Isolation and Characterization of Carcinogenic Chromium Reducing *Pseudomonas aeruginosa* From Water of Buriganga River

by

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A thesis submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
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Declaration

It is hereby declared that

1.The thesis submitted is my own original work while completing degree at Brac University.

2.The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3.The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4.Ihave acknowledged all the main sources of help.

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Approval

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Ethics Statement

The study does not involve any kind of animal trial or human trial.

Abstract

Hexavalent chromium is a highly toxic heavy metals which has the carcinogenic and mutagenic property. It is mobile in nature and causes many more diseases such as skin cancer, lung cancer, nasal infection etc. This hexavalent chromium is mainly used in diffrent industries like leather, tannery, dye, stainless steel etc. From these industries, chromium released in the environment without proper treatment which can be a dangerous for human and the environment. Hexavalent is more toxic than any other form of chromium.In this study, a bacterial strain was indentified named *Pseudomonas aeruginosa* which has the capability to reduce hexavalent chromium and produce trivalent form and show the resistance to Chromium. The strain was collected from Buriganga river and isolation was done in nutrient agar plates. Then purified the strain and used in different experiment such as DPCZ based bioassay. Bioassay method was used to find out their reduction capacity at three different temperature 25˚C, 37˚C, and 42˚C with three diffrentpH 5.5, 7, 8.5. From bioassay study, it was observed that the isolate has good Chromium reduction capacity at 37˚C pH 8.5 and 7. Highest reduction was found at 37˚C pH 8.5 which means the optimum reduction condition of the strain. It is also found from this study that the enzyme that bacteria used for reduction is exoenzyme. From this study it was also observed that this strain is mostly susceptible to Ciprofloxacin whereas strain is resistant to some other antibiotics such as Penicillin G, Amoxycillin, and Cefuroxime Sodium. MIC was also observed which showed that bacteria can tolerate upto 9mM of chromium concentration. Finally 16S rDNA squencing was used for identification and using the sequence data a phylogenetic tree was made which showed the similarity between the experimental strain and *Pseudomonas aeruginosa.*

Keywords: Bioremediation; Heavy metal; Toxicity; Resistance.

Dedication

I dedicate this work to my parents for their endless love and support.

Acknowledgement

First of all, I am very thankful to Almighty Allah for his blessing to me. I belive that he give me the devotion and strength that helps me to fullfil this project anddefeated all the difficultiesthataccompanied with it. It can not be possible to do all the work without his blessing.

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Table of Contents

List of Tables

List of Figure

List of acronyms

BLAST	Basic Local Alignment Search Tool
Cr	Chromium
CRB	Chromium Resistant Bacteria
DPCZ	DiphenylCarbazide
IARC	International Agency for Research on Cancer
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mL	Milliliter
mM	Millimolar
MOPS	3-(n-Morpholino)Propanesulfonic Acid
NA	Nutrient Agar
NB	Nutrient broth
O.D.	Optical density
OSHA	Occupation safety and health administration
Ppm	Parts per million
ROS	Reactive oxygen species
RPM	Revolution per minute
rRNA	Ribosomal ribonucleic acid
WHO	World Health Organization
ZI	Zone of Inhibition
µM	Micro molar
µg	Micro gram

Chapter1

Introduction

1.1 Background

In this advanced world, the environment becomes polluted with heavy metals continuously because of urbanization and industrialization. Nowadays it becomes global intimidation for the environment and human as well for its toxicity. Heavy metals apparently found in water or soil as solid or in liquid form by naturally or because of anthropogenic activity. Human activities like mining and smelting metalliferous ores, sewage sludge, abandoned waste from the industry, a large amount of using fertilizer and pesticides in agriculture are mainly responsible for the pollution of environmental soil and water and create a hazard for the human health (Yadav,Kumar,Gupta,J.kumar,2016). To restore the fertility of the soil and reducing the contamination of water there has a much more conventional method. But we need to get the result fast at a low cost. For this remediation of contaminated water and soil with microorganism is one of the fastest and easy ways at a low cost (Upadhyay et al,2017).

Heavy metals are called the most available and precarious contaminant in the environments. The density of these metals is5 g/cm3. Copper, Zinc, Manganese, Iron, Chromium, Nickel, Mercury are the most known heavy metals (Yadav et al, 2016). Between this, chromium is the dominant pollutants that mainly release from the industrial waste, pesticides, mining activities, petroleum industries (Fakruddin et al,2009). Chromium can be found in nature as Trivalent (Cr III) form which is nontoxic. But in the industries or in the other activities hexavalent chromium (Cr VI) form is widely used which is toxic and lack of proper control it can mix with water and soil and contaminate these. By dustinhalation, direct contact with the soil and water these heavy metals

entering into the human body which can cause a great hazard and can cause disease like cancer (Yadav et al, 2016). In Bangladesh, Leather tanning is the leading sector in the leather industry where chromium is for the tanning purpose and a huge amount of toxic chromium waste is produced which contaminate the water and soil (Fakruddin et al,2009).

For the reduction of toxicity of chromium from the wastewater reduction of chromium 6+ is essential. Among trivalent and Hexavalent chromium, the second one is 10-100 times more toxic than the first one. Nowadays for the reduction of hexavalent chromium, Bioremediation process is the most convenient way because it is less expensive and not time-consuming. Biological agents like yeasts, bacteria, fungi are used in the bioremediation process. For the chromium reduction microorganism is helpful because it can be found in nature and can grow rapidly (Fakruddin et al,2009).

Therefore, the present review is designed on the isolation and characterization of bacterial strains that have the chromate reducing and resistance property and for using in the unified bioremediation process, analyze the growth condition in various Cr concentration level.

1.2 Aims and Objectives

The hypothesis of this project is to isolate bacterial strain which is chromium resistant and is used in the bioremediation for carcinogenic and mutagenic heavy metals and search for the chromium reducing new enzyme. The objectives of this purpose are:

1. Analyze the reducing power of the isolated bacteria in the chromium affluent environment.
2. Identify the type of the working enzyme which can be exoenzyme or endoenzyme.

3. Evaluate the minimum inhibitory concentration(MIC) of Cr to find out the resistant and tolerance level of bacteria.
4. Find out the antibiotic resistance profile of the bacteria.
5. Identify the isolated bacteria and construct the phylogenetic tree of the isolated bacteria.

1.3 Literature review

Chromium present in the earth crust which is the seventh most bountiful element (0.1–0.3 mg/g), present in the group VI B in the periodic table as a 3D transition metal with a various oxidation state (Ahemad,2014). Chromium can be found in almost every oxidation states with a range from -2 to 6+. Though chromium has many oxidation states, only the Cr^{3+} and Cr^{6+} are more stable in the environment (Saranraj and Sujitha,2013). These two states of chromium mainly produced from the different industrial activities and chromium with 0 oxidation states are chemically inert and absent in the earth crust. Halides, Oxides, Sulphates are the most common form of chromium present in the environment and their oxidation states are +2 and these are not stable. So these become +3 chromium when they come in contact with air. (Shekhawat et al,2015). According to biological and physiological activity, Cr^{3+} and $Cr^{6+}$are different from each other. Hexavalent chromium is a mobile anion in nature and its dichromate form is highly aqueous soluble but the trivalent form is not much soluble and mobile (Saranraj,Sujitha,2013). The cause of the immobile nature of Cr^{3+} is that it is very reactive with inorganic ligands like humic acid and produce precipitation to create a complexes form (Madhavi et al,2012). For human Cr (VI) is more toxic, carcinogenic, mutagenic and teratogenic. On the other hand Cr(III) helps in the metabolism of amino acid, glucose,and lipid which mainly increases the efficiency of insulin (Saranraj,Sujitha,2013). 0.05 mg/L is the tolerance level of chromium in the water according to Indian Standard (Shekhawat et al,2015). The presence of hexavalent chromium in the

environment is mainly because of anthropogenic activities and less than 0.001% is for the geological input (Ahemad,2014). Cr(VI) mainly utilized in the tanning industry, paint production, silver and alloy factory which are the main source of Cr^{6+} that cause the contamination of soil and water. Cr(VI) can cause different types of disease like diarrhea, skin disease, eye irritation, lung carcinoma,and kidney dysfunction. it can also interfere in the growth of the plant and their morphology (Saranraj,Sujitha,2013). According to the International Agency for Research on Cancer(IARC) taking Cr(VI) by inhalation,theprocess can be carcinogenic for the human. At lower concentration Chromium (III) helps in the protein and lipid metabolism and we take chromium from our daily intake of food like grain foods, vegetables, seafood, mushrooms etc. But in the higher concentration, it may cause toxicity. In our daily intake chromium should not take more than 100µg (Achmad et al, 2017).

1.3.1 Historical perspective of chromium

More than 200 years ago history of chromium began in Beresof gold mines in Siberia. Johann Gottlob Lehmann found a sample with a color of orange-red and he named the sample "Siberian red lead". Five years later, after studying the sample Lehmann found that this lead also contains Fe particles and selenitic spar and changed it to be corocoit which is lead chromate ($PbCrO_4$).The study also showed that this sample makes a green solution with HCl. After the death of Lehmann, Vauquelin,thechemistry professor of School of Mines in Paris collect the corocoit core in 1797 (Jacobs and Teasta,2004). After that Vauquelin did some experiment with the sample such as boiled it with standard potash (K_2CO_3) and found a solution of yellow color which produce red and yellow precipitate with mercury salt and lead respectively. In 1798, he heated green precipitated produced from the reaction of lead and HCl with charcoal and he found a new compound is mixed with the corocoit and its produce different color. From these, Fourcroy and

Abbé René-Just Haüy proposed the name chromium which comes from theGreek word chromameans color and its a Greek word. In 1798, Vauquelin and Fourcroy along with two German chemists Louwitz and Klaproth discovered Cr in rocks in the Bersof mines (Jacobs and Testa,2004).

1.3.2 Chemistry of Chromium

Chromium situated in group VIB in the periodic table. It is found in the first row of the d-block. It is known as a transition metal (Wuana and Okieimen,2011). It can be found in different oxidation states ranging from -2 to 6+. Among them, Trivalent and hexavalent form are more stable in the environment (Saranraj,Sujitha,2013). It has some natural isotopes such as ^{50}Cr (4.3%),^{52}Cr (83.8%),^{53}Cr (9.6%), and ^{54}Cr (2.4%). The metal of chromium is glossy and the color is silver. Chromium is hard and brittle in nature (Jacobs and Teasta,2004). Chromium is tasteless and odorless (Saranraj,Sujitha,2013). Cr^{3+} is the leading form of chromium at less than pH 4. Cr(VI) is highly toxic and more soluble in water than Cr(III).

Table 1: Chemical properties of Chromium

Symbol	Cr
Chemical Name	Chromium
Atomic Number	24
Atomic Mass	52
Melting Point	1875◦C
Boiling Point	2665◦C
Density	7.19 g cm^{-3}

(Wuana and Okieimen,2011)

Cr(III) tranffers to Cr(VI) by oxidation reaction in the presence of oxygen and manganese at high concentration (Shadreck and Mugadza,2013). The reaction that is given below shows the reduction of Cr^{6+} into Cr^{3+} form in the acidic condition.

$$Cr_2O^{2-}_7 + 14H^+ + 6e^- = 2Cr^{3+} + 7H_2O \; E_0 = 1{:}33 \text{ V (Saha et al,2011)}$$

1.3.3 Occurrence and Sources of Chromium compound

Chromium is an essential element of the earth crust and it is naturally found as chromite ($FeCr_2O_4$) in serpentine and igneous rock. Chromium also found in a complex form in the crocoite ($PbCrO_4$), bentorite$Ca_6(Cr,Al)_2(SO_4)_3$ and tarapacaite (K_2CrO_4) and crocite.Chromium can be introduced with the environment by various industrial activities like metallurgy, refractory and chemical industries, wood preserving, tanning etc. From these industrial activities, chromium mix with the soil and water indirectly by atmospheric deposition(Shadreck and Mugadza,2013).

The main source of the chromium for mixing with soil is from the chromium plating wastes or ferrochromium slag which contains a different combination of Cr(III) and Cr(VI). Chromium that found in soil is mainly because of tanning, paint production, metal finishing,and other industrial activities(Chrysochoou et al,2016). Cr released in the surface through physical or chemical weathering of rocks. Cr can be introduced in the atmosphere by a volcanic eruption, windblown sand, forest fires, sea salt spray(Saha et al,2011). In the aqueous phase, most of the chromium is hexavalent because of its mobility and solubility characteristics.(Bielicka et al., 2005) Another source of the chromium for nature is fertilizer or pesticides which are used in the agricultural purpose.Around 40 million waste that contains Cr is released from the tanning industry (Saranraj,Sujitha,2013).

All over the world, chromium is present in a different concentration from the different source. Burning fossil fuels, industrial activities are the main source of chromium in China.(H. Cheng, Zhou, Li, Lu, & Lin, 2014).In India, Cr present in the underground water in a concentration rate of 12 mg/L and 550–1,500 ppm/L.(JaishankarM.et al,2014).In La Spezia, In Italy, the concentration of Cr(VI) in the groundwater is 73 μg/L and it is mainly because of ultramafic igneous rocks. In Mojavedesert, in the USA the concentration is upto 60 μg/L. Some other country like Brazil, Mexico have a high percentage of Cr in the soil because of ultramafic rocks(Chrysochoou et al,2016).

1.3.4 Chromium Cycle

In the environment, chromium is present in the surface, ground and sea water, soil, rocks, and air. This chromium comes from either naturally or from the anthropogenic activity. The cycle of the chromium happens in the environment which is a chemical process.(Saha et al,2011). It is started from the hexavalent form of the chromium.(Avudainayagam et al,2003). It is highly toxic, reactive and mobile in nature.A small or large concentration of Cr(VI) in the water and soil can be the result of Cr(VI) pollution or oxidation of Cr(III).(Dhal et al,2013). The concentration of chromate is pH dependent and the most available form is $HCrO_4^-$ or CrO_4^{2-}. Chromium can be removed by adsorption or precipitation reactions, uptake by plants, or leaching from the subsurface layers from the soil. In the photosynthesis process, with the help of carbon reduction of Cr(VI) to Cr(III) is occurred. Besides, the presence of Fe^{2+} or $S^{2-}$and organic matter from microbial decomposition products act as an electron donor. According to Bartlett and James, it is called dechromification and here Cr(III) is produced from the reduction of Cr(VI) which is important for the Cr cycle. After that Cr(III) binds with various ligands in the soil and make itself immobile, insoluble and unreactive and these ligands such as citrate ligand introduce Cr(III) in

the surface of MnO_2 where oxidation occurredboth of the ligand and Cr.(Avudainayagam et al,2003). In the presence of manganese oxides, oxidation of Cr(III) to Cr(VI) occurs. In this chromium cycle, these reduction and oxidation process of Cr is taken place at the same time in the soil and water. (Dhal et al,2013).

The oxidation reaction of Cr(III) to Cr(VI),in the presence of manganese oxides under the condition of neutral pH is:

$$Cr^{3+} + 1.5MnO_2 + H_2O \rightarrow HCrO_4^- + 1.5Mn^{2+} + H^+$$

Bsides, the reduction reaction of Cr(VI) to Cr(III) in the presence of organic compound is:

$C_6H_6O_2 + CrO_4^{2-} + 2H_2O \rightarrow 0.5Cr_2O_3 + 1.5C_6H_4O_2 + 2.5H_2O + 2OH^- \Delta G_{298}^{\circ} = -427$ kJ mol^{-1}(Dhal et al,2013).

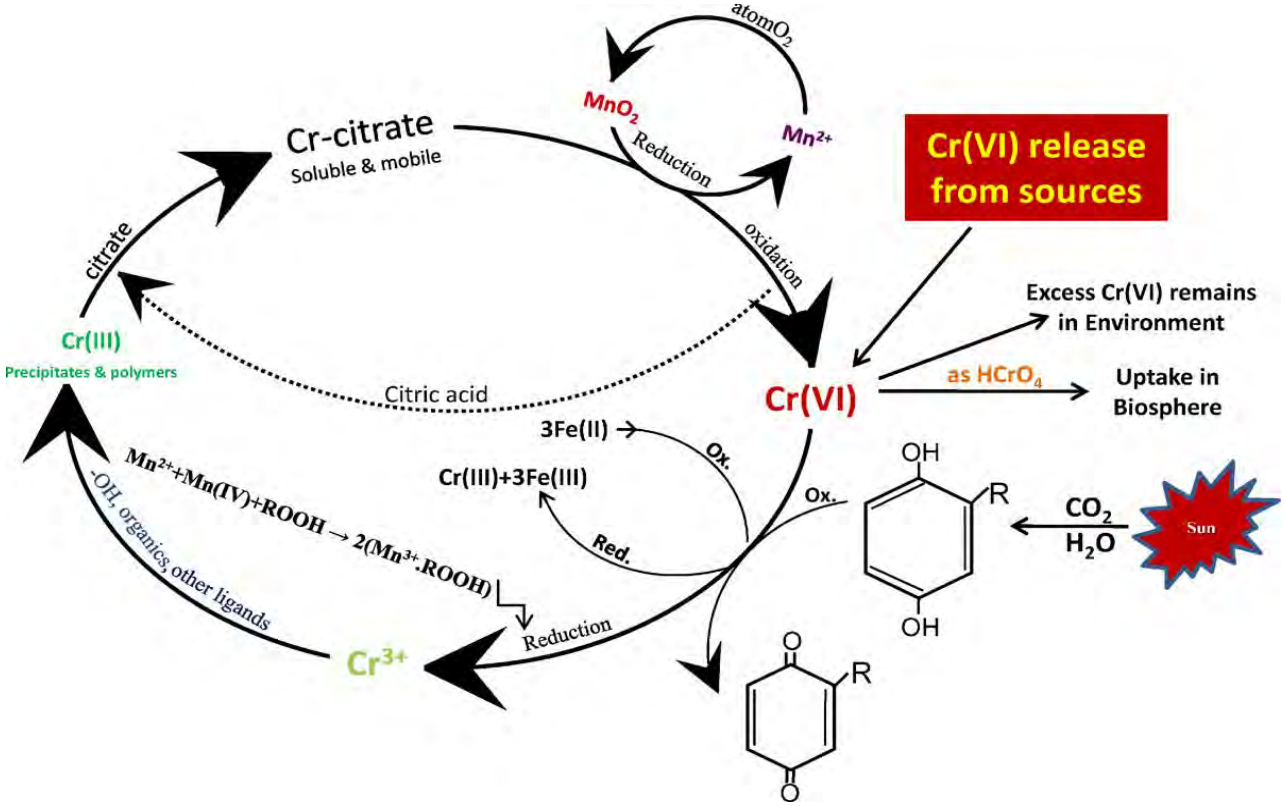

Figure 1: The natural chromium cycle in the environment.

(Image adapted from Dhal et al,2013).

1.3.5 Applications of chromium

Though chromium has a toxic effect on human health, it is used in various purpose in different sector. Chromium mainly used in the steel and alloys factories in the production of stainless steel. Chromium is also used in the leather tanning industries, wood preservation, paint production, paper pulp production,pigments,and ink production.(Focardiet al,2013). It is also used in metallurgy, refectories and chemical industries. For the making of bricks, mortar, gunning mixes chromite is used in the refectory industry.(Baldiris et al,2018). Chromium is also used in the production of the medical instrument which is mainly used by the dentist for the treatment purpose of teeth. Because of its high melting point, chromium is also used in the production of kitchen appliances. In the US and some other countries in the roadways chromium is used for the yellow lines that indicatevisitors lines.(EPA, 2010).

1.3.6 Regulation level of chromium

Chromium is a toxic and carcinogenic heavy metal for the human being and exceeds the level of chromium in the environment soil and water can be great hazards for the human. Because of the different industrial use of chromium, the contamination rate of chromium is increasing day by day. Ranges from 2000 and 5000 mg/L is the Cr concentration present in the tannery wastewater.But the bearable level is 2 mg/L for wastewater discharge.(Baldiris et al,2018). According to WHO, 50 µg/L is the maximum concentration that can be present in the drinking water and for the surface water it is 0.1 mg/l, above this can be considered as contamination.(Battashi et al,2016). At Oinofita area in Greece, the Cr concentration in drinking water is ranging from 41 to 156 ug/L which is highly contaminated.(Sun H. et al,2015)**.**All over the world mixing of Cr in the soil is observed 4.6 $x10^4$ metric $tonY^{-1}$. In the

remoteareas,abulkdeposition of chromium is below 0.2 $kgkm^{-2}Y^{-1}$, in rural areas it is 0.5–5 $kgkm^{-2}Y^{-1}$ and in urban areas it is more than 10 $kgkm^{-2}Y^{-1}$ (Saha et al,2011). 0.1mg/m^3 is the Permissible Exposure Limit (PEL) for the chromium according to the Occupational Safety and Health Administration (OSHA). According to NIOSH,the liability of Cr(VI) compound concentration in the airborne is limited to 0.2 µg Cr(VI)/m^3 for an 8 hour. (Baldiris et al,2018).

1.3.7 Chromium: A toxic heavy metal

1.3.7.1 Toxicity of chromium

Chromium in lower concentration is an important nutrient for the human that helps in the protein, lipid metabolism but in higher concentration chromium can create toxicity. From their different oxidative states, hexavalent chromium is more toxic and can cause carcinogenicity, mutagenicity, altered gene expression.(Battashi et al,2016). Continuous release of Cr from the modern agricultural system a large amount of Cr is introduced into the environment which mainly contaminated the soil and water and reduces the productivity of the soil. That actually reduce the quality of food product and vegetables that finallyaffect the human. Above the standard concentration presence of Cr in the soil can also affect the plant growth like reduce root growth, leaf chlorosis, prevent seed germination (JaishankarM.et al,2014).

On the basis of the report of IARC, hexavalent chromium is categorized as the carcinogen by the administration in the inhalation route.(Sun H. et al,2015). Because of its alikeness with the sulfate ion hexavalent Cr can penetrate the cell membrane through sulfate transport system which produces some intermediate such as Cr(V), Cr(IV), Cr (III) and free radicals react with the intracellular reductants like glutathione or ascorbatefor the time being inside the cell membrane. These all intermediate product produce Cr(VI) by electron exchange and form a complex form

with DNA which can prevent replication, transcription that occurs mutagenesis.(Joutey et al,2015) .

Cr(III) is less toxic and harmless because of its less membrane permeability. (JaishankarM.et al,2014). According to the United StatesEnvironmental Protection Agency (USEPA) for the Cr^{6+}chromium is one of the 17 hazardous chemicals.(Battashi et al,2016).

1.3.7.2 Toxicity effect in Human

Human exposure of chromium by inhalation from the air or working place is carcinogenic. People who are working in the chromate production industry or in chromate plating are prone to be effected in lung cancer. Irritation and inflammation is also a common disease for them.(Thompson et al,2013). Because of occupational purpose exposure of Cr(VI) ranging from 0.002–0.2mgCrVI/m^3 can cause a respiratory effect and severe nasal effect. (Salamaet al,2016). Cr(VI) exposure can alter the epigenetic modulation which is the cause of developing cancer. It mainly changes the gene expression and increases the DNA methylation that causesanerror in replication.(Sun H. et al,2015).

By the oral exposure, Cr(VI) can bring changes in the human hematological and biological parameters. A study showed that which is done in an 18 years old woman, exposure to potassium chromate can reduce the hematocrit and hemoglobin level but increases the white blood cell. Because of decreaseshematocrit, it causes microcytic and hypochromic anemia. Alteration in the activities of the liver enzyme is also happened because of Cr(VI) exposure by the oral route. (Sazakli.E et al,2014).

In human Cr^{6+}can also cause skin ulceration, asthma, dermatitis,andthecrack of nasal septum. Exposure of Cr(VI) can cause stomach damage that can cause cancer. Different lab analysis

showed that Cr(VI) toxicity reduces the male reproductive system and damages the fetus as well. (Joutey et al,2015). At Hubei in China, one study showed that the pregnant woman with a high level of maternal urinary Cr has a high risk of preterm birth.(Pan.X et al,2017).Chromium trioxide can increase bilirubin and serum lactic dehydrogenase(LDH) by the accidental ingestion of liquid which accommodates 300 $g \cdot L^{-1}$ chromium trioxide. (Sazakli.E et al,2014).

1.3.7.3 Toxicity effect in Animal

Hexavalent chromium has a toxic effect intheanimal as well.The different study shows that Cr(VI) has a carcinogenic effect in animal. In 2008, a study done by the NTP proves that Cr(VI) has the carcinogenic effect for both the mice and rates. For this study, they used F344/N rats and B6C3F1 mice and treated them with sodium dichromate dehydrate (SDD) in different concentration in oral routes with drinking water. Finally, they found that every mice and rats developed cancer in their oral cavity after 2 years. Tumors were also found in the small intestine of B6C3F1 mice. In rats, some other disease was observed like microcytic hypochromic anemia, epithelial hyperplasia in duodenum and jejunum(Sun H. et al,2015).

Chromium(VI) is also a great threat to the aquatic marine animal because of its high exposure in the water. It causes damaging of DNA and effecting on the reproductive system. It is proven from a study that Cr(VI) is cytotoxic for the sea turtle and whale cells.(Young et al,2015). It was established by a study that with a high dose of potassium dichromate(PDC) induced in intranasal route in rats, reduce the locomotor activity of the rats after 24 hours. (Salama et al,2016). Another study found that exposure of zinc chromate in 0.6 mg/ml concentration via the intranasal route in mice can cause peribronchiolar, alveolar and interstitial inflammation(Thompson et al,2013).

1.3.8 Pharmacokinetics of chromium:

1.3.8.1 Absorption:

Oral, dermal and inhalation route of administration is the main pathway for the absorption of chromium in the human body(Shekhawat et al,2015). We take chromium from diets as an inorganic or organic form but the hexavalent form is mainly induced by inhalation or from the anthropogenic activities. Absorption of Cr(VI) is higher than the Cr(III) because of its high solubility(Pechova and Pavlata,2007). Cr(VI) penetrate the cell membrane by facilitated diffusion method with the help of nonspecific anion channel, on the other hand, Cr(III) do it by passive diffusion(Shekhawat et al,2015). In our body Cr^{3+} mostly absorbed by the digestive system.(Pechova and Pavlata,2007). Throughout the body Cr concentration level is high in the liver,kidney,speelen and in the bone. Particle size, solubility,andoxidation state are the some factors that control the absorption of Cr in the body. Absorption also depends on the biomolecules present in the lungs(Shekhawat et al,2015). The presence of amino acids, the ascorbic acid,high carbohydrate, oxalate and aspirin levels in the diet help to increase the absorption of chromium in the human body. 40–240 μg/day is the stable condition for Cr absorption. (Pechova and Pavlata,2007)

1.3.8.2 Distribution:

Distribution of Cr is mainly depended on the chemical state and the dose of administration. But the reason behind the high concentration of Cr in the lung, speelen,and kidney is not known yet. It can be for overdose or physiological phenomenon. After the absorption Cr concentration is also found in the heart, pancreas and even in the brain. In ourbody Cr is highly present in the lung and is mostly from the airexposure(National Research Council,1974).

1.3.8.3 Metabolism:

After Absorption and distribution Cr transport through the blood by binding with the β-globulin plasma fraction for the metabolism process. After that Cr binds with the iron transport protein named transferrin to enters the tissue cell at the normal physiological concentration. This transferrin helps to increases the insulin level that ultimately helps the Cr to enter into the plasmatic membrane. Cr is released in the acidic pH after binding in the cell surface receptor with chromium-saturated transferrin. The release of Cr from different transferrin receptor separated the apochromodulin which produce the chromium-loaded chromodulin.Cr is rapidly absorbed by the bones from the blood and also stored in the kidneys, liver,andspeelen(Pechova and Pavlata,2007).

1.3.8.4 Excretion:

Urine and feces are the main two process of chromium excretion. 80% of the injected Cr is excreted through the urine pathway(National Research Council,1974).Cr is excreted with urine in the process of glomerular filtration or binding with low-molecular organic transporter. Some study said that 0.22 μg/day is the excreted amount of Cr through urine. Some amount of Cr is also excreted by the hair, perspiration,and bile. In stressful condition or in the Carbohydrate diet situation Cr release is increased 10-300 times than normal condition. Some factors like presence of chromium-oxide, chromium chloride, chromium acetate can change the Cr concentration that excreted with urine(Pechova and Pavlata,2007).

1.3.9 Carcinogenesis produced by Chromium

Cr is highly toxic and has a carcinogenic effect in human and enters the cell through sulfate or phosphate anion transporter. The potency of carcinogenicity of hexavalent chromium depends on the water-solubility complexes. With the help of ascorbic acid, GSH, and cysteine which are the reductive agent, Cr(VI) can damage the DNA. Cr(V),Cr(IV) are the intermediate products and Cr(III) is the most stable reactive form that is produced from Cr(VI) and these all things can bring changes in the DNA like single-strand breaks, DNA–amino acid cross-links, chromium–DNA adducts, formation of protein Cr(III) DNA cross-links which can induce carcinogenicity in the body. Cr(VI) in the zinc chromate has a high potentiality to produce carcinogenesis by breaking DNA double-strand(DSBs) and chromosome aberrations in WTHBF6 cells. By this same process lead chromate also induce carcinogenicity(Wang et al,2017).

Cr(VI) has the ability to produce oxidative stress and in the presence of endogenous superoxide anion and hydrogen peroxide, by the Haber-Weiss-like reactions, the intermediate product of hexavalent chromium produce hydroxyl radicals. Besides, it destroys cellular antioxidants and redox balance in the cell by the formation of Cr-Asc, Cr-GSH,and Cr-Cys crosslinks. Cytotoxicity and carcinogenicity produced in the oxidative stress by Cr(VI) and it depends on the ROS production. DNA generated lipid peroxidation is directly targeted by the high concentration of ROS which causes DNA damage by apoptosis and necrosis that can lead to cell death(Sun H. et al,2015).

Occupational Exposure of Cr has a major effect to cause cancer in the lung and sinonasal cavity. It is also the major cause of respiratory cancer. Exposure of Cr from the occupational site in the lung, can cause oxidative DNA damage in the *p53* gene, and also cause mutation in the adenine and guanine in the *p53* gene which leads to lung cancers(Wang et al,2017).Exposure of Cr(VI)

can also produce genomic changes. The microsatellite instability and chromosome aberrations are the reason behind the genomic changes in the biological system(Sun H. et al,2015). The different study shows that workers in the chromate industry have suffered from hypermethylation and MLH1 expression is reduced with the tumors formation. (Thompson et al,2013).

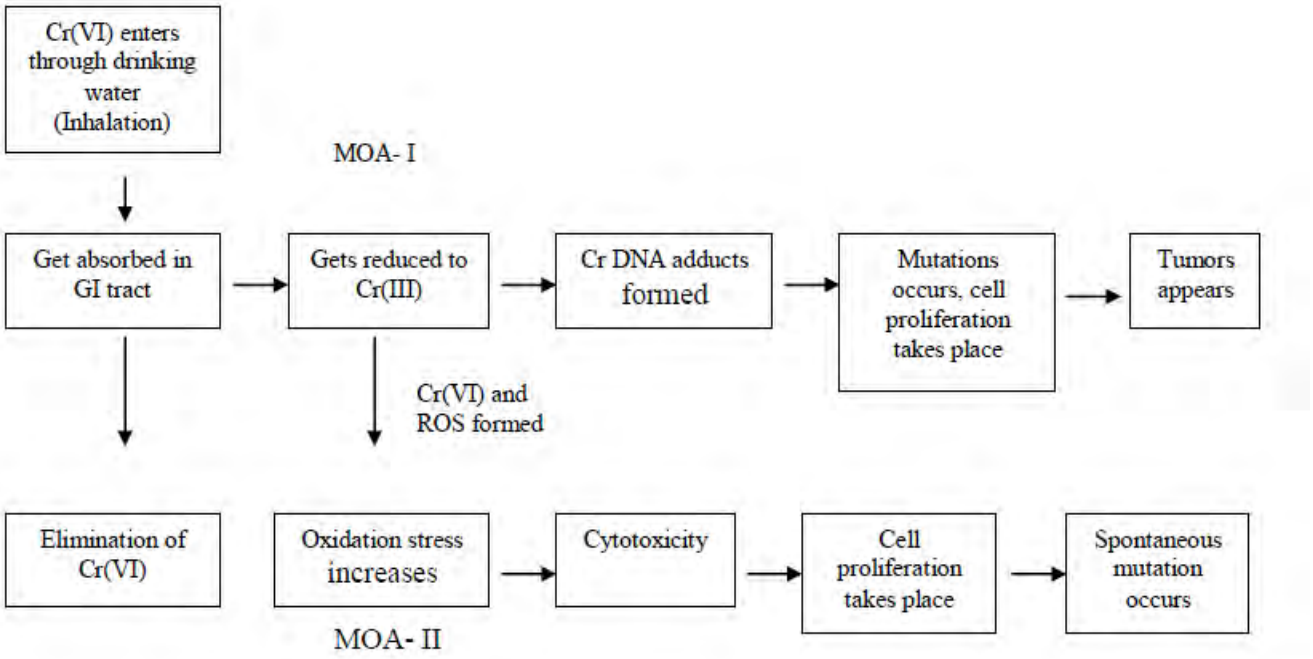

Figure 2:Mode of action of chromium to produce tumors.

(Image adapted from Shekhawat et al,2015).

1.3.10 Conventional methods for remediation of Chromium

Nowadays heavy metals pollution is one of the major concern in the world. From different anthropogenic activities, heavy metals get introduced into the environment which becomes dangerous for the environment and human life as well. This also the cause of the different disease and disorders happened in human life(Ibrahim & Mutawie, 2013).

For the reduction of Cr(VI) to Cr(III),there has various process. Most of the process done by the presence of an electron donor because Cr(VI) is a strong oxidant. Hexavalent chromium is most available in the water at the normal pH in the form of ions such as CrO_4^{2-}, $HCrO_4^-$ and $Cr_2O_7^{2-}$. These ions can easily reduce to Cr(III) in the presence of an electron donor. There is some established process for the remediation of Cr such as ion exchange, chemical precipitation, membrane filtration, electrochemical treatment etc. (Madhavi et al,2012).

1.3.10.1 Ion Exchange

Ion exchange is a physicochemical process that is widely used for chromium reduction because of its high efficiency, better treatment capacity and fast kinetics (Madhavi et al,2012). In this process for the separation of Cr(VI), various ion exchange resins are used. In column ion exchange, the anionic based resin is used which is in the hydroxide form (Kumar et al,2017). Some cationic resins are also used in this process and among theme strong acidic resins with sulphonic groups ($-SO_3H$) and weak acidic resins with carboxylic acid groups (-COOH) are most available. Hydrogen ion from both of the resins plays the role of the solution to passes the Cr metals in the exchange process. (Madhavi et al,2012).

$$nR\text{-}SO_3H + Mn^+ \rightarrow (R\text{-}SO_{3}\text{-})nMn^+ + nH+$$

$$nR\text{-}COOH + Mn^+ \rightarrow (R\text{-}COO\text{-})nMn^+ + nH+$$

When Cr contaminated water passing through the column resins bind with the Cr(VI) ion and separated the previous ion. Then reacting with NaOH, Cr(VI) changed into sodium chromate. After that resins beds again react with sodium chromate and form chromic acid which finally reacts with HCl acid for the regeneration of Cr. These resins are most effective in the normal pH range. Synthetic Dowex 2-X4 ion-exchange resin and Ambersep 132 are two most common resins used in the exchange process (Kumar et al,2017).

This process is commercially available for the removal of Cr(VI) from the Cr contaminated water for the recovery of Cr(III)(Hawley et al, 2004).

1.3.10.2 Activated carbon: used as an absorbent

For the heavy metals, pollution control and product purification activated carbon are used throughout the world. Because of their microporosity structure, it is used in the removal of Cr(VI) in chromium-contaminated water. According to their size and shape activated carbon are mainly four types:

1. Powder-activated carbon (PAC)
2. Granular-activated carbon (GAC)
3. Activated carbon fibrous (ACF)
4. Activated carbon cloth (ACC)

According to some study, different activated carbon hasa different capacity of Cr(VI) removal. PAC has the absorbent capacity of 390 mg/g at pH 2. Another study shows that GAC has the capacity of removal of Cr(VI) is 145 mg/g at pH range of 2.5–3.0. This absorbent capacity depends on the pretreatment of activated carbon. Chromium can be removed from the wastewater by using ACFs in the presence of copper metal and its capacity of the absorbent is 40 mg/g(Kumar et al,2017). This is an equilibrium process, so the absorbent capacity also depends on the Cr concentration in the contaminated water and the affinity of the carbon to the Cr. Furthermore, it also depends on some chemical bonding between the sorbents and Cr like ionic bonding, electrostatic attraction, and hydrogen bonding(Hawley et al, 2004).

1.3.10.3 Chemical Precipitation

This ismostly used and effective technique for the removal of Cr in the industry because of its simplicity and inexpensive to operate. In this chemical reduction process, Cr can be removed by two way and those are, in the acidic condition and as hydroxyl precipitation of Cr(III). Sodium sulfite, sodium metabisulphite, ferrous sulphate, barium sulphite are the reducing agent that is used in this technique (Madhavi et al,2012).

1.3.10.3.1 Hydroxide precipitation

In this process for the removal of Cr lime and limestone is used as the precipitating agent. in the presence of $Ca(OH)_2$ precipitation of Cr(III) is done in the pH of 8.7 and it also reduced the chromate concentration from 30mg/l. Ash-lime is used in this technique to increase the particle size of the precipitate which makes easy the process of Cr removal.

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

This process has some limitation like the presence of the complex agent in the wastewater can prevent the hydroxide precipitation formation, and also this process produce a large amount of sludge with is difficult to dispose of (Madhavi et al,2012).

1.3.10.3.2 Sulphide Precipitation

Sulphide precipitation process is similar to hydroxide precipitation but it is more advantageous than the previous one. This process has the ability to reduce the Cr(VI) into Cr(III) form following the same process that is needed for the metal precipitation. In the presence of the chelating agent, precipitation occurred without the change of pH.Sulphide precipitates are less

soluble than the hydroxide precipitates which make the separation process easier. Sulphide toxicity should be the major concern for this process(Madhavi et al,2012).

1.3.10.4 Membrane filtration

Membrane filtration means the separation of two phases by using a semi-permeable membrane which prevents the movement of ions. This separation process mainly depends on the particle size, electrical charge, solubility, diffusion coefficient(Madhavi et al,2012). According to pore size, membrane filtration can be divided into different section such as microfiltration, ultrafiltration(UF), nanofiltration (NF) and reverse osmosis (RO).

Between reverse osmosis and nanofiltration, recently nanofiltration is more popular because high pressure is needed to operate reverse osmosis. Thin film charged nanofiltration is used for the Cr(VI) removal and in this process, membrane surfacedrives away the Cr(VI) because of the negative charge of the surface(Hawley et al, 2004).

Removal of Cr(VI) from the wastewater, Polymer-enhanced ultrafiltration (PEUF)process is used.Presence of 17.5% polymer in the membrane increases the filtration rate of Cr(VI) and it can be up to 98%(Kumar et al,2017). Advantages of the PEUF process are high removal efficiency and high binding selectivity. In ultrafiltration, filtration is done according to the particle size. A study shows that membrane in ultrafiltration is modified by using NOx and used hydrazine hydrate for the Cr(VI) separation from wastewater have the two times more capacity to the separation of Cr(VI) than the unmodified membrane. Using chitosan also increases the membrane separation more than 6-10times(Madhavi et al,2012).Complexation-ultrafiltration is another technique where microligand bind with the heavy metals that make the metal bulky and separate from the wastewater(Madhavi et al,2012).

In reverse osmosis,thesemipermeable membrane is used and wastewater is passed through the membrane and heavy metal remain on the surface of the membrane. This process has a high efficiency to separate a large number of toxic metals(Kumar et al,2017).

1.3.10.5 Electrokinetics

This is also a Cr(VI) remediation process which is mainly used for the contaminated saturated and unsaturated soil. This remediation process is done by applying a low electric power between two or more anode and cathode.(Beretta et al,2018).Separation is done in two way:

a) Electromigration: According to the positive and negative charge attraction, ionic species are separated in a big amount.

b) Electroosmosis: All the neutral and ionic dissolved species is transferred from the anode to cathode electrode by fluid migration.(Beretta et al,2018).

50 to 150 V is applied in the electrokinetics remediation. Cr(VI) is gathered around the anode and transform into Cr(III) in the cathode. This is an in-situ process where an electrode is placed in the 3 to 5 m under the ground. But it can be applied as an ex-situ process where the electrode is placed in two different sides of the contaminated soil(Hawley et al, 2004).

There are some factors that increase the activity of electrokinetics remediation process such as, low cation exchange capacity, low salinity, low conductivity, and soluble Cr concentrations. A scientist named Reddy, with some of his friends, did an experiment with clay and glacial till the soil and found that Cr(VI) migration depends on the soluble fraction in both of the soil. But for Cr(III), it depended on the presence of soluble metal. (Hawley et al, 2004).

1.3.11 Bioremediation

Bioremediation is a process that is used natural and recombinant microorganism for the reduction of heavy metal toxicity from the environment. By this process, toxic metals transformed into non-toxic form by the intracellular accumulation or via enzymatic transformation.In order to make the compound non-toxic, bioremediation process using microorganism to change the oxidative state of the compound. This process mainly depends on the resistance profile of the microorganism to the heavy metals. (Mosa et al,2016). Besides this, bioremediation can change the solubility properties of the contaminant by changing pH, redox reaction or reducing absorption capacity of the contaminants. Chemically alteration of a toxic compound to nontoxic is done by a redox reaction especially for the As, Cr, Hg. Some contaminants removal process is dependent on the pH value, for example, the removal of pentachlorophenol(PCP) by enhancing the absorption capability of the *Aspergillusniger* and *Mycobacterium chlorophenolicum*fully dependent on pH value.(Ojuederie and Babalola,2017). Finally nowadays, for the removal of contaminants such as heavy metals, metalloids, radioactive waste, and oil products from polluted sites scientist used natural or modified microorganism in the bioremediation process as a continuous basis. (Mosa et al,2016).

1.3.11.1 Advantages of bioremediation

Nowadays for the heavy metal remediation, bioremediation process is widely used. In this study, we also used the bioremediation technique for the reduction of the hexavalent chromium to trivalent chromium that is non-toxic in nature. There have many reasons to use this technique. First of all, this technique is more eco-friendly and less expensive than the other remediation technique. (Mosa et al,2016). Furthermore, this technique can be administered at the actual site

where the contamination occurs and it has a low risk of exposure for the appointed personnel.(Mani and Kumar, 2013).

Besides this, it can remove contaminates permanently and actionability is longer than any other traditional process. This technique can be applied with some other physical or chemical process as well. Finally, it is an invasive technique which does not harm the environment.(Mani and Kumar, 2013).

1.3.11.2 Factors affecting bioremediation

Bioremediation process depends on some parameters which will affect the effectiveness of the process. These parameters are listed below.

- Nutrient: For the growth and cellular metabolism of the microorganism in the contaminated sites depends on the nutrient present in the sites. Organic carbon concentration should be high in the contaminant sites but because of microbial metabolism,this huge amount of carbon can also be diminished. For that supplemental nutrient like nitrogen, phosphate, potassium should be supplied in the sites for the proper microbial growth that can do the remediation. The ratio of carbon-nitrogen and carbon-phosphorus should remain10:1 and 30:1 respectively for bioremediation.
- pH range: pH is an important factor for the bioremediation technique. For the optimum growth of the microorganism pH range should be maintained between 5.5–8.0 and within this range microorganism can remove contaminates very effectively. (Mani and Kumar, 2013).

- Moisture content: Moisture content is an important factor for the growth of the microbe. Water should be present in the range of 12% to 25% in the environment for the optimum growth and proliferation of the microorganism.
- Contaminant concentration: Microbial activity can be affected by the contaminant concentration in the site. High concentration of contaminant can reduce the microbial activity and produce a toxic effect against the microorganism.(Adams et al.2015).
- Microbial diversity: Presence of various types of microorganism in the contaminated sites can increase theefficiency of the bioremediation process. Example of some microorganism is*Pseudomonas, Aeromonas, Flavobacteria, Chlorobacteria, Corynebacteria, Acinetobacter, Mycobacteria, Streptomyces, Bacilli, Arthrobacter, Aeromonas,*and*Cyanobacteria,* etc.
- Temperature: Biochemical reaction of microorganism depends on the temperature and its ideal range is 15-45˚C.
- Nature of the pollutants: Effectiveness of bioremediation process varies on the nature of the pollutants. Such as pollutants are a solid or semi-solid, organic or inorganic compound, heavy metals, polycyclic aromatic hydrocarbons these all affect the process. (Mani and Kumar, 2013).

1.3.11.3 Types of Bioremediation

Bioremediation technique mainly divided by their site of action. It can be applied in the actual contaminated site or draw out pollutants and treated it on another site. According to their site of action, bioremediation can be divided into two major groups. Such as 1)In-situ bioremediation 2)Ex-situ bioremediation.(Adams et al.2015).

Besides this, there has another major type of bioremediation which is called permeable reactive barrier. This technique is used to remediation of the groundwater.(Azubuikeet al.2016).

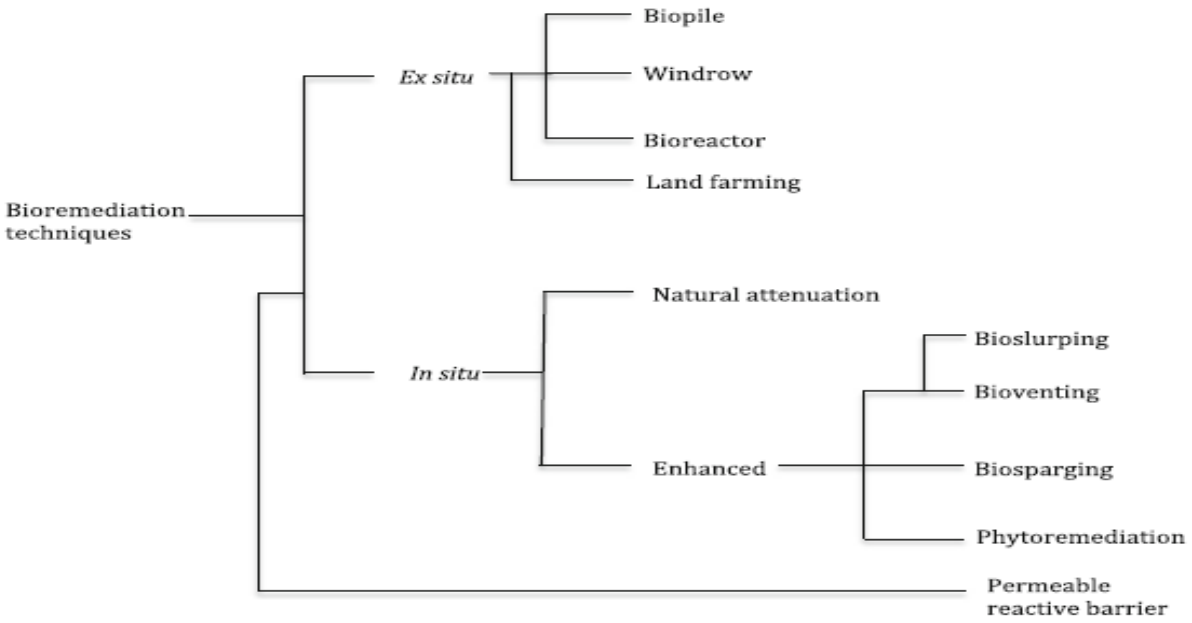

Figure 3: Types of Bioremediation Technique

(Image adapted from Azubuike et al,2016).

1.3.11.3.1 In situ bioremediation

This technique is applied in the contaminant sites for the reduction of pollutant. In this process, natural disturbance rate is very low because of no excavation of pollutants.(Azubuike et al,2016). Nutrients and oxygen should be supplied in liquid form to the bacteria for the remediation of contaminants.(Mani and Kumar, 2013).Pollutant sites like chlorinated solvents, dyes, heavy metals and hydrocarbon where in situ bioremediation technique is used.(Azubuike et al,2016).

In situ bioremediation further divided into two major groups such as natural attenuation and enhanced technique. (Azubuike et al,2016). Natural attenuation is also called intrinsic bioremediation. In this process,natural microorganism is used for the remediation process and to increase their activity nutrients and oxygen is supplied in the sites. In the enhanced technique modified microorganism is used for the remediation and introduced them in the contaminant sites with the proper physicochemical condition. For these process bacteria with rapid growthproperty should be more effective for the remediation.(Mani and Kumar,2013). Bioventing,

biospargingand phytoremediation are some enhanced technique that is used for the bioremediation.(Azubuike et al,2016).

Compare to the ex-situ process, in situ bioremediation is cheaper because it has no need totheexcavation of pollutants which is cost effective. Besides, it has no need for any kind of complicated machine.(Azubuike et al,2016). Production of dust and soil disruption also occurs in a very little amount. It has some limitation as well such as time-consuming, seasonal alteration of microbial activity and difficult to control in some cases.(Mani and Kumar, 2013).

1.3.11.3.2 Ex situ bioremediation

This technique is normally done by taking out the pollutants from the contaminants sites and treated them in another place.Biopile, land farming, bioreactor, windrow are some of the processes of ex situ bioremediation. (Azubuike et al,2016).

Biopile is used for the remediation of above ground polluted soil. In this process,soil is excavated from the sites and mixed them with nutrient amendments and used aeration system to increase the microbial activity. This process is suitable both for the aerobic and anaerobic

microorganism. (Mani and Kumar, 2013).Bioreactor is the process that produces some other products from the pollutant product by some biological reaction. According to their operating system,bioreactor can be different types such as Batch, fed-batch,sequencing batch, continuous and multistage. (Azubuike et al,2016). In-land farming, soil pollutants are removed by the biodegradative microorganism after excavating the soil from the pollutant sites. (Mani and Kumar, 2013).

1.3.12 Mechanism of Chromium reduction in Bacteria

Hexavalent chromium is the toxic heavy metals for the environment and human as well but the trivalent form of chromium is non-toxic. To reduce the toxic effect of chromium from the environment, changing the hexavalent chromium into trivalent form is the convenient way. Alkali precipitation, use of ion exchange and adsorption are some traditional mechanism that was used for the reduction of Cr^{6+} to Cr^{3+}. But the limitation of these process is that it produces a large amount of waste, more costly and in the presence of other anions these processesis less specific. For this reason, nowadays scientist chooses the remediation process by using microorganisms such as biotransformation and biosorption. For the transformation and absorption of heavy metals,scientistuse the potentiality of the microorganism and they considered as a better alternative.(Kanmani et al,2011).

Scientist use microorganism as a biotechnological tool for the remediation in the polluted area because of the need for less energy and toxic chemical reagents. Extracellular precipitation is a detoxification process of Cr in which Cr interact with the microorganism. Scientist found precipitation interaction with Cr and anaerobic*Clostridium* and sulfate-reducing bacteria. A transcriptional fusion ofchrchromate-resistance genes andluxreportergenes suggested as the chromate sensor that helps in the heavy metals resistance mechanism. (Cervantes et al,2001).

So it is clear that the transformation of Cr^{6+} to Cr^{3+} is a chromate detoxification process and not depend on the plasmid.(Viti et al,2013). Chromium reduction can be done by three main ways such as:

I. In aerobic condition: In the presence of oxygen chromium reduction is done by the soluble chromate reductases enzyme by the help of two cofactors NADH and NADPH. (Ramı´rez-Dı´az et al,2007).

II. In anaerobic condition: Under anaerobic condition bacteria acts as an electron donor and Cr(VI) as an electron acceptor. (Ramı´rez-Dı´az et al,2007). In this reduction process some compounds like carbohydrates, proteins, fats, hydrogen, NADH, NADPH supply electron to the Cr(VI).(Kanmani et al,2011).

III. Reductionis done by chemical reaction: In the presence of some redox intermediate organic compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione Cr(VI) reduction is done by the chemical reaction. (Ramı´rez-Dı´az et al,2007). It is an indirect process and the reaction mechanism is non-specific till now.(Viti et al,2013).

There isa various number of microbial species that have the ability of reduction of Cr(VI). The first enzyme that was used for the reduction of Cr(VI) to Cr(III) is Cr(VI) reductase from chromate-resistant Enterobacter cloacae HO1. this enzyme is membrane associated and with the NADH-dependent cytochromes, it reduced the Cr(VI) into Cr(III). According to the Ishibashi et al chromium reduction is the secondary function of the chromate reductaseenzyme. For an example, the enzyme nitroreductasesNfsA/NfsB from Vibrio harveyi has the ability to occupy a nitrofurazonenitroreductase which is its primary activity and Cr(VI) reduction is the secondary activity. (Ramı´rez-Dı´az et al,2007).

Scientist claimed that till now the best enzyme that is discovered for the Cr(VI) reduction is ChrR from *Pseudomonas putida.* It is a soluble flavin mononucleotide-binding enzyme and hexavalent chromium reduction is done by this enzyme which is a multistage process.(Viti et al,2013). ChrR is the ChrR-coding gene of the *P.putida*MK1 which was identified by the basis of sequence of amino acid in the N-terminal.(Das et al,2015). The activity of ChrR enzyme is NADH-dependent and has multiplefunctions such as reduction of quinones, prodrugs, Cr(VI), and U(VI) ions. In the full process of the reduction of Cr(VI) to Cr(III), chrR transferred one and two electrons from the Cr(VI) to produce an intermediate products Cr(V) which is not stable. ROS is produced from this unstable species by the reoxidation reaction. Finally,chrR transfer two electrons to change the hexavalent chromium into trivalent chromium which is non-toxic. During this reduction process, chrR of *P.putida* also shows quinonereductase activity and reduction of quinone produce quinols. These quinols neutralized the ROS that is produced by chrRreductase enzyme in the reduction process of Cr(VI). (Viti et al,2013).

Another enzyme named YieF from *Escherichia coli* has a similar sequence homology with theChrR enzyme from*P. putida*and also shows Cr(VI) reduction activity. Not only Cr(VI) this enzyme has also the reduction ability against ferricyanide, vanadium (V), molybdenum (VI), several quinones, 2,6-dichloroindophenol. By the help of the protein dimer (50 kDa), YieF enzyme reduces four-electron from the Cr(VI) in which three electrons for the production of Cr(III) and one electron is for the oxygen molecule generated by ROS. Because of the less amount of production of ROS, reduction process of Cr(VI) by the enzyme YieF from *Escherichia coli*is more effective towards chromium toxicity. (Ramı´rez-Dı´az et al,2007). Besides these, there have some other bacterial strains that have the Cr(VI) reduction ability such as *Vibrio fischeri, Aspergillusniger, Shewanella alga, Paracoccusdenitrificans, Pseudomonas*

sp., Bacillus subtilis, Bacillus sp., and Shewanellaoneidensis. (Long et al,2013).Under anaerobic condition, reduction of hexavalent chromium is done by the accepting of electrons from the carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves.(Das et al,2015).

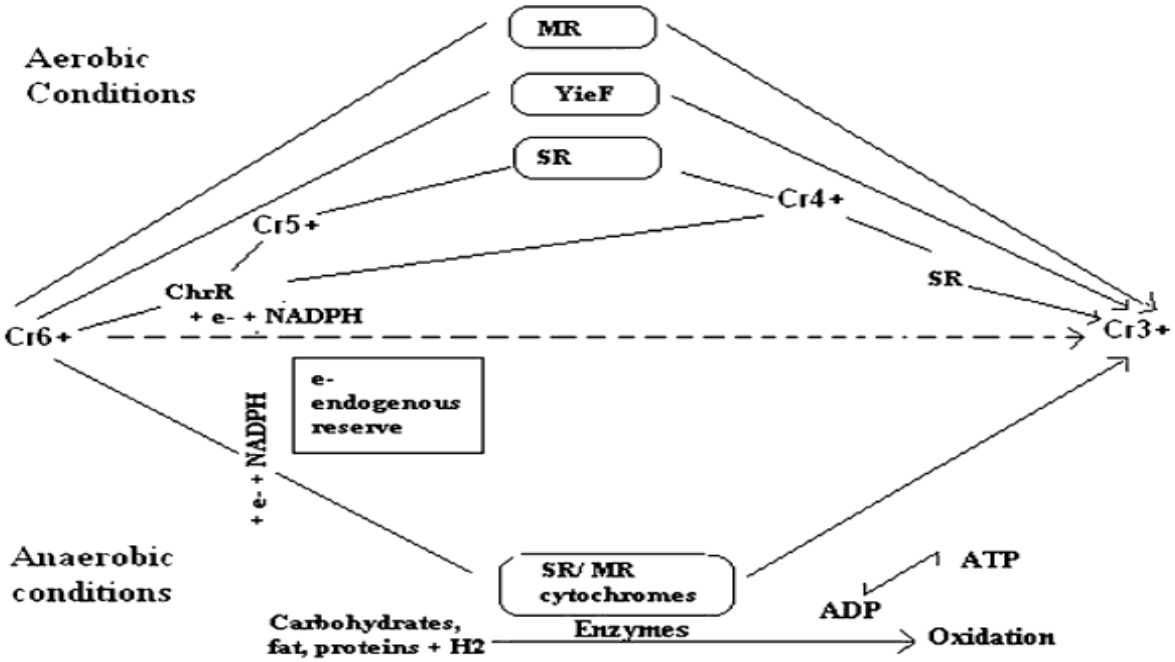

Figure 4: Mechanism of enzymatic reduction of Cr(VI) to Cr(III) in both aerobic and anaerobic condition.

(Image adapted from Kanmani et al,2011).

Chapter 2

Materials and Method

2.1 Introduction

In this part, the chemicals, reagents and equipments which is used in this study will be discussed. Besides that, in this section, the procedure of the full experiment will be discussed in details. Such as collection of the sample, different test which was done by the isolated sample, identification of the sample these all will be discussed.

2.2 Chemicals

All the chemicals or reagents that were used in this study is given below:

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

2.3. Glassware and instruments

Name of the instruments and glassware which was used in experiment that was done in this studyare given in table 2.

Table 2: Name of the instruments and their function

Name of instrument and glassware	Function
Autoclave machine	Sterilization
Incubator	Incubation of solid culture mediums
Digital Shaking incubator	Incubation of liquid culture mediums
Electric balance	Weight measurement
pH meter	pH measurement
Laminar Air Flow	To maintain the aseptic environment
Water Distillation Apparatus	Preparation of culture stock
UV-Vis Spectrophotometer	Measurement of absorbance
Electronic Centrifuge	Collection of supernatant
Micropipette	For withdrawing reagent and media in trace amount
SIEMENS Up-ride Freezer	For storing bacterial culture stock
Vortex Mixer	For proper mixing

2.4 Collection of sample

Bangladesh is surrounded by 230 small and large rivers and among these Buriganga is one of them (Uddin et al, 2018). Buriganga situated beside the southwest part of the Dhaka city. It is the most polluted river in Bangladesh. Dumping large amount of domestic and industrial waste make the river dead by hydrologically and biologically.Many tannery industriesare established on the bank of the buriganga without the proper drainage system. So all the waste is released in the river water (Mahmood et al,2017). Around 343 tannery is situated on the bank of the river buriganga and from these tanneries every day 21,600 square meters of wastewater is released to the river water. These tannery waste contains many heavy metals such as chromium, lead, Sulphur, Ammonium, Salts,etc which mixed with the river water and create pollution (Uddin et al, 2018). This untreated waste materials can mix with water, air, soil which can create dangerous hazard like health hazard, acid rain, global warming (Kibria et al,2015). In order to conduct the

experiment, we collected our samples from Buriganga River from three different locations: Showarighat, Pargandaria and Faridabadh. Water samples were collected from all three of the location and soil sample was collected from Showarighat and Pargandaria.

2.5 Isolation and subculture of sample

A standard protocol was followed for the isolation of microorganism from the collected sample. For the isolation purpose, inoculation of the sample was done in agar plates. The water sample firstly filtered with filter paper to remove the debris. Then 100µL water sample was added to the nutrient agar plates where K_2CrO_4 was mixed as a source of 2mM of Cr (VI). K_2CrO_4 wasused because only chromium resistant bacteria can grow. For soil sample, normal saline water (0.9% NaCl) was prepared and a small amount of soil was added to the saline water and 100µL from them was added to the agar plates. Then agar plates was kept for incubation at 37˚C for 24 hours. After 24 hours some bacterial colony was observed.

After that,2.8gm of agar was mixed in 100 ml water to prepare the agar plates. Then sterilization was done by running autoclave for 45 minutes at 121˚C under 15 Lb pressure. After that 15µL $K_2CrO_4$was added in the media and spread it into the petri dishes for solidifing. Isolated bacterial colony was taken by the help of asterilized toothpick and streaked on agar medium. Then the culture media was again kept for incubation for 24 hours at 37˚C. Specific strains that survived at this situation were selected for further inspection. 14 diffrentchromium resistant strain was isolated and mraked them with different letters like A, B, C,D, etc.

2.6 Stock Culture Preparation

For the stock culture preparation, 50% glycerol was prepared. To prepare 50% glycerol50 mL of water was added in 50mL of 100%glycerin. To sterilized the mixture, the solution was then autoclaved for 45 minutes at 121˚C, under 15Lb.pressure.The stock was to be stored in the eppendorff tubes. These tubes were also autoclaved to make sure no presence of microbes in the tubes.

To make Nutrient Broth, 2.8gm of nutrient broth was mixed to 100mL distilled water and placed it in the autoclave machine for the sterilization. After autoclaving, the bacteria to be stocked was taken from the subculture by using a loop and inoculated in the broth media. This was then placed in the shaking incubator at 37˚C overnight to incubate.

Next day, after keeping the culture overnight, 500 µL of glycerin was taken to an autoclaved eppendorfftube and then added 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 analyze the water sample, Diphenylcarbazide assay was used to estimate the hexavalent chromiumfollowing by standard procedure. (Greenberg et al., 1992).A standard curve was created to standardize the diminishment outline of the chromium reducing bacteria.

2.7.1 Preparation of chemicals

2.7.1.1 Preparation of 10mL 3M H_2SO_4

At first, a sterilized falcon tube was taken and transffered 8ml distilled water in it and added 1670µL of conc.H_2SO_4. Drop by drop it was added to water by using a micropipette. Finally, 330µL of distilled water was added to the solution to make thevolumeupto 10ml.

2.7.1.2 Preparation of DiphenylCarbazide

0.025gm of DPCZ was takenin a sterilized screw cap test tube. After that, 9.67ml of acetone was placed in the test tube and 330µl of 3M H_2SO_4 was also added. Finally, the tube was vortexed to make a consistent solution of DiphenylCarbazide. As DPCZ is light sensitive, testtube was wrapped by the aluminum foil to protect it from the contact of light.

2.7.1.3 Preparation of MOPS buffer

For the preparation of MOPS, we needed 1N NaOH for the pH adjustment purpose. So, at first 0.1 g NaOH was added in 50ml water to make 1N solution. After that, for 20mM MOPS preparation, 334.88mg MOPS powder was placed in a conical flask. 80ml of distilled water was mixed to it. After that, the MOPS buffer pH changed into constant to 7 by adding 1N NaOH dropwise within the buffer solutionand the pH was measured by a pH meter.

2.7.1.4 Preparation of 10mL 5mM Potassium Chromate

1.94gm of potassium chromate was taken in a falcon tube and 10ml of distilled water was added to it and vortex the solution for proper mixing. 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

For the standard curve, various sample solution was prepared in different concentration. The final amount of each sample is 1ml.

Table 3: Sample preparation for standard curve

Final Concentration (µM)	Amount of 5mM potassium chromate solution (µL)	Quantity of nutrient broth added (µL)	Final volume of 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 procedure for standard curve

Firstly, 600 µL of the sample was positioned right into a falcon tube.Then, 1200 µL, 20mM of MOPS, 99 µL of 3M H_2SO_4, 981µL distilled water and lastly 120µL DPCZ were placed

respectively to the sample solution. After that, the solution in the test tube was shaken and the reaction took place. After compliting the reaction, white color solution was changed to purple. Finally, the absorbance was taken at 540nm wavelength through UV-Visible spectrophotometer.

2.7.2.2 Evaluation of reduction profile of selected isolates

2.7.2.2.1 Procedure

Day 0:

First of all, six conical flasks were taken and prepared two 10ml and four 25ml nutrient broth media. 0.13gm and 0.325gm of nutrient broth were taken and added 10ml and 25ml water to make 10ml and 25ml broth media respectively. After that, between the four 25ml nutrient broth, two was taken to adjust the pH 8.5 and 5.5 by adding 1N NaOH and 1N HCl respectively. Then all the nutrient broth and glassware was placed in the autoclave machine for sterilization. After autoclaving all the things, 10ml broth media was kept for cooling for a few minutes. Then, with the help of a loop, bacteria was added to one of the 10ml broth media from the previously made stock solution and placed it in the shaking incubator at 37˚C for overnight at 120rpm. All the other things were kept inside the laminar flow.

Day 1:

The next day, 15µL of K_2CrO_4 was added to the all 25ml conical flask to make the 600µL potassium chromate (K_2CrO_4) solution. Then, 2mL of sample was taken from previous day's 10ml flask into a falcon tube and the absorbance was collected at 600nm through UV-spectrometer. After that calculation became completed to discover the amount that to be transferred into the 25ml culture from the 10ml culture to get 0.2 OD (Optical density). Then the

quantity discovers from the calculation was taken into 3 falcon tubes from the 10ml culture and centrifuged them for 5 minutes for the separation of cell from media and settled at the bottom.Collected the supernatant from the top and added same calculated amount of nutrient broth from the 25ml conical flask with the cell and vortex it for the proper mixing. Then all three of them were transferred into the three 25ml conical flasks which were marked as pH 5.5, 7, 8.5 from flacon tube. The otherone of 25ml was used as blank. After that, 2ml solution was taken from the all four conical flasks into four falcon tube and conical flasks were kept into the shaking incubator for the bacterial growth in the hexavalent Chromium condition. Then, with the 2ml solution absorbance was taken at 600nm in UV spectrometer which contains the bacteria sample. This process was continued throughout the day after every 1.5 hours for both the sample and blank as well and cell growth was measured by taking aborbance. For the collection of supernatant, centrifuged was done. With the supernatant the reaction was oocured to find out the chromium level.This process become persisted to the next day by using retaining the 25ml solution within the incubator overnight to see its overnight reduction of chromium. The entire system turned into repeated at three extraordinary temperatures: 25°C, 37°C and 42°C underneath three pH conditions of 5.5, 7 and 8.5.

2.8 Analyze the type of the enzyme: (Exo or Endo)

Day 0:

To determine the working type of the enzyme such as exo enzyme or endo enzyme, first of all 10ml nutrient broth was made in a conical flask and the isolated bacterial sample was inoculated into the broth media. Then the conical flask was kept into the shaking incubator at 37˚C for 24 hours.

Day 1:

The next day, two 25ml nutrient broth was made in two conical flasks. $K_2CrO_4$was added in one 25ml conical flask and another one was kept without K_2CrO_4. Then from the previous 10ml incubated bacterial sample, 2ml was withdrawn in a sterilized falcon tube to measure the absorbance at 600nm. According to the absorbance value calculated the amount that was transferred to both 25ml broth media from the 10ml flask to get 0.2 OD (Optical density). Then, both the 25ml conical flask was kept into the shaking incubator at 37˚C for overnight.

Day 2:

From both the 25ml conical flask, all the incubated sample was taken in falcon tube and centrifuged at 4000rpm for 5 minutes. Then supernatant was collected from the falcon tube and syringe filtration was done of the supernatant using membrane sieve of 0.45micron pore size and with and without chromium sample was kept into two sterilized conical flask. After that, 15µl $K_2CrO_4$was added in both the flask to make 600µL potassium chromate (K_2CrO_4) solution. Then, after every 5 minutes, absorbance was taken by withdrawing 2ml sample from both flasks till first 30 minutes and after that one reading was taken 30 minutes later and the next two was taken in 1hours difference. The absorbance was measured at 540nm.

2.9 Antibiotic resistance profiling of Chromium resistant bacteria

2.9.1 Strain culture preparation in nutrient broth (NB)

For the antibiotic resistance profiling, 10ml nutrient broth was prepared in a conical flask and the isolated bacteria were inoculated into the broth media. The conical flask was then kept into the shaking incubator at 37˚C for 24 hours at 120rpm. After 24 hours, the next day 2ml sample was

withdrawn from the conical flask and placed in a falcon tube. After that, absorbance was measured at 600nm wavelength. Finally calculated the amount that transferred to another 10ml media to make 0.5 McFarland suspensions.

2.9.2 Inoculation of test plates

For the preparation of plates, Muller Hinton Agar (MHA) was used. A cotton swab was used to swipe the sample culture. All the plates and cotton were autoclaved before for the sterilization. The cotton swab was dipped into the incubated strains of 0.5 McFarland suspension of culture and spread over the MHA plates surface. It was ensured that spreading was done all over the surface area of the media in the plate.

2.9.3 Application of antibiotic discs

To see antibiotic resistance profiling,14 antibiotic discs were used on the inoculated plates. Each plate contained 4 to 5 discs. The antibiotic discs that were used over the plate to see the antibiotic resistance profile are listed in Table 4 below:

Table 4: Antibiotics used in antibiotic resistance profiling

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

2.9.4 Incubation

After setting all the discs over the medium on the plate, all of them were kept in the incubator within 15 minutes at 37˚C for 24 hours.

2.10 Minimum Inhibitory Concentration (MIC) determination

Minimum inhibitory concentration(MIC) means the lowest concentration of heavy metals that inhibit the growth of bacteria (Baz.S et al,2014). To determine the MIC, bacteria was incubated in the nutrient broth media at different concentration of potassium chromate (5mM to 30mM). Before incubation of bacteria, 10 times serial dilution was done of the bacteria in saline water.

50µL of the diluted bacterial culture in saline was added to the nutrient broth by using a micropipette and all the test tubes that contained the bacterial culture was kept into the shaking incubator at 37˚C for 24 hours. The next day, absorbance was measured at 600nm of the incubated culture to determine the growth. According to the absorbance, no bacterial growth in a particular concentration was the minimum inhibitory concentration for that particular bacteria.

2.11 16S rDNA sequencing of sample D2

The isolated bacterial sample was identified by using 16S rDNA sequencing. Different software was used in the identification purpose. First of all, the chromatogram which was obtained from 16S rDNA sequencing opened by FinchTV and purified the sequencing data. Then, saved the purified data as FASTA file and BLASTwas performed of the purified sequence.From the result of BLAST similar microbial strain was found. Selected and downloaded some bacterial strain according to the similarity. All the sequence was compiled in FASTA format and then used BioEdit to check any kind of deletion. Finally, MEGA 7 was used for the construction of phylogenetic tree to analyze the origin of the bacteria.

Chapter 3

Results and Discussion

3.1 Isolation data of Chromium resistant bacteria

The nutrient agar plates contained chromium and isolation was done from these plates for individual colonies. Isolated bacteria was given different identical label such as D.

3.2 Chromium reduction profile of Chromium resistant bacteria

3.2.1 Standard Curve

According to the method stated in 2.7.2.1.2 standard curve was obtained. After that, values of the absorbance were plotted against the concentration of Chromium in micromole and graph was created by using Microsoft Excel Software. The obtained results are given below in Table 5.

Table 5: Chromium concentration with the corresponding absorbance

Cr Concentration (μM)	Absorbance at540nm
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 obtained data above, the standard curve was created which is shown below:

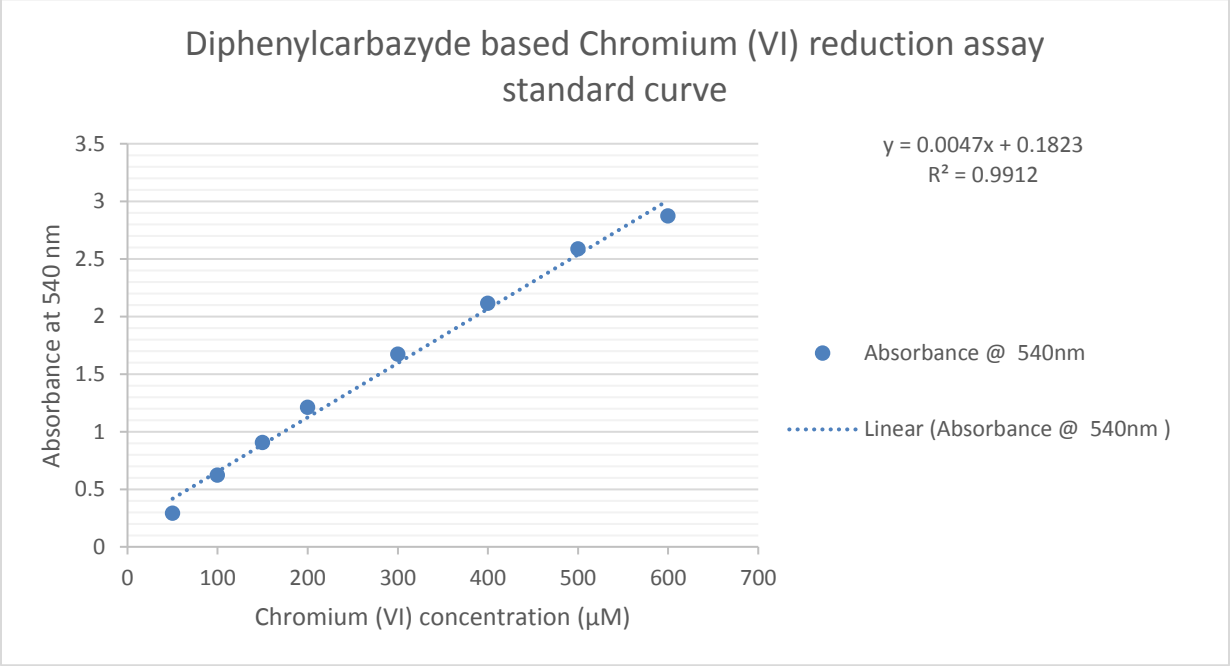

Figure 5: Standard curve of hexavalent Chromium

3.2.2 Reduction profile of isolate D2

Reduction profile or Bioassay was done in three different temperature such as 25˚C, 37˚C, and 42˚C and for each temperature three different pH was maintained such as pH 5.5, 7, and 8.5. From these experiments, the obtained results and graph was given in the subsection below.

3.2.2.1 Reduction profile at 25°C, pH 5.5

Table 6: Isolate D2: Chromium reduction profile vs. cell growth at 25°C, pH 5.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	520.4326241	0.203	490.0070922	0.002
1.5	508.1631206	0.19	505.4680851	0.004
3	422.6312057	0.315	512.3475177	0.002
4.5	366.4609929	0.533	486.7446809	0
6	272.7021277	0.935	489.7234043	0.001
7.5	250.1489362	1.117	490.5035461	0.001
24	170.1489362	1.563	490.5035461	0.001

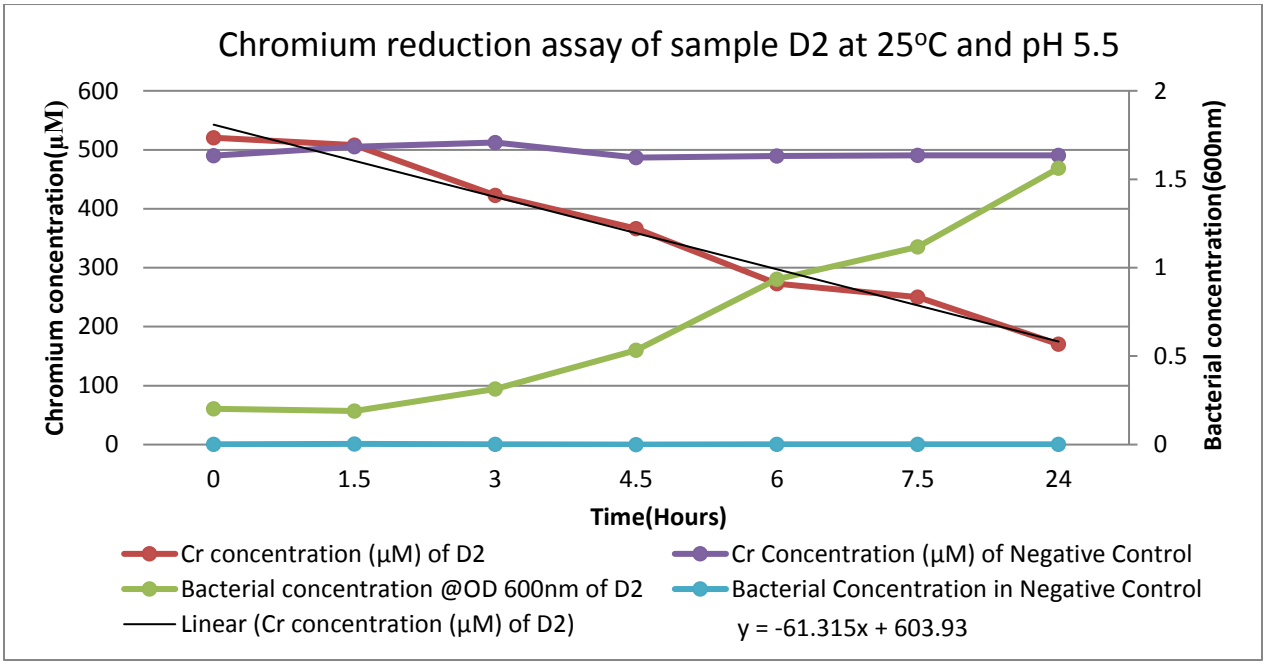

Figure 6: Chromium reduction assay of sample D2 at 25°C and pH 5.5

From figure 6, it can be found that chromium reduction was done and concentration reduced from 520.432µm to 170.148µm. The reduction is almost linear throughout the process. After 24 hours the total reduction of the chromium concentration was 68%. On the other hand, bacterial concentration increased from 0.203µm to 1.563 after 24 hours. In the negative control, no bacterial concentration was found and no reduction of chromium took place.

3.2.2.2 Reduction profile at 25°C, pH 7

Table 7: Isolate D2: Chromium reduction profile vs. cell growth at 25°C, pH 7

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	551	0.187	490.0070922	0.002
1.5	524.6879433	0.227	505.4680851	0.004
3	414.6879433	0.448	512.3475177	0.002
4.5	297.4539007	0.898	486.7446809	0
6	192.6312057	1.16	489.7234043	0.001
7.5	131.9929078	1.48	490.5035461	0.001
24	25.32624113	1.973	490.5035461	0.001

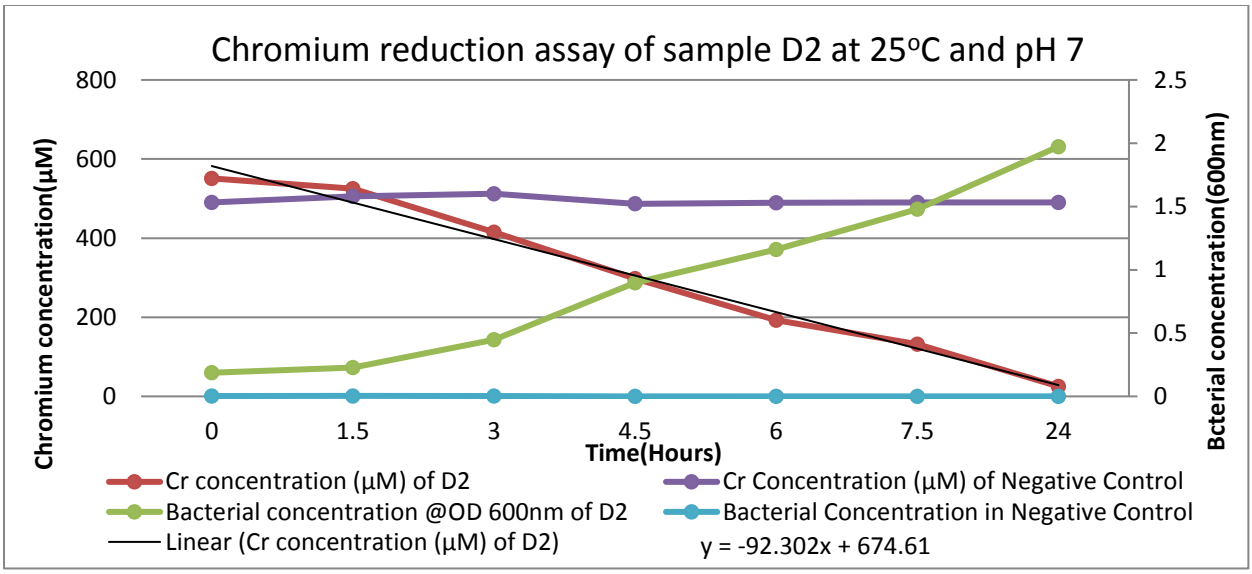

Figure 7: Chromium reduction assay of sample D2 at 25°C and pH 7

From figure 7, it can be observed that chromium reduction was done from524.687µM to 25.326µM till 24 hours. The reduction was almost linear after 3 hours till 24 hours. Total reduction percentage was 96%. With the reduction of chromium concentration, bacterial concentration increased from 0.187 to 1.973. In the negative control, chromium concentration was constant throughout the process till 24 hours and from 0 hours to 24 hours, no bacterial concentration was found in the negative control.

3.2.2.3 Reduction profile at 25°C, pH 8.5

Table 8: Isolate D2: Chromium reduction profile vs. cell growth at 25°C, pH 8.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	529.4397163	0.179	490.0070922	0.002
1.5	537.5957447	0.188	505.4680851	0.004
3	485.6808511	0.38	512.3475177	0.002
4.5	340.1489362	0.761	486.7446809	0
6	257.8794326	1.19	489.7234043	0.001
7.5	181.7092199	1.583	490.5035461	0.001
24	95.11347518	1.924	490.5035461	0.001

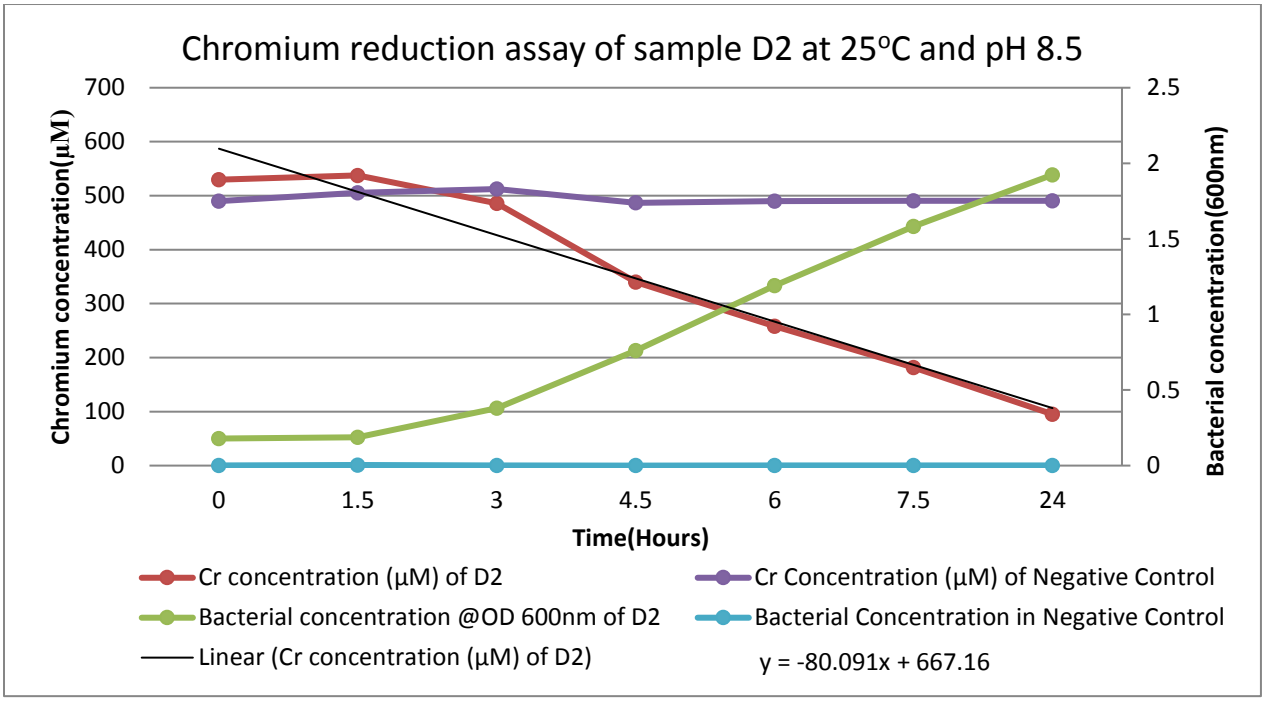

Figure 8: Chromium reduction assay of sample D2 at 25°C and pH 8.5

From figure 8, it can be obtained that from 0 hours to 24 hours chromium concentration was reduced and it starts from 529.439µM and ends at 95.113µM.The total reduction was 83%. Till 4.5 hours the reduction profile was not linear but from 4.5 hours to 24 hours the reduction was almost linear. Bacterial concentration increased with the time and from 0 to 24 hour it was 0.179 to 1.924. In the negative control, bacterial concentration was none 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 9: Isolate D2: Chromium reduction profile vs. cell growth at 37°C, pH 5.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	545.0425532	0.088	529.1560284	0.001
1.5	512.9148936	0.147	529.0141844	0.006
3	420.787234	0.415	513.1985816	0.005
4.5	359.0141844	0.78	523.2695035	0.007
6	287.7375887	1.239	519.8652482	0.005
7.5	247.3829787	1.462	521.5673759	0.005
24	166.7446809	1.881	522.9148936	0.001

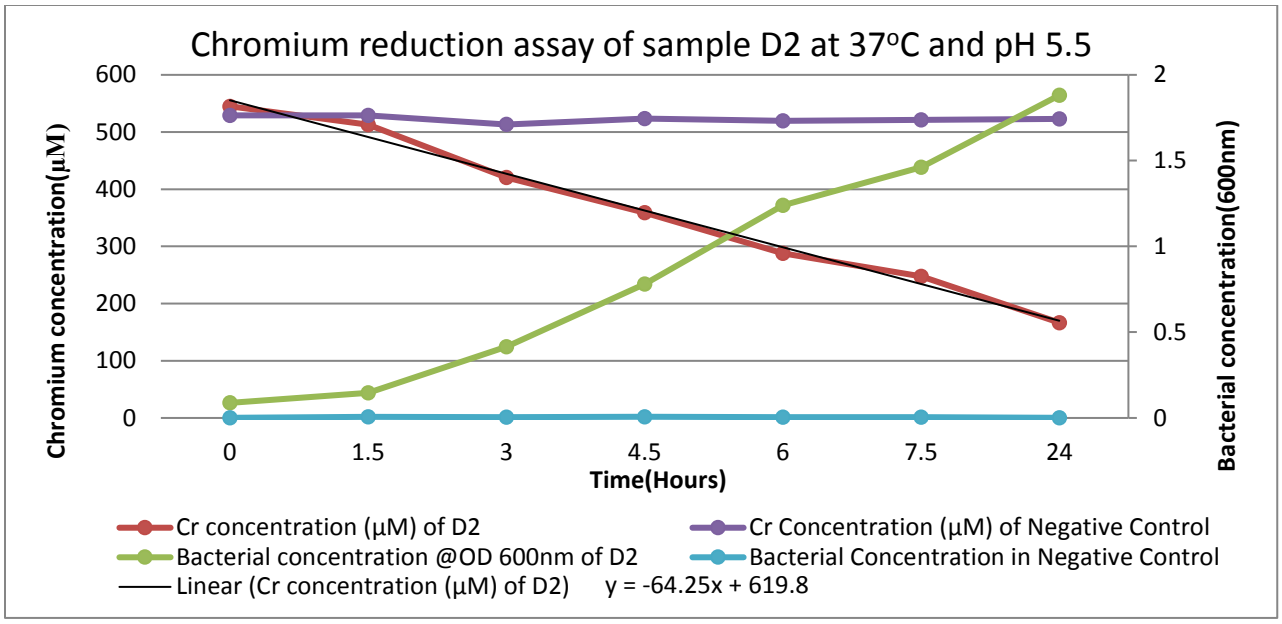

Figure 9: Chromium reduction assay of sample D2 at 37°C and pH 5.5

From figure 9, it is seen that chromium reduction was done in a small amount in the first 1.5 hours but after that, the reduction was almost linear throughout the process till 24 hours. At 7.5 hours the Cr concentration was 247.382µM and in overnight, after 24 hours it reduced to 166.744µM. On the other hand, bacterial concentration increased continuously with time. At 0 hours the concentration was 0.088 and after overnight, it was 1.881. In the negative control, bacterial growth was zero and chromium concentration remained same throughout the process which means no reduction was done because of the absence of bacteria.

3.2.2.5 Reduction profile at 37°C, pH 7

Table 10:Isolate D2: Chromium reduction profile vs. cell growth at 37°C, pH 7

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	489.3687943	0.139	529.1560284	0.001
1.5	432.5602837	0.354	529.0141844	0.006
3	370.787234	0.505	513.1985816	0.005
4.5	257.0992908	1.12	523.2695035	0.007
6	210.5744681	1.63	519.8652482	0.005
7.5	120.6453901	1.924	521.5673759	0.005
24	32.27659574	2.143	522.9148936	0.001

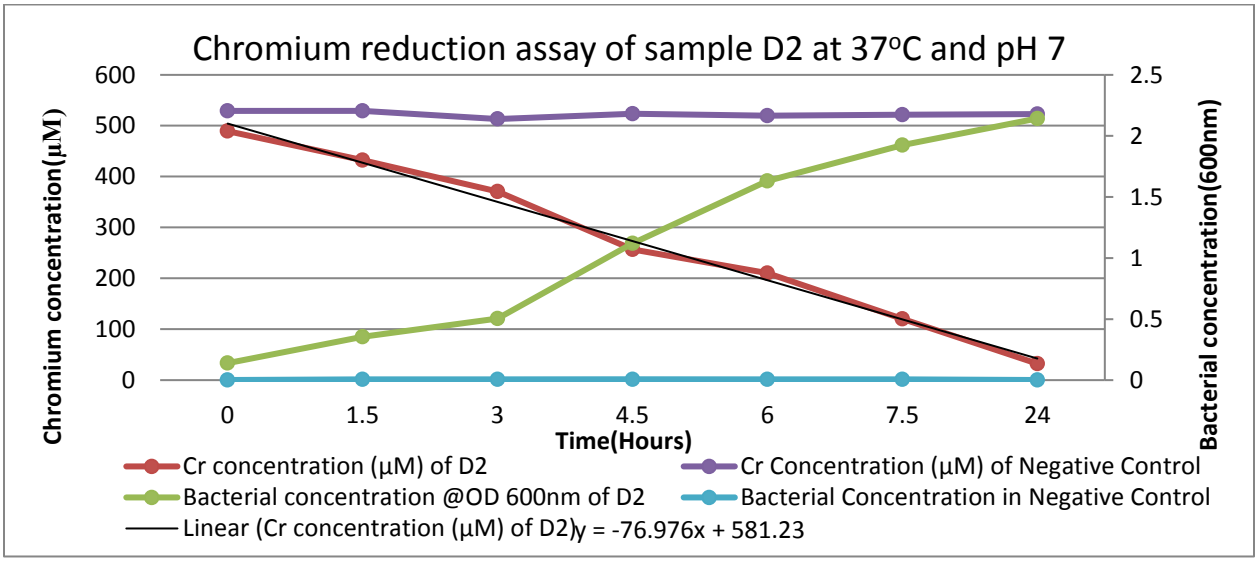

Figure 10: Chromium reduction assay of sample D2 at 37°C and pH 7

From figure 10, it can be seen that the reduction of chromium was almost linear from 0 hours to 24 hours. At 0 hours it was 489.368µM and after 24 hours it reduced to 32.276µM. The total reduction was 94% which means almost the full amount of chromium was reduced. Bacterial growth also increased with the reduction of chromium. At 0 hours the growth was 0.139 and at 7.5 hours it increased to 1.924. After overnight it slightly increased to 2.143. In the negative control, no bacterial growth was observed and no reduction to be seen because of the absence of bacteria.

3.2.2.6 Reduction profile at 37°C, pH 8.5

Table 11: Isolate D2: Chromium reduction profile vs. cell growth at 37°C, pH 8.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	499.9361702	0.114	529.1560284	0.001
1.5	479.1560284	0.361	529.0141844	0.006
3	400.5744681	0.52	513.1985816	0.005
4.5	308.8723404	1.263	523.2695035	0.007
6	151.070922	1.934	519.8652482	0.005
7.5	37.17021277	2.138	521.5673759	0.005
24	-6.5177305	2.42	522.9148936	0.001

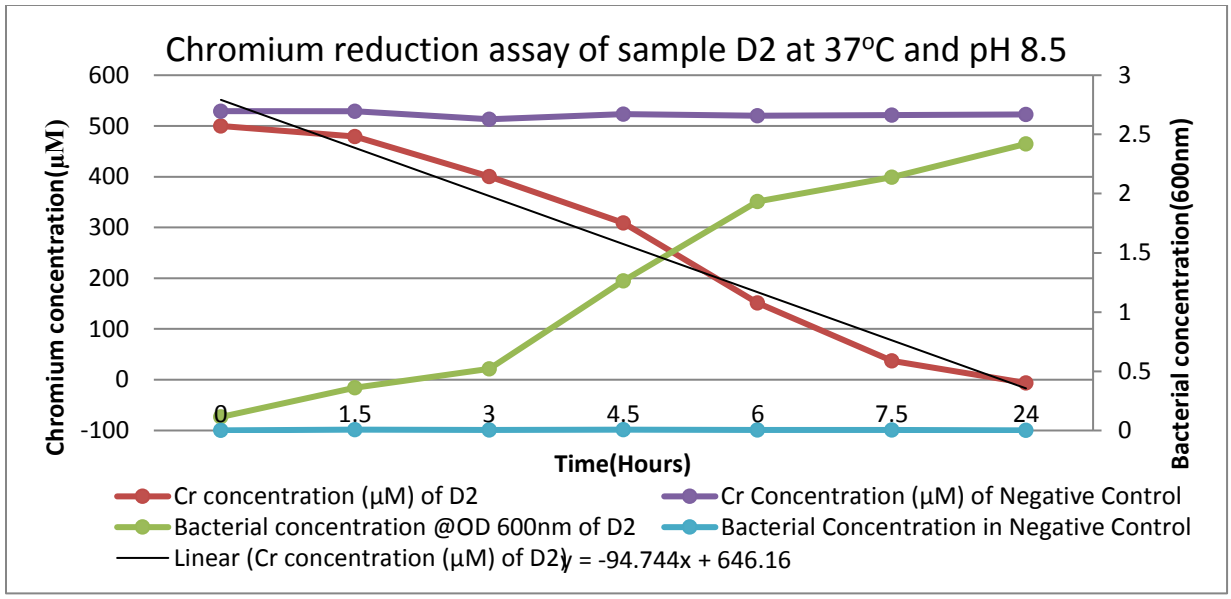

Figure 11: Chromium reduction assay of sample D2 at 37°C and pH 8.5

From figure 11, it can be observed that in the first three hours reduction rate was low and after that reduction rate increased. After 7.5 hours it became 37.170µM and after overnight at 24 hours, fully 100% reduction was done. Bacterial growth was also high at this temperature and pH. At the starting point, it was 0.114 and after 24 hours it increased to 2.42. In the negative control, chromium concentration remained constant and no reduction was observed and no bacterial growth was found.

3.2.2.7 Reduction profile at 42°C, pH 5.5

Table 12:Isolate D2: Chromium reduction profile vs. cell growth at 42°C, pH 5.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	572.9148936	0.197	562.4893617	0.006
1.5	544.5460993	0.291	522.4184397	0.003
3	490.0070922	0.472	527.2411348	0.003
4.5	423.1985816	0.811	526.6737589	0.005
6	369.7234043	0.992	532.6312057	0.001
7.5	297.3120567	1.108	530.4326241	0.001
24	133.6950355	1.774	533.9787234	0.001

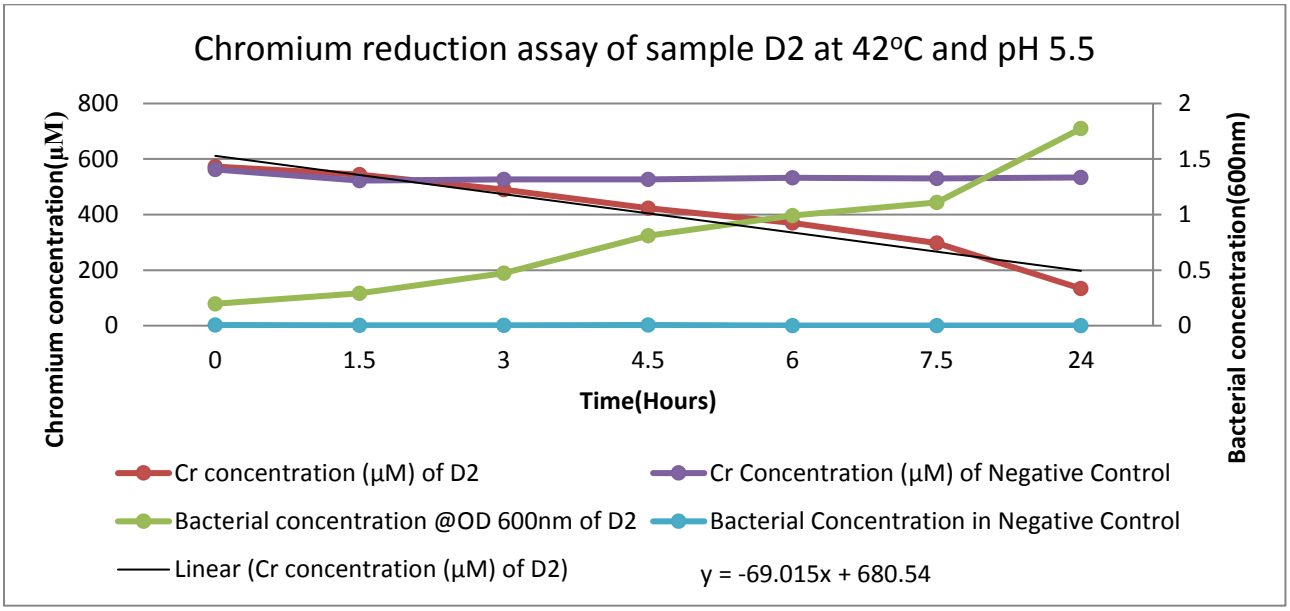

Figure 12: Chromium reduction assay of sample D2 at 42°C and pH 5.5

From figure 12, it can be said that from 0 hours to first 6 hours reduction was slightly low and at 6 hours it was 369.723µM. After that reduction rate increased and at 24 hours it became 133.695µM. The reduction was almost linear and the total reduction was 77%. Bacterial growth also increased and till 7.5 hours it was 1.108 and after overnight, it increased to 1.774. In negative control bacterial growth was none and no reduction was observed because of no bacterial growth.

3.2.2.8 Reduction profile at 42°C, pH 7

Table 13: Isolate D2: Chromium reduction profile vs. cell growth at 42°C, pH 7

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	546.3900709	0.182	562.4893617	0.006
1.5	530.5035461	0.305	522.4184397	0.003
3	474.6879433	0.667	527.2411348	0.003
4.5	391.4964539	0.996	526.6737589	0.005
6	355.1134752	1.138	532.6312057	0.001
7.5	233.9078014	1.482	530.4326241	0.001
24	87.5248227	1.982	533.9787234	0.001

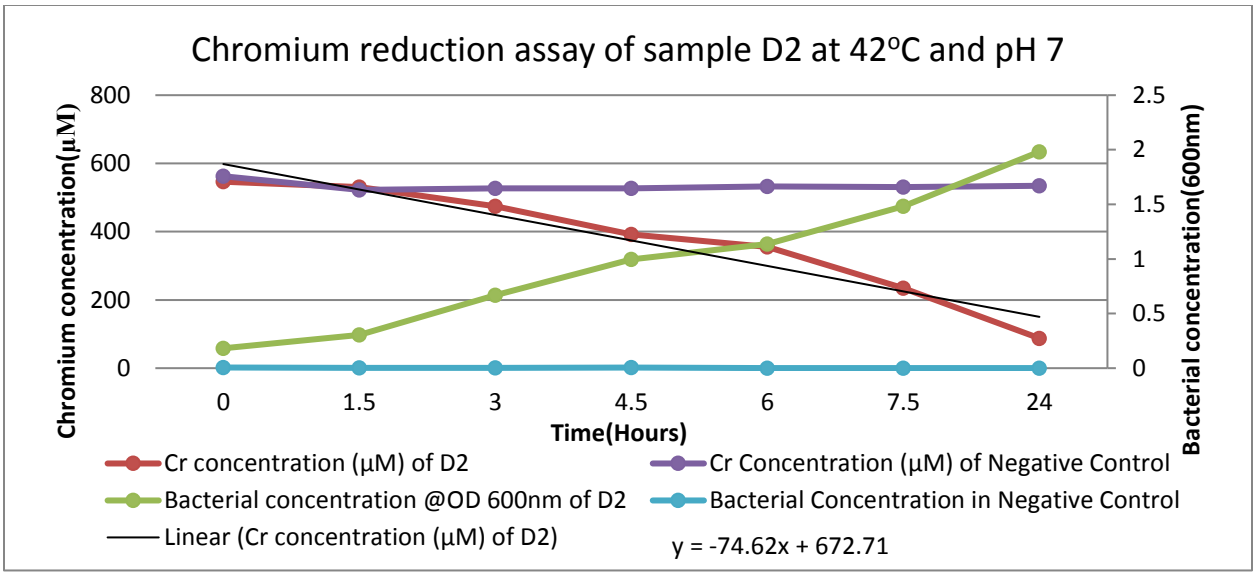

Figure 13: Chromium reduction assay of sample D2 at 42°C and pH 7

From figure 13, it can be seen that the first 1.5 hours chromium reduction rate was slow but after that, the reduction rate increased. After 3 hours, chromium concentration was 474.687µM and till 7.5 hours it reduced to 233.907µM. But after overnight chromium concentration drastically reduced to 87.524µM. The total reduction was 84%. On the other hand, bacterial concentration increased with time. At 0 hours it was 0.182 and after overnight, it increased to 1.982. In negative control, chromium reduction remained constant because there has no bcaterial growth.

3.2.2.9 Reduction profile at 42°C, pH 8.5

Table 14: Isolate D2: Chromium reduction profile vs. cell growth at 42°C, pH 8.5

	Sample		Negative Control	
Time (Hours)	Cr concentration (µM) at 540nm with D2	Bacterial concentrationat 600nm of D2	Cr Concentration (µM) in Negative Control	Bacterial concentration in Negative Control
0	584.4042553	0.18	562.4893617	0.006
1.5	575.1134752	0.283	522.4184397	0.003
3	488.8723404	0.62	527.2411348	0.003
4.5	433.9078014	0.968	526.6737589	0.005
6	366.7446809	1.067	532.6312057	0.001
7.5	329.7234043	1.116	530.4326241	0.001
24	136.035461	1.839	533.9787234	0.001

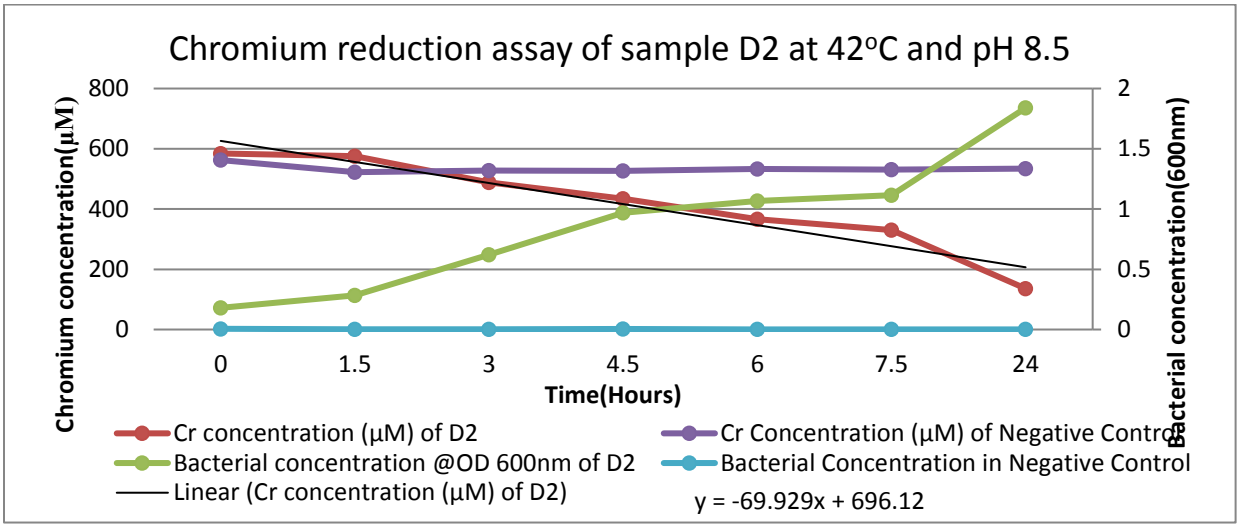

Figure 14: Chromium reduction assay of sample D2 at42°C and pH 8.5

From figure 14, it is shown that most of the reduction was done after overnight and till 7.5 the reduction percentage was 58%. But after overnight, the percentage became 77%. At 0 hours Cr concentration was 584.404µM and after 7.5 hours it reduced to 329.723µM. But after overnight

chromium concentration became very low and it was 136µM. On the other hand, bacterial concentration increased linearly. From 0.18 it increased to 1.839. 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 Identify the type of the enzyme of isolate D2

To identify the working type of the enzyme of the isolate sample D2 either exo enzyme or endo enzyme, the procedure was followed as described in section 2.8. The obtained data are given below in Table 15.

Table 15: Chromium reduction profile

	Sample		Negative control	
Time	With Cr^{6+}	Without $Cr^{6+)}$	With Cr^{6+}	Without Cr^{6+}
0 min	3.321	3.42	3.363	3.371
5 min	3.318	3.419	3.363	3.37
10 min	3.303	3.41	3.359	3.368
15 min	3.296	3.404	3.357	3.368
20 min	3.283	3.4	3.36	3.367
25 min	3.258	3.394	3.359	3.37
30 min	3.217	3.386	3.358	3.368
60 min	3.142	3.38	3.36	3.369
120 min	2.874	3.362	3.361	3.369
180 min	2.563	3.346	3.361	3.37

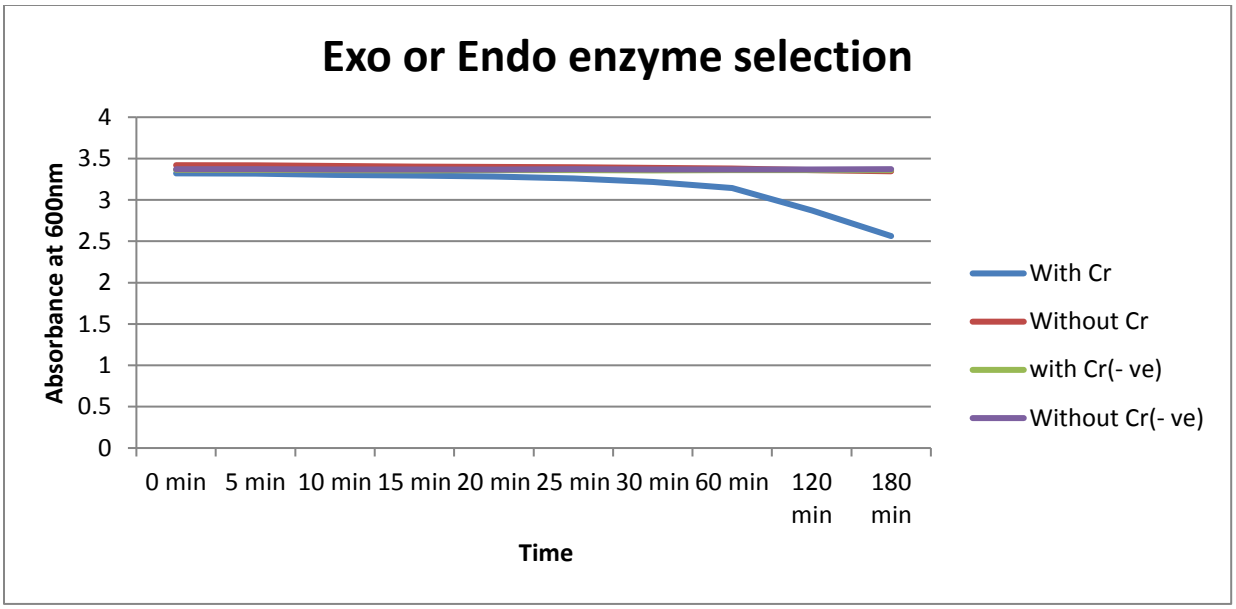

Figure 15:Chromium reduction profile to determine the enzyme

From figure 15, it can be observed that the bacteria which grow with chromium only shows the reduction. Which means these bacteria released its enzyme in the media. In the first 60 minutes a very small amount of chromium, reduction was done. But after 120 minutes the reduction rate was slightly high and it became 2.874 and after 180 minutes it reduced to 2.563. On the other hand, the bacteria which treated to grow without chromium did not show any significant reduction. In the negative control, no reduction was observed.

3.4 Antibiotic resistant among Chromium resistant isolate D2

According to the procedure which was discussed above in 2.9, antibiotic-resistant profiling was done and the data was obtained. The obtained data was given in Table 16 below.

Table 16: Zone of inhibition of antibiotics

Name of antibiotics	ZI/mm	ZI/mm	ZI/mm	average	standard deviation	Inference
Penicillin G (P10)	0	0	0	0	0	Resistant
Kanamycin (K30)	15	15	14	14.667	±0.5773502	Susceptible
Neomycin (N30)	21	20	21	20.667	±0.5773502	Susceptible
Vancomycin (VA30)	0	0	0	0	0	Resistant
Gentamycin (CN10)	20	20	21	20.333	±0.5773502	Susceptible
Cefixime (CFM5)	0	0	0	0	0	Resistant
Chloramphenicol (C30)	14	13	15	14	±1	Susceptible
Ceftriaxone (CRO30)	20	20	21	20.333	±0.5773502	Susceptible
Sulphamethoxazole (STX25)	0	0	0	0	0	Resistant
Cipropfloxacin (CIP5)	42	41	41	41.333	±0.5773502	Susceptible
Streptomycin (S10)	23	22	21	22	±1	Susceptible
Amoxycillin (AML10)	0	0	0	0	0	Resistant
Cefuroxime Sodium (CXM30)	0	0	0	0	0	Resistant
Azithromycin(AZM30)	17	16	15	16	±1	Susceptible

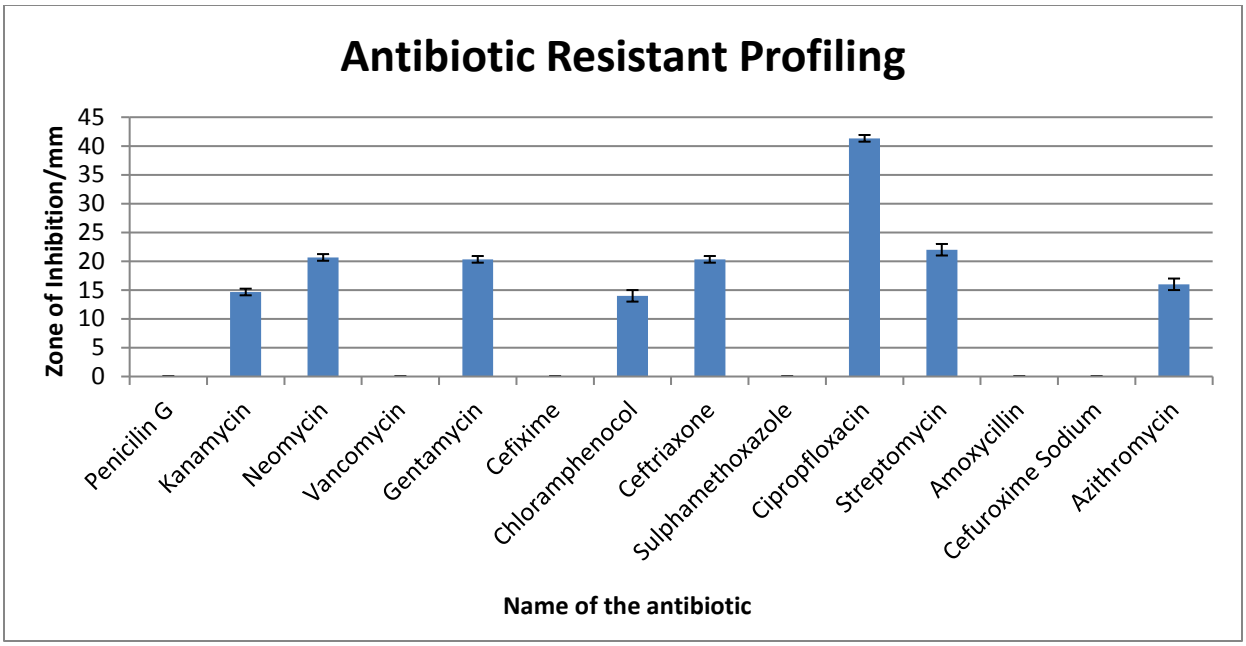

Figure 16: Zone of inhibition against the antibiotic disc

From figure 16, it is shown that Ciprofloxacin(CIP5) was more powerful against the isolated sample D2. Because Ciprofloxacin(CIP5) creates the highest zone of inhibition and it was 41mm in diameter after taking the average of triplicate data. Soit is safe to say that D2 was mostly susceptible to Ciprofloxacin. Among all the 14 antibiotics, the lowest zone of inhibition was seen for Chloramphenicol(C30). Zone of inhibition of Chloramphenicol was 14mm in diameter after taking the average of triplicate data. So isolated D2 sample was less susceptible against Chloramphenicol.D2was seen to be resistant against some of the antibiotics such as Penicillin G, Vancomycin, Cefixime, Sulphamethoxazole, Amoxycillin and Cefuroxime Sodium. Besides these, all the antibiotics showed the zone of inhibition which exceeds 10mm.

The picture of the plates that contain the all antibiotics are given below:

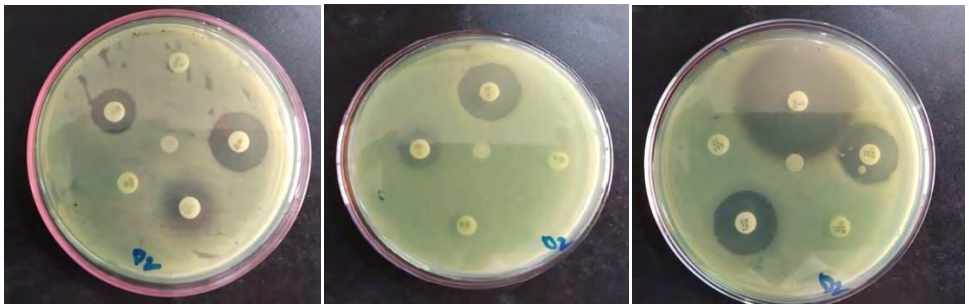

Figure 17: Zone of inhibition of antibiotic discs in isolate D2

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

According to the procedure discussed in section 2.10. Minimum inhibitory concentration was determined of the isolated sample D2 and the obtained result was tabulated below.

Table 17: MIC of Chromium resistant organism D2 against different Chromium concentration

Cr concentration	Absorbance at 600nm Test tube 1	Absorbance at 600nm Test tube 2	Absorbance at 600nm Test tube 3	Average	Standard Deviation
5mM	0.314	0.344	0.338	0.332	0.015875
6mM	0.227	0.235	0.229	0.23033	0.004163
7mM	0.138	0.163	0.147	0.149333	0.012662
8mM	0.055	0.041	0.056	0.050667	0.008386
9mM	0.028	0.025	0.023	0.025333	0.002517
10mM	0	0	0	0	0
11mM	0	0	0	0	0

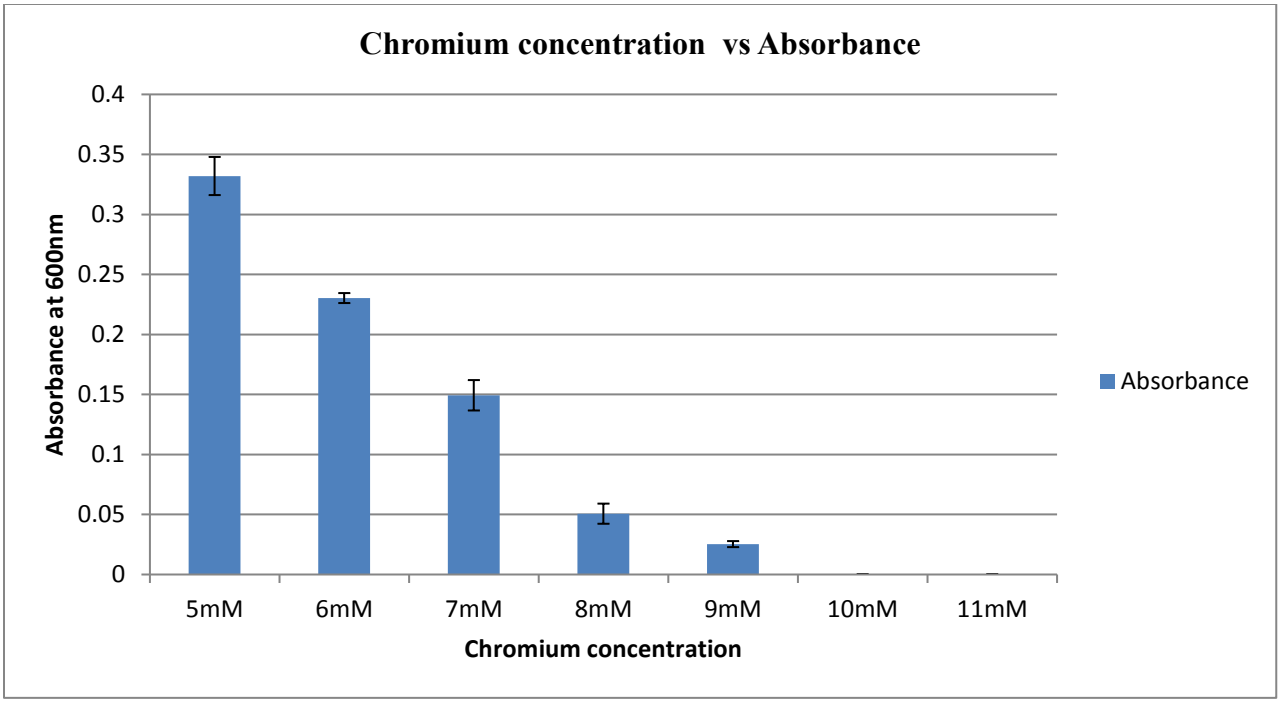

Figure 18: Minimum inhibitory concentration of the isolated sample D2

From figure 18, it was observed that the isolate D2 tolerates Chromium concentration up to 9mM. This means that D2 was able to demonstrate resistance till the Chromium concentration of 9mM. However, no growth was observed from the Chromium concentration of 10mM. Therefore, 10mM was the MIC for D2 isolate.

3.6 Identification of D2

The DNA sequence of the isolate D2 was obtained by Sanger sequencing (Heather et al., 2016).According to the procedure discussed in section 2.11, phylogenetic tree of the isolated sample D2 was obtained. The phylogenetic tree was given below in section 3.6.1. The sequence of the isolate D2 is shown in Appendix-A.

3.6.1 Phylogenetic tree of sample D2

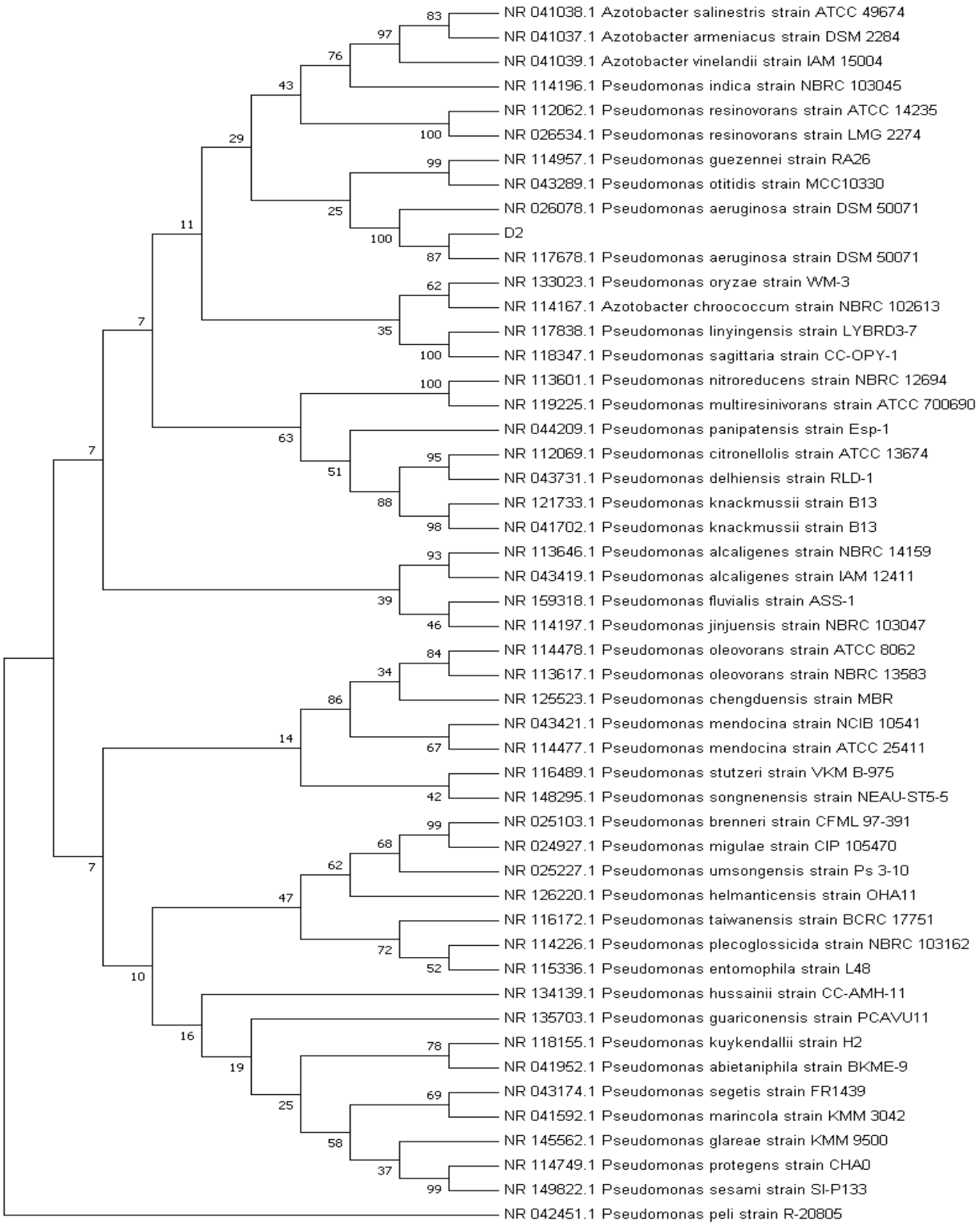

Figure 19:Phylogenetic tree of the isolated sample D2

The method of Neighbor-Joining was used to infer evolutionary history (Saitou &Nei, 1987). To represent the evolutionary history of the taxa analyzed, the bootstrap consensus tree inferred from 1000 replicates is taken. Branches that match partitions reproduced in bootstrap replicates of less than 50 percent will collapse. the share of mirror bushes wherein the related taxa clustered together within the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances had been computed using the maximum Composite chance technique (Tamura &Nei, 2004) and are inside the devices of the number of base substitutions in line with website online. The analysis involved fifty one nucleotide sequences. All positions containing gaps and missing data had been removed. There have been a complete of 820 positions in the final dataset. Evolutionary analyses were carried out in MEGA7 (Kumar et al., 2016).

3.7 Discussion

Chromium is highly toxic heavy metal which is disclosedintothe environment in varoius ways. It is mostly used in the tannery industry, dyeing industry which is the main source of Heavy metals. Presence of heavy metals in the environment in a large concentration has become a hazard to human life and animals. Especially $Cr^{6+}$is carcinogenic in nature. So, it has become a great concern to find out the solution to this life-threatening issue. The purpose of this study was to find out any biological strain which has the capability of reduction of toxic Chromium from the environment especially from water and soil.

For this experiment, the sample was collected from the showarighat, pargandaria area which is situated near the buriganga river. In Bangladesh most of the tannery industry is situated at these areas and their waste is released in the buriganga river which contains a large amount of heavy

metal like chromium. So that we collected our sample from these areas and then isolation and purification of the sample obtained. Then the experiment was carried out following the standard protocol and at three different temperature and pH the reducing capacity of the sample was analyzed. After obtaining all the data and graph, it was observed that the isolated sample D2 has the chromium reducing capacity. At 37˚C and pH 8.5, the isolate sample D2 showed the quickest reduction and also showed the 100% reduction of chromium. Besides these, at 37˚C, pH 7 and at 25˚C, pH 7 the isolate bacteria showed 94% and 96% chromium reduction respectively. Lowest reduction profile was seen at 25°C, pH 5.5 where only approximately 68% was reduced. At another pH value of 5.5 at 42°C only 77% was seen to be reduced approximately.

In exo or endo type of enzyme determination, it was observed that reduction was done after filtering out the bacterial cell from the media which means bacteria released its enzyme into the media during the incubation time. So that from this experiment we can say that the enzyme of the isolate sample D2 is exo enzyme which works outside the cell wall of the bacteria. We also observed that bacteria which was incubated overnight without chromium did not show any reduction. So it can be said that the presence of chromium actually triggers the bacteria to release the enzyme.

In antibiotic resistant profiling, it was found that the isolate was resistant to some of the antibiotics such as Penicillin G, Vancomycin, Cefixime, Sulphamethoxazole, Amoxycillin, Cefuroxime Sodium which means these antibiotics cannot destroy the isolate sample D2. The bacteria was most susceptible to Ciprofloxacin. Ciprofloxacin creates the highest zone of inhibition and it was 41 mm in diameter. The values taken by three-time and from them average value was taken with standard deviation.

In MIC, it was observed that sample D2 has the capability to tolerate up to 9 mM concentration of Chromium because after that no absorbance was observed in any concentration. So that the minimum inhibitory concentration of isolate D2 is 10 mM. From this result, it can be said that these bacteria can survive unto 9mM concentration of chromium.

Finally, identification of the isolate sample D2 was done by 16S rDNA sequencing and phylogenetic tree was contructed. From the phylogenetic tree it can be observed thatthere are three species in the clade which contains D2. These three texa are *Pseudomonas guezennei, Pseudomonas otitidis,* and *Pseudomonas aeruginosa.* Between thesethe isolate, D2 sample is more similar to the *Pseudomonas aeruginosa* than all other DNA sequence that was downloaded.

Chapter 4

Conclusion

4.1 Conclusion

After all the studies, it is clear that the isolated sample D2 which actually identified as *Pseudomonas aeruginosa* has the capability of reducing carcinogeneic hexavalent chromium. It has the potential to reduce chromium from water biologically. From this study it can be observed that, reduction rate is increased with time of the incubation. Besides this from this stutdy, it also state that the enzyme of the bacteria is exoenzyme and the bacteria can tolerate upto 9mM concentration of chromium. Moreover, after observing all the results it can be stated that, this strain is Chromium resistant. So it will be helpful to ascertain a better pathof treating the toxic effect of Chromium.

4.2 Future works

In *Pseudomonas aeruginosa,* chromium reductase enzyme is present. From this bacteria, some more experiment can be done to find out the interrelationship between antibiotic resistance profile and Chromium reduction assay. Besides this, plasmid analysis can be used to konow more information about this bacteriacan be done.

Chapter 5

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