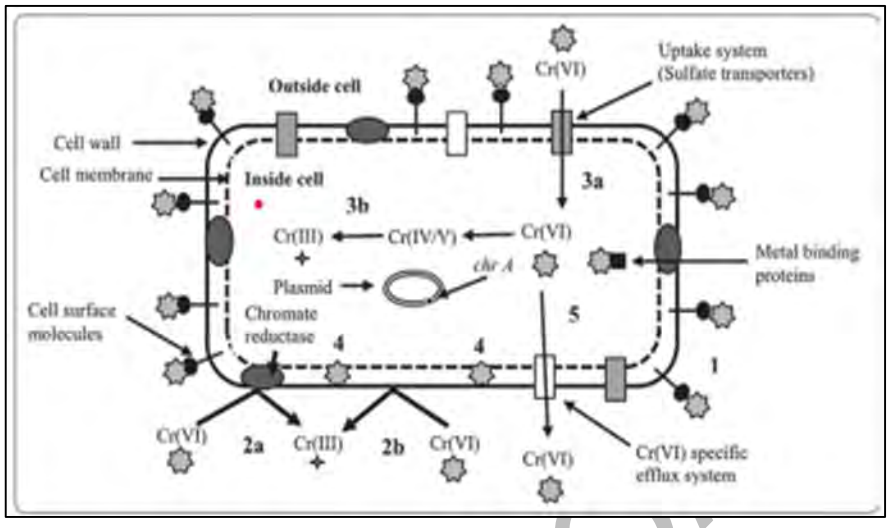


Fig 2.8 Molecular mechanism of chromate reductase

2.9 Lead (Pb) Occurrence and Chemistry

Lead metal appeared in bluish-gray color that is present in small amounts in the earth crust. In addition, lead is also released in high concentration through human activities including fossil fuels, burning, and mining. Lead is a member of group 14 of a periodic table. Pb exist in three oxidation states of lead. The Pb (II), is the most common form that is found in the environment, whereas Pb (IV) is only generated in severely oxidizing environments and is not present in everyday environments (Cullen & McAlister, 2017)

2.9.1 Toxicity of lead (Pb)

Exposure to lead has adverse effects on the health of children as well as adults. In children, its exposure is associated with blood level poisoning, poor intelligence, disrupted neurobehavioral development, low hearing acuity, speech and language impairments, growth retardation, short attention span, and antisocial and obsessive behaviors are only a few of the symptoms (Tchounwou et al., 2012). Lead is toxic because of its ability to intermingle with proteins. Lead can bind with a large group of biomolecules thus disrupting its activity. Sulfhydryl, amide, and group of enzymes

binding with lead lose their activities. It interferes with enzyme activity by competing with metallic cations for binding sites. Lead exposure also causes cell damage due to the production of reactive oxygen species (ROS). Workers exposed to lead have a high concentration and activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Flora et al., 2007).

2.9.2 Bacterial resistance mechanism to lead (Pb)

To prevent the harmful effects of lead, multiple defensive strategies are used by microbial strains without compromising their metabolism (Fig 2.9).

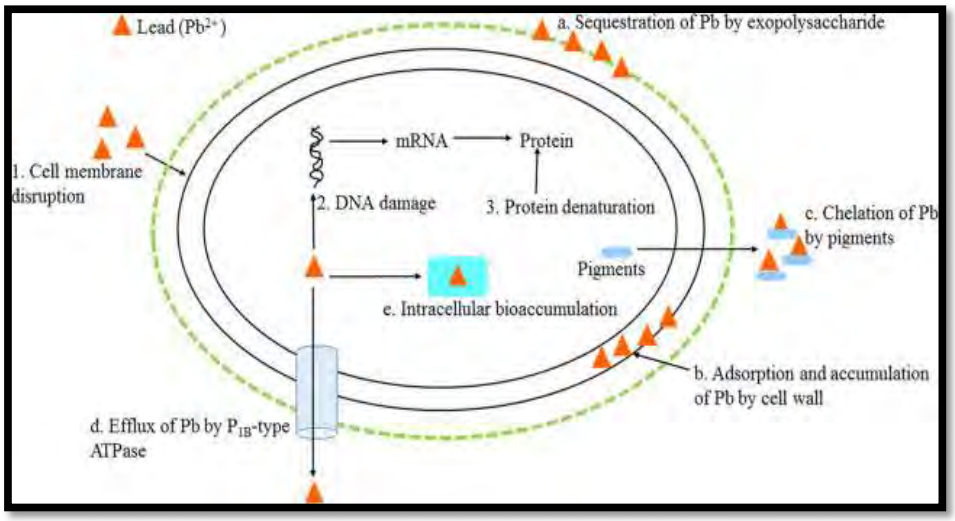


Fig 2.9 Bacterial resistance mechanism against lead (Kushwaha et al., 2018)

2.9.3. Biosorption by cell wall of lead (Pb)

The bacterial cell wall is one the important component for the biosorption of lead from the environment onto the surface of negatively charged groups such as carbonyl, hydroxyl, sulfonate, amine, sulfhydryl and phosphate groups. In this way, lead entry into the cell is prohibited. Carbonyl, phosphate, hydroxyl, amino groups in *P. aeruginosa* ASU6a and *Bacillus* sp. ATS2 are reported for the binding of Pb^{+2} (Kushwaha et al., 2018)

2.9.4. Extracellular sequestration of lead (Pb)

Microorganisms sequester metals outside the cell membrane to reduce the toxic effects of heavy metals because it depends upon the bioavailability of metals i.e., metals can penetrate the cell membrane. Extra polymeric substances (EPS) which are made up of polysaccharides, proteins, nucleic acids, humid substances, and lipids can sequester lead extracellularly due to the presence of charged functional groups (Bhaskar & Bhosle, 2006). Extra polymeric substances (EPS) bind with lead through electrostatic interactions. EPS of *P. aeruginosa* is reported to have binding affinity for lead in marine environment (Pérez-López et al., 2008).

2.9.5. Binding of Lead by Siderophores

Microorganisms' secrets distinct class of chelators called siderophores. They help in the transport of Fe to the cell. Apart from Fe, siderophores also have a binding affinity for other metals. Siderophores (pyochelin and providing), which form complex with Fe, are reported to be produced by *P. aeruginosa* 4EA. In addition to Fe, they also have an affinity for other heavy metals such as Pb, Cd, and Zn. After the formation of siderophore complexes, it is recognized by iron receptor proteins on the external surface and transported inside the cell. N and O atoms contain lone pair of electrons on the functional groups that aid in the formation of a polycyclic bond with the metal thus enhancing its chelation capability (Saha et al., 2016).

2.10 Application of Recombinant DNA Technology for the Removal of Heavy Metals

Heavy metals have a huge economic impact due to their uses in many industrial products such as dyes, coinage, and pharmaceuticals despite the fact that HMs are metal-inducing environmental pollutants with great toxicity. Heavy metals have properties of extreme toxicity, carcinogenicity, teratogenicity, and mutagenicity (Cheung & Gu, 2007). Researchers are focusing on bioremediation technology which is considered environmentally friendly, and easy to operate without soil destruction (Yang et al., 2013). Many studies have been conducted on the isolation of metal-resistant bacteria (Thatoi et al., 2014), whereas studies have been conducted on molecular mechanisms of genes and polypeptides associated with the removal of metals

(Liu et al., 2015). With the development of recombinant DNA technology, it is now possible to engineer microorganisms with characteristics that can enhance the uptake and removal of metals in much more high concentrations than wild strains (Kuroda & Ueda, 2010).

2.10.1 Microbial Cell-surface Engineering for Biosorption of Heavy Metals

Through microbial cell surface technology; proteins of interest are fused with microbial cell surface normal existing proteins. The cell surface protein was fused with carrier protein through genetic engineering for the first time in 1986 (Freudl et al., 1986). This technology is widely used in many biological processes i.e. polypeptides separation, microbial enzyme and vaccine production, and screening of proteins and whole-cell biosorbents (Kondo and Ueda, 2004). Microbial cell surface display technology for metal biosorption. The DNA gene sequence of metal-binding protein could be extracted through amplification of genomic or plasmid DNA. Then the gene of interest is transferred to a microbial host cell as a fusion protein after cloning (Li & Tao, 2015).

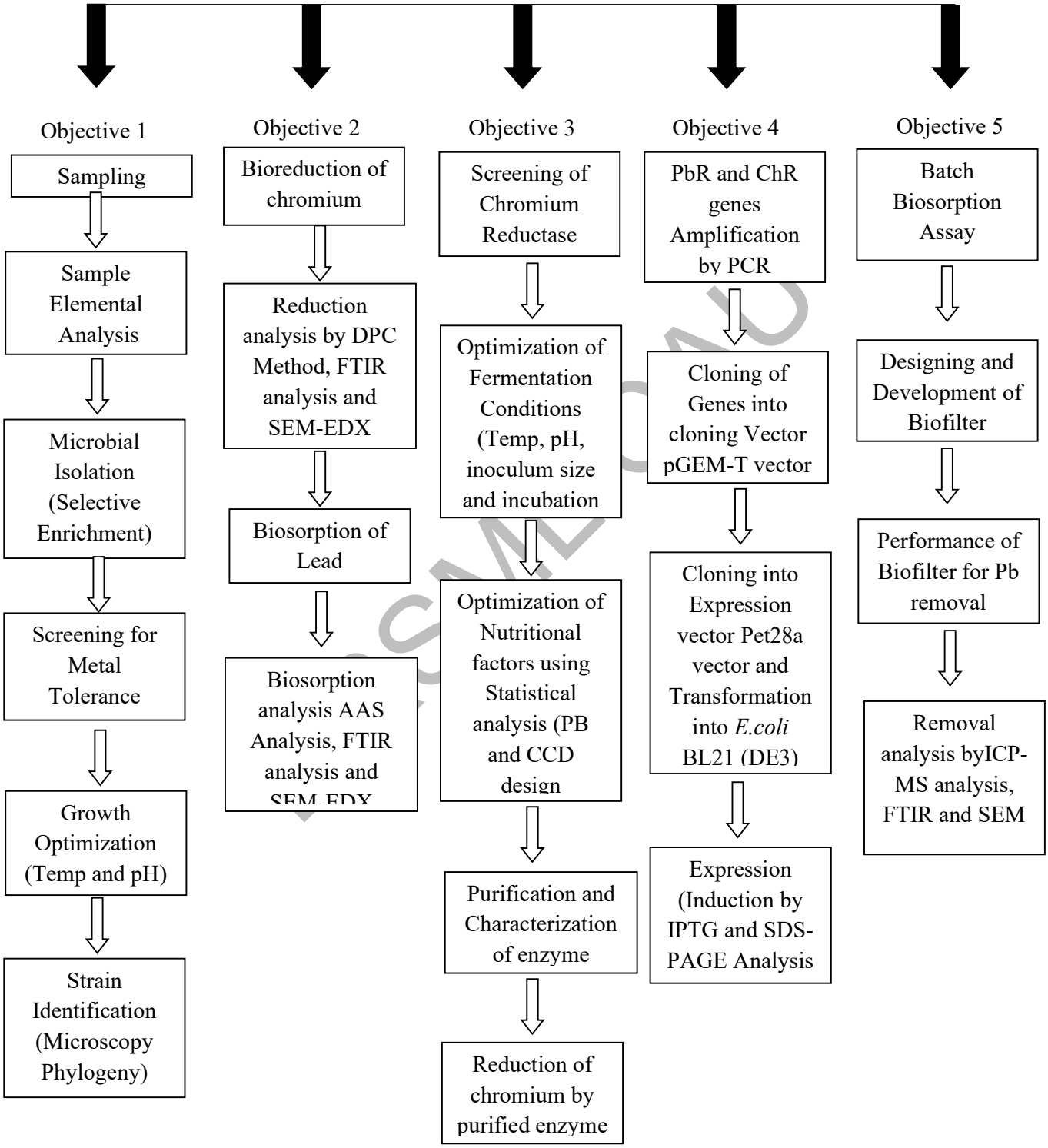
2.10.2 Bacterial Chromate Reductase Engineering for Reduction of Chromium

The studies on chromium bio-reduction are limited to some basic research including isolation, identification, and characteristics of chromium resistant strains such as *Serratia sp.*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acidophilic*, and *Bacillus sp. SFC 500-1E* (Zhou et al., 2017; Chihomvu et al., 2015; Huang et al., 2016; Gan et al., 2018; Ontanon et al., 2018), etc., but molecular mechanisms and genes behind metal resistance are less studied in these strains. Therefore, through recombinant DNA technology (Dong et al., 2018), the functional genes coding for chromium reduction, resistance, and removal were studied (He et al., 2018). Similarly, this technology is also applicable to microbial cells, which are pathogenic and could not release into the environment directly. Chromium reductase expressing gene was reported in the *Serratia*spp. S2 strain and pathogenic (Deng et al., 2015). Through genetic engineering technology ChrT-engineered bacteria were constructed and applied successfully (Tan et al., 2014).

CHAPTER 3

MATERIALS & METHODS

Role of Metallo-resistant Microorganisms in Remediation of Heavy Metals using Biofilter



Materials and Methods

The present study was conducted in the Applied, Environmental, and Geo-Microbiology Laboratory(AEG), Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan, and Molecular Research Laboratory, Department of Biology, Karadeniz Technical University, Trabzon, Turkey. Keeping in view the importance of Heavy metals contamination and its impact on health this study was designed to evaluate the potential role of Metallo-resistant microorganisms in the remediation of heavy metals using biofilter. The research work was divided into five phases; it includes Screening, identification and characterization of metallo-resistant microorganisms from industrial waste. Evaluation of selected strains for their bioremediation potential by optimization of process parameters. Production and characterization of chromium reductase from metal resistant microorganisms. Cloning and expression of Chromate reductase and lead binding protein genes in microbial host. Designing a lab-scale biofilter based on cloned bacterial strains and performance assessment for heavy metals removal. Analytical techniques used in the current study are atomic absorption spectrophotometer, electron microscopy and FTIR.

3.1 Sample Collection

Sludge samples were collected from two different sites, River bank of Lyari (24˚52′46.0″N 66˚59′25.7″E) and Korangi industrial area (24˚48′37.5″N 67˚06′52.6″E) located in Karachi city, Sindh province, Pakistan, which are highly polluted and contain considerable amount of heavy metals like chromium, lead, etc. Sludge samples were collected in triplicate down to 6 cm depth in sterile plastic bottles using standard sampling protocols (Carter & Gregorich 2007). Samples were kept at 4ºC and immediately transported to the Applied, Environmental and Geo-microbiology lab, Quaid-i-Azam University, Islamabad and analyzed within 72 hours of isolation.

3.2 Physical and Elemental Analysis of Sludge Samples

During samples collection, the temperature and pH were determined using thermometer and pH meter electrode respectively. For determination of moisture content, 5 gram of sludge sample was taken in a beaker and placed in an oven at

105°C overnight for drying. The beaker was then removed from the oven and allowed to cool. The following equation is used for calculating the moisture content of the soil sample.

% moisture content (MC) =

$$\frac{\boldsymbol{weight\ of\ moist\ soil\ (M)\ -\ weight\ of\ dry\ soil\ (D)}}{\boldsymbol{Weight\ of\ dry\ soil\ (D)}}$$

The sludge sample was prepared for elemental analysis using a standard protocol with minor modifications (Charles 1991). About 20g of sludge sample was dried overnight at 105ºC and grounded/sieved to small particles (450 µm pore size). About 0.5 gram of sample was mixed in 7.5 mL of HCl and HNO_3 in a ratio of (3:1) and boiled for maximum up to 30 minutes, and keep for 12 hours. After incubation about 5mL of $HClO_4$ was added to the mixture and boiled until the final volume was decreased to 3–5 mL. The residual mixture was then filtered by Whatman filter paper (No 42) and then the final volume was adjusted up to 20 mL by adding sterile deionized water. A simple garden soil sample was used and positive control. A negative control (blank) was run without addition of soil in the same manner. Metals concentration in all samples analyzed for the presence Chromium, Nickel, Copper, and Cobalt by atomic absorption spectroscopy (AA-7000, Shimadzu, Japan) (Din et al., 2020).

3.3 Isolation and Enumeration of Bacteria

A selective enrichment technique was followed for isolation of chromium and lead resistant bacterial strains. 10g of sludge was added to 90 mL nutrient broth (ThermoFisher, USA) amended with 100mg of potassium di-chromate (for chromium resistant bacteria) and lead nitrate (for lead resistant bacteria) separately and placed in shaker incubator for 7 days at 30°C and 120 RPM. After incubation, the enrichment culture was seven-fold diluted ranging from 10^{-1} to 10^{-7}.100µL of sample from each dilution was spread on nutrient agar plates and then incubated at 30°C for 24 hours. After incubation, the plates were observed for distinct colonies with varying colony morphology and culture characteristics. The pure bacterial colonies were kept at 4°C for further use.

3.4 Criteria for Strain Selection

Among the isolated strains, most potential strains were selected to achieve the objectives of our study, and two different criteria were set for strain selection based on our objectives. Bacterial isolates were screened for their maximum chromium tolerance and isolate (S48) with highest chromium tolerance was selected for chromium reduction, chromate reductase production and cloning and expression of chromate reductase. Whereas bacterial isolates were also screened for their lead tolerance and isolate (S54) with highest lead tolerance was selected for cloning of lead binding protein and lead removal using biofilter.

3.5. Screening of Chromium and Lead Resistant Bacterial Strains

A stock solution of 10,000 mg/L of potassium dichromate ($K_2Cr_2O_7$) and lead nitrate [$Pb(NO_3)_2$] was prepared in separately. A total of 53 pure bacterial isolates were screened for maximum resistance to chromium and lead. Bacterial isolates were spread separately on nutrient agar plates containing 100 to 1600 mg of $K_2Cr_2O_7$ and $Pb(NO_3)_2$ in the medium in separate. The plates were incubated at 30°C for 24 hours and observed for the growth of bacterial colonies after incubation. Furthermore, the resistance was confirmed in the broth medium considering the minimum inhibitory concentrations of chromium and lead against the isolates (Alam and Malik, 2008). $K_2Cr_2O_7$ and $Pb(NO_3)_2$ in concentration from 100-1600 mg were added to 100 mL nutrient broth in 250 mL Erlenmeyer flasks in separate. The bacterial strains were inoculated and incubated at 30°C for 24 hours. The bacterial growth was observed by spectrophotometer after 24 hours at 600 nm.

3.6 Identification and Characterization of Bacterial Isolates

3.6.1 Macroscopic and microscopic examination

Bacterial strains S48 and S54 were observed for colony morphology in terms of elevation, shape, margin and size. Gram staining was performed for microscopic identification. A single drop of normal saline was added on a sterile grease-free slide. A bacterial colony was spread on a slide. The slide was then passed through the flame about 2-3 times to heat fix the bacterial strains and then allowed to cool. Firstly, crystal violet dye was used to stain the smear for 1 minute and then washed gently for

2 seconds with tap water. After crystal violet, the slide was stained with gram's iodine for 1 minute and washed gently with water. Now, ethanol was added drop by drop add just for 15-20 seconds and washed gently with water. Finally, the slide was flooded with counterstain (safranin)for 1 min and then washed with water. The slide was dried with blotting paper and observed under a microscope using various magnifications (4X, 10X, 40X, and 100X).

3.6.2 Biochemical characterization

3.6.2.1 Catalase test

For catalase enzyme activity, hydrogen peroxide (H_2O_2) was used as a catalase reagent. A loopful bacterial colony ofstrainsS48 and S54 was taken and placed in the center of a glass slide and mixed along with a drop of hydrogen peroxide. The bubbles' formation due to oxygen indicated the catalase presence which was known as the catalase-positive.

3.6.2.2 Oxidase test

It is performed by filter paper test method.1% of tetramethyl-p-phenylenediamine dihydrochloride was added to distilled water to prepare Kovac's oxidase reagent. A small piece of filter paper (whatsmann No. 1) was soaked with 3 drops of 1% oxidase reagent and bacterial colonies of strain S48 and S54 were picked and rubbed on the filter paper. If the color changes to blue or purple within 30 seconds, then the results were noted as positive for oxidase activity.

3.6.2.3 MacConkey agar test

MacConkey agar (5g) was dissolved in 100mL distilled water. Sterilized by autoclaving the media. Under the aseptic condition, poured media into Petri plate. Inoculate MacConkey agar with fresh bacterial colonies S48 and S54 and incubate at 30˚C for 24 hours.

3.6.2.4 Triple Sugar Iron test (TSI)

The TSI agar slants were prepared in the test tube under sterile conditions. The fresh bacterial colonies of strainsS48 and S54 were first stabbed in the slant with a sterilized needle and then streaked on the slant surface. The test tubes were incubated at 37˚C for 24 hours and observed for gas production and color change after incubation.

3.6.2.5 Urease test

This test is used for the organisms that can utilize urea into ammonia and carbon dioxide. For this test, a urea agar base was prepared and sterilized. 40% urea solution was also prepared. Then 40% urea was added to the urea agar base through a syringe filter. Fresh bacterial colonies of S48 and S54 were inoculated separately onto the slants and incubated for 24 hours at 30°C. Slants were observed for color change after a period of incubation. Phenol red (indicator) was used in the urease test medium. The result of the urease test was considered positive based on the formation of ammonium carbonate and the pink-red color.

3.6.2.6 Simmon Citrate test

The simmon citrate slants were formed aseptically in the test tube. Fresh bacterial colonies of strainsS48 and S54 were selected and stabbed on the slant butt with a sterilized loop, and then streaking was performed on the slant and incubated for 24 hours at 37˚C. Slants were observed after a period of incubation for color change from green to blue.

3.6.2.7 Indole test

This test was performed by preparing tryptophan broth. 4mL of tryptophan broth was poured in test tubes. Colonies of strainsS48 and S54 were picked and inoculated in test tubes. The tubes were incubated for 24 hours at 37˚C. Kovac's reagent (0.5 ml) was added into 24 hours broth culture and color change such as absence or presence of a ring was observed.

3.6.3 Molecular identification

3.6.3.1 Molecular Analysis

DNA of strains S48 and S54was extracted for molecular identification using a DNA extraction kit as per manufacturer's instructions (Eco-Pure-Genome, Product Code # E1075). The concentration and purity of genomic DNA were determined using NanoDrop (Thermo Scientific, 2000). The 16S rRNA region of genomic DNA was amplified using universal primers Forward (ATTCTAGAGTTTGATCATGGCTCA) and reverse complement primers (TACACACCGCCCGTCACACGGTACCAT). The PCR mixture contained: primers (10mM)1µLeach; Go-Taq buffer10 µL; $MgCl_2$ (25 Mm)3 µL; dNTPs (10mM)1 µL; Taq polymerase0.5 µL; DNA template1 µL; and

deionized water32.5 µL, making total volume of reaction 50 µL. The PCR conditions were; denaturation temperature 95ºC for 3 min followed by annealing temperature 55ºC for 1 min, extension temperature for 36 cycles 72ºC for 1hr and 40sec, and one cycle of final elongation at 72ºC for7 minutes. PCR product amplification was confirmed by using 1% agarose gel. The 16S rRNA sequence of strains S48 and S54 were checked for similarity using the ezbiocloud program (https://www.ezbiocloud.net/) and all sequences showing similarity > 95% were downloaded. All downloaded and selected sequences were aligned using BioEdit.exe 7.2 Software, then the Neighbor-Joining phylogenetic tree was constructed using MEGA-X software (Kumar et al. 2018).

3.7 Optimization of Growth Condition

3.7.1 Inoculum preparation

During optimization parameters, uniform inoculum was used in all set up experiments. To prepare fresh bacterial inoculum, strain S48 (*Bacillus* Sp.*)* and strain S54 (*Escherichia* Sp.) were freshly grown in nutrient broth and incubated for an hour to initial exponential phase 0.2 OD at 600nm. 1% of inoculum was used in all experimental setups.

3.7.2 Effect of Temperature

The effect of temperature for maximum growth of strain S48 (*Bacillus* Sp.*)* and strain S54 (*Escherichia* Sp.*)* was determined at various temperatures ranging from 20-50 ºC. Bacterial strainsS48 and S54 were grown in nutrient broth amended with 300PPM of $K_2Cr_2O_7$ and Pb $(NO_3)_2$ respectively, for 72 hours in a shaking incubator at 150 rpm and growth was recorded in terms of optical density (OD) at 600 nm at different time intervals (24 to 72 hours).

3.7.3 Effect of pH

The effect of pH for maximum growth of strain S48 (*Bacillus* Sp.*)* and strain S54 (*Escherichia* Sp.*)* was determined at various pH ranging from 5.0-10.0. Bacterial strain S48 and S54 were grown in nutrient broth amended with 300PPM of $K_2Cr_2O_7$and $Pb(NO_3)_2$ respectively, for 72 hours in shaking incubator at 150 rpm and growth was calculated in terms of OD at 600 nm at different time intervals (24 to 72 hours).

3.8 Bioreduction Assay for Chromium Removal

1,5-diphenylcarbazide (DPC) method was used for hexavalent chromium reduction (Fulladosa et al., 2006). Fresh culture of strain S48 (*Bacillus* Sp.*)* was inoculated in 250 mL flask containing 100 mL of nutrient broth supplemented with $K_2Cr_2O_7$ (300ppm) and control was setup in separate without chromium. Flasks were then incubated at 30ºC for 72 hours in shaker incubator at 150 rpm.1 mL of sample was collected after every 24 hours and centrifuged at 14000rpm, 4˚C for 5 minutes in order to collect supernatant for Cr(VI) reduction. The reaction mixture contained; 10 mL distilled water, 400 µL cell free supernatant, 1 mL DPC (0.25g DPC in 100 mL acetone), and one drop of phosphoric acid in a test tube. The test tube was incubated for 10 minutes and then absorbance was measured at 540 nm using spectrophotometer (Agilent Technologies, G6860A, Malaysia). $K_2Cr_2O_7$ standard curve was plotted for measurement of chromium reduction.

Batch bioreduction experiment was performed under optimized conditions (pH 7.0 & temperature 35ºC) by varying Cr(VI) concentration (0–400 mg/L) and contact time (24–120 hrs), the abiotic control without inoculum was run in separate. The sample was taken after every 24 hours upto the maximum of 72 hours, centrifuged at 14000rpm, 4˚C for 5 minutes and supernatant was analysed for Cr(VI) reduction by 1,5-diphenylcarbazide method (Fulladosa et al., 2006).

3.9 Biosorption Assay for Lead

Strain S54 (*Escherichia* Sp.*)* was inoculated in nutrient broth in 500 mL Erlenmeyer flask supplemented with 500 mg/L of Pb and incubated for 72 hours at 30°C. Un-inoculated control was run in parallel to detect any possible abiotic Pb removal brought about by medium components. About 2 mL of sample was collected from each flask after 72 hour of incubation and centrifuged at 14,000 rpm. Cell free supernatant was analyzed by AAS to determine Pb concentration in the medium. The following equation was used to determine reduction in lead concentration in the medium:

Biological adsorption efficiency of (E %) = CI– CR/ CI × 100

CI = Initial concentration of metal added into the medium (mg/L), CR = Remaining concentration of metal after adsorption (mg/L)

3.10 Preparation of Cell Biomass for Chromium and Lead Biosorption

72 hours grown culture of *B* strain S48 (*Bacillus* Sp.) and strain S54 (*Escherichia* Sp.*)* was centrifuged at 8000 rpm for 10 minutes. Bacterial cells were washed with distilled water three times and dried in hot oven at 55°C for 48 hours. Dried biomass (200mg) was treated with 5 mL of 30% nitric acid and kept at 150°C for 5 hours to completely evaporate nitric acid. The dried biomass was again dissolved in 3 mL of 30% hydrogen peroxide and placed at 150°C for 2 hours in order to oxidize any remaining organic matter. The dried sample was resuspended in 1 mL of 30% nitric acid and diluted in 20 mL deionized water. Finally, the sample was filter using 0.20 Millipore syringe filter. The biological adsorption capacity and efficiency of biomass against chromium and lead was evaluated through inductively coupled plasma mass spectrometry (ICP-MS). The following equation was used to determine Adsorption capacity of biomass against chromium and lead concentration in the medium:

Biological adsorption capacity (Qe) = V (CI– CR)/M

Where Qe = Metal ion uptake of per gram of the total cell biomass (mg/g), CI =metal ion initial concentrations present in the medium (mg/L), CR=Metal ions residual concentration after bioadsorption (mg/L), M = dried cell biomass (g), and V = Reaction mixture total volum (ml).

3.11 Analysis of Biosorption

3.11.1 Fourier- Transform Infrared spectroscopy(FTIR) Analysis of bacterial biomass

FT-IR was used to observed the functional groups and overall nature of chemical bonds in strain S48 (*Bacillus* Sp.*)* and strain S54 (*Escherichia* Sp.*)*. The biomass from both test (bacteria grown with metal stress) and control (bacteria grown without metal

stress) samples were freeze-dried and then analyzed through FT-IR (PerkinElmer Spectrum Version 10.4.3). The spectral data was collected over the range of 450–4000 cm^{-1} at a resolution of 4 cm (Ramyakrishna and Sudhamani, 2017).

3.11.2 Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy(SEM-EDX) analysis of Biomass

Samples were prepared for SEM analysis by the method as previously described with minor modification (Kang et al.,2014). The bacterial strains S48 (*Bacillus* Sp.*)* and S54 (*Escherichia* Sp.*)* were cultured in chromium and lead containing medium for 72 hours. and then centrifuged for 10 minutes at 10,000 rpm to harvest bacterial cells in pellet. The obtained pellets were suspended in 0.1 M phosphate buffer and pH 7.5 containing 2.5 % glutaraldehyde and incubated for maximum up to 12 hours overnight. Afterward centrifugation was carried out in order to remove the obtained fixatives and washing was performed using phosphate buffer. The sample dehydration step was performed with different concentrations of ethanol, i.e., 20−100 % for 20 minutes. The dried samples were coated with gold and used for SEM-EDX analysis. For SEM-EDX was investigated by using Zeiss EVO MA15 with a Bruker X Flash EDX detector. The sample SEM images were taken using a High Definition Backscattered Detector (HDBSD).

3.12 Chromate Reductase Assay from strain S48 (*Bacillus* Sp.*)*

The enzyme chromate reductase activity was determined using method described by Thatoi et al. (2014) with some modifications. A reaction mixture contained: 0.2 mM $K_2Cr_2O_7$, 0.2 mL;0.2M buffer pH (7.2),0.2 mL;0.2 mM NADH, 0.2 mL;0.4 mL cell free supernatant, a total volume of 1 mL was incubated for 30 minutes at 30°C. After incubation, the reaction mixture was kept in water bath at 37°C for 30 minutes. About 0.5 mL of TCA (20%) was added in order to stooped the reaction. Finally, 2 mL of 0.5%(w/v) of DPC was added in acetone. Distilled water was added in blank instead of enzyme solution. The absorbance of hexavalent chromium was measured by spectrophotometer at 540 nm wavelength.

Enzyme Unit: The amount of enzyme that release 1 µmol/mL of chromate per min is stated as enzyme unit.

Specific Activity: Chromate specific activity was calculated by using the following formula:

Specific Activity = The number of enzyme units per mL divided by the concentration of protein in mg/mL

Unit of Enzyme specific activity = U/mg.

3.13 Potassium Dichromate Standard Curve

Standard curve of $K_2Cr_2O_7$was plotted by making 1mg/mL stock solution of $K_2Cr_2O_7$ in distilled water. The solution was further diluted so that the first tube contains 100 µg/mL and the final test tube contained 1000 µL/mL. A blank was prepared that contain 500 µL distilled water instead of potassium dichromate (Table 3.1). All the test tubes were kept at optimum temperature for 10 minutes. O.D of each solution was taken by spectrophotometer at 540nm wavelength. The graph was plotted by entering values in Microsoft excel 10 (Figure 3.1).

3.14 Reagents Composition and Solution Preparation for Protein Estimation

Preparation of solution A

Solution A was prepared by mixing 2.8g NaOH and 14.3g Na_2CO_3 in 300ml distilled water and kept the final volume of 500mL.

Solution B preparation

1 g potassium sodium tartrate was mixed in 100 mL distilled water.

Solution C preparation

Solution C was prepared by mixing 1.4g $CuSO_4$. $5H_2O$ into 100 mL distilled water.

Solution D preparation

Solution D was prepared by adding solutions A, B, and C with ratio 48:1:1.

Solution E preparation

Solution E was prepared by adding distilled water with Folin Phenol in a ratio 1:1.

3.14.1 Extracellular Protein determination

The protein content in crude sample was determined by Lowry's (1951) method. Estimation of protein content was done in a test tube by adding 1 mL sample and 1ml

solution D. Incubate for 10 minutes at room temperature. Following incubation, add 100 µL solution E and incubate in dark for 30 minutes at room temperature. After incubation, absorbance was measured by spectrophotometer at 650nmwavelength.

3.14.2 Potassium Phosphate Buffer

Potassium phosphate buffer (100mM) was prepared by dissolving 1.74g K_2HPO_4 and1.36g KH_2PO_4 in distilled water and making the final volume of 100 mL (pH 7.2).

Table 3.1 Data for plotting standard curve for potassium dichromate

Test Tube	Distilled water (mL)	$K_2Cr_2O_7$ (µg/mL)	$K_2Cr_2O_7$ Conc.(µg/mL)	Absorbance
1	0.00	1	1	0.073
2	0.1	0.9	0.9	0.172
3	0.2	0.8	0.8	0.253
4	0.3	0.7	0.7	0.389
5	0.4	0.6	0.6	0.531
6	0.5	0.5	0.5	0.631
7	0.6	0.4	0.4	0.775
8	0.7	0.3	0.3	0.930
9	0.8	0.2	0.2	1.081
10	0.9	0.1	0.1	1.473
11	1	0.00	0.00	1.581

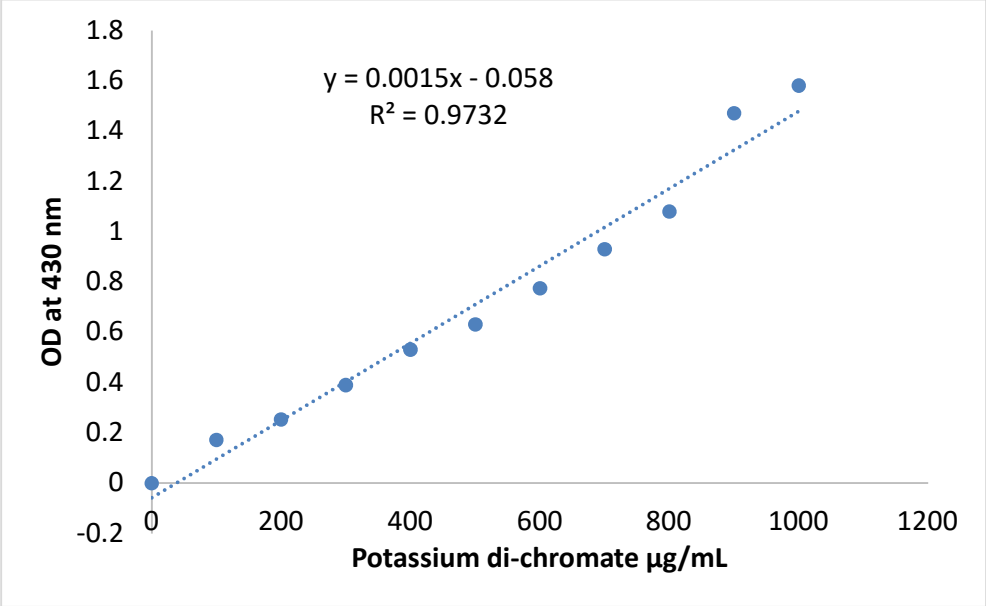


Figure 3.1 Standard curve for potassium dichromate. Y = 0.0015x + 0.058 was obtained from the slope of the graph.

X= Optical density of unknown chromium concentration in the sample and

Y= Concentration of known chromium in a known sample of potassium dichromate.

3.14.3 Preparation of Stock Solution of Potassium Dichromate

A stock solution of $K_2Cr_2O_7$ was prepared to have a concentration of 10,000 mg/L. To prepare stock solution 1.0 g of $K_2Cr_2O_7$ crystals were dissolved in 100 mL of sterilized deionized water and was stored at 4ºC for further use.

3.14.4 Bovine Serum Albumin Standard Curve

A stock solution of bovine serum albumin (BSA) was prepared to have a concentration of 1mg/mL. The BSA stock solution was diluted and checked for estimation of protein content (Table 3.2). The graph was plotted by entering the absorbance values in Microsoft excel 10. Pattern line was added to the graph and an equation was yielded from graph slop for determining unknown value of released sugar (Fig. 3.2).

Table 3.2 Date for standard curve of bovine serum albumin

Test Tube	Distilled water (mL)	BSA Stock Solution (mL)	Protein Conc. (mg/mL)	Absorbance
1	0.00	1	1	0
2	0.1	0.9	0.9	0.17
3	0.2	0.8	0.8	0.388
4	0.3	0.7	0.7	0.451
5	0.4	0.6	0.6	0.544
6	0.5	0.5	0.5	0.654
7	0.6	0.4	0.4	0.716
8	0.7	0.3	0.3	0.744
9	0.8	0.2	0.2	0.968
10	0.9	0.1	0.1	1.304
11	1	0.00	0.00	1.102

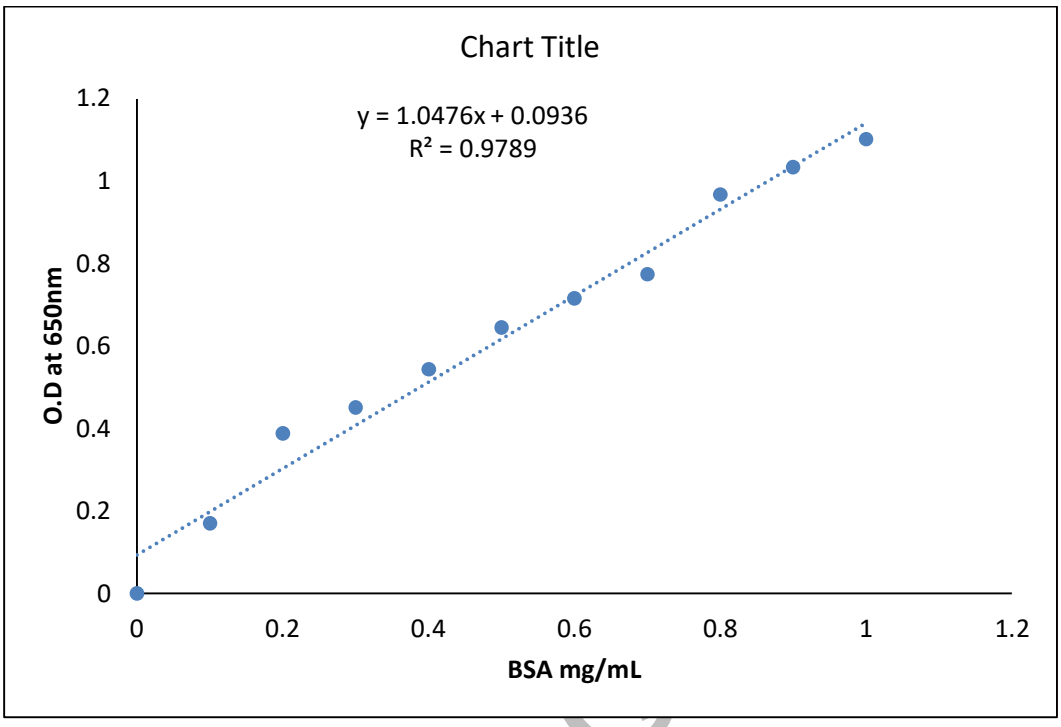


Fig 3.2: Standard curve development for bovine serum albumin Formula: Y = 1.0476x + 0.0936 was resulting from the slope of the graph where X= Optical density of unknown protein in the sample and Y= Concentration of known protein in a known sample of BSA.

3.15 Standardization of Chromate Reductase Assay Conditions

The enzyme production medium contained different components (g/L):KH_2PO_4, 3; Na_2HPO_4, 6; $CaCl_2$, 0.1; $MgSO_4.H_2O$, 0.1; NaCl, 0.5; sucrose, 10.05; Luria broth (LB), 20 and $K_2Cr_2O_7$, 0.1. The freshly grown culture of S48 was inoculated into production medium and incubated for 48 hours at 37˚C. After incubation, cell free supernatant was used for enzyme assay. Optimization of temperature and pH were determined by performing enzyme assay at different temperatures (20-50°C) and pH (3.0-9.0). The reaction mixture was incubated for 10-60 minutes in order to determine the optimum incubation time. Enzyme solution (100-500 µL) and substrate (100-500 µL) concentration was also optimized.

3.16 Optimization of Physico-chemical parameter for maximum chromate reductase production

The growth condition of strain S48 (*Bacillus* Sp.) for maximum production of chromate reductase was optimized via the method of submerged fermentation.

3.16.1 Preparation of inoculum

The inoculum was prepared by inoculating strain S48 (*Bacillus* Sp.*)* in nutrient broth and incubate it for 24 hours at 37˚C in a shaker incubator at 150rpm.

3.16.2 Effect of temperature

Optimum temperatures of chromate reductase of strain S48 (*Bacillus* Sp.*)* was achieved by incubating enzyme reaction solution at different temperature ranging from 20-45˚C for 1 hour. Sample was centrifuged and the supernatant was collected. The specific activity of chromate reductase was calculated by performing chromate reductase assay and protein content was determined.

3.16.3 Effect of pH

Optimum pH of chromate reductase from strain S48 (*Bacillus* Sp.) was achieved by incubating enzyme reaction solution at different pH values ranging from 5.0-9.0 for 1 hour. Samples were centrifuged and the supernatant was collected. The specific activity of chromate reductase was calculated by performing chromate reductase assay and protein content was determined.

3.16.4 Effect of time of incubation

The effect of incubation time on production of chromate reductase was calculated by growing strain S48 (*Bacillus* Sp.*)* at different time interval (24-96 hour). The specific activity was calculated by performing chromate reductase assay and protein quantification at 24-hour intervals upto the maximum of 96 hours.

3.16.5 Effect of Inoculum size

The effect of inoculum size on production of chromate reductase was observed by growing strain S48 (*Bacillus* Sp.*)* at different inoculum size (0.1-3%). The crude

enzyme activity and quantification of protein was determined at 24 hours interval upto the maximum of 96 hours and then the specific activity of chromate reductase was measured.

3.17 Optimization of Media Constituents for Chromate Reductase Production

3.17.1 The Plackett-Burman Design (PBD)

The PBD was used to figure out the most important parameters influencing enzyme production. Different constituents of the production medium were optimized by using Placket-Burman. Mostly, Design Expert 7 software (Stat-Ease Inc.) was used to plan experiments. strain S48 (*Bacillus* Sp.*)* inoculum was prepared from 24 h fresh culture and incubated in production medium. 11 factors were analyzed (Na_2HPO_4, yeast extract, $MgSO_4$.$7H_2O$, KH_2PO_4, $CaCl_2$, NaCl, sucrose, $K_2Cr_2O_7$, Na_2HPO_4, K_2HPO_4, and $(NH_4)_2SO_4$)] to optimize the composition of production medium for maximum enzyme production. For mathematical modeling, first order polynomial model used was described below.

$$Y = \beta_0 + \sum \beta_i X_i$$

In this equation Y is representing specific activity of enzyme in term of predicted response where β_0 , β_i and X_i represent the intercept of the model, the level of self-regulating variable and linear coefficient respectively. The effect of these different factors was observed at two different stages; -1 for the low concentration and +1 for the maximum concentration. Design Expert 7 software was used to run 15 sets with various concentrations of compounds. All the 15 sets of selected strains were run at 37°C through submerged fermentation at 150 rpm. Crude and specific chromate reductase activity was calculated after 72 hours by performing enzyme assay and protein quantification. The important factors were observed by analyzing the responses. Factors having p-value<0.05 for the production of chromate reductase were regarded as important and further optimized by central composite design (CCD).

