Isolation and Characterization of Chromium reducing *Pseudomonas aeruginosa* from Buriganga Riverbed

A project submitted

by

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To my parents

Acknowledgement

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Certification Statement

This is to certify that this project titled “Isolation and Characterization of Chromium reducing *Pseudomonas aeruginosa* from Buriganga River bed” is submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy (Hons.) from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Professor Dr. Eva Rahman Kabir, Chairperson, and 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

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Countersigned by the supervisor

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ABSTRACT

The hexavalent form of Chromium is known to possess carcinogenic and mutagenic property and causes various serious diseases such as skin cancer, lung cancer, pulmonary congestion, etc. Salts containing hexavalent form of Chromium are an important component in various sectors including leather, tannery, dye, stainless steel etc.; and are being discharged into the natural environment every day without any treatment, thus making it very hazardous for human health. The following study aimed to discover the ability of bacterial strain C_2 to reduce hexavalent Chromium to its less toxic trivalent form. Water and soil samples were collected from the Buriganga River. Bioremediation assay was carried out on samples of the bacterial isolate containing K_2CrO_4 (Potassium Chromate) at combination of temperatures- 25^0C, 37^0C and 42^0C and pH 5.5, 7 and 8.5. Therefore, a total of 9 samples were prepared and monitored for their Chromium reducing ability, by checking for their absorbance using a UV spectrometer. It was observed that, the bacterial isolate was able to reduce Chromium completely at 37^0C and 42^0C, both at pH 7. The isolate failed to show any significant reduction at all pH values in 25^0C. Furthermore, Antibacterial Susceptibility Assay and Minimum Inhibitory Concentration (MIC) tests were performed. The isolate showed greatest susceptibility to Ceftriaxone (201.7± 1.89 ZI/mm), Neomycin (19± 1.00 ZI/mm), Gentamycin (18.67± 0.5867 ZI/mm) and Ciprofloxacin (18.5± 0.867 ZI/mm) and least susceptibility to Vancomycin (8.33± 0.57 ZI/mm) and Penicillin (0 ZI/mm); and showed moderate susceptibility to other test antibiotics. In addition, the MIC of K_2CrO_4 at 16mM. No significant correlation was observed between the reduction capacity, MIC and Antibiotic Resistance property of the isolate. Finally, 16s rRNA sequencing was conducted to identify the unknown isolate, in accordance with the utilization of nucleotide BLAST, Finch TV, BioEdit and Mega 7 tools to identify and trace the origin of bacterial isolate C_2. Consequently, the identity of the isolate was confirmed to be *Pseudomonas aeruginosa.*

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
Cr	Chromium
DPCZ	Diphenyl Carbazide
EPA	Environmental Protection Agency
mL	Milliliter
mM	Millimolar
NADH	Nicotinamide Adenine Dinucleotide
NB	Nutrient Broth
NIOSH	National Institute for Occupational Safety and Health
nm	Nanometer
O.D.	Optical Density
OSHA	Occupation Safety And Health Administration
PEL	Permissible Exposure Limit
ppm	Parts per million
RPM	Rotation Per Minute
rRNA	Ribosomal Ribonucleic Acid
SCHER	Scientific Committee on Health and Environmental Risks
WHO	World Health Organization
ZI	Zone of Inhibition
µM	Micro Molar

Chapter 1: Introduction

The industrial sector has integrated into galvanizing, leather, textile, electroplating, garments, locomotive manufacture and mining. These sectors utilize considerable amounts of toxic heavy metal ions on a daily basis. Among these wide range of ions, Chromium plays a dominant role of being lethal for human species and other cellular entities (Thacker & Madamwar, 2005). Chromium along with its accommodating metal ions are leached into rivers via mouth of factory exits, spills or leakages, through uncontrolled leaching from waste dumps and heaps (Moncur et al, 2005) surrounding the water body perimeter.

