Isolation and Characterization of carcinogenic Chromium reducing *Pseudomonas soli* from soil of Buriganga riverbed

A project submitted

by

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ID: 13346013

Session: Summer 2013

to

The Department of Pharmacy

in partial fulfillment of the requirements for the degree of

Bachelor of Pharmacy (Hons.)

Dhaka, Bangladesh

September 2018

This work is dedicated to my parents for their selfless love and utmost support.

Certification Statement

This is to certify that this project titled "Isolation and Characterization of carcinogenic Chromium reducing *Psuedomonas soli* from soil of Buriganga river bed" is submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Md. Samiul Alam Rajib, Senior Lecturer, Department of Pharmacy, BRAC University. This project is the result of the author's original research and has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the project contains no material previously published or written by another person except where due reference is made in the project itself.

Signed

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Countersigned by the supervisor

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Acknowledgement

First and foremost, I want to thank Almighty Allah for giving me the strength and dedication to finish the project and to overcome all the obstacles that accompanied with it. It would not have been possible to accomplish the purpose of the work without His mercy.

I would also like to thank my supervisor, Md. Samiul Alam Rajib, Senior Lecturer, Department of Pharmacy, BRAC University for his constant support and help regarding any problems that I faced or any questions that I had. I am thankful to him for his guidance, eagerness and supervision throughout the project work. This work would not have been completed without his continuous instructions and versatile knowledge.

I also want to express my gratitude to Professor Dr. Eva Rahman Kabir, Chairperson, Department of Pharmacy, BRAC University, for giving me a chance and necessary support to do this project at an individual level. Furthermore, I want to give thanks to all the Lab Officers and Lab Assistants for their utmost help and concerns.

Last but not the least; I would like to thank all the Faculty Members of Department of Pharmacy, BRAC University, my parents and my fellow mates, Tashfeea Islam and Fatima Tabassum for their belief in me. It would have been really hard to complete the project work without their nonstop support and prayer.

Abstract

Hexavalent Chromium is a heavy metal which possesses carcinogenic and mutagenic property and causes various serious diseases such as skin cancer, lung cancer, pulmonary congestion etc. It is an important component in various industries like leather, tannery, dye, stainless steel etc. and so they are being freed every day without any treatment and making the natural environment very hazardous for the human health. It has become an obligation to reduce the hexavalent Chromium from the natural environment to preserve the earth. A strain from soil sample named *Pseudomonas soli* has been identified as being capable of reducing carcinogenic hexavalent Chromium to trivalent Chromium. The strain was isolated from Buriganga River bed soil. Nutrient agar medium was employed which was supplemented with hexavalent Chromium in the form of potassium chromate. By purification and subculture on nutrient agar plates containing different concentrations of Chromium, the isolate was obtained. 16S rRNA gene analysis was done to find out its identity. The strain was then subjected to further experimental procedures such as DPCZ base bioremediation assay. Bioassay was carried out at three temperatures: 25°C, 37°C and 42°C and at each temperature, Chromium reduction was seen at three different pH 5.5, 7 and 8.5. It was observed that, the bacterial strain was able to reduce Chromium completely at temperature and pH of 37°C, pH 8.5 and at temperature 42°C, in both pH 7 and 8.5. However, the highest rate of reduction was seen at 42°C at pH 7. Therefore, 42°C, pH 7 is its optium temperature for reduction. Minimum Inhibitory Concentration (MIC) of Chromium (VI) on isolate's growth pattern and antibiotic resistance profile of the isolate were also deduced. MIC was found to ne 70 mM. The strain demonstrated multiple resistances to antibiotics which included Penicillin, Amoxycillin, Cefixime and Cefuroxime sodium. Moreover, it was seen after study that the bacteria under consideration can withstand up to high concentration of Chromium (upto 69mM) which demonstrates its capacity to survive in very adverse condition. Information gathered from this project confirms that the bacteria is capable of reducing hexavalent Chromium.

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List of acronyms

BCM	biologically controlled mineralization
BIM	biologically induced mineralization
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
CNO	Carbon nano onion
Cr	Chromium
CRB	Chromium Resistant Bacteria
DPCZ	Diphenyl Carbazide
DTPA	Diethylene tri-amine penta acetic acid
EDDS	Ethylenediamine-N,N'-disuccinic acid
EDTA	Ethylene diamine tetra acetatic acid
IARC	International Agency for Research on Cancer
Kg	Kilogram
LDL	Low density lipoprotein
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mL	Milliliter
mM	Millimolar
MOPS	3-(n-Morpholino)Propanesulfonic Acid
NA	Nutrient Agar
NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth
NIOSH	National Institute for Occupational Safety & Health
Nm	Nanometer
O.D.	Optical density
OSHA	Occupation safety and health administration
PEL	Permissible exposure limit

Ppm	Parts per million
ROS	Reactive oxygen species
RPM	Revolution per minute
rRNA	Ribosomal ribonucleic acid
SCHER	Scientific Committee on Health & Environmental Risk
WHO	World Health Organization
ZI	Zone of Inhibition
µM	micro molar
µg	Micro gram

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1 INTRODUCTION

1.1 Chromium: a brief study

Chromium belongs to the group of transition metals and is considered a heavy metal which has harmful effects in the human body when exposed to it. The metal has an atomic number of 24 and atomic mass of 51.966amu. (Focardi, Pepi, & Focardi, 2013) It occurs naturally in the Earth's environment, the concentration of which is increasing till date. (Bielicka, Bojanowska, & Wiśniewski, 2005) The concentration ranged from 100 to 300 $\mu g\ g^{-1}$ in the year 2013. (Focardi et al., 2013)

Chromium can be found in the environment from two main sources: first being ferrochromite ($Fe_2Cr_2O_4$) and the second one are linked with the use of chromium in industries, predominantly in metallurgy, tanning and textile pigment production. (Augustynowicz et al., 2010) Chromium has oxidation states ranging from +2 to +6; among which the hexavalent forms (+6) are more toxic than trivalent forms (+3) because they are mutagenic and carcinogenic and is known to cause cancer. (Yrjälä & Hasnain, 2012)

Besides the many uses of chromium in medicine, chromium is also used industrially. Chromium has been used extensively in metallurgical, chemical and refractory industries. It is used for production of stainless steel, chrome-plating and also for leather tanning. (Mädler et al., 2016) It is also said to be used for preparation of alloys, production of refractories, dye industry, industrial water cooling, production of paper pulp, petroleum refining, preservation of wood and nuclear power. (Focardi et al., 2013)

Where a significant amount of Chromium is considered to be toxic for the body, trace amounts have certain benefits. (Lewicki et al., 2014) It was found out in a study that Chromium treatment lowered mood symptoms in pre-menstruating women and showed marked improvement in health. The Chromium supplements taken with antidepressant showed synergistic effect compared to antidepressants or chromium supplements taken alone. (Brownley et al., 2013) Chromium is said to be associated with the proper activity of the immune system. Chromium (III) helps in the metabolism of lipids, glucose and proteins. It was shown in a study that presence of dietary supplements containing chromium in rats and cattle caused lower cholesterol level, LDL, triglycerides and non-essential fatty acid and thus can be used in obesity treatment.

Since chromium is associated with metabolism of glucose, it has shown to improve insulin action and regulate diabetes. (Lewicki et al., 2014)

So use of chromium in trace amount is essential for the body whereas the same metal in a higher concentration is proven to be harmful for life. It is said that if concentration of hexavalent Chromium into the environment goes above 0.05 mg/L, it may have an effect on the human physiology. (Bharagava & Mishra, 2018) Water soluble hexavalent chromium is tremendously irritating and lethal to tissues of the human body as the solubility causes active transport of chromate across biological membranes. (Ray, R. R. 2016) Hexavalent chromium causes irritation to skin and mucous membrane and some of these hexavalent compounds are also known to be strong corrosive agents. These compounds also produce an allergic contact dermatitis which is characterized by eczema. (Barceloux, D. G., & Barceloux, D. 1999)

1.2 Why Bioremediation?

There are ways in which the toxic state of the Cr (VI) can be converted to less toxic state. Some of them being: Use of nano filtration and reverse osmosis membrane, biosorption, bioremediation by microbial activity, use of carbon nano onions (CNOs), Use of electrosynthesized 4-amino-3-hydroxynaphthalene-1-sulfonic acid doped polypyrrole as adsorbent and use of Zr(IV) cross-linking chitosan magnetic microspheres. In this project, we will look into the bioremediation process and how microorganisms can be used in reduction of the heavy metal form its toxic oxidation state to less toxic oxidation state.

Bioremediation is the process of conversion of hazardous substances into non-hazardous and harmless substances by the use of organisms. The process of bioremediation for the reduction of chromium +6 to +3 has been chosen for a number of reasons. To start with, presently, bioremediation is recognized as a cost-effective remediation technology and also environmental friendly. It is potentially suitable for vast polluted areas, like contaminated water and soil. Since our sample was collected from the river Buriganga, bioremediation is the proper approach for our aim. Its probable safety and effectiveness will positively accelerate further development of the technology. (Cheng, Y., Holman, H. Y., & Lin, Z. 2012)

1.3 Background of the study

Maintaining high quality of water is necessary for ensuring a healthy ecosystem. In recent years, the quality of water has been greatly affected by human activities like agriculture and industrial activity. Heavy metal contamination of the water resulting by this increase in human activity has become a major threat to the health of ecosystems. (Zhaoyong, Abuduwaili, & Fengqing, 2015) The contamination of aquatic systems by heavy metals, especially in sediments, has turned out to be one of the most challenging pollution issues on account of subsequent bio-accumulation, the toxicity, abundance and persistence, and of these materials. (Tang et al., 2014) Human activity is a significant driver of heavy metal contamination, and this contamination comes from exhaust emissions from automobile, industrial pollution, and use of fertilizers and pesticides. Heavy metals do not only directly affect the physical and chemical properties of the water and its surrounding environment, inhibit microbial activity in the water, and hamper the supply of nutrients but also be absorbed by the human body through food or air intake, and, thus, pose a threat to human health. In addition, heavy metals in aquatic environments can easily spread to other regions (Zhaoyong et al., 2015) Environmental pollution caused by sewage irrigation and sludge application, discharge from tanneries, together with chemical fertilizers and pesticides, municipal and animal waste, has created a potential hazard to human health. (Dwivedi & Vankar, 2014)

1.4 Aims and Objectives

The rationale of the project is to isolate Chromium resistant bacteria which are capable of performing bioremediation of carcinogenic and mutagenic heavy metal, Chromium and to characterize them. It was aimed at studying the isolated bacteria. For this purpose, the objectives of the study were:

1. Evaluation of the performance of the isolated bacteria in Chromium contaminated environment
2. Study of antibiotic resistance profiling of the isolated bacteria
3. To find out the minimum inhibitory concentration of Cr (VI) to outgrow selected isolate
4. To construct the phylogenetic tree of the bacteria for its identification and genetic evaluation.

1.5 Literature Review

1.5.1 Introduction

Chromium is transition metal in the periodic table and is considered to be a heavy metal. The metal can be found as several chemical states in the environment: from -2 to +6. The states that are abundantly found in the nature are trivalent and hexavalent states. These two forms show distinctive chemical and physical properties in addition to different influence on living organisms. The hexavalent state of Chromium, Cr (VI), mainly originates from anthropogenic sources and is found in water as $HCrO_4^-$, $Cr_2O_7^{2-}$ and CrO_4^-. These are extremely mobile anions. The dominant forms of trivalent chromium, Cr (III) are mostly cationic: $CrOH^{2+}$ and $Cr(OH)_2^+$. In comparison to hexavalent chromium, trivalent chromium is not as mobile. The reason behind being that trivalent chromium has the ability to precipitate and form complexes with inorganic ligands like humic acid. (Augustynowicz et al., 2010) While Chromium has beneficial aspects in the body in trace amounts, increasing level has been proven to be toxic. The intoxicating nature of Chromium to pollute the nature has been observed from long time. (Sellamuthu, Umbright, Chapman, Leonard, & Li, 2011) Hexavalent Chromium is fatal and mutagenic to humans and other living beings. Pollution of Chromium is occurring in the environment due to the entrance of untreated manufacturing drainage into water bodies. This is mixed into the common asset like sediment, water, air and soil. It is happening in underdeveloped countries as well as in developed countries. (Thacker & Madamwar, 2005) Hexavalent chromium compounds are utilized in chromium plating and cement and paint production industries. (Bielicka et al., 2005) This presents elevated potential for contamination of aquatic environments around which this industries are located. It was seen in a study that the gills of Channa punctatus exposed to Cr^{6+} showeded hyperplasia of the lamellar epithelium, lamellar fusion and necrosis of tissues. (Castro et al., 2014) Cr (III) is needed as a fundamental necessity for the living beings whereas Cr (VI) is thought to be the reason for various types of cancer. (Bielicka et al., 2005) This trace element, Chromium (III) is involved in the metabolism of carbohydrates, lipids, and proteins which mainly works by increasing the efficiency of insulin. (Lewicki et al., 2014)

1.5.2 Chemistry

Chromium is a transition metal situated in the first series in group VIB. (Cohen, Kargacin, Klein, & Costa, 1993) The metal is an odorless hard metal which has a silverish gray color. The name Chromium is derived from the Greek origin 'chroma' which means color. (Horn, 2013) Chromium has an atomic number of 24 and its symbol is Cr. Its boiling point is 2672°C and melting point is 1097°C. (Das, Dhundasi, & Das, 2011) Chromium can exist in more than a few chemical forms, displaying oxidation numbers ranging from -2 to + 6. (Cohen et al., 1993) Cr (III) is the most thermodynamically stable and thus as a result, it is most abundant in the trivalent state. (Katz, 1989) In acidic medium, hexavalent chromium compounds are known to be powerful oxidizing agents. Cr (VI) is readily reduced to Cr (III) at low pH:

$$Cr_20_7^{2-} + 14H^+ + 6e^- \rightarrow 2Cr^{3+} + 7H_20$$

In alkaline medium, trivalent chromium compounds are easily oxidized to the yellow (hexavalent) chromate ion:

$$CrO_2^- + 4OH^- \rightarrow CrO_4^{2-} + 2H_2O + 3e^-$$

Many chromates show low solubility in aqueous medium. Another oxidation state of Chromium: tetravalent chromium, compounds of which are ill-defined. Considering the acid-base properties of the metal, Cr (III) oxide is amphiprotic. In acidic medium, trivalent chromium is reduced to the divalent state with zinc amalgam and in alkaline medium, trivalent chromium is oxidized to the hexavalent state with peroxide or chlorine. (Katz, 1989)

1.5.3 Sources and Occurrence of Chromium compounds

Chromium mainly exists as a mineral chromite ($FeOCr_2O_3$ or $FeCr_2O_4$) in the Earth's crust and it is introduced into the environment via various industrial processes, like as metallurgic, and wood preserving, tanning and plating industry. (Room, Lauderdale, & Goldberg, 2015) In soil, concentration of chromium varies from 1-3000 mg/kg, 5-800 µg/L in sea water, and 26 µg/L to 5.2 mg/L in lakes and rivers. Chromite is the most fundamental mineral ore. Moreover, it can

also be found as outcomes of both marine or earthly volcanic eruptions.(Aslam & Yousafzai, 2017)

The major source of Chromium in soil is weathering of their parent compounds. Chromium that is found in soil is generally trivalent and is absorbed in small quantities in plants. Chromium can be found in water due to weathering of rocks, dry fallout from the atmosphere and wet precipitation, and washed out from earth. An increase in Chromium concentration in soil and water bodies might be due to fallout and washout of particles containing Chromium being dumped from industries which makes heavy use of the metal. Chromium that is present in the atmosphere originates from anthropogenic sources. This account for 60-70% and the remaining is from natural source which accounts for the 30-40%. (Bielicka et al., 2005)

Chromium is fundamentally found in different countries in the world. In China, hexavalent Chromium along with its compounds are released into the atmosphere as by-products of burning of fossil fuels, incineration of waste and a variety of industrial processes. (H. Cheng, Zhou, Li, Lu, & Lin, 2014) In South Africa, Ferric Chromite ($FeCr_2O_4$) is mostly found. The chromite metal store addresses around 72% of the earth's perceived sources in South Africa. (Papp, 2006) Distinctive countries with vulnerable stocks of mineral consolidate Brazil, India, Zimbabwe, Finland, Kazakhstan and Philippines. (Papp, 2006)

1.5.4 Chromium as a useful metal

Many Chromium compounds like sodium chromate, ferrochromate, dichromates are widely made use of in commercial scale in industries. It can be used as an anticorrosive agent in boilers (Aslam & Yousafzai, 2017) Chromium and its compounds are functional in leather tanning, paints, pigments, inks, timber preservation, industrial dyes and many more. (Löv et al., 2017) Chromium is opposed to ordinary corrosive agents at room temperature and thus can used as an electroplated, protective coating. It is also used in ferrous and nonferrous alloys, in refractories, and in chemicals. Ferrous alloys of chromium- mainly stainless steels account for most of its utilization. Chromite (Iron Chromium Oxide) is employed in the refractory industry for making bricks, mortar, and ramming and gunning mixes. Chromite improves their thermal shock, slag resistance, strength and volume stability. (Bielicka et al., 2005)

1.5.5 Chromium regulation levels

The presence of Chromium in water bodies and near water surfaces in amounts which exceeds the standard concentration imposes threat to human, animals as well as plants. Chromium concentration in water is increasing due to human activities like dumping of tannery waste in water bodies near it. The permissible limit for tannery wastewater discharge is only 2 mg/L. However, concentration of Chromium in tannery wastewater ranges from 2000-5000 mg/L. This exceeds the limit by a great range. (Bharagava & Mishra, 2018) The reason behind the high concentration of Chromium is the dumping of industrial effluents into water bodies which is estimated to be $6.7X10^6$ kg yearly. (Bharagava & Mishra, 2018) It is said that if concentration of hexavalent Chromium into the environment goes above 0.05 mg/L, it may have an effect on the human physiology. (Bharagava & Mishra, 2018)

As per rule set by the World Health Organization (WHO) of drinking water, the highest level of concentration that is permissible for hexavalent Chromium to be present is 0.05 mg/L and for total Chromium which includes both hexavalent, trivalent along with other forms of Chromium to be present is 2mg/L. (V. K. Gupta & Rastogi, 2009) The acceptable amount of Chromium in filtered water is 0.1 mg/L. Level of Chromium must exceed 50ppm in dyes and color additives. The Permissible Exposure Limit (PEL) for hexavalent Chromium was declared to be $0.1mg/m^3$ by Occupational Safety and Health Administration (OSHA).

1.5.6 Chromium toxicity

Chromium, if exposed in amounts which exceeds the standard, may cause hazardous reactions in the body such as skin irritation, ulceration, lung carcinoma, nasal irritation, eardrum perforation and a lot more. (Bharagava & Mishra, 2018) Chromium has the capacity to accumulate into placenta and thus causing damage to the enlargement of fetus. (Poopal & Laxman, 2009) There are numerous studies which reported heavy metal deposition in soil, crops, and vegetables grown in the locality of industrial areas **(J. U. Ahmad & Goni, 2010)** Accumulation of hexavalent Chromium in the environment changes the structure of communities of microbes in soil, reducing its growth and its correlated enzymatic activities. (Focardi et al., 2013)

Hexavalent Chromium has the potential to penetrate cellular membranes through the sulfate transport system in cell membranes and damage it by creating oxidative stress. This causes inhibition of electron transport chain addition to loss of the membrane integrity of the cell. Once

hexavalent Chromium enters the cell, it can bind to cellular materials and alter their regular physiological functions. In vivo, Cr (VI) species and hydroxyl radicals can cause DNA lesions. The intermediates originated from Cr (VI) action are lethal to cell organelles, proteins and nucleic acids. Hexavalent Chromium is said to induce carcinogenic, teratogenic and mutagenic effects in the body due to its precarious chemical form. The cell membrane is almost impermeable to Cr (III). Thus the toxicity of Cr (VI) is only about one thousandth of the toxicity of Cr (VI). Taking all these into account, it is safe to say that, Chromium can have different biological effects depending on its oxidation state; with Cr (VI) being highly toxic to most organisms, and Cr (III) being relatively harmless. (Focardi et al., 2013)

Due to the numerous health hazards caused by Cr (VI), US Environmental Protection Agency (USEPA) has categorized hexavalent Chromium as a class A human carcinogen and a priority pollutant. (Polti, Amoroso, & Abate, 2010)

1.5.7 Effects of Chromium on Health

1.5.7.1 Effects in humans:

Hexavalent Chromium is a strong epithelial irritant and is a confirmed human carcinogen. The heavy metal is deadly to humans, plants and aquatic animals. Acute inhalation exposure of hexavalent Chromium in humans, such as in a job-related set-up, can cause respiratory irritation, like as dyspnea, cough, wheezing, sneezing, rhinorrhea, choking sensation, etc. Exposure due to occupation, hexavalent Chromium in the form of chromium trioxide in the electroplating industry resulted in upper respiratory problems among the workers. A case history showed that a group of nine men in a chrome plating facility showed as many as seven cases of nasal septum ulceration. Cases of dizziness and headache at high concentrations have also been reported, and this can trigger asthmatic attacks in sensitive patients. It was reported in a study that a patient became anorexic and lost around 9.09–11.36 kg of bodyweight within a period of 3 months after continuous exposure to hexavalent Chromium. An elevated risk of death from non-cancer respiratory disease was reported in retrospective mortality studies among workers in a chrome plating plant. On the other hand, studies of workers in the chromium pigment, chrome plating, and ferrochromium industries had shown a statistically major connection between worker exposure to hexavalent Chromium and lung cancer. (Das et al., 2011) The mechanism by which

hexavalent Chromium causes cancer is unknown but it has been hypothesized that Cr (VI) binds to double stranded DNA thereby altering the process of gene replication, repair and duplication. (Bielicka et al., 2005)

Another significant way by which human exposure to Chromium can take place is through skin penetration. (Annangi, Bonassi, Marcos, & Hernández, 2016) Acute exposure of hexavalent Chromium to skin can lead to skin burns and can also have similar continuation that can ultimately lead to death. No concrete data were found relating to systemic effects in humans after acute exposure to trivalent Chromium compounds by any route. (Das et al., 2011) Astudy showed the correlation between content of Chromium in cement (as a contaminant) and occurrence of contact dermatitis seen among the construction workers in numerous countries. (Sellamuthu et al., 2011)

1.5.7.2 Effects in animals:

It was found in experiments that rats treated with hexavalent chromium showed hyperglycemia or diabetes: symptoms of which were associated with a decline in liver glycogen concentration and diabetic oral glucose tolerance test response. As an outcome of heavy metal exposure, such as hexavalent Chromium, it was shown that it fascilitated lipid peroxidation, DNA damage, altered calcium, and sulfhydryl homeostasis. On top of these, marked disorder of the antioxidant defense system in various metabolically active tissues also took place. (Das et al., 2011)

The gills of *Channa punctatus* exposed to Cr (VI) displayed hyperplasia of the lamellar epithelium, lamellar fusion and even necrosis. (Castro et al., 2014) It was established in a study that acute toxic concentrations of Cr (VI) brought about changes in gills, kidneys and stomach of rainbow trout along with changes in plasma osmolarity and hematocrit values of blood. Another study showed hevalent Chromium induced hepatotoxicity and carcinogenicity in fishes. (Svecevičius, 2006)

1.5.8 Kinetics

Studies have shown the toxic nature of Chromium and its effect as a mutagenic and carcinogenic agent. The dumping of waste from industries especially tannery industries into nearby water bodies contain large amount of Chromium. The microbes in the water have developed various

resistance mechanisms to endure chromate toxicity that facilitate them to survive in such toxic environmental conditions. Some of the detoxification strategies of these microbes are biosorption, bioaccumulation and biotransformation through enzymatic reduction, diminish intracellular accumulation by either direct obstruction of ion uptake system or active chromate efflux, precipitation, and reduction of hexavalent Chromium to less lethal and less mobile trivalent Chromium. (Bharagava & Mishra, 2018)

Two types of enzymatic mechanisms for reduction of hexavalent Chromium has been put forward; one being the aerobic activity and the other, anaerobic activity. Aerobic activity of reduction of hexavalent Chromium is usually linked with a soluble protein fraction which uses an electron donor such as NADH. The use of the electron donor is considered to be a necessity for the activity or it is used to maximize the activity. Also, an increase in concentration of H^+ inside cells can also increase the rate of reduction of hexavalent Chromium through the respiratory-chain-linked activity through the reaction:

$$CrO_4^{2-} + 8H^+ + 3e^- \rightarrow Cr^{3+} + 4H_2O$$

1 mole of hexavalent Chromium requires 8 mol of H^+ to be reduced. Therefore, Cr (VI) reduction which occurrs on the surface of the cell results in a vast reduction of the H^+ gradient across the membrane. This is the driving force needed for oxidative phosphorylation in cells. Hence, the energy produced through the reduction of Cr (VI) on the cell surface by the soluble Cr(VI) reductase activity cannot be preserved. The enzyme responsible for reduction is called chromate reductase. In anaerobic conditions, haxavalent Chromium can act as an electron acceptor through a membrane bound reductase activity. It was shown that in certain strain of microbe, the anaerobic reduction takes place at a quicker rate than aerobic reduction process. It was found out in a study that the reductase activity of a strain is completely destroyed in high heat like all enzymes. It was also found out that the reducing ability was not diminished by the presence of respiratory inhibitors like azide, cyanide and rotenone. (Shen & Wang, 1993)

Kinetics of reduction of hexavalent Chromium makes it possible for the design of bioreactors to be used in industrial scale application of these organisms in wastewater treatment. (Narayani & Vidya Shetty, 2014)

1.5.9 Metabolism of Chromium in body

The pathways through which Chromium enters into the body are skin, digestive tract and respiratory system. The metabolism of Chromium is significantly influenced by its route of entry. However, it is also dependent on its level of oxidation and nature of its ligands. (Ducros, 1992) The main site of absorption of Chromium is intestinal mucosa. (Ducros, 1992) Chromate can easily diffuse through cell membranes and causes chromate induced toxicity by their intracellular reduction process. Once inside the cell, Cr (VI) is partially reduced to Cr (V) which is greatly unstable. This leads to the formation of reactive oxygen species which are said to be the main causes of Cr (VI) toxicity and carcinogenesis. (Abdelnasser S. S. Ibrahim, 2012) Cr (VI) undertakes a chain of reductions forming intermediate Chromium species: Cr (V), CR (IV) and Cr (III) once they enter the cells. The formation of reactive oxygen species (ROS) in the reduction of Chromium causes oxidative damage in DNA. The intermediates formed in the reduction process are all very reactive and results in Cr-DNA adducts and genomic changes. (Clementino, Shi, & Zhang, 2018) Studies have shown that Cr (VI) can bring about enzymes inactivation, disruption of the plasma membrane and protein denaturation. All these disturb the normal metabolism in the body. (Feng et al., 2017)

Chromate (CrO_4^{2-}) which is a strong oxidizing agent is reduced intracellularly to Cr (V) which produces reactive species and free radicals that could impair DNA molecules and other biomolecules, thus generating a wide spectrum of genomic changes like DNA strand breaks, alkali-labile sites, DNA-protein, and DNA-DNA crosslinks, and Cr (III)-DNA adducts which are connected with the mutagenic effects in biological systems. (Baldiris, Acosta-Tapia, Montes, Hernández, & Vivas-Reyes, 2018) Transferrin has two protein binding sites A and B. In blood, Cr^{3+} binds to transferrin exclusively to site B. (Ducros, 1992) Studies have revealed that Cr^{3+} readily binds to the two metal-binding sites in the two lobes of apotransferrin. The Cr^{3+} binding is accompanied by strong changes in the transferrin's ultraviolet spectrum. This intense change is a result from chromic ion binding to two tyrosine residues in the two iron-binding sites of transferring. (DENG et al., 2016) At very high concentrations, Chromium can bind nonspecifically to other plasma proteins as well such as γ- and β-globulins and lipoproteins. (Ducros, 1992)

1.5.10 Carcinogenesis and mutagenesis induced by Chromium

Chromium is said to be a mutagenic and carcinogenic agent owing to its toxicity. (Narayani & Vidya Shetty, 2014) The approximated potency of hexavalent Chromium for causing cancer in humans is 0.5 $(mg/kg/day)^{-1}$. Numerous organs can be target of Chromium carcinogenicity. These include: liver, bladder, gastrointestinal tract, kidney, hematopoietic systems and even bone. (Linos et al., 2011)

Compounds of hexavalent Chromium are mutagenic as proven from numerous bacterial and mammalian mutagenesis studies through reliable data. Base substitution mutations in *Escherichia coli* are detected subsequent to treatments with K_2CrO_4. Chromate mainly yielded base substitution mutations in the *his* locus of the *Salmonella* tester strains. (Nestmann et al., 1979) Nevertheless, some frameshift mutations were also observed. Base substitution mutagenesis is more recurrent in the Ames strains that hold the pKM101 plasmid, and mutations prevailed at A-T rather than G-C sequences. (Petrilli, L., & DeFlora, 1979) In assays conducted for mammalian mutagenesis, hexavalent Chromium compounds are found to be frequently less mutagenic than in the bacterial assays. It yields mutants in most assays but not in all of them. (Majone & Levis, 1979) Mammalian mutagenesis data propose that nature of Chromium compounds and the experimental cell line that is examined as well as different genetic loci contributes to Chromium mutagenesis. It has been said that Chromium compounds do not cause mutations in all mammalian loci. However, low levels of Chromium induced mutagenesis were seen in Na^+K^+/ ATPase gene in the cells of ovary in Chinese hamster. The mutagenesis at this locus is limited to just base substitution mutations. (Cohen et al., 1993)

The IARC Working Group on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, after performing studies by analyzing all the accessible animal study data, concluded that there was adequate confirmation for the carcinogenicity of soluble calcium chromate and numerous relatively insoluble hexavalent chromium compounds in laboratory rodents. (Cohen et al., 1993)

According to the research, cancer results from chronological attainment of mutations in genes whose protein products regulate cell growth and apoptosis. (Hahn & Weinberg, 2002) Hexavalent Chromium interacts weakly with DNA and other biomolecules. However, many species produced in the process of its intracellular reduction are very reactive towards them. The

reactive species include the final product of hexavalent Chromium reduction: trivalent Chromium, the unstable pentavalent Chromium and tetravalent Chromium reduction intermediates, reductant-specific thiyl and carbon-based radicals and, perhaps, reactive oxygen species (ROS). (AM, Rodrigues CFD, & MC, 2008) All these together induce an extensive spectrum of DNA lesions like adducts, gaps, breaks, crosslinks, oxidized bases and abasic sites. Genetic defects like sister chromatid exchanges, microsatellite instability, micronuclei and chromosomal aberrations are also seen. (AM et al., 2008) In humans with occupational exposure to hexavalent Chromium has shown disrupted TP53 gene. The protein product of TP53, p53 is a transcription factor which is conventionally seen as the guardian of genome. The recognition of microsatellite instability in these cancers is in succession with the reported repression of DNA repair genes, a result that was also seen in cultured cells which were exposed to this carcinogen-hexavalent Chromium. (Abreu, Ferreira, Alpoim, & Urbano, 2014)

1.5.11 Detoxification of Chromium in body

The body has its own defense mechanism against compounds that are harmful to the body. In the case with hexavalent Chromium, its entrance into the body also follows certain detoxifying mechanisms. It was shown in a study on rats that in lungs, Cr (VI) is reduced to Cr (III) by ascorbate. (Y & K., 1990) This results in smaller time of residence of the toxic metal in the lungs and this comprises the initial defense against oxidizing reagents in lungs. It was also found out that in absence of ascorbate, glutathione can also perform the reduction, however in a much slower rate. In humans, capacities for reducing Cr (VI) were estimated to be 0.7-2.1mg/day in saliva, 8.3-12.5mg/day in gastric juice, 11-24 mg for intestinal bacteria excreted daily with feces, 3300mg/h in liver, 234mg/h and 187mg/h in whole blood for males and females respectively, 128mg/h and 93mg/h in red blood cells for males and females respectively, 0.1-1.8mg/h in epithelial lining fluid, 136mg/h in pulmonary alveolar macrophages and 260mg/h in peripheral lung parenchyma. (Das et al., 2011)

Extracellular reduction of Cr (VI) to Cr (III) in gastric fluid is a well-known detoxification method. (Petrilli et al., 1986) The competency of reduction of Cr (VI) in different body fluids and cell compartments is probable to comprise of a threshold mechanism limiting the *in vivo* activity of Chromium and a significant factor in the selection of target cells. For example,

reduction in the alimentary tract delivers a primary defense mechanism against Chromium that has been ingested. (S. De Flora et al., 1987)

1.5.12 Different methods for remediation of Chromium

One of the main environmental problems in several countries is pollution by heavy metals. Discharge from many industries contains dissolved heavy metals which exceeds the permissible sanitary standards. Heavy metals are considered to be non-biodegradable and have a tendency to accumulate in living organisms causing numerous diseases and disorders, at the same time, can cause harmful ecological effects. (Ibrahim & Mutawie, 2013)

To facilitate lowering the level of heavy metals in the environment, certain ways has been eshtablished. Chelation technology is one of the modern approaches. Other ways include membrane separation, electrotreatment and photcatalytic processes etc. (Chauhan, Pant, & Nigam, 2015)

1.4.12.1 Chelation Technology

Chelation can be defined as formation of stable and water soluble metal ligand complexes. Metal extraction with the help of chelating agents does not require elevated temperature and the chelating agent can be recycled to be used again. For this reason, this process is much more economical than other conventional process to removing metals. It is also eco-friendly as no toxic byproducts are produced and for its easy recovery of the chelating agent in use. High effectiveness of metal extraction and reasonable thermodynamic stabilities of the metal complexes is what makes this technology more approving than any other technology for metal recovery. (Chauhan et al., 2015)

An ideal chelator should possess high solubility in water, should be resistance to biotransformation, and must have ability to reach the sites of metal storage, retain chelating ability at the pH of fluids and the property of forming metal complexes which are less toxic than the free metal ion. (S. J. S. Flora & Pachauri, 2010) The international market is observing the increasing demand of chelating agents and appearance of many new chelating products. Chelation concept has been also been engaged, in current era, for metal extraction from industrial wastes. Some common chelating agents used are EDTA, EDDS and DTPA. It was concluded in

a study that extraction of metal is dependent on the complexing affinity of a chelating agent for metal under consideration and on the affinity between solid and metal. (Chauhan et al., 2015)

Some of the drawbacks of chelation technology includes: redistribution of toxic metal under consideration, essential metal loss and no removal of metal from intracellular sites. (S. J. S. Flora & Pachauri, 2010)

1.5.12.2 Membrane Separation by nanofiltration and reverse osmosis

Membrane separation has become more and more useful for treatment and recovery of heavy metals because of its high effectiveness, easy operation, and low cost. The equipment used for the procedure can be prepared with commercial spiral wound reverse osmosis membrane RO-SG-2514 and nanofiltration membrane NF-HL-2514 supplied by Osmonics. Both modules used for study are approximately 64mm in diameter and 356mm in length. (Mnif, Bejaoui, Mouelhi, & Hamrouni, 2017) Process of filtration using a membrane has become a very important consideration for the treatment of effluent water. Aquatic hydrolytic force is utilized for separation with the help of a semi permeable membrane. Membranes of various categories like aquatic membrane, inorganic membrane and polymeric membranes are being used for the clearing of hexavalent Chromium. (Pugazhenthi et al., 2005) To take apart hexavalent Chromium from the preparation of liquid, the membrane is applied. Portion examination of the caustic strategy of chromic was concluded by utilizing 96% basic membrane discharge, 84% nitrated carbon membrane discharge and 88% aminated membrane discharge. Absorption of film provender solution and adjustment of pH had occurred for the ejection of hexavalent Cr through the use of diverse polyamide films of nano-filtration compounds (Muthukrishnan & Guha, 2008). Escalation of the pH of provender solution raises the discharge rate of Chromium. Immense vivacity, poor discharge of metal, ingestion of chemical and dangerous overflow or effluent generation which needs interchange are the snags which are termed as some of the problems of this framework insulated from being economically exaggerated. High measure of vitality is used ceaselessly. It is being used reluctantly and is proving to be harmful and expensive are some of the negative aspects of this strategy which is being utilized to treat hexavalent Chromium polluted water of ground. These strategies are very expensive and occasionally the auxiliary squanders need suitable management. (Mnif et al., 2017)

1.5.12.3 Removal of hexavalent Chromium by Adsorption

The traditional method of chromium treatment by adsorption mainly consists of four steps: (Vinod K. Gupta, Rastogi, & Nayak, 2010)

1. Reduction of hexavalent chromium to trivalent chromium.
2. Precipitation of trivalent Chromium as Chromium hydroxide at high pH.
3. Allowing the insoluble metal hydroxide to settle down.
4. Disposal of the dewatered sludge.

Process like adsorption is thought to be a suitable method for reduction of hexavalent Chromium due to major limitations of conventional treatment methods such as which makes use of activated carbon which includes high cost of safely disposal, requirement of costly chemicals, and incomplete reduction of hexavalent Chromium. (Vinod K. Gupta et al., 2010) Adsorption is an efficient and multipurpose technique for elimination of heavy metals which when combined with appropriate desorption steps, solves the trouble of sludge disposal. Multiple adsorbents of low cost have been used previously for the removal of toxic pollutants from water wastes. (Bailey, Olin, Bricka, & Adrian, 1999) During current years, a variety of naturally available adsorbents such as wool, hazelnut shell, olive cake, sawdust, cactus leaves, soot, coconut shell charcoal, pine needles, banana peel, seaweed, charcoal used tyres, dead fungal biomass, almond shells, cyanobacterium, and green alga were utilized for the elimination of Chromium. (Lakatos, Brown, & Snape, 2002) Nevertheless, many of these available adsorbents which are available in nature have shown low chromium adsorption capacity and slow process kinetics. Thus, there is still a necessity to develop innovative adsorbents of lower cost which will be convenient for both industry and the environment. (Kobya, 2004)

1.5.12.4 Ion exchange method

Ion exchange method was applied for removal of heavy metal quite later as compared to other methods that were used. (Sapari, Idris, & Hamid, 1996) Even so, the method has proved to be efficient in removing Chromium from water waste. During the procedure, any targeted metal species are able to be removed by insoluble exchange with a substitute species. Upon inflowing of Chromium, it passes alongside a segment, then crosses through a resin bed and is lastly expelled. As the amount of the resins start to decrease, all the accumulated solids are emptied.

Resins like synthetic Dowex 2-X4 and Amberlite IR-120 are used to filter hexavalent Chromium from water waste. (Sapari et al., 1996)

Although this procedure has proven useful, it comes with specific limitations and difficulties. The exchange of fluid is very specific for a heavy metal ion that has to be separated. The preferred resin must be able to remove the heavy metal in consideration completely. The ion exchange machine is very expensive and furthermore, the system can be hampered by other organic sludge and different solid residues in the contaminated water sample. However, an ion exchange can treat and purify contaminated water sample to a very high standard despite the disadvantages that come along with the procedure. (Sapari et al., 1996)

1.5.12.5 Electrochemical precipitation

Electrochemical hexavalent Chromium reduction techniques can be utilized through numerous approaches. The approach depends on pH of the aqueous solution, the strength of the current density, and the electrode material that is being used. In this sense many instantaneous processes could take place on the surface of the electrode or in the aqueous solution. (Barrera-Díaz, Lugo-Lugo, & Bilyeu, 2012)

The method makes use of electric potential to help heavy metal removal from contaminated water over traditional methods (Kurniawana, Chana, Loa, & Babelb, 2006). This process has been seen to be functional in treating either of both industrial water and portable water. By utilizing the similar principle of an electrotype cell, the actions of a cathode, anode and a DC powered house were applied to the contaminated sample electrolyte solution (Kongsricharoern and Polprasert, 1996). The hexavalent Chromium concentration can be reduced from 3.860 mg\L to 0.2 mg\L with this method. The Method has been established to be effective and versatile as numerous removals of metals including ferric chlorids, ferric hydroxide, arsenic and phosphate can be done by electrochemical precipitation method. (Pinisakul, Polprasert et al, 2002).

1.5.13 Bacterial Resistance to heavy metals

Survival of microorganisms in polluted soils depends on a number of factors like: (Abou-Shanab, van Berkum, & Angle, 2007)

1. Intrinsic biochemical and structural properties,
2. Physiological, and/or genetic adaptation which includes morphological and other changes of cells
3. Environmental modifications of metal speciation

These microorganisms apply numerous kinds of resistance mechanisms in response to heavy metals. These mechanisms may be encoded by chromosomal genes. However, more typically loci conferring resistance are positioned on plasmids. (Abou-Shanab et al., 2007)

The occurrence of high concentrations of chromate in the environment slows down the growth of most microorganisms. (Cervantes et al., 2001) Chromate promotes the selection of resistant variants as well. (Yang et al., 2007) Bacterial resistance to chromate may be the cause of chromosomal mutations, which usually affects the sulfate transport or plasmid borne. It seems to be that chromosomal and plasmid determinants work by different mechanisms, such as resistances to chromate are additive in cells that possess both determinants. Plasmid-determined bacterial resistance to chromate has been seen in *Streptococcus, Pseudomonas* and *Alcaligenes* genes. Chromate resistance results from declined chromate accumulation by the resistant cells in consideration. Even though chromate ions enter the cells by the pathway of sulfate transport, the kinetic parameters of uptake of sulfate are unaffected by the presence of chromate resistance plasmids. (Cervantes et al., 2001)

It has been discovered that the process by which bacteria deals with chromate are diverse, such as: (Yang et al., 2007)

1. Retreating intracellular accumulation through either creating direct barrier for the ion uptake system or active chromate efflux,
2. Biosorption, and
3. Reduction of toxic hexavalent Chromium to less-toxic trivalent Chromium by using chromate as the terminal electron acceptor in their respiratory chains or by making use of enzymes to catalyze the reduction of chromate.

During the last decade, most of the mechanisms were projected on the basis of the macroscopic experimental results of microbiology or molecular biology. (Yang et al., 2007)

Investigations resulted in the finding of a gene for a hydrophobic polypeptide which was designated as ChrA. The composition of amino acid of ChrA has similarities with those of other transport related polypeptides. As a result, even though ChrA seems to be a membrane transport protein, its role in transport and the mechanism of energy coupling is still unidentified. ChrA may be responsible for the decreaseded uptake of chromate ions by directly reducing initial uptake. However, more probable explanation would be that there is a chromate efflux process for which laboratory conditions has not yet been found. (Aguilar-Barajas, Paluscio, Cervantes, & Rensing, 2008)

Chromate resistance mechanisms involve efflux that has been characterized from a number of different microorganisms. The efflux protein, ChrA is encoded on plasmids in bacteria like *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* and transports Cr^{+6} to exterior of the cell membrane using a force known as the proton motive force. (Alvarez et al., 1999) Other microorganisms react to exposure to Chromium by stimulating the expression of genes that fight against oxidative stress as a defense mechanism. For example, *Escherichia coli* increase production of ROS (reactive oxygen species), detoxification enzymes such as superoxide dismutase (SOD) and catalase. (Ackerley, Gonzalez, Keyhan, Blake, & Matin, 2004) On the other hand, *Shewanella oneidensis* MR-1 generates elevated concentrations of thioredoxins and glutaredoxins. (Chourey et al., 2006) DNA repair systems are also generated in response to aerobic chromate exposure which includes components of the DNA SOS repair system and the Rec system. (Thorgersen et al., 2017)

1.5.14 Mechanism of Chromium reduction in microorganisms

Resistance and reduction of hexavalent Chromium: Cr(VI) have been shown in a number of aerobic, facultative, and anaerobic bacterial strains, like *Vibrio fischeri, Aspergillus niger, Shewanella alga, Paracoccus denitrificans, Pseudomonas sp., Bacillus subtilis, Bacillus sp., and Shewanella oneidensis. (Long et al., 2013)*

Frequently, reduced uptake or expulsion of Cr (VI) compounds through the transmembrane sulphate shuttle in membranes, biosorption, and the upregulation of genes connected with oxidative stress response is regarded as different modes of resistance in bacterial cells to surmount the Cr (VI) stress. In most scenarios, metal resistance mechanisms have been

recognized to plasmid genes and are supposed to have evolved by horizontal gene transfer in response to the selective pressure in the infected environment. (Ahemad, 2014) In fact, Cr^{6+} resistance is independent from Cr^{6+} reduction which was originally suggested as a mechanism for bacterial Cr^{6+} resistance by decreasing the conventration of intracellular hexavalent Chromium. Evidently, the degree of Cr^{6+}-reducing activity was found comparable in both sensitive (wild-type) and resistant (mutant) strains of *Pseudomonas fluorescens* LB300 under sublethal concentrations of Cr^{6+}. (Ahemad, 2014)

Microbial reduction of Cr^{6+} to Cr^{3+}may be considered to be a chromate detoxification mechanism and is generally not plasmid-associated. Two direct hexavalent Chromium reduction mechanisms are described below: (Viti, Marchi, Decorosi, & Giovannetti, 2014)

1. Cr (VI) is reduced in the presence of oxygen, i.e. under aerobic conditions normally associated with soluble chromate reductases that make use of NADH or NADPH as cofactors.
2. Cr (VI) can be utilized as an electron acceptor in the electron transport chain without the presence of oxygen i.e. under anaerobic conditions by some bacteria.
3. Cr (VI) can also be reduced indirectly by nonspecific reactions that are linked with redox intermediate organic compounds like amino acids, nucleotides, sugars, vitamins, organic acids or glutathione.

The finest studied chromate reductase among many is ChrR from *P. putida*, a soluble flavin mononucleotide-binding enzyme which is able to catalyze the reduction of Cr (VI) to Cr (III). The ChrR enzyme works as a NADH-dependent reductase which have a broad substrate specificity and permits the NAD(P)H dependent reduction of quinones, prodrugs, Cr(VI), and U(VI) ions. ChrR catalyzes a combination of one- and two-electron transfers to Cr(VI) with the formation of the unstable species Cr(V) before further reduction to Cr(III) which has been summarized in the figure below (Figure: 1.1). Even though a percentage of the Cr (V) intermediate is instinctively reoxidized to generate ROS, its reduction to Cr (III) through two-electron transfer decreases the production of damaging radicals. Studies with purified *P. putida,* ChrR exposed that this enzyme has a quinone reductase activity during chromate reduction. Quinols produced by quinone reduction present tolerance to ROS. As a result, ROS generated by ChrR activity during Cr^{6+} reduction should be neutralized by quinols created by the quinone

reductase activity of the same enzyme. Therefore, although ChrR activity produces ROS during Cr^{6+} reduction, it reduces quinones that provide protection against ROS. (Viti et al., 2014)

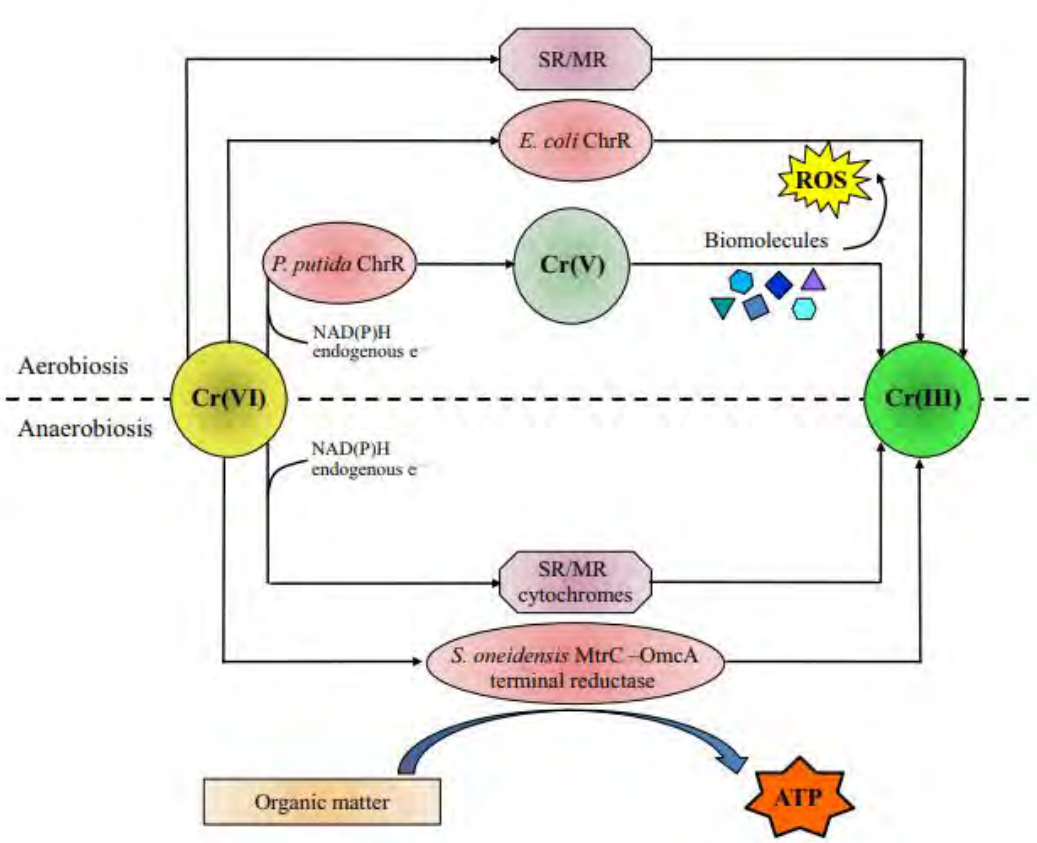

Figure 1.1: Mechanisms of reduction of hexavalent Chromium enzymatically. ChrR of *Pseudomonas putida* catalyzes a mixture of one- and two-electron transfers to Cr (VI) with the momentary production of Cr (V). Cr (V), in the company of more than a few biomolecules is reduced to Cr (III). This process produces ROS. ROS is eliminated by a system of scavenging of ROS. ChrR of *Escherichia coli* intervene a four electron shuttle for the direct reduction of Cr (VI) to Cr (III) using the remaining electron transferred to oxygen. MtrC and OmcA are considered to be the terminal reductases of Cr (VI) in *Shewanella oneidensis* MR-1. Furthermore, both in aerobiosis and in anaerobiosis, a multitude of soluble reductase (SR) or membrane-bound reductase (MR) enzymes can reduce Cr (VI) using NADH or endogenous electron reserves as electron donors. (Image adopted from Viti et al., 2014)

1.5.15 Microbial Reduction of hexavalent Chromium

The conventional treatment technologies used for removal of Cr (VI) from heavily polluted water bodies are frequently accomplished by physical and chemical procedures which include precipitation, reduction, membrane processes, ion exchange, and adsorption etc. These processes can sometimes prove to be inefficient or highly expensive especially when the metals in solution are in the low concentration range of 10-100 g/m^3 but still above the tolerable range by humans. Keeping the disadvantages of these traditional methods in mind, there has always been a search for alternative methods. Bioremediation of Cr (VI) has gained popularity since the last thirty years after the isolation of the first hexavalent Chromium reducing strain in the late 1970s which could reduce more toxic form of chromium (Cr(VI)) to less toxic form (Cr(III)). (Narayani & Vidya Shetty, 2014)

Nevertheless, microbial reduction of toxic hexavalent Chromium to non-toxic trivalent Chromium by chromium resistant bacteria (CRB) is the most realistic approach that proposes an economical as well as eco-friendly alternative for chromate detoxification and bioremediation. Microbes are equipped with various resistance mechanisms to deal with chromate toxicity that allow them to survive in harsh environmental circumstances. These detoxification methods include biosorption, bioaccumulation and biotransformation by enzymatic reduction, reduced intracellular accumulation through either creating direct obstacle in ion uptake system or active chromate efflux, precipitation, and reduction of Cr (VI) to less toxic and less mobile Cr(III). (Bharagava & Mishra, 2018)

The swift industrialization and growth in industrial processes led to the occurrence of heavy metals in industrial effluents and water bodies surrounding the industries. These components are extremely poisonous and are being released in massive quantities by numerous industries. Among these toxic constituents, chromium compounds are present in large amounts. (Mnif et al., 2017) Therefore, it has become imperative that nature has very low or no amount of the toxic Chromium present in it. Conservative techniques for removing toxic hexavalent Chromium include chemical reduction following precipitation, ion exchange, and adsorption on activated

coal, alum, kaolinite, and ash. Nonetheless, most of these processes require high amount of energy or huge quantities of chemical reagents. Microbial reduction of deadly hexavalent chromium has practical importance, as, biological strategies present green technology that is cost effective. (Camargo, Bento, Okeke, & Frankenberger, 1997) So, even though there are numerous ways to removing toxic hexavalent Chromium, bioremediation by making use of microorganisms is very popular for certain reasons. Currently, bioremediation is considered to be a cost-effective remediation technology. It is potentially suitable for vast polluted areas, such as contaminated water and soil. Its potential safety and efficiency will surely accelerate further development of the technology. (Y. Cheng, Holman, & Lin, 2012) Dissimilatory metal reducing bacteria are excellent bioremediation candidates for their ability to reduce iron, sulfate, chromate, or uranyl ions as a form of anaerobic respiration. (Jin et al., 2012) Microorganisms are competent enough to tolerate and reduce hexavalent chromium in order to detoxify the contaminated environment. (Frankenberger et al, 2003) Reduction changes the valence and may as a result change the toxicity and environmental mobility of a metal. For that reason, conversion of Cr^{+6} to Cr^{+3} by microbial reduction could be a valuable method of combating Cr (VI) pollution. (Polti et al., 2010) Bioremediation processes show promising outcomes for the elimination of metals, even when these metals under consideration are present in very low concentrations where physicochemical removal methods falls short to function. (Focardi et al., 2015) In recent times, microbially mediated reduction of Cr (VI), in other words use of bioremediation represents a probable development not only for detoxification or neutralization, but also as an essential step towards the definitive removal of chromium from contaminated aqueous environment. (Pattanapipitpaisal & Reakyai, 2013)

1.5.15.1 Biomineralization

Biomineralization is a method by which living organisms impact the precipitation of metals and generate inorganic minerals. It has a significant connection between the lithosphere and the biosphere of the environment. The procedure can take place through either of the two methods: biologically controlled mineralization (BCM) or biologically induced mineralization (BIM). (Y. Cheng et al., 2012) There are certain points of difference between BCM and BIM. In BCM, the microorganism under consideration intentionally synthesizes minerals that meet up specialized functions. On the other hand, in BIM, the microorganism under condition unintentionally

predicates the metal ions in consequence of its metabolism or as a form of self protection against specific adverse environmental conditions. (Y. Cheng et al., 2012) Usually, the mineral produced through BIM is an inorganic precipitate. This means of biomineralization is shown to be common in prokaryotes and fungi. The reduction and precipitation of the metals chromium and uranium induced by microorganisms is such an example. (Y. Cheng et al., 2012)

1.5.15.2 Biosorption & Bioaccumulation

Bioremediation can be classified into biosorption and bioaccumulation where microorganism such as bacteria, fungus, yeast and mold have been most extensively used. The microorganisms can detoxify or neutralize metals through physic-chemical retention between the metal and functional groups that are located on the exterior of the cell, based on several factors like pH ionic strain, biomass concentration and temperature. (Luciene et al, 2015). A principal example of natural microorganisms is *lactobacillus* which has the ability to bind with metals including Cr (VI) to detoxify them in different partss of the human body (Monachese, Burton, &Reid, 2012). Only living biomass can contribute to bioaccumulation. On the other hand, both living and dead biomass can participate in biosorption (Luciene et al, 2015). The bioaccumulation of Chromium depends upon size of microorganism. With succeeding increase in size and dimension, the concentration of Chromium present in soft tissues is reduced considerably. (Aslam & Yousafzai, 2017)

1.5.15.3 Phycoremediation

Phycoremediation is a type of bioremediation technology which makes use of photosynthetic microorganisms such as microalgae, macroalgae and cyanobacteria for the elimination of pollutants as metals. In addition, it is required to understand the allocation of the metal adsorbed onto the surface of the microbe in relation to the metal which has been accumulated inside the cell, so that we can understand the mechanisms of predominant removal and can make decisions of the viability of the recovery of the adsorbed metals. (Focardi et al., 2015) The knowledge of using microalgae for generating low cost wastewater treatment and also bioenergy feedstock source is a well-recognized fact. Utilization of wastewater as a low cost nutrient source to alga is said to reduce 90% water demand and makes it a lot less expensive for biomass production. (Pathak, Kothari, Chopra, & Singh, 2015)

Noteworthy work on algal wastewater treatment has been done and some effort has been made towards establishment of anaerobic digestion of algal biomass. However, very less or no report exists on integration of phycoremediation with the algal biogas production.(Prajapati, Kaushik, Malik, & Vijay, 2013)

1.5.15.4 Bio-augmentation-assisted phyto-extraction

The competence of bioremediation can be amplified with the assistance of bio-augmentation and in combination with phyto-extraction. (Anna et al, 2016) To work with plants, it enables certain bacteria and/or fungus to gather or accumulate metal and eventually break them down. The end goal of heavy metal reduction to be executed by plants with the help of microorganism such as bacteria is highly recommended (Lebeau, Braud & Jezequel, 2008).

2 MATERIALS AND METHODS

2.1 Introduction

This section will portray the experiments that were carried out and the equipments and reagents used to for the experiments. The section will provide detailed explanation starting from collection of the sample, isolation of bacteria to different tests that were carried out and also the identification of the sample on which the paper is designed on. Both water and soil samples were collected from a number of locations in the Dhaka city: Showari Ghat, Pargandaria and Faridabadh. Several studies like antimicrobial resistance profiling, minimum inhibitory concentration (MIC) and DPCZ based Chromium reduction bioassay of the isolated bacteria from collected sample were carried out those of which are discussed in this section.

2.2 Chemicals

The reagents used were made sure to be of analytical grade and untainted. Below is the list of chemicals and reagents that were used for the entire study:

1. Nutrient Agar (NA)
2. Agar powder
3. Muller-Hinton Agar (MHA)
4. Nutrient Broth
5. Potassium Chromate
6. Diphenyl Carbazide (DPCZ)
7. MOPS Buffer
8. Sulfuric acid

2.3. Glassware and instruments

Throughout the entire time of study, the following tabulated instruments and glassware was used:

Table 2.1: Instruments used along with their function

Name of instrument and glassware	Function	Origin	Model
Autoclave machine	Sterilization	Vision Scientific, Korea	VS-1221
Incubator	Incubation of solid culture mediums (agar plates)	Thermo Scientific, Germany	Heratherm IGS60
Digital Shaking incubator	Incubation of liquid culture mediums (broth)	OVAN, Spain	I10-OE+OL30-ME
Electric balance	Measurement of weight	CSC Balance, Japan	JT2003D
pH meter	Measurement of pH	Mettler Toledo, Switzerland	S220
Laminar Air Flow	Maintenance of aseptic environment	Labtech, Korea	LCB-1101VE
Water Distillation Apparatus	Preparation of culture stock	BOECO,Germany	BOE-8704000
UV-Vis Spectrophptometer	Measurement of absorbance pertaining to the bacterial growth and reduction of hexavalent Chromium	Shimadzu, Japan	UV- 1800
Electronic Centrifuge	Collection of supernatant	China	80-2
Micropipette	For withdrawing reagent and media in minute amount	Eppendorf	N32164B, M34137B
SIEMENS Up-ride Freezer	For storing bacterial culture stock	SIEMENS, Germany	GS36NVW30G
Vortex Mixer	For proper mixing	Hwashintech, Korea	260VM

	during serial dilution in MIC		

2.4 Collection of sample

The Buriganga River with a magnitude of 17 km which is flowing by the border of Dhaka city- the capital of Bangladesh is considered to be one of the most contaminated rivers in Bangladesh. Numerous industries have been established around the city and the number is continuously increasing. Therefore, the quantity of untreated waste water that is being discharged into the Buriganga River has risen with time. (Kawser Ahmed et al., 2016) This led to absolute deterioration of the quality of water of Buriganga River. The main problem is the dumping of enormous volume of toxic waste into the river on a continuous basis. This amount increases with increase in population each year. The contaminants include heavy metals, glass, textiles, polythene materials, cardboard etc. (M. K. Ahmad, Islam, Rahman, Haque, & Islam, 2010) The elevated usage of heavy metals in industries has led to the elevated release of dangerous heavy metals into the river water. Metals and metalloids enter into the aquatic environment where they impose a grave threat to humans and ecological health, due to their toxicity, extensive persistence, bioaccumulation and biomagnifications in the food chain. (Kawser Ahmed et al., 2016) In order to conduct experiment, we collected our samples from Buriganga River from three different locations: Showari ghat, Pargandaria and Faridabadh, GPS coordinates of those areas are listed in the table 1.2 below. Water samples were collected from all three of the location and soil sample was collected from Showari ghat and Pargandaria. Isolate F2 was collected from Showari ghat with coordinates of 23°42’29.32” N 90°23,31.65” E.

Table 2.2: GPS coordinates of the sample collection area

	Area	GPS Coordinates	Sample collected
1.	Showari Ghat	23°42’29.32”N 90°23,31.65” E	soil & water
2.	Faridabad	23°41’44.19”N 90°25’17.63”E	water
3.	Par Gandaria	23°41’48.01”N	soil & water

		90°25’11.67”E	

Figure 2.1: Showari Ghat area

2.5 Isolation and subculture of sample

Microorganisms from the collected sample were isolated by following standard protocol. The microorganisms that were chromium resistant were isolated by inoculation of sample in agar medium. The collected water sample was first filtered with filter paper to remove the sand debris and 15 µL of water was added to culture medium. For soil sample, normal saline water (0.9% NaCl) was prepared and a small spoonful of from collected soil was added to it. The test tube containing the soil sample and saline was vortexed for even distribution. It was then filtered as was done for water sample and from there, 15 µL was added to culture medium containing agar medium. The agar plates contained potassium chromate (K_2CrO_4) supplementation complementary to 2mM of Cr (VI) so that only those microorganisms that are chromium resistant could survive. The cultures were incubated for 24 hours at 37˚C to allow the microorganisms to grow.

After the first 24 hours, Nutrient agar medium was prepared by adding 2.8 gm of Nutrient agar in 100 ml of water. It was then autoclaved for 45 minutes at 121˚C, under 15 Lb. pressure for sterilization. This was followed by incorporation of potassium chromate into the media and transfer into the petri dishes for settlement. Isolated bacterial colony was then picked up with sterilized toothpick and streaked on agar medium comprising of potassium chromate containing hexavalent Chromium. Once more, the nutrient medium was incubated for 24 hours at 37˚C. Specific strains that survived at this condition were chosen for further investigation. 14 single colonies were isolated from various nutrient agar plates on the basis of the morphological features of the colonies containing Chromium resistance and fixation property after performing respective procedures. Different name tags were given to the isolated bacteria at this point like A, B, C etc.

2.6 Stock Culture Preparation

50% glycerol was produced by adding 50 mL of water in 50mL of glycerin. The solution mixture was then autoclaved for forty-five minutes at 121˚C, under fifteen Lb. pressure for sterilization. This ensured no place for cross contamination. The eppendorff tubes in which the stock was to be stored were also autoclaved to ensure no presence of microbes in them.

Nutrient Broth was made by adding 2.8gm of nutrient broth to 100mL distilled water and autoclaving it. The bacteria to be stocked was then scoped from the subculture with a loop and inoculated in the broth. This was then incubated at 37˚C overnight in a shaking incubator.

After keeping the culture overnight, 500 µL of glycerin was added to autoclaved eppendorff tube followed by 500 µL of nutrient broth containing the bacterial culture. The lid was closed and the tube was shaken for even distribution. The tubes were then stored at -4˚C for 4 hours and then transferred to -70˚C.

2.7 Chromium reduction profile of chromium resistant bacteria

To identify the amount of Cr (VI) in the effluent water, Diphenyl Carbazide was used (Greenberg, et al., 1992) and the tasks were recorded. Additionally, to compare the performance of microorganisms, a standard curve was established.

2.7.1 Preparation of chemicals

2.7.1.1 Preparation of 10mL 3M H_2SO_4

For the preparation of 10mL 3M H_2SO_4, an autoclaved falcon tube was taken and 8mL of distilled water was added to it. 1670µL of concentrated sulfuric was added drop wise to the 8mL of water present in the falcon tube. The transfer was done by making use of a micropipette. After that, the solution was made 10mL by adding 330µL of distilled water to the solution already in the falcon tube.

2.7.1.2 Preparation of Diphenyl Carbazide

For the preparation of Diphenyl Carbazide, 0.025gm of DPCZ was taken in an autoclaved screw cap test tube. Then, 9.67mL of acetone is added to it followed by 330µL of 3M sulfuric acid. The test tube was shaken to give a uniform solution of DPCZ. Since DPCZ is light sensitive, it was wrapped in aluminum foil to protect it from exposure to light.

2.7.1.3 Preparation of MOPS buffer

Before preparing MOPS buffer, 1N sodium hydroxide has to be prepared for adjusting the pH of MOPS buffer. For that, 0.1gm of sodium hydroxide was added to 50mL distilled water to make 50mL 1N sodium hydroxide. Next, in order to prepare 20mM of MOPS buffer, 334.88mg of MOPS powder was taken in a clean container and 80mL of distilled water added. pH of the MOPS buffer was adjusted to 7 by adding 1N sodium hydroxide to the solution of MOPS buffer and measuring its pH with a pH meter.

2.7.1.4 Preparation of 10mL 5mM Potassium Chromate

To 10mL distilled water, 1.94gm of potassium chromate was added and mixed. If lumps were seen it was mixed using a vortex. Once no lumps were visible, the solution was filtered using membrane sieve of 0.45micron pore size. Once all these were done, it was diluted up to 5mM by addition of distilled water and stored for use later.

2.7.2 Experiment Procedure

2.7.2.1 Preparation of Standard Curve

2.7.2.1.1 Sample Preparation for reaction

Several solutions of sample of subsequent concentrations was prepared. Absolute volume of every sample was one milliliter.

Table 2.3: Sample preparation for standard curve

Final Concentration (µM)	Quantity of 5mM K2CrO4 solution (µL)	Amount of NB added (µL)	Final volume to solution (mL)
50	10	990	1
100	20	980	1
150	30	970	1
200	40	960	1
300	60	940	1
400	80	920	1
500	100	900	1
600	120	880	1

2.7.2.1.2 Reaction protocol for standard curve

Firstly, 600 µL of sample was added into a falcon tube. Then into the sample, 1.2ml, 20mM buffer of MOPS, 99 µL of 3M sulfuric acid, 981 µL purified water and lastly 120µL of diphenyl carbazide were taken progressively and thus a uniform blend was made. (Jain, Amatullah, Rajib, & Reza, 2012) The falcon tube was shaken in order for the reaction to occur. It was observed that the solution changed its color to a shade of purple. Finally, the absorbance of the solution was measured through UV-Visible spectrophotometer at the wavelength of 540nm.

2.7.2.2 Evaluation of reduction profile of selected isolates

2.7.2.2.1 Procedure

Day 0:

Firstly, six conical flasks were taken of which two conical flasks were used to prepare 10mL nutrient broth and the rest four to prepare 25mL nutrient broths. To make 10mL nutrient broth, 0.38gm of nutrient broth was added to 10mL of water and to make 25mL nutrient broth, 0.7gm of nutrient broth was added to 25ml water. Two of the four 25mL nutrient broth containing conical flasks were taken to adjust the pH: one 5.5 and the other 8.5. pH was adjusted using 1N NaOH and 1N HCl. All the glassware were autoclaved. After autoclaving, the desired bacterium from the previously prepared stock was added to one of 10mL nutrient broth flask. The nutrient broth was allowed to cool down first before adding the stock culture. Then the conical flask containing sample was then kept inside the shaking incubator to culture bacteria at 37 °C for 24 hours at 60rpm speed. All the conical flasks were labeled properly. Lastly, all five conical flasks were them kept inside the laminar air flow to be used for conducting bioassay the next day.

MOPS, DPCZ and 3M H_2SO_4 were also prepared according to protocol described in 2.7.1.

Day 1:

To prepare 600µL potassium chromate (K_2CrO_4), 15µL K_2CrO_4 was pipetted all conical flasks containing 25mL of nutrient broth. At first, 2mL of sample was withdrawn into a falcon tube from the 10mL conical flask which was kept for the incubation of bacteria the previous day. Absorbance of the 2mL sample was taken in UV-spectrometer at wavelength of 600nm. From the absorbance value, the amount of culture that has to be added to 25mL conical flasks containing nutrient broth to get 0.2 OD (optical density) were calculated. Then the calculated amount was withdrawn from the 10ml culture into three falcon tubes and centrifuged for five minutes. This caused the bacteria to get settled at the bottom of the falcon tube leaving the supernatant at the top. The supernatant was withdrawn leaving the bacterial cells in the falcon

tube. Again the same calculated amount of nutrient broth was withdrawn from one 25 ml nutrient solution and mixed with the cell in the falcon tube and vortexed to mix them properly. Then they are transferred into the three 25ml conical flask marked ph 5.5, 7 and 8.5 from the falcon tube. The other 25ml was counted as the blank. 2ml solution from each 25ml conical flasks was withdrawn in four falcon tubes and then the conical flasks were put into the shaking incubator to culture microorganisms in the hexavalent Chromium condition. Then the absorbance of the bacteria containing sample is taken at 600 nm in UV spectrometer. This procedure is repeated throughout the day after every 1.5 hours for both the sample and the blank to see the bacterial growth for the positive control (sample) and negative control (without sample). Then it was centrifuged to collect the supernatant with which the reaction stated in 2.7.2.1.2 previously was performed to check the chromium level. 7 such readings were taken in every 90 minutes interval. This process was continued to next day by keeping the 25ml solutions in shaking incubator overnight to see overnight Chromium reduction pattern with the bacterial growth in the culture. The whole procedure was repeated at three different temperatures: 25°C, 37°C and 42°C under three different pH conditions of 5.5, 7 and 8.5

2.8 Antibiotic resistance profiling of Chromium resistant bacteria

2.8.1 Strain culture preparation in nutrient broth (NB)

20mL nutrient broth was prepared in a conical flask and the bacterium of concern was inoculated in it. The conical flask was kept overnight in a shaking incubator at 37ºC at 60 rpm speed in order for the bacteria to grow. After it was kept overnight, 2mL form the broth is taken in an autoclaved falcon tube and absorbance was measured. The culture was allowed to grow more or nutrient broth added to the culture to make it 0.5 McFarland suspensions. (Driscoll, Bhat, Karron, Brien, & Murdoch, 2018) This ensures the amount of bacteria in each disc that were to be swapped by the suspension.

2.8.2 Inoculation of test plates

Muller Hinton Agar (MHA) was used for the preparation of test plates or petri dishes. One cotton swab which was autoclaved beforehand was taken and dipped in the incubated strains of 0.5 McFarland suspension of culture. Inoculation was done on dry exterior of MHA plates by Lawn culture method. (Dubourg, Elsawi, & Raoult, 2015)

2.8.3 Application of antibiotic discs

A total of 14 antibiotic discs were applied on the inoculated plates; each plate containing 4 to 5 discs. The antibiotic discs that were placed over the plate to see the antibiotic resistance profile are shown in the Table 2.4 below:

Table 2.4: Antibiotics used in antibiotic resistance profiling

Serial number	Antibiotics	Symbol	Strength
1.	Penicillin	P:10	10mg/ml
2.	Kanamycin	K:30	30 mg/ml
3.	Neomycin	N:30	30mg/ml
4.	Vancomycin	VA:30	30mg/ml
5.	Gentamycin	CN:10	10mg/ml
6.	Cefixime	CFM:5	5mg/ml
7.	Chloramphenicol	C:30	30mg/ml
8.	Ceftriaxone	CRO:30	30mg/ml
9.	Trimethoprim	STX:25	25mg/ml
10.	Cipropfloxacin	CIP:5	5mg/ml
11.	Streptomycin	S:10	10mg/ml
12.	Ofloxacin	OF:5	5mg/ml
13.	Amoxycillin	AML:10	10mg/ml
14.	Cefuroxime Sodium	CXM:30	30mg/ml

2.8.4 Incubation

The test plates were kept in incubator for 24 hours at 37ºC within 15 minutes from the beginning of the work.

2.9 Minimum Inhibitory Concentration (MIC) determination

Minimum inhibitory concentration or MIC is the lowest concentration at which visible bacterial growth is inhibited. (Papich, 2013) The test was carried out by incubating the bacteria in agar medium with increasing concentration of potassium chromate (2mM to 30mM) after serial dilution of 10times with saline. 50µL of the diluted bacterial culture in saline was spread through an autoclaved spreader onto the agar medium in petri dishes. The petri dishes were kept at 37ºC for a period of 48 hours. Then, finally colonies were counted in order to determine the minimum inhibitory concentration. The concentration in which there was no visible bacterial growth was termed as the minimum inhibitory concentration of the bacteria in concern.

2.10 16S rDNA sequencing of sample F2

16rDNA sequencing was performed to identify the bacterial strain that was isolated. The obtained sequence data was purified and saved as FASTA file. FinchTV was used to read chromatogram files. The file containing the sequence was simply draged and dropped in the interface. (Geospiza.com, 2015) BLAST (Basic Local Alignment Search Tool) (Blast.ncbi.nlm.nih.gov, 2015) was performed of the purified sequence. Similar strains were identified and downloaded on account of similarity. The sequences were compiled in a single file in FASTA format and checked for any deletion using the software BioEdit. So, basically BioEdit is used for editing the sequences before construction of the phylogenetic tree. (Hall, 1999) Then, phylogenetic tree was constructed to study the evolution and origin of the bacteria under consideration by using MEGA7. (Kumar, Stecher, 2015)

3 RESULTS AND DISCUSSION

3.1 Isolation data of Chromium resistant bacteria:

Isolation was carried out for the individual colonies from the nutrient agar plates with Chromium. The isolate was labeled as F for our convenience.

3.2 Chromium reduction profile of Chromium resistant bacteria

3.2.1 Standard Curve

The standard curve was acquired by the method stated in 2.7.2.1.1. Microsoft Excel Software was used to create the graph where values of absorbance were plotted against concentration of Chromium in micro mole. The results obtained are tabulated in Table 3.1 below:

Table 3.1: concentration of Chromium with corresponding absorbance

Concentration µM	**Absorbance @ 540nm**
50	0.294
100	0.624
150	0.907
200	1.214
300	1.675

400	2.117
500	2.587
600	2.875

From the tabulated data above, the standard curve was obtained which is shown below:

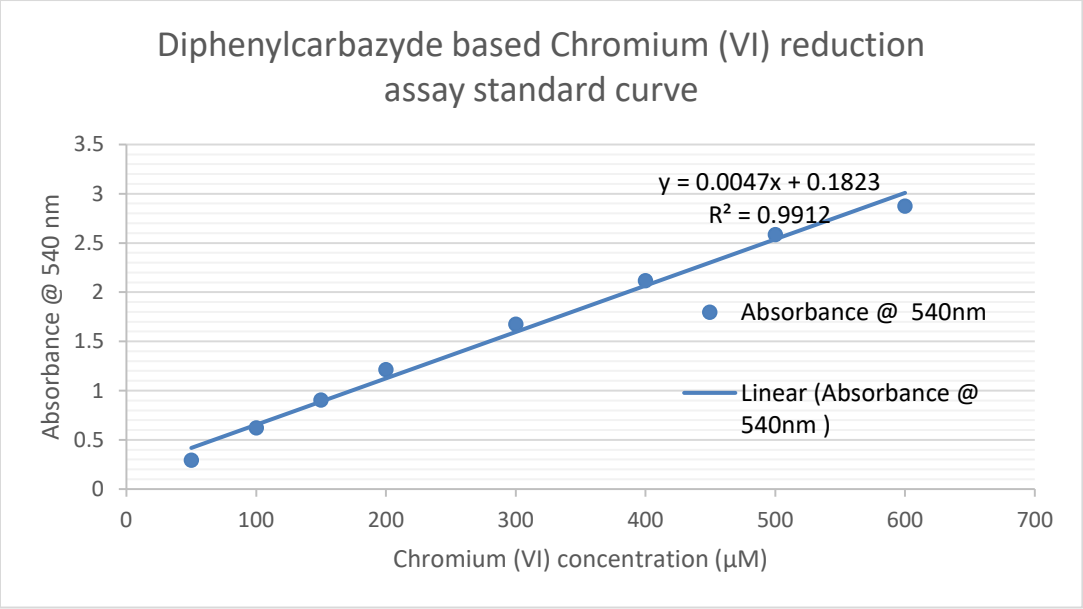

Figure 3.1: Standard curve of hexavalent Chromium with R^2 of 0.9912 showing high confidence level.

3.2.2 Reduction profile of isolate F2

Bioassay was carried out at three different temperatures, and at each temperature, three different pH conditions were provided. The results of the experiment along with necessary graphs and tables are listed below in the subsections below.

3.2.2.1 Reduction profile at 25°C, pH 5.5

Table 3.2: Isolate F2: Chromium reduction outline vs. cell growth at 25°C, pH 5.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control
0	567.0283688	0.614	580.4326241	0.079
1.5	455.3262411	0.782	522.3475177	0.071
3	356.9574468	0.942	555.4680851	0.074
4.5	245.2553191	1.584	544.4751773	0.068
6	217.7375887	1.746	562.9858156	0
7.5	231.7092199	1.812	581.7092199	0
24	243.7092199	1.974	530.2907801	0

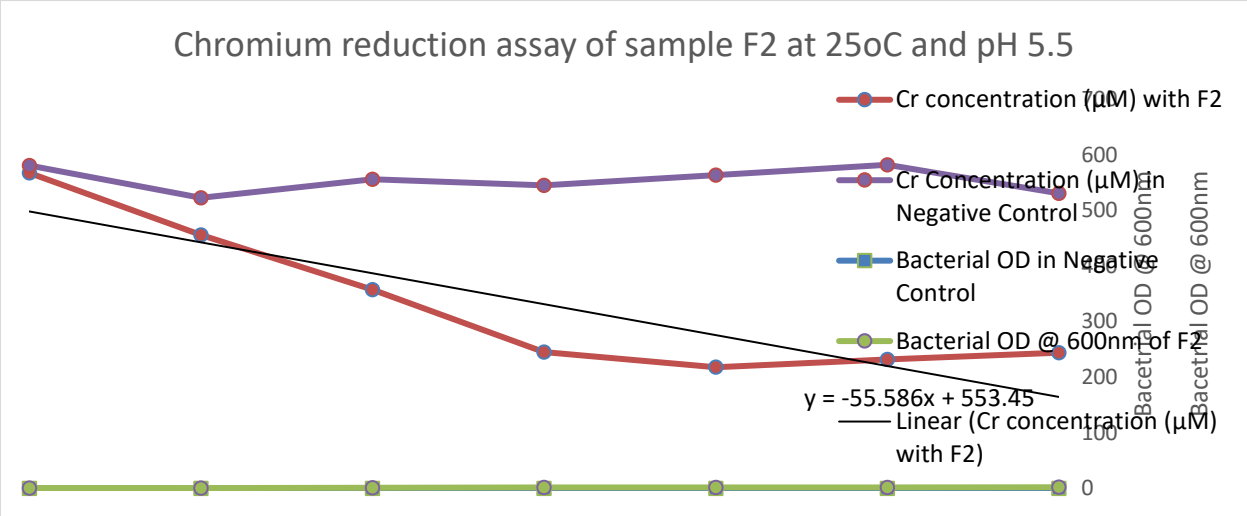

Figure 3.2: Bioremediation assay curve of sample F2 at 25ºC, pH 5.5

From Figure 3.2, it can be observed that reduction of Chromium was almost linear till 6 hours, concentration reduced from 567µm to 217 µm. Then after that Chromium concentration showed

slight increase. Total of 57% Chromium was seen to be reduced after 24 hours. On the other hand, bacterial OD increased from 0.614 to 1.812 in 7.5 hours and after 24 hours, OD was seen to be 1.974. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.2 Reduction profile at 25°C, pH 7

Table 3.3: Isolate F2: Chromium reduction outline vs. cell growth at 25°C, pH 7

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (μM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (μM) of Negative Control	Bacterial OD in Negative Control
0	575.1134752	0.677	580.4326241	0.079
1.5	508.8723404	0.954	522.3475177	0.071
3	408.6595745	1.417	555.4680851	0.074
4.5	368.1631206	1.695	544.4751773	0.068
6	304.6170213	2.12	562.9858156	0.079
7.5	291.2836879	2.161	581.7092199	0
24	275.4680851	2.234	530.2907801	0

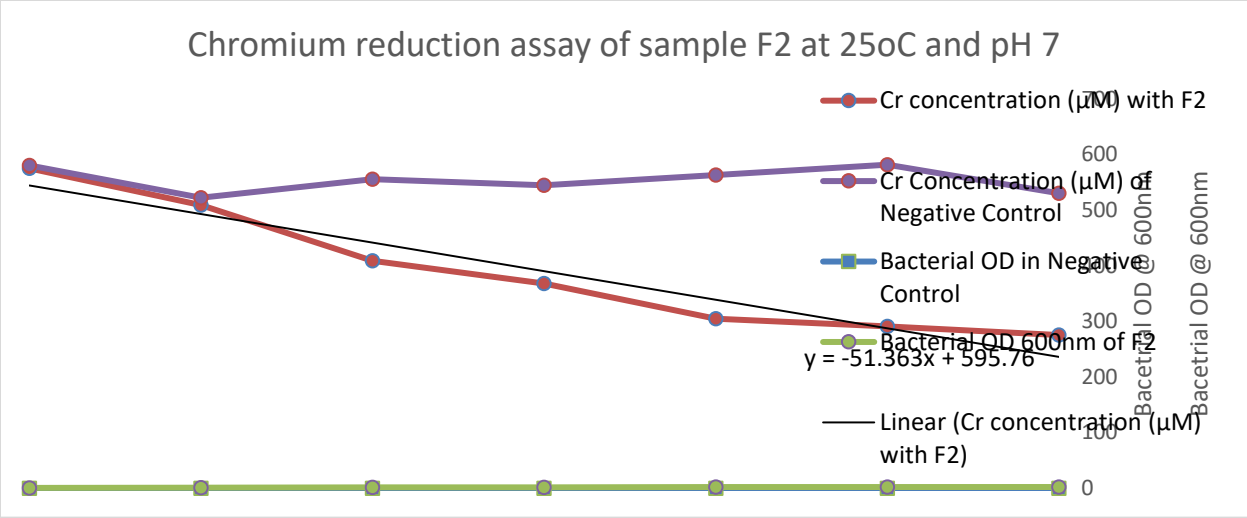

Figure 3.3: Bioremediation assay curve of sample F2 at 25ºC, pH 7

From Figure: 3.3, it is observed that the chromium concentration is decreasing over time. At 0 hour, the concentration is 575µm and it reduces to become 291µm after 7.5 hours. However, after 24 hours the reduction is seen to be less and the Chromium concentration reduced to only 275µm when the concentration was 291µm after 7.5 hours. After overnight, a total of only 52% Chromium was reduced. The bacterial OD was seen to increase from 0.677 at 0 hour to 2.161 at 7.5 hours. After 24 hours, the bacterial OD is seen to be only 2.234. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.3 Reduction profile at 25°C, pH 8.5

Table 3.4: Isolate F2: Chromium reduction outline vs. cell growth at 25°C, pH 8.5

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control
0	586.8156028	0.675	580.4326241	0.079

1.5	535.1134752	0.921	522.3475177	0.071
3	517.7375887	1.461	555.4680851	0.074
4.5	501.9219858	1.839	544.4751773	0.068
6	240.787234	2.18	562.9858156	0
7.5	191.070922	2.375	581.7092199	0
24	188.8014184	2.397	530.2907801	0

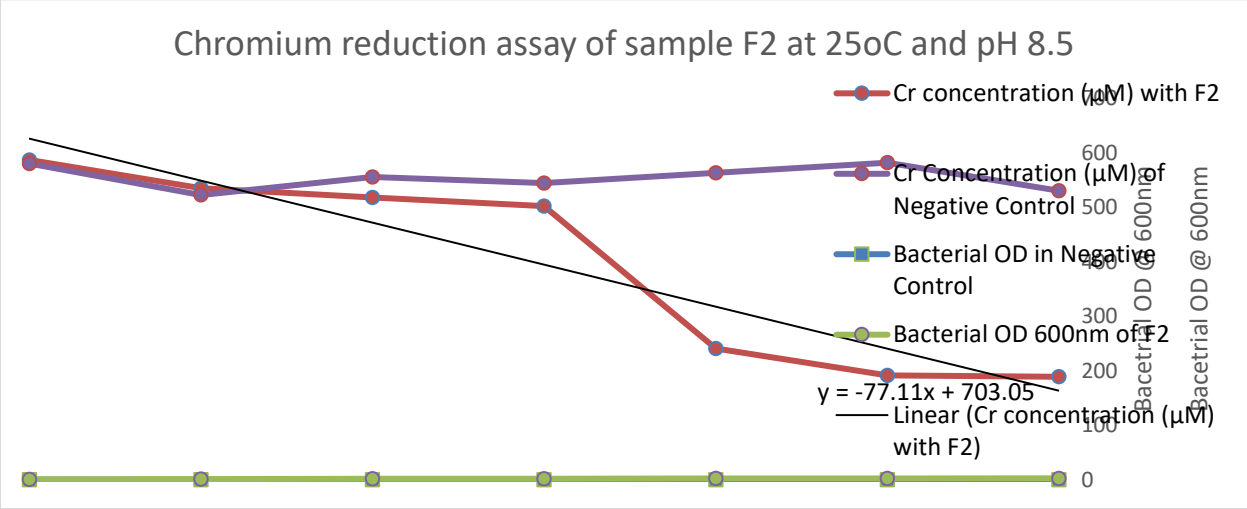

Figure 3.4: Bioremediation assay curve of sample F2 at 25ºC, pH 8.5

From Figure: 3.4, it is seen that concentration of Chromium reduced by a small amount in the first three hours, from 586 µm to 517 µm. However, there was a drastic decrease in Chromium concentration after 4.5 hours where the concentration was seen to be 240 µm. After 24 hours, concentration of Chromium recorded was 188 µm. Total of 67% Chromium was reduced. The bacterial OD on the other hand showed almost linear increase. OD at 0 hour was seen to be 0.675 and at 7.5 hours, 2.375. However, bacterial OD was almost the same after overnight. After 24 hours, bacterial OD was seen to be just 2.397. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.4 Reduction profile at 37°C, pH 5.5

Table 3.5: Isolate F2: Chromium reduction outline vs. cell growth at 37°C, pH 5.5

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control
0	473.5531915	0.475	509.0851064	0.009
1.5	440.3617021	0.385	495.1134752	0
3	524.7588652	0.435	503.3404255	0.012
4.5	438.9432624	0.428	508.2340426	0.008
6	432.9858156	0.465	461.070922	0.015
7.5	373.1985816	0.446	525.5390071	0.016
24	208.4468085	0.433	486.248227	0

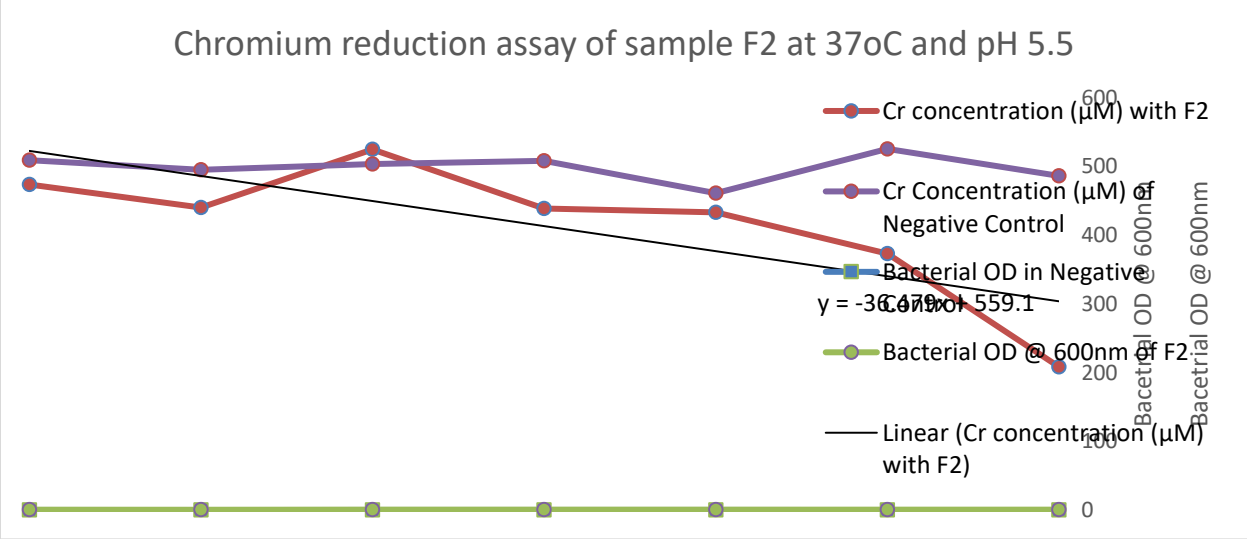

Figure 3.5: Bioremediation assay curve of sample F2 at 37ºC, pH 5.5

From the Figure: 3.5, it is observed that the Chromium concentration decreased from 473 µm to 440 µm in the first 1.5 hours. However, the concentration was seen to increase at 3 hours and became 524 µm. The concentration then seen to decrease and after 24 hours, concentration of Chromium was 208 µm. After overnight, total of 56% Chromium was reduced. The bacterial growth was seen to be very irregular at this particular temperature and pressure. Concentration of bacteria initially decreased after 1.5 hours and then increased at 3 hours. It then decreased at 4.5 hours only to increase again at 6 hours. Finally after 24 hours, the concentration of bacteria was found to be 0.433. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.5 Reduction profile at 37°C, pH 7

Table 3.6: Isolate F2: Chromium reduction outline vs. cell growth at 37°C, pH 7

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control
0	375.5390071	0.567	509.0851064	0.009
1.5	402.7730496	0.897	495.1134752	0
3	363.3404255	1.4	503.3404255	0.012
4.5	229.2269504	1.542	508.2340426	0.008
6	185.964539	1.567	461.070922	0.015
7.5	120.0070922	1.737	525.5390071	0.016

24	50.57446809	2.549	486.248227	0

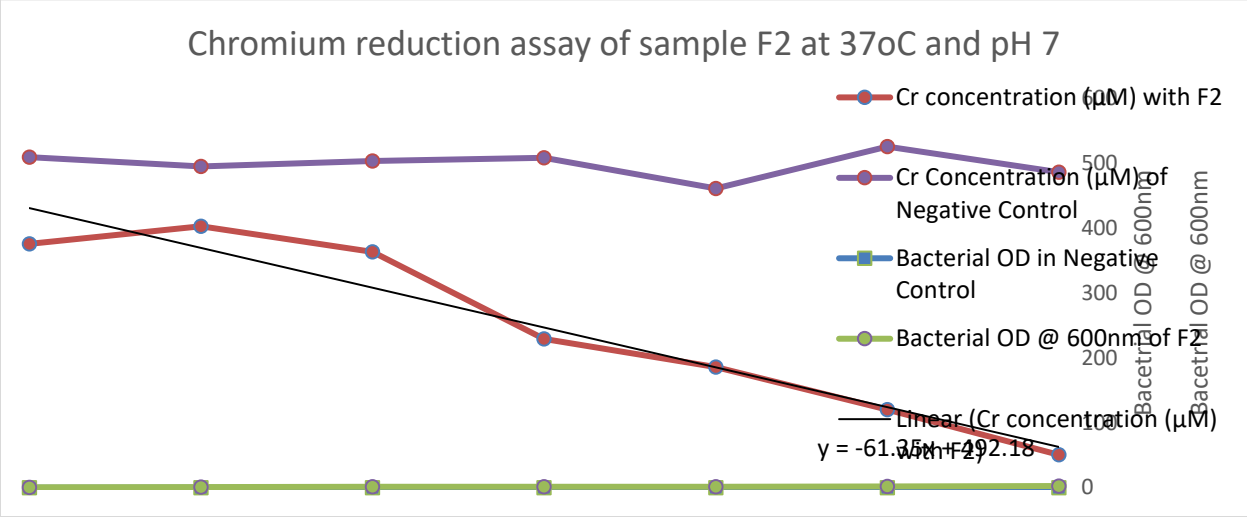

Figure 3.6: Bioremediation assay curve of sample F2 at 37ºC, pH 7

From the Figure: 3.6, it is observed that Chromium concentration increased in the first 1.5 hours from 375 µm to 402 µm. However, after that, the reduction in concentration was almost linear until 7.5 hours. After 24 hours, concentration of Chromium was seen to be only 50 µm. After overnight. 87% of Chromium was seen to be reduced. The bacterial growth was seen to increase over time. At 0 hours, bacterial OD was 0.567 and at 24 hours, bacterial OD was 2.549. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.6 Reduction profile at 37°C, pH 8.5

Table 3.7: Isolate F2: Chromium reduction outline vs. cell growth at 37°C, pH 8.5

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control

0	294.6170213	0.721	509.0851064	0.009
1.5	245.2553191	0.938	495.1134752	0
3	132.4893617	1.235	503.3404255	0.012
4.5	39.0141844	1.485	508.2340426	0.008
6	-2.40425532	1.617	461.070922	0.015
7.5	-13.822695	1.735	525.5390071	0.016
24	-21.2695035	2.304	486.248227	0

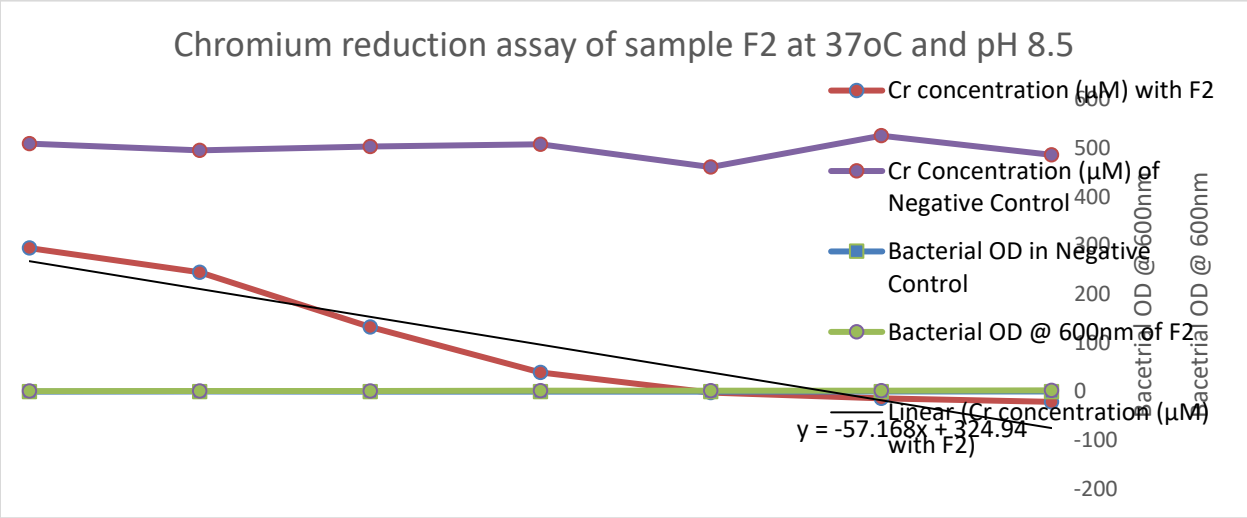

Figure 3.7: Bioremediation assay curve of sample F2 at 37ºC, pH 8.5

From the graph 3.7, it is observed that concentration of Chromium decreased almost linearly and all of hexavalent Chromium was reduced by 6 hours. The initial concentration of Chromium was 294 µm and at 4.5 hours it was only 39 µm. 65% was reduced by 4.5 hours. On the other hand, Bacterial OD was seen to be almost linear as well. At 0 hour, OD of bacteria was 0.721 and at 24 hours, it was 2.304. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.7 Reduction profile at 42°C, pH 5.5

Table 3.8: Isolate F2: Chromium reduction outline vs. cell growth at 42°C, pH 5.5

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (μM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (μM) of Negative Control	Bacterial OD in Negative Control
0	329.1560284	0.372	380.787234	0.017
1.5	298.6595745	0.413	394.7588652	0
3	273.9078014	0.382	394.0496454	0
4.5	248.3758865	0.397	402.0638298	0
6	207.1702128	0.403	407.5248227	0.002
7.5	178.9432624	0.47	382.2765957	0
24	30.21985816	0.515	375.1134752	0.002

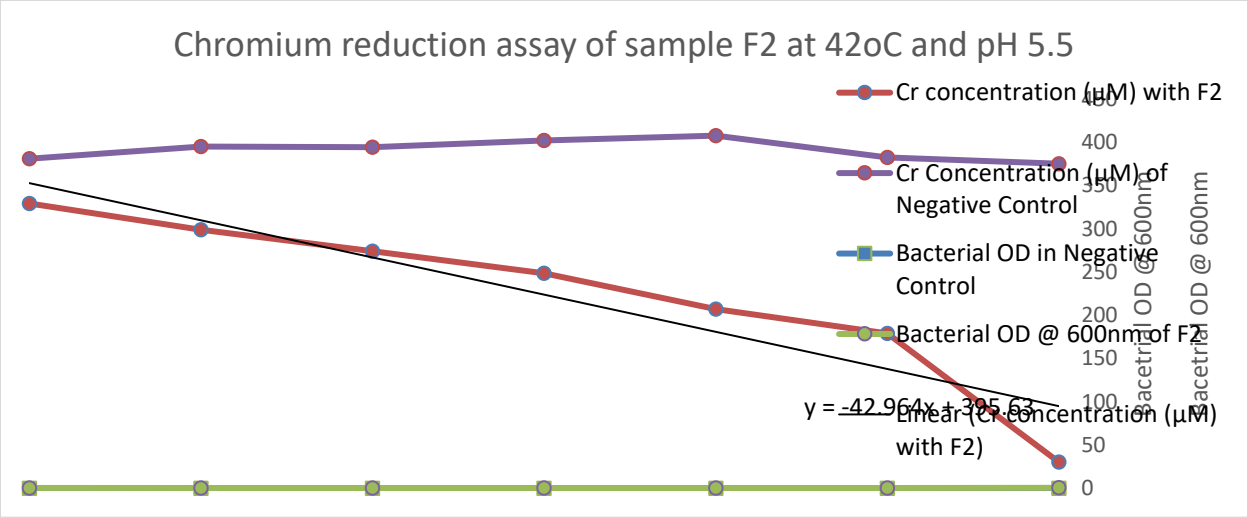

Figure 3.8: Bioremediation assay curve of sample F2 at 42ºC, pH 5.5

From the graph 3.8, it is observed that the trend line almost overlaps the Chromium concentration. This shows that the reduction of Chromium was linear. It decreased from 329 µm at 0 hour to 178 µm at 7.5 hours. After 24 hours, concentration was 30µm. After overnight, 90% of the Chromium was reduced. On the other hand, bacterial growth was seen to be very less and OD varied from 0.587 to 1.97 till 7.5 hours. After 24 hours, the OD was recorded as 2.144. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.8 Reduction profile at 42°C, pH 7

Table 3.9: Isolate F2: Chromium reduction outline vs. cell growth at 42°C, pH 7

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (µM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (µM) of Negative Control	Bacterial OD in Negative Control

0	384.4751773	0.587	380.787234	0.017
1.5	363.1985816	0.694	394.7588652	0
3	301.4255319	1.082	394.0496454	0
4.5	73.34042553	1.426	402.0638298	0
6	-3.46808511	1.574	407.5248227	0.002
7.5	-24.035461	1.97	382.2765957	0
24	-36.5886525	2.144	375.1134752	0.002

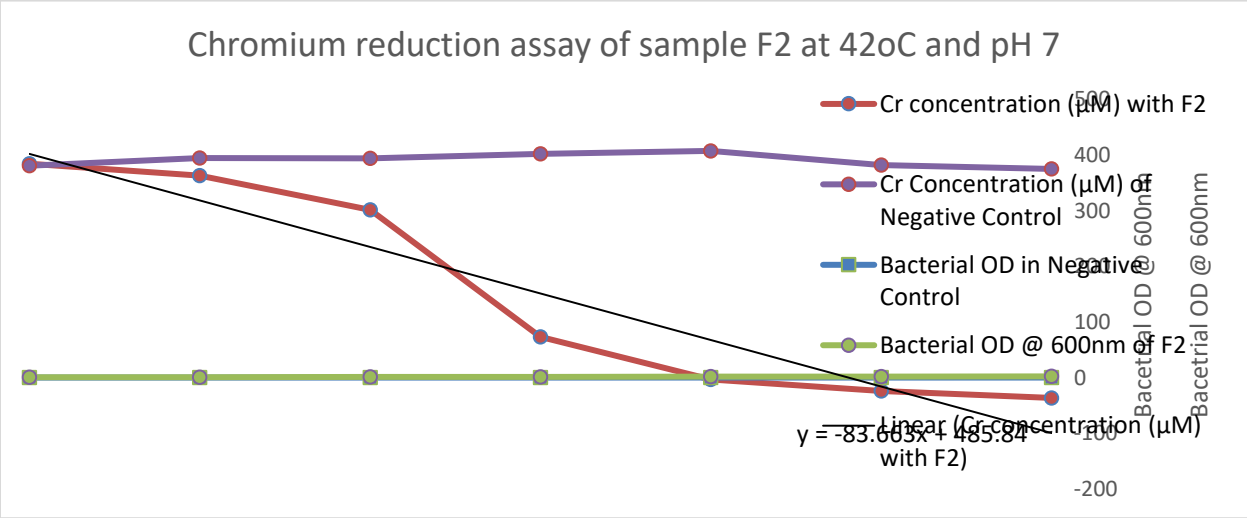

Figure 3.9: Bioremediation assay curve of sample F2 at 42ºC, pH 7

From the graph 3.9, it is seen that Chromium concentration decreases a little in the first three hours, from 384µm to 301µm and then falls drastically at 4.5 hours, the value of concentration being reduced to only 73 µm. By 4.5 hours, approximately 80% of the Chromium was reduced. At 6 hours all of hexavalent Chromium has been reduced. On the other hand, bacterial OD was seen to increase from 0.587 at 0 hour to 2.144 at 24 hours. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.9 Reduction profile at 42°C, pH 8.5

Table 3.10: Isolate F2: Chromium reduction outline vs. cell growth at 42°C, pH 8.5

	Sample		**Negative Control**	
Time (Hours)	Cr concentration (μM) with F2	Bacterial OD @ 600nm of F2	Cr Concentration (μM) of Negative Control	Bacterial OD in Negative Control
0	304.1914894	0.515	380.787234	0.017
1.5	256.9574468	0.857	394.7588652	0
3	171	1.369	394.0496454	0
4.5	72.34751773	1.71	402.0638298	0
6	39.22695035	1.748	407.5248227	0.002
7.5	-6.30496454	1.949	382.2765957	0
24	-22.4042553	2.301	375.1134752	0.002

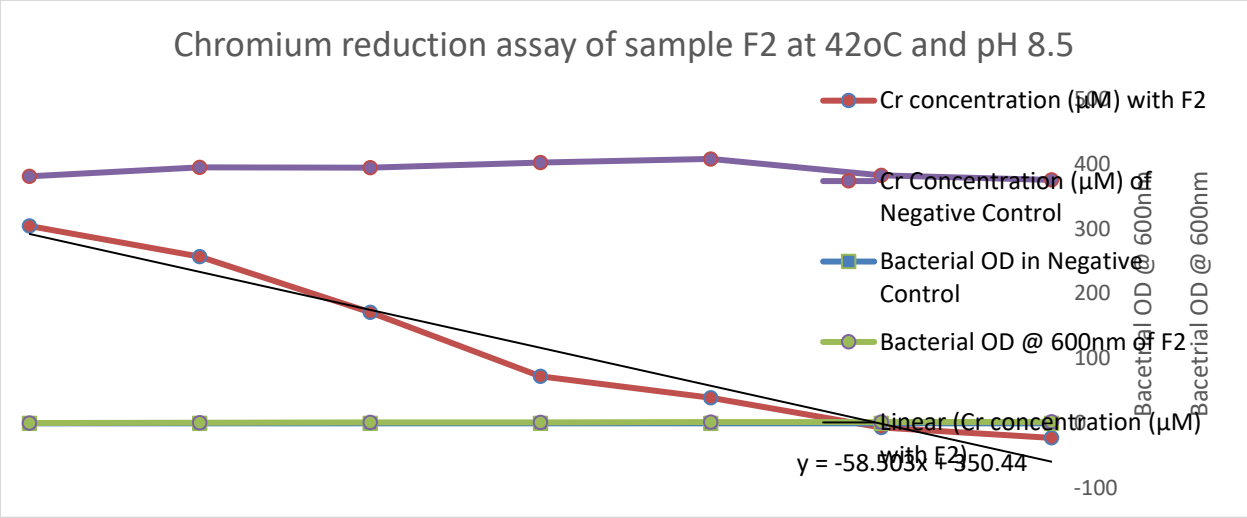

Figure 3.10: Bioremediation assay curve of sample F2 at 42ºC, pH 8.5

From the graph 3.10, it is shown that Chromium concentration has reached zero by the time it was 7.5 hours. The Chromium concentration had decreased over the time form 304 µm at 0 hour to 39µm at 6 hours. Percentage reduced by 6 hours is approximately 87%. On the other hand, bacterial OD was seen to increase almost linearly till 4.5 hours. Then at 6 hours the OD increased very slightly from 1.71 to 1.748. On the other hand, bacterial OD was seen to increase from 0.515 at 0 hour to 2.301 at 24 hours. In negative control no bacterial growth was observed and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.3 Antibiotic resistant among Chromium resistant isolate F2

The method of antibiotic resistant profiling has been discussed in 2.8. According to the method stated in 2.8, the test was carried out and the following result was obtained:

Table 3.11: zone of inhibition of antibiotics

Name of antiobiotics	ZI/mm	ZI/mm	ZI/mm	average	standard deviation	Inference
Penicillin (P10)	0	0	0	0	0	Resistant
Kanamycin (K30)	20	20	18.5	19.5	±0.866025404	Susceptible

Neomycin (N30)	13	12.5	12	12.5	±0.5	Susceptible
Vancomycin (VA30)	9.5	11	10.5	10.33	±0.763762616	Susceptible
Gentamycin (CN10)	18	19	18.5	18.5	±0.5	Susceptible
Cefixime (CFM5)	0	0	0	0	0	Resistant
Chloramphenicol (C30)	17	18	16	17	±1	Susceptible
Ceftriaxone (CRO30)	10	10.5	11	10.5	±0.5	Susceptible
Trimethoprim (STX25)	10	10	9	9.67	±0.577350269	Susceptible
Cipropfloxacin (CIP5)	12	10	11	11	±1	Susceptible
Streptomycin (S10)	9	9	10	9.33	±0.577350269	Susceptible
Ofloxacin (OF5)	13	11.5	13.5	12.67	±1.040833	Susceptible
Amoxycillin (AML10)	0	0	0	0	0	Resistant
Cefuroxime Sodium (CXM30)	0	0	0	0	0	Resistant

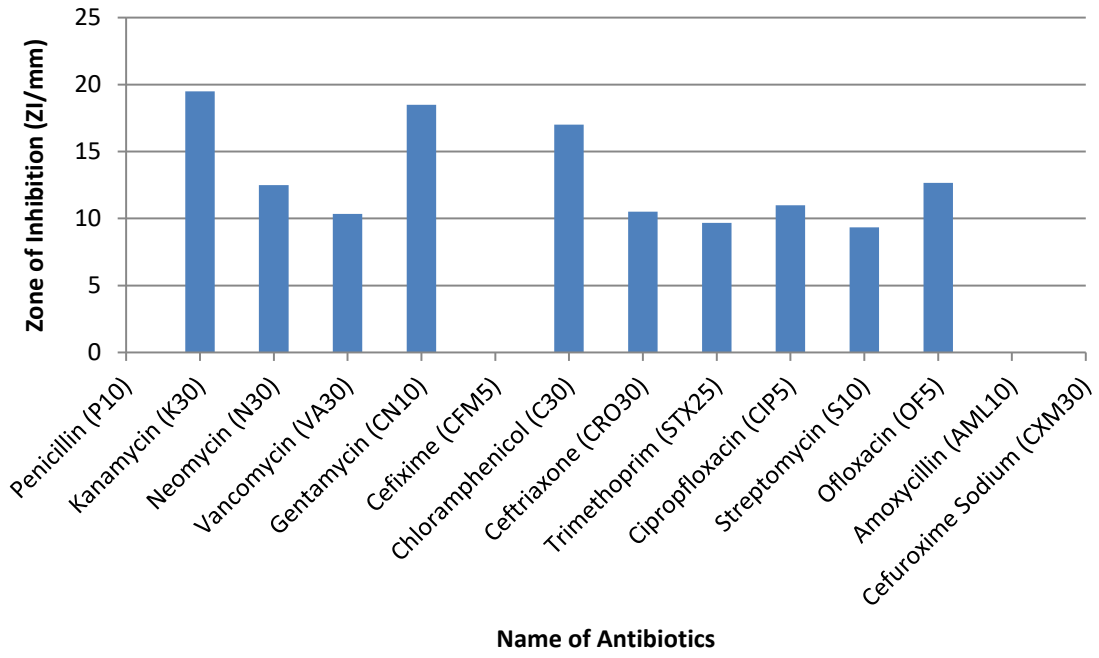

Figure 3.11: Graph showing the zone of inhibition against antibiotic discs.

From the Figure 3.11, it can be concluded that, for bacterial isolate F2, the highest zone of inhibition was seen in Kanamycin (K30) which was 19.5mm on average after taking triplicate data. Therefore, the action of Kanamycin was more powerful than all the other antibiotics against the bacterial isolate of F2. So, it is safe to say that F2 was mostly susceptible against Kanamycin. On the other hand, the lowest zone of inhibition was seen for Streptomycin which was 9.33 after taking triplicate reading. Therefore, action of Streptomycin was very poor than all other antibiotics against F2 isolate. This is because Streptomycin could not inhibit the bacterial growth of F2 strain significantly. F2 was seen to be resistant against certain antibiotics like Penicillin, Cefixime, Amoxicillin and Cefuroxime sodium. Only two of the antibiotics showed zone of inhibition which was below 10mm and they are: Trimethoprim and Streptomycin. All the other antibiotics showed zone of inhibition which exceeds 10mm.

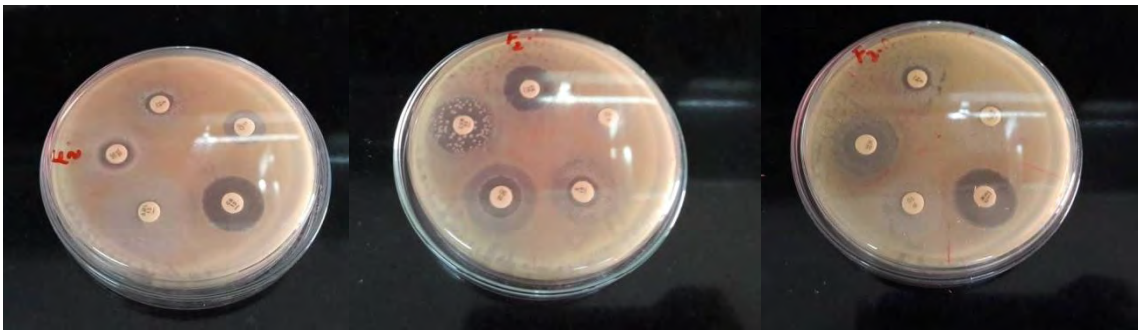

Figure 3.12: Zone of inhibition of the antibiotic discs in F2 swabbed HMA plate

3.4 Minimum Inhibitory Concentration of Chromium to inhibit the growth of Chromium resistance bacteria

The bacteria under consideration, F2 was subjected to different concentration of Chromium in order to find out its minimum inhibitory concentration. The result obtained is tabulated below:

Table 3.12: MIC of Chromium resistant organism F2 against different Chromium concentration

Concentration of Chromium (mM)	Colony forming unit (CFU)
5	93
10	90
15	87
20	78
25	69
30	63
35	57
40	45
45	38
50	31
55	27
60	25

65	6
66	3
67	3
68	2
69	2
70	0
71	0

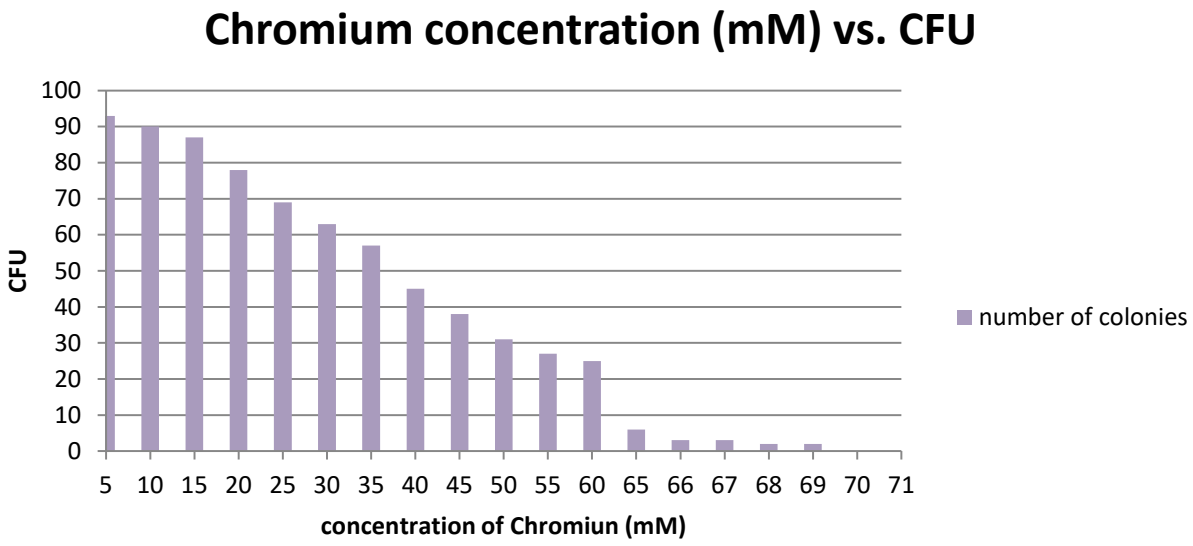

Figure 3.13: MIC of isolate F2

From Figure 3.13, it was observed that, the isolate F2 tolerates Chromium concentration up to 69mM. This means that F2 was able to demonstrate resistance till the Chromium concentration of 69mM. However, no growth was observed from the Chromium concentration of 70mM. Therefore, 70mM was the Minimum Inhibitory Concentration for F2 isolate.

3.5 Identification of F2

3.5.1 Obtained sequence by Sanger sequencing

The DNA sequence of the isolate was found out by Sanger sequencing. (Heather et al., 2016). The sequence of the isolate F2 is shown in Appendix-A.

3.5.2 BLAST analysis of F2

The table below, table 3.13 contains the BLAST (Altschul et al., 1997) result:

Table: 3.14: BLAST result of isolate F2

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pseudomonas flavescence strain NBRC 103044 16S ribosomal RNA gene, partial sequence	1081	1081	100%	0.0	99%	NR 114195.1
Pseudomonas oryzihabitans strain NBRC 102199 16S ribosomal RNA gene, partial sequence	1076	1076	100%	0.0	99%	NR 114041.1
Pseudomonas borbori strain R-20821 16S ribosomal RNA gene, partial sequence	1072	1072	100%	0.0	99%	NR 042450.1
Pseudomonas alcaligenes strain ATCC 14909 16S ribosomal RNA gene, partial sequence	1068	1068	100%	0.0	99%	NR 114472.1
Pseudomonas	1076	1076	100%	0.0	99%	NR 042191.1

psychrotolerans strain C36 16S ribosomal RNA gene, partial sequence						
Pseudomonas anguilliseptica strain S 1 16S ribosomal RNA gene, partial sequence	1072	1072	100%	0.0	99%	NR 029319.1
Pseudomonas marincola strain AB251f 16S ribosomal RNA gene, partial sequence	1067	1067	100%	0.0	99%	NR 117186.1
Pseudomonas composti strain C216S ribosomal RNA gene, partial sequence	1067	1067	100%	0.0	99%	NR 116992.1
Pseudomonas segetis strain FR1439 16S ribosomal RNA gene, partial sequence	1067	1067	100%	0.0	99%	NR 043174.1
Pseudomonas punonensis strain LMT03 16S ribosomal RNA gene, partial sequence	1063	1063	100%	0.0	99%	NR 109583.1

3.5.3 Phylogenetic tree of sample F2

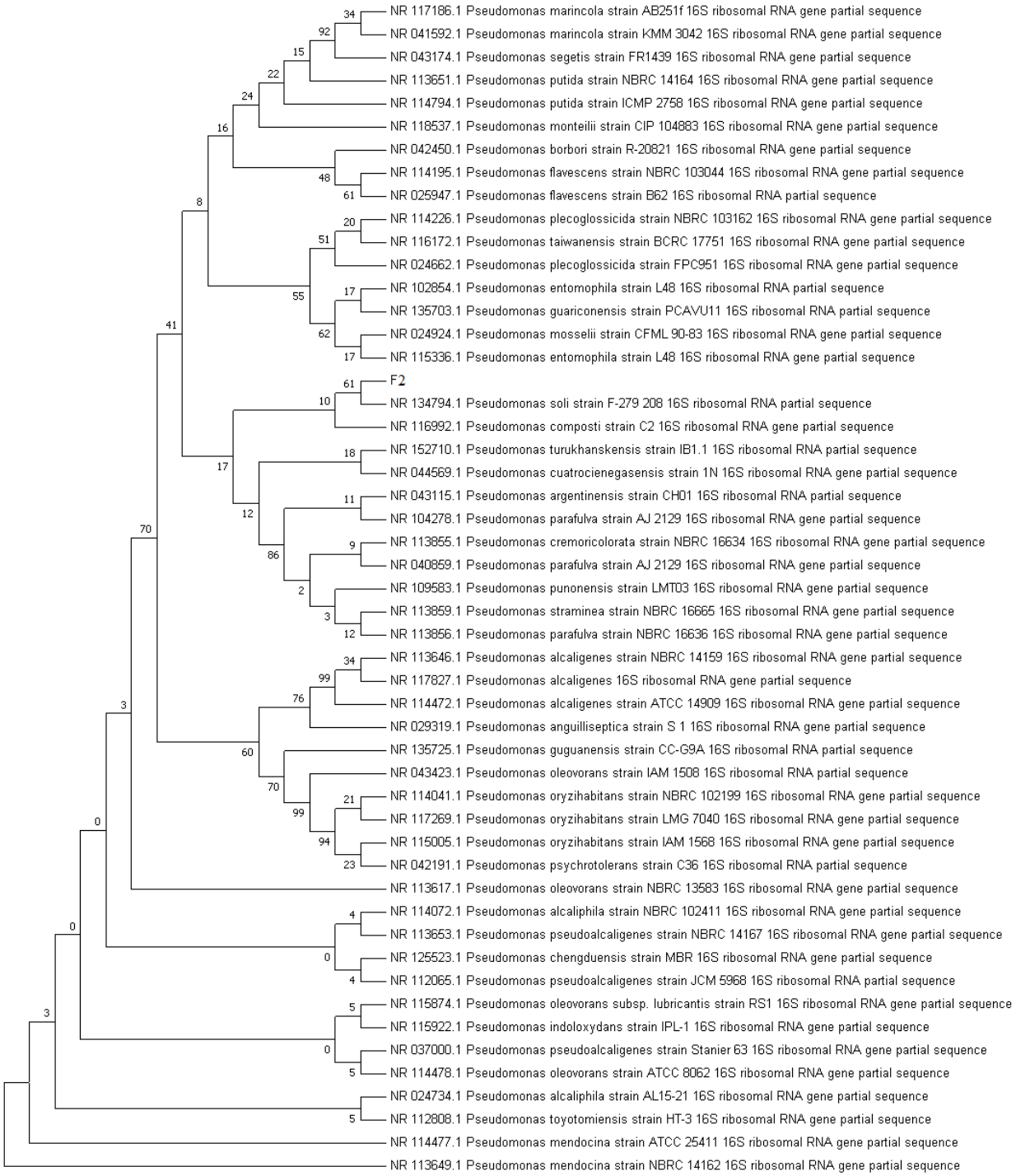

Figure 3.14: The evolutionary history of the sample was deduced by means of the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to characterize the evolutionary history of the taxa analyzed. Branches correlatinging to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura & Nei, 2004) and are in the units of the number of base substitutions per site. The analysis involved a total of 51 nucleotide sequences. All positions which contained gaps and missing data were eliminated. There were a total of 565 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016)

3.6 Discussion

In this research, isolation was done for only those bacteria which showed resistance against hexavalent Chromium and had the capacity for reducing the carcinogenic and mutagenic Chromium effectively.

The analysis was conducted with F2 with various temperature and pH. The obtained data was tabulated and graph generated accordingly. 9 different graphs were generated (three different temperatures and for every temperature, experiment was carried out in three pH values) were and results were examined. There was also a blank prepared for better conduct of the experiment. After studying the graphs, it was evident that the isolate possessed Chromium reducing capacity. It was also seen that the reduction capacity of isolate F2 increased with increasing temperature. The quickest reduction was seen after 6 hours which was seen at 37°C, pH of 8.5 and also at 42°C, pH 7. At 42°C, pH 5.5, after 24 hours, 90% of the Chromium was seen to be reduced. Lowest reduction profile was seen at 25°C, pH 7 where only approximately 52% was reduced. At two other pH values of 5.5 and 8.5 at 25°C, only 57% and 56% was seen to be reduced approximately, respectively.

The most linear reduction pattern was observed at 42°C, pH 5.5. The the graph of reduction of concentration of hexavalent Chromium was seen to almost overlap the trend line curve. This

showed very good remediation pattern as reduction occurred by almost the same amount in a certain time period.

Even though almost all the graphs showed reduction profile over time, sometimes, reoxidation was observed for which the reason behind is not known. Also, sometimes, it was seen that the reduction occurs very quickly and concentration of Chromium fell drastically within 1.5 hours period. The cause of this is also unknown.

In the antibiotic resistance profiling, it was seen that the isolate was resistant to most of the antibiotics that contained beta lactam rings. It showed resistance against four antibiotics: Penicillin, Cefixime, Amoxicillin and Cefuroxime sodium. The bacteria must contain certain elements because of which it can grow despite the presence of these antibiotics. The isolate was most susceptible to Kanamycin and least susceptible to Streptomycin. All the zone of inhibition values were seen to be in between 9.33mm to 19.5mm. The values of zone of inhibition were taken three times and the n average and standard deviation calculated to be more precise in the final output.

In minimum inhibitory concentration (MIC), it was seen that the isolate grew up till 69mM concentration of Chromium. So, its MIC was 70mM. As the concentration of Chromium was gradually increased, it was seen that number of colonies decreased with increase in Chromium concentration. The decrease is not linear but a prominent decrease in number of colonies is observed as concentration of Chromium was increased. The possible explanation for its survival up to such a high value of concentration might be due to presence of an endospore, however, it cannot be confidently said so without further investigation.

The phylogenetic tree that was constructed for the project has multiple outgroups which means a taxon outside the group of interest. In this tree, the outgroup is *Pseudomonas chengduensis* strain strain and *Pseudomonas mendocina* strain. There are three species in the clade which contains F2. The other two taxa are *Pseudomonas composti* and *Pseudomonas soli.* Since the bacteria under consideration and *Pseudomonas soli* has a common node, it can be concluded that the DNA sequence of F is most similar to *Pseudomonas soli* than all the other DNA sequences that we downloaded to find out its identification and thus F is identified as *Psuedomonas soli*. *Pseudomonas composti* strain is sister sister taxon to clade containing F and *Psuedomonas soli.*

4 Conclusion

4.1 Conclusion

After multiple studies that were carried out, it is assertive that the isolate F2, which was later identified as *Pseudomonas soli*, possesses hexavalent Chromium reducing capabilities. The microorganism carries an astonishing potential to biologically reduce Chromium. It was observed that, the bacterial strain was able to reduce Chromium if provided its optimum condition for reduction. The rate of reduction was seen to increase in most cases with increase in time. Moreover, it was seen after study that the bacteria under consideration can withstand up to high concentration of Chromium which demonstrates its capacity to survive in very adverse condition. It must have some defense mechanism with which it survives such high concentration of Chromium. Information gathered from this project confirms for sure that the bacteria is capable of reducing hexavalent Chromium and that it can survive up to quite high concentration of Chromium.

4.2 Future works

Since after the study it was concluded that the bacteria under consideration can survive up to high concentration of Chromium, the bacteria could be further studied to find out the bacteria's defense mechanism and how it reduced the hexavalent Chromium.

Research can be further conducted to find out the following:

1. Correlation between antibiotic resistance profile and Chromium reduction assay.
2. Further studies such as cell free extract will help to elucidate the mechanisms responsible for the reduction of Chromium and whether the enzyme responsible for the reduction is an exoenzyme or endoenzyme.

3. Isolate F2 can be a potential source for Chromium reductase enzyme. Comparative genomic studies might be carried out to find out best candidate for Chromium reduction activity.
4. Plasmid analysis could also be done in order to find out more about the bacteria.

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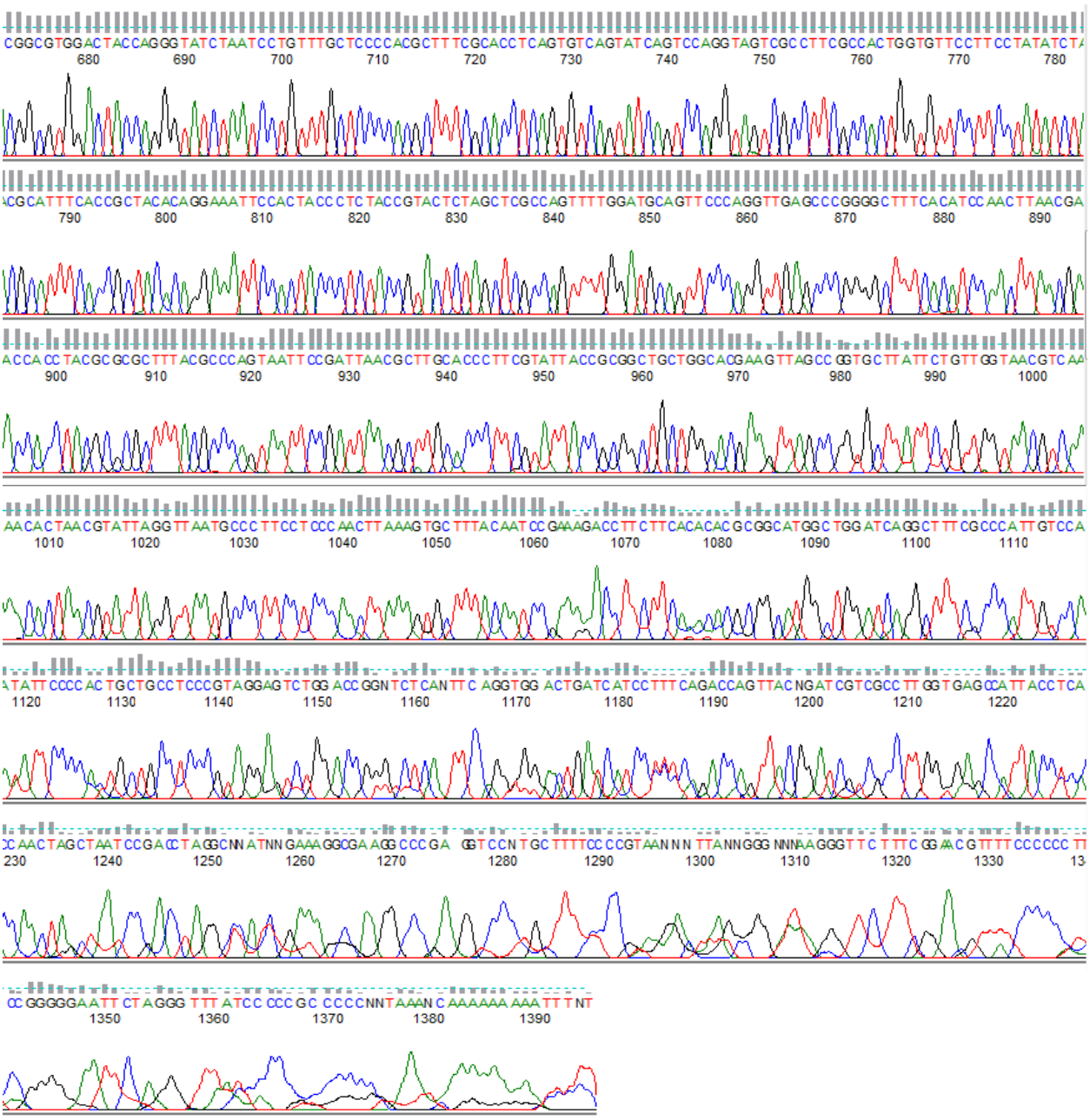
6 Appendix- A

Chromatogram of F2 (*Pseudomonas soli*) is given below :



Obtained partial sequence of F2 after performing Sanger sequencing:

AAAAACTNCGGGTNANNNCCCCCCCGNAGGTGNNNATTATTTTTGGGCCAACCNNNC
CCCNNNGGGNNGGGGGGNNTNCAAGGCCCGGGAANNTTTCCCCGGNCTTTTGTTTC
CGATTNTAGGGTTCGGNNTCCCGCAGGGAGTTCAGCATNGATCCCGACTCCGTTCGG
TTTTATGGGATTAGCTCCACCTCCCGGCTTGGCAACCCTTTGTACCGACCATGNAGC

ACGNGGTAGCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCC
GGTTTNTCACCGGCAGTCTCCTTAGAGTGCCCACCATAACGTGCTGGTAACTAAGGA
CAAGGGTTGCGCTCGTTACGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAGCCATGCAGCACCTGTGTCTGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGT
TCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATG
CTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTAC
TCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAAATCTCAAGGATTCCAA
CGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTC
CCCACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTAGTCGCCTTCGCCACTGG
TGTTCCTTCCTATATCTACGCATTTCACCGCTACACAGGAAATTCCACTACCCTCTAC
CGTACTCTAGCTCGCCAGTTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCAC
ATCCAACTTAACGAACCACCTACGCGCGCTTTACGCCCAGTAATTCCGATTAACGCT
TGCACCCTTCGTATTACCGCGGCTGCTGGCACGAAGTTAGCCGGTGCTTATTCTGTTG
GTAACGTCAAAACACTAACGTATTAGGTTAATGCCCTTCCTCCCAACTTAAAGTGCT
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CTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCC
CCTGCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCGGAACGTTATCCCC
CACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTAAATCAGAGAGCA
AGCTCTCTTCATCCGCTCGACTGCATGTGTA