3.17.2 Central Composite Design (CCD)

The important factors provided by Placket-Burman were further optimized by CCD, having both positive as well as the negative effect on production of chromate

reductase from strain S48 (*Bacillus* Sp.*)*. The optimum reading of factors, their significant effect, and their interaction with one another was determined for enzyme production. Four varying factors (KH_2PO_4, $K_2Cr_2O_7$, Sucrose, and $MgSO_4$.$7H_2O$) obtained from PBD were further checked for their effect and analyzed in runs at two different levels. Specific activity of the enzyme was evaluated using second order polynomial equation and multiple regressions were used for fitting data. Furthermore, the effect of significant independent variables (linear, quadratic and mutual interactions) on dependent variable (Enzyme Specific Activity) was evaluated using given quadratic polynomial equation;

$$Y = \beta_0 + \sum_{i=1}^{6} \beta_i X_i + \sum_{i=1}^{6} \beta_{ii} X_i^2 + \sum_{i<j=2}^{6} \beta_{ij} X_i X_j$$

In this equation Y; X_i,β_i,β_{ii},β_{ij}, β_0 are representing the specific activity; significant independent variables, linear regression coefficients, quadratic regression coefficients, interactive regression coefficients and a constant term, respectively. The model suitability was assessed using ANOVA.

3.18 Bulk Production of Chromate Reductase under Optimized Culture Conditions

500 ml of production medium (pH 7.0) was prepared and inoculated with 1% fresh culture of strain S48 (*Bacillus* Sp.*)* incubated for 72 hours at 37˚C in a shaking incubator at 150rpm. The composition of enzyme production medium was [g/L; KH_2PO_4,1.0; Na_2HPO_4,4.0; NaH_2PO_4, 3.0; $CaCl_2$, 0.1; NaCl, 0.6; $MgSO_4.7H_2O$, 5.0; Sucrose, 15.0; $K_2Cr_2O_7$, 0.002; K_2HPO_4,3.0; Yeast extract, 8.0 and $(NH_4)_2SO_4$ 2.5)]. The supernatant was collected after centrifugation and protein quantification and enzyme assay was performed.

3.19 Chromate Reductase Purification from strain S48 (*Bacillus* Sp.*)*

The chromate reductase enzyme from strain S48 (*Bacillus* Sp.*)* was purified in three steps; ammonium sulfate precipitation, dialysis, and column chromatography.

3.19.1 Ammonium Sulfate Precipitation

Cell-free supernatant was collected after centrifugation at 10,000 x g at 4˚C, in a separate flask. 30% of ammonium sulfate using online calculator was added slowly into the supernatant with continuous stirring on magnetic stirrer. The solution was store overnight at 4˚C and then centrifuged was performed to collect the supernatant and pellet. The pellet was dissolved in potassium phosphate buffer having pH 7.5. Enzyme assay and protein quantification of both supernatant and pellet was performed to calculate their specific activities. Lowery's method was used for protein estimation. The same protocol was repeated and ammonium sulphate concentration was gradually increased in the supernatant upto maximum 80%

3.19.2 Dialysis of Crude Enzyme

Dialysis was performed in order to remove ammonium sulfate from enzyme in buffer solution recovered after ammonium sulphate precipitation. The crude enzyme re-suspended in potassium phosphate buffer (pH 7.5) was transferred to a dialysis bag and placed in the same phosphate buffer at 4˚C for 24 hours.

3.19.3 Protein Purification (Sephadex G-100) Column Chromatography

The gel permeation chromatography technique was used for the separation of proteins on the basis of its sizes (Determann, 2012). 2.3 g of Sephadex G-100 was soaked in 150 mL potassium phosphate buffer for 24 hours at 45˚C.0.04g fluconazole (antifungal)and 0.04g sodium-azide (anti-bacterial) was mixed to control any contamination. Buffer and gel solution were sonicated for 10-15 minutes for degasing. The glass column (3cm × 27cm) was filled with gel solution and then left untouched at room temperature for 24 hours, resulted into tight packing of gel in column. Approximately 4mL of crude enzyme was loaded to the Sephadex column. Potassium phosphate buffer was passed through the column at flow rate 3mL per 15 minutes. About 30 fractions were collected and absorbance was noted at 280nmwavelength to determine total protein concentration in each fraction. The protein quantification and enzyme assay was performed for all fractions. Fractions with high chromate reductase activity were collected and stored at 4˚C for further study.

3.20 Molecular Weight Determination by SDS-PAGE

Chromate reductase molecular size was determined by SDS-PAGE. 12% gel was used in SDS-PAGE to run the sample and Coomassie brilliant blue R-250 dye was used to stain gel. (Laemmli, 1970).

3.20.1 Resolving Gel preparation (12%)

The following ingredients were added to make resolving gel; Distilled water, 1.6 ml; 30% acrylamide, 2mL; 10% SDS, 50µL; 10 % APS, 50µL; Tris-HCl 1.5 M, 1.3mL with pH 8.8 and TEMED, 4 µL. All these ingredients were mixed thoroughly. The gel assembly was then stuffed with gel leaving 0.5 inches unfilled for stacking gel.100 µL of isopropanol was added to eliminate bubbles that were interfering with gel polymerization. The gel was then left at room temperature for 30 minutes to obtain complete polymerization.

3.20.2 Stacking Gel Preparation (8%)

The stacking gel was comprised of the following chemicals: 1M Tris-HCl, pH 8.8; 10 % APS, 30µL; 30% polyacrylamide gel, 500µL; TEMED, 3µL and distilled water, 2.1 ml. All the ingredients were mixed properly, and poured over the polymerized resolving gel. The comb was inserted into stacking gel to create wells and gel was left unaffected for 30 minutes for solidification.

3.20.3 Electrophoresis Buffer Preparation and protein loading

Tris-glycine buffer 5X, also known as electrophoresis buffer, was prepared by adding the following chemicals: glycine, 21.6g; 25 mM of Tris base, 4.5g; and 10% SDS,15 mLto distilled water, a final volume of 1.5L was achieved. The gel tank was kept in the electrophoresis apparatus for a long time and then fixed. To avoid the formation of bubbles, carefully poured the Tris-glycine electrophoresis buffer (1X) into the tank. About 15µL of Gel column purified protein fractions with highest enzyme activity were mixed with loading dye of 15µL, heated at 95˚C for 5 minutes and then placed at ice pad for 5 minutes before loading in wells. Then, the wells were loaded with 25µL of denatured protein fractions. The apparatus was connected with electric cables and a current of 60V was provided for 30 minutes. After that, the voltage was increased up to 100 volts and maintained for 40 min.

3.20.4 Staining of Gel

After running the protein fractions in electrophoresis, gel was moved to a box containing coomassie brilliant blue staining solution and slowly agitated continuously for 60 min. The gel was removed and transferred to destaining solution and kept for another 1 hour. After destaining, the gel was observed with a naked eye for protein bands.

3.21 Characterization of Purified Chromate Reductase

The purified chromate reductase was characterized by observing the effect of various physical and chemical factors and relative activity of enzyme was determined by the following formula (Hora et al., 2015)

Residual Activity (%) = (Activity (U/mg) of partially purified enzyme / Activity (U/mg) of the purified enzyme) × 100

3.21.1 Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on activity of purified chromate reductase enzyme was measured at various temperatures ranging from 30-60˚C for 30 minutes. The thermal stability of enzyme was determined by incubating it at varying temperatures from 30-60˚C in the absence of substrate and activity was monitored after every 30 minutes interval upto the maximum of 120 minutes.

The effect of temperature was determined in terms of relative activity.

3.21.2 Effect of pH on Enzyme Activity and Stability

The effect of pH on activity of purified chromate reductase enzyme was measured at various pH ranges 3.0-9.0 using different buffers i.e. 100mM acetate buffer (pH 3.0-5.0), phosphate buffer for (pH 6.0 – 8.0), and Glycine NaOH buffer (pH 9.0 – 11.0) for 30 min. The pH stability of enzyme was determined by incubating it at varying pH from 3:0 -9:0 in the absence of substrate and activity was monitored after every 30 minutes interval up to the maximum of 120 minutes The effect of pH was determined in terms of relative activity.

3.21.3 Metal Ion effects on Enzyme Activity

The effect of various metal ions i.e. barium sulfate, calcium chloride, copper sulfate, magnesium sulfate, calcium sulfate, zinc sulfate, copper chloride, potassium chloride, sodium chloride, mercury sulfate, ferrous sulfate, nickel chloride, potassium sulfate, cobalt chloride, urea, nickel sulfate, and sodium sulfate on activity of purified chromate reductase enzyme was determined using two different metals concentration (2 mM and 10 Mm) for 30 minutes. The effect of metal ions was determined in terms of relative activity.

3.21.4 Surfactant effects on the Activity of the Enzyme

The effect of surfactants i.e. Tween 80, Tween 20, SDS, CTAB, Triton X-100, and Polyethylene Glycol (PEG) on activity of purified chromate reductase enzyme was measured at two different concentrations 0.5% and 1% for 30 minutes. The effect of surfactants was determined in terms of relative activity.

3.21.5Organic Solvent effects on the Activity of the Enzyme

The effects of organic solvents (15% each) include methanol, acetonitrile, acetone, propanol, butanol, ethanol, formaldehyde, glycerol, and ethyl acetate on purified chromate reductase activity were carried out for 30 minutes. The effect of organic solvents was determined in terms of relative activity**.**

3.21.6 Determination of Kinetic Parameters

Chromate reductase activity was determined at 40°C, pH 7.2 in a reaction mixture with various concentrations of substrate (0.1-1 mM). The V_{max} and K_m values were determined by plotting the results on the Lineweaver-Burk software.

3.22 Bioreduction of Hexavalent Chromium by Purified Chromate Reductase

Effluent was prepared by adding 500 mg/L of $K_2Cr_2O_7$to sterile distilled water and then treated with varying concentration of crude and purified enzyme in terms of percentage (1-10 v/v). The samples were incubated at 35˚C for 72 hour and concentration of remaining chromium in the effluent was measured using DPC method after every 24 hours.

3.23 Cloning and Expression of Chromate Reductase and Lead Binding Protein Genes

3.23.1Primer Designing and Gene Amplication

The primers for chromate reductase (ChR) and Lead binding protein (PbR) genes were designed on complete sequences of *Bacillus subtilis* FMN reductase and *Enterobacter cloacae* lead binding protein PbrR obtained from GenBank containing NCO1 and *HindIII* restriction sites in forward and reverse primer (Table 3.3). The conserved regions were identified by aligning sequence with the help of Clustal W program implemented in MEGA 4.0. The ChR and PbR genes were amplified through PCR. A basic PCR reagents and component mentioned in table 3.4. For gene amplification sterilized PCR tubes were taken and labeled as ChR and PbR. 50 µl of PCR reaction mixture were prepared for each sample**.** The reaction mixture contained; Go Taq buffer (5X), 10µl; $MgCl_2$ (25mM), Forward primer (10mM) 1µl, 3µl; dNTPs (10mM), 1µl; Reverse Primer, 1µl; distilled water (Dnase free PCR water), 32 ml Taq Polymerase, 0.5µl; and template DNA of each sample, 1 µl (Table 3.4)

Table 3.4: Components of Bulk Reaction Mixture used for molecular analysis

Stock	**1RXN**
Vol/RXN=50 µl	(µl)
ddH2O	32.5
Primer-F (10mM)	1
Primer-R (10mM)	1
Taq Polymerase	0.5
Go Taq Buffer (5X)	10
Template DNA	2
$MgCl_2$ (25mM)	3
dNTPs (10mM)	1

Table 3.3 Primers detail with PCR conditions used for PbR and ChR Genes

Primers	Gene	PCR Conditions	Sequence
ChR- R	ChR	94°C 3 min	5'-CCATGGGCATATTAGTGATCAGC-3'
ChR- F		58°C 1 min 72°C 1 hour: 40 sec 4°C 5 min	5'-CCATGGGCATATTAGTGATCAGC-3'
PbR- R	PbR	94°C 3 min	5'-AAgCTTCCCAGATGTTTGACTGTTCG-3'
PbR-F		52°C 1 min 72°C 1 hour: 40 sec 4°C 5 min	5'-CCATGGAAATCAGAATTGGCGACC-3'

3.23.2 Ligation of PCR Amplicon into pGEM-T Vector and Transformation into *E.coli* JM101

Ligation of ChR and Pbr genes was performed according to the standard protocol using T4 DNA ligase enzyme. *E.coli* (JM101) competent cells were prepared for the transformation of gene of interest (ChR and Pbr). Total of 200 µL competent cells were transferred separately to two 1.5 mL sterile eppendorf tubes containing 10 µL of ChR and PbR genes. Then the reaction mixtures were kept on ice pad for 30 minutes followed by heating at 42°C for 2 min and then quickly shifted to the ice again for 10 minutes. Afterward 200 µL of Luria broth was added to each Eppendorf and put in shaking incubator for 60 min at 37°C. Then 200 µL sample from each tube was

inoculated on Ampicillin-LB agar plates (100 µg/mL of Amp) and incubated for 14-16 hours at 37°C to observed transformed colonies.

3.23.3 Extraction of Cloned pGEM-T Vector from Transformed Competent Cells

Thermo Scientific GeneJET Plasmid Miniprep Kit was used for plasmid extraction. Pellet of recombinant cells was obtained by centrifugation at 12000 rpm for 3 minutes and 250 µL of re-suspension buffer was added. The solution was quickly vortexed to dissolve pellet and avoids bacterial clumps formation. About 250 µL of lysis solution was added to the tube containing cells suspension and thoroughly mixed by inverting the Eppendorf 10-15 times till the solution appeared cleared and viscous. Neutralization solution (350µL) was added and mixed quickly. The reaction mixture was centrifuged at 13000rpm for 15 minutes to obtained cell debris and chromosomal DNA in pellet. The obtained supernatant was then transferred to GeneJET spin column and again centrifuged for 1 minute at 13000 rpm, the flow-through was discarded. The spin column was placed back,500 µL of wash solution was added and flow-through was discarded after centrifugation for 1 minute at 13000 rpm and flow-through was discarded. The spin column (GeneJET) was shifted to new sterile (1.5 mL) microcentrifuge tube and 50µL of elution buffer was added to elute the plasmid DNA through centrifugation at 13000 rpm for 2 minutes. The eluted plasmid DNA was confirmed by loading on to 1% agarose gel and DNA bands were observed under transilluminator.

3.23.4 Digestion of pGEM-T Cloned Genes and pET28a Vector with Restriction Enzymes

The 500 ng of extracted pGEM-T cloned vector and pET28a expression vector was first digested with restriction enzyme *HindIII*. The reaction mixture contained; *HindIII* (10 U/µL), 1.5µL; rCutSmarttm buffer*, 6*µL and *Plasmid52.5*µL. The reaction mixture of both vectors for digestion was incubated at 37°C for 2 hours and digestion products i.e. pGEM-T vector, insert and pET28a were confirmed on 1% agarose gel. After confirmation on agarose gel both inserts and pET28a plasmids were further treated with ethanol to remove impurities. For this, sodium acetate buffer (3M) having

pH 5.2 (1/10 times of total volume) was added to the tubes containing pET28a and Inserts separately and tubes were inverted 10-15 times. 2.5 volume of chilled 96% ethanol was added and mixed by vortexing, then were kept in -20°C overnight followed by centrifugation at maximum speed for 5 min to discard supernatant. The pellet in the tube was dissolved in 70% ethanol, and then centrifuged at maximum speed for 5 min, lastly Supernatant was removed and pellet was dried at 37°C. The pellet was resuspended in TE buffer (pH 8.0). The ethanol precipitated pET28a vector and insert were then digested with second restriction enzyme *NCO1* by keeping at 37ºC for maximum 2 hour. The digestion of pET28a vector and insert was confirmed with 1% agarose gel. The reaction mixture for second digestion contained; *NCO1* (10 U/µL), 1µL; *rCutSmart*^{tm}*buffer, 2.5*µL; *Pet28a/Insert, 18*µL; and NP *Water,* 3.5 µL.

3.23.5 Ligation of the Digested Genes into pET28a Vector and Transformation into *E.coli* BL21 (DE3)

The double digested inserts (PbR and ChR genes) were ligated separately to the double digested pET28a vector using T4 Ligase Enzyme and mixtures were incubated at 16°C for 12 hours. The composition of ligation mixture;

10 XT4 DNA Ligase Buffer	**1µL**
PbR/ ChR gene insert	*4/3*µL
T4 DNA Ligase	*1*µL
pET28a plasmid	*5* µL

Then 200µL competent cells of expression host *E. coli* BL21 (DE3) were prepared in $CaCl_2$ suspensions and transferred to each of two 1.5 mL eppendorf tube and added 10 µL of each pET28a ligated ChR and PbR genes separatly. Then the reaction mixtures were kept on ice pad for 30 minutes followed by heating at 42°C for 2 min and then quickly shifted to the ice again for 10 minutes. Afterward 200 µL of Luria broth was added to each Eppendorf and put in shaking incubator for 60 min at 37°C. Then 200µL sample from each tube was inoculated on Kanamycin-LB agar plates (100 µg/mL of Kan) and incubated for 14-16 hours at 37°C to observed transformed colonies. Single recombinant colony was picked via sterile toothpicks and inoculated

in kanamycin (100µg/mL) LB broth and incubated at 37°C in orbital shaker for 16 hours.

3.23.6 Isolation of Cloned Pet28a Vector from Transformed *E.coli* BL21 Competent Cells

Overnight broth culture of *E.coli* BL21 was centrifuged at 13000 rpm for 3 minutes, Supernatant was discarded. Then 250 µL of resuspension buffer was added to the pellet and vortexed to resolve the pellets completely (so no bacterial clumps remain). About 250 µL of lysis solution was added to the tube containing cells suspension and thoroughly mixed by inverting the Eppendorf 10-15 times till the solution appeared cleared and viscous. Neutralization solution (350µL) was added and mixed quickly. Then the whole mixture was centrifuged at 13000 rpm for 15 min. The supernatant was transferred to GeneJET spin column with the help of pipette without disturbing white precipitates. The column was again centrifuged for 1 min at 13000 rpm and discarded the flow-through. The spin column was placed back and 500 µL of wash solution was added, centrifuged at 13000 rpm for 1 minute and discarded the flow-through. The spin column (GeneJET) was then shifted to new sterile microcentrifuge tube (1.5 mL). The plasmid DNA was eluted by adding 50µL of elution buffer to spin column, centrifuged at 13000 rpm for 2 minutes. The eluted plasmid DNA was confirmed on 1% agarose gel.

3.23.7 Double digestion of pET28a containing Pbr and ChR genes

The pET28a Vectors containing ChR and PbR genes separately were treated with restriction enzymes *HindIII and Nco1*. The reaction mixture of each gene was mixed well by inverting the tube and incubated at 37°C for 2 hours. The digestion of pET28a vectors containing gene of interest was confirmed on 1% agarose gel followed by electrophoresis. The reaction mixture was contained of; *NCO1* (10 U/µL), 0.2µL; *rCutSmart^{tm}buffer,* 1µL; *HindIII,* 0.2µL*; Plasmid,* 6µL and *NP Water,* 2.6 µL.

3.23.8 Sequencing of Cloned ChR and PbR gene

For the determination of sequences of Chromate reductase gene (ChR) and lead binding Protein gene (PbR), plasmids containing ChR and PbR gene were purified using PCR Clean-Up System Kit (Promega Corp., Madison, WI, USA) and Wizard®

SV Gel. Then plasmids were sequenced using Taq Dye Deoxy Terminator Cycle Sequencing Kit according to the manufacturer's instructions and analyzed with an Applied Biosystems (Macrogen, Korea, Model370A automatic sequencer).

3.23.9 Bioinformatics analysis

Nucleotides analysis their deduced amino acids were carried out by using BLASTp and BLASTn programs respectively (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment analysis was carried out by using CLUSTALW program for ChR and PbR (http://www.ebi.ac.uk/clustalW). Dendrogram construction and phylogenetic analysis for chromium reductase and lead pbrwas performed using MEGA 6.0 software.

3.23.10 Expression of ChR and PbR gene *E. coli* BL 21

The transformed *E. coli* BL21 (DE3) competent cells harboring pET28a-ChR vector and pET28a-PbR vector respectively were cultured overnight in 10 mL LB medium amended with kanamycin (50 ug/mL) at 37 ◦C. then OD at 600nm was calculated and 2ml of culture was inoculated to 30 mL fresh LB medium, placed in shaking incubator at 37 ◦C. When an OD reached to 0.6 at 600 nm, then 0.5 mM IPTG was added and cultures were induced and incubated at 20 ◦C in shaking incubator for 16 h. The induced cells were collected by centrifugation at 13,000 rpm for 10 min and re-suspended into 0.2M Potassium phosphate buffer (pH 7.5). Cells were sonicated to release intracellularly produced chromate reductase and lead binding proteins separately using ultrasonic homogenizer (Sartorius Labsonic M, 80 % amplitude, 0.6 cycles for 5 min). Centrifugation was performed for 15 minutes at 13000rpm in order to remove the cell debris and clear supernatants were obtained.

3.24 Chromate Reductase Purification from Recombinant Strain BL21 (DE3)

The chromate reductase enzyme from recombinant BL21was purified used 3 steps. Precipitation by ammonium sulfate, dialysis, and DEAE Sepharose chromatography.

3.24.1 Ammonium Sulfate Precipitation

Cell-free supernatant was recovered after centrifugation at 4˚C and 10,000 rpm, in a separate flask. 80% of ammonium sulfate using online calculator was added slowly

into the supernatant with continuous stirring on magnetic stirrer. The solution mixture was stored overnight at 4˚C and then centrifuged to collect the supernatant and pellet separately. The pellet was resuspended in phosphate buffer having pH 7.5. Enzyme assay and protein quantification of both supernatant and pellet was performed to calculate their specific activities. Lowery's method was used for protein estimation. The same protocol was repeated and ammonium sulphate concentration was gradually increased in the supernatant upto maximum 80%.

3.24.2 Dialysis of Crude Enzyme

Dialysis was performed in order to remove ammonium sulfate from enzyme in buffer solution recovered after ammonium sulphate precipitation. The crude enzyme was re-suspended in phosphate buffer (pH 7.5) was transferred to a dialysis bag and placed in the same phosphate buffer at 4˚C for 24 hours.

3.24.3 DEAE Sepharose Chromatography

The ammonium sulphate precipitated proteins were further purified using DEAE Sepharose anion-exchange column chromatograph (1.5 × 50 cm). Sepharose Fast Flow ion exchange media were pre-swelled in 20% ethanol and then this gel slurry was transferred to the column. The DEAE-Sepharose column was pre-equilibrated with 10 mM sodium phosphate buffer pH 6.0. Then the partially protein proteins were eluted with linear gradient of 0.55M NaCl in 10mM sodium phosphate buffer (pH 6.0) at flow rate 0.5 mL/min. Total of 25 fractions were collected and checked for chromate reductase activity. The fractions contained enzyme were then combined and concentrated by using ultrafiltration (Sartorious, 30000 MWCO filters).

3.24.4 Determination of Molecular Weight through SDS-PAGE

The purity and molecular size of Chromate reductase was determined by SDS-PAGE. 12% gel was used in SDS-PAGE to run the sample and Coomassie brilliant blue R-250 dye was used to stain gel. (Laemmli, 1970).

3.25 Characterization of Purified Recombinant Chromate Reductase

The purified chromate reductase was characterized by observing the effect of various physical and chemical factors and relative activity of enzyme was determined by the following formula (Hora et al., 2015)

Residual Activity (%) = (Activity (U/mg) of partially purified enzyme / Activity (U/mg) of the purified enzyme) × 100

3.25.1 Effect of Temperature on the Activity and Stability

The effect of temperature on activity of purified chromate reductase enzyme was measured at various temperatures ranging from 30-60˚C for 30 minutes. The thermal stability of enzyme was determined by pre-incubating it at varying temperatures from 30-60˚C in the absence of substrate ($K_2Cr_2O_7$) and activity was monitored after every 30 minutes interval upto the maximum of 120 minutes. The effect of temperature was determined in terms of relative activity.

3.25.2 Effect of pH on the Activity and Stability

The effect of pH on activity of purified chromate reductase enzyme was measured at various pH ranges 3.0-9.0 for 30 min. The pH stability of enzyme was determined by pre-incubating it at varying pH from 3:0 -9:0 in the absence of substrate and activity was monitored after every 30 minutes' interval up to the maximum of 120 minutes. The effect of pH was determined in terms of relative activity.

3.25.3 The Effect Metal Ions, Surfactants and organic solvent on Enzyme Activity

The effect of various metal ions i.e. barium sulfate, calcium chloride, copper sulfate, magnesium sulfate, calcium sulfate, zinc sulfate, copper chloride, potassium chloride, sodium chloride, mercury sulfate, ferrous sulfate, nickel chloride, potassium sulfate, cobalt chloride, urea, nickel sulfate, and sodium sulfate on activity of purified chromate reductase enzyme was determined using two different metals concentration (2 mM and 10 Mm). Similarly the effect of surfactants i.e. Tween 80, Tween 20, SDS, CTAB, Triton X-100, and Polyethylene Glycol (PEG) was measured using two different concentrations 0.5% and 1% and the effects of organic solvents (15% each) include methanol, acetonitrile, acetone, propanol, butanol, ethanol, formaldehyde, glycerol, and ethyl acetate were also determined. The effect of organic solvents was determined in terms of relative activity**.** For determination the effects of these chemicals enzyme was incubated with all mentioned chemicals for 30 min and activity was checked. The effects of chemicals were determined in terms of relative activity.

3.26 Bioreduction of Hexavalent Chromium by Recombinant Chromate Reductase

Effluent was prepared by adding 100-500 mg/L of $K_2Cr_2O_7$to sterile distilled water and then treated with 10mg/mL of recombinant purified enzymes in term of percentage. The samples were incubated at 35˚C for 72 hours and concentration of chromium was analyzed after every 24 hours using DPC method.

3.27 Batch Biosorption Assay for Lead Removal using Ceramic Bioballs

The lead biosorption assay was carried out using ceramic balls as supporting material, obtained from local market. Ceramic balls were taken in250-mL Erlenmeyer flask containing 150 mL LB medium with pH 7.5 and sterilized at 120 °C for 20 min to discharge air from balls pores. The flask was then inoculated with recombinant strain S54 (*Escherichia* Sp.) and incubated in shaking incubator at 30°C and 150 rpm for 72 hours for exponential growth and biofilm formation. After incubation, the media was discarded and the ceramic balls harboring biofilms were washed twice with 0.1 M NaCl solution. The ceramic balls harboring biofilms were then shifted to new sterilized Erlenmeyer flasks (250-mL) containing 100 mL of Pb (100 mg/L) solution and incubated at 30 °C for 48 hours in shaking incubator. After incubation Pb solution was filtered using O.22 mm cellulose filtered membrane and 1 mL of filtrate was acidified with 1 mL 30% nitric acid and diluted into 20 mL deionized water. Then the final solution was filtered using 0.20 Millipore syringe filter and analyzed through inductively coupled plasma mass spectrometry (ICP-MS). Whereas adsorption of lead onto ceramic balls harboring biofilms were determined through FTIR, and SEM analysis.

3.27.1 Fourier-Transform Infrared Spectroscopy (FTIR) Analysis of strain S54 (*Escherichia* Sp.)

Fourier- transform infrared spectroscopy (FT-IR) was used to analyze the functional groups and overall nature of chemical bonds groups on the ceramic support. The balls were dried at 50°C for 16 hours and then ground in an agate mortar until obtaining a homogeneous powder. The FT-IR analysis of the homogeneous powder with and without bacterial biofilms was performed using PerkinElmer Spectrum Version

10.4.3. The spectral data was collected over the range of 450–4000 cm^{-1} at a resolution of 4 cm^{-1}.

3.27.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) (Carl Zeiss, Oberkochen, Germany) was performed to observe the biofilm formation of the strain S54 (*Escherichia* Sp.) on ceramic balls. Two ceramic balls obtained after biosorption experiment were twice washed with PBS buffer. The balls were dried at 60°C overnight then ground in an agate mortar until obtaining a homogeneous powder. The samples were coated with gold and used for SEM-EDX analysis.

3.28 Removal of Lead using Column Biofilter

The column biofilter (10 × 76 cm) was designed and developed for removal of heavy metals. The glass column was filled with Ceramic bioballs (500g) and inoculum of strain S54 (*Escherichia* Sp.) was prepared in 500 mL of nutrient broth, which were then pumped through tube to the column followed at flow rate 0.4 mL/min. The total biomass immobilized and retained was 1.9519 g before the start of the biosorption cycles. The bed was washed twice with sterile deionized water at a flow rate of 4 mL/min. Afterward Continuous biosorption experiment was started by slowly pumping the metal solutions (50 mg/L) the column followed at flow rate 0.4 mL/min at room temperature. Samples (5 mL) were drawn at different time intervals for upto 96 hours and analyzed for Pb concentration by ICP-MS. The column experiment, metals effluents concentration reached to constant value then it was stopped. When all Pb solution in the column was eluted, the column was washed with 0.1 M NaCl until the pH stabilized at a value of 7.0. The cleaned column was used for next adsorption cycle to determine the potential reusability of the biofilm in many operation processes. The samples collected at different time interval were analyzed through ICP-MS and changes in bacterial biomass were determined through analytical techniques i.e. FT-IR and SEM.

CHAPTER 4

RESULTS

Results

4.1 Samples Collection and their Physico-Chemical Analysis

Sludge samples were collected from two different sites, River bank of Lyari (24˚52′46.0″N 66˚59′25.7″E) and Korangi industrial area (24˚48′37.5″N 67˚06′52.6″E) located in Karachi city, Sindh province, Pakistan, which are highly polluted and contain considerable amount of heavy metals like chromium, lead, etc. Three different parameters (temperature, metal concentration and pH,) were determined through chemical and physical analysis. Temperature and pH of the sludge samples from Korangi were recorded as 30ºC and 8.0, whereas for Lyari sample, it was recorded as 30ºC and 7.0. The chemical analysis of industrial waste samples was examined by mean of atomic absorption spectroscopy (AAS) (AA-7000, Shimadzu, Japan), which showed that chromium and lead were at high concentration in Korangi as compared to Lyari samples. The total chromium and lead concentration in Korangi sludge was 2648.64and 239.889 mg/Kg whereas in Lyari sludge was about 2460.97and 339.13mg/Kg, respectively (Table 4.1)

Table 4.1 Physico-chemical parameters of samples from Korangi and Lyari regions, Karachi

Physical parameters			**Metals concentrations mg/kg**				
Sample	**Temp**	**Ph**	**Cu**	**Cr**	**Pb**	**Ni**	**Co**
Korangi sludge	30^0C	8	797.66	2648.64	239.88	983.02	28.55
Lyari sludge	45^0C	7	829.48	2460.97	339.13	209.79	62.11

4.2 Isolation and Enumeration of Chromium and Lead Resistant Bacteria

Total of 53 bacterial strains from sludge samples were isolated on nutrient agar plate. 40 bacterial strains were isolated from Korangi sludge sample and 13 bacterial strains from Lyari sludge sample. Colonies forming unit/ml were calculated as 2×10^{-6} for Korangi sludge whereas 5×10^{-4} for Lyari sludge.

4.3 Primary Screening for Chromium and Lead Resistant bacteria

All 53 bacterial strains were found resistant up to 100mg/L of Potassium dichromate ($K_2Cr_2O_{7)}$ and Lead nitrate $Pb(NO_3)_2$ on a nutrient agar plate. Out of 53 isolates, seven bacterial isolates designated as (S9, S13, S17, S18, S30, S35, and S48) were capable to grow in the presence of 300mg/L of $K_2Cr_2O_{7,}$ while 5 bacterial isolates (S29, S35, S46, S51 and S54) could grow in the presence of 300mg/L of $Pb(NO_3)_2$. The resistance level of all isolates was further confirmed through both plate and broth assay by increasing of $K_2Cr_2O_7$ and $Pb(NO_3)_2$ concentration from 300 -1600mg/L. Two of the bacterial isolates designated as S48 and S54 were selected on the basis of their growth on 1500 mg/L of $K_2Cr_2O_7$and $Pb(NO_3)_2$ showed prominent growth on plates, respectively (Fig 4.1 A, B). Broth assay further confirmed resistance of bacterial strain towards increasing concentration of$K_2Cr_2O_7$and $Pb(NO_3)_2$ (Fig 4.2 and 4.3).

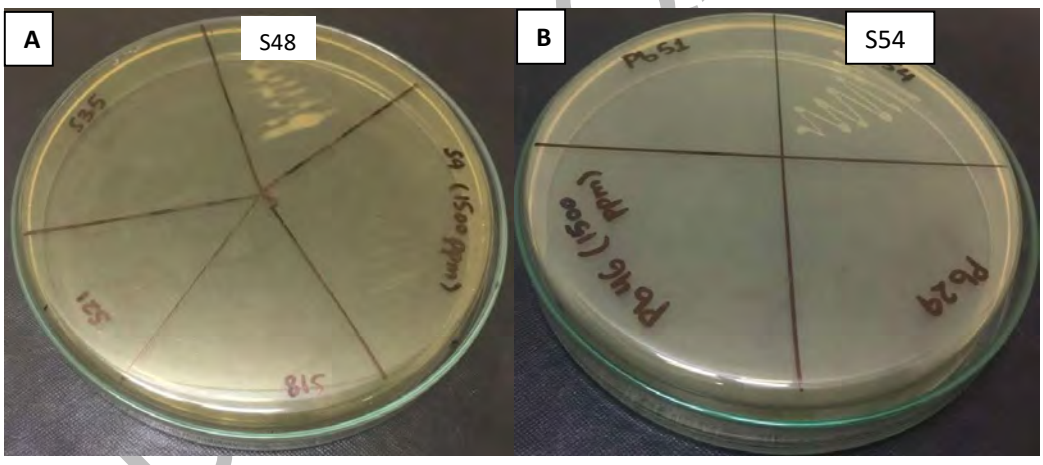


Fig 4.1 Metal Resistance limit of bacterial strain on nutrient agar plate supplemented with heavy metals after 48 hours of incubation. (A) S48 supplemented with1500 mg/L of $K_2Cr_2O_7$ (B) S54 supplemented with 1500 mg/L of $Pb(NO_3)_2$

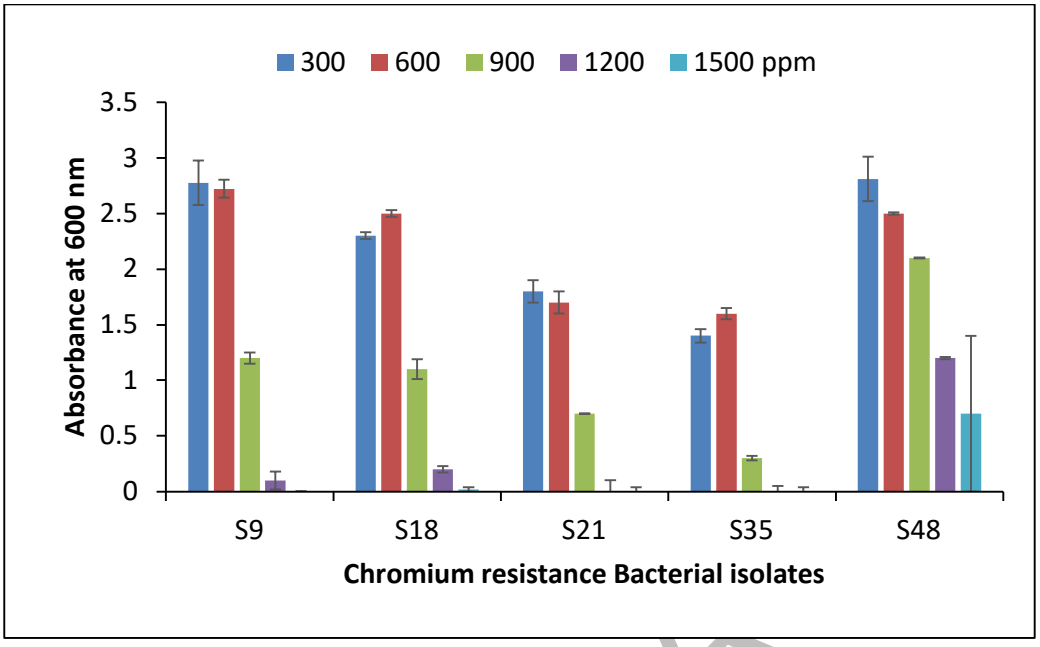


Fig 4.2 Resistance limit determination of bacterial strain 48 via broth assay after 72 hours against varying concentration of $K_2Cr_2O_7$(300-1500 ppm)

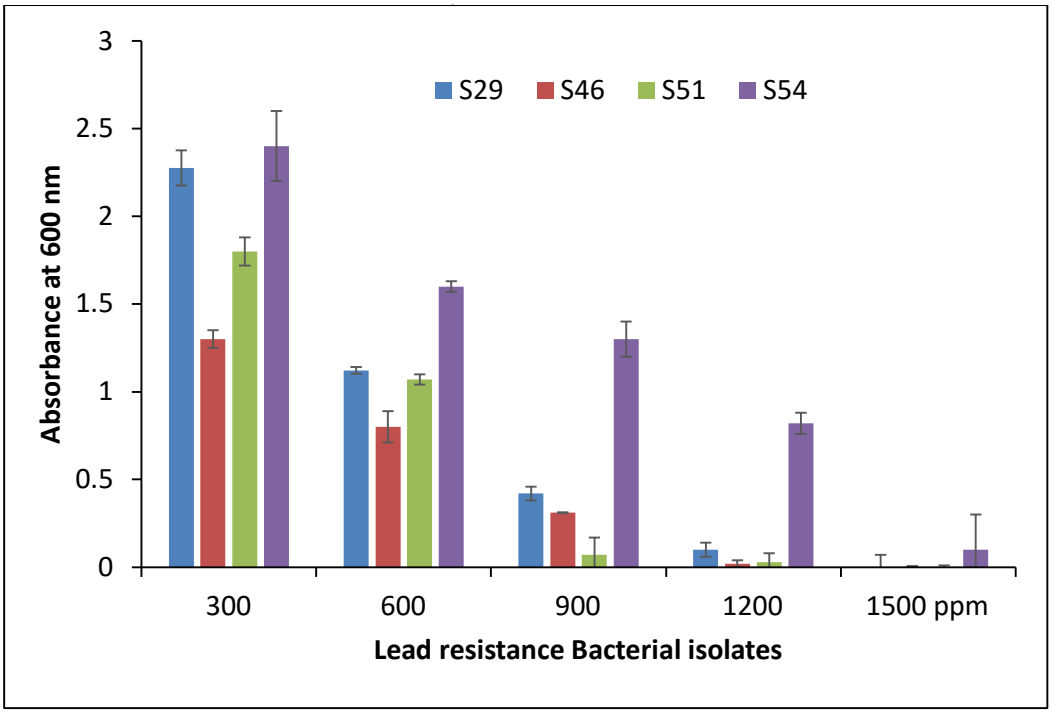


Fig 4.3 Resistance limit determination of bacterial strain S54 via broth assay after 72 hours against varying concentrations of $Pb(NO_3)_2$(300-1500 ppm)

4.4 Identification of Chromium and Lead Resistant Bacterial Strains

4.4.1 Cultural Characteristics

Bacterial strain S48 was observed with flat and off white and rough edges of the colonies while bacterial strain S54 has round, circular, sticky, and whitish to light yellow colonies on nutrient agar plate. Rapid and prominent growth was observed by the strains S48, S54, at temperature 30°C, and 35°C respectively.

4.4.2 Microscopy

Through gram staining, the cellular morphology of bacterial strain S48 was observed gram-positive, while bacterial strain S54 was gram-negative.

4.4.3 Biochemical Characterization

Different Biochemical tests i.e. motility, oxidase, urease, indole, citrate utilization, catalase and triple sugar iron tests were performed for identification and characterization of bacterial strains S48 and S54. Bacterial strain S48 was found positive for oxidase, catalase and motility and was negative for indole, citrate utilization test and triple iron sugar. Whereas S54 strain showed negative results for oxidase, catalase and motility and positive for indole, citrate utilization and triple iron sugar (Table 4.2).

4.4.4 Molecular Identification of Strain S48 and S54

Bacterial strain S48 and S54 were identified by 16S rRNA gene sequencing by comparing the nucleotide sequences present in NCBI databases using search BLAST analysis. The ITS sequence analysis of strain S48 and S54 showed close homology (99%) to *Bacillus paramycoides* and *Escherichia fergusonii* respectively. The S48 and S54 were identified as *Bacillus* Sp. Strain S48 and *Escherichia* Sp. Strain S54. Furthermore, the nucleotide sequence of S48 and S54 was submitted to NCBI database under accession number ON338038 and ON337871 respectively (Figure 4.4 and 4.5).

Table 4.2 Physiological and biochemical characteristics of strain S48 and S54

Tests	Results	
	Strain S48	**Strain S54**
Celullar morphology	Flat and half white	Sticky, and whitish to light pink
Gram staining	Gram positive	Gram negative
Oxidase	Positive	Negative
Catalase	Positive	Negative
Motility	Positive	Negative
Indole	Negative	Positive
Citrate Utilization	Negative	Positive
Triple Sugar Iron	Negative	Positive

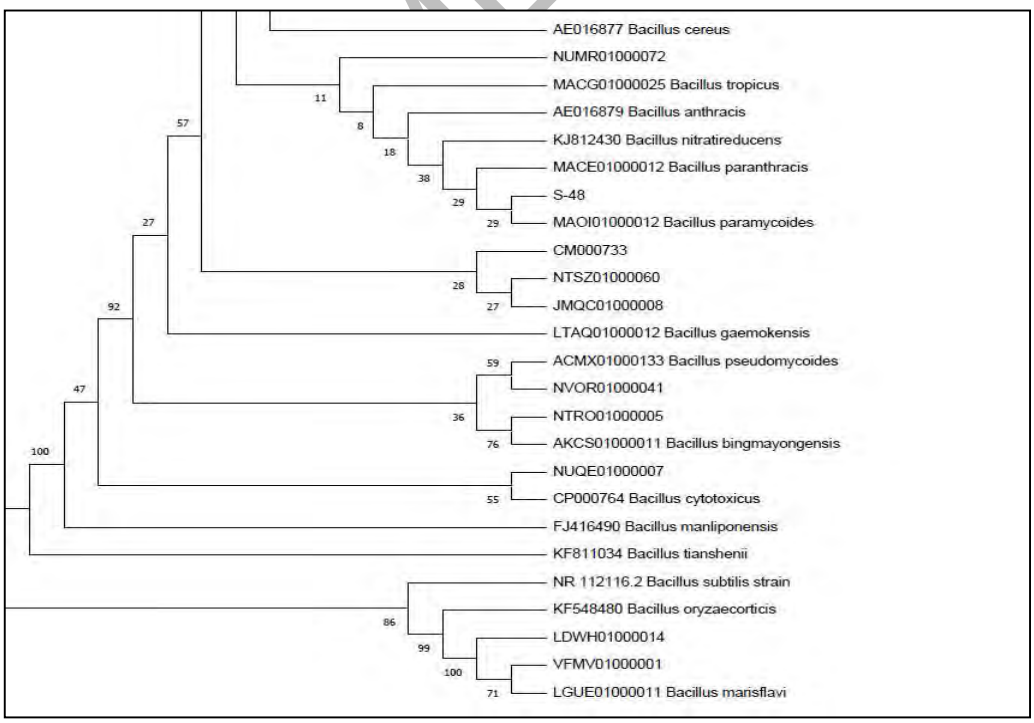


Fig. 4.4 Phylogenetic tree of isolate S48 constructed by maximum likelihood method using Mega X software

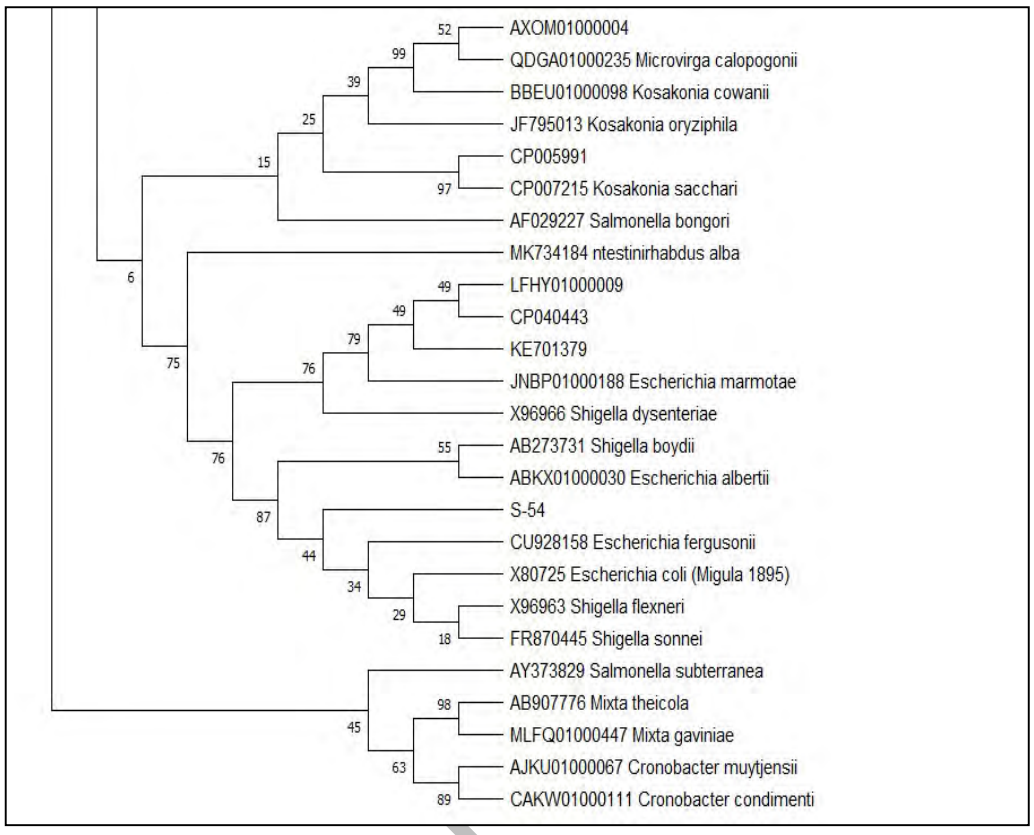


Fig. 4.5 Phylogenetic tree of isolate S54 constructed by maximum likelihood method using Mega X software

4.5 Optimization of Conditions for Chromium and Lead Removal *Bacillus* Sp. Strain S48 and *Escherichia* Sp. Strain S54

4.5.1 Effect of Temperature

The effect of temperature on growth of chromium and lead resistant bacterial strains S48 and S54 were analyzed by incubating at various temperatures ranging from 25-45°C. The highest growth OD (600nm) of strain S48 was achieved at 35°C with more than 50% chromium reduction after 72 hours (Fig 4.6). Similarly, the optimum growth temperature for strain S54was determined at 35°C along with 55% removal of lead was observed after 48 hours of incubation (Fig. 4.7).

4.5.2 Effect of pH

The effect of pH on growth of chromium and lead resistant strainsS48 and S54 were determined using various pH ranges; 5.0-9.0. Maximum growth OD (600nm) of strain S48 was observed at pH 8.0 with 52% reduction of chromium observed after 72 hours (Fig 4.8). Similarly, optimum growth pH for strain S54was observed at pH 8.0 and more than 50% lead removal was achieved after 48 hours of incubation (4.9).

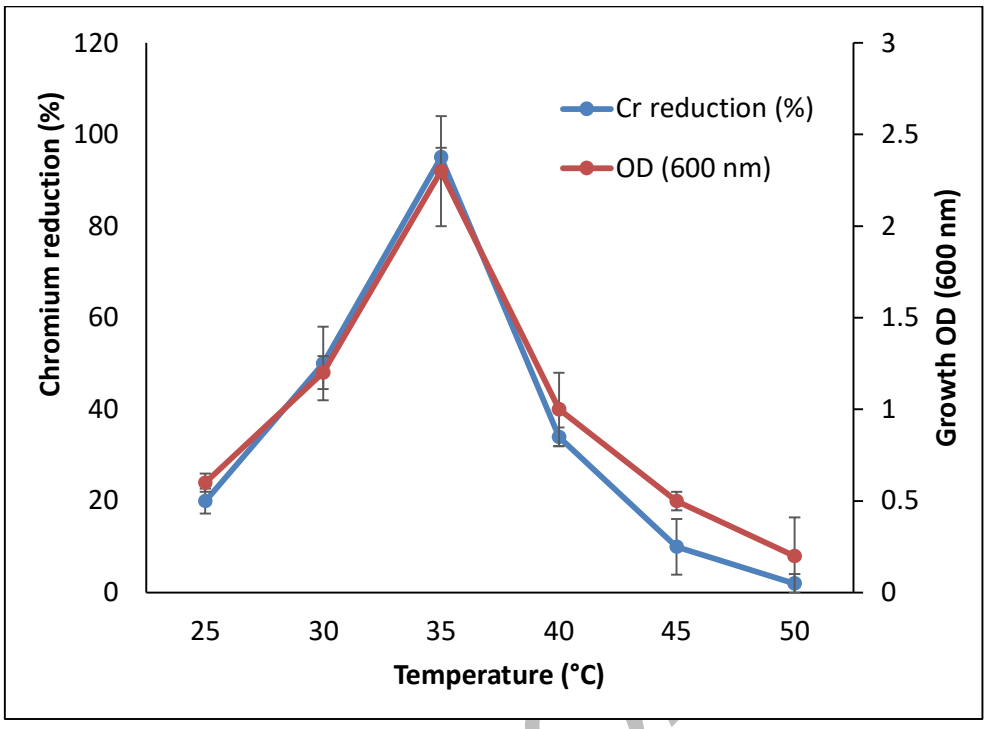


Fig 4.6 Effect of temperature on strain *Bacillus* Sp. Strain growth and chromium removal using (100 mg/L of chromium)

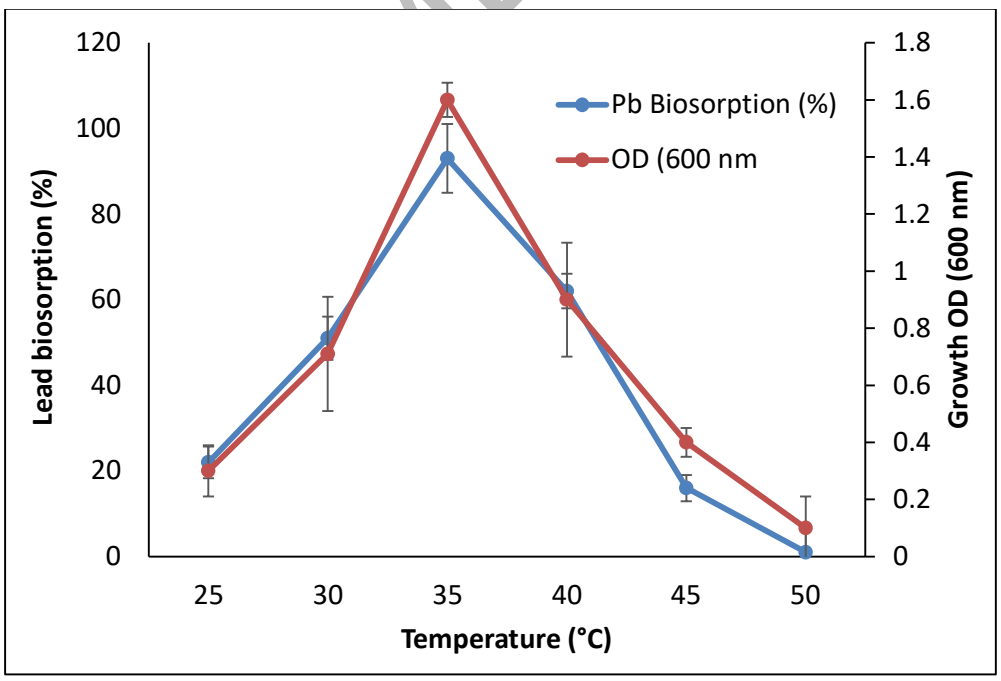


Fig 4.7 Effect of temperature on strain *Escherichia* Sp. Strain S54 growth and lead biosorption using (100 mg/L of Lead)

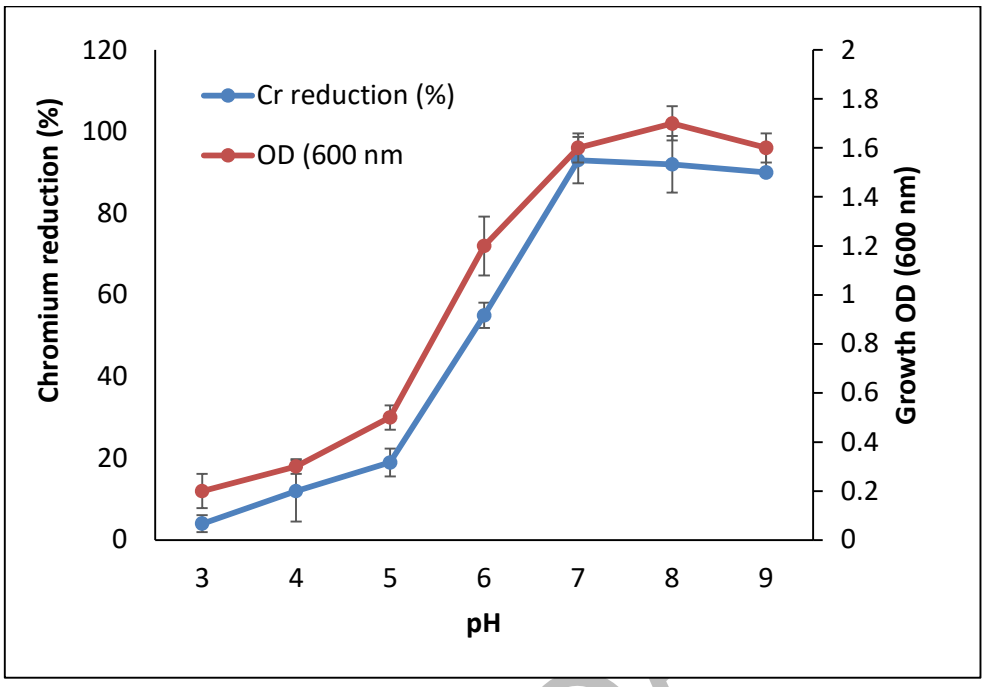


Fig 4.8 Effect of pH on *Bacillus* Sp. Strain S48 growth and chromium reduction using (100 mg/L of chromium)

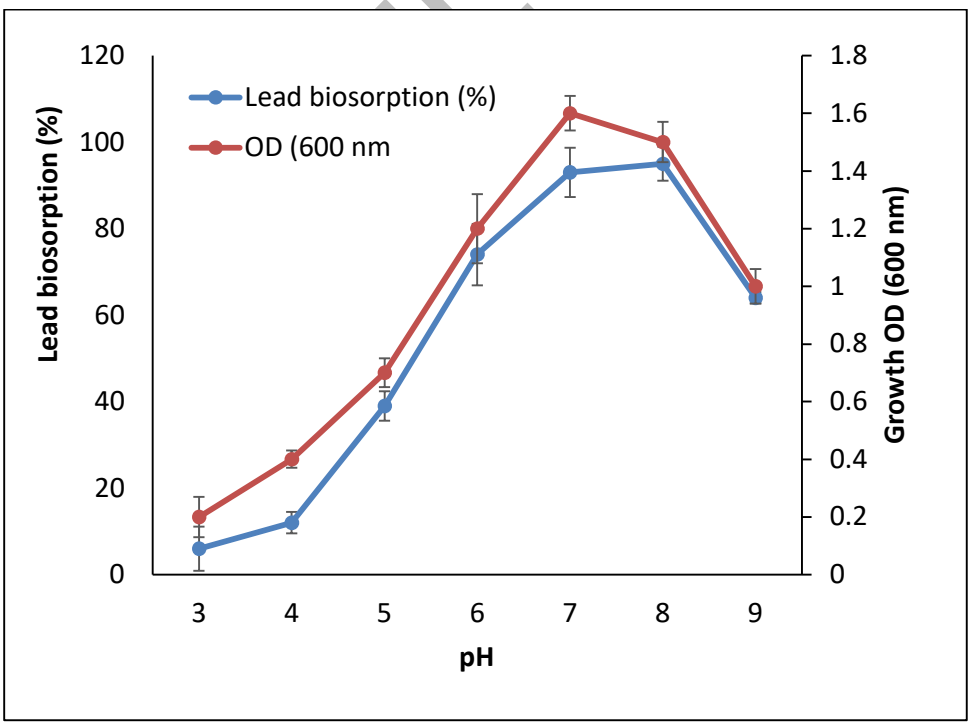


Fig 4.9 Effect of pH on *Escherichia* Sp. Strain S54growth and lead biosorption using (100 mg/L of Lead)

4.6 Reduction of Hexavalent Chromium by *Bacillus* Sp. strain S48

The reduction of Chromium (VI) by *Bacillus* Sp. Strain S48 was determined at varying initial concentrations ranging from 25-500 mg/L using $K_2Cr_2O_7$ as a source of Cr. Strain S48 showed potential to successfully reduce Cr (VI) to Cr (III). Rapid bioreduction of chromium with a concentration of 25 and 50 mg/L was determined after 48 hours of incubation. With an increase concentration K_2Cr_2O7 (100-500 mg/L), more than 60% bioreduction was achieved after 96 hours respectively. The experiment results clearly show the potential ability of strain S48 in the bioreduction of chromium (Fig 4.10)

4.6.1 Functional Group Analysis of *Bacillus* Sp. S48 by FTIR

FTIR analysis of biomass after metal treatment was performed in the range of 400-4000 cm^{-1} to determine the functional groups involved in metal binding. The untreated biomass (control) was also analyzed for comparison. The FTIR spectra of *Bacillus* Sp. strain S48 after treatment with Cr(VI) are shown in Figures 4.11. The peak in the range of 3500-3280 cm^{-1} showed -OH group of glucose and -NH stretching of protein. The peak obtained at 2923.80 cm^{-1} was moved to 2929.89 cm^{-1}after chromium adsorption depicting stretching of CH_3 groups. Similarly, other peaks at1633.96 cm^{-1}, 1525.08 cm^{-1}, 1394.59 cm^{-1}, 1039.74 cm^{-1} and 610.88 cm^{-1} shifted to 1626.81 cm^{-1}, 1516.40 cm^{-1}, 1395.08 cm^{-1}, 1047.01 cm^{-1} and 610.40cm^{-1}, respectively, showed the presence of stretching vibration at C=O, amid bond, COO, phosphate groups and deformation of S=O and –C-C- stretching. In control, two peaks were obtained at 574.67 and 554.95 cm^{-1} which are completely disappeared in Cr(VI) treated sample, furthermore in the chromium treated sample new peak at 539.47 cm^{-1} appeared which was not seen in control sample. The FTIR analysis clearly indicates the effect of chromium metal on different functional groups attached to cell wall of strain S48 and their role in chromium binding (Fig 4.11).

4.6.2 SEM and EDX Analysis of *Bacillus* Sp. Strain S48

The SEM analysis of both chromium treated as well as untreated biomass was performed in order to check the cellular morphology. The micrograph of metal untreated biomass (control) showed elongated, rod shaped, and uniform sized bacterial cells with smooth surface (Fig. 4.12 A), whereas, cells exposed to Cr(VI) were observed with marked changes in its appearance such as increase in width, reduce in length, with rough appearance and clumping of cells (Fig. 4.12 B). EDX analysis of biomass exposed to chromium showed a prominent peak of chromium at a binding energy of 5.4, which attributed to intracellular absorption of chromium by bacterial strain (Fig. 4.12B). However, no peak of chromium was observed in control samples (Fig. 4.12A). Therefore, EDX analysis further confirmed the presence of Cr on bacterial cell surface in comparison of non-treated cells.

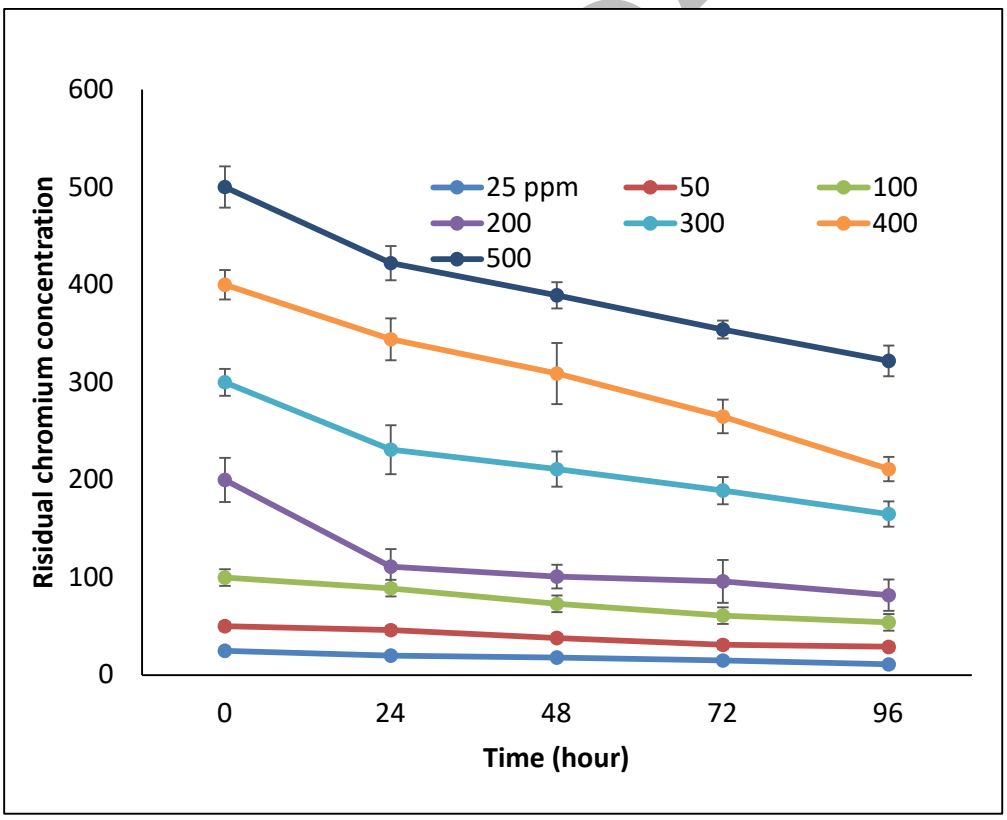


Fig 4.10 Chromium reduction by *Bacillus* Sp. Strain S48 with different initial concentration (25- 500 mg/L)

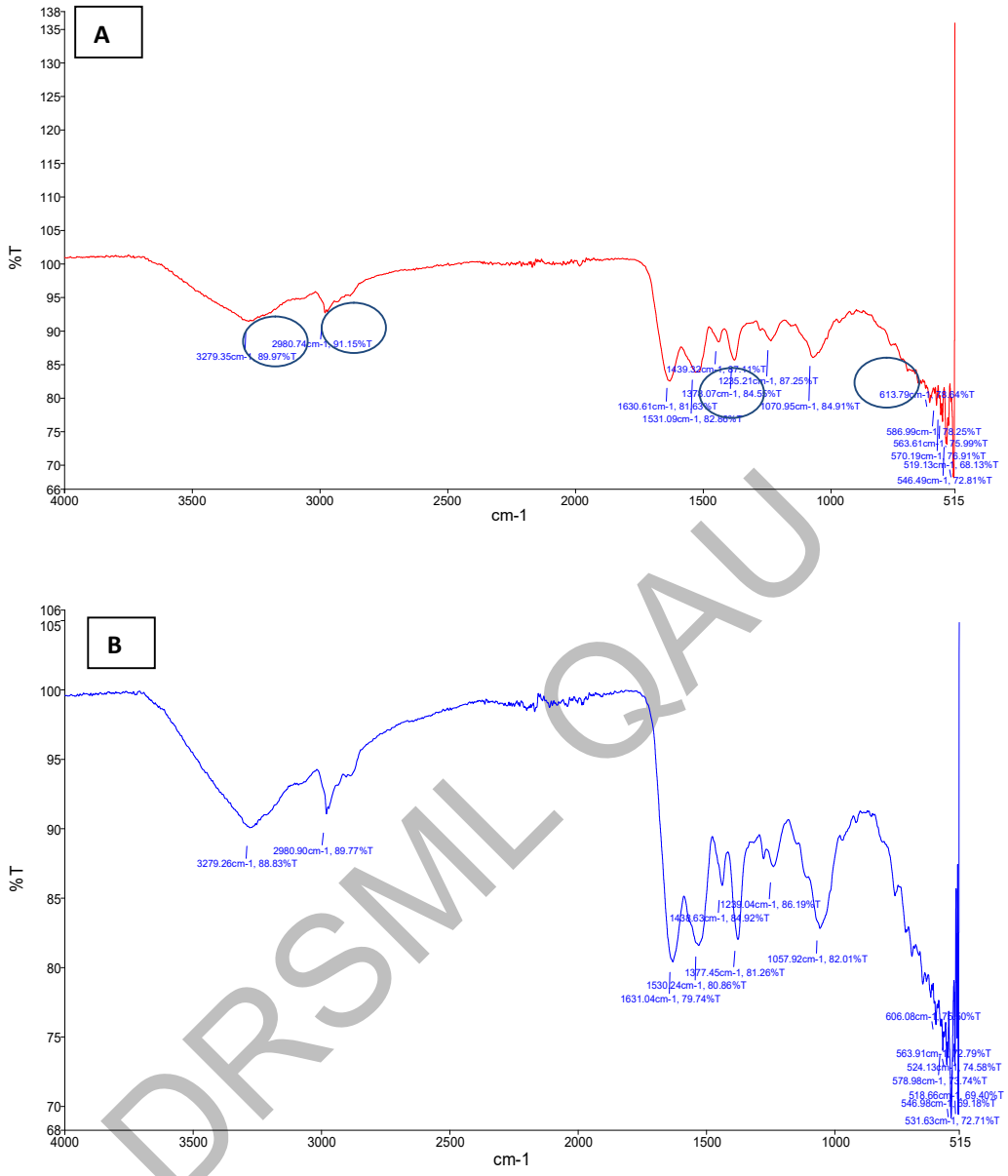


Fig 4.11 FTIR spectra of *Bacillus* Sp. S48 strain **A)** Cr treated **(B)** Cr untreated

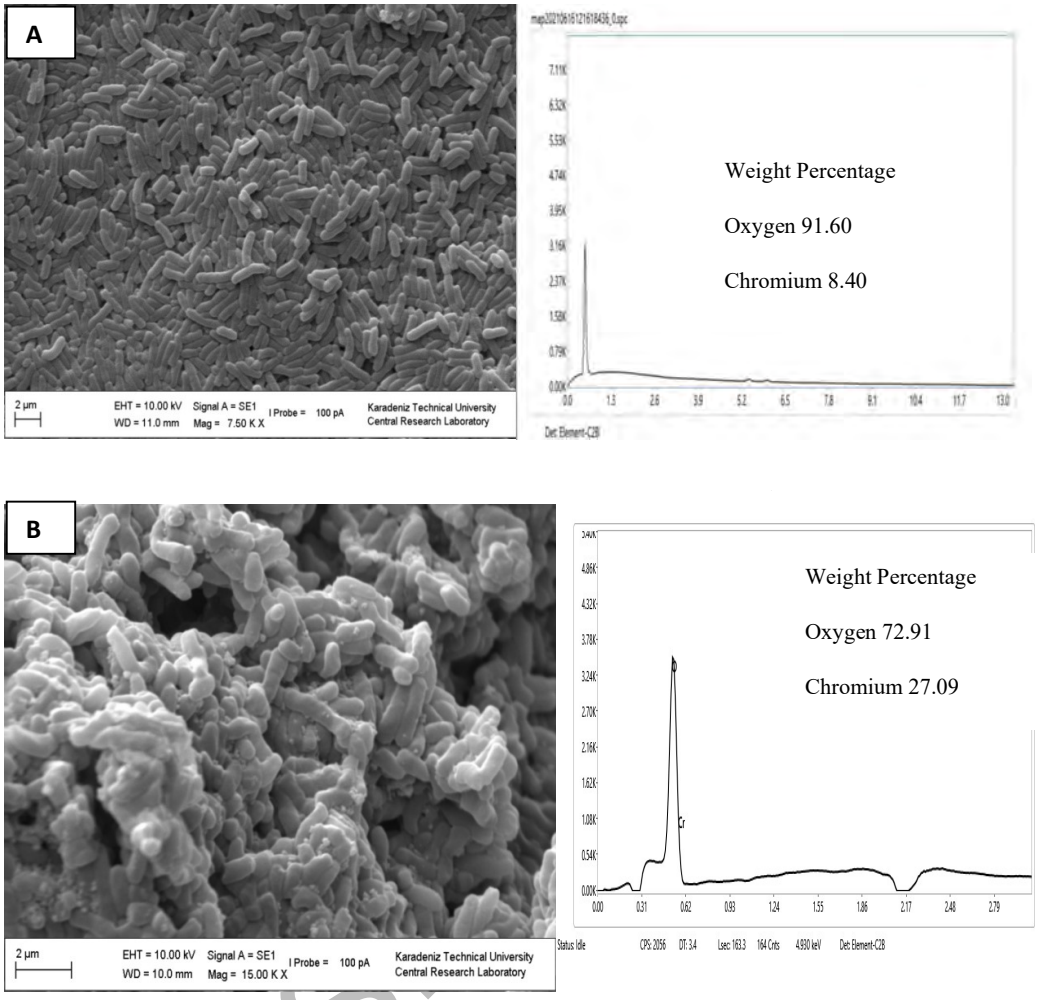


Fig 4.12 SEM images of *Bacillus* Sp. strain S48 after Cr reduction(A) Control S48 without Cr treatment and EDX Spectrum of control containing no detectable Cr Peak (B)S48 treated with Cr and EDX Spectrum of S48 containing detectable Cr Peak

4.7 Biosorption of Lead by *Escherichia* Sp. strain S54

Lead (Pb) biosorption by *Escherichia* Sp. strain S54 was determined by incubation of bacterium in a medium containing different concentration of lead ranges from 25-500 up to 96 hours. The *Escherichia* Sp. strain S54was showed the potential to successfully remove lead from supernatants. The amount of residual lead present in supernatant after the process of biosorption was measured by atomic absorption spectroscopy (AAS). Complete biosorption of lead with concentrations of 25 and 100 mg/L was achieved after 72 hours of incubation. With an increased concentration lead nitrate (200-500 mg/L), more than 60% biosorption was observed after 120hours respectively (Fig. 4.13).

4.7.1 Functional Groups Analysis of *Escherichia* Sp. strain S54

For determination the role of functional groups presents on cell wall surfaces in the binding of Pb metal, FTIR analysis for S54 strain treated with Pb and without treatment with Pb were carried out in the range of 400-4000 cm-1. The peak at position 2900cm^{-1} represents the stretching vibrations of CH2. The increase in the value of the peaks indicates its role in the binding of lead. The peak position at 1500 cm^{-1}, showing starching of the amide group, slightly altered their position after the biosorption. An increase in the intensity at 1394cm^{-1} showed binding with -COO functional group of amino acid and fatty acid derivatives. The peak at 3276.43 cm-1 represents -NH stretching of protein and slightly moved to 3276.90. The peak at 2924.48 cm-1 largely moved to2923.60 cm-1after lead treatment, whereas other peaks at1630.81 cm-1, 1533.24 cm-1, 1395.14 cm-1, 1228.95 cm-1 and 1059.47 cm-1 shifts to 1625.98 cm-1, 1533.28 cm-1, 1398.84cm-1, 1232.18 cm-1 and 1055.90cm-1 respectively, it showed the stretching vibration at -SH, N=N, N-H, -SO3 groups and deformation of C–H groups stretching. In the sample, one additional peak was obtained at 1452.74, which disappeared in control (Fig 4.14).

4.7.2 SEM and EDX Analysis of *Escherichia* Sp. strain S54

The SEM result of *Escherichia* Sp. Strain S54also showed surface morphological changes after Pb Biosorption as shown in Fig. 4.15. In a control without treatment of Pb, the S54 cells were clear rod-shaped and intact with a smooth surface. Whereas the

sample treated with Pb, cells were found dense, distorted and stuck to each other due to the toxic nature of Pb metal. The adherence physical disintegration of the bacterial cells showed the shrinkage of the total surface area exposed to Pb metal. Whereas, The. EDX analysis of Pb metal treated cells of S54 showed high peaks of Pb at binding energy ranges 0.5-5.4, which showing the total load of the presence of Pb metal onto the S54 cell surfaces. No such peaks were observed for control S54 without treatment of Pb metal.

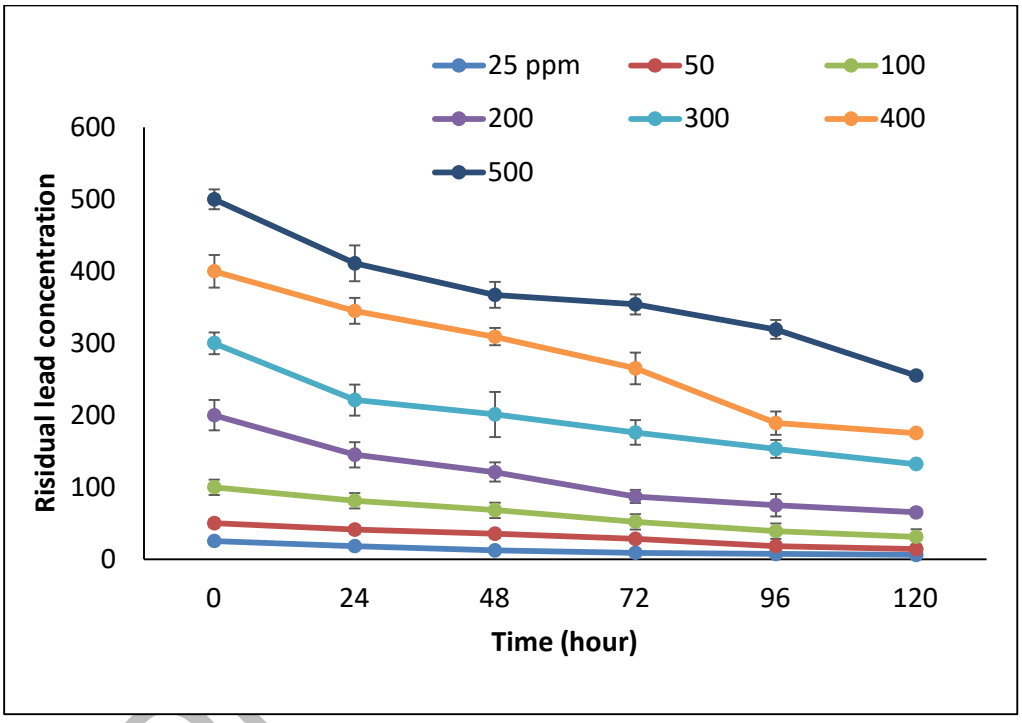


Fig 4.13 Lead biosorption by *Escherichia* Sp. Strain S54 with different initial concentration (25- 500 mg/L)

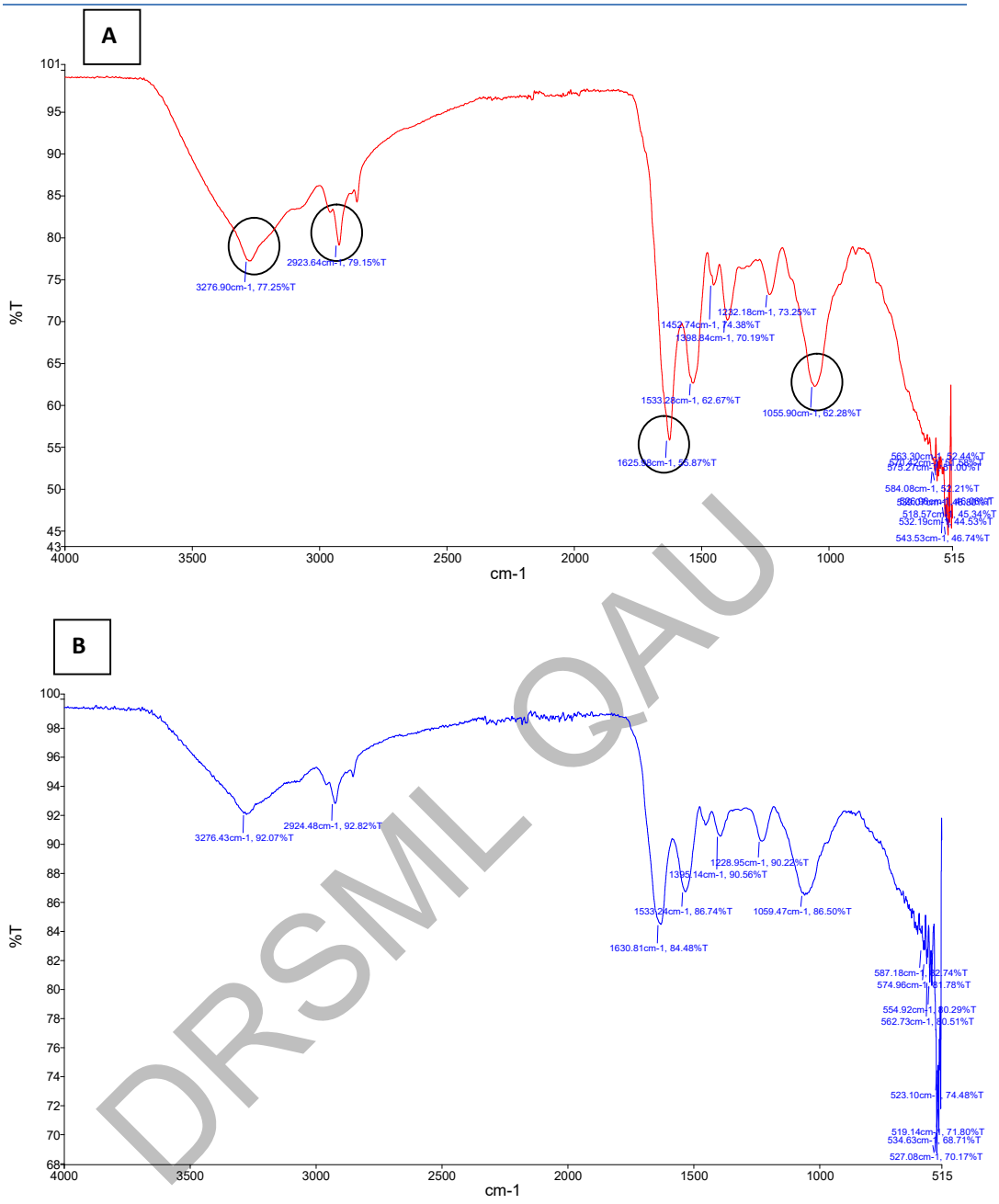


Fig 4.14 FTIR spectra of *Escherichia* Sp. strain S54 (A) treated with Pb (B) without Pb treatment

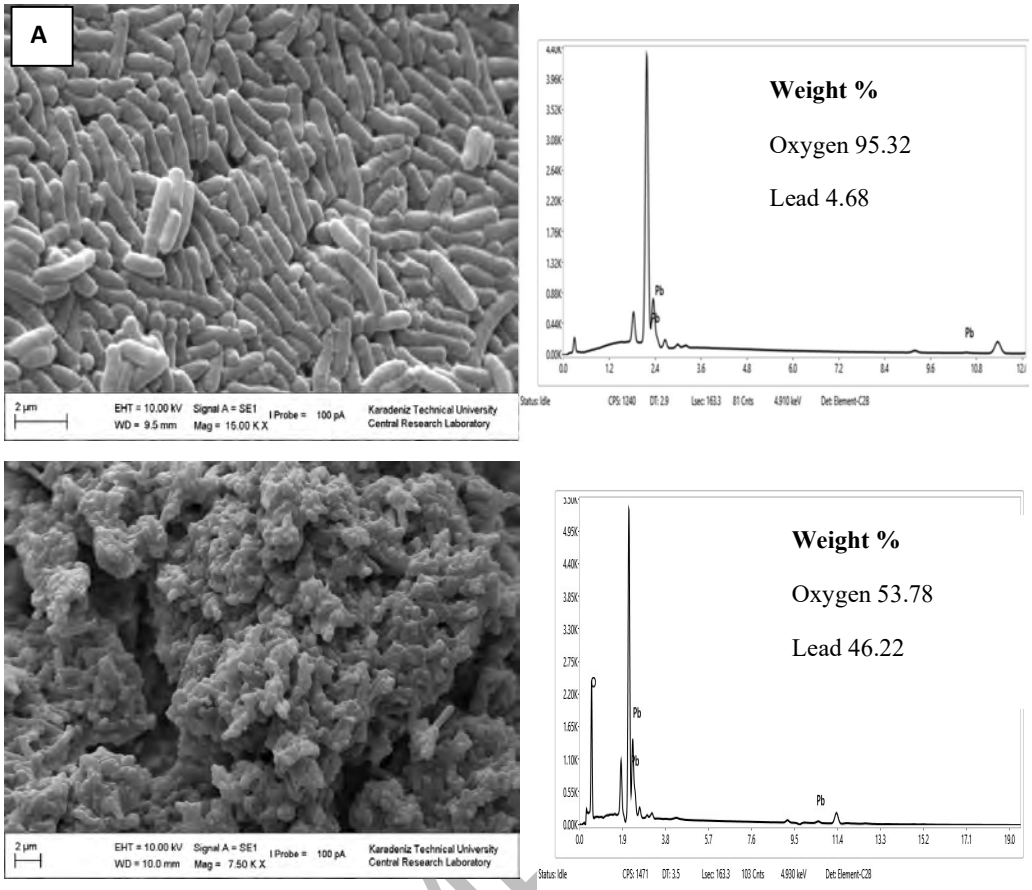


Fig 4.15 SEM images of *Escherichia* Sp. Strain S54 after Lead biosorption (A) S54 without lead and EDX Spectrum of S54 containing no detectable Pb Peak(B) S54 with Lead and EDX Spectrum of S54 containing detectable Pb Peak.

4.8 Production of Chromate Reductase Producing *Bacillus* Sp. Strain S48

Bacillus Sp. strain S48 was screened for chromate reductase activity. *Bacillus* Sp. strains S48 was grown in a defined medium (g/L: KH_2PO_4 3g/L, $CaCl_2$ 0.1g/L, Na_2HPO_4 6g/L, NaCl 0.5g/L, Sucrose 10.5g/L, $MgSO_4.7H_20$ 0.1g/L, and $K_2Cr_2O_7$ 100mg/L) for production of chromate reductase. Strain S48 showed maximum activity (211.32 U/mg) for chromate reductase after 48 hours of incubation.

4.9 Standardization of Chromate Reductase Enzyme Assay

After confirmation of chromate reductase production by *Bacillus* Sp. strain S48, the assay parameters were optimized. The enzyme assay was determined using the optimized assay conditions, i.e., enzyme concentration 400 µL, substrate concentration 200 µL at 35°C and pH 7.0 incubated for 50 min.

4.10 Optimization of Conditions for Chromate Reductase Production

4.10.1 Effect of Temperature on Production of Chromate Reductase

The effect of temperature on production of chromate reductase was determined by incubation of production medium inoculated with strain S48 at various temperatures from 25 to 45°C. Samples were collected after every 24 hours upto maximum 72 hours for protein estimation and enzyme assay. Maximum specific activity (190.37 U/mg) was achieved at 35°C after 48 hours of incubation (Fig. 4.16).

4.10.2 Effect of pH on Chromate Reductase Production

The effect of pH on production of chromate reductase was determined by incubation of production medium inoculated with strain S48 at various pH ranges (5.0–10.0). Samples were collected after every 24 hours up to maximum 72 hours for protein estimation and enzyme assay. Maximum specific activity (258.71 U/mg) was achieved at 35°C after 48 hours of incubation. Enzyme activity was decreased below pH 6.0 and above pH 8.0 (Fig. 4.17).

4.10.3 Effect of Incubation Time on Chromate Reductase Production

The effect of incubation time on chromate reductase activity was analyzed after every 24 hours up to 72 hrs. Maximum specific activity (161.92 U/mg) was achieved after 48 hours of incubation (Fig. 4.18).

4.10.4 Effect of Inoculum Size

The effect of inoculum size on chromate reductase was determined after every 24 hours up to the maximum of 72 hours. The maximum specific activity (232.8 U/mg) was achieved at 1% inoculum size after 48 hours of incubation (Fig. 4.19).

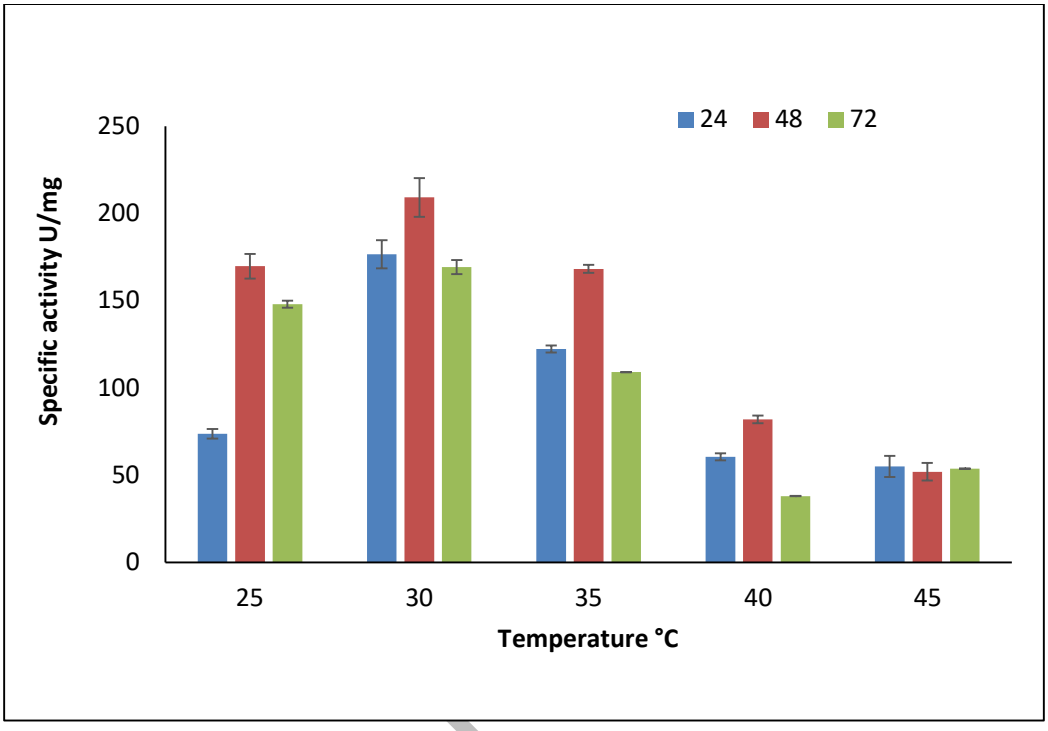


Fig 4.16 Effect of different temperatures on production of the chromate reductase by *Bacillus* Sp. strain S48

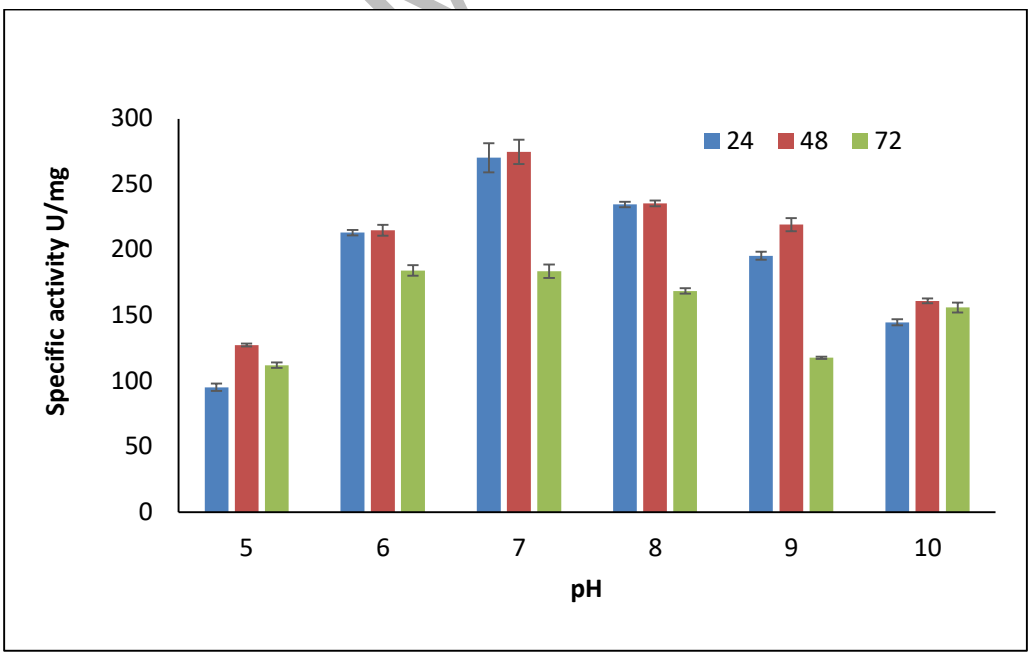


Fig 4.17 Effect of different pH on the production of the chromate reductase by *Bacillus* Sp. strain S48

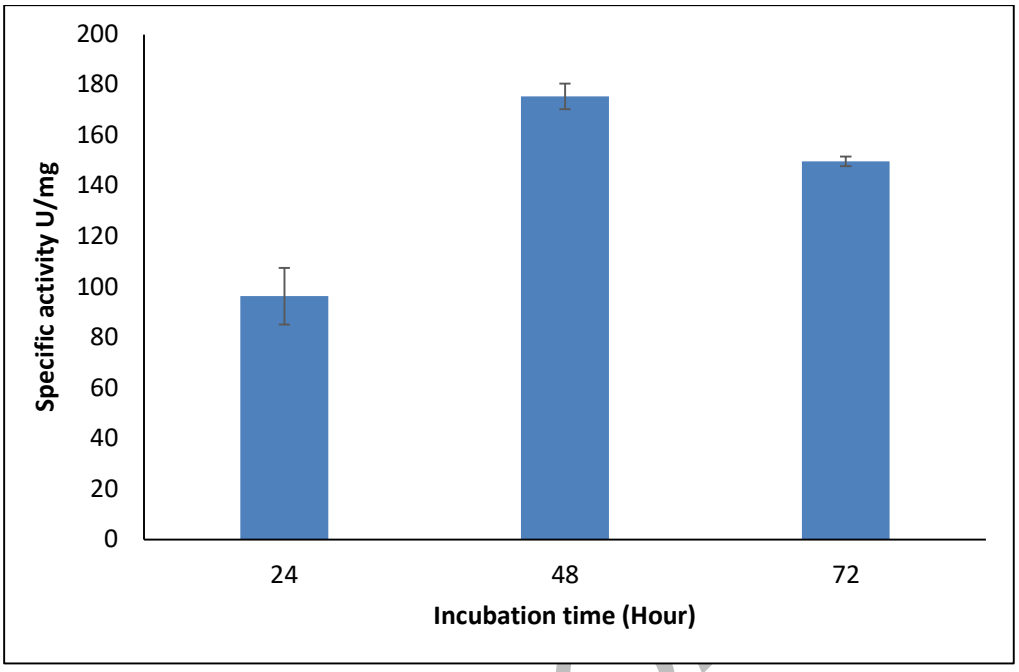


Fig 4.18 Effect of incubation time on the production of chromate reductase by *Bacillus* Sp. strain S48

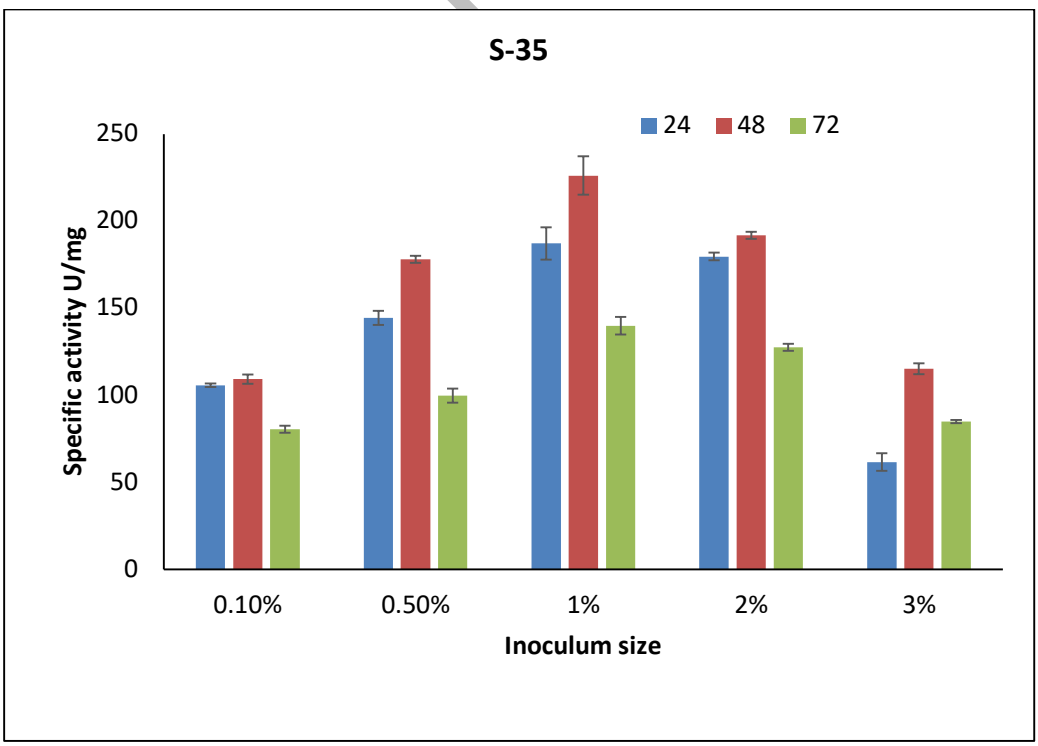


Fig 4.19 Effect of inoculum size on production of the chromate reductase by *Bacillus* Sp. strain S48

3.11 Optimization of the Nutritional factors for Chromate Reductase production using Plackett-Burman and Central Composite Design

3.11.1 Plackett-Burman Design (PBD)

The factors optimized through Plackett-Burman design showed a significant effect on production and yield of chromate reductase. Total 11 factors were used in 15 different experimental runs. The significant factors on production of chromate reductase were achieved by PBD. Maximum specific activity 305.386 U/mg was achieved in run number 08 and the lowest activity (60.162 U/mg) was observed in run number 3 (Table 4.3**).** All experiments were performed in triplicate. Furthermore, Pareto chart obtained from Placket-Burman explain the effect of each individual factor on production of chromate reductase (Fig 4.20). 05 out of 11 factors were found to have significant effect towards chromate reductase production including B: KH_2PO_4, G; $K_2Cr_2O_7$, F; Sucrose, C; $MgSO_4.7H_2O$ and K; Yeast extract, as evident by the values of "Prob > F" of each factor (Table 4.4). The enzyme activity was highly influenced by KH_2PO_4 as indicated by largest coefficient followed by $K_2Cr_2O_7$, sucrose and $MgSO_4.7H_2O$. KH_2PO_4 and $K_2Cr_2O_7$ had negative influence while Sucrose and $MgSO_4.7H_2O$ had positive influence on enzyme production. The chromate reductase yield was enhanced by decreasing KH_2PO_4 and $K_2Cr_2O_7$ concentration and increasing sucrose and $MgSO_4.7H_2O$ in production medium.

The model equation for chromium reductase specific activity (U/mg) can be written as:

Specific activity: (R)= (+196.15) + (-7.32 A) + (-39.30B) + (30.20C) + (5.32D) + (-13.48E) + (31.49F) + (-34.30G) + (7.26H) + (-5.29J) + (-22.06K)

The Model F-value of 12.11 implies that the model is significant.Value of "Prob > F" less than 0.0500 indicates model terms are significant. In this case B, C, F, G, K are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms.

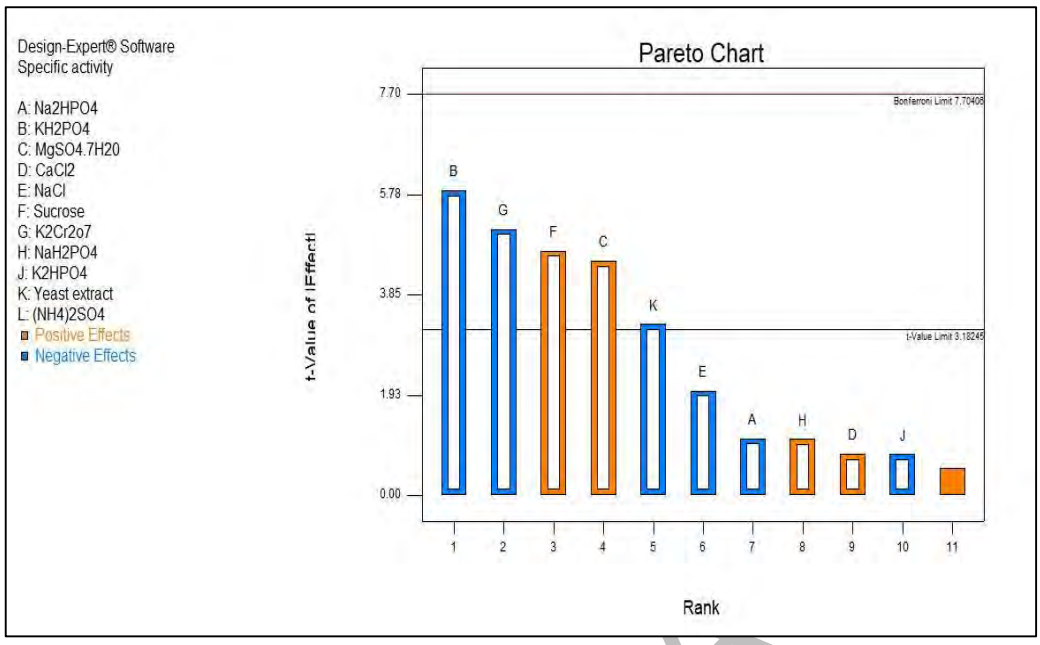


Fig. 4.20: Pareto chart showing the effect of different factors generated by Plackett Burman design on the rate of enzyme production.

Table 4.3. Placket Burman design of factors with specific enzyme activity (U/mg) of *Bacillus* Sp. Strain S48 as a response.

Run No	Factor Na_2HPO_4 %	Factor KH_2PO_4	Factor C $MgSO_4.7H2O$%	Factor CaCl2%	Factor NaCl%	Factor sucrose %	Factor $K_2Cr_2O_7$ %	Factor NaH_2PO_4 %	Factor K_2HPO_4 %	Factor Yeast extract %	Factor $K(NH_4)_2SO_4$ %	Response 1 specific activity U/mg
1	0.4	0.1	0.01	0.01	0.02	0.5	0.01	0.1	0.3	0.3	0.3	240.07
2	0.4	0.5	0	0.04	0.02	0.5	0.01	0.3	0.3	1.3	0.5	210.01
3	0.6	0.3	0.03	0.03	0.04	1	0.02	0.2	0.4	1.05	0.4	60.162
4	0.8	0.5	0.04	0.01	0.002	0.5	0.03	0.1	0.5	1.3	0.3	83.93
5	0.8	0.1	0.04	0.04	0.06	0.5	0.01	0.1	0.5	0.8	0.5	266.04
6	0.4	0.1	0.04	0.01	0.06	1.5	0.01	0.3	0.5	1.3	0.3	296.32
7	0.4	0.5	0.04	0.01	0.08	1.5	0.03	0.1	0.3	0.8	0.5	196.39
8	0.8	0.1	0.04	0.04	0.02	1.5	0.03	0.3	0.3	0.8	0.3	305.33
9	0.8	0.5	0.01	0.01	0.02	1.5	0.01	0.3	0.5	0.8	0.5	220.85
10	0.4	0.5	0.01	0.04	0.08	0.5	0.03	0.3	0.5	0.8	0.3	80.495
11	0.4	0.1	0.01	0.04	0.02	1.5	0.03	0.1	0.5	1.3	0.5	197.49
12	0.6	0.5	0.03	0.03	0.04	1	0.02	0.2	0.4	1.05	0.4	106.88
13	0.6	0.3	0.03	0.03	0.04	1	0.02	0.2	0.4	1.05	0.4	105.09
14	0.8	0.5	0.01	0.04	0.06	1.5	0.01	0.1	0.3	1.3	0.3	149.39
15	0.8	0.1	0.01	0.01	0.06	0.5	0.03	0.3	0.3	1.3	0.5	107.35

Table 4.4 ANOVA of*Bacillus* Sp. strain S48for Placket-Burman Design

Source	Sum of square	Df	Mean square	F value	p-value probe> F
Model	65474.78	10	6547.48	12.11	0.0320
A-Na_2HPO_4	642.69	1	642.69	1.19	0.3553
B-KH_2PO_4	18533.99	1	18533.99	34.28	0.0099
C-$MgSO_4.7H_2O$	10946.10	1	10946.10	20.25	0.0205
D-$CaCl_2$	339.99	1	339.99	0.63	0.4857
E-NaCl	2180.55	1	2180.55	4.03	0.1382
F-sucrose	11902.13	1	11902.13	22.01	0.0183
G-$K_2Cr_2O_7$	14121.19	1	14121.19	26.12	0.0145
H-NaH_2PO_4	632.16	1	632.16	1.17	0.3587
K_2HPO_4	335.51	1	335.51	0.62	0.4884
Yeast Extract	5840.46	1	5840.46	10.80	0.0462

3.11.2 Central Composite Design (CCD)

For further optimization, two-level CCD was used on the significant factors achieved from PBD that have a positive effect on chromate reductase production. For strain S48, the CCD was performed on four factors obtained from PBD and the effect of these factors study in 30 runs at two levels in 30 runs (Table 4.5). The acceptability of the model was evaluated using ANOVA (Table 4.6). *Bacillus* Sp. strain S48, among the quadratic and linear terms, only interactive term AB and AC were significant model terms, whereas A represent KH_2PO_4 and B represent $K_2Cr_2O_7$ while, C represent sucrose. Response surface plot (AB) shows that increase in $K_2Cr_2O_7$ and a decrease in KH_2PO_4 enhanced enzyme activity (Fig 4.21). The response plot (AC) shows that Chromate reductase activity was increased by decreasing the concentration of KH_2PO_4 and increasing sucrose concentration (Fig 4.22). Second polynomial

equation was obtained after multiple regression analysis data. Final Equation in terms of coded factors:

Specific activity = + 512.56-25.86A-22.90B-39.80C + 6.36D-57.57AB + 41.05AC + 11.34AD-7.66BC-3.85 BD-27.91CD-6.47A^2-104.05B^2-61.15C^2-29.41D^2

The Model F-value of 8.64 implies that the model is significant.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, C, AB, AC, B^2, C^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.

Table 4.5CCD for *Bacillus* Sp. strain S48 showed significant factors with specific enzyme activity (U/mg) as a response

S.No	Factor 1 KH_2PO_4%	Factor $2K_2Cr_2O_7$%	Factor 3 sucrose	Factor 4 $MgSO_4$. $7H_207H20$	Response Specific Activity
1	0.10	0.01	2.00	0.53	545.993
2	0.20	0.01	2.00	0.53	543.614
3	0.20	0.02	1.00	0.06	355.134
4	0.20	0.01	1.50	0.41	484.383
5	0.20	0.01	2.00	0.53	232.155
6	0.20	0.01	2.50	0.53	545.156
7	0.20	0.01	2.00	0.53	530.135
8	0.20	0.01	2.00	0.53	350.879
9	0.30	0.01	2.00	1.00	379.947
10	0.40	0.01	1.50	0.53	483.266
11	0.10	0.02	2.50	1.00	173.723
12	0.20	0.03	2.00	0.53	130.879
13	0.20	0.01	2.00	0.53	401.259
14	0.20	0.01	2.00	1.47	483.983
15	0.20	0.00	2.00	0.53	117.755
16	0.30	0.01	1.50	0.06	334.172
17	0.10	0.01	2.50	1.00	203.872
18	0.20	0.01	2.00	0.53	542.752
19	0.10	0.02	2.50	0.06	337.551
20	0.10	0.01	1.50	0.06	411.373
21	0.30	0.02	2.50	0.06	216.033
22	0.30	0.02	1.50	1.00	205.913
23	0.10	0.02	1.50	1.00	533.00
24	0.30	0.01	2.50	0.06	317.88
25	0.30	0.01	2.50	1.00	406.358
26	0.10	0.01	1.50	1.00	352.019
27	0.30	0.02	2.50	1.00	113.006
28	0.20	0.01	2.00	0.53	528.449
29	0.30	0.02	1.50	0.06	156.981
30	0.10	0.01	2.50	0.06	259.353

Table 4.6 ANNOVA for central composite design

Source	Sum of square	Df	Mean square	F value	p-value probe> F
Model	5.523E+005	14	39452.65	8.64	0.0001
A-KH_2PO_4	16051.11	1	16051.11	3.52	0.0804
B- $K_2Cr_2O_7$	12581.36	1	12581.36	2.76	0.1177
C- sucrose	38024.40	1	38024.40	8.33	0.0113
D- $MgSO_4.7H_20$	696.53	1	696.53	0.15	0.7016
AB	53025.59	1	53025.59	11.61	0.0039
AC	26958.97	1	26958.97	5.90	0.0281
AD	2058.29	1	2058.29	0.45	0.5122
BC	938.56	1	938.56	0.21	0.6568
BD	237.10	1	237.10	0.052	0.8228
CD	12463.30	1	12463.30	2.73	0.1193

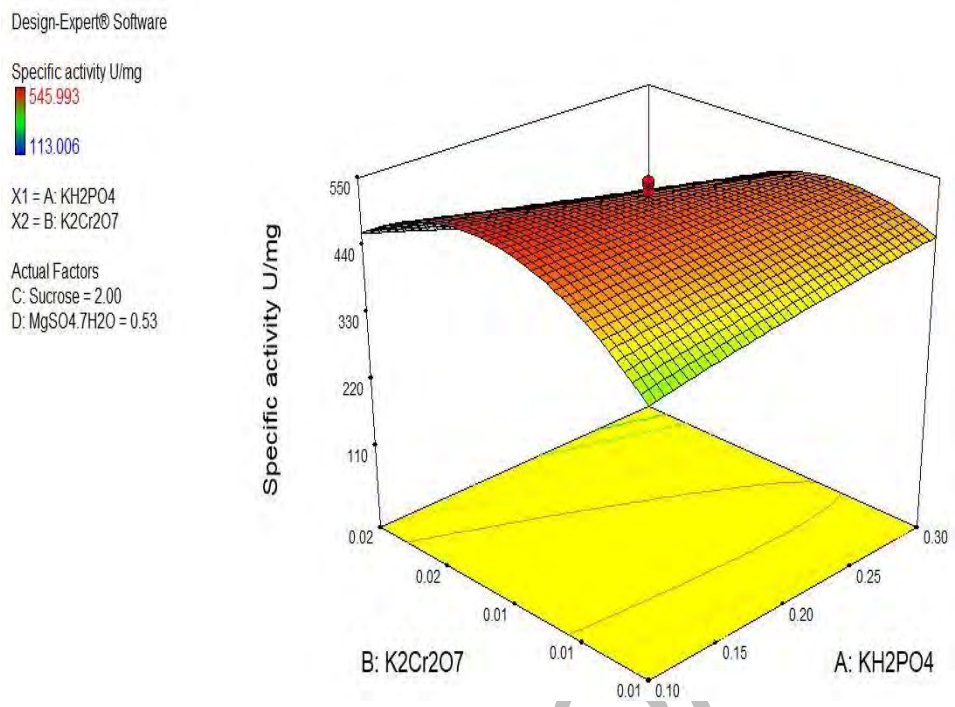


Fig 4.21 Response surface plots showing the combined effects of A; KH_2PO_4 B; $K_2Cr_2O_7$

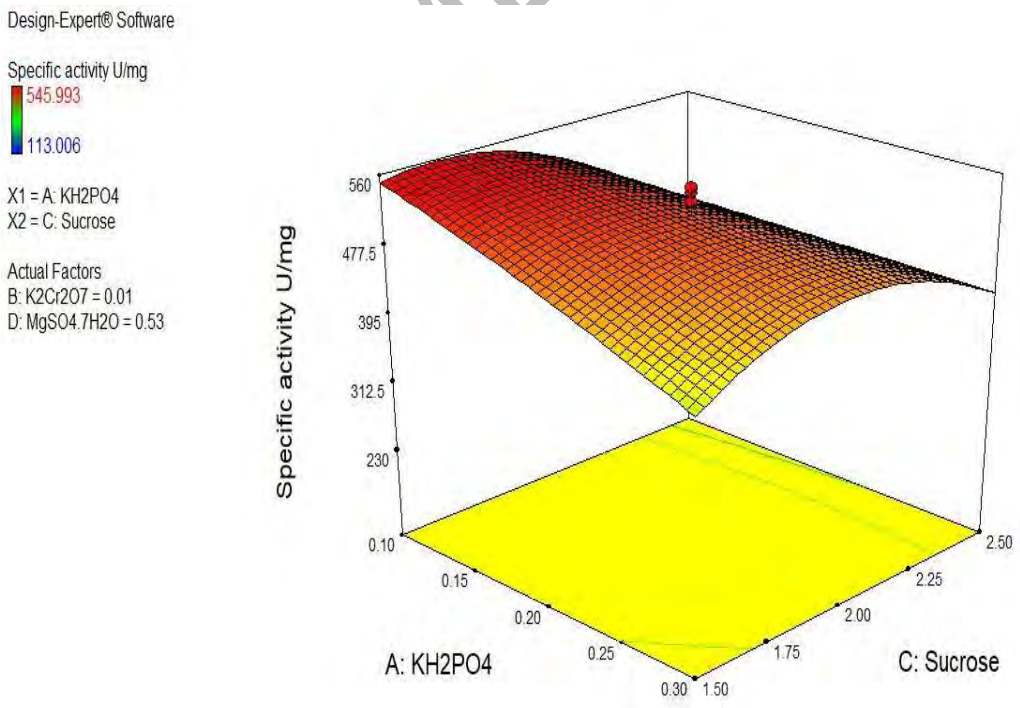


Fig 4.22 Response surface plots showing the combined effects of A, KH_2PO_4; B, Sucrose

4.12 Purification of Chromate Reductase from *Bacillus* Sp. strain S48

4.12.1 Ammonium sulfate Precipitation of Crude Chromate Reductase

The enzyme was precipitated using ammonium sulfate in different concentrations. The highest enzyme activity 5.09 U/mg was achieved at 70% saturation, further addition of ammonium sulfate resulted into drop-in enzyme activity, while below 70% saturation chromate reductase activity was low (Fig. 4.23).

4.12.2 Gel Filtration Chromatography

A partially purified chromate reductase was subjected for further purification by Gel Filtration Chromatography. A total of 30 fractions were collected and maximum activity of chromate reductase was observed in fractions 11– 22 with a maximum specific activity of 1171.5 U/mL at fraction 11 (Fig. 4.24).

4.12.3 Total Yield of Purified Chromate Reductase

The total yield and purification fold for precipitated and gel filtered chromate reductase from *Bacillus* Sp. strain S48 was calculated. The total yield and purification fold were 59.5% and 6.6 as given in table 4.7.

4.12.4 Molecular Weight Determination

The protein size of chromate reductase was determined using SDS-PAGE. On 12% gel, a protein band was observed after staining with Coomassie Brilliant Blue R-250. The molecular weight was observed approximately 35kDa against standard denaturing marker protein (Fig. 4.25).

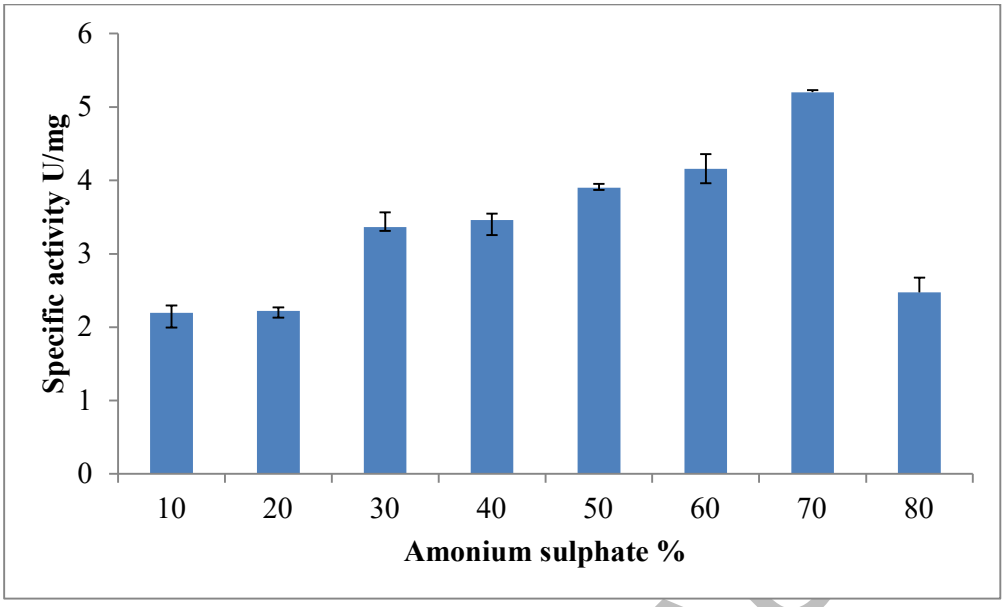


Fig 4.23 Ammonium sulfate precipitation of *Bacillus* Sp. strain S48 and evaluation of specific activities of the supernatant at various percentages of ammonium sulfate precipitation.

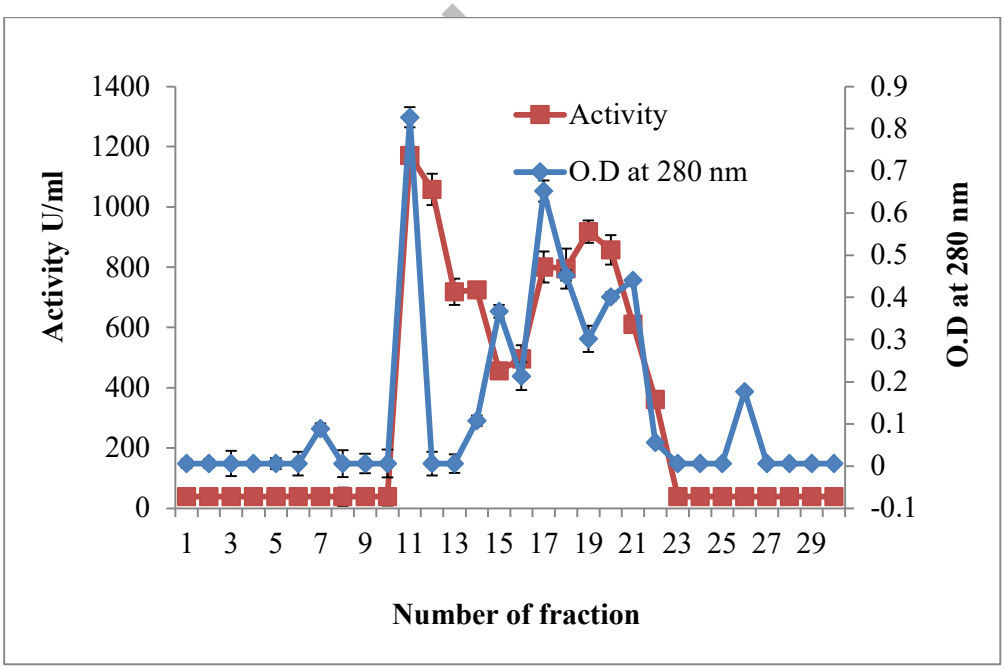


Fig. 4.24 Total protein and specific activity profile of chromate reductase from *Bacillus* Sp. strain S48 in fractions from column chromatography.

Table 4.7 Purification steps of chromate reductase from *Bacillus* Sp. strain S48

Protein purification steps	Activity U/mL	Protein mg/mL	Specific Activity U/mg	Yield (%)	Purification fold
Crude extract	432.9	2.02	214.0	100	1
Precipitation	797.3	1.54	516.7	92.0	2.4
Sephadax-G100	1171.5	0.82	1416	59.5	6.6

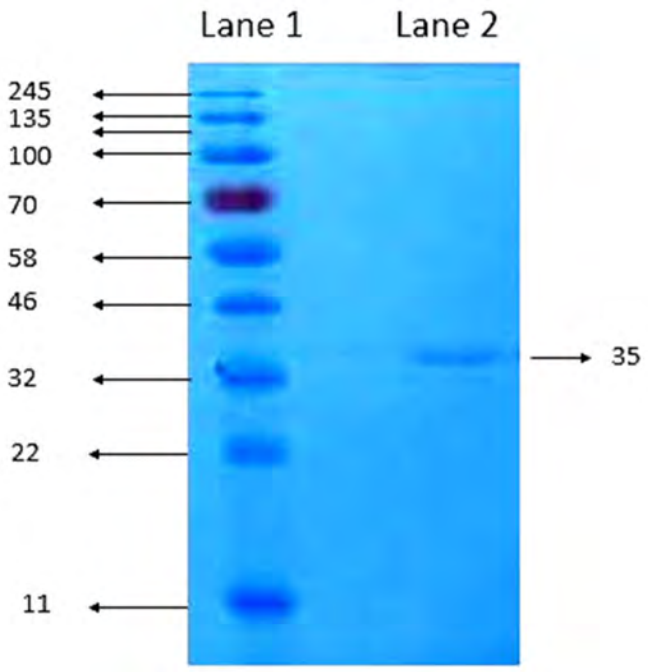


Fig. 4.25 SDS-PAGE of chromate reductase form *Bacillus* Sp. S48 after purification by column chromatography. **Lane 1**, (Color Prestained Protein Standard) 11-245 kDa; **Lane 2**, Chromate reductase of 35 kDa

4.13 Characterization of Chromate Reductase from *Bacillus* Sp. strain S48

The purified chromate reductase from *Bacillus* Sp. strain S48 was characterized for different parameters known to effect on enzyme activity.

4.13.1 Effect of Temperature on Activity and Stability from *Bacillus* Sp. Strain S48

The effect of temperature on the activity of purified chromate reductase from *Bacillus* Sp. strain S48 was calculated by incubating enzyme with $K_2Cr_2O_7$ (substrate) at different temperatures ranging from 30-60˚C for a maximum up to 30 min. The optimum temperature for maximum enzyme activity turned out to be 40˚C (Fig. 4.26). The stability of purified chromate reductase was calculated by incubating the enzyme without substrate at different temperature ranging from 30-60˚C for 120 min. The maximum stability was achieved at 35°C and enzyme retained 100% of its activity upto 120 min, whereas 90% activity at temperatures 30˚C to 40˚C for the same duration. The activity was reduced to only 4% and 3% when incubated at 55˚C and 60˚C for 120 minutes (Fig. 4.27).

4.13.2 Effect of pH on Activity and Stability from *Bacillus* Sp. Strain S48

The effect of pH on the activity of purified chromate reductase from *Bacillus* Sp. strain S48 was calculated by incubating enzyme with $K_2Cr_2O_7$ substrate at various pH ranging from3.0-9.0 for 30 min. The optimum activity was observed at pH 7.0 (Fig 4.28).

The stability of purified chromate reductase was calculated by incubating the enzyme without substrate at a different pH ranging from 3.0-10.0 at 35°C. The enzyme was stable at pH 7.0 and retained more than 90% of its activity for 120 minutes, followed by retaining 80% of its activity at pH 6.0. The activity was reduced to only 5% when incubated at pH 10.0 for 120 minutes (Fig. 4.29).

4.13.5 Effect of Metal Ions

The effect of different metals on chromate reductase activity was analyzed at 2 mM and 10 mM concentrations. Metals such as $CaSO_4$, $BaSO_4$, $FeSO_4$, NaCl, K_2SO_4, KCL, and $MgSO_4$ have shown positive effect on the activity of chromate reductase at 10mM concentration and increased by 10% of its activity. A strong inhibitory effect was observed for $HgCl_2$and $CdCl_2$at both 2 mM and 10 mM concentrations (Fig 4.30).

4.13.6 Effect of Surfactants

Different surfactants were used to analyze their effect on the activity of the purified chromate reductase. SDS completely inhibited enzyme activity, while it retained 100% activity in the presence of Tween 20 and Tween 60. Tween 80, and PEG, potentially enhanced chromate reductase activity at both concentrations (0.5% and 1.0%) (Fig 4.31).

3.13.7 Effect of Organic Solvent

The effect of different organic solvent on activity of purified enzyme was determined by incubating with organic solvent for 120 mins. Acetonitrile, ethyl acetate and n-Hexane reduced enzyme activity, while glycerol, methanol, propanol, and DMSO increased enzyme activity upto 120% and retained it for 120 min. (Fig 4.32).

4.13.8 Determination of Kinetic Parameters

The Michaelis Menten constant (K_m) and rate of reaction (V_{max}) values of purified chromate reductase from *Bacillus* Sp. strain S48 were calculated by Lineweaver and Burk's plot. The enzyme was incubated with varying concentrations (1 µM-10 µM) of substrate (potassium dichromate). The K_m and V_{max} of purified enzyme were found to be 2.33 µM and 222.22 $\mu molmg^{-1}min^{-1}$(Fig 4.33).

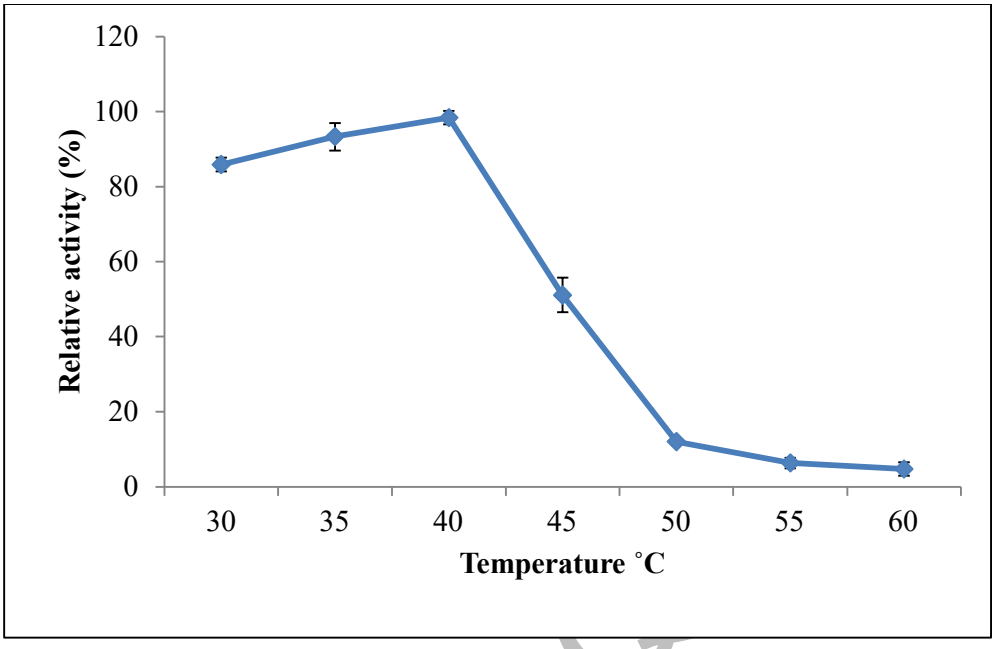


Fig 4.26 Effect of temperature on the specific activity of chromate reductase from *Bacillus* Sp. strain S48

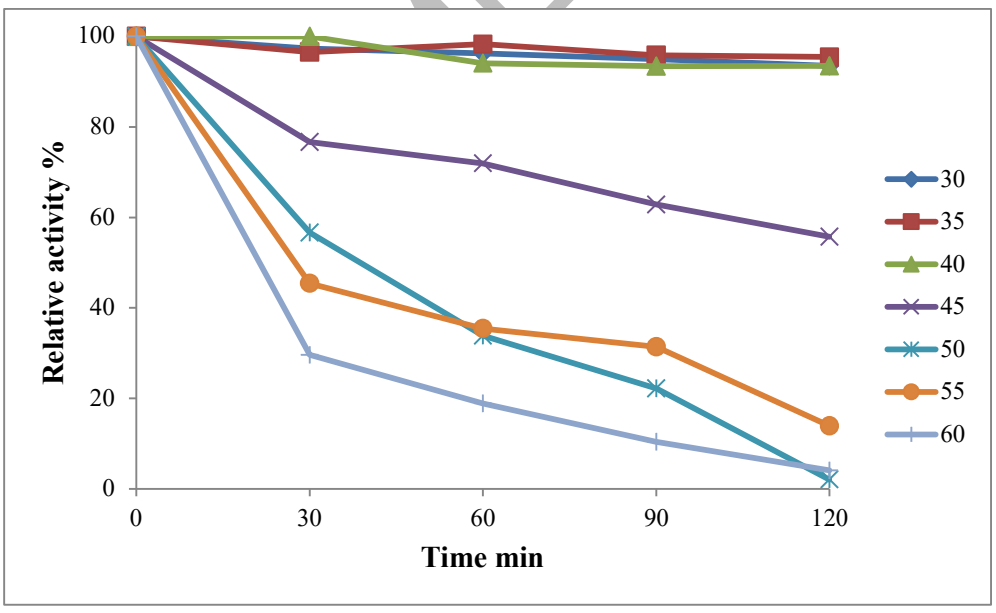


Fig. 4.27 Temperature stability of chromate reductase from *Bacillus* Sp. strain S48

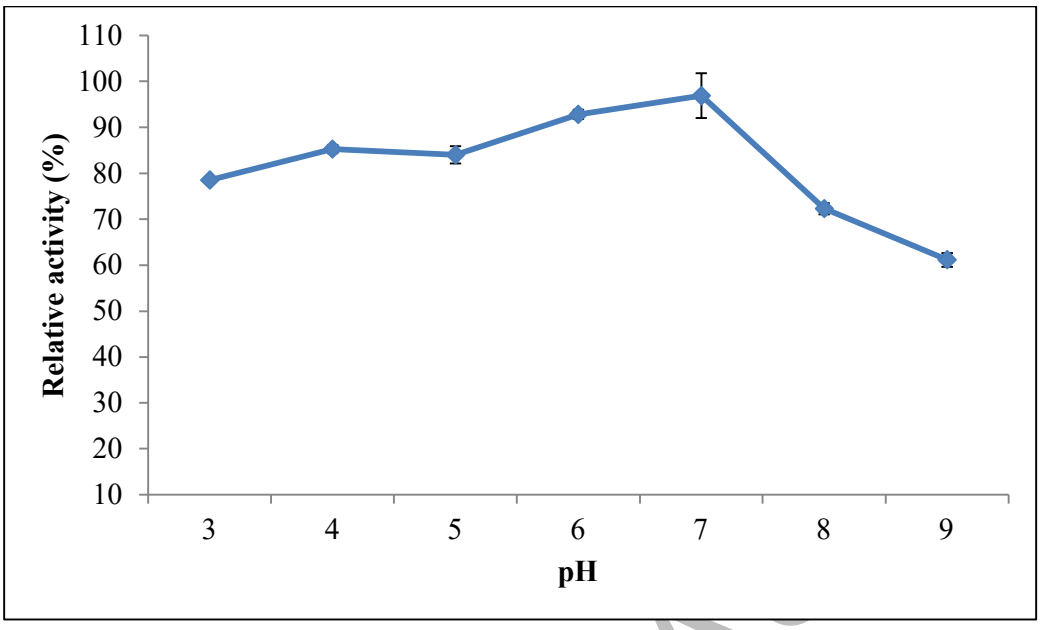


Fig 4.28 Effect of pH on the activity of chromate reductase from *Bacillus* Sp. strain S48

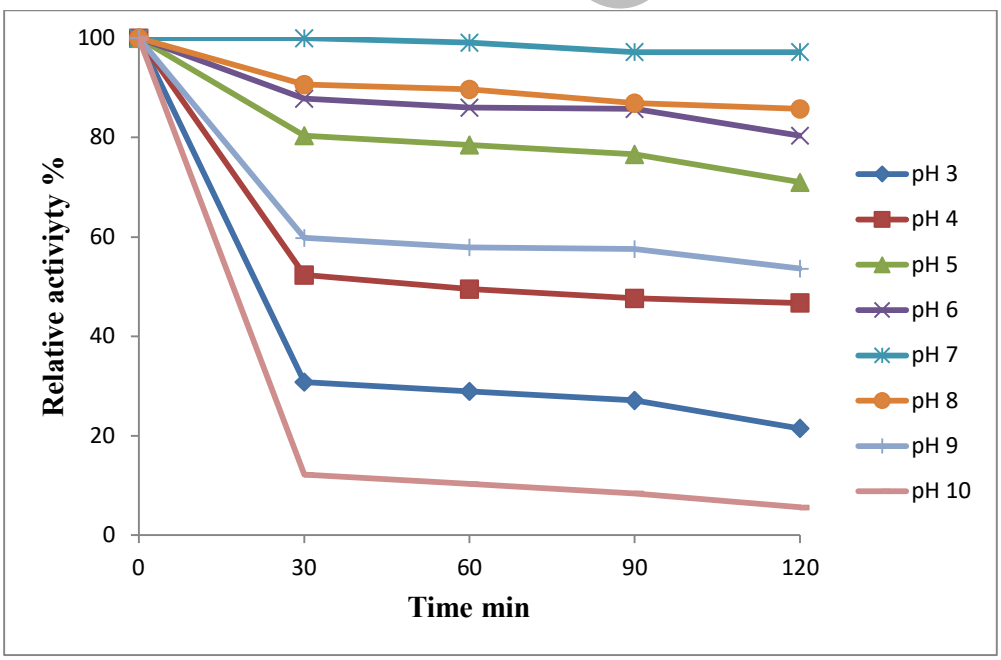


Fig. 4.29 pH stability profile of chromate reductase from *Bacillus* Sp. strain S48

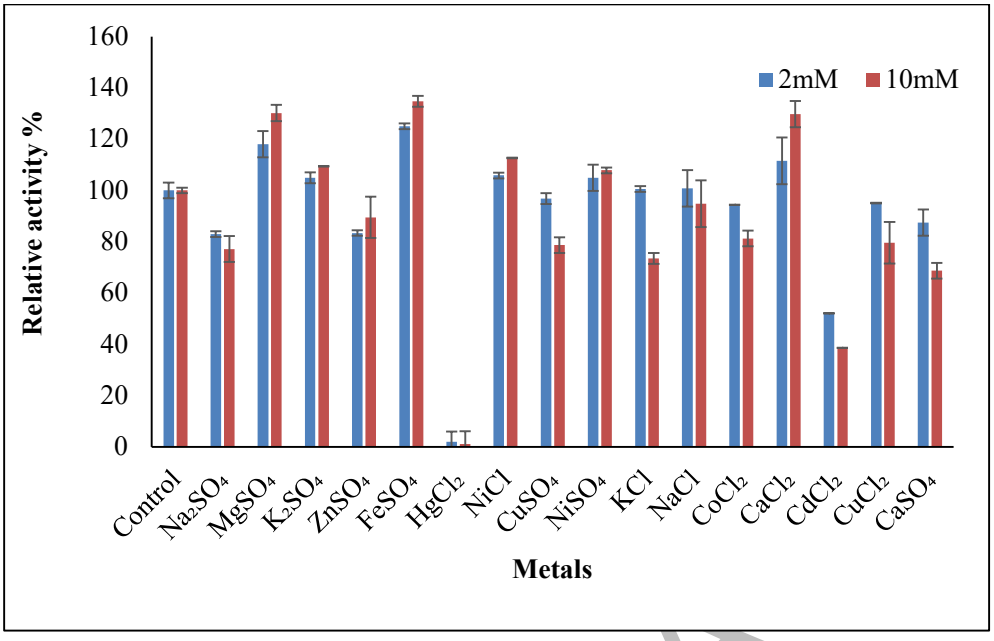


Fig. 4.30 Effect of metals on the activity of chromate reductase from *Bacillus* Sp. strain S48

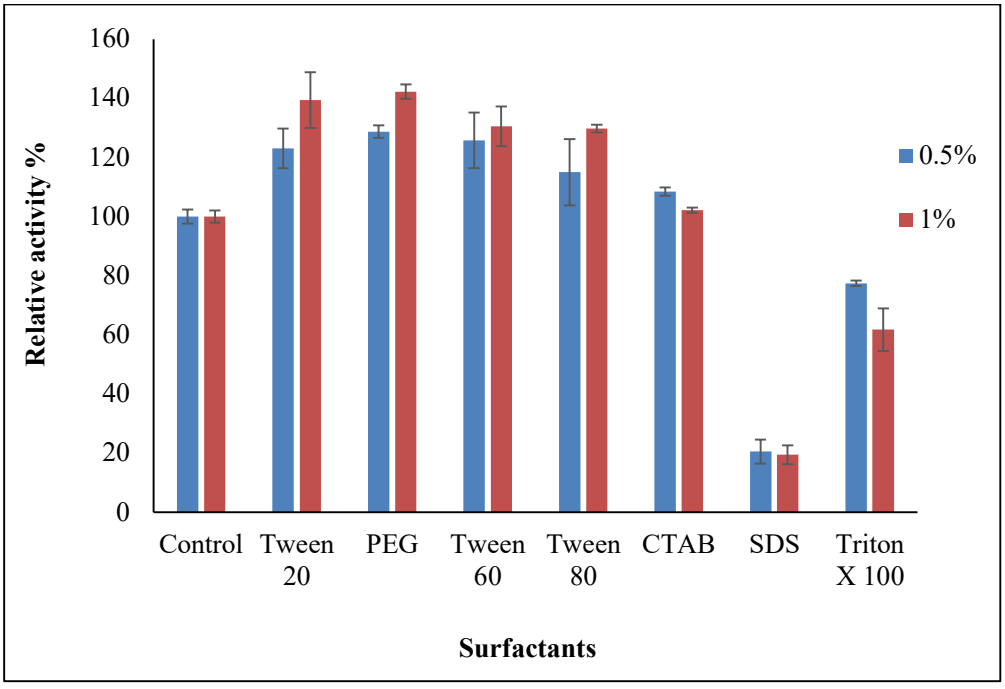


Fig. 4.31 Effect of surfactants on activity of chromate reductase from *Bacillus* Sp. strain S48

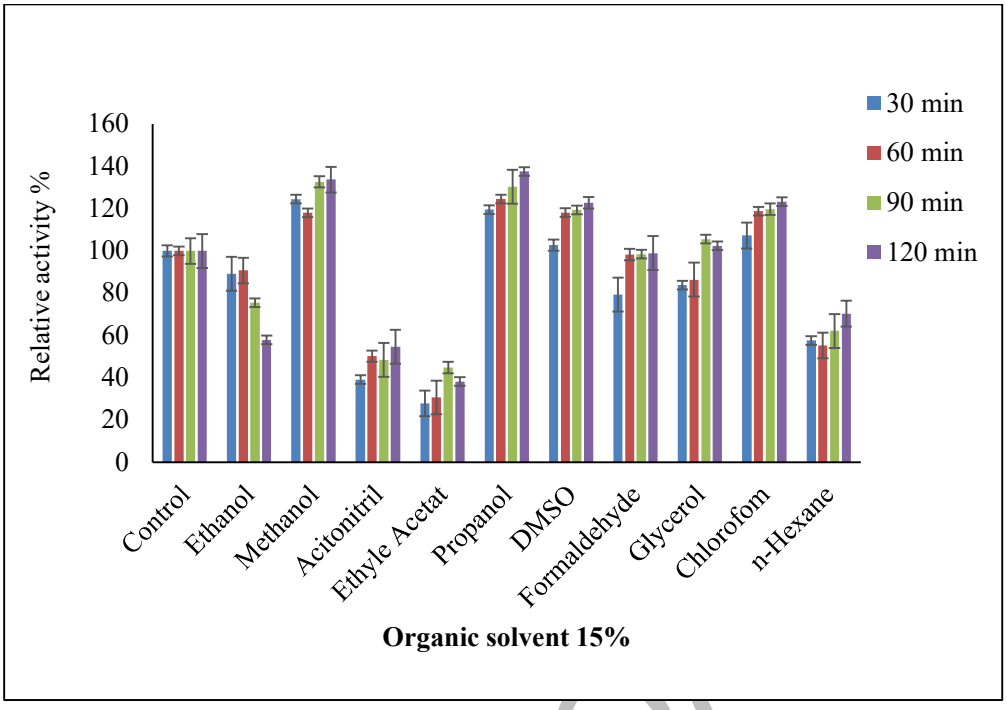


Fig. 4.32 Effect of organic solvents on the activity of chromate reductase from *Bacillus* Sp. strain S48

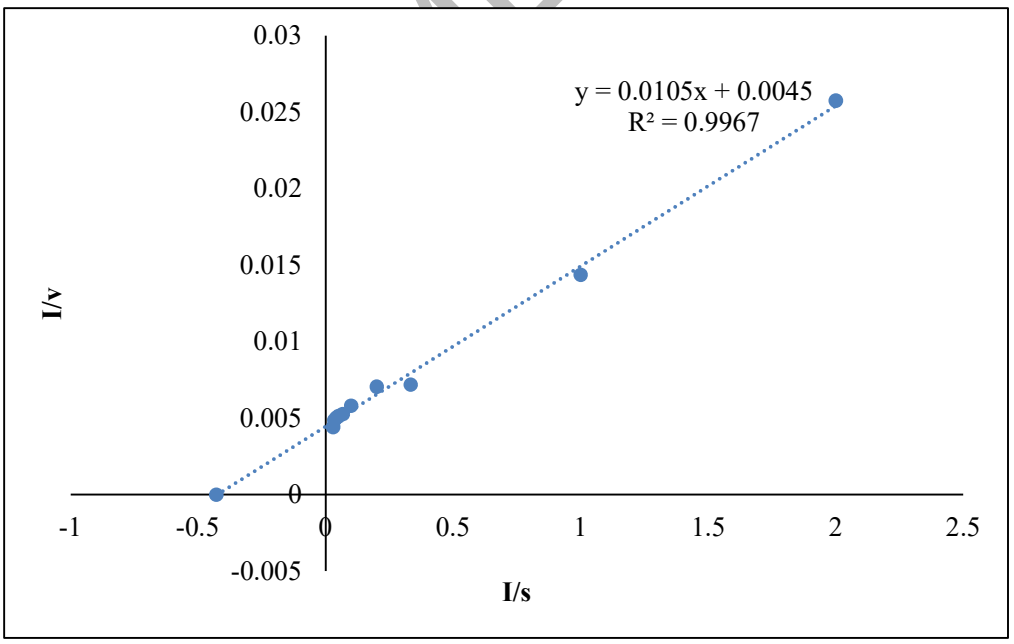


Fig. 4.33 Kinetics analysis of chromate reductase from *Bacillus* Sp. strain S48

4.14 Application of Chromate Reductase in Bioreduction of Hexavalent chromium

100mg/L of $K_2Cr_2O_7$ was dissolved in 100 ml deionized water in 250 ml Erlenmeyer flask, treated with 0.1,1, and 5 mg/Ml of both crude and purified enzyme. Crude enzyme showed 20, 45, and 75% chromate reduction with 0.1, 1 and 5 mg/mL, respectively, after 120 hours. Purified chromate reductase of strain S48 showed 42, 64, and 87% chromate reduction with 0.1, 1 and 5 mg/mL of enzyme after 120 hours (Fig 4.34).

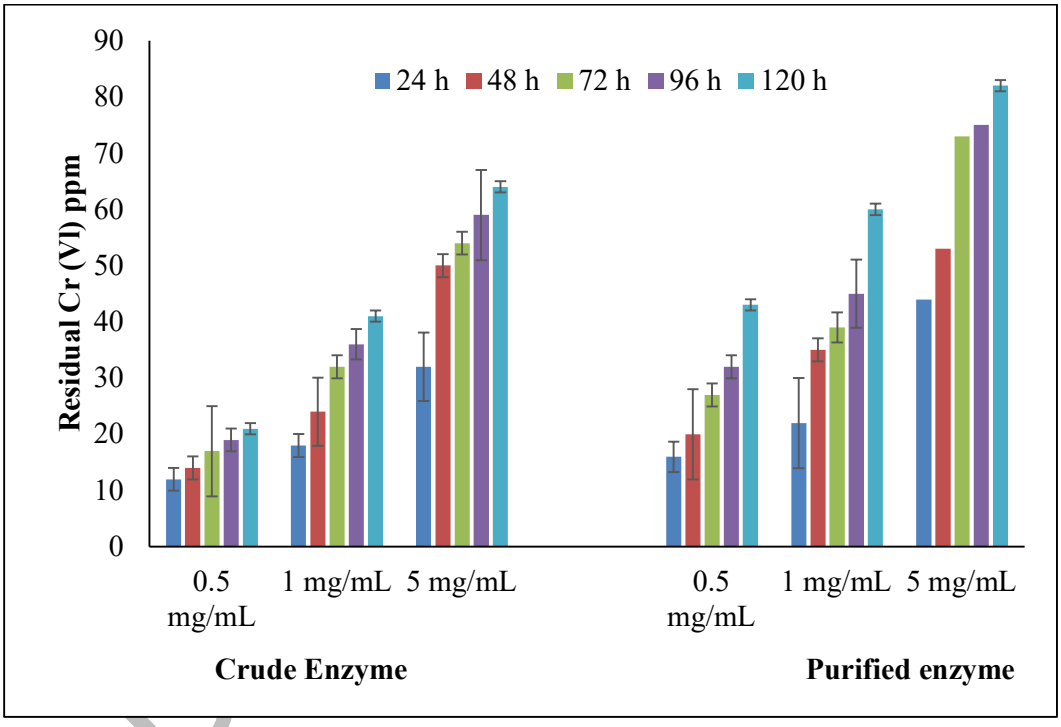


Fig 4.34 Enzymatic reduction of Cr(VI) reduction with crude and purified chromate reductase from *Bacillus* Sp. strain S48

4.15 Chromate Reductase and Lead Binding Protein Genes cloning and Expression

4.15.1 Isolation of genomic DNA

The genomic DNA of *Bacillus* Sp. strain S48 and *Escherichia* Sp. Strain S54 was extracted using DNA extraction kit (Eco-Pure-Genomic, Product Code # E1075). The DNA was run on 1% agarose gel using gel electrophoresis and visualized by staining it with ethidium bromide. The extracted DNA served as a template for amplification of chromate reductase (ChR) and lead binding protein (PbR) genes in PCR reaction.

4.15.2 Optimization of PCR Conditions

Annealing temperature for ChR and PbR genes of strain S48 andS54 were optimized at various ranges 45-65°C.The optimum annealing temperature for *Bacillus* Sp. strain S48 and *Escherichia* Sp. strain S54 turned out to be 55 and 58ºC, respectively.

4.15.3 PCR Amplification of ChR and PbR genes

ChR gene from S48 and PbR gene S54 were amplified separately by using their genomic DNA as template in the presence of newly designed primers. Approximately 0.6 kb size of chromate reductase encoding gene from *Bacillus* Sp. strain S48 and approximately 0.43 kb lead binding gene from *Escherichia* Sp. strain S54 were amplified (Fig. 4.35 A, B).

4.15.4 Gel Purification of Amplified ChR and PbR genes

The amplified ChR and PbR genes fragments were extracted using QIAquick Gel Extraction Kit (QIAGEN), run on 1% agarose gel and visualized by staining it with ethidium bromide.0.6 kb Chromate reductase and 0.43 kb lead binding gene was observed using gel documentation system.

4.15.5 Ligation, Transformation and Confirmation of ChR and PbR gene into pGEM-T Easy Vector

Chromate reductase (ChR) and lead binding protein (PbR)genes were ligated separately in the pGEM®-TEasy vector. The ligated product was then transformed in *Escherchia coli* JM101cell. The blue and white screening methods were used for validation of cloning gene, containing ligated chromate reductase and lead binding genes (Fig 4.36 A &Fig 4.36 B). One blue and 10 white colonies were selected from LB-Ampacillin agar and re-inoculated in LB-Amp broth for extraction of plasmid. Plasmids were extracted from transformed *Escherchia coli* JM101 and cloning was

confirmed by digesting plasmids with *EcoR1* restriction enzyme. The recombinant pGEM®-TEasy vectors separately containing ChR and PbR genes were sequenced by Macrogen, Netherland and analyzed by GenBank BLASTn program (http://www.ncbi.nlm.nih. gov/). About 0.6 bp ChR and 0.43 pb PbR gene were observed from pGEM®-T cloned plasmid digested with restriction enzyme (Fig 4.37 A &Fig 4.37 B).

4.15.6 Cloning and Transformation of *BparChR*and *EferPbR*Geneinto Expression Vector pET-28a

Chromate reductase gene of *Bacillus* Sp. strain S48 was designated as *BparChR* gene. Similarly, from *Escherichia* Sp. Strain S54 lead binding protein gene was designated as *EferPbR.* Whereas, the recombinant pGEMT-Easy vector containing ChR and PbR genes were named as pGEMT- *BparChR* and pGEMT-*EferPbR.*

pGEMT-*BparChR* and pGEMT-*EferPbR* and pET28a vectors were extracted and examined by 1% agarose gel electrophoresis and visualized by staining it with ethidium bromide for their confirmation. pGEMT-*BparChR,* pGEMT-*EferPbR* vectors andpET28a expression vector were double digested with restriction enzyme HindIII and NcoI. The digested pGEMT- *BparChR* and pGEMT-*EferPbR* showed two bands of about 3015 bp and 650 bp (pGEMT+ ChR) and 3015 bp and 430 bp (pGEMT+ PbR), while pET28a showed about 5300 bp product size under gel doc system. The digested pGEMT-*BparChR* and pGEMT-*EferPbR* were then ligated into expression vector pET28a and transformed into *E.coli* JM101. Ten white was selected from LB-kan agar and re inoculated in LB-kan broth for extraction of the plasmid. Recombinant expression vectors*PET28BparChR*and *PET28EferPbR*were extracted from transformed *E. coli* JM101 and cloning was confirmed by digesting vectors with restriction enzyme. The digested pET28a vector showed the desired *BparChR* and *EferPbR* genes product size along with the size of pET28a vector. The extracted *PET28BparChR*and *PET28EferPbR* vectors were then transformed into expression host system *E.coli* BL-21 (DE3) and the newly transformed cells were designated as*ChREcoli BL21*and *PbREcoli BL21* (Fig 4.38 A & B)

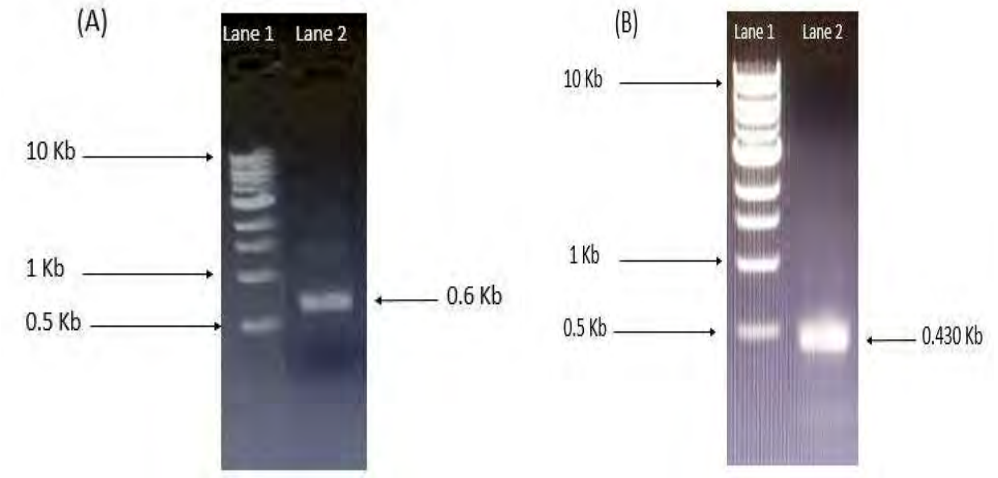


Fig. 4.35 Amplification of Chromate reductase (ChR) and lead binding protein (PbR) gene (A) Lane 1, DNA ladder (NEB, B7025); Lane 2, ChR PCR amplicon (B) Lane 1, DNA ladder (NEB, B7025); Lane 2, PbR PCR amplicon

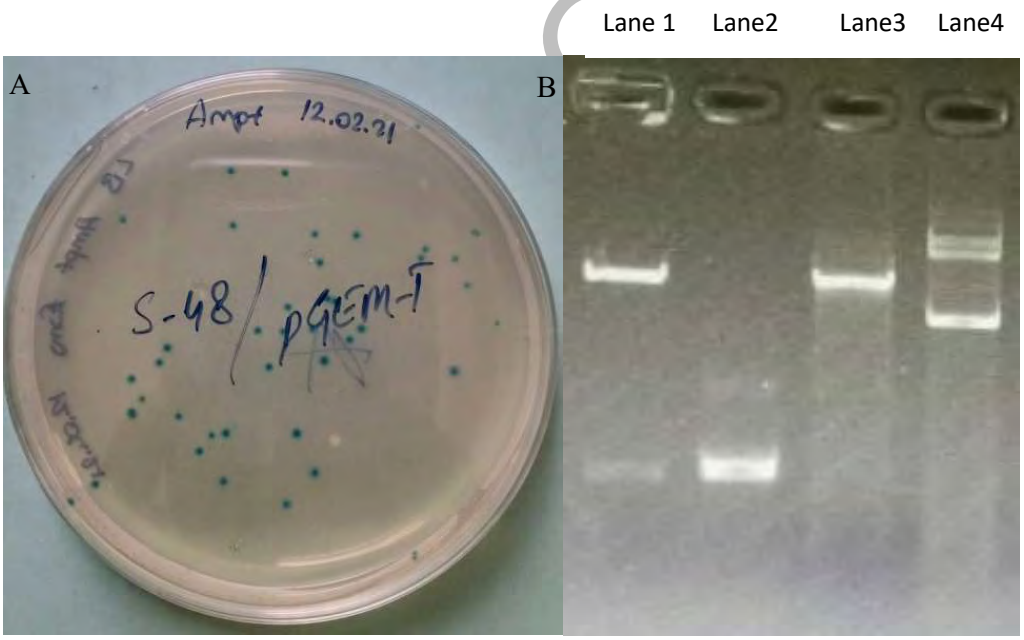


Fig. 4.36 Ligation, Transformation and confirmation of ChR gene into *p*GEM-T Easy Vector (A) Transformation of *pGEM-T* easy vector carrying ChR gene PCR product into *E.coli* JM101cell (B) Lane 1 Digested ChR Clone, Lane 2 ChR gene Amplicon, Lane 3 *pGEM-T* easy vector digested, Lane 4 Undigested ChR Clone

A

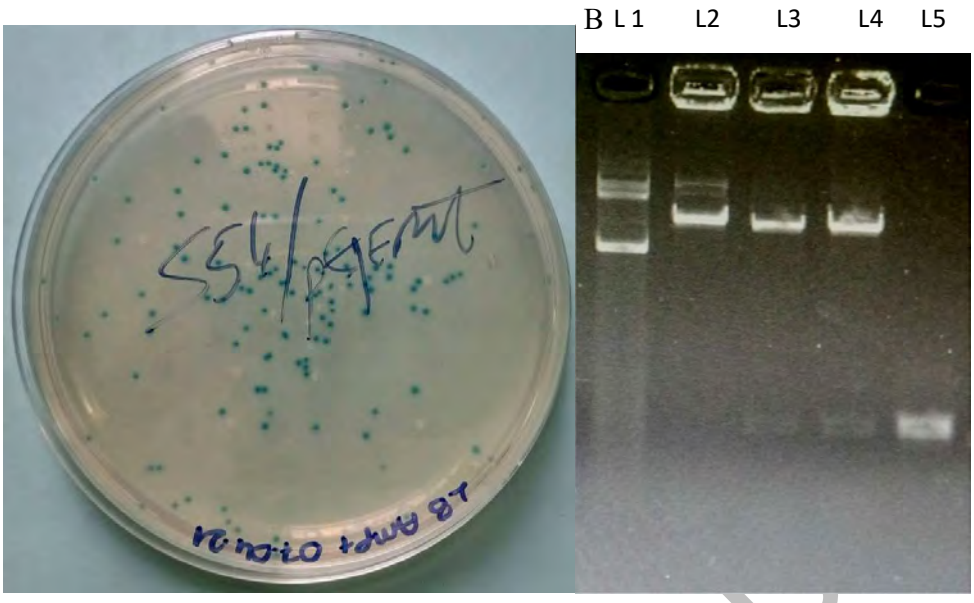


Fig. 4.37 Ligation, Transformation and confirmation of PbR gene into *p*GEM-T Easy Vector(A) Transformation of *pGEM-T* easy vector carrying PbR gene PCR product into *E.coli* JM101 cell (B) L1 Undigested PbR Clone, L2 *pGEM-T* easy vector digested, L3 and L4 Digested PbR Clone, L5 PbR gene Amplicon

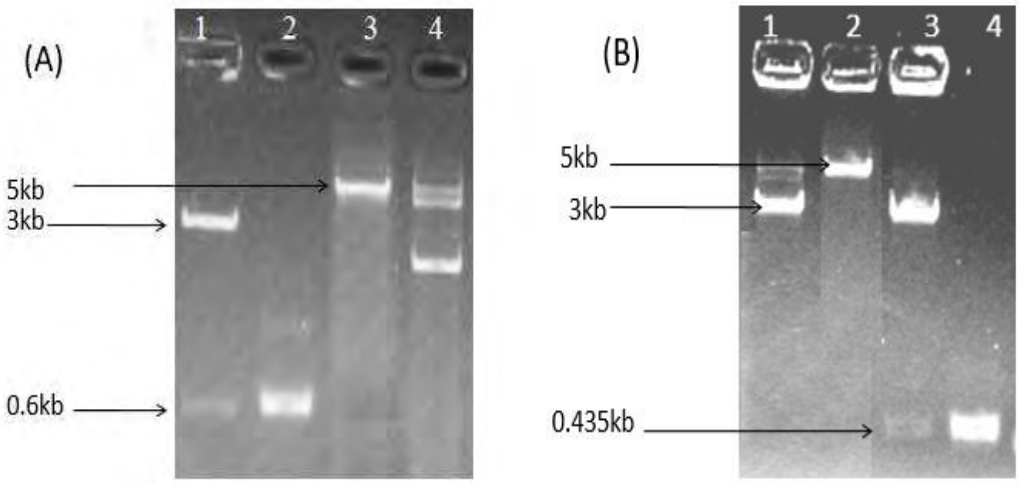


Fig 4.38 Double digestion and purification of pET28a vector and ChR and PbR gene product (A) 1. *Nco1* and *Hind 111* digested *pGEM-T-ChR* Clone *2*: ChR PCR product 3: *Nco1* and *Hind 111* digested *pET* 28 4: Undigested *pET* 28a (B)1: Undigested *pET* 28a 2*: Nco1* and *Hind 111* digested *pET* 28a 3: *Nco1* and *Hind 111* digested *pGEM-T-PbR* clone 4: *PbR* PCR product

4.16 Bioinformatics Analysis

Phylogenetic relationship of *BparChR* of *Bacillus* Sp. strain S48 with other chromate reductase available at NCBI database showed highest identity with chromate reductase of *Bacillus paramycoides* sp OV166 (Fig 4.39). Similarly, the phylogenetic relationship of *EferPbR* of *Escherichia* Sp. strain S54 with other lead binding gene available at NCBI database showed highest identity with lead binding gene of *Escherichia coli* strain CE1537 (Fig 4.40).

4.17Induction and Over Expression of Recombinant Chromate Reductase and Lead Binding Protein

The induction of recombinant chromate reductase and lead binding protein was carried out using0.5 mM IPTG and expression was performed by SDS-PAGE. SDS-PAGE clearly showed expressed band of chromate reductase and lead binding protein (Fig 4.41).

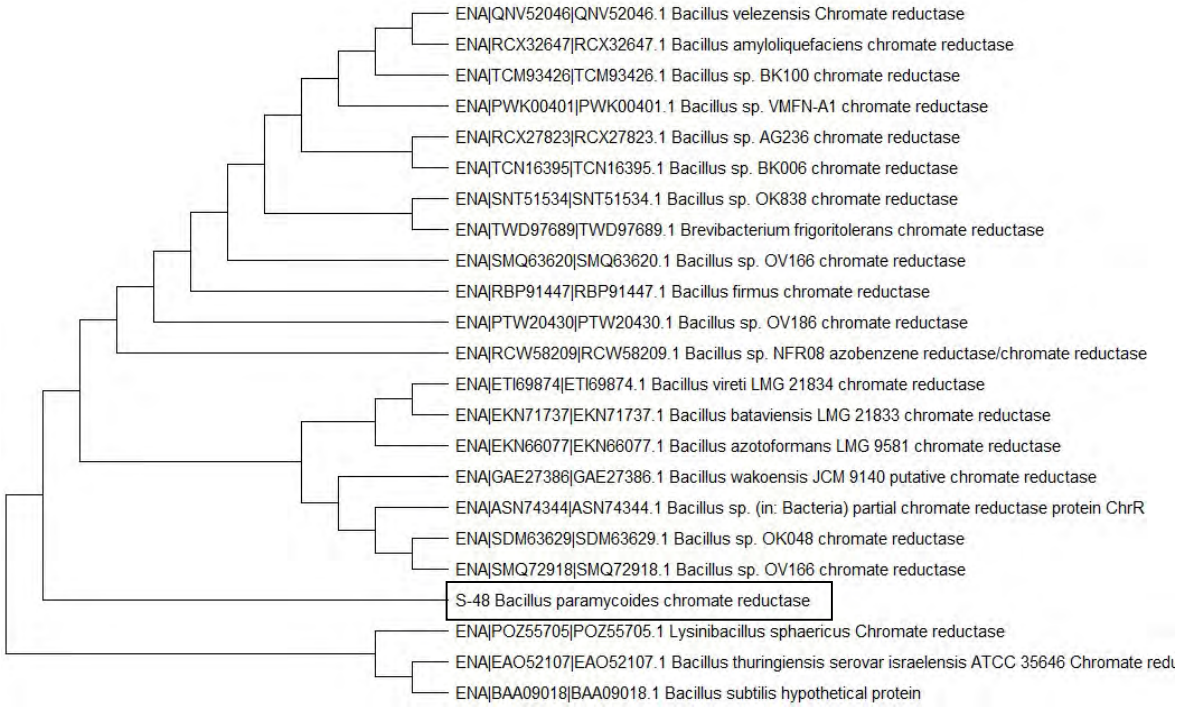


Fig 4.39 Phylogenetic relationship of *Bacillus* Sp. S48chromate reductase with other chromate reductase available in NCBI database, Neighbor-joining tree showed maximum identity with chromate reductase of *Bacillus paramycoides* sp OV166.

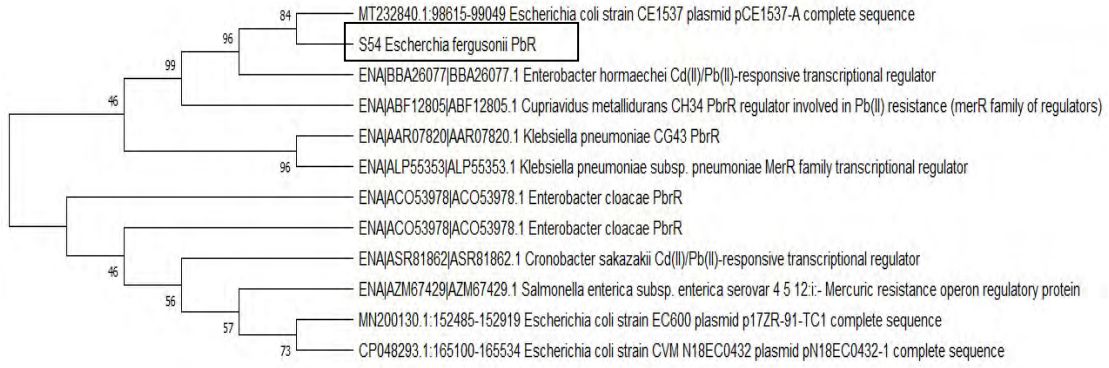


Fig 4.40 Phylogenetic relationship of (*EferPbR*) of *Escherichia* Sp. Strain S54 with other lead binding gene available at NCBI database showed highest identity with lead binding of *E. coli* strain CE1537

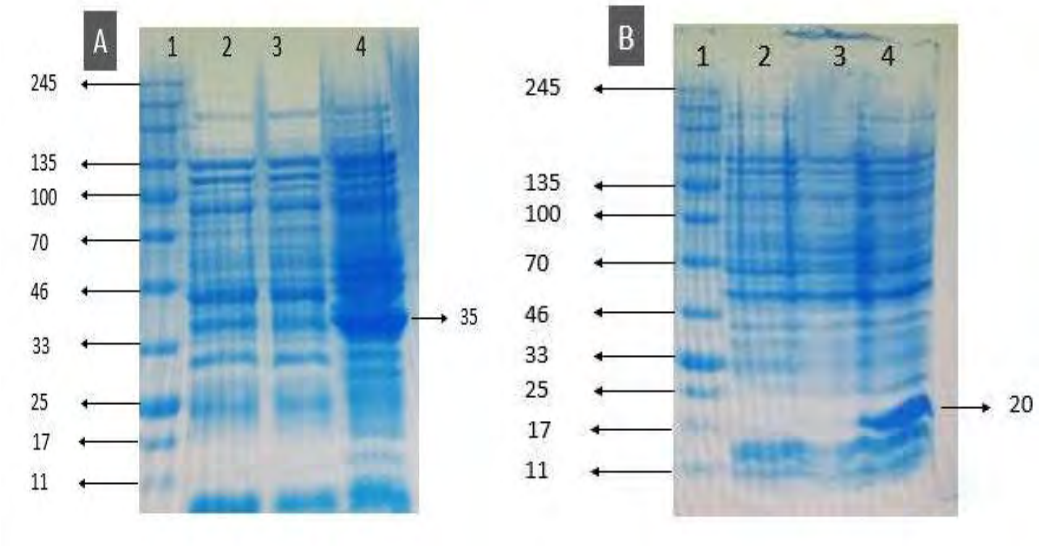


Fig 4.41 Recombinant chromate reductase and lead binding protein expression by SDS-PAGE (A) SDS-PAGE for chromate reductase1: Protein marker (thermofisher 11 – 245 kDa2,3: Induced protein of *E.coli* BL 21 (DE3) with *pET-28a*4: Induced protein of *E.coli* BL 21 (DE3) with *pET28BparChR.* (B) SDS-PAGE for Lead binding protein (PbR) 1: Protein marker (thermofisher 11 – 245 kDa 2,3: Induced protein of *E.coli* BL 21 (DE3) with *pET-28a* 4: Induced protein of *E.coli* BL 21 (DE3) with *pET28EferPbR*

4.18Purification of Recombinant Chromate Reductase

4.18.1 Ammonium Sulfate Precipitation of Recombinant Crude Chromate Reductase

Maximum enzyme activity was achieved at 70% saturation (5.09 U/mg), whereas further addition of ammonium sulfate resulted into drop-in enzyme activity, while the activity was low at ammonium sulfate concentration below 70% saturation.

4.18.2 Filtration Chromatography

After dialysis partially purified chromate reductase was subjected for further purification by DEAE Sepharose ion-exchange chromatography technique, eluted by NaCl 0.9 M concentration along with 100mM phosphate buffer. A total of 30 fractions with 3 mL of each fraction, were collected. All fractions were assayed for chromate reductase activity and total protein was assayed at 280 nm. Maximum activity of recombinant chromate reductase was observed in fractions 09-17(Fig. 4.42).

4.18.3 Total Yield of Purified Recombinant Chromate Reductase from *Bacillus* Sp. strain S48

The total yield and purification fold for precipitated recombinant chromate reductase from *Bacillus* Sp. strain S48 was calculated. Total yield was 34.19% and the purification fold was 5.73%, respectively. The purification fold and yield are given in (Table 4.8).

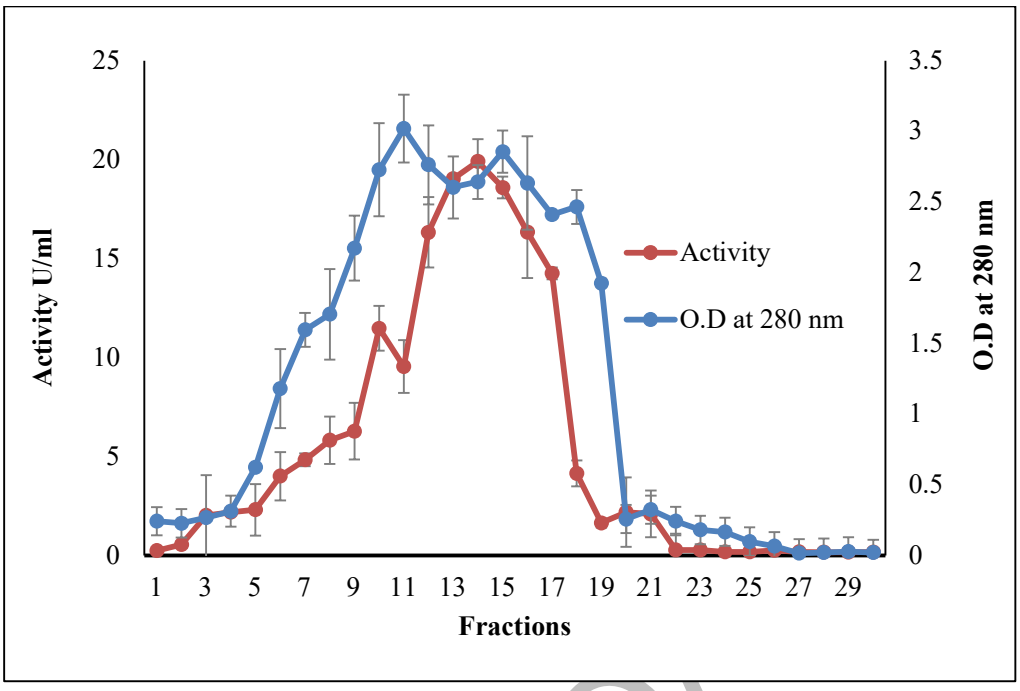


Fig 4.42 Total protein and activity profile of ammonium sulfate precipitated recombinant chromate reductase from *Bacillus* Sp. S48 through DEAE Sepharose ion-exchange chromatography technique.

Table 4.8 Purification steps of recombinant chromate reductase from *Bacillus* Sp. S48

Purification steps	Total Activity (U/ml)	Total Protein (mg/ml)	SA (U/mg)	Yield (%)	Purification Fold
Crude Extract	63866.67	218	292.9664	100	1
Sephadex(G 100)	21840	13	1680	34.19	5.73

4.19 Characterization of Purified Recombinant Chromate Reductase

4.19.1 Effect of Temperature on Activity and Stability

The effect of temperature on the activity of purified recombinant chromate reductase from *Bacillus* Sp. Strain S48was calculated by incubating enzyme with $K_2Cr_2O_7$(substrate) at different temperatures ranging (30 to 60˚C) for maximum 30 min. The optimum enzyme activity was achieved at 35°C, while activity was observed to drop at temperatures above and below 35˚C (Fig. 4.43) while, the stability of purified recombinant chromate reductase was calculated by incubating enzyme without substrate at different temperature ranges (30 to 60˚C) for 120 min. The maximum enzyme stability was achieved at 35°C and retained 94% of the stability for 120 min. More than 75% of the stability was achieved at temperatures 30˚C and 40˚C for 120 mins. Exposure to a temperature 50˚C recombinant chromate reductase retained only 20% stability for 120 minutes (Fig. 4.44).

4.19.2 Effect of pH on the Activity and Stability

The effect of pH on the activity of purified recombinant chromate reductase from *Bacillus* was calculated by incubating enzyme with $K_2Cr_2O_7$ substrate at various pH ranging (3.0-12.0) for 30 min. The optimum enzyme activity was achieved at pH 6, while activity was observed to drop at pH above and below 6 (Fig. 4.45) while, the stability of purified recombinant chromate reductase was calculated by incubating enzyme without $K_2Cr_2O_7$ (substrate) at various pH ranging (3.0-12.0) for 120 min. The highest enzyme stability was achieved at pH 6.0 and retained more than 90% of its stability for 120 minutes. More than 70% of the stability was retained after exposure to (pH 8.0- 10.0) for 120 mins. Whereas, almost 30% of stability was recorded at pH 3.0-5.0 and 10.0 (Fig. 4.46).

4.19.3 Effect of Metal Ions

Various metal ions at (2mM and 10mM) concentrations were used to analyze the effect on the activity of recombinant chromate reductase. In general, S48 enzyme activity was observed to increase with the increase of metal concentration. Strong inhibition of recombinant chromate reductase enzyme was observed in presence of $HgCl_2$ at both concentrations (2mM and 10mM). 100% of activity retained at $CuSO_4$,

KCl, NaCl, $CoCl_2$, $CuCl_2$ and $CaSO_4$ at both concentrations while $CaCl_2$, $NiCl_2$, $FeSO_4$, $NiSO_4$, K_2SO_4 and $MgSO_4$ potentially enhanced 27% enzyme activity at (10 Mm) concentration and 10% activity at 2 mM concentration. Enzyme retained 52% of activity in the occurrence of $CdCl_2$ at concentration 2mM and retained 38% of activity at 10mM concentration. Whereas, $ZnSO_4$ and $Na2SO_4$ decreased 15% the recombinant chromate reductase activity at both (2mM and 10 Mm) concentrations (Fig 4.47).

4.19.4 Effect of Surfactants on the Activity of Recombinant Chromate Reductase

Different surfactants at 0.50% and 1.0% concentrations were used to analyze their effect on the activity of purified recombinant chromate reductase enzyme. In general, SDS completely inhibited enzyme activity at both concentrations (0.50 and 1.0%), while in the occurrence of Triton X 100 enzyme retained 77% activity at low concentration (0.50%) and 61% at high concentration (1.0%). Comparatively, Tween 20, Tween 60, Tween 80, PEG and CTAB potentially enhanced recombinant chromium reductase activity at both concentrations (0.5% and 1.0%). (Fig. 4.48).

4.19.5 Effect of Organic solvents on the Activity of Recombinant Chromate Reductase

Different organic solvent (15%) ultimate concentration was used to determine the effect on the activity of recombinant chromate reductase for 120 mins. In general, DMSO enhanced 50% enzyme activity for 120 minutes while propanol increased 30% enzyme activity for 120 mins. While Ethanol, acetonitrile, ethyl acetate and n-hexane reduced the activity of enzyme with an increase of time. recombinant chromate reductase retained 100% activity in the presence of glycerol, methanol, formaldehyde and chloroform for 120 mins. (Fig. 4.49).

4.19.6 Determination of Kinetic Parameters

The kinetic parameter of S48 recombinant chromate reductase, Lineweaver and Burk (1934) plot was used to calculate the V_{max} and K_m constant. The same concentration of recombinant chromate reductase was incubated with various concentration 1 µM to 10 µM of K_2Cr_2O7 (substrate) and specific activity was determined. The recombinant chromate reductase was recorded 1.36 mg/ml. Respectively, V_{max} of recombinant chromate reductase was recorded 909.90 $\mu molmg^{-1}min^{-1}$ for K_2Cr_2O7 (Fig. 4.50).

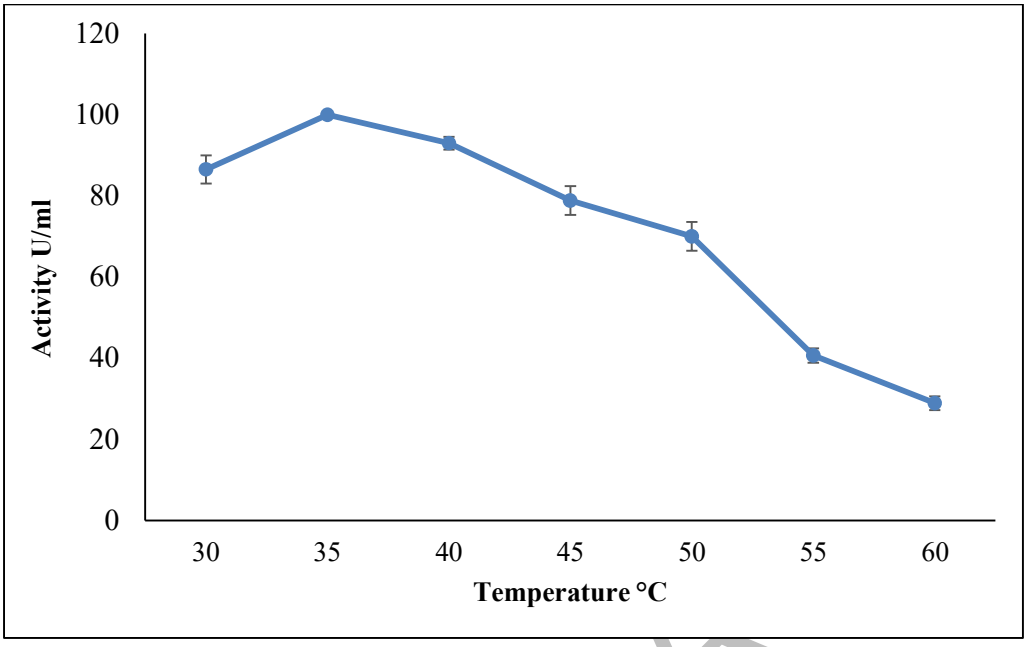


Fig. 4.43: Effect of temperature on activity of purified recombinant chromate reductase from *Bacillus* Sp. strain S48

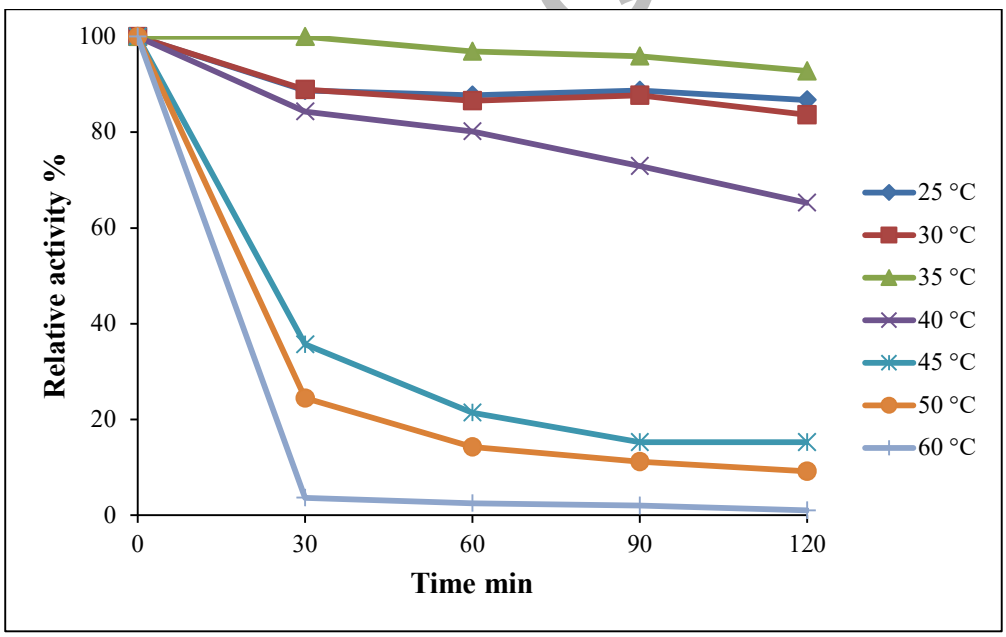


Fig. 4.44: Effect of temperature on stability of purified recombinant chromate reductase from *Bacillus* Sp. strain S48

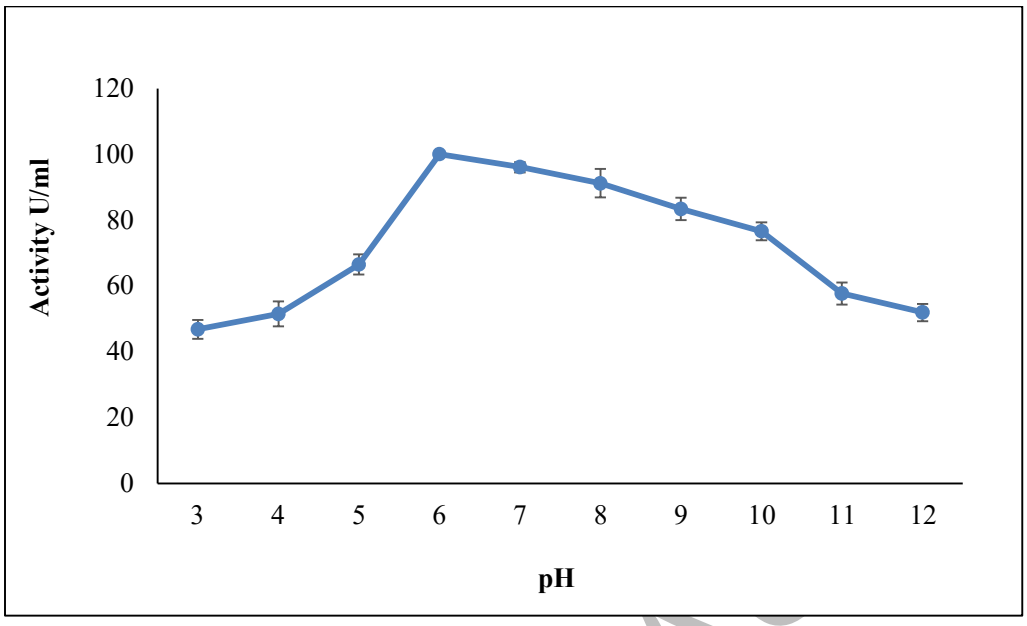


Fig. 4.45: Effect of pH on activity of purified recombinant chromate reductase from *Bacillus* Sp. strain S48

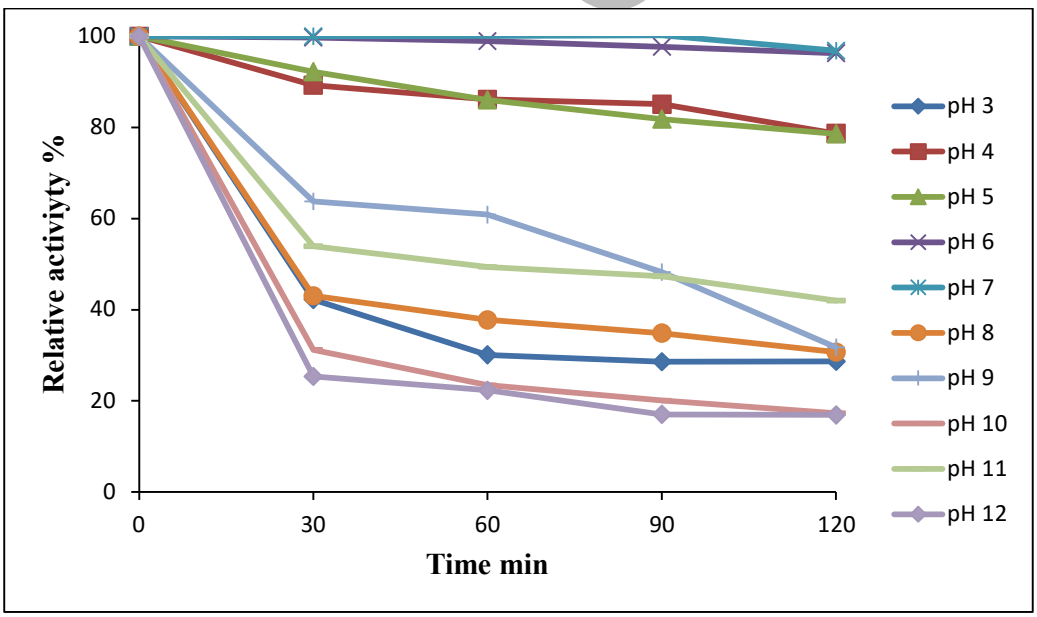


Fig. 4.46: Effect of pH on stability of purified recombinant chromate reductase from *Bacillus* Sp. strain S48

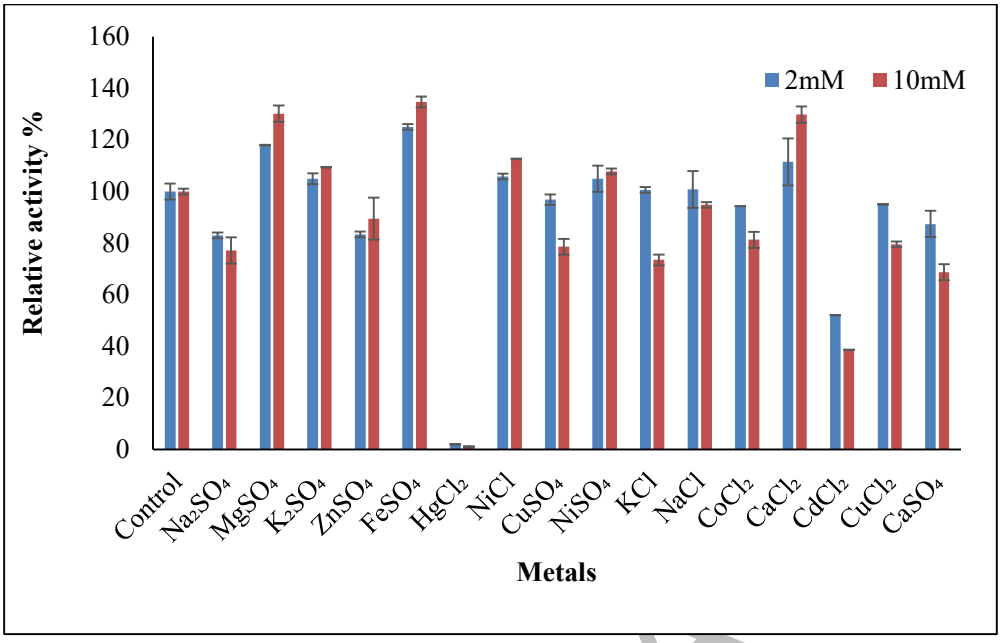


Fig. 4.47: Effect of metal ions on purified recombinant chromate reductase from *Bacillus* Sp. strain S48

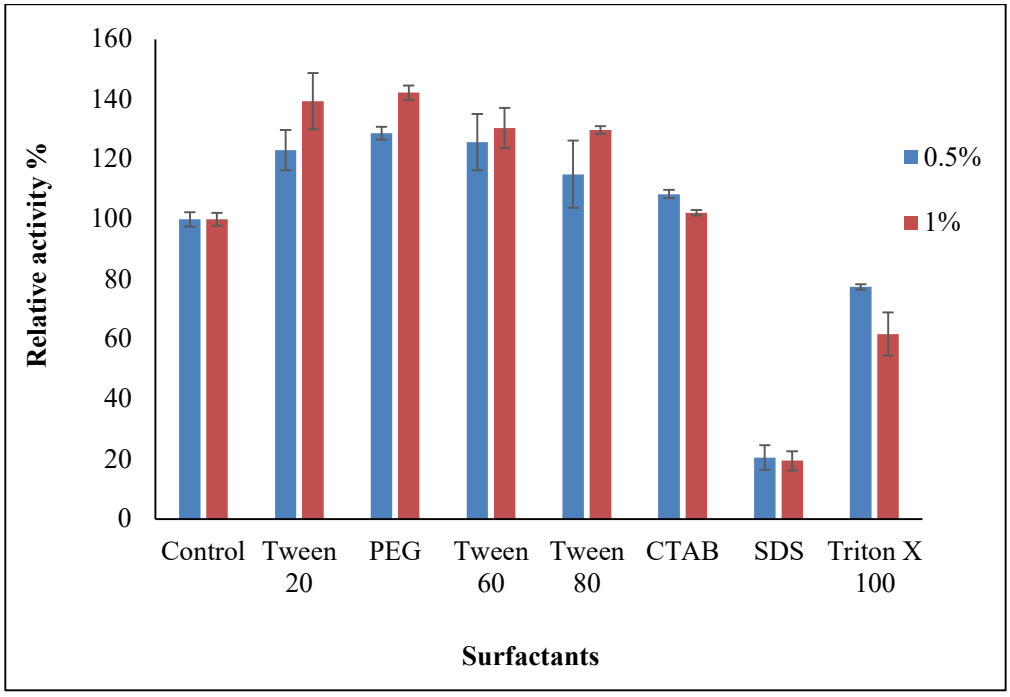


Fig. 4.48: Effect of surfactants on purified recombinant chromate reductase from *Bacillus* Sp. strain S48

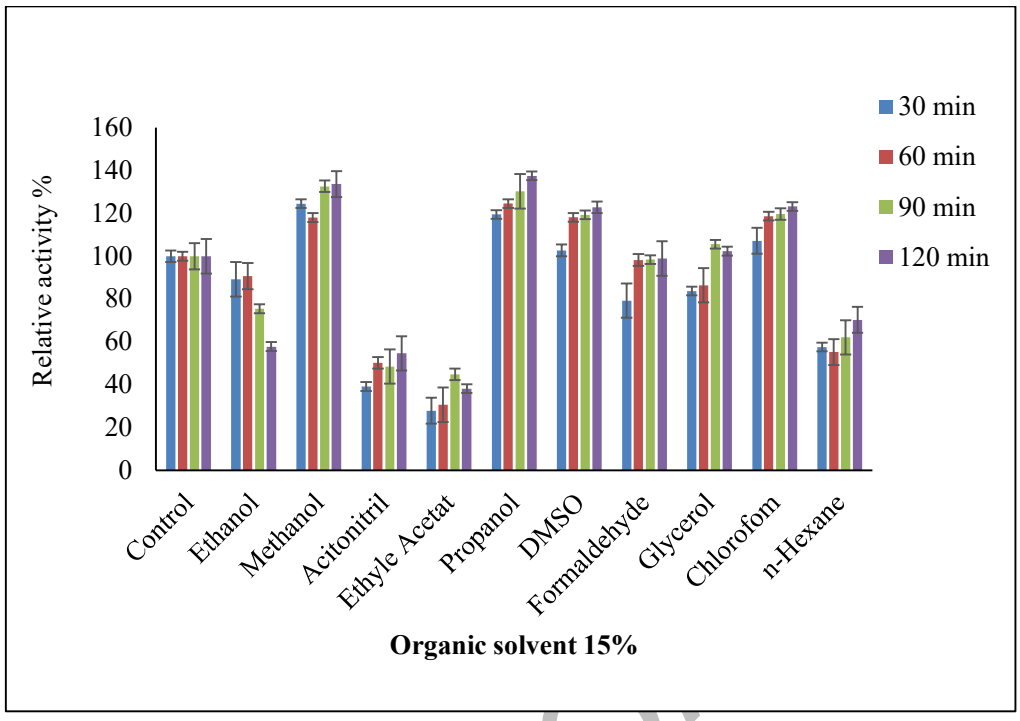


Fig. 4.49: Effect of different organic solvents on purified recombinant chromate reductase from *Bacillus* Sp. strain S48

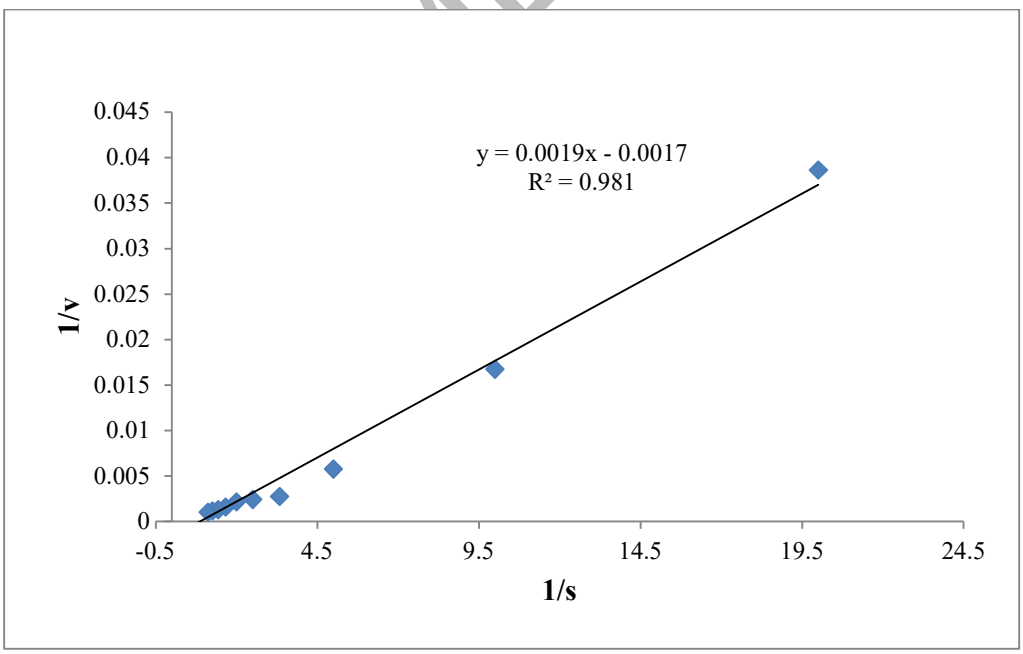


Fig. 4.50: Kinetics analysis of recombinant chromate reductase from *Bacillus* Sp. strain S48. K_m and V_{max} value were observed using Lineweaver-burk plot

4.20 Application of Purified Recombinant Chromate Reductase in Bioreduction of Hexavalent Chromium

Deionized water containing different concentration 100-500mg/L of chromium was treated with 10mg/mL of recombinant enzyme. The recombinant enzyme of *Bacillus* Sp. strain S48 showed 100 % chromate reduction at 24 hours with 25-100mg/L of chromium. Similarly, 200-500mg/L of chromium was completely reduced after 96 hours by recombinant chromate reductase isolated *Bacillus* Sp. strain S48 (Fig 4.51).

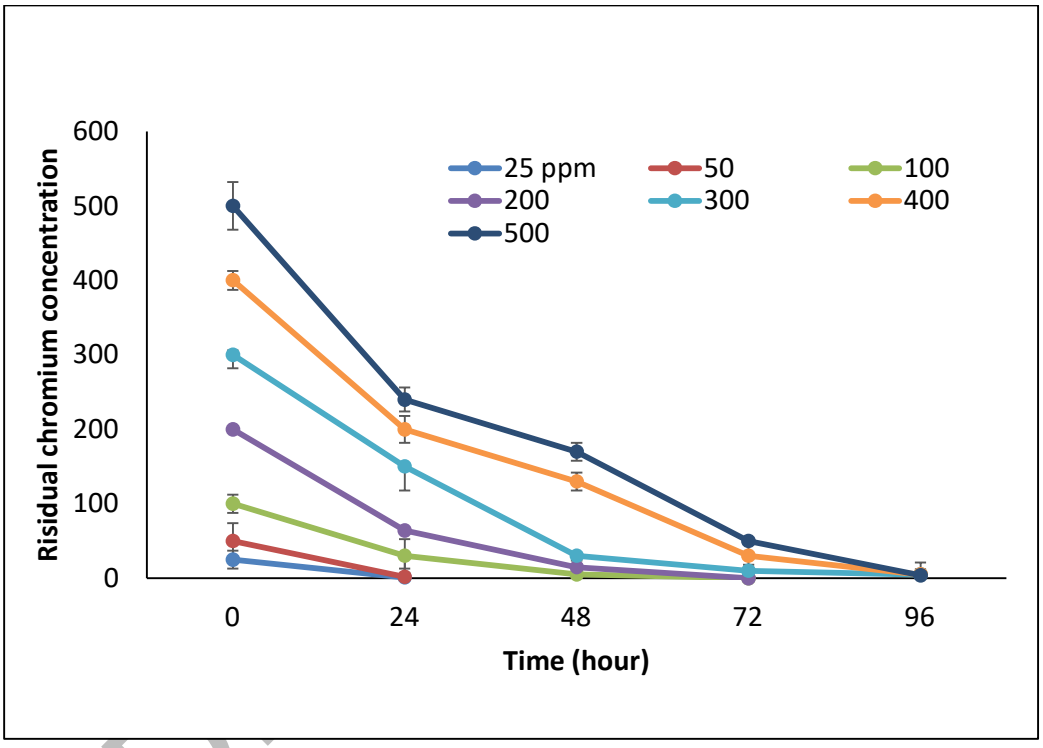


Fig 4.51: Aqueous solution containing different concentration of Cr (100-500 mg/L) treated with recombinant chromate reductase (10mg/ml).

4.21 Optimization of Conditions for Lead Biosorption by Recombinant Strain *PbREcoliBL21*

4.21.1 Effect of Temperature

Effect of temperature on growth and biosorption of lead by recombinant strain *PbREcoliBL21* was analyzed using various temperatures ranging from 25-50°C. Highest growth OD (600nm) of recombinant strain *PbREcoliBL21* was achieved at

35°C while more than 90% Pb biosorption was observed after 72 hours of incubation (Fig 4.52).

4.21.2 Effect of pH

The effect of pH on growth and biosorption of Pb by recombinant strain *PbREcoli BL21*was analyzed at various pH ranges from 5.0-9.0. Maximum growth OD (600nm) of recombinant strain *PbREcoli BL21*was observed at pH 7.0 and more than 90 % of Pb was removed after 72 hours of incubation (Fig 4.53).

4.21.3 Biosorption of Lead by Recombinant Strain *PbREcoliBL21*

Lead (Pb) biosorption by recombinant strain *PbREcoli BL21*was determined using Pb $(NO_3)_2$ as a source for maximum 96 hours of incubation. Strain *PbREcoliBL21* formed biofilm over the ceramic balls, after formation of biofilm the ceramic balls were washed with an electrolyte solution of 0.1 M NaCl. The recombinant strain *PbREcoliBL21* showed potential to successfully removed Pb from the supernatant. The amount of residual Pb present in supernatant after the process of biosorption was measured by AAS. 95 % biosorption of Pb with concentration of 100 mg/L was achieved after 72 hours of incubation (Fig. 4.54).

4.21.4 SEM Analysis of Ceramic Balls after Batch Biosorption of Lead by Recombinant *PbREcoliBL21*

The micrographs of ceramic balls obtained after batch biosorption of Pb clearly showed different stages of biofilm formation onto ceramic balls and attachment of Pb metal ions onto biofilm surfaces in form of white patches in comparison to control (Fig. 4.55B). This could be attributed to the fact that the biofilm matrix produced by recombinant strain *PbREcoli BL21* allows the binding of the biofilm with divalent cations.

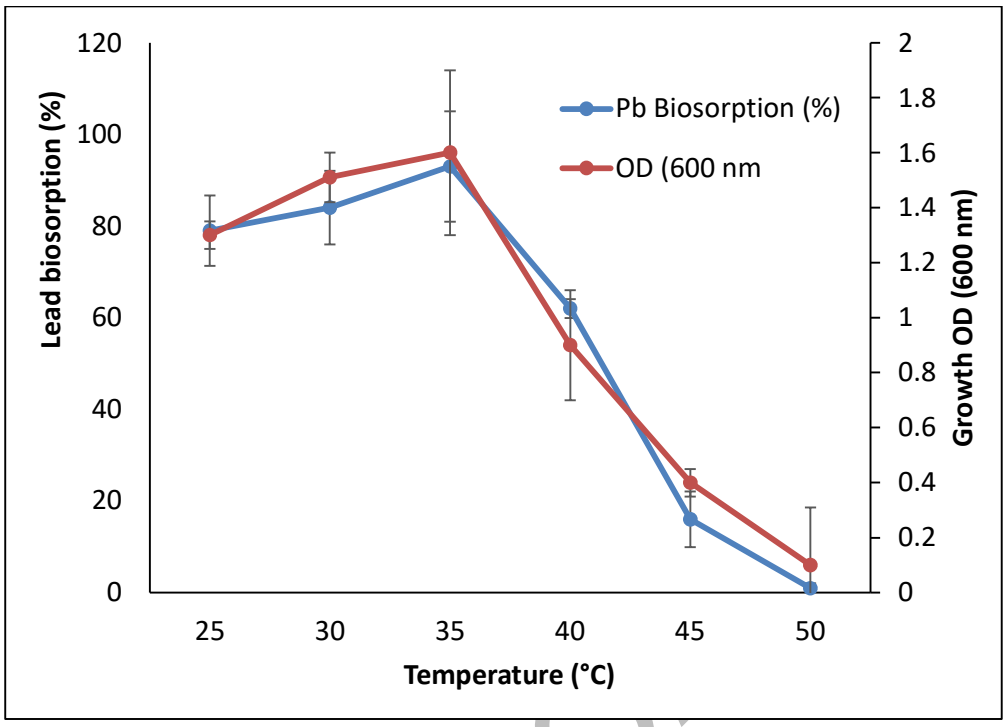


Fig 4.52 Effect of temperature on recombinant strain *PbREcoliBL21* growth and lead biosorption using (100 mg/L) of Pb

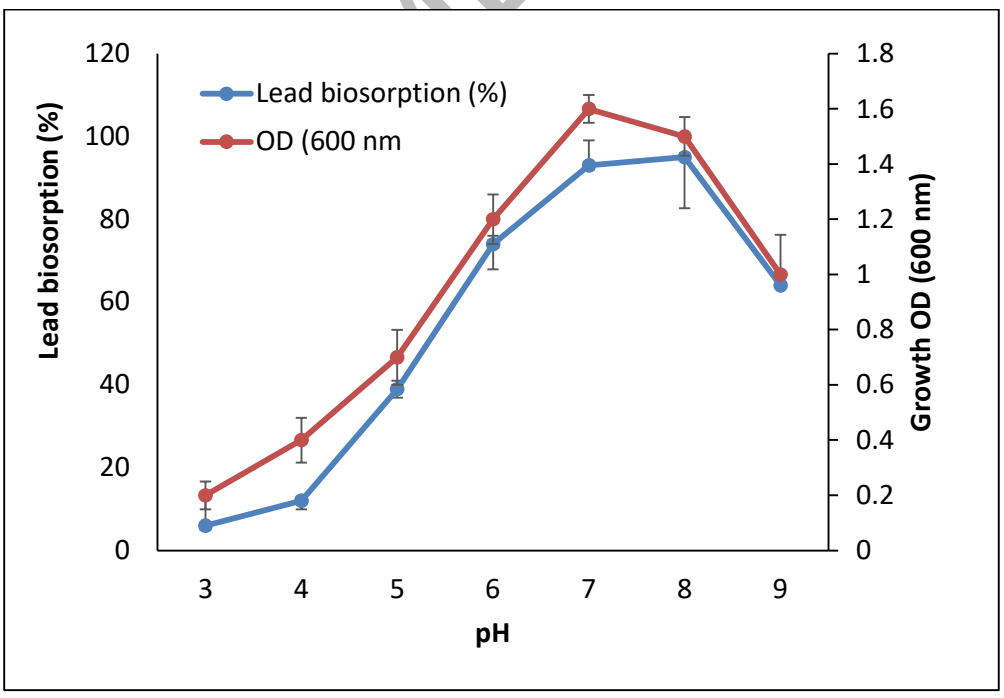


Fig 4.53 Effect of pH on recombinant strain *PbREcoliBL21* growth and lead biosorption using (100 mg/L) of Pb

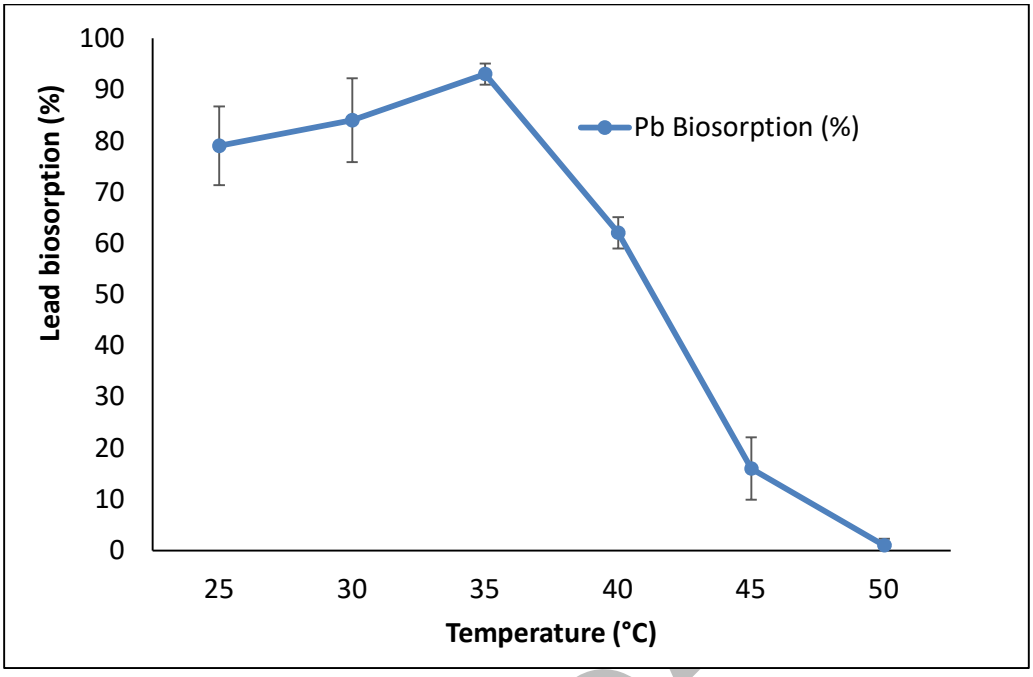


Fig 4.54 Biosorption of Pb by recombinant strain *PbREcoliBL21* using (100 mg/L) of Pb

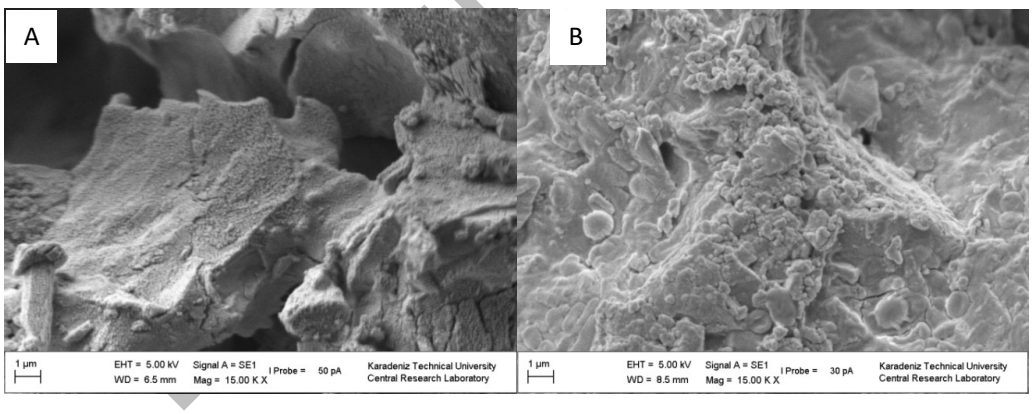


Fig 4.55 SEM images of ceramic balls obtained after Pb removal (A) Abiotic control (x15.0K) (B) Micrographs that show the presence of the retained metal (x15.0K).

4.22 Removal of Lead by Designed Biofilter Using Recombinant *PbREcoli BL21* Immobilized on Ceramic Balls

Two setup of columns were used for Pb(II) biosorption in a continuous sequence, one column for the ceramic balls (control) without biofilm, and another column for the ceramic balls covered with *PbREcoliBL21* biofilm (Fig 4.56). The operating parameters for column are presented in Table 4.9. Pb(II) biosorption by recombinant *PbREcoliBL21* was determined using 100 mg/L of $Pb(NO_3)_2$ as a source of lead for a maximum upto 72 hours of incubation. About 4 mL of sample was taken at different time intervals and Pb biosorption was measured by ICP-MS analysis. The column contained ceramic balls covered by recombinant *PbREcoliBL21* successfully removed 95% of Pb(II) with a concentration of 100 mg/L in the supernatant after 72 hours, while no biosorption was observed in a column with without biofilm (Fig 4.57).

4.22.1 Scanning Electron Microcopy of Ceramic Balls after Removal of Lead by Lab Scale Biofilter

The micrograph of ceramic balls obtained after a continuous column experiment for Pb removal clearly showed different stages of biofilm formation on ceramic balls and attachment of Pb particles to biofilm surfaces can easily be observed in the form of white patches (Fig. 4.58B).Whereas no binding of Pb to ceramic balls was observed in micrograph from control experiment (Ceramic balls+ Pb) (Fig. 4.58A).This could be attributed to the fact that the biofilm matrix produced by recombinant *PbREcoli BL21*allow the binding of biofilm with divalent cations.

4.22.2 FTIR Analysis of Ceramic Balls after Biosorption of Lead

FTIR analysis was performed for the determination of functional groups and their role in the removal of metals on the ceramic ball surfaces. The results from control samples demonstrated the presence of very few functional groups on ceramic support, which reduces the capacity of ceramic balls for adsorption of Pb metal. Whereas more functional groups were observed on ceramic balls with immobilized *PbREcoli BL21.*Peak positions at 2922 and 3277 cm^{-1}, γC-H bond of $-CH_2$ groups combined with CH_3 groups, altered their position after the biosorption of Pb. Increase in the intensity at 1647cm^{-1} showed C=O of amide stretching (Fig. 4.59)

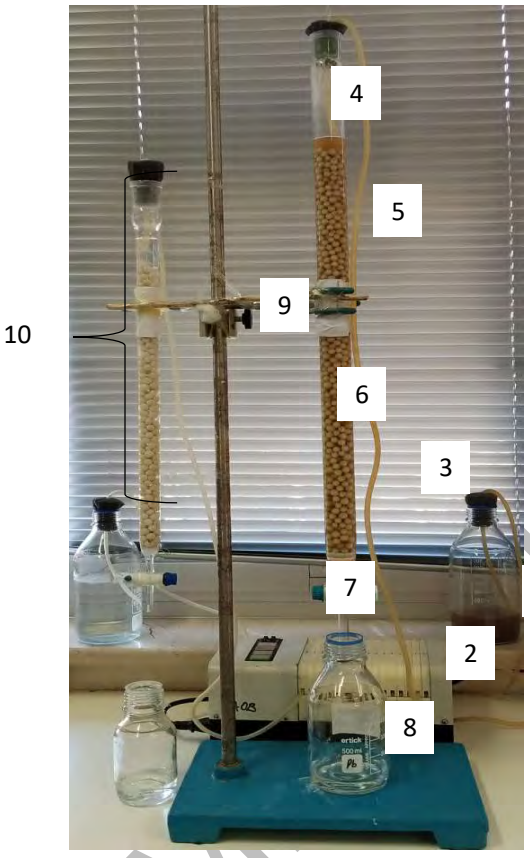


Fig. 4.56 Development of biofilter using recombinant *PbREcoli BL21* cell immobilized on Ceramic balls. The description of numbers pointed in this figure is mentioned in table 4.9

Table 4.9 Column operating parameters for Pb(II) biosorption by using recombinant *PbREcoliBL21* cell immobilized on ceramic balls

S.No	Parts	Purpose
1	Metal Solution (100 mg/L in MSM)	Supplied to support microbial growth (1L)
2	Control volve	Control flow of MSM and lead solution (100 ppm/L) 4 mL/min
3	Tube	Flow of solution to column
4	Column	Glass tube (length 76 cm) (diameter 10cm)
5	Packing material	Composed of ceramic ball (500g) Column fill 65cm
6	Packaging media	Composed of MSM solution (70 cm)
7	Outlet sampling port	Sample outlet
8	Collection Tank	Receive treated solution
9	Stand	Support for column
10	Control	No bacterial cell (Abiotic control)
11	pH	7.0
12	Temperature	35 °C

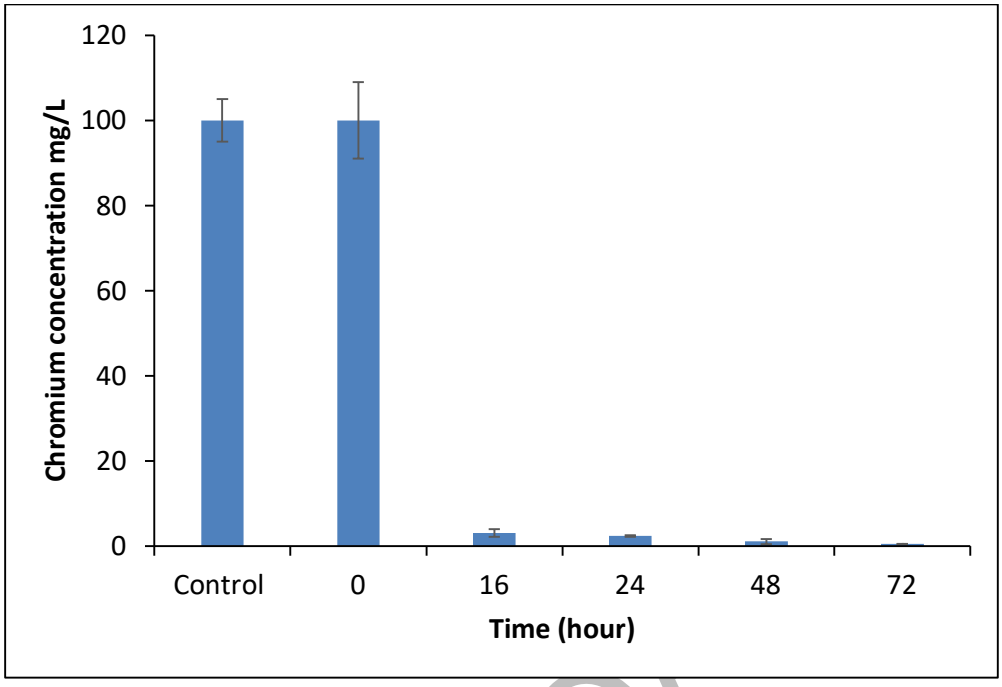


Fig. 4.57 Biosorption of Pb (100 mg/L) in lab scale bio filter using *PbREcoli BL21* cell Immobilized on Ceramic balls.

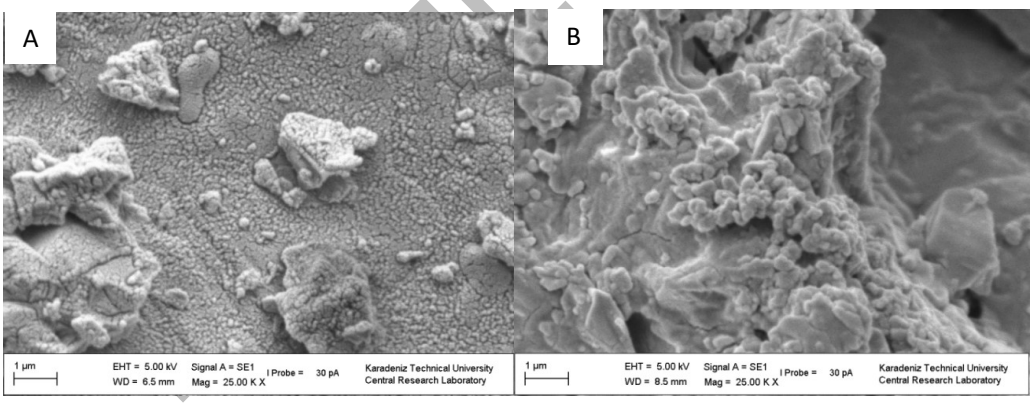


Fig 4.58 SEM images of ceramic bioball obtained after lead biosorption **(A)** Abiotic control **(B)** Micrograph that shows the presence of the retained metal.

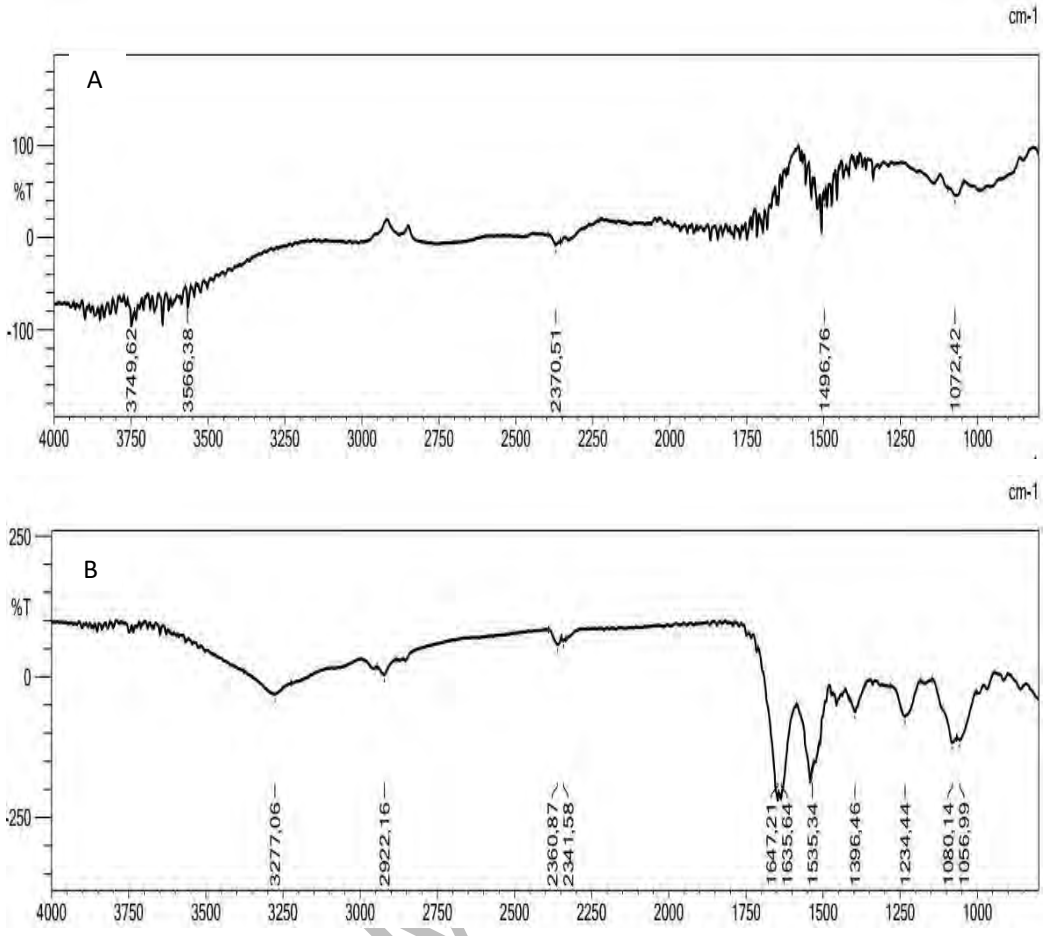


Fig. 4.59 FTIR spectra of ceramic balls (A) Abiotic control (B) Balls with immobilized *PbREcoli BL21* cells.

CHAPTER 5
DISCUSSION

Discussion

In the last few decades, an increase in concentration of different heavy metals caused environmental contamination and public health problems. Human exposure to metals increased due to increase in use of heavy metals in various industries such as textile, painting, leather etc. Effluents releasing from these industries have profound effect on human health as well as environment (Raskinand Ensley, 2000). Accumulation of potentially toxic heavy metals i.e. Hg, Cd, Cr, Cu and Zn in a body causes mental abnormalities, neuromuscular disorders, cancer and growth abnormalities (Wuana and Okieimen, 2011). A large number of microorganisms are found naturally in waters and soil receiving industrial effluents. These microorganisms have adopted and developed various strategies as a defense mechanism to protect themselves from the toxicity of heavy metals (adsorption, uptake, methylation, oxidation, and reduction). Accumulation of Cr (VI) and Pb in agriculture lands reduces soil productivity by decreasing the population of various soil microbes (Karthik et al., 2017; Wani et al., 2018).

Elemental analysis of sludge and soil samples were performed and high concentration of different metals were observed, chromium (Cr) and lead (Pb) were found in very high concentration approximately 2648.644 and 339.137 mg/L, respectively, which is above US-EPA standard values. Total Cr and Pb in industrial effluents were found varying from 0.005 to 1423.05 mg/L (Hossain and Islam, 2019). The levels of Cr and Pb (40.06 ± 0.21) were the highest and the water comprehensive pollution index was 1000 times greater than the critical values (Chan et al 2021). Karachi is very largest and important city due to its dimensions and economic worth. There are approximately 11000 industrial units including 2571 industrial setups in Korangi (Aziz and Khan, 2014; Ahmed et al. 2018; Mujeeb et al. 2020), these industries are linked to coastal area due to continuous shipping through Port of Karachi increasing pollution day by day and major source of contamination of forest and marine life (Ahmed et al., 2018; Ali et al., 2019).

The current study was aimed to isolate the most potent Cr and Pb resistant bacteria from industrial effluents, and qualitative test was performed for the initial screening of Cr and Pb resistant strains. Two bacterial strains *Bacillus* sp. strain S48 from Korangi sludge and *Escherchia* sp. strain S54 from Lyari soil were found to be the best Cr and Pb resistant strains respectively, Similar study was conducted in which high concentration of Cr and Pb in the industrial waste was recorded (Wani and Khan, 2010). Bacterial population in industrial effluents were present approximately 200 – 300 colonies per 100 mL at different sample location (Mustapha and Halimoon, 2015). The industrial effluent contains Cr and Pb, which makes it an ideal medium for many bacterial species to grow (Saranraj et al., 2013).

Bacillus sp. strain S48 and *Escherchia* sp. strain S54 were screened for Cr and Pb resistance, S48 and S54 exhibited highest Cr (1500 mg/L) and Pb (1200 mg/L) tolerance level at temperature 35°C and pH 7.0, respectively. In a previous study the minimum inhibitory concentration (MIC) for heavy metal i.e. Cr and Pb were examined ranging from 50 to 1900 µg/ml (Marzan et al., 2017). A bacterium *Cellulosi microbium* (KX710177) previously reported from tannery effluent, survived in the presence 100 mg concentration of the Cr (Bharagava and Mishra, 2018).

Bacillus sp. strain S48 showed highest growth and Cr (VI) reduction at temperature 35°C and pH 7.0. Whereas Cr (VI) reduction was significantly decreased above and below optimum temperature. *Bacillus* sp. strain S48 showed 60% of Cr (VI) reduction at 35 °C and pH 7.0 whereas 30% reduction was observed at 30°C and pH 5.0. Rapid reduction of Cr by *Bacillus* Sp. strain S48 was observed upto 50 mg/L within 48 hours of incubation (chromate reduction rate 1 mg/L/hour). Approximately 60% of reduction was achieved with increase in initial concentration of Cr around 100-500 mg/L after 96 hours. Our result showed significant bioreduction of Cr within short period of time in comparison to the previous report where 80% of Cr (VI) reduction by *Pseudomonas sp.* MAI4 was observed with initial concentration 150µg/mL after 120 hours (Wani et al., 2019). Das et al., (2014) also reported pH 7.0 to be the optimum for maximum reduction of Cr (VI). Optimum pH for chromate reductase falls between the range of pH 6.5 and 9, because secretion of chromate reductase

changes with fluctuation in pH from optimum point (Wani, et al., 2018). The most suitable parameters as previously reported are considered to be 100 mg/L, pH 7 and 35°C for maximum Cr (VI) reduction (Das et al., 2014). Almost complete reduction of Cr by *B. amyloliquefaciens* has already been reported at 35°C after 144 hours (Dhal et al., 2010). Whereas bacterial growth and Cr (VI) reduction was negatively affected at high temperature due to inhibition of cellular physiological activity as a result of damage to bacterial macromolecules (DNA and proteins) as well as outer membrane structure. An increase in incubation time resulted in to an increase in reduction (Soni et al. 2012).

The FTIR spectrum of *Bacillus* sp. strain S48 after incubation with Cr metal indicated a visible shift in peaks. These results correspond to attachment of metal to the functional group present in the cell wall of bacterium. The peak at position 2929cm^{-1} represents to stretching vibrations of C-H bond. The increase in value of peaks indicates its role in binding of Cr. The peak position at 1633 cm^{-1} corresponds to vibration of C=O group. A stretching vibration of C=*O* and C-H groups was characterized that represent their role in Cr binding (Srinath et al., 2002). The scanning electron micrographs of *Bacillus* sp. strain S48 after incubation with metal were also observed for morphological changes. The surface of bacterial strain S48 with Cr appeared distorted, dense and attached to each other in comparison to control with clear smooth surface. According to the previous reports, several bacterial strains, such as *Pseudomonas* sp. B50D, *Acinetobacter* sp. B9 and *Bacillus* sp. strain PZ-1 were observed with similar morphological changes in the presence of toxic heavy metals (Giovanella et al., 2017; Bhattacharya and Gupta,2013). These results proposed that configuration of bacterial cell wall and surface properties play the most imported role in survival of bacteria under metal stress.

In this study, *Escherchia* sp. strain S54 was observed with potential to remove Pb from samples through biosorption. The optimum growth and biosorption by strain S54 was found at 35°C and pH 7.0 and 55% reduction were achieved after 96 hours. The results of Pb biosorption studies suggested that bacterial biomass increased with an increase in incubation time, Likewise Pb biosorption also increased due to the large

surface area which can improve the biosorption of Pb. (Edris et al. 2014). Therefore, our results clearly demonstrated that the optimum growth of our bacterial strain is not inhibited in the presence of different temperatures and pH. The current results suggested that our bacterial strain is a strong candidate for metal removal in future applications.

The FTIR pattern and scanning electron images of *Escherchia* Sp. strain S54 showed that COO, CH_2 and O-H are involved in the binding of Pb A similar result of the FTIR spectrum by *Bacillus* sp. MN3-4 was also reported by Kushwaha et al., (2018), suggested the function of carbonyl and amide groups present in the bacterial cell wall for Pb adsorption. A stretching vibration of O-H and C*OO* groups was found that suggested their role in Pb binding (Kushwaha et al., 2018). Moreover, several bacteria have been reported, such as *Pseudomonas* sp. B50D, *Acinetobacter* sp. B9 and *Bacillus* sp. strain PZ-1 with similar morphological changes after incubation with various toxic heavy metals (Giovanella et al., 2017; Bhattacharya and Gupta, 2013). Bueno et al., (2008) reported, FT-IR and SEM analysis for bacterial strain *Rhodococcus opacus* that the, –NH, –OH and –COOH groups have major role in uptake of Pb and the essential mechanism may contain electrostatic biosorption (Bueno et al. 2008).

Maximum production of chromium reductase by *Bacillus* Sp. strain S48 was achieved at temperature 35°C, pH 7.0. The nutritional parameters for maximum enzyme production were statistically optimized using Plackett-Burman and central composite designs, and the total yield was increased up to 545.993 U/mg. Mala et al., (2015) in obtained an initial chromate reductase activity of 212.84 U/mg protein at 48 h in a low-cost defined medium amended with 0.25 mM chromate and increase upto 312.99 U/mg at 48 h by addition of inducer Mala, (2015), reported chromium reductase production significantly increase by using Plackett-Burman and CCD. Very few studies are available where statistical design has been used for optimization of chromate reductase production and reduction of Cr(VI). Plackett-Burman design is an efficient statistical tool for optimization of medium components (Mabrouk et al., 2008; Mabrouk et al., 2014). Chromate reductase was purified to homogeneity by gel

filtration column chromatography and its molecular size was determined to be approximately 35kDa. Chromate reductase enzyme from a novel bacterium *Ochrobactrum* sp. strain Cr-B4 was partially purified and characterized and its molecular mass was found to be approximately 31.53 kDa, (Hora and Shetty, 2015).

We further characterized the purified chromate reductase by determining physic-chemical parameters for optimum activity and stability. The optimum activity was obtained at 40˚C and pH 7.0 while retained 100% activity for 150 minutes. Its activity was enhanced in the presence of various metal except Hg^{+2}. Chromate reductase from *Rhodopseudomonas palustris* KU003 was strongly inhibited by metals such as Cd^{+2}, Zn^{+2} and Cu^{+2} (Merugu et al., 2013). Purified chromate reductase by *Bacillus* Sp. strain S48 retained 100% activity in the presence of various surfactants strong inhibition was observed by SDS and CTAB. The non-ionic surfactants react with enzyme hydrophobic region and lead to modification in 3-D structure (Gulcin et al., 2003). Organic solvents such as acetonitrile, *n*-hexane and ethyl acetate reduced enzyme activity with increase time. The effect of organic solvents on chromium reductase on enzyme activity depend on the polarity of the active site which could lead to activity inhibition by the orientation of the bound substrate (Merugu et al. 2013). The chromium reductase exhibited promising low *K*m and *high V*max values which indicated a high viability of the reaction system in the presence of $K_2Cr_2O_7$ as a substrate.

Chromate reductase (ChR gene) and Lead binding protein gene (PbR) from *Bacillus* Sp. strain S48 and *Escherchia* Sp. strain S54 were cloned and expressed in *E. coli* BL21 host. Interestingly, chromate reductase and lead binding protein activity in *E. coli* was found to be higher than in the native strains S48 and S54, possibly due to higher level of protein expression. The enzyme produced by *E. coli* was functionally active and capable of removing toxic Cr and Pb even. Several researchers have reported their success stories on cloning and expression of ChR and PbR genes in *E. coli* host (Wei et al., 2014; Erez et al., 2007). The cloned Chromate reductase (ChR gene) and Lead binding protein gene (PbR) from *Bacillus* Sp. strain S48 and *Escherchia* Sp. strain S54 are of 0.6 kb and 0.43 kb like those of *Bacillus* sp (Chenet

al., 2012; He et al., 2018). Baldiris et al., (2018) reported previously 468 bp chromium reductase gene in *S. maltophilia*. Similarly, Patra et al., (2010) reported partially purified chromate reductase that was 268 bp. The ChrR gene was mostly identified in Gram-positive bacteria such as *Bacillus atrophaeus Rhodococcus erythropolis and Arthrobacter aurescens* have a potential role in bioremediation of toxic Cr(VI) into Cr(III) (Baldiris et al., 2018; Patra et al., 2010; Chaney and Baklanov et al., 2017).). Consequently, Shehata, (2018) constructed different recombinant *E. coli* encoding AT, PbrR-AT, SmtB-AT and PbrR/SmtB-AT for expressing of PbrR and determined their Pb removal efficiency. These results showed the successful expression of PbrR-AT and SmtB-AT recombinant proteins in the *E. coli to remove toxic metals from wastewater effluents (Shehata, 2018).*

The recombinant *BparChR* was observed to retain 100% stability at temperature range 25-35 with optimum activity at 35°C and optimal pH at 7.0. The highest activity at neutral pH may offer sustainability of the *BparChR* for bioremediation of industrial effluents. Enzyme activity increase in the presence of acidic pH (3.0) and alkaline pH (10.0) represent that the enzyme conformation was best at that pH, however not expressing the optimum activity. Zhou et al., (2017) reported ChrT-engineered bacteria that have the capability to remove metals such as Cr (VI) at optimal temperature 37˚C and pH 7.0, which may be associated with the type of the host bacteria. Furthermore, the optimum pH and temperature for recombinant *E. coli* BL21 (DE3) is pH 7.2 and 37˚C (Fan. Et al., 2009). Sometime Unsuitable environmental conditions affect the growth and survival of host bacteria, as a result removal Cr (VI) may be effected to change in environmental conditions. In our study, the enzyme was active in the presence of metal ions except Hg^{+2}, inhibited *BparChR* activity. In bioremediation technology the main issue associated in the presence of other pollutants that effect the process of Cr (VI) removal the main problems to be solved are the effects of other pollutants on reducing Cr (VI), most commonly is react other metal ions ions (Chovanec et al., 2012). Chovanec et al., (2012) demonstrated in previous study that most of the metal ions did not affect the Cr (VI) reduction process in engineered strain, although Cu^{2+}, Pb^{2+} and Co^{2+} enhanced the removal ability, which may be helpful to the actual application in the environment. The present result

is suitable for wastewaters contain in accumulation of Cr. The enhanced activity of chromate reductase in presence of metal ions is significant for effective bioremediation of wastewater polluted.

Effluent containing 100-500mg/L of $K_2Cr_2O_7$ was treated with purified recombinant *BparChR* showed 98% chromate reduction after 96 hours. This reduction in Cr is greater than earlier reports on most of the bacterial chromate reductase (Magnuson et al. 2010). In the previous study (Zhou et al., (2017) reported that ChrT-engineered bacteria and their enzyme chromium reductase have the capability to detoxify the Cr (VI) into Cr (III), which provided the basis and developmental value to the practical application of engineered bacteria and ChrT in Cr (VI) polluted water and soil (Zhou et al., (2017). The recombinant *BparChR* is the most potent for removal of toxic Cr(VI) l from solution and thus has the most potential and efficient method for Cr treatment in industrial effluents before release into water. In the ICP-MS analysis a total of 95% biosorption of Pb was recorded after 96 hours by recombinant PbR*Ecoli*BL21 in batch biosorption and column experiment containing 100 mg/L of Pb The biosorption potential of recombinant strain was found better than *Bacillus cereus (85.4%) (*Murthy et el., 2012), and *Streptomyces* VITSVK5 (84%) (Saurav and Kannabiran, 2011) The remarkable metal uptake is depending of the strong biofilm of recombinant PbR*Ecoli*BL21 on ceramic ball. A different behavior also found in the previously reported literature for metal absorption in the column (Volesky, 2003; Vijayaraghavan et al., 2005; Muñoz et al., 2016).

The FTIR pattern and scanning electron images of *Escherchia* Sp. strain S54 showed that *Si–O–Si*, C-H, CH_2, CH_3, O-H, Si–OH are involved in the binding of Pb A similar result of the FTIR spectrum by *Bacillus* sp. MN3-4 was also reported by Kushwaha et al., (2018), suggested the function of carbonyl and amide groups present in the bacterial cell wall for Pb adsorption. At suitable pH circumstances, these links could increase to functional groups such as aluminol (Al–OH) from the silanol (Si–OH) and octahedral layers from the tetrahedral layers of the stratified alumino silicate (Saikia et el., 2010). The spectrum of FTIR by *Klebsiella* sp. 3S1 found in the previous study (Munoz et el., 2015) was also included for relative purposes. This could be attributed

to the fact that the biofilm matrix produced by recombinant *PbREcoli*BL21 allow the binding of the biofilm with divalent cations. This could be concluded that most of the gram negative bacteria produced EPS matrix typically neutral (uronic acids) or polyanionic polysaccharides. This anionic behavior could provide the binding of the bacterial biofilm with divalent cations (Chmielewski, and Frank, 2003; Kurniawan and Yamamoto, 2013; Giovanella et al., 2017). Munoz et al., (2015) reported, FT-IR and SEM analysis for bacterial strain *Klebsiella* sp. 3S1 that the, *Si–O–Si,* –NH, –OH and Si–OH groups have major role in uptake of Pb and the essential mechanism may contain electrostatic biosorption (Munoz et el., 2015).

CONCLUSION

Conclusions

i. Total 53 bacterial strains were isolated from both sludge and soil sample. 40 bacterial strains from Korangi sludge and 13 bacterial strains from Liyari soil samples. Among which the most putative strains were identified, characterized and further investigated for bioremediation.

ii. *Bacillus* sp. strain S48 and *Escherichia*sp.strain S54 exhibited the highest tolerance to Cr (1500 mg/l) and Pb (1200 mg/l), respectively.

iii. Native *Bacillus* sp. strain S48 can effectively reduce Cr (VI) to Cr(III) with maximum reduction potential more than 60% after 96 h. Whereas native*Escherchia*sp.strainS54 has proved Pb Biosorption potential and can affectively remove 60% of Pb after 72 h. The bioreduction and biosorption potential was confirmed through SEM-EDX and FTIR.

iv. Native *Bacillus* sp. strain S48 was investigated for production of chromate reductase. Maximum chromate reductase production was achieved at 35°C and pH 7.0. The purified enzyme was stable at a temperature 35°C and pH 7.0 and retained 100% of its activity for 150 min. More than 70% reduction in chromium was achieved after treatment with purified enzyme.

v. Chromium reductase (ChR) and Lead binding protein (PbR) genes from *Bacillus* sp. strain S48 and *Escherchia* sp. strain S54 were successfully cloned into intracellular PET-28a and expressed into BL 21 (DE3) and used for removal of metals. The efficiency of chromium and lead removal were enhanced to 90% and 95%, respectively when treated with recombinant cells.

vi. Both batch and continuous column experiments clearly demonstrated that the recombinant *PbREcoliBL21* significantly improved the adsorption properties of the support and FTIR spectra showed that *PbREcoliBL21* provides binding sites for the retention of the metal cations. A total of 95% biosorption in lead were achieved by *PbREcoliBL21* in fixed bed biofilter.

FUTURE PROSPECTS

Future Prospects

Future prospects include

i. Diversity of sampling sites for isolation of more significant strains.
ii. Cost-effective removal of lead and chromium on large scale.
iii. Chromate reductase and lead binding protein are capable of removing metals, which provided the basis and developmental value to the practical application of more engineered bacteria in metals polluted soil and water.
iv. The biofilter column is found stable for varied input loads of metal concentration, which signifies that this system may be well suited for actual industrial operations. It gives an insight for scale-up of biofilter operation to pilot and industrial levels.

REFERENCES

References

1. Abd-Elwahed, M. S. (2018). Influence of long-term wastewater irrigation on soil quality and its spatial distribution. *Annals of Agricultural Sciences*, *63*(2), 191-199.
2. Adhikari, K., & Pal, S. (2016). Assessment of pollution potential of soil and groundwater in a non-engineered MSW landfill site. *International Journal of Environmental Science and Development*, *7*(3), 207.
3. AHMED, Q., Levent, B. A. T., ÖZTEKİN, A., & ALI, Q. M. (2018). A review on studies of heavy metal determination in mackerel and tuna (Family-Scombridae) fishes. *Journal of Anatolian Environmental and Animal Sciences*, *3*(3), 107-123.
4. Alam, M. Z., & Malik, A. (2008). Chromate resistance, transport and bioreduction by Exiguobacterium sp. ZM-2 isolated from agricultural soil irrigated with tannery effluent. *Journal of basic microbiology*, *48*(5), 416-420.
5. Ali, N., Khan, M. U., &Khoso, S. A. (2019). Identification of biological threats zones along the coastline of Karachi. *International journal of environmental science and technology*, *16*(7), 3557-3564.
6. Alves, C. F., Melo, L. F., & Vieira, M. J. (2002). Influence of medium composition on the characteristics of a denitrifying biofilm formed by Alcaligenesdenitrificans in a fluidised bed reactor. *Process Biochemistry*, *37*(8), 837-845.
7. Asati, A., Pichhode, M., & Nikhil, K. (2016). Effect of heavy metals on plants: an overview. *International Journal of Application or Innovation in Engineering & Management*, *5*(3), 56-66.
8. Azimi, S., &Moghaddam, M. S. (2013). Effect of mercury pollution on the urban environment and human health. *Environment and Ecology Research*, *1*(1), 12-20.
9. Aziz, I., & Khan, F. (2014). Distribution, Ecology and Ecophysiology of Mangroves in Pakistan. In *Sabkha ecosystems* (pp. 55-66). Springer, Dordrecht.

10. Baatrup, E. (1991). Structural and functional effects of heavy metals on the nervous system, including sense organs, of fish. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, *100*(1-2), 253-257.
11. Bae, W. C., Lee, H. K., Choe, Y. C., Jahng, D. J., Lee, S. H., Kim, S. J., ... &Jeong, B. C. (2005). Purification and characterization of NADPH-dependent Cr (VI) reductase from Escherichia coli ATCC 33456. *Journal of Microbiology*, *43*(1), 21-27.
12. Baldiris, R., Acosta-Tapia, N., Montes, A., Hernández, J., & Vivas-Reyes, R. (2018). Reduction of hexavalent chromium and detection of chromate reductase (ChrR) in Stenotrophomonas maltophilia. *Molecules*, *23*(2), 406.
13. Barupal, T., Meena, M., & Sharma, K. (2019). Inhibitory effects of leaf extract of Lawsoniainermis on Curvularialunata and characterization of novel inhibitory compounds by GC–MS analysis. *Biotechnology Reports*, *23*, e00335.
14. Berenjian, A., Chan, N., &Malmiri, H. J. (2012). Volatile organic compounds removal methods: A review. *American Journal of Biochemistry and Biotechnology*, *8*(4), 220-229.
15. Bharagava, R. N., & Mishra, S. (2018). Hexavalent chromium reduction potential of Cellulosimicrobium sp. isolated from common effluent treatment plant of tannery industries. *Ecotoxicology and Environmental Safety*, *147*, 102-109.
16. Bhaskar, P. V., &Bhosle, N. B. (2006). Bacterial extracellular polymeric substance (EPS): a carrier of heavy metals in the marine food-chain. *Environment international*, *32*(2), 191-198.
17. Bhat, S. A., Hassan, T., & Majid, S. (2019). Heavy metal toxicity and their harmful effects on living organisms–a review. *International Journal of Medical Science And Diagnosis Research*, *3*(1).
18. Bhattacharya, A., & Gupta, A. (2013). Evaluation of Acinetobacter sp. B9 for Cr (VI) resistance and detoxification with potential application in bioremediation of heavy-metals-rich industrial wastewater. *Environmental Science and Pollution Research*, *20*(9), 6628-6637.

19. Boey, A., & Ho, H. K. (2020). All roads lead to the liver: metal nanoparticles and their implications for liver health. *Small*, *16*(21), 2000153.
20. Bornhorst, J., Chakraborty, S., Meyer, S., Lohren, H., GroßeBrinkhaus, S., Knight, A. L., &Aschner, M. (2014). The effects of pdr1, djr1. 1 and pink1 loss in manganese-induced toxicity and the role of α-synuclein in C. elegans. *Metallomics*, *6*(3), 476-490.
21. Borremans, B., Hobman, J. L., Provoost, A., Brown, N. L., & van Der Lelie, D. (2001). Cloning and functional analysis of the pbr lead resistance determinant of Ralstoniametallidurans CH34. *Journal of bacteriology*, *183*(19), 5651-5658.
22. Browning, C. L., & Wise Sr, J. P. (2017). Prolonged exposure to particulate chromate inhibits RAD51 nuclear import mediator proteins. *Toxicology and applied pharmacology*, *331*, 101-107.
23. Bueno, B. Y. M., Torem, M. L., Molina, F. A. L. M. S., & De Mesquita, L. M. S. (2008). Biosorption of lead (II), chromium (III) and copper (II) by R. opacus: Equilibrium and kinetic studies. *Minerals engineering*, *21*(1), 65-75.
24. Burghate, S. P., &Ingole, N. W. (2013). Fluidized bed biofilm reactor–a novel wastewater treatment reactor. *International Journal of Research in Environmental Science and Technology*, *3*(4), 145-155.
25. Cao, Y. R., Zhang, X. Y., Deng, J. Y., Zhao, Q. Q., & Xu, H. (2012). Lead and cadmium-induced oxidative stress impacting mycelial growth of Oudemansiellaradicata in liquid medium alleviated by microbial siderophores. *World Journal of Microbiology and Biotechnology*, *28*(4), 1727-1737.
26. Cargnelutti, D., Tabaldi, L. A., Spanevello, R. M., de Oliveira Jucoski, G., Battisti, V., Redin, M &Schetinger, M. R. C. (2006). Mercury toxicity induces oxidative stress in growing cucumber seedlings. *Chemosphere*, *65*(6), 999-1006.
27. Carter, M. R., &Gregorich, E. G. (2007). *Soil sampling and methods of analysis*. CRC press.

28. Chandran, H., Meena, M., Barupal, T., & Sharma, K. (2020). Plant tissue culture as a perpetual source for production of industrially important bioactive compounds. *Biotechnology Reports*, *26*, e00450.
29. Chaney, R. L., & Baklanov, I. A. (2017). Phytoremediation and phytomining: status and promise. In *Advances in botanical research* (Vol. 83, pp. 189-221). Academic Press.
30. Charbit, A., Boulain, J. C., Ryter, A., &Hofnung, M. (1986). Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface. *The EMBO journal*, *5*(11), 3029-3037.
31. Chaudhary, D. S., Vigneswaran, S., Ngo, H. H., Shim, W. G., & Moon, H. (2003). Biofilter in water and wastewater treatment. *Korean Journal of Chemical Engineering*, *20*(6), 1054-1065.
32. Chen, L., & Hoff, S. J. (2012). A two-stage wood chip-based biofilter system to mitigate odors from a deep-pit swine building. *Applied Engineering in Agriculture*, *28*(6), 893-901.
33. Cheung, K. H., &Gu, J. D. (2007). Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. *International Biodeterioration& Biodegradation*, *59*(1), 8-15.
34. Cheung, K. H., Lai, H. Y., &Gu, J. D. (2006). Membrane-associated hexavalent chromium reductase of Bacillus megaterium TKW3 with induced expression. *Journal of Microbiology and Biotechnology*, *16*(6), 855-862.
35. Chihomvu, P., Stegmann, P., & Pillay, M. (2015). Characterization and structure prediction of partial length protein sequences of pcoA, pcoR and chrB genes from heavy metal resistant bacteria from the Klip River, South Africa. *International Journal of Molecular Sciences*, *16*(4), 7352-7374.
36. Chovanec, P., Sparacino-Watkins, C., Zhang, N., Basu, P., & Stolz, J. F. (2012). Microbial reduction of chromate in the presence of nitrate by three nitrate respiring organisms. *Frontiers in Microbiology*, *3*, 416.
37. Chrysargyris, A., Papakyriakou, E., Petropoulos, S. A., &Tzortzakis, N. (2019). The combined and single effect of salinity and copper stress on growth and quality of Menthaspicata plants. *Journal of hazardous materials*, *368*, 584-593.

38. Coetzee, J. J., Bansal, N., &Chirwa, E. (2020). Chromium in environment, its toxic effect from chromite-mining and ferrochrome industries, and its possible bioremediation. *Exposure and health*, *12*(1), 51-62.
39. Cohen, Y. (2001). Biofiltration–the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. *Bioresource technology*, *77*(3), 257-274.
40. Crini, G., &Lichtfouse, E. (2019). Advantages and disadvantages of techniques used for wastewater treatment. *Environmental Chemistry Letters*, *17*(1), 145-155.
41. Cullen, J. T., & McAlister, J. (2017). Biogeochemistry of lead. Its release to the environment and chemical speciation. *Met. Ions. Life Sci*, *17*, 21-48.
42. Das, S., Mishra, J., Das, S. K., Pandey, S., Rao, D. S., Chakraborty, A., ... &Thatoi, H. (2014). Investigation on mechanism of Cr (VI) reduction and removal by Bacillus amyloliquefaciens, a novel chromate tolerant bacterium isolated from chromite mine soil. *Chemosphere*, *96*, 112-121.
43. Das, S., Raj, R., Mangwani, N., Dash, H. R., & Chakraborty, J. (2014). Heavy metals and hydrocarbons: adverse effects and mechanism of toxicity. *Microbial biodegradation and bioremediation*, 23-54.
44. de Paris Júnior, O., Scapini, T., Camargo, A. F., Venturin, B., Dalastra, C., Kubeneck, S., &Treichel, H. (2019). Removal of chromium from wastewater by swine hair residues applied as a putative biofilter. *Environmental Science and Pollution Research*, *26*(32), 33014-33022.
45. Deng, P., Tan, X., Wu, Y., Bai, Q., Jia, Y., & Xiao, H. (2015). Cloning and sequence analysis demonstrate the chromate reduction ability of a novel chromate reductase gene from Serratia sp. *Experimental and therapeutic medicine*, *9*(3), 795-800.
46. Deschatre, M., Ghillebaert, F., Guezennec, J., & Colin, C. S. (2013). Sorption of copper (II) and silver (I) by four bacterial exopolysaccharides. *Applied biochemistry and biotechnology*, *171*(6), 1313-1327.
47. Dhal, B., Thatoi, H., Das, N., & Pandey, B. D. (2010). Reduction of hexavalent chromium by Bacillus sp. isolated from chromite mine soils and

characterization of reduced product. *Journal of Chemical Technology & Biotechnology*, *85*(11), 1471-1479.

48. Dialynas, E., &Diamadopoulos, E. (2009). Integration of a membrane bioreactor coupled with reverse osmosis for advanced treatment of municipal wastewater. *Desalination*, *238*(1-3), 302-311.
49. Din, G., Hassan, A., Rafiq, M., Hasan, F., Badshah, M., Khan, S., ... & Shah, A. A. (2020). Characterization of organic acid producing Aspergillus tubingensis FMS1 and its role in metals leaching from soil. *Geomicrobiology Journal*, *37*(4), 336-344.
50. Dixit, R., Malaviya, D., Pandiyan, K., Singh, U. B., Sahu, A., Shukla, R., ... & Paul, D. (2015). Bioremediation of heavy metals from soil and aquatic environment: an overview of principles and criteria of fundamental processes. *Sustainability*, *7*(2), 2189-2212.
51. Dizge, N., Keskinler, B., &Barlas, H. (2009). Sorption of Ni (II) ions from aqueous solution by Lewatit cation-exchange resin. *Journal of hazardous materials*, *167*(1-3), 915-926.
52. Dong, L., Zhou, S., He, Y., Jia, Y., Bai, Q., Deng, P., ... & Xiao, H. (2018). Analysis of the genome and chromium metabolism-related genes of Serratia sp. S2. *Applied biochemistry and biotechnology*, *185*(1), 140-152.
53. Edelstein, M., & Ben-Hur, M. (2018). Heavy metals and metalloids: Sources, risks and strategies to reduce their accumulation in horticultural crops. *ScientiaHorticulturae*, *234*, 431-444.
54. Edris, G., Alhamed, Y., & Alzahrani, A. (2014). Biosorption of cadmium and lead from aqueous solutions by Chlorella vulgaris biomass: equilibrium and kinetic study. *Arabian Journal for Science and Engineering*, *39*(1), 87-93.
55. Efome, J. E., Rana, D., Matsuura, T., & Lan, C. Q. (2018). Metal–organic frameworks supported on nanofibers to remove heavy metals. *Journal of Materials Chemistry A*, *6*(10), 4550-4555.
56. Engwa, G. A., Ferdinand, P. U., Nwalo, F. N., &Unachukwu, M. N. (2019). Mechanism and health effects of heavy metal toxicity in humans. *Poisoning in the modern world-new tricks for an old dog*, *10*, 70-90.

57. Enniya, I., Rghioui, L., &Jourani, A. (2018). Adsorption of hexavalent chromium in aqueous solution on activated carbon prepared from apple peels. *Sustainable Chemistry and Pharmacy*, *7*, 9-16.
58. Fang, X., Li, J., Li, X., Pan, S., Zhang, X., Sun, X., ... & Wang, L. (2017). Internal pore decoration with polydopamine nanoparticle on polymeric ultrafiltration membrane for enhanced heavy metal removal. *Chemical Engineering Journal*, *314*, 38-49.
59. Flora, S. J. S., Saxena, G., Gautam, P., Kaur, P., & Gill, K. D. (2007). Response of lead-induced oxidative stress and alterations in biogenic amines in different rat brain regions to combined administration of DMSA and MiADMSA. *Chemico-biological interactions*, *170*(3), 209-220.
60. Fomina, M., & Gadd, G. M. (2014). Biosorption: current perspectives on concept, definition and application. *Bioresource technology*, *160*, 3-14.
61. Freudl, R., MacIntyre, S., Degen, M., & Henning, U. (1986). Cell surface exposure of the outer membrane protein OmpA of Escherichia coli K-12. *Journal of molecular biology*, *188*(3), 491-494.
62. Fu, Z., & Xi, S. (2020). The effects of heavy metals on human metabolism. *Toxicology mechanisms and methods*, *30*(3), 167-176.
63. Fulladosa, E., Desjardin, V., Murat, J. C., Gourdon, R., &Villaescusa, I. (2006). Cr (VI) reduction into Cr (III) as a mechanism to explain the low sensitivity of Vibrio fischeri bioassay to detect chromium pollution. *Chemosphere*, *65*(4), 644-650.
64. Gallardo-Rodríguez, J. J., Rios-Rivera, A. C., & Von Bennevitz, M. R. (2019). Living biomass supported on a natural-fiber biofilter for lead removal. *Journal of environmental management*, *231*, 825-832.
65. GALLUCCI, F., VAN SINTANNALAND, M., &Kuipers, J. A. M. (2009). Comparison of packed bed and fluidized bed membrane reactors for methane reforming. *6th Inter. Sympos. on Multiphase Flow, Heat Mass Transfer and Energy Conversion, Xi'an, China*, 11-15.
66. Gan, M., Li, J., Sun, S., Ding, J., Zhu, J., Liu, X., &Qiu, G. (2018). Synergistic effect between sulfide mineral and acidophilic bacteria

significantly promoted Cr (VI) reduction. *Journal of environmental management*, *219*, 84-94.

67. Gang, H., Xiao, C., Xiao, Y., Yan, W., Bai, R., Ding, R., & Zhao, F. (2019). Proteomic analysis of the reduction and resistance mechanisms of Shewanellaoneidensis MR-1 under long-term hexavalent chromium stress. *Environment international*, *127*, 94-102.
68. Garbisu, C., &Alkorta, I. (2003). Basic concepts on heavy metal soil bioremediation. *ejmp& ep (European Journal of Mineral Processing and Environmental Protection)*, *3*(1), 58-66.
69. Garbisu, C., Llama, M. J., & Serra, J. L. (1997). Effect of heavy metals on chromate reduction by Bacillus subtilis. *The Journal of General and Applied Microbiology*, *43*(6), 369-371.
70. Gauthier, P. T., Norwood, W. P., Prepas, E. E., & Pyle, G. G. (2014). Metal–PAH mixtures in the aquatic environment: a review of co-toxic mechanisms leading to more-than-additive outcomes. *Aquatic toxicology*, *154*, 253-269.
71. Genchi, G., Sinicropi, M. S., Lauria, G., Carocci, A., & Catalano, A. (2020). The effects of cadmium toxicity. *International journal of environmental research and public health*, *17*(11), 3782.
72. Ghosh, A., Dastidar, M. G., &Sreekrishnan, T. R. (2017). Bioremediation of chromium complex dyes and treatment of sludge generated during the process. *International Biodeterioration& Biodegradation*, *119*, 448-460.
73. Giovanella, P., Cabral, L., Bento, F. M., Gianello, C., & Camargo, F. A. O. (2016). Mercury (II) removal by resistant bacterial isolates and mercuric (II) reductase activity in a new strain of Pseudomonas sp. B50A. *New biotechnology*, *33*(1), 216-223.
74. Gómez-Borraz, T. L., González-Sánchez, A., Bonilla-Blancas, W., Revah, S., &Noyola, A. (2017). Characterization of the biofiltration of methane emissions from municipal anaerobic effluents. *Process Biochemistry*, *63*, 204-213.
75. Gopinath, M., Pulla, R. H., Rajmohan, K. S., Vijay, P., Muthukumaran, C., &Gurunathan, B. (2018). Bioremediation of volatile organic compounds in

biofilters. In *Bioremediation: Applications for Environmental Protection and Management* (pp. 301-330). Springer, Singapore.

76. GracePavithra, K., Jaikumar, V., Kumar, P. S., &SundarRajan, P. (2019). A review on cleaner strategies for chromium industrial wastewater: present research and future perspective. *Journal of Cleaner Production*, *228*, 580-593.
77. Grzegorzek, M., Majewska-Nowak, K., & Ahmed, A. E. (2020). Removal of fluoride from multicomponent water solutions with the use of monovalent selective ion-exchange membranes. *Science of the Total Environment*, *722*, 137681.
78. Gu, R., Gao, J., Dong, L., Liu, Y., Li, X., Bai, Q., ... & Xiao, H. (2020). Chromium metabolism characteristics of coexpression of ChrA and ChrT gene. *Ecotoxicology and Environmental Safety*, *204*, 111060.
79. Gulcin, İ., Buyukokuroglu, M. E., & Kufrevioglu, O. I. (2003). Metal chelating and hydrogen peroxide scavenging effects of melatonin. *Journal of pineal research*, *34*(4), 278-281.
80. Gunatilake, S. K. (2015). Methods of removing heavy metals from industrial wastewater. *Methods*, *1*(1), 14.
81. Gupta, V. K., &Nayak, A. (2012). Cadmium removal and recovery from aqueous solutions by novel adsorbents prepared from orange peel and Fe2O3 nanoparticles. *Chemical engineering journal*, *180*, 81-90.
82. Hamdaoui, O. (2009). Removal of copper (II) from aqueous phase by Purolite C100-MB cation exchange resin in fixed bed columns: Modeling. *Journal of hazardous materials*, *161*(2-3), 737-746.
83. He, Y., Dong, L., Zhou, S., Jia, Y., Gu, R., Bai, Q., ... & Xiao, H. (2018). Chromium resistance characteristics of Cr (VI) resistance genes ChrA and ChrB in Serratia sp. S2. *Ecotoxicology and Environmental Safety*, *157*, 417-423.
84. Hora, A., & Shetty, V. K. (2015). Partial purification and characterization of chromate reductase of a novel Ochrobactrum sp. strain Cr-B4. *Preparative Biochemistry and Biotechnology*, *45*(8), 769-784.
85. Hossain M, Islam M. 2019. Ship breaking activities and its impact on the coastal zone of Chittagong. http://ypsa.org/publications/Impact.pdf

86. Hu, P., Brodie, E. L., Suzuki, Y., McAdams, H. H., & Andersen, G. L. (2005). Whole-genome transcriptional analysis of heavy metal stresses in Caulobactercrescentus. *Journal of bacteriology*, *187*(24), 8437-8449.
87. Huang, J., Li, J., & Wang, G. (2016). Production of a microcapsule agent of chromate-reducing Lysinibacillus fusiformis ZC1 and its application in remediation of chromate-spiked soil. *SpringerPlus*, *5*(1), 1-10.
88. Ibanga, I. E., Fletcher, L. A., Noakes, C. J., King, M. F., & Steinberg, D. (2018). Pilot-scale biofiltration at a materials recovery facility: The impact on bioaerosol control. *Waste Management*, *80*, 154-167.
89. Igiri, B. E., Okoduwa, S. I., Idoko, G. O., Akabuogu, E. P., Adeyi, A. O., &Ejiogu, I. K. (2018). Toxicity and bioremediation of heavy metals contaminated ecosystem from tannery wastewater: a review. *Journal of toxicology*, *2018*.
90. Jacob, J. M., Karthik, C., Saratale, R. G., Kumar, S. S., Prabakar, D., Kadirvelu, K., &Pugazhendhi, A. (2018). Biological approaches to tackle heavy metal pollution: a survey of literature. *Journal of environmental management*, *217*, 56-70.
91. Javed, M., &Usmani, N. (2019). An overview of the adverse effects of heavy metal contamination on fish health. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *89*(2), 389-403.
92. Jeong, S., Cho, K., Jeong, D., Lee, S., Leiknes, T., Vigneswaran, S., & Bae, H. (2017). Effect of engineered environment on microbial community structure in biofilter and biofilm on reverse osmosis membrane. *Water research*, *124*, 227-237.
93. Jobby, R., Jha, P., Yadav, A. K., & Desai, N. (2018). Biosorption and biotransformation of hexavalent chromium [Cr (VI)]: a comprehensive review. *Chemosphere*, *207*, 255-266.
94. Jobby, R., Shah, K., Shah, R., Jha, P., & Desai, N. (2016). Differential expression of antioxidant enzymes under arsenic stress in Enterobacter sp. *Environmental Progress & Sustainable Energy*, *35*(6), 1642-1645.

95. Kalogerakis, N., Fava, F., &Corvini, P. (2017). 6th European Bioremediation Conference, Chania, Crete, Greece, 29 June-2 July, 2015. *New Biotechnology*, *38*(Part B), 41-106.
96. Karthik, C., Oves, M., Sathya, K., Sri Ramkumar, V., &Arulselvi, P. I. (2017). Isolation and characterization of multi-potential Rhizobium strain ND2 and its plant growth-promoting activities under Cr (VI) stress. *Archives of Agronomy and Soil Science*, *63*(8), 1058-1069.
97. Kondo, A., & Ueda, M. (2004). Yeast cell-surface display—applications of molecular display. *Applied microbiology and biotechnology*, *64*(1), 28-40.
98. Kumar, K. V., Sridevi, V., Harsha, N., Lakshmi, M. C., & Rani, K. (2013). Biofiltration and its application in treatment of air and water pollutants-A review. *International Journal of Application or Innovation in Engineering & Management 2 (9)*, 226-231.
99. Kumar, M., Giri, B. S., Kim, K. H., Singh, R. P., Rene, E. R., López, M. E., & Singh, R. S. (2019). Performance of a biofilter with compost and activated carbon based packing material for gas-phase toluene removal under extremely high loading rates. *Bioresource technology*, *285*, 121317.
100. Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), 1547.
101. Kurniawan, A., & Yamamoto, T. (2013). Biofilm polymer for biosorption of pollutant ions. *Procedia Environmental Sciences*, *17*, 179-187.
102. Kuroda, K., & Ueda, M. (2010). Engineering of microorganisms towards recovery of rare metal ions. *Applied microbiology and biotechnology*, *87*(1), 53-60.
103. Kushwaha, A., Hans, N., Kumar, S., & Rani, R. (2018). A critical review on speciation, mobilization and toxicity of lead in soil-microbe-plant system and bioremediation strategies. *Ecotoxicology and environmental safety*, *147*, 1035-1045.
104. Li, C., Zhou, K., Qin, W., Tian, C., Qi, M., Yan, X., & Han, W. (2019). A review on heavy metals contamination in soil: effects, sources, and

remediation techniques. *Soil and Sediment Contamination: An International Journal*, *28*(4), 380-394.

105. Li, P. S., & Tao, H. C. (2015). Cell surface engineering of microorganisms towards adsorption of heavy metals. *Critical reviews in microbiology*, *41*(2), 140-149.

106. Lim, H. S., Lim, W., Hu, J. Y., Ziegler, A., & Ong, S. L. (2015). Comparison of filter media materials for heavy metal removal from urban stormwater runoff using biofiltration systems. *Journal of environmental management*, *147*, 24-33.

107. Liu, S. H., Zeng, G. M., Niu, Q. Y., Liu, Y., Zhou, L., Jiang, L. H., & Cheng, M. (2017). Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. *Bioresource technology*, *224*, 25-33.

108. Liu, X., Wu, G., Zhang, Y., Wu, D., Li, X., & Liu, P. (2015). Chromate reductase YieF from Escherichia coli enhances hexavalent chromium resistance of human HepG2 cells. *International journal of molecular sciences*, *16*(6), 11892-11902.

109. Lonergan, D. J., Jenter, H. L., Coates, J. D., Phillips, E. J., Schmidt, T. M., &Lovley, D. R. (1996). Phylogenetic analysis of dissimilatory Fe (III)-reducing bacteria. *Journal of bacteriology*, *178*(8), 2402-2408.

110. Luo, J., Ye, Y., Gao, Z., & Wang, W. (2014). Essential and nonessential elements in the red-crowned crane Grus japonensis of Zhalong Wetland, northeastern China. *Toxicological & Environmental Chemistry*, *96*(7), 1096-1105.

111. Lwin, C. S., Seo, B. H., Kim, H. U., Owens, G., & Kim, K. R. (2018). Application of soil amendments to contaminated soils for heavy metal immobilization and improved soil quality—A critical review. *Soil science and plant nutrition*, *64*(2), 156-167.

112. Mabrouk, M. E. (2008). Statistical optimization of medium components for chromate reduction by halophilic Streptomyces sp. MS-2. *Afr. J. Microbiol. Res*, *2*, 103-109.

113. Mabrouk, M. E., Arayes, M. A., &Sabry, S. A. (2014). Hexavalent chromium reduction by chromate-resistant haloalkaliphilicHalomonas sp. M-Cr newly isolated from tannery effluent. *Biotechnology & Biotechnological Equipment*, *28*(4), 659-667.

114. Mačaitis, K., Misevičius, A., Paškevičius, A., Raudonienė, V., &Repečkienė, J. (2014). Effectiveness research on a wavy lamellar plate-type biofilter with a capillary system for the humidification of the packing material applying introinduced microorganisms. *Journal of Environmental Engineering and Landscape Management*, *22*(4), 254-263.

115. Magnuson, T. S., Swenson, M. W., Paszczynski, A. J., Deobald, L. A., Kerk, D., & Cummings, D. E. (2010). Proteogenomic and functional analysis of chromate reduction in Acidiphiliumcryptum JF-5, an Fe (III)-respiring acidophile. *Biometals*, *23*(6), 1129-1138.

116. Majumder S (2015) Studies on removal of heavy metals from wastewater using biofiltration. (Thesis, https://shodhganga.inflibnet.ac.in/ bitstream/10603/124741/1/thesis.pdf).

117. Majumder, S., Gupta, V., Raghuvanshi, S., & Gupta, S. (2016). Simultaneous sequestration of ternary metal ions (Cr6+, Cu2+ and Zn2+) from aqueous solution by an indigenous bacterial consortium. *Process Safety and Environmental Protection*, *102*, 786-798.

118. Majumder, S., Raghuvanshi, S., & Gupta, S. (2016). Application of a hybrid biofilter column for the removal of Cr (VI) from aqueous solution using an indigenous bacterial strain Pseudomonas taiwanensis. *Bioremediation Journal*, *20*(1), 10-23.

119. Mala, J. G. S., Sujatha, D., & Rose, C. (2015). Inducible chromate reductase exhibiting extracellular activity in Bacillus methylotrophicus for chromium bioremediation. *Microbiological research*, *170*, 235-241.

120. Martin, E. M., & Fry, R. C. (2018). Environmental influences on the epigenome: exposure-associated DNA methylation in human populations. *Annu Rev Public Health*, *39*(1), 309-333.

121. Megharaj, M., Avudainayagam, S., & Naidu, R. (2003). Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. *Current microbiology*, *47*(1), 0051-0054.

122. Metcalf, W. (2003). Metcalf and Eddy wastewater engineering: treatment and reuse. *Wastewater Engineering: Treatment and Reuse McGraw Hill. New York, NY*, *384*.

123. Mujeeb, A., Aziz, I., Ahmed, M. Z., Alvi, S. K., &Shafiq, S. (2020). Comparative assessment of heavy metal accumulation and bio-indication in coastal dune halophytes. *Ecotoxicology and environmental safety*, *195*, 110486.

124. Muñoz, A. J., Espínola, F., & Ruiz, E. (2016). Removal of Pb (II) in a packed-bed column by a Klebsiella sp. 3S1 biofilm supported on porous ceramic Raschig rings. *Journal of industrial and engineering chemistry*, *40*, 118-127.

125. Murthy, S., Bali, G., & Sarangi, S. K. (2012). Biosorption of lead by Bacillus cereus isolated from industrial effluents. *British Biotechnology Journal*, *2*(2), 73.

126. Nagajyoti, P. C., Lee, K. D., &Sreekanth, T. V. M. (2010). Heavy metals, occurrence and toxicity for plants: a review. *Environmental chemistry letters*, *8*(3), 199-216.

127. 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.

128. Nanda, M., Kumar, V., & Sharma, D. K. (2019). Multimetal tolerance mechanisms in bacteria: The resistance strategies acquired by bacteria that can be exploited to 'clean-up'heavy metal contaminants from water. *Aquatic toxicology*, *212*, 1-10.

129. Ngwenya, N., &Chirwa, E. M. N. (2011). Biological removal of cationic fission products from nuclear wastewater. *Water Science and Technology*, *63*(1), 124-128.

130. Ontañon, O. M., Fernandez, M., Agostini, E., & González, P. S. (2018). Identification of the main mechanisms involved in the tolerance and

bioremediation of Cr (VI) by Bacillus sp. SFC 500-1E. *Environmental Science and Pollution Research*, *25*(16), 16111-16120.

131. Osredkar, J., &Sustar, N. (2011). Copper and zinc, biological role and significance of copper/zinc imbalance. *J Clinic Toxicol S*, *3*(2161), 0495.

132. Oves, M., Saghir Khan, M., Huda Qari, A., NadeenFelemban, M., &Almeelbi, T. (2016). Heavy metals: biological importance and detoxification strategies. *Journal of Bioremediation and Biodegradation*, *7*(2), 1-15.

133. Pandey, N., & Tiwari, A. (2021). Human health risk assessment of heavy metals in different soils and sediments. In *Heavy Metals in the Environment* (pp. 143-163). Elsevier.

134. Patra, R. C., Malik, S., Beer, M., Megharaj, M., & Naidu, R. (2010). Molecular characterization of chromium (VI) reducing potential in Gram positive bacteria isolated from contaminated sites. *Soil Biology and Biochemistry*, *42*(10), 1857-1863.

135. Patra, R. C., Malik, S., Beer, M., Megharaj, M., & Naidu, R. (2010). Molecular characterization of chromium (VI) reducing potential in Gram positive bacteria isolated from contaminated sites. *Soil Biology and Biochemistry*, *42*(10), 1857-1863.

136. Pechova, A., &Pavlata, L. (2007). Chromium as an essential nutrient: a review. *Veterinárnímedicína*, *52*(1), 1.

137. Pepi, M., Borra, M., Tamburrino, S., Saggiomo, M., Viola, A., Biffali, E., ... &Casotti, R. (2016). A Bacillus sp. isolated from sediments of the Sarno River mouth, Gulf of Naples (Italy) produces a biofilm biosorbingPb (II). *Science of the Total Environment*, *562*, 588-595.

138. Pérez, A. A., Gajewski, J. P., Ferlez, B. H., Ludwig, M., Baker, C. S., Golbeck, J. H., & Bryant, D. A. (2017). Zn2+-inducible expression platform for Synechococcus sp. strain PCC 7002 based on the smtA promoter/operator and smtB repressor. *Applied and environmental microbiology*, *83*(3), e02491-16.

139. Pérez-López, M., de Mendoza, M. H., Beceiro, A. L., & Rodríguez, F. S. (2008). Heavy metal (Cd, Pb, Zn) and metalloid (As) content in raptor

species from Galicia (NW Spain). *Ecotoxicology and environmental safety*, *70*(1), 154-162.

140. Phookphan, P., Navasumrit, P., Waraprasit, S., Promvijit, J., Chaisatra, K., Ngaotepprutaram, T., &Ruchirawat, M. (2017). Hypomethylation of inflammatory genes (COX2, EGR1, and SOCS3) and increased urinary 8-nitroguanine in arsenic-exposed newborns and children. *Toxicology and Applied Pharmacology*, *316*, 36-47.

141. Popovych, V. V., Stepova, K. V., Voloshchyshyn, A., &Bosak, P. V. (2019). *Physico-chemical properties of soils in LvivVolyn coal basin area* (Doctoral dissertation, EDP Sciences).

142. Prasad, S., Yadav, K. K., Kumar, S., Gupta, N., Cabral-Pinto, M. M., Rezania, S., &Alam, J. (2021). Chromium contamination and effect on environmental health and its remediation: A sustainable approaches. *Journal of Environmental Management*, *285*, 112174.

143. Punia, A. (2021). Role of temperature, wind, and precipitation in heavy metal contamination at copper mines: a review. *Environmental Science and Pollution Research*, *28*(4), 4056-4072.

144. Pushkar, B., Sevak, P., Parab, S., &Nilkanth, N. (2021). Chromium pollution and its bioremediation mechanisms in bacteria: A review. *Journal of Environmental Management*, *287*, 112279.

145. Qian, J., Zhou, J., Wang, L., Wei, L., Li, Q., Wang, D., & Wang, Q. (2017). Direct Cr (VI) bio-reduction with organics as electron donor by anaerobic sludge. *Chemical Engineering Journal*, *309*, 330-338.

146. Qian, X. Y., Shen, G. X., Wang, Z. Q., Chen, X. H., Zhao, Q. J., Bai, Y. J., & Tang, Z. Z. (2020). Application of dairy manure as fertilizer in dry land in East China: field monitoring and model estimation of heavy metal accumulation in surface soil. *Environmental Science and Pollution Research*, *27*(29), 36913-36919.

147. Raghuvanshi, S., &Babu, B. V. (2009). Experimental studies and kinetic modeling for removal of methyl ethyl ketone using biofiltration. *Bioresource technology*, *100*(17), 3855-3861.

148. Rahman, K. S. M., & Murthy, M. A. V. (2011, April). Stabilization of chromium by reductase enzyme treatment. In *Stabilisation/Solidification Treatment and Remediation: Proceedings of the International Conference on Stabilisation/Solidification Treatment and Remediation, 12-13 April 2005, Cambridge, UK* (p. 347). CRC Press.

149. Rai, P. K., Lee, S. S., Zhang, M., Tsang, Y. F., & Kim, K. H. (2019). Heavy metals in food crops: Health risks, fate, mechanisms, and management. *Environment international*, *125*, 365-385.

150. Rajyalaxmi, K., Merugu, R., Girisham, S., & Reddy, S. M. (2019). Chromate reduction by purple non sulphur phototrophic bacterium Rhodobacter sp. GSKRLMBKU–03 isolated from pond water. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, *89*(1), 259-265.

151. Ramírez-Díaz, M. I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., & Cervantes, C. (2008). Mechanisms of bacterial resistance to chromium compounds. *Biometals*, *21*(3), 321-332.

152. Ramyakrishna, K., &Sudhamani, M. (2017). The metal binding potential of a dairy isolate. *Journal of Water Reuse and Desalination*, *7*(4), 429-441.

153. Raskin, I., & Ensley, B. D. (2000). *Phytoremediation of toxic metals*. John Wiley and Sons.

References

154. Remenar, M., Kamlarova, A., Harichova, J., Zámocký, M., &Ferianc, P. (2018). The heavy-metal resistance determinant of newly isolated bacterium from a nickel-contaminated soil in Southwest Slovakia. *Polish Journal of Microbiology*, *67*(2), 191-201.

155. Rene, E. R., Sergienko, N., Goswami, T., López, M. E., Kumar, G., Saratale, G. D., &Swaminathan, T. (2018). Effects of concentration and gas flow rate on the removal of gas-phase toluene and xylene mixture in a compost biofilter. *Bioresource technology*, *248*, 28-35.

156. Rubio, J., Souza, M. L., & Smith, R. W. (2002). Overview of flotation as a wastewater treatment technique. *Minerals engineering*, *15*(3), 139-155.

157. Ruggaber, T. P., & Talley, J. W. (2006). Enhancing bioremediation with enzymatic processes: a review. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management*, *10*(2), 73-85.

158. Sagar, S., Bajaj, S., Gola, D., Malik, A., Khankhane, P., Kaur, R., & Singh, D. K. (2017). A qualitative approach to nickel and lead uptake by heavy metal resistant bacteria Klebsiella sp. 10KN. *IJAR*, *3*(7), 878-885.

159. Saha, M., Sarkar, S., Sarkar, B., Sharma, B. K., Bhattacharjee, S., &Tribedi, P. (2016). Microbial siderophores and their potential applications: a review. *Environmental Science and Pollution Research*, *23*(5), 3984-3999.

160. Samuelson, P., Gunneriusson, E., Nygren, P. Å., &Ståhl, S. (2002). Display of proteins on bacteria. *Journal of biotechnology*, *96*(2), 129-154.

161. Saurav, K., &Kannabiran, K. (2011). Biosorption of Cd (II) and Pb (II) ions by aqueous solutions of novel alkalophillic Streptomyces VITSVK5 spp. biomass. *Journal of Ocean University of China*, *10*(1), 61-66.

162. Saxena, R., Azad, C. S., &Shrinet, K. (2016). Effect of heavy metal pollutants on human population: An Overview.

163. Sfakianakis, D. G., Renieri, E., Kentouri, M., &Tsatsakis, A. M. (2015). Effect of heavy metals on fish larvae deformities: a review. *Environmental research*, *137*, 246-255.

164. Shahid, M., Shamshad, S., Rafiq, M., Khalid, S., Bibi, I., Niazi, N. K., ... & Rashid, M. I. (2017). Chromium speciation, bioavailability, uptake, toxicity and detoxification in soil-plant system: A review. *Chemosphere*, *178*, 513-533.

165. Shahot, K., Idris, A., Omar, R., &Yusoff, H. M. (2014). Review on biofilm processes for wastewater treatment. *Life Sci J*, *11*(11), 1-13.

166. Shanker, A. K., Djanaguiraman, M., &Venkateswarlu, B. (2009). Chromium interactions in plants: current status and future strategies. *Metallomics*, *1*(5), 375-383.

167. Shao, W., Li, M., Teng, Z., Qiu, B., Huo, Y., & Zhang, K. (2019). Effects of Pb (II) and Cr (VI) stress on phosphate-solubilizing bacteria

(Bacillus sp. strain MRP-3): oxidative stress and bioaccumulation potential. *International Journal of Environmental Research and Public Health*, *16*(12), 2172.

168. Sharma, A., Johri, B. N., Sharma, A. K., & Glick, B. R. (2003). Plant growth-promoting bacterium Pseudomonas sp. strain GRP3 influences iron acquisition in mung bean (Vignaradiata L. Wilzeck). *Soil Biology and Biochemistry*, *35*(7), 887-894.

169. Sharma, J., Shamim, K., & Dubey, S. K. (2018). Phosphatase mediated bioprecipitation of lead as pyromorphite by Achromobacterxylosoxidans. *Journal of environmental management*, *217*, 754-761.

170. Sharma, S., Tiwari, S., Hasan, A., Saxena, V., & Pandey, L. M. (2018). Recent advances in conventional and contemporary methods for remediation of heavy metal-contaminated soils. *3 Biotech*, *8*(4), 1-18.

171. Shehata, M. (2018). *Using recombinant E. coli displaying surface heavy metal binding proteins for removal of Pb2+ from contaminated water* (Doctoral dissertation, 北海道大学).

172. Shehata, M. (2018). *Using recombinant E. coli displaying surface heavy metal binding proteins for removal of Pb2+ from contaminated water*.

173. Singh, M., Kumar, J., Singh, S., Singh, V. P., Prasad, S. M., & Singh, M. P. V. V. B. (2015). Adaptation strategies of plants against heavy metal toxicity: a short review. *Biochemistry & Pharmacology: Open Access*, *4*, 2167-0501.

174. Soni, S. K., Singh, R., Awasthi, A., Singh, M., &Kalra, A. (2013). In vitro Cr (VI) reduction by cell-free extracts of chromate-reducing bacteria isolated from tannery effluent irrigated soil. *Environmental Science and Pollution Research*, *20*(3), 1661-1674.

175. Speer, R. M., Wise, C. F., Young, J. L., Aboueissa, A. M., Bras, M. M., Barandiaran, M., ... & Wise Sr, J. P. (2018). The cytotoxicity and genotoxicity of particulate and soluble hexavalent chromium in leatherback sea turtle lung cells. *Aquatic Toxicology*, *198*, 149-157.

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

177. Srivastava, N. K., &Majumder, C. B. (2008). Novel biofiltration methods for the treatment of heavy metals from industrial wastewater. *Journal of hazardous materials*, *151*(1), 1-8.

178. Tan, X., Deng, P., Wu, Y., Bai, Q., Jia, Y., & Xiao, H. (2014). In vitro synthesis and activity identification of Chromate reductase T. *Chongqing YikeDaxueXuebao*, *39*, 973-977.

179. Tang, C. Y., Fu, Q. S., Criddle, C. S., &Leckie, J. O. (2007). Effect of flux (transmembrane pressure) and membrane properties on fouling and rejection of reverse osmosis and nanofiltration membranes treating perfluorooctane sulfonate containing wastewater. *Environmental science & technology*, *41*(6), 2008-2014.

180. Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. *Molecular, clinical and environmental toxicology*, 133-164.

181. Thatheyus, A. J., &Ramya, D. (2016). Biosorption of chromium using bacteria: an overview. *Science International*, *4*(2), 74-79.

182. Thatoi, H., Das, S., Mishra, J., Rath, B. P., & Das, N. (2014). Bacterial chromate reductase, a potential enzyme for bioremediation of hexavalent chromium: a review. *Journal of Environmental Management*, *146*, 383-399.

183. Tran, A. T., Zhang, Y., Jullok, N., Meesschaert, B., Pinoy, L., & Van der Bruggen, B. (2012). RO concentrate treatment by a hybrid system consisting of a pellet reactor and electrodialysis. *Chemical engineering science*, *79*, 228-238.

184. Trellu, C., Mousset, E., Pechaud, Y., Huguenot, D., van Hullebusch, E. D., Esposito, G., &Oturan, M. A. (2016). Removal of hydrophobic organic pollutants from soil washing/flushing solutions: a critical review. *Journal of Hazardous materials*, *306*, 149-174.

185. Tripathi, A., &Ranjan, M. R. (2015). Heavy metal removal from wastewater using low cost adsorbents. *J BioremedBiodeg*, *6*(6), 315.

186. Uwitonze, A. M., &Razzaque, M. S. (2018). Role of magnesium in vitamin D activation and function. *Journal of Osteopathic Medicine*, *118*(3), 181-189.

187. 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*(4), 327-338.

188. Vardhan, K. H., Kumar, P. S., & Panda, R. C. (2019). A review on heavy metal pollution, toxicity and remedial measures: Current trends and future perspectives. *Journal of Molecular Liquids*, *290*, 111197.

189. Verma, S., &Kuila, A. (2019). Bioremediation of heavy metals by microbial process. *Environmental Technology & Innovation*, *14*, 100369.

190. Vigneswaran, R., Aitchison, S. J., McDonald, H. M., Khong, T. Y., & Hiller, J. E. (2004). Cerebral palsy and placental infection: a case-cohort study. *BMC pregnancy and childbirth*, *4*(1), 1-7.

191. Vikrant, K., Kailasa, S. K., Tsang, D. C., Lee, S. S., Kumar, P., Giri, B. S., ... & Kim, K. H. (2018). Biofiltration of hydrogen sulfide: Trends and challenges. *Journal of cleaner production*, *187*, 131-147.

192. Viti, C., Pace, A., &Giovannetti, L. (2003). Characterization of Cr (VI)-resistant bacteria isolated from chromium-contaminated soil by tannery activity. *Current microbiology*, *46*(1), 0001-0005.

193. Wang, G., Wang, S., Sun, W., Sun, Z., & Zheng, S. (2017). Synthesis of a novel illite@ carbon nanocomposite adsorbent for removal of Cr (VI) from wastewater. *Journal of Environmental Sciences*, *57*, 62-71.

194. Wang, J. J., Zhang, H., Schroder, J. L., Udeigwe, T. K., Zhang, Z., Dodla, S. K., &Stietiya, M. H. (2011). Reducing potential leaching of phosphorus, heavy metals, and fecal coliform from animal wastes using bauxite residues. *Water, Air, & Soil Pollution*, *214*(1), 241-252.

195. Wang, Z., van Loosdrecht, M. C., &Saikaly, P. E. (2017). Gradual adaptation to salt and dissolved oxygen: strategies to minimize adverse effect of salinity on aerobic granular sludge. *Water research*, *124*, 702-712.

196. Wani, P. A., Hussaini, N. A., Garba, S. H., Wahid, S., Damilola, F. K., Adeola, A. A., &Wasiu, I. A. (2018). Prospective of chromium (VI) reduction

under in vitro and in vivo conditions and stimulation of antioxidant defense of Cowpea under the exposure of Cr (VI). *Applied Soil Ecology*, *132*, 187-193.

197. Wei, W., Liu, X., Sun, P., Wang, X., Zhu, H., Hong, M., ... & Zhao, J. (2014). Simple whole-cell biodetection and bioremediation of heavy metals based on an engineered lead-specific operon. *Environmental science & technology*, *48*(6), 3363-3371.

198. White, C., Sayer, J. A., & Gadd, G. M. (1997). Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS microbiology reviews*, *20*(3-4), 503-516.

199. Wuana, R. A., &Okieimen, F. E. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *International Scholarly Research Notices*, *2011*.

200. Yang, F., & Massey, I. Y. (2019). Exposure routes and health effects of heavy metals on children. *Biometals*, *32*(4), 563-573.

201. Yang, W., Wang, J., Mu, Q., Zhen, J., & Chen, G. (2013). Screening of Cr (VI)-resistant strains and condition optimization. *Henan Science*, *8*, 1175-1179.

202. Yin, K., Li, B., Wang, X., Zhang, W., & Chen, L. (2015). Ultrasensitive colorimetric detection of Cu2+ ion based on catalytic oxidation of l-cysteine. *Biosensors and Bioelectronics*, *64*, 81-87.

203. Yin, K., Wang, Q., Lv, M., & Chen, L. (2019). Microorganism remediation strategies towards heavy metals. *Chemical Engineering Journal*, *360*, 1553-1563.

204. Zhitkovich, A. (2011). Chromium in drinking water: sources, metabolism, and cancer risks. *Chemical research in toxicology*, *24*(10), 1617-1629.

205. Zhou, S., Dong, L., Deng, P., Jia, Y., Bai, Q., Gao, J., & Xiao, H. (2017). Reducing capacity and enzyme activity of chromate reductase in a ChrT-engineered strain. *Experimental and Therapeutic Medicine*, *14*(3), 2361-2366.

APPENDICES

Appendix 1: Composition of Chromium nutrient agar

$K_2Cr_2O_7$	1500 mg
Nutrient broth	O.8 gram
Agar	1.5 gram
Water	100 ml

Appendix 2: Composition of lead nutrient agar

Pb $(NO_3)_2$	1500 mg
Nutrient broth	O.8 gram
Agar	1.5 gram
Water	100 ml

Appendix 3: Composition of production media

Yeast extract	8 gram
KH_2PO_4	1 gram
Na_2HPO_4	4 gram
$MgSO_4.7H_2O$	5 gram
$CaCl_2$	0.1 gram
NaH_2PO_4	3 gram
NaCl	0.6 gram
Sucrose	15 gram
$(NH_4)SO_4$	2.5 gram
$K_2Cr_2O_7$	0.002 gram
K_2HPO_4	3 gram
Water	1000 ml

Appendix 4: Phosphate buffer

Components	100 Mm
K_2HPO_4	17.4 gram
KH_2PO_4	13.6 gram
Water	1000 ml

Appendix 5: Potassium Dichromate solution

$K_2Cr_2O_7$	1 gram
Water	100 ml

Appendix 6: Running gel

Serial number	Gel %	15%	12.5%
1	30% Acrylamide/bis	4.9 ml	4.2 ml
2	Tris (1.5 M pH 6.8)	2.5 ml	2.5 ml
3	SDS 10%	100 µl	100 µl
4	TEMED	20 µl	20 µl
5	10% APS	50 µl	50µl
6	Water	2.4 ml	3.4 ml

Appendix 7: Stacking gel

Serial number	Gel %	Volume
1	30% Acrylamide/bis	696 µl
2	Tris (1.5 M pH 6.8)	650 µl
3	SDS 10%	100µl
4	TEMED	10 µl
5	10% APS	50 µl
6	Water	3.65 ml

Appendix 8: Protein dye solution

Water	40 ml
Methanol	50 ml
Acetic acid	10 ml
Commasi brilliant blue R-250	0.125 gram

Appendix 9: Distaining solution 1

Water	40 ml
Methanol	50 ml
Acetic acid	10 ml

Appendix 10: Distaining solution 2

Water	132 ml
Methanol	7.5 ml
Acetic acid	10.5 ml

Appendix 11: Potassium dichromate standard curve

Concentration	O.D at 540 nm
100	0.073
200	0.172
300	0.253
400	0.389
500	0.531
600	0.631
700	0.775
800	0.930
900	1.081
1000	1.473

Appendix 12: BSA standard curve

Protein Conc. (µg/ml)	Absorbance
100	0.17
200	0.388
300	0.451
400	0.544
500	0.654
600	0.716
700	0.744
800	0.968
900	1.304
1000	1.102

Appendix 13: Sephadex (G-100) profile of S48 chromium reductase

Fraction	O.D at 280 nm	O.D at 540 nm
1	0.1	0
2	0.1	0
3	0.1	0
4	0.1	0
5	0.1	0
6	0.1	0
7	0.187	0
8	0.1	0
9	0.1	0
10	0.1	0
11	0.96	1.699
12	0.1	1.53
13	0.1	1.02
14	0.206	1.03
15	0.478	0.627
16	0.317	0.686
17	0.777	1.143
18	0.567	1.136
19	0.41	1.32
20	0.514	1.228
21	0.555	0.859
22	0.152	0.484
23	0.1	0
24	0.1	0
25	0.1	0
26	0.279	0
27	0.1	0
28	0.1	0
29	0.1	0
30	0.1	0

Appendix 14: S48 chromium reductase temperature profile

Temperature (°C)	O.D	Relative Activity
30	1.025	85.91785
35	1.113567	93.34172
40	1.1744	98.44091
45	0.61	51.1316
50	0.143667	12.04247
55	0.075467	6.325789
60	0.0567	4.752724

Appendix 15: S48 chromium reductase pH profile

pH	O.D	Relative Activity
3	1.05	78.29978
4	1.156	86.20433
5	1.1	82.02834
6	1.24	92.46831
7	1.341	100
8	0.951	70.91723
9	0.8	59.65697

Appendix 16: S48 chromium reductase temperature stability profile

S48 Relative activity							
Time (minutes)	Temperature (°C)						
	30	35	40	45	50	55	60
0	100	100	100	100	100	100	100
30	88.77	88.97	100	84.38	35.71	24.48	3.649
60	87.75	86.63	96.93	80.20	21.42	14.28	2.505
90	88.77	87.75	95.91	72.95	15.30	11.22	2.003
120	86.73	83.67	92.85	65.30	15.30	9.18	1.043

Appendix 17: S48 chromium reductase pH stability profile

S48 Relative activity								
Time (minutes)	**Ph**							
	3	4	5	6	7	8	9	10
0	100	100	100	100	100	100	100	100
30	42.27	89.25	92.22	99.73	100	43.06	63.80	31.26
60	30.14	86.13	86.03	98.94	100.10	37.80	60.89	23.43
90	28.61	85.10	81.85	97.72	100.21	34.86	48.25	20.05
120	28.67	78.67	78.62	96.29	96.82	30.69	31.74	17.30

Appendix 18: S48 chromium reductase metal ions profile

Metals	2mM	10mM
Control	100	100
Na_2SO_4	82.9703	77.12871
$MgSO_4$	118.0198	130.198
K_2SO_4	104.9505	109.4059
$ZnSO_4$	83.36634	89.50495
$FeSO_4$	125.0495	134.7525
$HgCl_2$	1.960396	1.128713
NiCl	105.8416	112.6733
$CuSO_4$	96.83168	78.61386
$NiSO_4$	104.9505	107.8218
KCl	100.5941	73.46535
NaCl	100.7921	94.85149
$CoCl_2$	94.35644	81.28713
$CaCl_2$	111.4851	129.802
$CdCl_2$	52.07921	38.61386
$CuCl_2$	95.0495	79.60396
$CaSO_4$	87.42574	68.71287

Appendix 19: S48 chromium reductase surfactants profile

Surfactants	0.50%	1%
Control	100	100
Tween 20	123.0693	139.4059
PEG	128.7327	142.2772
Tween 60	125.7426	130.495
Tween 80	115.0495	129.7624
CTAB	108.4158	102.1782
SDS	20.49505	19.40594
Triton X 100	77.42574	61.74257

Appendix 20: S48 chromium reductase organic solvents profile

S48 Relative activity				
Time	30	60	90	120
Control	100	100	100	100
Ethanol	89.24051	90.73418	75.51899	57.8481
Methanol	124.5316	118.0759	132.7089	133.7468
Acitonitril	39.18987	50.20253	48.50633	54.65823
Ethyle Acetat	27.87342	30.65823	44.81013	38.17722
Propanol	119.519	124.6076	130.3544	137.5696
DMSO	102.7595	118.1772	119.3418	122.8
Formaldehyde	79.34177	98.27848	98.48101	98.96203
Glycerol	83.82278	86.4557	105.6456	102.3797
Chloroform	107.2911	118.7848	119.7722	123.2658
n-Hexane	57.67089	55.26582	62.12658	70.32911

Appendix 21: S48 chromium reductase kinetic profile

S48 kinetics					
substrate	**Absorbance at 540 nm**	**Activity (U/ml**	**Specific activity U/mg**	**I/s**	**I/v**
				-1.36364	0
0.05	0.044	7.984848	31.93939	20	0.031309
0.1	0.09	14.95455	59.81818	10	0.016717
0.2	0.171	27.22727	108.9091	5	0.009182
0.3	0.249	39.04545	156.1818	3.333333	0.006403
0.4	0.359	55.71212	222.8485	2.5	0.004487
0.5	0.363	56.31818	225.2727	2	0.004439
0.6	0.41	63.43939	253.7576	1.666667	0.003941
0.7	0.58	89.19697	356.7879	1.428571	0.002803
0.8	0.78	119.5	478	1.25	0.002092
0.9	0.88	134.6515	538.6061	1.111111	0.001857

Appendix 22: *Escherichia* Sp. strain S54 16S gene

GATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAACAGCTTGCTGTTT
CGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCCGATGGAGGGGGATAACTACT
GGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCA
TCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAG
CTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGGGGTATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTT
CGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTAC
CCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAA
TCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTC
AACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGT
AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACT
GACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCT
GGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA
TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACGGAAGTTTTCAGAGAT
GAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCA
AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGA
CCAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACC

TCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTA
ATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTG

Appendix 23: *Bacillu*s Sp. strain S48 16S gene

GGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTAT
GAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGG
GAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCT
GTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGA
AGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT
GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCC
ACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATT
CCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTC
TGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCA
CTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAAC
CCTAGAGATAGGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGT
CGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC
CTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGA
GCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCG
CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG

Appendix 24: *Bacillu*s Sp. strain S48 chromate reductase gene

ATCTGGAACAGGAACCGGAGCTTTCCAATTGACACCACAGGATCCGATTATTACTTTCGTTGTTAATT
CGAATATTGTTCGTATACCAGTTCAATTTGTAACTGCGACAGTCACGAAAAACGCTTCCGTCAGCTCA
GCTTATTTAAATCAATATTTTGATTACACGGTGCGTATTACGAATACTTCCGAGATTTCACTCTTAAAT
ATTTCTTTACAGGATACTATTCCAGTAGGTTTACAATTTATGAGCGGCACCGCCTCCATTAACGGAGA
ACGCTCTCCACTAGCGAATCCGAATATCGGTTTCCTAGTTGCTACTAATTTAGAACCAAGCGAAACAA
TTATCGTGTTATTCACCGTACAAGTGATAAGTCCACCTGTTACTAATGAGTTTAAAAATACGGCTAAT
ATTTCGTTACAACTTCAAGCCTCACCTACCGATCCACCAATTACAGTAACCGTTACAAGTAACGAAAA
CATCGTCACCTTTGTTCCAGAAAATCCGGATAAAACACTTCCAAATTTCAATTGCTTCTTTGACGGTGA
GCGCTTCATACGGATTACTCCTCGAAATGTAGGCAATTACCTTTGGACTTGGATTTGGTGGAATTAAT
TAAAACAAAAAAGAGATTGTGATTTGGTTTCAACCAAATTACAATCTCTTTTTCATTACTTTTATCGTG
CTACATTATAACCCGGCCAATGATATACGATTAAGCGTCATCTAAAGTTATATTGTAAATAAAACAAA
AATATCCATATTCCGAATCAATTCTCTTCCATACAAATAAGACCAATGGTTTTAGTCA

Appendix 25: *Escherichia* Sp. strain S54 lead binding protein PbR gene

AAGCTTCCCAGATGTTTGACTGTTCAATATCGATAATCTTCAAATAAACGTCTTGCTGTGCAGCAAGC
CATTCAGCCCATACCGTGGTTTTGGTAAAGCAATAAACCATAATATTTAATGACGAATCAGCAAACTG

GTTGAAATAAACCAGTAAGGTTTGTCGCTGGTCGATGGCCGGGTGATTTTTCAGCATCTCACGTACA
GCTTCGACAATAACGCCCACTTTTGCCGCATCCTCATAACGTAAACCAATGGTCGTGGTAATGCGCCG
GTTGGTCATTCGTCCAGGATTTTCTACGCTGATCGACGAAAACAGCGAGTTCGGTACGTACAATGGA
CGATTATCAAAGGTCTTAATTTTGGTAATTCGCCAGCCAATTTCCGCTACTGTACCTTCGATATTTCTG
TCCGGTGAACGGATCCAGTCGCCAATTCTGATTTCCATGG

Appendix 26: *Bacillu*s Sp. strain S48 chromate reductase Amino acid sequence

MTKTIGLICGSLRKNSYNRAIAQSLFDFDNAAQFRWVEIDNLPFFNEDLEIAG
APDSVTSFRADIQDVDGVIIVSPEYNSGIPGVLKNALDWASRPRTSSVLNRKP
VGLIGATPGGFGTAFGQTQMREVLEAMQVNVLPFQKMLISQVHEKIDSAQNI
LTDEQTKRYLQRYLQQFIHWIDHAPVPVPD

Appendix 27: *Escherichia* Sp. strain S54 lead binding protein Amino acid sequence

MEIRIGDLAKRSGCEVVTIRYYEKEGLLPKPARSGGNFRLYGEAHIERLQFIRH
CRSLDMTLSEIRALLGLRDNPMQDCGEVITLLEAHIQQVEMRVSALLQLKRH
LVDLREKCSGSRSVEACGILQGLGNCHGESATNSQTSG

Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Isolation and screening of chromium resistant bacteria from industrial waste for bioremediation purposes

Isolamento e triagem de bactérias resistentes ao cromo de resíduos industriais para fins de biorremediação

Kalsoom[a], Afshan Batool[a], Ghufranud Din[a], Salah Ud Din[a], Johar Jamil[a], Fariha Hasan[a], Samiullah Khan[a], Malik Badshah[a], Aamer Ali Shah[a*]

[a]Quaid-i-Azam University, Department of Microbiology, Faculty of Biological Sciences, Islamabad, Pakistan

Abstract

Chromium (VI) a highly toxic metal, a major constituent of industrial waste. It is continuously release in soil and water, causes environmental and health related issues, which is increasing public concern in developing countries like Pakistan. The basic aim of this study was isolation and screening of chromium resistant bacteria from industrial waste collected from Korangi and Lyari, Karachi (24°52′46.0″N 66°59′25.7″E and 24°48′37.5″N 67°06′52.6″E). Among total of 53 isolated strains, seven bacterial strains were selected through selective enrichment and identified on the basis of morphological and biochemical characteristics. These strains were designated as S11, S13, S17, S18, S30, S35 and S48, resistance was determined against varying concentrations of chromium (100-1500 mg/l). Two bacterial strains S35 and S48 showed maximum resistance to chromium (1600 mg/l). Bacterial strains S35 and S48 were identified through 16S rRNA sequence and showed 99% similarity to *Bacillus paranthracis* and *Bacillus paramycoides*. Furthermore, growth condition including temperature and pH were optimized for both bacterial strains, showed maximum growth at temperature 30°C and at optimum pH 7.5 and 6.5 respectively. It is concluded that indigenous bacterial strains isolated from metal contaminated industrial effluent use their innate ability to transform toxic heavy metals to less or nontoxic form and can offer an effective tool for monitoring heavy metal contamination in the environment.

Keywords: chromium resistance, industrial waste, *Bacillus paramycoides*, *Bacillus paranthracis*

Resumo

O cromo (VI), metal altamente tóxico, é um dos principais constituintes dos resíduos industriais. É liberado no solo e na água, causa problemas ambientais e de saúde de crescente preocupação pública em países em desenvolvimento como o Paquistão. O objetivo básico deste estudo foi o isolamento e a triagem de bactérias resistentes ao cromo de resíduos industriais coletados em Korangi e Lyari, Karachi (24°52'46,0"N 66°59'25,7"E e 24°48'37,5"N 67°06'52,6"E). Do total de 53 cepas isoladas, sete cepas bacterianas foram selecionadas por enriquecimento seletivo e identificadas com base em características morfológicas e bioquímicas. Essas cepas foram designadas como S11, S13, S17, S18, S30, S35 e S48, apresentaram alta resistência aos metais contra concentrações variáveis (100-1500 mg / l) de cromo. Já as cepas S35 e S48 foram identificadas por meio da sequência 16S rRNA e apresentaram 99% de similaridade com *Bacillus paranthracis* e *Bacillus paramycoides*. Além disso, as condições de crescimento incluindo temperatura e pH foram otimizadas e ambas as cepas bacterianas apresentaram crescimento máximo na temperatura de 30 ºC, enquanto seu pH ótimo foi observado em 7,5 e 6,5, respectivamente. Conclui-se que o potencial de resistência dessas bactérias resistentes ao cromo pode ser efetivamente utilizado na remoção de cromo de efluentes industriais contaminados. Técnicas de base biológica usando bactérias ajudarão a fornecer métodos mais baratos e ecológicos de remoção, recuperação e desintoxicação de cromo.

Palavras-chave: resistência ao cromo, resíduos industriais, *Bacillus paramycoides*, *Bacillus paranthracis*

1. Introduction

In last few decades, the increase concentration of different heavy metals causes environmental contaminations and devastating effect on public health. Human expose to metals due to increase of heavy metals in various industry such as textile, painting, leather etc. Effluents releasing from these industries have profound effect on human health as well as environment (Raskin and Ensley, 2000). Accumulation of potentially toxic heavy

*e-mail: alishah@qau.edu.pk.
Received: August 21, 2020 – Accepted: January 21, 2021

metals i.e. Hg, Cd, Cr, Cu and Zn in a body causes mental abnormalities, neuromuscular disorders, cancer and growth abnormalities (Wuana and Okieimen, 2011). Chromium is used in electroplating, tanning, textile dyeing, and metal processing industries. Industrial wastewater and effluents contain chromium, which has toxic effects on the microbial consortia of wastewater treatment systems (Stasinakis et al., 2003).

Naturally, Chromium exists in different oxidation states varying from Cr(II) to Cr(VI). Cr(III)is not highly toxic and has high absorbance capacity in water and soil while, Cr(VI) is the toxic form, highly soluble and not easily adsorbed in natural bodies (Kotas and Stasicka, 2000). The permissible limit of Cr in drinking water is 0.05 mg/L according to the World Health Organization (WHO, 1993).

To reduce the toxicity of chromium in the environment, industrial effluents must be treated before release. Many conventional methods have been adopted for removing metals from industrial effluents i.e. chemical (precipitation, oxidation or reduction, ion exchange) and physical(filtration and membrane technologies) (Ahluwalia and Goyal, 2007).These technologies are effective only when high concentration is needed to be treated, ineffective, and highly expensive for treating metal concentration in wastewater below the range of 100 mg/L (Nourbakhsh et al., 1994). Therefore, these technologies need to be replaced with highly effective, cheaper and eco-friendly techniques, which can effectively remove very small concentration of toxic heavy metals. A large number of microorganisms are found naturally in waters and soil receiving industrial effluents. These microorganisms have adopted and developed various strategies as a defense mechanism to protect themselves from the toxicity of heavy metals (adsorption, uptake, methylation, oxidation, and reduction). Accumulation of Cr (VI) in agriculture lands reduces soil productivity by decreasing the population of various soil microbes (Karthik et al., 2017; Wani et al., 2018). Chromium is carcinogenic and mutagenic, designated as priority pollutant or Class A pollutant by the United States environmental protection agency (USEPA, 1996).

Microbial reduction of hexavalent chromium to trivalent chromium is an important and cost effective remediation technology (Wang et al., 2013), necessary for glucose metabolism (Vincent, 2000), enzymatic activation and DNA and RNA stabilization (Karuppanapandian et al., 2009). Chromium (VI) remediation has been reported in both soil and water contaminated with metal by bacterium *Pannonibacter phragmitetus* BB (Wang et al., 2014). Removal of Cr(VI) either by reduction or via biosorption can significantly reduce the risks to human health (Kamaludeen et al., 2003).The reduction of hexavalent Cr to trivalent Cr is a viable process, mediated by chromate reductase under aerobic conditions via its cytosolic form and in anaerobic respiration through its membrane-bound component (Camargo et al. 2004).

Main water pollution sources in Pakistan are the industrial waste released by different industries i.e. of textile, paints, chemical, leather and paper etc. (Waseem et al., 2014). In Pakistan, Karachi is the largest contributor for heavy metal pollution as approximately 8000 industries running in 9 industrial estates released 362 million gallons per day sewage. Total of 60% of this industrial waste released directly into Korangi and Lyari River without treatment and disposed-off into coastal belt of Karachi (World Bank, 2006). Lacking the waste treatment facility in industries of billion-dollar business is responsible for ruining environment by releasing excessive chemicals in the water and soil which in turn harmfully affect the public health (Sundar et al., 2010). The solution to this pollution control is the installation of effective treatment plant, which is efficient, cost-effective, simple and ecofriendly.

Keeping in view the toxicity of chromium, it needs to analyze industrial wastes for the isolation and screening of indigenous bacteria and to explore their role in bioremediation of toxic chromium in such industrial wastes. The objectives of this study are; isolation and screening of indigenous chromium resistant bacteria from industrial waste and optimization of growth condition for resistant strains.

2. Materials and Methods

2.1. Collection of chromium contaminated Industrial Waste Samples

Samples were collected from two different sites, River bank of Lyari (24°52′46.0″N 66°59′25.7″E) and Korangi industrial area (24°48′37.5″N 67°06′52.6″E) located in Karachi, Pakistan. Soil samples were collected in triplicate down to 10 cm depth in sterile zippered bags and water/sludge samples were collected in sterile plastic bottles using standard sampling protocols (Carter and Gregorich, 2007). Samples were kept at 4°C and immediately transported to the Applied, Environmental and Geomicrobiology lab, Quaid-i-Azam University, Islamabad.

2.1.1. Physico-chemical and elemental analysis of samples

During sample collection temperature and pH were determined by mean of thermometer and pH meter electrode respectively. Moisture content in soil and sludge samples was monitored by dry oven method. For elemental analysis samples were prepared following standard protocol with minor modification (Cantle, 1986). All soil samples (20 g) were dried over night at 60°C and grounded/sieved to small particles. Then one gram of each grounded soil samples was mixed in 15 ml of HNO_3 and HCl (1:3), boiled for 30 minutes, and then left overnight. Then 5ml of $HClO_4$ was added and boiled until the total volume was reduced to 3–5 ml. After cooling, the mixture was filtered through Whatman filter paper (No 42) and then volume was brought up to 20 ml with sterile deionized water. As a control, an ordinary garden soil sample was similarly collected and processed. A blank was processed in the same manner without the addition of any soil and its values were subtracted from the rest of the experimental values in order to remove procedural errors. All samples were processed and analyzed for presence of heavy metals concentration i.e Chromium, Copper, Nickel and cobalt

Turnitin Originality Report

Role of Metallo-resistant Microorganisms in Remediation of Heavy Metals using Biofilter by Kalsoom .

From CL QAU (DRSML)

- Processed on 02-Aug-2022 12:14 PKT
- ID: 1878017025
- Word Count: 30007

Similarity Index
16%
Similarity by Source

Internet Sources:
8%
Publications:
12%
Student Papers:
5%

sources:

1 1% match (student papers from 08-Feb-2017)
Submitted to Higher Education Commission Pakistan on 2017-02-08

2 1% match (publications)
Babita Sharma, Pratyoosh Shukla. "Lead bioaccumulation mediated by Bacillus cereus BPS-9 from an industrial waste contaminated site encoding heavy metal resistant genes and their transporters", Journal of Hazardous Materials, 2021

3 1% match (publications)
Antonio Jesús Muñoz, Francisco Espínola, Encarnación Ruiz. "Removal of Pb(II) in a packed-bed column by a Klebsiella sp. 3S1 biofilm supported on porous ceramic Raschig rings", Journal of Industrial and Engineering Chemistry, 2016

4 1% match (publications)
Sadia Mehmood Satti, Amina Mumtaz Abbasi, Salahuddin, Qurrat ul Ain Rana et al. "Statistical optimization of lipase production from Sphingobacterium sp. strain S2 and evaluation of enzymatic depolymerization of Poly(lactic acid) at mesophilic temperature", Polymer Degradation and Stability, 2019

5 1% match (publications)
Sanam Islam Khan, Numan Saleh Zada, Miray Sahinkaya, Dilsat Nigar Colak et al. "Cloning, expression and biochemical characterization of lignin-degrading DyP-type peroxidase from Bacillus sp. Strain BL5", Enzyme and Microbial Technology, 2021

6 < 1% match (student papers from 13-Dec-2017)
Submitted to Higher Education Commission Pakistan on 2017-12-13