1.1 Background

The River Buriganga is a notorious landmark that runs in Dhaka City, the capital of Bangladesh. The city comprises of dominant river streams namely Tongi Khal (due North), Turag (West) and Buriganga (South) and further relies on the major drainage channels i.e. Dholai Khal and Begunbari Kahal. These drainage channels collect all local dumps, industrial effluents as well as waste water and discharge them into the peripheral rivers (Ahmed et al, 2011). In recent times, the increase in waste production emerged in Dhaka City, due to an exponential increase in industrial growth, as witnessed in Hazaribagh, a chief industrial hub for the city (Islam et al, 2014). The Hazaribagh area comprises of 227 tannery industries, 15 dye industries, 3 salt factories, 1 pharmaceutical establishment, 2 match box factories and 2 Lead-Zinc factories (Islam et al, 2014). Therefore Buriganga River; a major haven for aquatic organisms and food sources, is plagued with a constant inflow of heavy metal and solid wastes, along with other natural canals low lying areas lagoons, and these waste have been reported to contain toxic concentrations of Cr, Pb, Cd and As (Bhuiyan et al, 2015).

Numerous techniques are used all over the globe to treat the increasing heavy metal effluent and reduce their toxicity. Preliminary methods include abiotic techniques such as pump-and-treat methods, where the effluent is pumped out from water and collected as a residual precipitate. Further intricate and cutting- edge technologies include the use of nano-filters in a reverse osmosis membrane (Mnif et al, 2017) and biosorption- a Chromium removal process that is not only cost effective but also environmentally friendly(Majumder et al, 2017).

Bioremediation technology involves in the effective use for Cr^{6+} treatment in both industrial and healthcare sectors. A range of diverse techniques including electrolytic reduction, membrane filtration, reverse osmosis, liquid- liquid extraction, electrocoagulation and adsorption, etc. Bioremediation technology has proven have a wide scope for the future, since it has the advantages of being cost-effective, can be potential applied for large polluted areas; for both water and soil and has remarkable degrees of potential safety and accuracy, which further exponents its scope of usage (Cheng et al, 2012).

Chromium exists in abundance among the group VIA family of elements. It is the 13^{th} most common element and originates among several other minerals in the Earth's core at concentrations of 400 parts per million. Chromium has several oxidation states ranging from +2 to +6 and even though proven to be beneficial in leather, tanning, textile, dyeing and electroplating (McGrath &Smith, 1990), it has been proven to be mutagenic in nature. The hexavalent form of Chromium is also known to cause cancer. Aside from the Earth's crust, Chromium is also found in volcanic dust, rocks and soils, plants and animals. It exists in the environment predominantly in trivalent and hexavalent forms, where the hexavalent Cr (VI) state is deemed a potential carcinogen and a common soil, deep water and surface water contaminant (Cervantes et al, 2001). A slight raise in Cr^{6+} levels create numerous health and environmental issues due to its high toxicity (Sharma et al, 1995), carcinogenicity (Venitt and Levy, 1974) and mutagenicity (Nishioka, 1975). On the contrary, the Cr^{6+} state is insoluble, less toxic and a vital nutritive component of the human body.

1.2 Aims and Objectives

The aim of this research was to investigate, analyze and determine a bacteria or multiple bacteria, capable of reducing and neutralizing the toxic hexavalent Chromium and to further assess a potential source for the chromate reductase enzyme. To accomplish this, the following objectives were carried out:

1) Evaluation of performance of isolated bacteria.
2) Investigation of the Minimum Inhibitory Concentration (MIC) of the isolated bacteria; therefore evaluating its tolerance limit to Chromium.
3) Investigation of antibiotic resistant profile of the isolated bacteria.

Chapter 2: Chromium

2.1 Chemistry

Chromium is an odorless, hard and a silvery gray metal. They become solubilized in dilute hydrochloric acid and sulfuric acid, though they remain insoluble in strong acids and alkalis (Merck, 1989). In the Periodic Table, Chromium has the symbol Cr, and its atomic number is 24. It has a well-polished appearance yet brittle in nature. The boiling point of Chromium is 2672°C and its melting point is 1907°C ("Chemical properties of Chromium - Health effects of Chromium – Environmental effects of Chromium," 1998-2016). Different forms of Chromium bear unique distinct colors. Therefore, its name is derived from the Greek term "Chroma" (Horn, 2013). Chromium is used in the production of alloys and stainless steel due to its ability to exhibit solidarity and imperviousness towards erosion and metallic corrosion, and this renders Cr an extremely valuable element (Rifkin et al., 2004).

In addition, Cr is an essential trace mineral that is necessary for the regulation of nucleic acid, metabolism of glucose and contributes to the fundamental framework of certain chemical compounds. Even though its deficiency may cause disease, increased levels of Chromium is poisonous (Thacker & Madamwar, 2005). Cr (VI) is a deadly and mutagenic agent to all cellular life forms and it further causes lung cancer in individuals along with respiratory tract and skin diseases (Thacker & Madamwar, 2005). On a global scale, the extraordinary discharge of poisonous Chromium impacts the biosphere. Therefore, reduction of Chromium through natural mechanisms is of utmost importance.

Table 2.1: Chromium's widespread data ("American Elements," 2016).

Emblem	Cr
Term	Chromium
Class	Transition metal
Group, period, block	6, 4, d
Atomic number	24
Atomic weight	51.996
Atomic radius	128 pm
Appearance	Silver-grey
Electronic configuration	[Ar] 3d5 4s1
Thermal conductivity	93.9 $W.m^{-1}. K^{-1}$
Oxidation state	-2, -1, 1, 2, 3, 4, 5, 6
Crystal structure	Body-centered cubic

2.2 Sources of Chromium Compounds

Chromium in its natural state is sited amongst hardened rocks, living organisms, soil and water bodies. Both trivalent and hexavalent states exist as precipitates in water and geologic environments.

Chromium (VI) can be found in uncommon materials and a potential source to be ground water in addition to man made contributions (Chandra & Khulshreshta, 2016). Sectors such as development and production are chiefly responsible for the utilization of significant quantities of Chromium (Chromium trioxide, Ammonium dichromate, Potassium dichromate) along with discharge of chromium mixes through industrial waste.

An extraction from the United States Environmental Protection Agency (US-EPA) determines the Cr content in soil at 100-2000 mg/kg soil, as opposed to the normal levels of 40 mg/ kg of soil; 0.2-50 µg/L in ocean water and 0.1- 6.0 µg/ L in remote water channels (EPA, 2010). Only at 30 µg/L in water.

2.3 Chromium Utilization

Chromium is as essential element in the production of stainless steel. Chrome based steel bodies consist of approximately 18 percent Chromium due to its hardened metallic nature that contributes to the prevention of rust, decay and resistance to extreme heat. This is the fundamental basis or numerous appliances, kitchenware, gadgets, processed food containers, locomotive and healthcare facilities and institutions. In addition, various parts such as motors, trimmer blades, hub-caps and embellishments are chromium plated Chromium can be categorized as a super metal; capable of tolerating excessive thermal and tensile stress from jet engines. The streets and highways used to indicate road signs contain chromium. Chromium containing metals also situate themselves amongst cosmetic products; and even in molds for brocks due to its ability to tolerate high temperatures. Chromium is an essential element for health: its inadequate amounts resulting in the human body to inhibit tolerance of glucose. Therefore the body is replenished with adequate levels of chromium from dietary sources including mushrooms, meat, and wheat amongst many potential sources. Other sectors where chromium is highly utilized include leather, textile, electroplating, synthesis of chemicals and erosion inhibitors (EPA, 2010). Other uses include treatment of wood, particularly timber using copper dichromate.

2.4 Regulation regarding safe levels of Chromium

In recent days, the presence of Chromium has led to a toxic impact among people and animals and plants due to its drastic increase in the water bodies of lakes and rivers beyond their natural concentrations. As a result, strict policies have been implemented numerous organizations to bring down the Chromium levels to the desired constraint levels. According to the analysis showed by the World Health Organization (WHO) in light of regulation of safe drinking water, it is recommended for that Chromium levels are reduced to a range from 0.05 mg/L to 2 mg/L for all Cr (VI) and all its valent states (Gupta & Rastogi, 2009). The Act of Non-toxic Intake Liquid has recommended safe concentrations of Chromium in water for intake, to be at 0.1 mg/L. In dyes for textile, if Chromium levels cross 50 ppm, it is considered to be toxic. In addition, the maximum permissible levels for Chromate in leather tannery sector is 2.75 percent by weight. According to the Occupational Safety and Health Administration., the maximum Permissible Exposure Limit (PEL) for Chromate and Chromic acid is $0.1 mg/m^3$.

Furthermore, the National Institute for Occupational Safety and Health (NIOSH) has stated lethal levels or IDLH (Immediately Dangerous to Life and Health) levels of Cr (VI) to be 15 mg/m^3. For safe standard levels of Chromic acid, Chromyl Chloride and all forms the proposed levels are up to 0.001 mg/m^3.

2.5 Toxicity of Chromium and its effects on health

2.5.1 Chromium toxicity

Individuals exposed to lethal levels of Chromium suffer from conditions including ulceration, dermatitis, hypersensitivity, nasal & dermal disorders, infection in the lungs, and disruption of the ear drum membrane (Poopal & Laxman, 2009). In addition, Cr (VI) accumulates in the placenta and disrupts the growth of the fetus (Poopal & Laxman, 2009). As a pollutant in the environment, hexavalent chromium interferes with the morphological structure of microbes by accumulating and hampering growth and division of bacteria and further interfering with their enzymatic reactions (Shi, Becker, Bischoff, Turco & Konokpa, 2002). The toxic abilities of Cr (VI) in cellular life forms occur due to their ability to pass directly across the plasma membrane. This adverse effect can be observed in both prokaryotic and eukaryotic cells, as the Chromate progresses on, leading to oxidizing organelles, disrupting protein cross links, interfering with the electron transport chain and damaging the molecular structure and cross-links of the DNA. (Codd, Rillon, Levina & Lay, 2001). To pass through the plasma membrane, Cr (VI) utilizes the sulfate transport pathway, situated in the lipid bilayer (Ohtake, Cerventes & Silver, 1987). On the contrary, the cell membrane shows resistance towards the trivalent form of Chromium and therefore Cr (III) is less toxic than Cr (VI) by relatively a thousandth level (Polti, Amoroso & Abate, 2010). Ultimately, it can be said that hexavalent Chromium is exceptionally deadly to most living things. In contrast, the trivalent Chromium can be considered safe for living beings (Katz & Salem, 1993; Wong & Trevors, 1988).

2.5.2 Effects on health

2.5.2.1 Kinetics

Patients with orally administered Chromium dosage forms have extended pharmacokinetic models. In addition, numerous literature and data from various studies were observed with respect to toxicokinetics. A combination of second order and pH derived process complied with the consistency of toxicokinetic parameters, as observed in patients chromium. Cr (VI) can easily pass through biological barriers and reduce to Cr (III) state. Acidic condition along with associated biological chemicals increases the of hexavalent chromium reduction from Cr^{6+} to Cr^{3+} conversion process. Cr^{6+} is absorbed more rapidly compared to Cr^{3+} due to an intra deficiency of Cr^{6+} and competitive ingestion.

2.5.2.2 Mode of Action

It is established that Chromium has the ability to cause mutagenicity and genotoxicity. The hexavalent form of Chromium is capable of hampering the cell regulation process (IARC, 2012). It can penetrate easily through cell membranes and provoke oxidation of DNA, proteins and lipids. Genomic components associate with Cr^{6+} to form DNA adducts, Chromium-DNA complexes, DNA-Chromium-DNA complex, DNA-protein-Chromium-DNA complexes, oxidized nitrogenous bases, unwound DNA helix and intra strand crosslinks between protein and DNA (Wise, Holmes &Wise, 2008). These forms of mutations are observed in in-vitro studies of human and bacteria. Upon reduction, Cr^{6+} causes damage by forming Reactive Oxygen Species (ROS). On the contrary, Cr^{3+} is less mobile and harmful to the body tissues. If produced intracellularly, Cr^{6+} readily mobilizes and develops crosslinks with DNA, further inhibiting activities of DNA polymerase as a consequence. This causes the ROS levels to rise, which is produced as the outcome of conversion of Cr (VI) to Cr (III). In addition, presence of hydrogen peroxide aggravates Cr^{3+} to increase the ROS levels in cells producing 8-hydroxyguanosine within the DNA. Ultimately, these compounds also emerge from within the cell, thereby hampering the entire cellular life cycle (Hadjiliadis, 2012).

2.5.2.3 Effects in Humans

For humans, maximum records of health impact by Cr (VI) has been observed from regular exposure to high levels of Chromate through inhalation in industrial facilities. In general, workers are secluded in a Chromate area such as electroplating and pigment sectors, where they are exposed to several forms of Chromium compounds. Inflammation, rashes and ulcers develop on the skin once exposed to lethal levels of hexavalent chromium. A Fix test revealed that 2 μg concentration of Cr (VI) was sufficient to generate a positive hypersensitive skin reaction. An investigation in several European countries revealed the levels of Chromium to be in a rage of 0.5 to 1.7 percent in water (Peltonen & Fraki, 1983; Hartwig, 2007; Hartwig, 2010). Experts have revealed the impact of Chromium commonly on nasal routes such as the sinus orifice, where mucosal ulceration develops. In addition, the lungs were observed for any alteration in parameters. It was observed that hexavalent Chromium contributed to damaging the DNA; leading to abnormalities in the chromosome, micronuclei, strands of sister chromatids, DNA-protein crosslinks and bonds within the DNA double helix. These were observed inside lymphocytes. (WHO/IPCS, 2013). Regarding effects of hexavalent Chromium on nasal infections and sinus inflammation, epidemiological studies indicate its occurrence but fails to provide a proper statistical record (IARC, 2012). Similarly, a comprehensive review conducted on 12 countries including UK, Iceland and Japan could not reveal proper correlated results of the effects of Chromium in the gastrointestinal tract of the employees, because the study lacked the parameter of employees using face masks during breathing (Gatto et al., 2010). However, there are cases of correlation between the presence of Cr and stunted growth of gut flora in the abdominal tract in one of the polluted regions in China (Zhang & Li, 1997). In separate yet related investigations, there were other parameters lacking that influenced the effect of Chromium activity in humans, i.e. data regarding dietary factors, smoking, alcohol consumption, financial status, etc.

2.5.2.4 Effects in animals

Several studies have been conducted to analyze the intense lethal effects of Cr (VI). The nature of the Chromium compound, age, sex, and type of breed of rodent were taken into factor, as Cr (VI) was administered orally at lethal doses to rats. Doses varied from 12 to 30 mg/kg b.w depending on the weight of the rodents. As a result of this administration, certain significant changes were observed including reduction in immune strength and body weight; and alteration in hematological parameters. To understand the daily intake of Chromium levels and its impact on the body in healthy people, mice and guiena pigs were treated to sodium dichromate for a prolonged period. These test subjects were introduced to Chromium compound via nasal pathway, leading to aspiratory irritation and neutrophil count. Several studies provide evidence for Chromium to cause disease by administration via oral route, intra thecal, intra muscular and intra-peritoneal pathways. In most cases, carcinogenesis is the ultimate outcome at the effective site of origin and increased inhalation of Chromium (VI) leads to lung cancer in mice. In addition, benign sarcomas were observed via administration of a number of Chromium infusions (lead chromate, zinc chromate, calcium chromate). Orally administered potassium chromate led to skin cancer, showing systemic tumor growths.

2.6 Carcinogenesis induced by Chromium

The hexavalent form of Chromium is notorious for being a lethal carcinogenic agent that has led to alterations in the human DNA. Unlike its trivalent form which binds to DNA polymerase, the hexavalent form remains as an oxyanion by utilizing the sulfate anion framework. Upon entry into cells, the Cr^{6+} is reduced to Cr^{3+} species, forming oxygen radicles as a result (Conet, 1983; Mattagajsingh, 1995) and these Reactive Oxygen Species commence the cancer causing activity by modifying the DNA molecular structure (Kawanishi, 1986). Intermediate valence forms of Cr^{6+} and Cr^{3+} are known to cause DNA damage as well. In addition, proteins; either individually or in conjunction with other proteins are also involved in crosslinking and accelerating DNA damage (Stein, 1979). These crosslinks between DNA and protein is different and abnormal; contrary to regular DNA- protein incorporation; and results in misinterpreted hereditary outcomes and interrupted gene expressions. Therefore, a potential mechanism of suppressing Cr carcinogenesis is to identify the protein responsible for creating crosslinks with the DNA.

2.7 Traditional methods for reducing Cr toxicity

There are particular efficient process designed to reduce Cr^{6+} in contaminated water; techniques that are either stand-alone or mixed such as filtration, sedimentation, electrochemical treatment, development of films (Ahluwalia & Goyal, 2007, Al-Sou'd, 2012).

2.7.1 Electrochemical Precipitation

This system utilizes potential difference to enhance removal of several metals from water and is preferred over traditional processes (Kurniwarna et al, 2006). This method analyzes the causative heavy metal's concentration in the water at parts per million (ppm). Using electrochemical precipitation, Cr^{6+} concentration can be reduced from 386 mg/L to 0.21 mg/L.

2.7.2 Ion Exchange

Even though the ion- exchange process has taken a delay to initiate, the technique is now a significant method for reducing Chromium in waste water. Particles of any size and distinct shape are removed by being exchanged with substituted species during the course of action in the process. Chromium passes through one channel of the segment, then along a bed of resin and finally expelled. By decreasing the amount of resin in the bed, additional solids have accumulated and removed.

Manufactured Dowex 2-X4 resins are used to monitor the in-flux of Chromium from the incoming waste water (Sapari, Idris & Hamid, 1996). For an anionic exchange, a major anion sap containing hydroxide pellets of specific concentration is used. Another type of resin used is Ambersep 132 which is used to analyze the concentration of chromic acid during the manufacturing of metal plates; and this technique utilizes a four phase ion-exchange method.

There are several limitations to ion-exchange. A major limitation to the ion-exchange technique for Chromium removal is that the resin responsible for the exchange must be specific. The selected resin must be able to remove the targeted metal at the desired successive rate. In practical aspects, Chromium is not removed thoroughly and even further, the equipment for ion- exchange can be expensive. In addition, the method cannot handle the separation of heavy metals at concentrated levels. The ion-exchange system also attracts a significant amount of unwanted solid debris that accumulate with the metals in the water.

2.7.3 Biosorption

It is a newly introduced concept that aids in the recovery of Chromium from waste water. For this process, low cost and economic agricultural waste material can be used (Basso et al, 2002, Park et al, 2006). This process also bears a few limitations- there are several variables for metal sorption using agricultural waste. In addition, the process itself is complex. The concept of Biosorption is synonymous with other methods including sorption of chemicals, exchange of ions, micro-precipitation and adsorption of surface.

2.7.4 Utilizing activated carbon for adsorption

Chromium can be adsorbed well into rich carbon containing materials such as coconut husks and charcoal. It was observed that actuated carbon was more proficient in the adsorption of Chromium. The actuated carbon consists of different unrefined materials including husks of coconut any, hard based shells of nuts, saw dirt and so on (Mohan & