# Isolation and Characterization of Hexavalent Chromium Reducing *Proteus mirabilis* isolated from the water of Buriganga River

**A project submitted**

**by**

**Most. Nasrin Aktar**

**ID: 14146062**

**Session: Spring 2014**

**to**

**The Department of Pharmacy**

**in partial fulfillment of the requirements for the degree of**

**Bachelor of Pharmacy (Hons.)**


**Dhaka, Bangladesh**

**February, 2019**

This work is dedicated to my beloved mother and my elder sister for their continuous support and affection.

## Certification Statement

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

Signed,

_____

Countersigned by the supervisor,

_____

## Acknowledgement

Firstly, I want to thank Almighty Allah for giving me the strength and ability to overcome all the hindrance and fulfil this project. It would never be possible to accomplish the purpose of the work without His mercy.

I would also like to thank my supervisor, Professor Dr. Eva Rahman Kabir, Chairperson, Department of Pharmacy and Md. Samiul Alam Rajib, Senior Lecturer, Department of Pharmacy, BRAC University for their continuous support and guidance. I am grateful to them and this work would not be completed without their continuous instruction and versatile knowledge.

Moreover, I want to give thanks to all the Lab Officers for their help and concerns. Lastly, I would like to thank my mother and my fellow mates for helping me to complete this project work.

## Abstract:

This study was conducted by considering the hazardous effects of hexavalent Chromium which is a highly carcinogenic, mutagenic and a teratogenic substance. In this study the sample was collected from Swari Ghat near the Buriganga River in Dhaka. After collecting the sample, it was isolated and purified. Then, Chromium reduction bioassay, antibiotic resistant profile, Minimum Inhibitory Concentration (MIC) and identification of the isolated sample were done. Di-phenyl Carbazide (DPCZ) and 3-(N-morpholino)propanesulfonic acid (MOPS) were used in the Chromium reduction bioassay. Also, in the bioassay, the experiment was done in three different temperature- 25˚C, 37˚C, 42˚C and three different pH 5.5, 7, and 8.5. In the result of bioassay it was observed that the strain had the best capacity to reduce the Chromium concentration at 37˚C and pH 8.5. Moreover, the strain had gone through the antibiotic resistant profile study to observe their resistance towards antibiotics. From this study it was found that cipropfloxacin (CIP5) and ceftriaxone (CRO30) had a strong effect on this strain whereas some other antibiotics such as penicillin (P10), vancomycin (VA30), trimethoprim (STX25), amoxycillin (AML10) did not have any effect on the strain. The Minimum Inhibitory Concentration method was used which showed that the strain could tolerate up to 16 mM of Chromium concentration in its surroundings and its MIC was 17 mM. Moreover, to identify the strain, 16s rDNA sequencing was done. A phylogenetic tree was made by using the sequence data and different software which showed that our isolated strain is *Proteus mirabilis.*

## Table of Contents



## Chapter 1

## Chapter 2

## List of Tables:

## List of Figures:

## List of Acronyms:

| | |
|---|---|
| ATSDR | Agency for Toxic Substances and Diseases Registry |
| BLAST | Basic Local Alignment Search Tool |
| Cr | Chromium |
| CRB | Chromium Reducing Bacteria |
| CFU | Colony Forming Unit |
| DPCZ | Diphenyl Carbazide |
| DNA | Deoxyribonucleic Acid |
| EPA | Environmental Protection Agency |
| ETP | Effluent treatment plant |
| GTF | Glucose Tolerance Factor |
| HDL | High Density Lipoprotein |
| IDLH | Immediately Dangerous to Life and Health |
| Kg | Kilogram |
| LDP | Low Density lipoprotein |
| MCL | Maximum Contamination Level |
| Mg | Miligram |
| MHA | Muller Hinton Agar |
| MIC | Minimum Inhibitory Concentration |
| mL | Mililitre |
| MIC | Minimum Inhibitory Conetration |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| NADH | Nicotinamide adenine dinucleotide |
| NB | Nutrient Broth |
| NCBI | National Centre of Biotechnology Information |
| NIOSH | National Institute for Occupational Safety and Health |
| Nm | Nanometer |
| OD | Optical Density |

| | |
|---|---|
| Ppm | Parts per million |
| PCR | Polymerase Chain Reaction |
| ROS | Reactive Oxygen Species |
| RPM | Rotation Per Minute |
| rRNA | Ribosomal ribonucleic acid |
| USEPA | United State Environmental Protection Agency |
| WHO | World Health Organization |
| ZI | Zone of Inhibition |

# Chapter 1

## 1. Introduction:

## 1.1 Background

The two main sources of Chromium in the environment are ferrochromate-$Fe_2Cr_2O_4$ and minerals in the earth's crust. For industrial uses like tanning, metallurgy, electroplating, (Jeyasingh & Philip, 2005) and textile pigment production the minerals of the Chromium are involved (Augustynowicz et al., 2010).The tannery industries are considered the main source of Chromium contamination due to the discharge of high amount of Chromium containing wastewater ranging from 2000-5000mg/L, which is much higher than the permissible limit of 2mg/L for waste water discharge(Belay, 2010). From the transition group VI-B, Chromium exists in several oxidation states in which Cr (III) and Cr (VI) are the most stable forms. These two stable states of Chromium have different chemical and physical properties as well as they have distinct differences in the influence in the environment including all the living organisms (Kotaś & Stasicka, 2000a).

The hexavalent Chromium originates from the anthropogenic sources and as highly mobile anions such as $HCrO_4^-$, $CrO_4^-$ and $Cr_2O_7^-$ are found in water. On the other hand, the most abundant form of trivalent Chromium are hydroxides of Chromium - $CrOH^{2+}$ (aq) and $Cr(OH)_2^+$(aq) or neutral $Cr(OH)_3$ , which all are cationic forms of hydroxides. Because of having the ability to precipitate and form complexes with organic ligands, the trivalent Chromium is less mobile than hexavalent Chromium (Nakayama, Kuwamoto, Tsurubo, Tokoro, & Fujinaga, 1981). As a substance of glucose tolerance factor (GTF), trivalent Chromium is used in human diet as it is useful in the metabolism of sugar. Here, the hexavalent Chromium shows severe toxicity due to having high redox potential activity, higher solubility in water (Baldiris, Acosta-Tapia, Montes, Hernández, & Vivas-Reyes, 2018) and the ability to penetrate the cell membranes (Kotaś & Stasicka, 2000b).

According to the Agency for Toxic Substances and Diseases Registry, hexavalent Chromium is considered as one of the seventeen most toxic substance (ATSDR, 2008). Similarly, it is included as a grade 'A' mammal carcinogen by the United States Environmental Protection Agency (U.S. EPA) (Maqbool et al., 2012). All the compounds of hexavalent Chromium are considered as an

occupational carcinogen according to National Institute for Occupational Safety and Health (NIOSH)(Baldiris et al., 2018). Moreover, many studies have reported that exposure and release of hexavalent Chromium into the environment is a result of severe health problems which include accumulation in the placenta. This causes impairment in the development of the fetus, skin allergies, vomiting, brain damage, diarrhea and premature death in mammals (Pattanapipitpaisal, Brown, & Macaskie, 2001). Apart from these side effects in mammals, it has also severe effects on plants which includes metabolic alterations of the plants, poor seed germination, stop root growth, impairment of photosynthesis and death of the plants. This extreme situation has indicated that the world is going to face an alert to the intense toxicity of Cr (VI). So it is very essential to find out the most efficient and effective treatments for reducing the concentration of the Chromium in the environment.

Recently, there are many technologies such as anion exchange resins and electrolysis (Baldiris et al., 2018), phytoremediation, bioremediation, physico-chemical extraction, stabilization or solidification, soil removal or land filling, soil washing, flushing etc. which are available for reducing the concentration of Chromium in the contaminated sites. Among all these techniques, bioremediation is one of the most important and effective technique, because other techniques do not result in a permanent solution or simply immobilize the contaminants. Furthermore, bioremediation is very cost effective (Jeyasingh & Philip, 2005) and safe for the environment (Lin et al., 2009). Through the bioremediation techniques, the most toxic hexavalent Chromium is detoxified by reduction reaction and turned into trivalent Chromium which is an immobilized form of Chromium. As a result of reducing the toxic effects of Cr (VI) by turning it to Cr (III) which form insoluble $Cr(OH)_3$ with the pH range of 6-9 ($k_{sp}$, $6.7 \times 10^{-31}$). That is why, hexavalent Chromium can not undergo to the ground water (Benefield, Judkins, & Weand, 1982). A wide variety of bacteria like *Bacillus sp.* (Camargo, Bento, Okeke, & Frankenberger, n.d.), *Exiguobacterium sp.*(Alam, Hossain, Yonge, Peyton, & Petersen, 2006), *Staphylococcus aureus* and *Pediococcus pentosaceus* (Cheung & Gu, 2005), *Stenotrophomonas maltophilia*, *Pantoea sp.* and *Aeromonas sp*.(Ahemad, 2014), *Acinetobacter* and *Ochrobactrum* , *Arthrobacter* (Megharaj, Avudainayagam, & Naidu, 2003), *Pseudomonas sp.* (Guha, Rajkumar, Ashok Kumar, & Mathew, 2010), *Serratiamarcescens* (Cervantes et al., 2001) *Ochrobactrum* sp.(Thacker & Madamwar, 2005), *Bacillus* sp. (Elangovan, Abhipsa, Rohit, Ligy, & Chandraraj, 2006), *Desulfovibrio vulgaris* (Goulhen, Gloter, Guyot, & Bruschi, 2006) ,*Cellulomonas* spp. (Viamajala et al., 2007) etc. are

used in bioremediation techniques to reduce the Chromium concentration from the contaminated sites. These microorganisms were found to reduce hexavalent Chromium by different mechanisms either by utilizing the hexavalent Chromium as final electron acceptor or by secreting certain soluble enzymes (Samuel et al., 2013). The soluble enzymes include ChrR, YieF, NemA and LpDH which are used to catalyze the reduction of Cr (VI) to Cr (III), mediating the transfer of electrons from electron donors like NAD(P)H to Cr(VI) (Baldiris et al., 2018).

## 1.2 Aim

The main purpose of this paper was to identify, separate, screen and select the specific number of bacteria from the Chromium contaminated water sources that are Cr (VI) resistant and also evaluate their extent or capability to reduce or detoxify the hexavalent Chromium at the optimum temperature and pH.

## 1.3 Objectives

- To identify the bacterial strains which potentially reduce Cr (VI) ions.
- To do the plasmid analysis.
- Assay of antibiotic resistance profiling of the Chromium resistance bacteria.
- To calculate the Minimum Inhibitory Concentration (MIC).
- To do the bioassay.
- To determine the optimum temperature and pH for bacterial activity.

## 1.4 Chromium

### 1.4.1 Importance

Among the two most stable forms of Chromium, the trivalent Chromium is considered an essential element in human diet. These trace elements increase the efficiency of insulin and thus are involved in the metabolism of lipids, proteins and carbohydrates. Due to the effects of these trace elements the normal lipid and glucose level are affected when there is deficiency of these elements (Lewicki et al., 2014). According to the Food and Nutrient Board at the Institution of Medicine, the daily uptake of Chromium level for men and women is 35 µg and 25µg. There are different studies suggesting that dietary supplements with $Cr^{3+}$ decreased the level of Low Density Lipoprotein

(LDP) and increase the level of High density of Lipoprotein (HDL) which is beneficial for health. Also, it lowers the level of non-esterified fatty acids and triglycerides (Lewicki et al., 2014).

### 1.4.2 Chemistry

Chromium is an odorless, silvery gray hard metal. In dilute hydrochloric acid and sulfuric acid, it gets solubilized, but insoluble in alkali and strong alkalis (Merck, 1989). The atomic number of Chromium is 24 and it is symbolized as Cr. It is brittle and highly polished. The boiling point of Chromium is 2672°C and the melting point is 1907°C ["Chemical properties of Chromium - Health effects of Chromium – Environmental effects of Chromium,"1998-2016]. Chromium has different colored compounds. It was derived from the Greek word "chroma" (Appenroth, Teller, & Horn, 1996). Chromium is utilized to produce alloys and stainless steel because the solidity and imperviousness towards erosion and corrosion turns Chromium into an extremely valuable element (Rifkin, Gwinn, & Bouwer, 2004). Regardless of the way that Chromium is an essential trace mineral for adjustment of nucleic acid and metabolism of glucose and stimulus framework of chemical. Elevated levels of Chromium is poisonous, however its deficiency may cause disease (Thacker & Madamwar, 2005). Hexavalent Chromium is deadly and mutagenic to living creatures. It causes lung cancer, respiratory tract infection and skin diseases (Thacker & Madamwar, 2005). This poison extremely impacts on the biosphere. Therefore, the natural reduction of Chromium is significantly important.

**Table 1.1.** Basic information of Chromium ("American Elements," 2016; "Chromium," 2013)

| | |
|---|---|
| Symbol | Cr |
| Term | Chromium |
| Class | Transition metal |
| Group, period ,block | 6,4,d |
| Atomic weight and number | 51.996 and 24 |
| Atomic radius | 128pm |

| | |
|---|---|
| Appearance | Silver gray |
| Electronic configuration | [Ar] $3d^5$ $4s^1$ |
| Thermal conductivity | 93.9 $Wm^{-1}.K^{-1}$ |
| Oxidation state | -2, -1, 1, 1, 2, 3, 4, 5, 6 |
| Crystal structure | Centered cubic |

## 1.4.3 Sources of Chromium

### 1.4.3.1. Environmental sources

Environmental sources of Chromium includes:

- Decay of asbestos lining
- Mote of cement
- Vitiated landfill
- Nicotine and tobacco smoke
- Airborne discharge from the chemical plants and burning facilities
- Rocks and topsoil (ATSDR, 2008)

### 1.4.3.2. Occupational sources:

- Discharge of the water and wastage that contain high concentration of Chromium into the river water and soil.
- Different agents including- cement, anti-freezing agents, anti- algae agents, substance used in chrome alloy production, wood preservatives etc.
- Activities like chrome electroplating, glassmaking, copier servicing, leather tanning, painting, photoengraving, manufacturing of porcelain, ceramics and magnetic audio tapes, inserting pigments into punctures in the skins (ATSDR, 2008).

## 1.4.4 Toxicity

### 1.4.4.1. Carcinogenic, mutagenic and teratogenic action of hexavalent Chromium

According to the United States Environmental Protection Agency (USEPA), hexavalent Chromium was among seventeen chemicals which is the most hazardous chemical constituents to human health. It is also considered as carcinogenic and mutagenic agent. This is because it can cross the cell membrane and enter into the cell, has high solubility and tendency to be absorbed in organic carbon and mineral surfaces (Cervantes et al., 2001). After entering into the cell, hexavalent Chromium reacts with reducing agents like NAD(P)H, $FADH_2$ cysteins, pentose and antioxidants like ascorbate and glutathione etc. and produce unstable $Cr^{5+}$ and $Cr^{4+}$ intermediates including the free radicals like hydroxyl free radicals and super oxides free radicals. These two free radicals are collectively known as Reactive Oxygen Species (ROS) which results in DNA damage, DNA protein cross links and Cr-DNA adducts etc. Thus these unstable intermediates and free radicals can cause oxidative degradation to the proteins and DNA (Cervantes et al., 2001) and causes cancer. This is why, unstable intermediates $Cr^{5+}$ and $Cr^{4+}$ and free radicals are considered as vital agents responsible for carcinogenesis (Zhitkovich & Costa, 1992) and apoptosis. Again, in DNA sequence, the negative charged phosphate ions bind with the trivalent and tetravalent Chromium and hinder the DNA replication as well as inhibit the RNA transcription (Ramírez-Díaz et al., 2008). According to many animal experiments, both the trivalent and hexavalent Chromium are considered teratogenic substances (Danielsson, Hassoun, & Dencker, 1982). These accumulate in the fetus and cause impairment in the development of the fetus (Pattanapipitpaisal et al., 2001).

### 1.4.4.2. Brain damage and respiratory effects of hexavalent Chromium

The workers involved in the chromate production and chromate electroplating are more prone to inhale the Chromium dust, which results in the perforation of the nasal septum and respiratory diseases such as hyperplasia of the bronchial epithelium and fibrosis. The amount of the Chromium that is absorbed through the nasal route is rapidly delivered to the brain within a minute via the olfactory and terminal nerves (Salama, Hegazy, & Hassan, 2016). The inhaled Chromium dust causes serious health hazards problems like chronic bronchitis and irritation, asthma, hyperemia, ulceration of the nasal mucosa, polyps of the upper nasal mucosa etc.(ATSDR, 2008).

#### 1.4.4.3. Effects of hexavalent Chromium on skin

Due to the cytotoxic property of Chromium, skin irritation and dermatitis are the common problems in people who are exposed to the Chromium (Bruynzeel, Hennipman, & van Ketel, 1988). Here, two mechanisms are involved in causing skin irritation and dermatitis - one is induction and another is sensitization. Through induction, penetration of Chromium occurs through the skin. On the other hand, sensitization of Chromium is involved with the immune response and depends on the upper threshold level of the Chromium (EPA, 1998). There are a lot of symptoms of Chromium irritancy and dermatitis such as insipidity of the skin, cracking, rash, swelling etc.(Babula et al., 2008)

#### 1.4.4.4. Renal and hepatic effects of hexavalent Chromium

Renal Chromium poisoning can lead to severe renal failure and renal tubular necrosis (Sharma, Singhal, & Chugh, 1978). Chromate poisoning in the kidney can be found by measuring the elevated level of β2-microglobulin which is a marker of renal tubular damage and this result is more prominent in younger people who are more exposed to the Chromium (Lindberg & Vesterberg, 1983). Chromate poisoning in the liver can be found by estimating the increased level of Kupffer cells, necrosis and alienation in the liver cells, lymphocytic and histolytic infiltration in the liver (Pascale, Waldstein, Engbring, Dubin, & Szanto, 1952).

#### 1.4.4.5. Hematological effects of hexavalent Chromium

Ingestion of lethal or sub lethal dose of hexavalent Chromium causes hematological effects which results the reduction of hemoglobin contents and hematocrit. It has also been found in a study that total white blood cell count, reticulocyte and hemoglobin has increased after four days of ingestion of few grams of $K_2Cr_2O_7$ which has indicated that intravascular hemolysis has ensued(Sharma et al., 1978). In addition, another study which has been conducted in the laboratory which showed that a 35 years old woman died after ingestion of 50 ml of fresh chromic acid containing 25g of hexavalent Chromium which elicited the result of anemia- hemoglobin 56g/L, hematocrit 17% and thrombocytopenia (Loubières et al., 1999).

### 1.4.5 Chromium utilization

Many Chromium compounds such as sodium chromate, ferrochromate, dichromates are utilized in industries widely. In addition, some of Chromium compounds are implemented in stainless steel welding process, tanning leather, dyes and pigments, timber preservation. Chromium also has anti-corrosive property and used in boiler (World Bank., Nationally Coordinated Research Projects (Nigeria), & University of Port Harcourt. Faculty of Science., 2010.).

### 1.4.6 Chromium resistance in bacteria

For avoiding harmfulness of metals, numerous microorganisms create a framework surrounding them and thus they may survive against the hazardous metal-contaminated circumstances. They create this framework by using different methods like sorption, methylation of deoxyribonucleic acid, outflow of metal and biologically conversion of metal (Pei, Yu, Ji, Khan, & Li, 2018). Conversion of hexavalent Chromium by bacteria to trivalent Chromium is mainly hazardous from biological remediation's perspective that may be counted as an additional system of the resistance of chromate (Cervantes et al., 2001). A collection of microbes that are resistant to Chromium with greater reducing potential of hexavalent Chromium are *Deinococcus, Enterobacter*, *Pseudomonas, Thermus, Eschericia, Agrobacterium, Bacillus, Shewanella* and various species (Yamamoto, Kato, Yano, & Ohtake, 1993). Strains that are resistant and non-impervious to chromate have the power to decrease the concentration of chromate. However, at the higher concentration of Chromium, the growth of the bacteria are going to be stop (Bopp & Ehrlich, 1988). By this manner, the property of microorganism which is chiefly beneficial towards the potent methodology for bioremediation, connects a strength by converting hexavalent Chromium to trivalent Chromium (Dhal, Thatoi, Das, & Pandey, 2010). Chromium Reducing Bacteria (CRB) are those bacteria which may reduce the quantity of hexavalent Chromium. Gram-positive bacteria is one sort of CRB and it decreases the poisonous effects of hexavalent Chromium at a great extent. However, gram-negative bacteria shows a smaller quantity of resistance towards hexavalent Chromium (Nriagu & Nieboer, 1988).

Microbes that originate within the spoiled condition of metal are very harmless for those metals. Microscopic organisms gathered from the mine dirt of chromate are protected to hexavalent Chromium nearby other metal elements (Thatoi, Das, Mishra, Rath, & Das, 2014).

Along these line, both diminishment and imperviousness of Chromium are microorganism's autonomous assets (Bopp & Ehrlich, 1988). Microbes use characteristic segments so that it can overcome the quantity of hexavalent Chromium in ground that fuse the attenuate take-up of hexavalent Chromium. It modifies hexavalent Chromium extracellularly, diminishes hexavalent Chromium intracellularly through cleansing by ROS (Reactive Oxygen Species), healing of DNA by enzymes. Hexavalent Chromium flow outside of the cell and releasing of ROS are depicted in the figure 1.1,

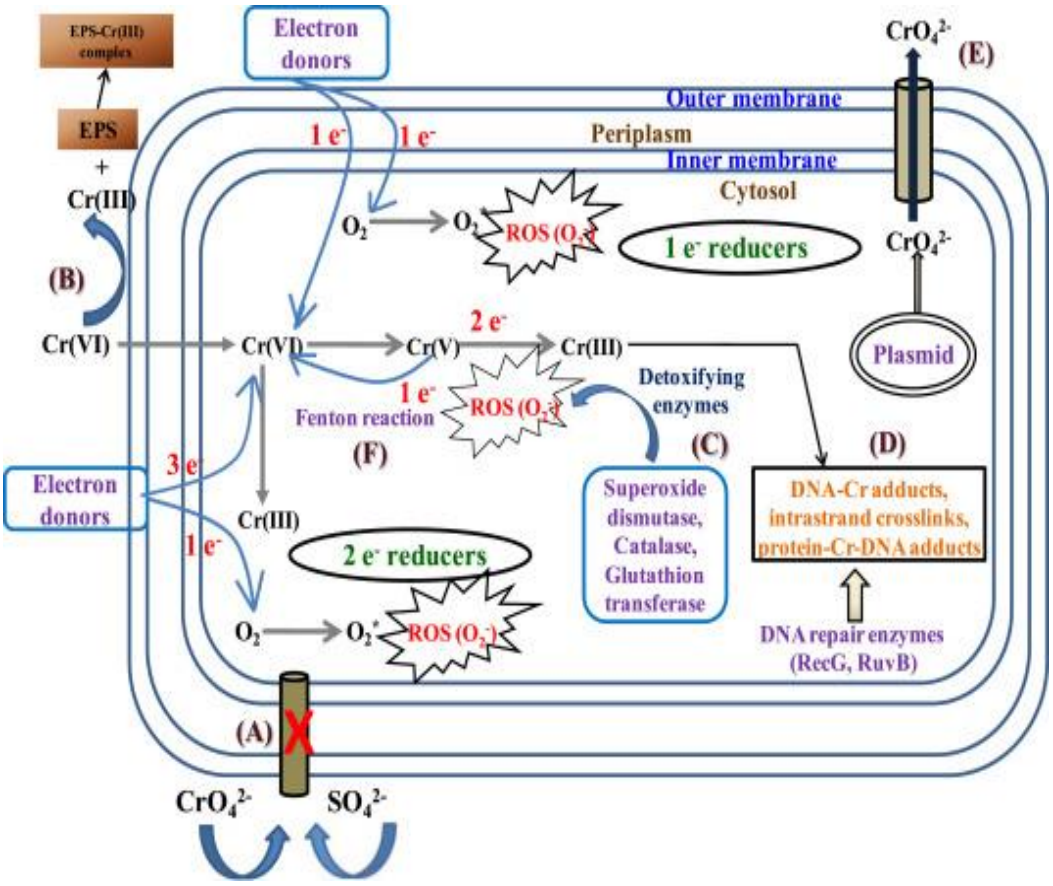


**Figure 1.1.** Resistance of chromate mechanisms into the cells of bacteria. A. Transformation in the chromosome-encrypted take-up transporter of sulfate, B: Lessening of hexavalent Chromium extracellularly into trivalent Chromium, C: Lessening of hexavalent Chromium intracellularly to trivalent Chromium through chromate reductase, D: Utility of the healing method of SOS in diminishing oxidative pressure, E: Chromate efflux from cytoplasm, F:ROS foraging enzyme activity to diminish oxidative pressure (Thatoi et al., 2014).

### 1.4.6.1. Reduce uptake of Cr (VI) by the microorganism

Decreased take-up of hexavalent Chromium such as take-up passageway of sulfate and through the homeostasis of iron or sulfur are the capable protective frameworks in contrast to the destructive effects of hexavalent Chromium.

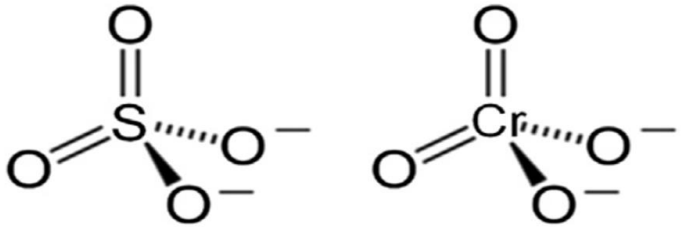


**Figure1.2.** Resemblance among structures of the ions of sulfate and chromate

It is permeable to the membrane of cell through the $SO_4^{2-}$ transportation passageway, by the assistance of the non-specific anionic carriers like $PO_4^{3-}$ and $SO_4^{2-}$. If mutation occurs within the microbe's sulfate take-up passageway that is encrypted by chromosome, then diminishment occurs within the chromate movement (Ramírez-Díaz et al., 2008). Microorganism that exist within the spoiled condition of metal encounter expedient change to form impermeableness of hexavalent Chromium that stimulates reduced take-up of hexavalent metal through sulfate transportation passageway (Kümmerer, 2009).

### 1.4.6.2. Extracellular reduction of Cr (VI)

An additional framework of resistance is the lessening of hexavalent Chromium into trivalent Chromium extracellularly straggled through attachment of it with functional group on the cell exterior of bacteria (Ngwenya & Chirwa, 2018). Parts of peptidoglycan are the extraordinary trivalent Chromium binder and that they exist into the partitions of cell of those life forms (McLean & Beveridge, 2001). It may be seen that some styles of microorganisms have the power of surface assimilation that boost the removal of the species of metal from the solution of water. Unremarkably, these skills select the movement of responsive practical teams' equivalent to phosphate, amine, carboxyl, sulfhydryl and hydroxyl group upon the partition of cell exterior of microorganisms. Thusly, cell becomes impermeable for hexavalent Chromium at the time of its extracellular change occurs.

### 1.4.6.3. ROS detoxifying enzymes or intracellular reduction of Cr (VI)

In the midst of the depletion of hexavalent Chromium into trivalent Chromium a transient, exceptionally responsive transitional radical of hexavalent Chromium is generated that undergoes redox reaction. By this way, pentavalent Chromium goes for oxidization and turns once more into hexavalent Chromium. Thus di-oxygen receives the electron of it and ROS (Reactive Oxygen Species) are formed. As a result, oxidative strain is originated in the bacteria. By this methodology, proteins situated in the bacteria are in like manner actuated through chromate into the shield in contrast to oxidative pressure provoking extra preparation of chromate's imperviousness(Ramírez-Díaz et al., 2008). In any case, oxidative strains are removed on account of reactive oxygen species through purifying enzymes such as, catalase, superoxide dismutase, glutathione transferase and so forth (Ackerley, Gonzalez, Park, Blake, Keyhan, & Matin, 2004).

### 1.4.6.4. Enzymes for DNA repairing

One of the other way of protection made by means of hexavalent Chromium which is safety of the cells of microorganism through the healing enzymes of DNA of that DNA which is harmed. Hexavalent Chromium transfers into the cell of microbe that is speedily decreased into trivalent Chromium via the motion of a range of exercise of non-catalyst or catalyst which delivers reactive oxygen species (ROS). In this way that does unfavorable consequences upon the cell's DNA and protein. Reactive oxygen species cause the alteration of base, disruption of one strand and disruption of two-fold strands and thus it disrupts the DNA formation. Such DNA damage be healed by restoration mechanism of DNA such as, the reaction catalysts of SOS which are RuvB, RecA and RecG (Hu, Brodie, Suzuki, McAdams, & Andersen, 2005). For example, Hexavalent Chromium into E.coli has not been acknowledged as the recovery device of SOS which from the oxidative pressure defends DNA. Moreover, helicases of DNA such as, RuvB, RecG, sections of recuperation framework of recombinant DNA are seemed to appreciate the response to the damage of DNA obtained via chromate into *Pseudomonas aeruginosa* (Miranda et al., 2005). Hexavalent Chromium diminishment of cell is performing technique, making pentavalent or tetravalent Chromium like intermediates of redox that is energetic and constant trivalent Chromium enclosing adducts of DNA and Chromium that is the utmost abundant sort of destruction of DNA which is accountable for alterations and also disruptions of chromosome (Zhitkovich & Costa, 1992).

### 1.4.6.5. Efflux of Cr (VI)

Chromate efflux is a profitable and broad resistance system and it inhibits accumulation of poisonous particles in the cell of bacteria (Ramírez-Díaz et al., 2008). *P. aeruginosa* has the greatest appreciated resistance mechanism of chromate. Superfamily of CHR of the particle provider of chromate is connected with the protein of ChrA. CrhA is a protein of membrane that tends to repel and no longer absorb water, encrypted by pMOL28 and P. aeruginosa's plasmids known as pUM505 (Cervantes et al., 2001) which is incorporated in imperviousness of chromate through efflux section of chromate (Ramírez-Díaz et al., 2008). Protein of ChrA executes as chemi-osmotic propel system. From the periplasm or cytoplasm it then excretes chromate so that external driven can take place through proton held method energy (Alvarez, Moreno-Sánchez, & Cervantes, 1999). Proteins of CHR of a few microorganisms are incorporated into imperviousness of chromate through efflux framework of chromate (Ramírez-Díaz et al., 2008).

### 1.4.6.6. ROS scavenging

Consequent of hexavalent Chromium for inflowing mobile may additionally be reduced into pentavalent Chromium. Donors of electrons such as NADPH or glucose donates the electrons towards pentavalent Chromium and for this reason it provokes the enchantment of comparative unpredictable noxious reasonable pentavalent Chromium. However, through the semi-tight instrument, reinstructs of chromate in addition decrease pentavalent Chromium into trivalent Chromium with an alternate between two electrons, once in a while this response is not noticeably brisk. In this way a fragment of the intermediate of pentavalent Chromium is right away reoxidized into hexavalent Chromium thusly delivering reactive oxygen species via one Fenton-like reaction. In the midst of this methodology radicals of hydroxyl are fashioned into the mobile of bacteria (Shi & Dalal, 1990) and it is represented into following situation:

$$Cr\ (V) + H_2O_2 \rightarrow Cr\ (VI) + OH^- + OH^-$$

**Table 1.2.** Outlines the techniques of bacteria & they are associated with the resistance of chromate

| Enzyme/System | Species | Function | Reference |
|---|---|---|---|
| **Transport** | | | |
| ChrA transporter | *Pseudomonas Aeruginosa* | Efflux of cytoplasmic chromate | (Alvarez et al., 1999) |
| Cys operon products | *Shewanella Oneidens* | Sulfate transport | (Brown et al., 2006) |
| TonB receptor, hemin Transporter | *S.oneidensis* | Iron Transport | (Brown et al., 2006) |
| **Reduction** | | | |
| Chromate Reductases | Diverse species | Reduction of Cr (VI) to Cr(III) | (Cervantes et al., 2001) |
| SOD, catalase | *Eschericia coli* | Combat of oxidative stress | (Ackerley, Gonzalez, Park, Blake, Keyhan, & Matin, 2004) |
| Outer membrane Proteins | *Caulobacter Crescentus* | General stress response | (Hu et al., 2005) |
| **DNA repair** | | | |
| RecG and RuvB DNA Helicases | *Pseudomonas Aeruginosa* | Repair of DNA damage | (Miranda et al., 2005) |
| SO0368, UvrD and HrpA helicases | *Shewanella Oneidensis* | Repair of DNA damage | (Chourey et al., 2006) |
| **Other Mechanisms** | | | |
| Cys operon products | *S. oneidensis* | Sulfur metabolism | (Brown et al., 2006) |
| Adenylyl sulfate Kinase | *S. oneidensis* | Sulfur metabolism | (Brown et al., 2006) |
| Ferritin | *S. oneidensis* | Iron binding | (Brown et al., 2006) |

## 1.4.7. Chromium transport

According to Nies, heavy metals enter into the cell through the cell membrane for showing its toxicity or its physiological activity. Hexavalent Chromium ($Cr^{6+}$) with anionic substance of the microbial membrane it is unable to bind. On the other hand, trivalent Chromium ($Cr^{3+}$) is freely bind with the microbial envelops (Cervantes et al., 2001). Hexavalent Chromium ($Cr^{6+}$) and dichromate ($Cr_20_7^{2-}$) or $SO_4^{2-}/PO_4^{3-}$ has the similar structure and for this reason in both prokaryotes and eukaryotes an active sulfate transport is needed through which they can easily cross the cell membrane(Daulton, Little, Jones-Meehan, Blom, & Allard, 2007). On the contrary, for trivalent Chromium ($Cr^{3+}$) the biological membrane is impenetrable because it is not soluble in water.

## 1.4.8. Reduction of Cr (VI) in the bacteria

### 1.4.8.1. Enzymatic method (direct process)

According to Chueng and Gu and many other authors, there are three main mechanisms of hexavalent Chromium reduction which are as follows:

1. Under anaerobic conditions, various elements of the cell's protoplasm like amino acids, nucleotides, carbohydrates, vitamins, organic acids, glutathione, hydrogen NADH (NADPH in some species), flavoproteins, and hemoproteins that act as electron donors cut back hexavalent Cr (Ahemad, 2014).

2. Soluble reductase: In the presence of oxygen, NAD(P)H dependent extracellular soluble reductases are unit made on purpose by the cell to cut back $Cr^{6+}$ to $Cr^{3+}$ that is removed by reacting with functional groups which present on the cell surface. Again, genus *Pseudomonas putida* PRS2000, genus *Pseudomonas ambigua* G-1, *Desulfovibrio vulgaris*, and *E. coli* ATCC 33456 are reportable to supply soluble $Cr^{6+}$ reductases (Shen & Wang, 1995) that utilize numerous electron donors and may be situated either within or outside the microorganism's cell (Chen & Hao, 1998). Since reduction mediated by such reductases is an energy-requiring and extremely regulated method, these enzymes are unit made constitutively. Due to independence from transport mechanisms for $Cr^{6+}/Cr^{3+}$ intake and expulsion, extracellular $Cr^{6+}$ reduction is advantageous for bacterial cell because it protects the cell from $Cr^{6+}/Cr^{3+}$ induced DNA injury (Ahemad, 2014). Some

oxidoreductase enzymes are available in the bacteria that catalyze the hexavalent Chromium reduction. Some examples of oxidoreductase enzymes are given below in figure 1.3:

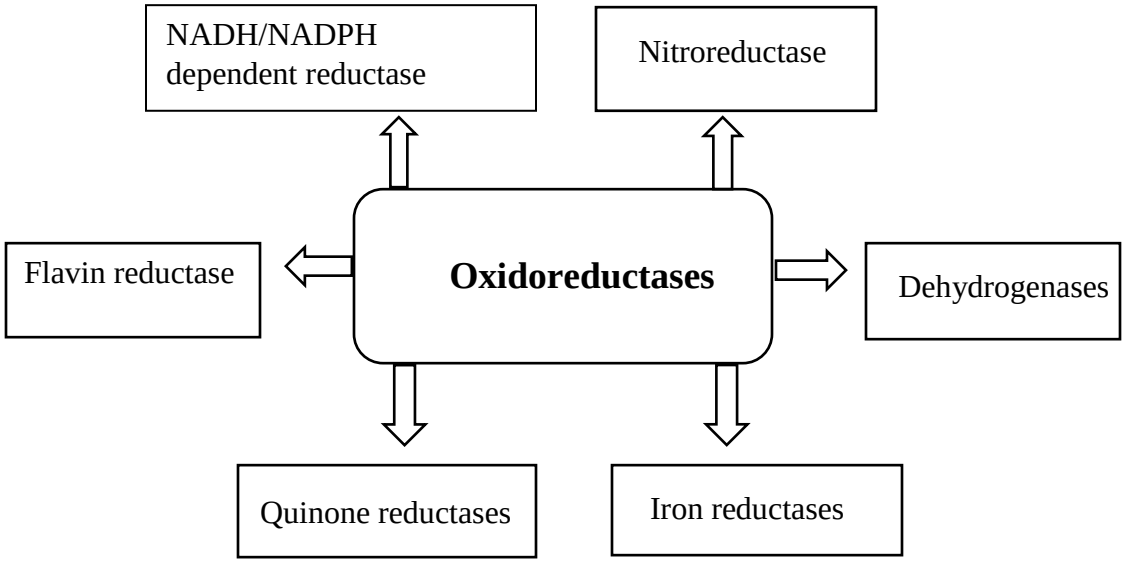


**Figure 1.3.** Oxidoreductase enzymes found in the bacteria that catalyze Cr(VI) reduction (Thatoi et al., 2014).

**3.** Membrane associated reductases: In the absence of oxygen, membrane associated reductases are involved for hexavalent Chromium reduction which requires the hydrogen or glucose so that they can donate electrons (Ibrahim, El-Tayeb, Elbadawi, Al-Salamah, & Antranikian, 2012). Again, in the same condition *Pseudomonas maltophilia* strain O-2, *Bacillus megaterium* strain TKW3, and *Amphibacillus sp*. KSUCr3 are the microorganisms that provide cell fractions for reduction of hexavalent chromium (Cheung & Gu, 2005). In case of electron donor, glucose plays an important role by donating electrons externally (Ibrahim et al., 2012).

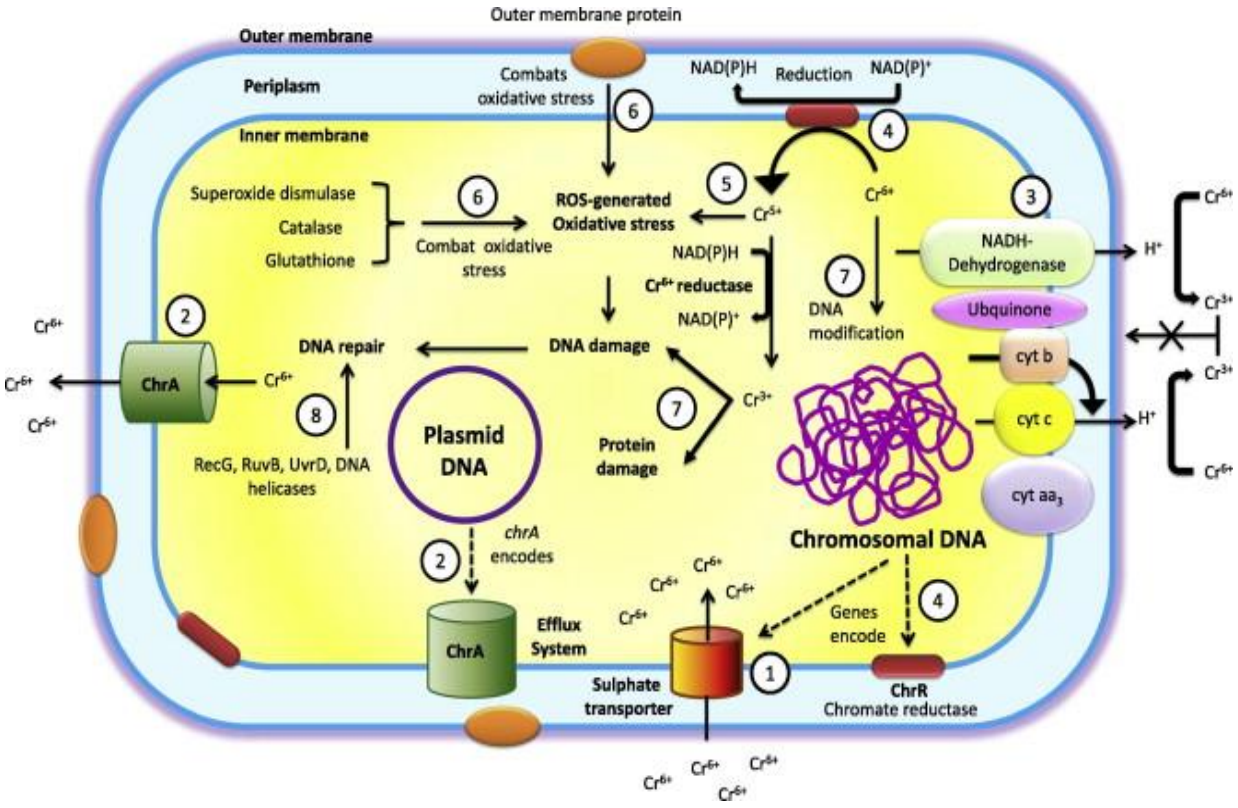


**Figure 1.4.** Schematic depiction of Chromium resistance and toxicology in bacterial cell

In the above figure 1.4, the following points are described,

(1) Chromate due to the structural similarity with sulfate enters the bacterial cell through sulfate transporter encoded by the chromosomal DNA. (2) Plasmid DNA encoded efflux systems are used to expel the intracellular chromates outside the bacterial cell to resist the chromate toxicity. (3) Aerobic $Cr^{6+}$ reduction into $Cr^{3+}$ involves soluble reductase which requires NAD(P)H as an electron donor while anaerobic $Cr^{6+}$ reduction occurs in the electron transport pathway by cytochrome b (cyt b) or cytochrome c (cyt c) along the respiratory chains in the inner membrane; $Cr^{3+}$ cannot pass the bacterial cell membranes due to the insolubility of $Cr^{3+}$ derivatives. (4) Membrane-embedded chromate reductase which is encoded by the chromosomal DNA, reduces $Cr^{6+}$ anaerobically in the presence of electron donors. (5) $Cr^{5+}$ produced during the redox cycle of $Cr^{6+}$ produces oxidative stress by the production of reactive oxygen species (ROS). (6) To combat the ROS generated oxidative stress, protective metabolic enzymes superoxide dismutase, catalase and glutathione are secreted. Some outer membrane proteins are also involved to counter the oxidative stress. (7) $Cr^{6+}$ and principally $Cr^{3+}$ not only negatively affects DNA replication and

RNA transcription by damaging DNA but also alters gene expression. In addition, $Cr^{3+}$ also damages proteins by impairing their functions. (8) DNA repair system is activated in order to repair the damaged DNA (Ahemad, 2014).

### 1.4.8.2. Non-enzymatic reduction

In the presence of different chemical compounds hexavalent chromium is transform to the trivalent Chromium. For example, Iron (II) and $HS^-$ reduce the hexavalent Chromium which are the metabolic end products of iron and sulfate reducing bacteria. Furthermore, ascorbic acids, glutathione, hydrogen peroxides, ascorbate, etc. are the chemical compounds that reduce the hexavalent Chromium (Poljsak, Pócsi, Raspor, & Pesti, 2010).

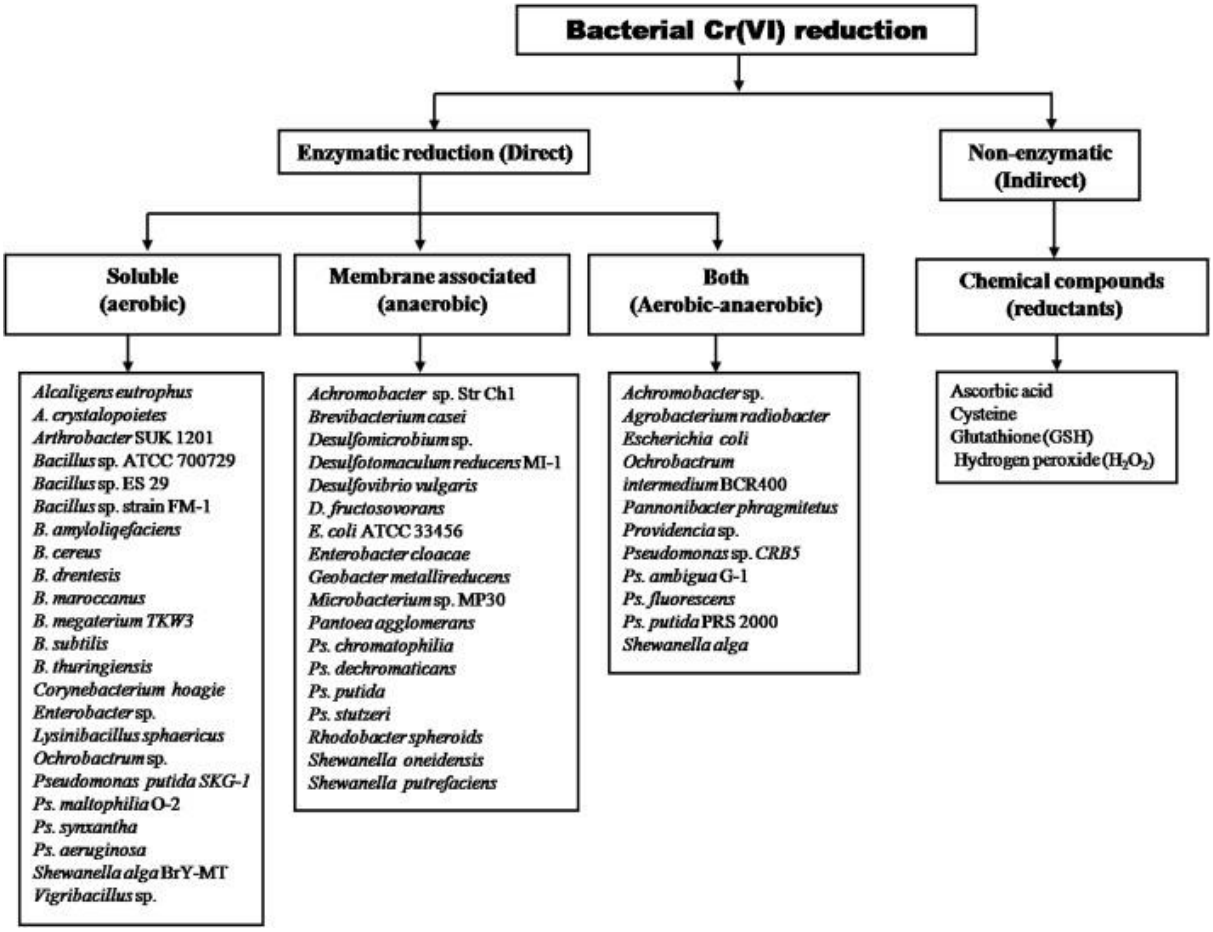


**Figure 1.5.** A schematic diagram of bacterial Cr(VI) reduction (Direct and Indirect reduction) (Thatoi et al., 2014).

## 1.4.9. Classification of chromate reductase enzymes

Based on the sequence homologies, there are two types of chromate reductase enzymes.

**a) Class I chromate reductase**

According to Ackerley, class I chromate reductase includes ChrR and YieF. YieF is a unique enzyme isolated from the E. coli which is a dimer of a flavoprotein and directly reduces Cr(VI) to Cr(III) through 4 electron transfer. Here, three electrons are consumed by the reducing Cr (VI) and 1 electron is transferred to the oxygen (Ackerley, Gonzalez, Park, Blake, Keyhan, Matin, et al., 2004). On the contrary, YieF is considere the most effective enzyme after ChR as the production of the ROS by the YieF in the Cr (VI) reduction is less. In contrast to the ChR, during chromate reduction YieF never showed the generation of the semiquinone flavoprotein and only 25% NADH electrons are consumed to molecular oxygen in ROS generation (Li & Krumholz, 2009).

**b) Class II chromate reductase**

In the class II chromate reductase family, NfsA protein of *E.coli* and ChfN protein of *B. subtilis* are included. Class II chromate reductase possess nitroreductase function and it has no homology to the class I reductase. By an obligatory two electron system NsfA reduces the nitrocompound and the quinone. It is more effective as it can detoxify the nitro compound, possess the therapeutic activity and has the ability to activate the prodrugs which are used in the cancer chemo therapy (Darnowski, Carroll, Płachno, Kabanoff, & Cinnamon, 2006).

## 1.4.10. Potential of chromate reductase in anticancer therapy

It has been suggested that, with some modification the chromate reductase enzymes can play a vital role in anticancer therapy. For example Y6 is such kind of this enzyme which is the modified version of the *E.coli* YieF that result due to the error prone Polymerase Chain reaction (PCR). Y6 acts as a prodrug and when it is kept in the native form it is non-toxic but becomes toxic when it is reduced. This drug can target the both growing and non-growing tumor cells and kill by generating DNA adducts (Nachtigal et al., 2005). Y6 has been tested with Hela cells and proved its improved activity in prodrug reduction (Ackerley, Barak, Lynch, Curtin, & Matin, 2006). In contrast to NfsA, Y6 showed 5 fold greater efficiency to kill the Hela cells (Ackerley et al., 2006). Thus the chromate reductase enzyme YieF has the potential use in anticancer therapy. Bacterial

nitrosoreductases such as NfsA and NfsB from *E.coli* is a class of enzyme that has already been well studied in gene-delivered enzyme prodrug therapy (Grove et al., 2003).

# Chapter 2

# 2. Material and Method

## 2.1 Chemicals

Pure chemicals and analytical tools were applied into every investigations comprising organization of media for advancement. Following chemicals have been applied into this investigation:

**Media used in the experiments:**

**a) Nutrient Agar media:**

1. 0.5% Peptone
2. 0.3% beef extract/yeast extract
3. 1.5% agar
4. 0.5% NaCl ("Nutrient Agar: Composition, Preparation and Uses," 2015)

**b) Nutrient Broth media:**

1. D(+)-glucose: 1 g/L
2. Peptone: 15g/L
3. NaCl: 6g/L
4. Yeast extract: 3g/L ("Nutrient Broth No. 1, for microbiology | Sigma-Aldrich," 2010.)

**c) Muller Hinton Agar media:**

1. Beef extract: 2.0 g/L
2. Acid Hydrolysate of Casein: 17.5 g/L
3. Starch: 1.5 g/L
4. Agar: 17.0 g/L ("Mueller Hinton Agar (MHA): Composition, preparation and uses -," 2013)

**Chemical used:**

1. Potassium chromate ($K_2CrO_4$)
2. Diphenyl Carbazide (DPCZ)
3. MOPS buffer

## 2.2 Glassware and Apparatus

The devices and tools applied during the research are itemized in Table 2.1

**Table 2.1.** Total number of tools utilized throughout the research and their role

| **Instruments** | **Functions** |
|---|---|
| Vertical Autoclave | Sterilization |
| Analytical Balance | Measurement of weight |
| Laminar airflow | Aseptic atmosphere |
| pH meter | pH measurement |
| BOD incubator | Culture incubation |
| Water system | Stock solution preparation |
| UV-visible spectrophotometer | Growth of the cell measurement and degradation of hexavalent Chromium measurement. |
| Centrifuge | Pellet collection and measurement of hexavalent Chromium |
| Water bath | Solubilization of media |
| Microscope | Observation of bacterial morphology |
| Shaker | To incubate the culture of bacteria at the temperature of room |
| Micro pipette | For withdrawing chemical and media's trace quantity |

## 2.3 Collection of samples

Dhaka is situated near the Buriganga river which flows through the south- western part of Dhaka city. Surrounding the river Buriganga, there are lot of tannery industries, leather industries, garment industries etc. These industries throw their wastage into the river water without any pre-treatment and this is increasing day by day as the number of industries are also being increased. Moreover, this is a result of increasing the different heavy metals and different pollutants into the water of the river and thus the living organisms in the water and other lives are being facing

threatened. For conducting this study the water sample was collected from the Swarighat in the Buriganga River (Rahman, Rahman, & Bakri, 2010.)

## 2.4 Isolation and condition of the Culture

Segregation of microorganisms from samples of the water had been finished by standard framework. For the withdrawal of microorganism that show imperviousness to Chromium, inoculation was done for one hundred micro-liter specimen of effluent liquid and one hundred micro-liter specimen of water was obtained from unspoiled sample through scattering framework into agar dish of nutrient towards media comprising two millimolar of hexavalent Chromium complemented as potassium chromate ($K_2CrO_4$ ) and then incubation was done. Some colonies of bacteria had been observed taking after incubation for twenty-four hours at 37ºC temperature. Establishment of the media of nutrient agar was accomplished through melting 2.8gram powder of nutrient agar into one hundred milliliter of $H_2O$. Sterilization was done for media at 121ºC for forty-five minute with keeping fifteen Lb pressure. By then potassium chromate was incorporated into media and finally that media had been occupied throughout the dish for setting the arrangement of agar dish of nutrient. Bacterial colony that had been segregated and contracted with toothpick which were sterilized and speckled upon the agar dish of nutrient on medium comprising two, three, four and five mili molar hexavalent Chromium. Once more its incubation was done for twenty-four hours at 37 ºC. This system had been repeated with continuously greater focuses of six, seven, ten, fifteen, twenty, twenty five, thirty, thirty-five, forty millimolar of hexavalent Chromium till MIC (Minimum Inhibitory Concentration) of the restrain of bacteria had been procured. Enormous improvement & rapid debasement energy of hexavalent Chromium of specific species of bacteria inside the forty millimolar hexavalent Chromium in the midst of the incubation of 24 hours at 37 ºC had been reflected as impervious to hexavalent Chromium.

## 2.5 Chromium reduction profile of Chromium resistant bacteria

To investigate $H_2O$ and effluent $H_2O$, test of Diphenyl Carbazide was done to estimate hexavalent Chromium which was attuned through standard procedures (Eaton et al., 1998) and likewise these approaches were documented (Turick, Apel, & Carmiol, 1996). Arrangement was done for one curve that is standard for institutionalization of depletion chart of the microorganism that showed imperviousness to Chromium.

### 2.5.1 Chemical preparation

#### 2.5.1.1. 10mL 3M $H_2SO_4$ preparation

At first, 8mL purified $H_2O$ was poured into one falcon tube. After that, into falcon tube, 1670 µL conc. $H_2SO_4$ had been poured through droplet comprising 8mL purified $H_2O$. Next, solution was prepared to the equal of 10mL by adding 330µL purified $H_2O$.

#### 2.5.1.2. Diphenyl Carbazide preparation

0.025 Diphenyl Carbazide powder was added into one falcon tube. Then into falcon tube, 9.67mL acetone and afterward 3M sulphuric acid of 330µL were taken comprising Diphenyl Carbazaide (DPCD) powder. Mixing was finely done of that falcon tube to prepare uniform solution of DPCZ.

#### 2.5.1.3. MOPS Buffer preparation

At first, into 50mL $H_2O$, 0.1g sodium hydroxide had been taken to prepare 50mL 1N sodium hydroxide. Then, to prepare 20mM buffer of MOPS, MOPS powder of 334.88mg had been mixed with purified $H_2O$ of 80mL. MOPS buffer's pH was attuned into 7 through the addition of adequate quantity of 1N sodium hydroxide inside the solution of buffer.

#### 2.5.1.4. 5mM 10mL $K_2CrO_4$ preparation

At first, for the solution preparation of 1M potassium chromate, 19.4g potassium chromate had been melted into 10mL purified $H_2O$. Then, filtration was done for this solution utilizing membrane sieve containing 0.45micron size of the pore. At last, dilution was done for this solution up to 5mM and it was preserved so that it could be utilized in future.

### 2.5.2 Processes of experiment

#### 2.5.2.1. Standard Curve preparation

#### 2.5.2.2. Sample preparation for reaction

Subsequent solutions of specimen of various strength had been arranged. Individual specimen's absolute volume was one milliliter.

**Table 2.2.** Sample preparation for standard curve:

| Concentration Final | Quantity of 5mM $K_2CrO_4$ solution | Amount of NB added | Final volume to solution |
|---|---|---|---|
| 50µM | 10µL | 990µL | 1ml |
| 100µM | 20µL | 980µL | 1ml |
| 150µM | 30µL | 970µL | 1ml |
| 200µM | 40µL | 960µL | 1ml |
| 300µM | 60µL | 940µL | 1ml |
| 400µM | 80µL | 920µL | 1ml |
| 500µM | 100µL | 900µL | |
| 600µM | 120µL | 880µL | |

## 2.6 Reaction protocol for standard curve

Firstly, sample of 600 µL was added into one falcon tube. Then into the sample, 1.2ml 20mM buffer of MOPS, 99 µL 3M sulfuric acid, 981 µL purified $H_2O$ and diphenyl carbazide of 120µL were taken progressively and thus a uniform blend has been made. When the response occurs into the sample and solution forms, then the solution becomes purple. Finally, the response solution's absorbance was measured through UV-Visible spectrophotometer at 540nm.

## 2.7 Evaluation of reduction profile of selected isolates at room temperature

### 2.7.1. Process:

Day 1:

Nutrient broth was made into two distinct 10 ml of conical flasks. Then, from stored culture, specimen was added and inoculation was done. Control reflected that broth medium of nutrient where inoculation was not done. Finally, upon the rotating incubator, both of the 10mL conical flasks were given for incubation for 24 hours.

Day 2:

On the next day, nutrient broth medium of 25ml were prepared in 2 conical flasks and were autoclaved. Then in the laminar airflow 15 microliter of potassium chromate was added in the conical flask to make the concentration 600 microliter. Then 2ml of sample was withdrawn into a falcon tube from the 10ml conical flask which was kept for the incubation of bacteria on previous day and the absorbance of sample was taken at 600nm to see the growth of culture. After that calculation was done to identify the amount to be added into the 25ml culture from the 10ml culture to get 0.2 OD. Then the calculated amount was withdrawn from the 10ml culture into a falcon tube and centrifuged by which the cell got separated from the liquid. The liquid was withdrawn leaving the cell in the falcon tube. Again the same calculated amount was also withdrawn from one 25 ml nutrient solution and mixed with the cell in the falcon tube and vortexed to mix them properly. Then they were transferred into the 25ml conical flask again from the falcon tube. The other 25ml was counted as the blank. 2ml solution from each 25ml conical flask was withdrawn in two falcon tubes and then the conical flasks were put into the shaking incubator to culture microorganisms in the hexavalent Chromium condition. Then, the absorbance of the bacteria containing sample was taken at 600 nm. Like previously taken 2ml solution from each conical flask into falcon tube, after every 1.5 hours the sample and the blank were collected throughout the day and absorbance was taken to see the cell growth. Then it was centrifuged to collect the supernatant with which the reaction was performed to check the Chromium level. This process was continued until the next day. The 25ml solution was kept in incubator overnight to observe its overnight activity towards Chromium reduction.

## 2.8 Antibiotic resistance among Chromium resistant isolate

### 2.8.1. Strain Culture preparation in Nutrient Broth (NB)

For the sensitivity investigation of antibiotic, nutrient broth was made for culturing strains. Into one conical flask, nutrient broth of 20ml was added and inoculation was done for the subsequent strains in the individual conical flasks comprising 20ml nutrient broth and incubation was done for overnight at 37 degree Celsius. Labeling was done into the conical flasks.

### 2.8.2. Inoculation of test plates

For the preparation of test plates, Mueller Hinton Agar (MHA) was utilized. Incubated strains of culture were withdrawn after overnight for their inoculation in the plates of MHA. One cotton swab which were sterilized was immersed in conical flask comprising the preparation of culture strain. Inoculation was done on MHA plate's dry exterior through moving the cotton swab upon whole sterile surface of the MHA. This method has been reiterated through moving 2 more periods, for each period, circling that plate about 60º for ensuring inoculum's uniform spread. Finally, the agar's rim was mopped. For three to five minutes, the cover was opened, however, it could not be left open after fifteen minutes so that no extra moisture from the surface can be absorbed before giving antibiotic disks.

### 2.8.3. Application of Antibiotic discs

Antibiotic disc's fixed battery was distributed on MHA plate's surface. Individual discs were pushed down for ensuring of the thorough contact within the surface of agar. Following antibiotic discs were utilized in this test:

**Table 2.3.** Antibiotic discs with their symbol and strength which were used in the antibiotic profiling test:

| Name of the antibiotics | Symbol | Strength (mg) |
|---|---|---|
| Penicillin | P10 | 10 |
| Kanamycin | K30 | 30 |
| Neomycin | N30 | 30 |
| Vancomycin | VA30 | 30 |

| | | |
|---|---|---|
| Gentamycin | CN10 | 10 |
| Cefixime | CFM5 | 5 |
| Chloramphenicol | C30 | 30 |
| Ceftriaxone | CRO30 | 30 |
| Trimethoprim | STX25 | 25 |
| Cipropfloxacin | CIP5 | 5 |
| Streptomycin | S10 | 10 |
| Ofloxacin | OF5 | 5 |
| Amoxycillin | AML10 | 10 |
| Cefuroxime Sodium | CXM30 | 30 |

### 2.8.4. Incubation

Into the incubator, within fifteen minutes, the test plates were positioned at 37ºC for 24 hours after disc application had been done.

## 2.9 Minimum Inhibitory Concentration (MIC) test

In microbiology, MIC is an anti-bacterial minimum concentration which will prevent one bacteria's observable development after the incubation of 24 hour. Chromate's Minimum Inhibitory Concentration for individual isolate was expressed through counting technique of colony. Inoculation of the plates comprising agar media of nutrient complemented with various concentrations of potassium chromate ($K_2CrO_4$) from 2mM – 30mM was done. Then all of these plates were given for incubation at the temperature 37ºC for 48 hours. Finally, the bacterial development was measured through counting of the colony.

## 2.10 Identification of the isolate A

Sequence data file of isolate A was obtained by 16s rDNA sequencing. The sequence was observed by Chromas tool and purified sequence data. The file format was saved as FASTA. BLAST (Basic Local Alignment Search Tool) of the query sequence was done with existing database from NCBI (National Center for Biotechnology Information). Bacterial strain was obtained on the basis of maximum similarity score.

# Chapter 3

# 3. Result

## 3.1 Isolation data of Chromium resistant microorganism

Isolation was done for the individual colonies of two from the agar dishes of nutrient with various concentrations of Chromium. Labelling was done conferring toward distinctive concentrations of Chromium of them such as A.

## 3.2 Chromium reduction profile of Chromium resistant microorganism

### 3.2.1 Standard curve

According to the method standard curve was prepared. After that, utilizing the value of absorbance, a graph of absorbance against concentration was generated using Microsoft Excel Software of 2016. Results which had been obtained are given below:

**Table3.1.** Data of standard curve of hexavalent Chromium:

| Concentration (µM) | Absorbance at 540nm |
|---|---|
| 50 | 0.294 |
| 100 | 0.624 |
| 150 | 0.907 |
| 200 | 1.214 |
| 300 | 1.675 |
| 400 | 2.117 |
| 500 | 2.587 |
| 600 | 2.875 |

From these data, following standard curve was formed:

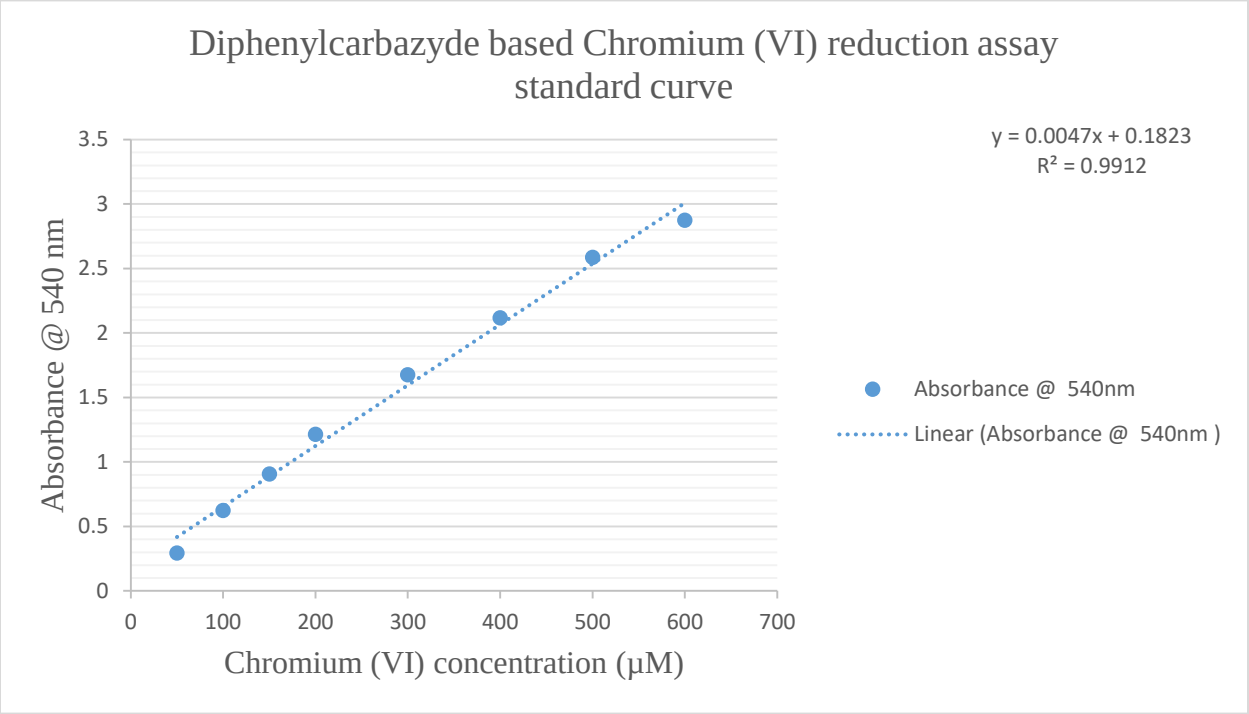


**Figure 3.1.** Standard curve for DPCZ-base Chromium bioremediation assay

### 3.2.2 Reduction profile of Isolate: A

At various conditions, this test was done such as, altering the pH and temperature and the outcomes which were attained precisely in table 3.2:

**Table 3.2.** Isolate A: Chromium reduction profile vs. Cell Growth at 25ºC, pH 5.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 414.82 | 0.552 | 479.01 | 0.042 |
| 1.5 | 381.63 | 0.586 | 483.41 | 0.036 |
| 3 | 368.51 | 0.808 | 450.43 | 0.041 |
| 4.5 | 352.98 | 1.06 | 490.57 | 0 |
| 6 | 316.88 | 1.253 | 458.94 | 0.001 |
| 7.5 | 279.36 | 1.411 | 452.27 | 0.033 |
| 24 | 163.48 | 2.445 | 493.26 | 0.002 |

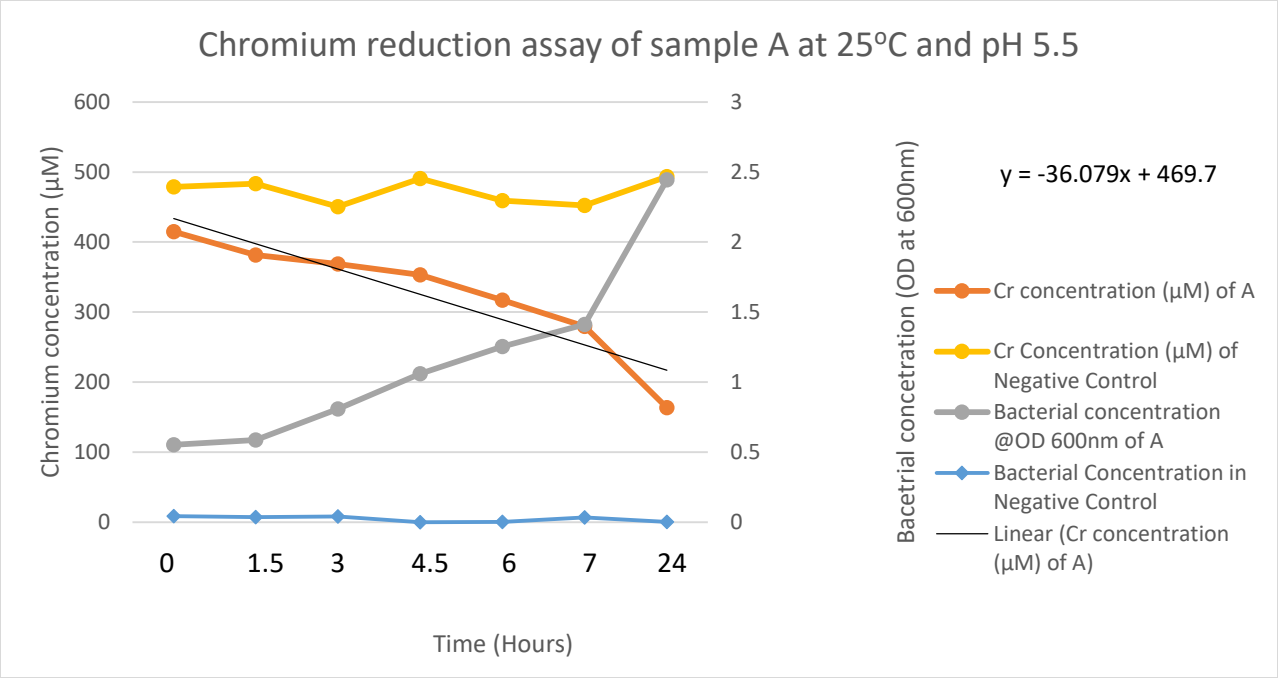


**Figure 3.2.**Chromium reduction vs. Cell Growth in isolate A at 25ºC, pH 5.5

In the figure 3.2, it was found that in sample A at 0 hour, Chromium concentration was around 414.82 µM at 540nm. Then, a gradual fall of the concentration of Chromium was found after 1.5hour, which was 381.63 µM at 540nm. Chromium concentration was continued to decrease in the following hours up to 7.5 hours which was 279.36 µM. Finally, a marked reduction of Chromium concentration had been observed after 24 hours, which was 163.48 µM. Furthermore, in sample A, a sharp growth of bacterial concentrations was observed at 600nm from 0.552 µM to 2.445 µM within 24 hours. But no significant reduction of Chromium concentration or bacterial growth was obtained in the negative control.

**Table 3.3.** Isolate A: Chromium reduction profile vs. Cell Growth at 25ºC, pH 7

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 389.6 | 0.468 | 479.01 | 0.042 |
| 1.5 | 384.97 | 0.674 | 483.41 | 0.036 |
| 3 | 320.71 | 1.218 | 450.43 | 0.041 |
| 4.5 | 287.45 | 1.748 | 490.57 | 0 |
| 6 | 236.95 | 1.912 | 458.94 | 0.001 |
| 7.5 | 216.24 | 2.018 | 452.27 | 0.033 |
| 24 | 152.06 | 2.547 | 493.26 | 0.002 |

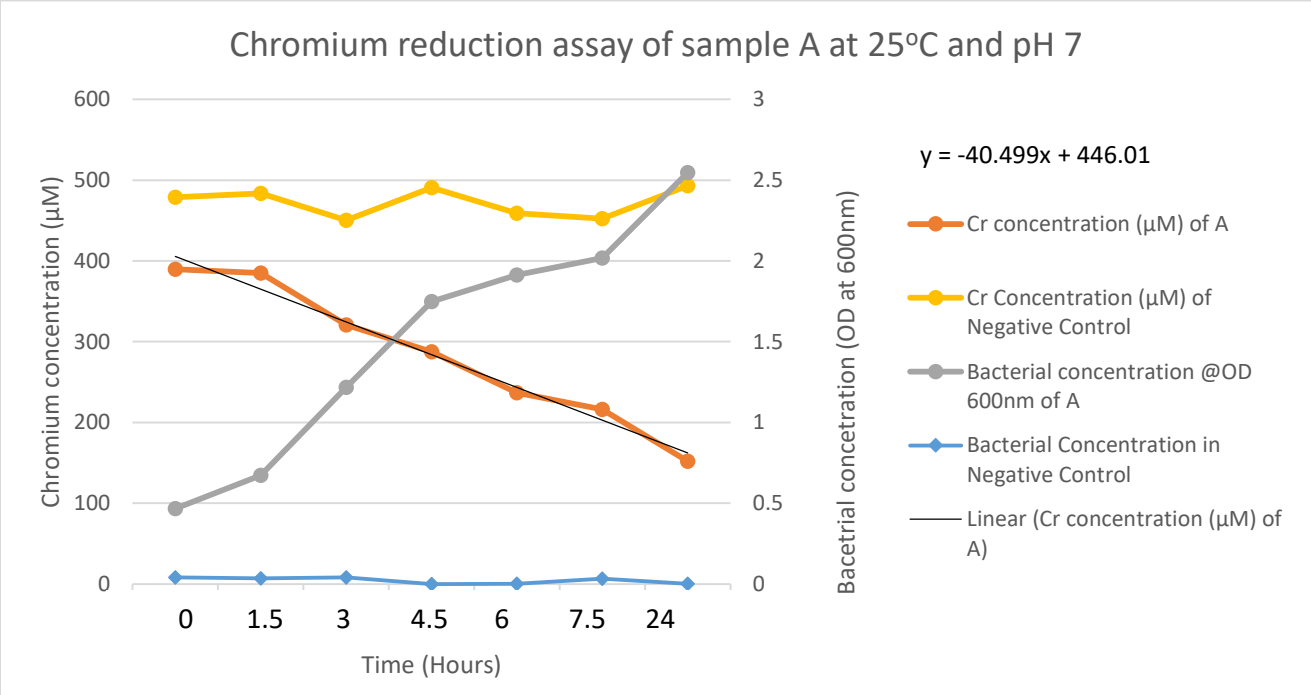


**Figure 3.3.** Chromium reduction vs. Cell Growth in isolate A at 25ºC, pH 7

In the figure 3.3 it was observed that, in sample A at 0 hour, Chromium concentration was around 389.65 µM at 540nm. Then, gradually, decrease in Chromium concentration was seen in the

following hours from 1.5 to 7.5 and found 384.97, 320.71, 287.45, 236.95, 216.24 µM at 540nm. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 152.06 µM. Furthermore, lower growth of bacterial concentration was observed in sample A from 0.468 to 2.547 µM within 24 hours at 600nm. But no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.4.** Isolate A: Chromium reduction profile vs. Cell Growth at 25ºC, pH 8.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 345.89 | 0.584 | 479.01 | 0.042 |
| 1.5 | 332.91 | 0.607 | 483.41 | 0.036 |
| 3 | 234.47 | 1.161 | 450.43 | 0.041 |
| 4.5 | 297.59 | 1.739 | 490.57 | 0 |
| 6 | 245.75 | 1.948 | 458.94 | 0.001 |
| 7.5 | 161.42 | 2.052 | 452.27 | 0.033 |
| 24 | 128.94 | 2.186 | 493.26 | 0.002 |

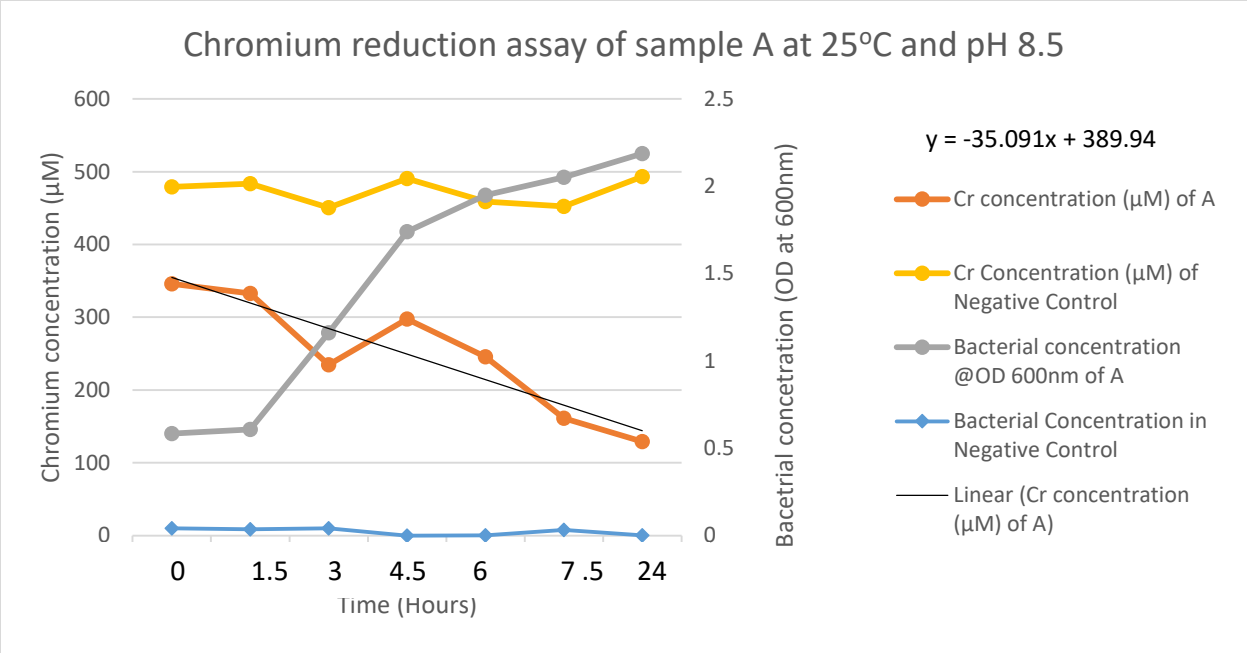


**Figure 3.4.** Chromium reduction vs. Cell Growth in isolate A at 25ºC, pH 8.5

In the figure 3.4 it was seen that, in sample A at 0 hour, Chromium concentration was around 345.89 µM at 540nm. Then, gradually, decrease in Chromium concentration was seen in the following hours from 1.5 to 7.5 and found 332.91 to 161.42 µM at 540nm. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 128.94 µM. Moreover, minimal growth of bacterial concentration was observed in sample A from 0.584 to 2.186 in 24 hours at 600nm. But no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.5.** Isolate A: Chromium reduction profile vs. Cell Growth at 37ºC, pH 5.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 425.11 | 0.329 | 377.80 | 0 |
| 1.5 | 393.62 | 0.332 | 458.44 | 0.007 |
| 3 | 316.81 | 0.335 | 455.32 | 0 |
| 4.5 | 341.56 | 0.345 | 407.31 | 0.003 |
| 6 | 319.58 | 0.332 | 430.64 | 0 |
| 7.5 | 281.70 | 0.45 | 428.80 | 0.008 |
| 24 | 97.52 | 0.36 | 499.51 | 0 |

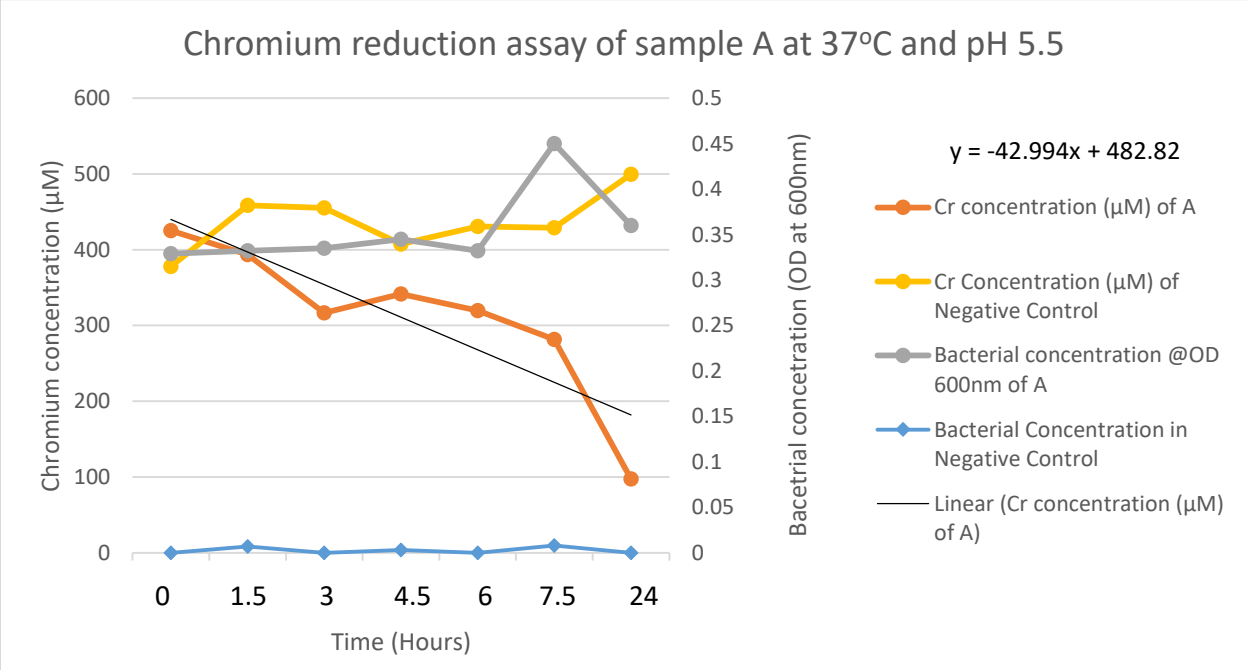


**Figure 3.5.** Chromium reduction vs. Cell Growth in isolate A at 37ºC, pH 5.5

In the figure 3.5 it was seen that, in isolate A at 0 hour, Chromium concentration was around 425.11 µM at 540nm. Then, gradually the decreases in Chromium concentration was seen in the following hours from 1.5 to 7.5 hours at 540nm which were 393.62 to 281.70 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 97.52 µM. Moreover, minimal growth of bacterial concentration was observed in sample A from 0 to 7.5 hour and after 24 hours it was found 0.36 µM at 600nm which showed that there was no significant increase of the bacterial growth and no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.6.** Isolate A: Chromium reduction profile vs. Cell Growth at 37ºC, pH 7

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 410.29 | 0.419 | 377.80 | 0 |
| 1.5 | 363.90 | 0.963 | 458.44 | 0.007 |
| 3 | 189.93 | 1.325 | 455.32 | 0 |
| 4.5 | -32.19 | 1.518 | 407.31 | 0.003 |
| 6 | -38.78 | 1.508 | 430.64 | 0 |
| 7.5 | -38.78 | 1.561 | 428.80 | 0.008 |
| 24 | -25.24 | 1.591 | 499.51 | 0 |

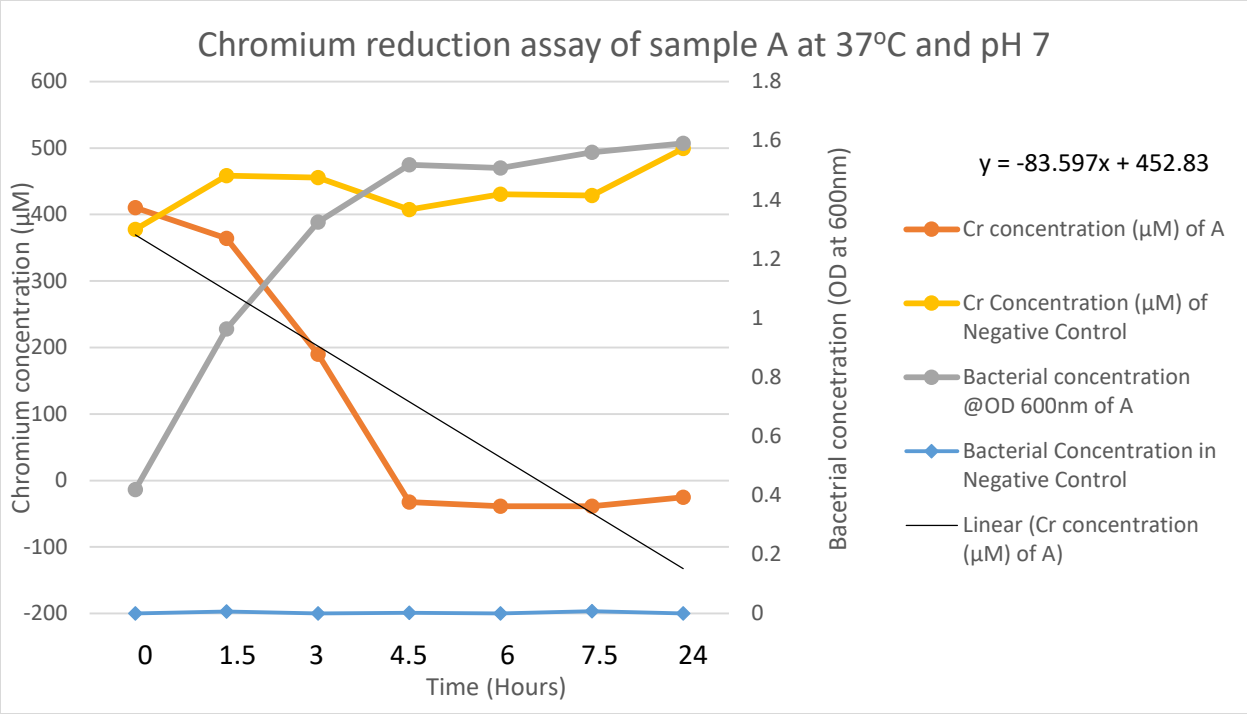


**Figure 3.6.** Chromium reduction vs. Cell Growth in isolate A at 37ºC, pH 7

From the above figure 3.6 it was seen that, in sample A at 0 hour, Chromium concentration was around 410.29 µM at 540nm. Then, gradually, the decreases in Chromium concentration was seen up to 3 hours and it was 189.93 µM at 540nm. After that, at 4.5 hours Chromium concentration was found -32.19 µM and at 6 and 7.5 hours it was constant, -38.78 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was -25.24 µM. Moreover, minimal growth of bacterial concentration was observed in sample A from 0 to 7.5 hours and found gradually 0.419 µM to 1.561 µM and after 24 hours it was 1.591 µM at 600nm. No significant bacterial growth or Chromium reduction was obtained in the negative control which was found almost 0.

**Table 3.7.** Isolate A: Chromium reduction profile vs. Cell Growth at 37ºC, pH 8.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 387.80 | 0.706 | 377.80 | 0 |
| 1.5 | 318.30 | 1.463 | 458.44 | 0.007 |
| 3 | 25.11 | 1.69 | 455.32 | 0 |
| 4.5 | -9.63 | 1.902 | 407.31 | 0.003 |
| 6 | -31.62 | 1.962 | 430.64 | 0 |
| 7.5 | -38.78 | 2.036 | 428.80 | 0.008 |
| 24 | -26.73 | 2.538 | 499.51 | 0 |

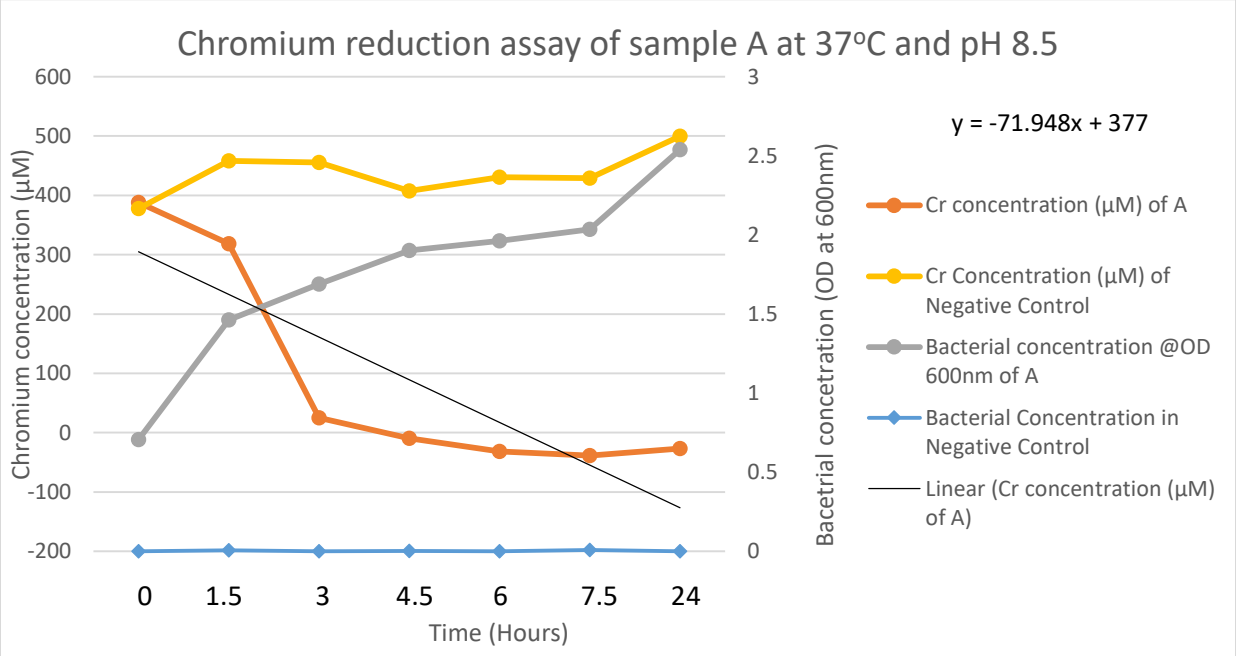


**Figure 3.7.** Chromium reduction vs. Cell Growth in isolate A at 37ºC, pH 8.5

From the figure 3.7 it was seen that, in sample A at 0 hour, Chromium concentration was around 387.80 µM at 540nm. Then, gradually the decreases in Chromium concentration was seen up to 3 hours, 25.11 µM at 540nm. After that, at 4.5 hours Chromium concentration was found -9.63 µM, at 6 hours it was -31.62 µM and at 7.5 hours it was -38.78 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was -26.73 µM. But no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table3.8.** Isolate A: Chromium reduction profile vs. Cell Growth at 42ºC, pH 5.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 409.936 | 0.254 | 498.23 | 0.005 |
| 1.5 | 349.93 | 0.253 | 450.21 | 0.004 |
| 3 | 339.58 | 0.167 | 463.12 | 0.0014 |
| 4.5 | 284.04 | 0.168 | 451.07 | 0.004 |
| 6 | 267.098 | 0.17 | 444.61 | 0.012 |
| 7.5 | 258.16 | 0.166 | 460.361 | 0.013 |
| 24 | 21.212 | 0.179 | 493.26 | 0.012 |

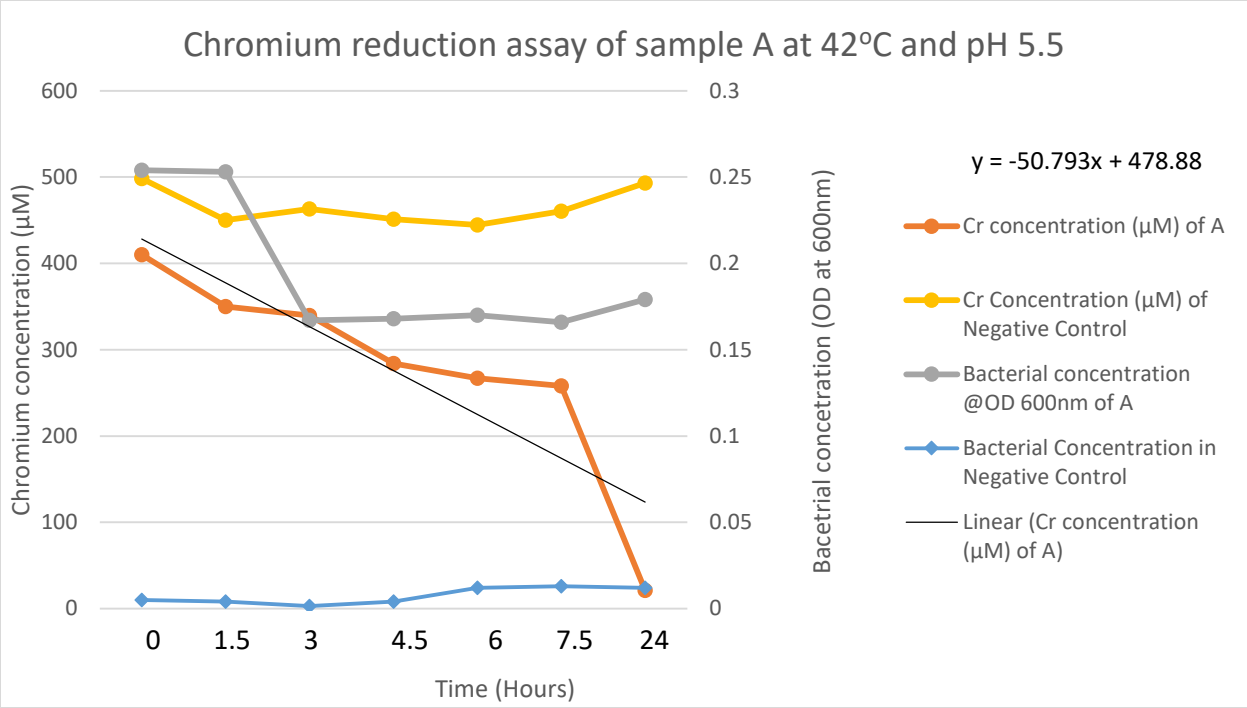


**Figure 3.8.** Chromium reduction vs. Cell Growth in isolate A at 42°C, pH 5.5

In the fiigure 3.8 it was seen that, in sample A at 0 hour, Chromium concentration was around 409.93 µM at 540nm. Then, gradually, decrease in Chromium concentration was seen in the following hours from 1.5 to 7.5 hours at 540nm which were 349.93 µM to 258.16 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 21.21 µM. Again, the decreases growth of bacterial concentration was observed in sample A from 0.254 µM to 0.179 µM within 24 hours at 600nm and no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.9.** Isolate A: Chromium reduction profile vs. Cell Growth at 42ºC, pH 7

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 403.41 | 0.218 | 498.23 | 0.005 |
| 1.5 | 387.59 | 0.252 | 450.21 | 0.004 |
| 3 | 381.42 | 0.295 | 463.12 | 0.0014 |
| 4.5 | 358.37 | 0.323 | 451.07 | 0.004 |
| 6 | 336.31 | 0.42 | 444.617 | 0.012 |
| 7.5 | 282.06 | 0.432 | 460.36 | 0.013 |
| 24 | 118.16 | 1.087 | 493.26 | 0.012 |

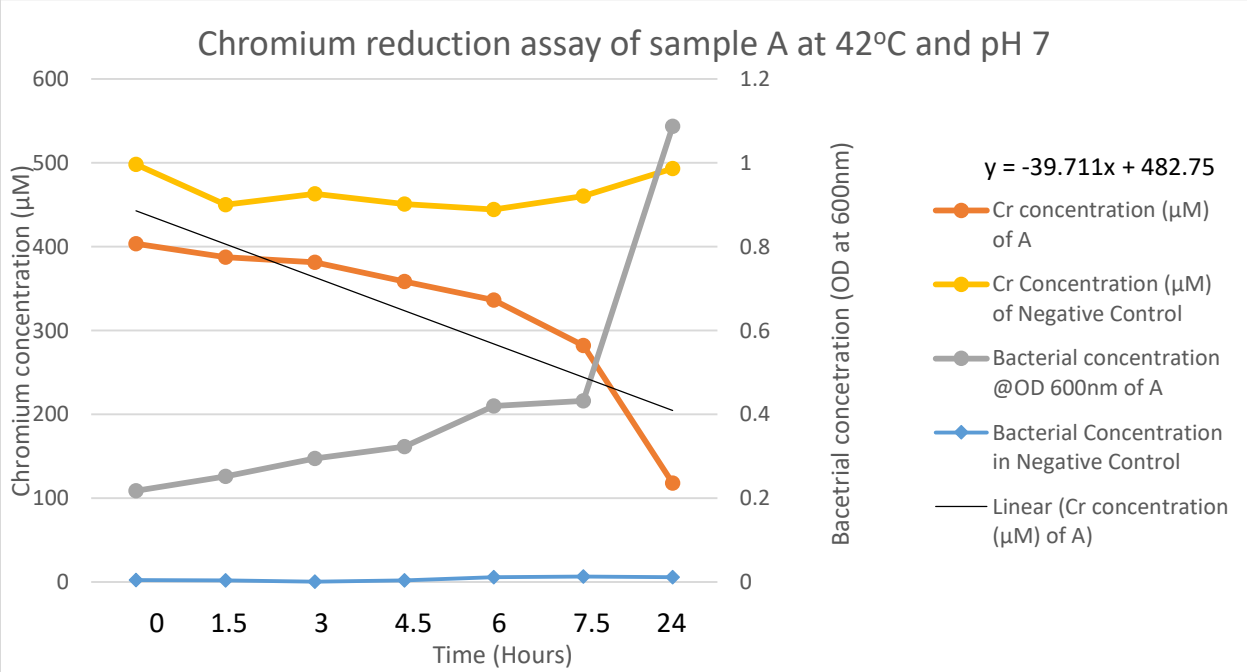


**Figure 3.9.** Chromium reduction vs. Cell Growth in isolate A at 42ºC, pH 7

In the figure 3.9 it was seen that, in sample A at 0 hour, Chromium concentration was around 403.41 µM at 540nm. Then, gradually, the decreases in Chromium concentration was seen in the following hours from 1.5 hours to 7.5 hours at 540nm which were 387.59 µM to 282.06 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 118.16 µM. Moreover, minimal growth of bacterial concentration was observed in sample A from 0.218 µM to 0.432 µM within 0 to 7.5 hours and bacterial growth 1.087 was found in 24 hours at 600nm. But no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.10.** Isolate A: Chromium reduction profile vs. Cell Growth at 42ºC, pH 8.5

| Sample | | | Negative control | |
|---|---|---|---|---|
| Time (Hour) | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm | Chromium concentration on (µM) at 540 nm | Bacterial concentration at 600 nm |
| 0 | 416.31 | 0.231 | 498.23 | 0.005 |
| 1.5 | 368.37 | 0.321 | 450.21 | 0.004 |
| 3 | 322.20 | 0.387 | 463.12 | 0.0014 |
| 4.5 | 301.63 | 0.399 | 451.07 | 0.004 |
| 6 | 273.05 | 0.585 | 444.61 | 0.012 |
| 7.5 | 226.24 | 0.712 | 460.36 | 0.013 |
| 24 | 80.21 | 1.047 | 493.26 | 0.012 |

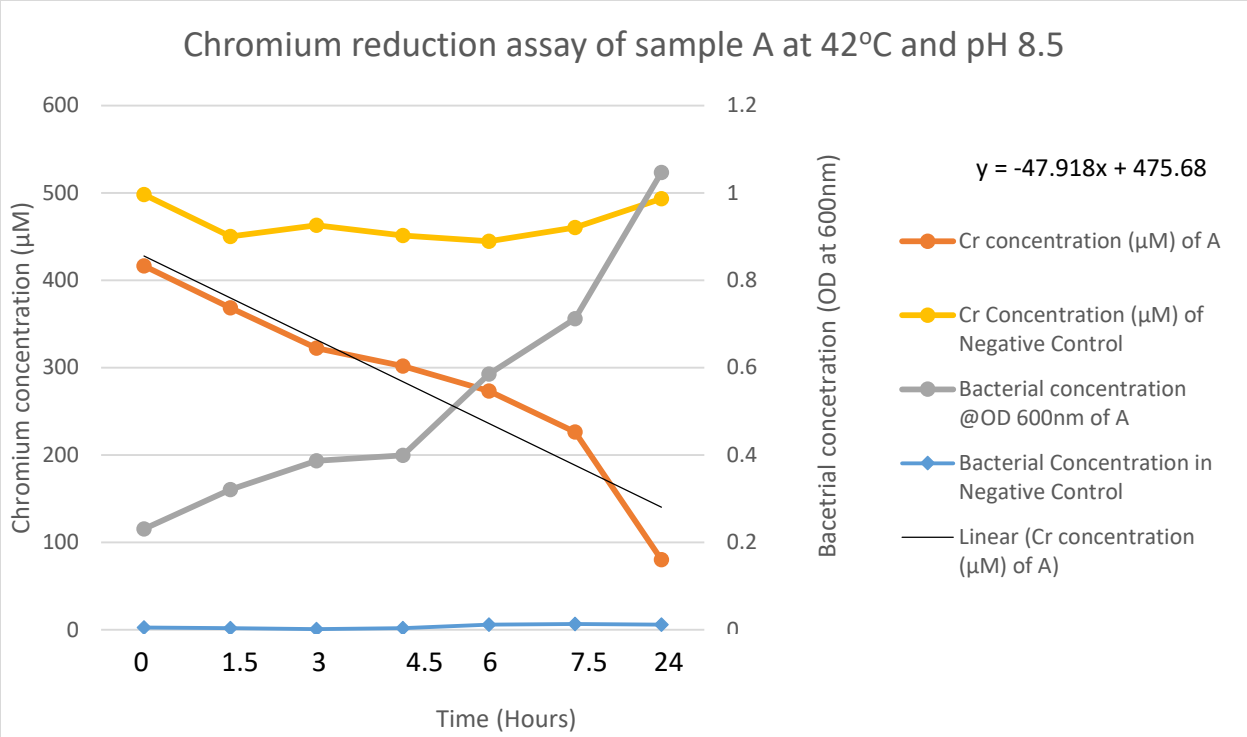


**Figure 3.10.** Chromium reduction vs. Cell Growth in isolate A at 42ºC, pH 8.5.

From the figure 3.10 it was clear that, in isolate A at 0 hour, Chromium concentration was around 416.31 µM at 540nm. Then, gradually, the decreases in Chromium concentration was seen in the following hours from 1.5 hours to 7.5 hours at 540nm. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 80.21 µM. Moreover, minimal growth of bacterial concentration was observed in sample A from 0.231 µM to 1.047 µM in 24 hours at 600nm. But no significant bacterial growth or Chromium reduction was obtained in the negative control.

**Table 3.11.** Summary of the result of Chromium reduction bioassay

| Temperature (˚C) | pH | Time (Hours) | Percentage (%) of Chromium reduction |
|---|---|---|---|
| 25 | 5.5 | 7.5 | 32.66 |
| | | 24 | 60.59 |
| | 7 | 7.5 | 44.50 |
| | | 24 | 60.97 |
| | 8.5 | 7.5 | 53.32 |
| | | 24 | 62.72 |
| 37 | 5.5 | 7.5 | 33.73 |
| | | 24 | 77 |
| | 7 | 7.5 | 109.45 |
| | | 24 | 106.15 |
| | 8.5 | 7.5 | 110 |
| | | 24 | 106.89 |
| 42 | 5.5 | 7.5 | 37.02 |
| | | 24 | 94.83 |
| | 7 | 7.5 | 30.08 |
| | | 24 | 70.70 |
| | 8.5 | 7.5 | 45.66 |
| | | 24 | 82.72 |

## 3.3 Antibiotic resistance among Chromium resistant isolate A

Antibiotic resistance test was done by distributing and fixing eleven discs of antibiotic on the surface of autoclaved MHA plates. After that, the activities of antibiotic discs against isolate A were determined by measuring the diameter of zone of inhibition in millimeter with the help of a transparent scale.

The plates including with all the antibiotic discs are given below:

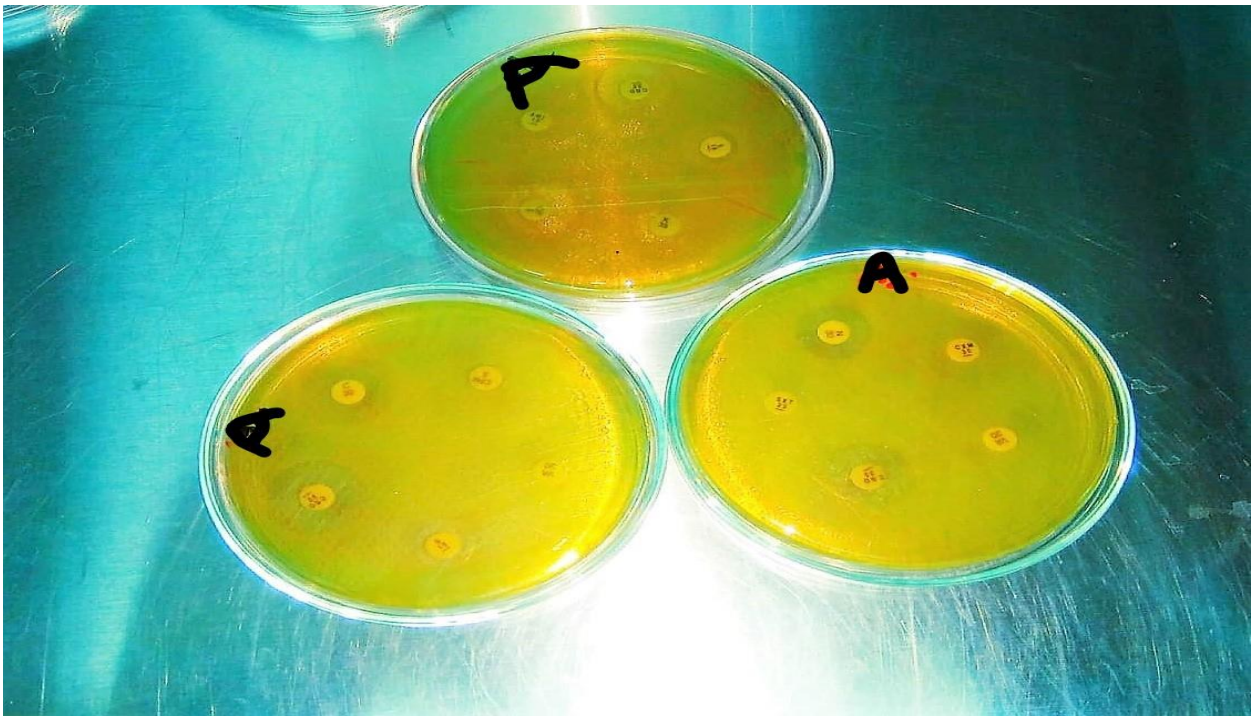


**Figure 3.11.** Zone of Inhibition of Antibiotic discs in isolate A

**Table 3.12.** Isolate-A: Antibiotic resistance profile

| **Name of Antibiotic disc** | **Average Zone of Inhibition (ZI) of isolate A (mm)** | **Standard deviation (SD)** |
|---|---|---|
| Penicillin (P10) | 0 | 0 |
| Kanamycin (K30) | 11.17 | 0.288 |
| Neomycin (N30) | 15.67 | 0.577 |
| Vancomycin (VA30) | 0 | 0 |
| Gentamycin (CN10) | 16 | 1 |
| Cefixime (CFM5) | 8 | 1 |
| Chloramphenicol (C30) | 14.67 | 1.15 |
| Ceftriaxone (CRO30) | 19 | 0 |
| Trimethoprim (STX25) | 0 | 0 |
| Cipropfloxacin (CIP5) | 19.67 | 0.577 |

| | | |
|---|---|---|
| Streptomycin (S10) | 11.5 | 0.5 |
| Ofloxacin (OF5) | 18.67 | 0.577 |
| Amoxycillin (AML10) | 0 | 0 |
| Cefuroxime Sodium (CXM30) | 17.83 | 1.607 |

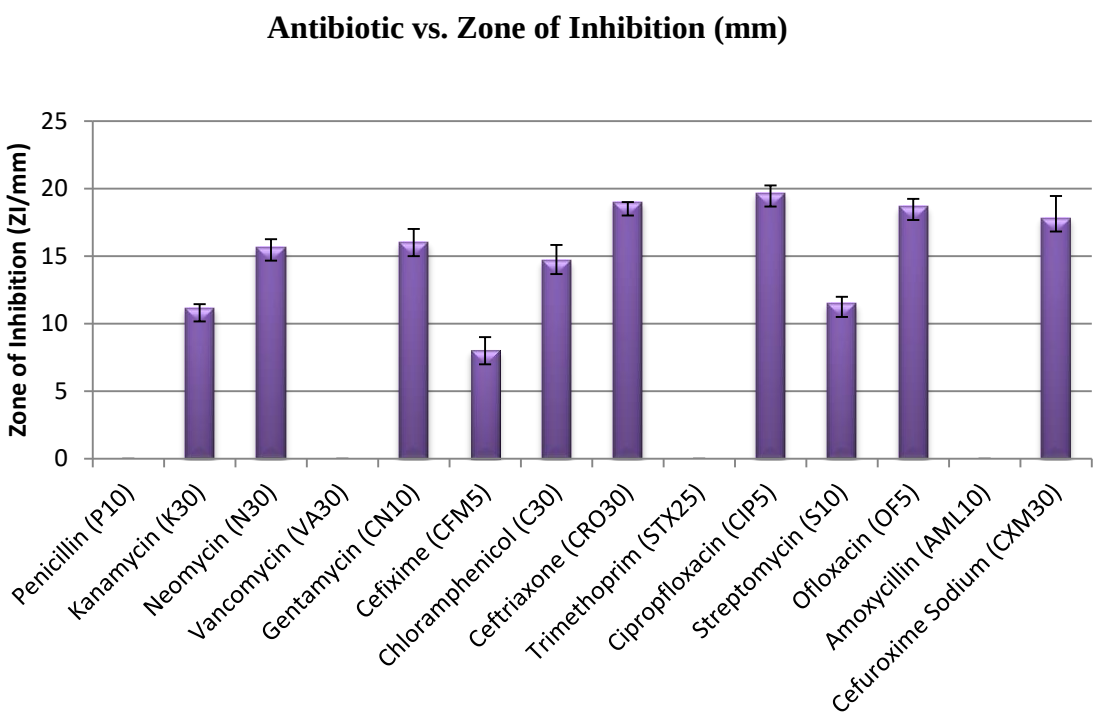


**Figure 3.12.** Antibiotic vs. zone of inhibition (mm)

By observing the above graph and table it was seen that, the bacterial isolate of sample A showed resistant to 4 antibiotics which were Penicillin (P10), Vancomycin (VA30), Trimethoprim (STX25) and Amoxycillin (AML10) as there were no zone of inhibition was found. Among other antibiotics, Cipropfloxacin (CIP5) showed the highest zone of inhibition 19.67mm. Therefore, the performance of Cipropfloxacin (CIP5) was more potent than all other antibiotics against the bacterial isolate of A because this antibiotic disc was capable for killing the most bacterial cells of A strain. After Cipropfloxacin (CIP5), Ceftriaxone (CRO30) was more potent to kill the bacteria and its zone of inhibition was recorded 19mm, then Ofloxacin (OF5) which had provided the zone of inhibition, 18.67mm. On the contrary, the lowest zone of inhibition was provided by the

Kanamycin (K30) and it was recorded 11.17 mm. So we could say that A was mostly susceptible to the Cipropfloxacin (CIP5) and less susceptible to Kanamycin (K30).

## 3.4 Minimum Inhibitory Concentration of Chromium of isolate A

Different number of colonies had been found at different Chromium concentration which had been recorded in the following table:

**Table 3.13.** MIC of isolate A

| Concentration of Cr(VI) | Colony Forming Unit(CFU) |
|---|---|
| 2 | 31 |
| 3 | 29 |
| 4 | 25 |
| 5 | 24 |
| 6 | 20 |
| 7 | 18 |
| 8 | 15 |
| 9 | 12 |
| 10 | 10 |
| 11 | 8 |
| 12 | 6 |
| 13 | 4 |
| 14 | 3 |
| 15 | 3 |
| 16 | 2 |
| 17 | 0 |

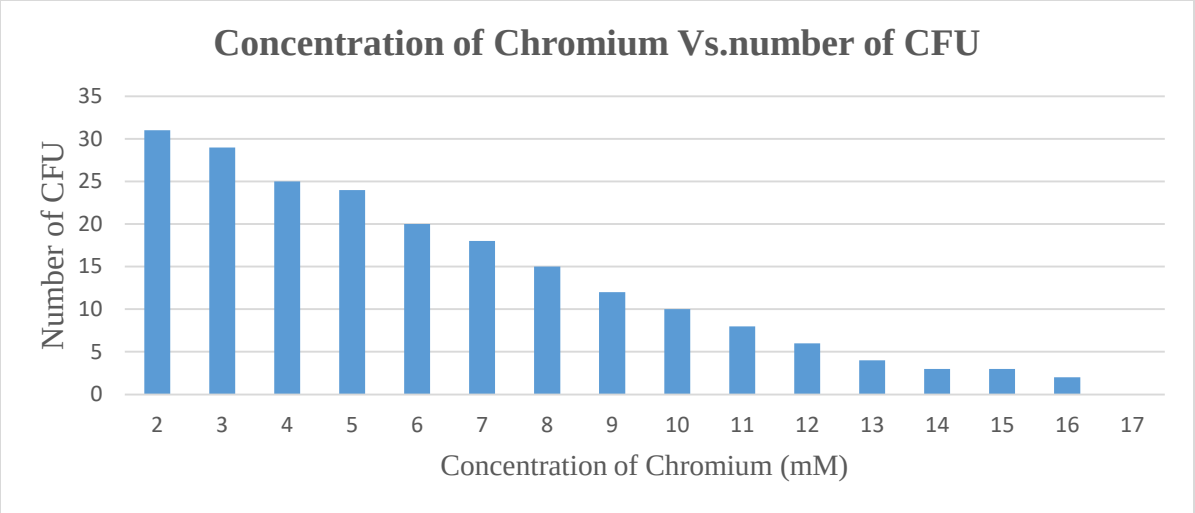


**Figure 3.13.** MIC of Cr(VI) to prevent the growth of isolate A

In the figure 3.13 it was seen that the isolate A could tolerate the Chromium concentration up to 16 mM. After 16 mM of Chromium concentration it could not tolerate Chromium. At 2 mM of Chromium concentration it showed maximum number of colonies of the bacteria and this number of bacterial colonies were gradually decreased with increasing Chromium concentration. Finally, at 17 mM concentration of Chromium no bacterial colonies were found. So, 17mM was the Minimum Inhibitory Concentration of isolate A.

## 3.5 Identification of the isolate A

Sequence information file of isolate A was obtained via 16s rDNA sequencing and the sequence was observed by Chromas tool. The file format was saved as FASTA. BLAST (Basic Local Alignment Search Tool) of the query sequence was achieved with present database from NCBI (National Center for Biotechnology Information). Bacterial strain was confirmed on the basis of most similarity score. For the identification of isolate A different software like Finch TV, MEGA7 and BioEdit tools etc were used. By using these software a phylogenetic tree was established which showed that isolate A was 100% similar to the strain of *Proteus mirabilis.*

The phylogenetic tree was given bellow:

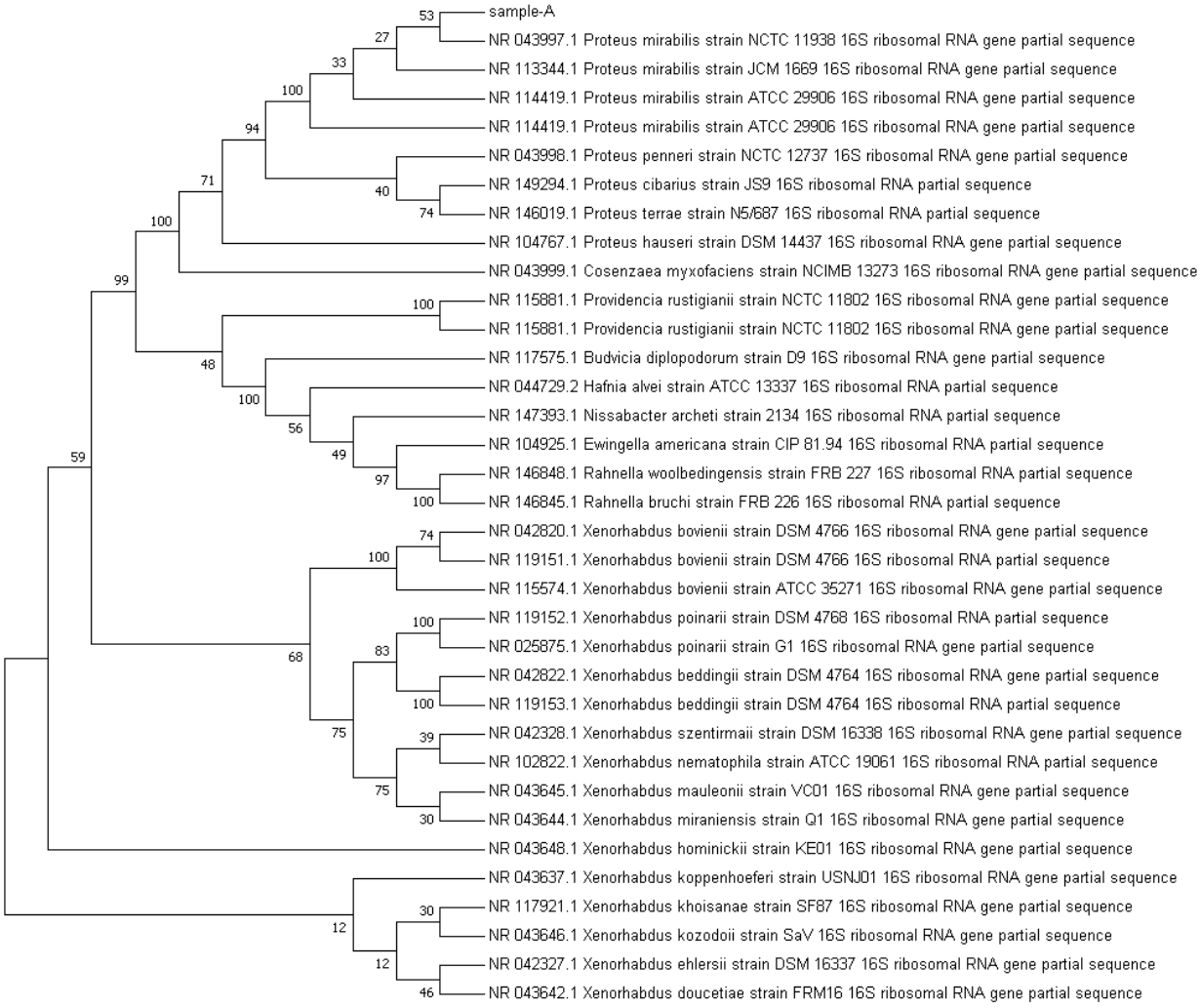


**Figure 3.14.** Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method(Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches(Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 35 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1220

positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, Stecher, & Tamura, 2016).

# Chapter 4

## 4.1 Discussion

The main purpose of this paper is to identify, separate, screen and select the specific number of bacteria which are Cr (VI) resistant. In this study, the severity of Chromium toxicity was discussed in details in chapter two which showed that Chromium implies its toxic effects on skin, kidney, liver etc. and also it is considered as a teratogenic, carcinogenic and mutagenic substance. So, it is clearly understood that how much important this study is to identify and find out the Chromium resistant bacteria and also identify their optimum environment for their maximum activity to reduce or detoxify the hexavalent Chromium.

After collection, isolation and purification the pure sample was stored. For conducting the bioassay of the isolate sample A, a standard curve was prepared by following the standard protocol in which the absorbance of Chromium was recorded. Chromium reduction assay was done at 25˚C, 37 ˚C and 42 ˚C with three different pHs- 5.5, 7 and 8.5. Next, the results of bioassay were compared by analyzing table 3.11 which showed the summary of the bioassay profile. By observing this table the comparisons were made among the results and the optimum temperature and pH of the isolate A. Within 7.5 hours 110% of Chromium was reduced by the isolate A at 37˚C and in the same temperature and pH within 24 hours 106.89% of Chromium concentration was reduced. After that, the optimum temperature was 42 ˚C and optimum pH was 5.5 and 8.5. At pH 5.5, within 24 hours 94.83% of Chromium concentration was reduced. Lastly, the isolate A showed the lowest capability to reduce or detoxify the Chromium concentration at 25 ˚C temperature. So, the optimum temperature and pH of the isolate A strain was 37 ˚C and pH 8.5.

Now, from the antibiotic resistance profile it was found that the bacterial isolate of sample A showed resistant to four antibiotics which were Penicillin (P10), Vancomycin (VA30), Trimethoprim (STX25) and Amoxycillin (AML10) as no zone of inhibition was found there. Among other antibiotics, Cipropfloxacin (CIP5) showed the highest zone of inhibition, 19.67mm. Therefore, the performance of Cipropfloxacin (CIP5) was more susceptible than all other antibiotics against the bacterial isolate of A because this antibiotic disc was capable for killing most bacterial cells of A strain surrounding the antibiotic discs. After Cipropfloxacin (CIP5), Ceftriaxone (CRO30) was more susceptible to kill the bacteria and its zone of inhibition was

recorded 19 mm, then Ofloxacin (OF5) which had provided the zone of inhibition 18.67mm. On the contrary, the lowest area of zone of inhibition was provided by the Kanamycin (K30) and it was recorded 11.167mm. The more susceptible antibiotics with increasing their zone of inhibition is shown below:

Cipropfloxacin (CIP5> Ceftriaxone (CRO30)> Ofloxacin (OF5)> Cefuroxime Sodium (CXM30)> Gentamycin (CN10)> Neomycin (N30)> Chloramphenicol (C30)> Streptomycin (S10)> Kanamycin (K30)> Cefixime (CFM5)> Penicillin (P10), Vancomycin (VA30), Trimethoprim (STX25), Amoxycillin (AML10)

So, it could be said that the isolate strain A was mostly susceptible to Cipropfloxacin (CIP5) and less susceptible to Kanamycin (K30) and resistant to Penicillin (P10), Vancomycin (VA30), Trimethoprim (STX25) and Amoxycillin (AML10) antibiotics.

From the result of Minimum Inhibitory Concentration (MIC), the MIC was obtained 17mM for the isolate A. So, it could be said that the isolate sample A could not tolerate the 17mM of Chromium(VI) concentration.

Lastly,16s rDNA sequencing was done to identify the bacterial strain and a phylogenetic tree was prepared by using different softwares.. By studying the phylogenetic tree it was confirmed that the strain of the isolate A was *Proteus mirabilis* as they were situated in the same branch of the phylogenetic tree.

# Chapter 5

## 5.1 Conclusion

From this study, it can be concluded that isolate A has the potential to reduce Chromium concentration biologically. As a result, the Chromium reductase enzyme, which is present in the microorganism can be synthesized for the treatment of Chromium toxicity in human. Also, it can be used in the water purification plants for the purpose of purifying the waste water which contains a large amount of hexavalent Chromium.

## 5.2 Further work

Further studies can be done in which Chromium reductase enzyme can be isolated and identified. It can also be correlated with the antibiotic resistance and Chromium reduction assay. Further, it can be found whether the enzyme, responsible for the reduction of Chromium is an exoenzyme or an endoenzyme and plasmid analysis can also be done in order to find out more about the bacteria and chromate reductase enzyme.

# Chapter 6

## 6.1 References


Ackerley, D. F., Barak, Y., Lynch, S. V, Curtin, J., & Matin, A. (2006). Effect of chromate stress on Escherichia coli K-12. *Journal of Bacteriology*, *188*(9), 3371–3381. https://doi.org/10.1128/JB.188.9.3371-3381.2006

Ackerley, D. F., Gonzalez, C. F., Park, C. H., Blake, R., Keyhan, M., & Matin, A. (2004). Chromate-reducing properties of soluble flavoproteins from Pseudomonas putida and Escherichia coli. *Applied and Environmental Microbiology*, *70*(2), 873–882. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14766567

Ackerley, D. F., Gonzalez, C. F., Park, C. H., Blake, R., Keyhan, M., Matin, A., & Matin, A. (2004). Chromate-reducing properties of soluble flavoproteins from Pseudomonas putida and Escherichia coli. *Applied and Environmental Microbiology*, *70*(2), 873–882. https://doi.org/10.1128/AEM.70.2.873-882.2004

Ahemad, M. (2014). Bacterial mechanisms for Cr(VI) resistance and reduction: An overview and recent advances. *Folia Microbiologica*, *59*(4), 321–332. https://doi.org/10.1007/s12223-014-0304-8

Alam, M., Hossain, M. A., Yonge, D. R., Peyton, B. M., & Petersen, J. N. (2006). Bioreduction of Hexavalent Chromium in Flow-Through Quartz Sand Columns. *Journal of Environmental Engineering*, *132*(3), 358–366. https://doi.org/10.1061/(ASCE)0733-9372(2006)132:3(358)

Alvarez, A. H., Moreno-Sánchez, R., & Cervantes, C. (1999). Chromate efflux by means of the ChrA chromate resistance protein from Pseudomonas aeruginosa. *Journal of Bacteriology*, *181*(23), 7398–7400. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10572148

Appenroth, K.-J., Teller, S., & Horn, M. (1996). Photophysiology of turion formation and germination inSpirodela polyrhiza. *Biologia Plantarum*, *38*(1), 95–106. https://doi.org/10.1007/BF02879642

ATSDR. (2008). Chromium Toxicity, 1–67. Retrieved from https://www.atsdr.cdc.gov/csem/chromium/docs/chromium.pdf

Augustynowicz, J., Grosicki, M., Hanus-Fajerska, E., Lekka, M., Waloszek, A., & Kołoczek, H. (2010). Chromium(VI) bioremediation by aquatic macrophyte Callitriche cophocarpa Sendtn. *Chemosphere*, *79*(11), 1077–1083. https://doi.org/10.1016/j.chemosphere.2010.03.019

Babula, P., Adam, V., Opatrilova, R., Zehnalek, J., Havel, L., & Kizek, R. (2008). Uncommon heavy metals, metalloids and their plant toxicity: a review. *Environmental Chemistry Letters*, *6*(4), 189–213. https://doi.org/10.1007/s10311-008-0159-9

Baldiris, R., Acosta-Tapia, N., Montes, A., Hernández, J., & Vivas-Reyes, R. (2018). Reduction of hexavalent chromium and detection of chromate reductase (ChrR) in stenotrophomonas maltophilia. *Molecules*, *23*(2). https://doi.org/10.3390/molecules23020406

Belay, A. A. (2010). Impacts of Chromium from Tannery Effluent and Evaluation of Alternative Treatment Options. *Journal of Environmental Protection*, *01*(01), 53–58. https://doi.org/10.4236/jep.2010.11007

Benefield, L. D., Judkins, J. F., & Weand, B. L. (1982). *Process chemistry for water and wastewater treatment*. Englewood Cliffs: Prentice-Hall. Retrieved from https://www.worldcat.org/title/process-chemistry-for-water-and-wastewater-treatment/oclc/440936818

Bopp, L. H., & Ehrlich, H. L. (1988). Chromate resistance and reduction in Pseudomonas fluorescens strain LB300. *Archives of Microbiology*, *150*(5), 426–431. https://doi.org/10.1007/BF00422281

Brown, S. D., Thompson, M. R., VerBerkmoes, N. C., Chourey, K., Shah, M., Zhou, J., … Thompson, D. K. (2006). Molecular Dynamics of the *Shewanella oneidensis* Response to Chromate Stress. *Molecular & Cellular Proteomics*, *5*(6), 1054–1071. https://doi.org/10.1074/mcp.M500394-MCP200

Bruynzeel, D. P., Hennipman, G., & van Ketel, W. G. (1988). Irritant contact dermatitis and chrome-passivated metal. *Contact Dermatitis*, *19*(3), 175–179. https://doi.org/10.1111/j.1600-0536.1988.tb02889.x

Camargo, F. A. O., Bento, F. M., Okeke, B. C., & Frankenberger, W. T. (n.d.). Chromate reduction by chromium-resistant bacteria isolated from soils contaminated with dichromate. *Journal of*

*Environmental Quality*, *32*(4), 1228–1233. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12931876

Cervantes, C., Campos-García, J., Devars, S., Gutiérrez-Corona, F., Loza-Tavera, H., Torres-Guzmán, J. C., & Moreno-Sánchez, R. (2001). Interactions of chromium with microorganisms and plants. *FEMS Microbiology Reviews*, *25*(3), 335–347. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11348688

Chen, J. M., & Hao, O. J. (1998). Microbial Chromium (VI) Reduction. *Critical Reviews in Environmental Science and Technology*, *28*(3), 219–251. https://doi.org/10.1080/10643389891254214

Cheung, K. H., & Gu, J.-D. (2005). Chromate reduction by Bacillus megaterium TKW3 isolated from marine sediments. *World Journal of Microbiology and Biotechnology*, *21*(3), 213–219. https://doi.org/10.1007/s11274-004-3619-9

Chourey, K., Thompson, M. R., Morrell-Falvey, J., Verberkmoes, N. C., Brown, S. D., Shah, M., … Thompson, D. K. (2006). Global molecular and morphological effects of 24-hour chromium(VI) exposure on Shewanella oneidensis MR-1. *Applied and Environmental Microbiology*, *72*(9), 6331–6344. https://doi.org/10.1128/AEM.00813-06

Danielsson, B. R. G., Hassoun, E., & Dencker, L. (1982). Embryotoxicity of Chromium - Distribution in Pregnant Mice and Effects on Embryonic-Cells Invitro. *Archives of Toxicology*, *51*(3), 233–245.

Darnowski, D. W., Carroll, D. M., Płachno, B., Kabanoff, E., & Cinnamon, E. (2006). Evidence of Protocarnivory in Triggerplants (Stylidium spp.; Stylidiaceae). *Plant Biology*, *8*(6), 805–812. https://doi.org/10.1055/s-2006-924472

Daulton, T. L., Little, B. J., Jones-Meehan, J., Blom, D. A., & Allard, L. F. (2007). Microbial reduction of chromium from the hexavalent to divalent state. *Geochimica et Cosmochimica Acta*, *71*(3), 556–565. https://doi.org/10.1016/J.GCA.2006.10.007

Dhal, B., Thatoi, H., Das, N., & Pandey, B. D. (2010). Reduction of hexavalent chromium by Bacillus sp. isolated from chromite mine soils and characterization of reduced product. *Journal of Chemical Technology & Biotechnology*, *85*(11), n/a-n/a.

https://doi.org/10.1002/jctb.2451

Eaton, A. D., Clesceri, L. S., Greenberg, A. E., Franson, M. A. H., American Public Health Association., American Water Works Association., & Water Environment Federation. (1998). *Standard methods for the examination of water and wastewater*. American Public Health Association.

Elangovan, R., Abhipsa, S., Rohit, B., Ligy, P., & Chandraraj, K. (2006). Reduction of Cr(VI) by a Bacillus sp. *Biotechnology Letters*, *28*(4), 247–252. https://doi.org/10.1007/s10529-005-5526-z

EPA, U. (1998). Chromium (VI) (CASRN 18540-29-9). *IRIS, US EPA ORD NCEA Integrated Risk Information System*, (Vi), 1–33. Retrieved from http://www.epa.gov/iris/subst/0144.htm

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, *39*(4), 783. https://doi.org/10.2307/2408678

Goulhen, F., Gloter, A., Guyot, F., & Bruschi, M. (2006). Cr(VI) detoxification by Desulfovibrio vulgaris strain Hildenborough: microbe–metal interactions studies. *Applied Microbiology and Biotechnology*, *71*(6), 892–897. https://doi.org/10.1007/s00253-005-0211-7

Grove, J. I., Lovering, A. L., Guise, C., Race, P. R., Wrighton, C. J., White, S. A., … Searle, P. F. (2003). Generation of Escherichia Coli Nitroreductase Mutants Conferring Improved Cell Sensitization to the Prodrug CB1954 1, 5532–5537.

Guha, G., Rajkumar, V., Ashok Kumar, R., & Mathew, L. (2010). Aqueous extract of Phyllanthus amarus inhibits chromium(VI)-induced toxicity in MDA-MB-435S cells. *Food and Chemical Toxicology*, *48*(1), 396–401. https://doi.org/10.1016/j.fct.2009.10.028

Hu, P., Brodie, E. L., Suzuki, Y., McAdams, H. H., & Andersen, G. L. (2005). Whole-genome transcriptional analysis of heavy metal stresses in Caulobacter crescentus. *Journal of Bacteriology*, *187*(24), 8437–8449. https://doi.org/10.1128/JB.187.24.8437-8449.2005

Ibrahim, A. S. S., El-Tayeb, M. A., Elbadawi, Y. B., Al-Salamah, A. A., & Antranikian, G. (2012). Hexavalent chromate reduction by alkaliphilic Amphibacillus sp. KSUCr3 is mediated by copper-dependent membrane-associated Cr(VI) reductase. *Extremophiles*, *16*(4), 659–668. https://doi.org/10.1007/s00792-012-0464-x

Jeyasingh, J., & Philip, L. (2005). Bioremediation of chromium contaminated soil: Optimization of operating parameters under laboratory conditions. *Journal of Hazardous Materials*, *118*(1–3), 113–120. https://doi.org/10.1016/j.jhazmat.2004.10.003

Kotaś, J., & Stasicka, Z. (2000a). Chromium occurrence in the environment and methods of its speciation. *Environmental Pollution (Barking, Essex : 1987)*, *107*(3), 263–283. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15092973

Kotaś, J., & Stasicka, Z. (2000b). Chromium occurrence in the environment and methods of its speciation. *Environmental Pollution*. https://doi.org/10.1016/S0269-7491(99)00168-2

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Kümmerer, K. (2009). Antibiotics in the aquatic environment - A review - Part II. *Chemosphere*, *75*(4), 435–441. https://doi.org/10.1016/j.chemosphere.2008.12.006

Lewicki, S., Zdanowski, R., Krzyzowska, M., Lewicka, A., Debski, B., Niemcewicz, M., & Goniewicz, M. (2014). The role of chromium III in the organism and its possible use in diabetes and obesity treatment. *Annals of Agricultural and Environmental Medicine*, *21*(2), 331–335. https://doi.org/10.5604/1232-1966.1108599

Li, X., & Krumholz, L. R. (2009). Thioredoxin is involved in U(VI) and Cr(VI) reduction in Desulfovibrio desulfuricans G20. *Journal of Bacteriology*, *191*(15), 4924–4933. https://doi.org/10.1128/JB.00197-09

Lin, C.-C., Wu, M.-L., Yang, C.-C., Ger, J., Tsai, W.-J., & Deng, J.-F. (2009). Acute Severe Chromium Poisoning After Dermal Exposure to Hexavalent Chromium. *Journal of the Chinese Medical Association*, *72*(4), 219–221. https://doi.org/10.1016/S1726-4901(09)70059-0

Lindberg, E., & Vesterberg, O. (1983). Monitoring exposure to chromic acid in chromeplating by measuring chromium in urine. *Scandinavian Journal of Work, Environment & Health*. Scandinavian Journal of Work, Environment & HealthFinnish Institute of Occupational HealthDanish National Research Centre for the Working EnvironmentNorwegian National

Institute of Occupational Health. https://doi.org/10.2307/40964444

Loubières, Y., Lassence, A. de, Bernier, M., Vieillard-Baron, A., Schmitt, J.-M., Page, B., & Jardin, F. (1999). Acute, Fatal, Oral Chromic Acid Poisoning. *Journal of Toxicology: Clinical Toxicology*, *37*(3), 333–336. https://doi.org/10.1081/CLT-100102431

Maqbool, F., Wang, Z., Xu, Y., Zhao, J., Gao, D., Zhao, Y.-G., … Xing, B. (2012). Rhizodegradation of petroleum hydrocarbons by Sesbania cannabina in bioaugmented soil with free and immobilized consortium. *Journal of Hazardous Materials*, *237–238*, 262–269. https://doi.org/10.1016/J.JHAZMAT.2012.08.038

McLean, J., & Beveridge, T. J. (2001). Chromate reduction by a pseudomonad isolated from a site contaminated with chromated copper arsenate. *Applied and Environmental Microbiology*, *67*(3), 1076–1084. https://doi.org/10.1128/AEM.67.3.1076-1084.2001

Megharaj, M., Avudainayagam, S., & Naidu, R. (2003). Toxicity of Hexavalent Chromium and Its Reduction by Bacteria Isolated from Soil Contaminated with Tannery Waste. *Current Microbiology*, *47*(1), 51–54. https://doi.org/10.1007/s00284-002-3889-0

Miranda, A. T., González, M. V., González, G., Vargas, E., Campos-García, J., & Cervantes, C. (2005). Involvement of DNA helicases in chromate resistance by Pseudomonas aeruginosa PAO1. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *578*(1–2), 202–209. https://doi.org/10.1016/J.MRFMMM.2005.05.018

Mueller Hinton Agar (MHA): Composition, preparation and uses -. (n.d.). Retrieved December 14, 2018, from https://microbeonline.com/why-mueller-hinton-agar-is-used-in-routine-antibiotic-susceptibility-testing/

Nachtigal, M. C., Patterson, R. E., Stratton, K. L., Adams, L. A., Shattuck, A. L., & White, E. (2005). Dietary supplements and weight control in a middle-age population. *Journal of Alternative and Complementary Medicine (New York, N.Y.)*, *11*(5), 909–915. https://doi.org/10.1089/acm.2005.11.909

Nakayama, E., Kuwamoto, T., Tsurubo, S., Tokoro, H., & Fujinaga, T. (1981). Chemical speciation of chromium in sea water: Part 1. Effect of Naturally Occurring Organic Materials on the Complex Formation of Chromium(III). *Analytica Chimica Acta*, *130*(2), 289–294.

https://doi.org/10.1016/S0003-2670(01)93006-5

Ngwenya, N., & Chirwa, E. M. N. (2018). Biological removal of cationic fission products from nuclear wastewater. https://doi.org/10.2166/wst.2011.021

Nriagu, J. O., & Nieboer, E. (1988). *Chromium in the natural and human environments*. Wiley. Retrieved from https://books.google.com.bd/books?hl=en&lr=&id=49oGjgz8riQC&oi=fnd&pg=PA335&dq=Chromium+toxicity:+effects+on+microorganisms+with+special+reference+to++the+soil+matrix.&ots=WvyGIP4C5P&sig=YMrwyJsxSDRdcIK24NkaFjg7R4A&redir_esc=y#v=onepage&q=Chromium toxicity%3A effects on microorganisms with special reference to the soil matrix.&f=false

Nutrient Agar: Composition, Preparation and Uses. (n.d.). Retrieved December 14, 2018, from https://microbiologyinfo.com/nutrient-agar-composition-preparation-and-uses/

Nutrient Broth No. 1, for microbiology | Sigma-Aldrich. (n.d.). Retrieved December 14, 2018, from https://www.sigmaaldrich.com/catalog/product/sial/70122?lang=en&region=BD

Pascale, L. R., Waldstein, S. S., Engbring, G., Dubin, A., & Szanto, P. B. (1952). CHROMIUM INTOXICATION. *Journal of the American Medical Association*, *149*(15), 1385. https://doi.org/10.1001/jama.1952.02930320025008

Pattanapipitpaisal, P., Brown, N. L., & Macaskie, L. E. (2001). Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. *Applied Microbiology and Biotechnology*, *57*(1–2), 257–261. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11693930

Pei, Y., Yu, Z., Ji, J., Khan, A., & Li, X. (2018). Microbial community structure and function indicate the severity of chromium contamination of the Yellow River. *Frontiers in Microbiology*, *9*(JAN), 1–12. https://doi.org/10.3389/fmicb.2018.00038

Poljsak, B., Pócsi, I., Raspor, P., & Pesti, M. (2010). Interference of chromium with biological systems in yeasts and fungi: a review. *Journal of Basic Microbiology*, *50*(1), 21–36. https://doi.org/10.1002/jobm.200900170

Rahman, M. A., Rahman, M. A., & Bakri, D. Al. (n.d.). A Study on Selected Water Quality

Parameters along the River Buriganga, Bangladesh. Retrieved from http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.668.6247

Ramírez-Díaz, M. I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., & Cervantes, C. (2008). Mechanisms of bacterial resistance to chromium compounds. *BioMetals*, *21*(3), 321–332. https://doi.org/10.1007/s10534-007-9121-8

Rifkin, E., Gwinn, P., & Bouwer, E. (2004). Peer Reviewed: Chromium and Sediment Toxicity. *Environmental Science & Technology*, *38*(14), 267A–271A. https://doi.org/10.1021/es040575m

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Salama, A., Hegazy, R., & Hassan, A. (2016). Intranasal chromium induces acute brain and lung injuries in rats: Assessment of different potential hazardous effects of environmental and occupational exposure to chromium and introduction of a novel pharmacological and toxicological animal model. *PLoS ONE*, *11*(12), 1–20. https://doi.org/10.1371/journal.pone.0168688

Samuel, J., Paul, M. L., Ravishankar, H., Mathur, A., Saha, D. P., Natarajan, C., & Mukherjee, A. (2013). The differential stress response of adapted chromite mine isolates Bacillus subtilis and Escherichia coli and its impact on bioremediation potential. *Biodegradation*, *24*(6), 829–842. https://doi.org/10.1007/s10532-013-9631-8

Sharma, B. K., Singhal, P. C., & Chugh, K. S. (1978). Intravascular haemolysis and acute renal failure following potassium dichromate poisoning. *Postgraduate Medical Journal*, *54*(632), 414–415. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/683912

Shen, H., & Wang, Y.-T. (1995). Hexavalent Chromium Removal in Two-Stage Bioreactor System. *Journal of Environmental Engineering*, *121*(11), 798–804. https://doi.org/10.1061/(ASCE)0733-9372(1995)121:11(798)

Shi, X., & Dalal, N. S. (1990). One-electron reduction of chromate by NADPH-dependent glutathione reductase. *Journal of Inorganic Biochemistry*, *40*(1), 1–12.

https://doi.org/10.1016/0162-0134(90)80034-U

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*(30), 11030–11035. https://doi.org/10.1073/pnas.0404206101

Thacker, U., & Madamwar, D. (2005). Reduction of Toxic Chromium and Partial Localization of Chromium Reductase Activity in Bacterial Isolate DM1. *World Journal of Microbiology and Biotechnology*, *21*(6–7), 891–899. https://doi.org/10.1007/s11274-004-6557-7

Thatoi, H., Das, S., Mishra, J., Rath, B. P., & Das, N. (2014). Bacterial chromate reductase, a potential enzyme for bioremediation of hexavalent chromium: A review. *Journal of Environmental Management*, *146*, 383–399. https://doi.org/10.1016/j.jenvman.2014.07.014

Turick, C. E., Apel, W. A., & Carmiol, N. S. (1996). Isolation of hexavalent chromium-reducing anaerobes from hexavalent-chromium-contaminated and noncontaminated environments. *Applied Microbiology and Biotechnology*, *44*(5), 683–688. https://doi.org/10.1007/BF00172503

Viamajala, S., Smith, W. A., Sani, R. K., Apel, W. A., Petersen, J. N., Neal, A. L., … Peyton, B. M. (2007). Isolation and characterization of Cr(VI) reducing Cellulomonas spp. from subsurface soils: Implications for long-term chromate reduction. *Bioresource Technology*, *98*(3), 612–622. https://doi.org/10.1016/J.BIORTECH.2006.02.023

World Bank., Nationally Coordinated Research Projects (Nigeria), & University of Port Harcourt. Faculty of Science. (n.d.). *Journal of applied science & environmental management : JASEM*. National Agricultural Research Project. Retrieved from http://www.bioline.org.br/request?ja03012

Yamamoto, K., Kato, J., Yano, T., & Ohtake, H. (1993). Kinetics and modeling of hexavalent chromium reduction inenterobacter cloacae. *Biotechnology and Bioengineering*, *41*(1), 129–133. https://doi.org/10.1002/bit.260410117

Zhitkovich, A., & Costa, M. (1992). A simple, sensitive assay to detect DNA-protein crosslinks in intact cells and in vivo. *Carcinogenesis*, *13*(8), 1485–1489. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1499101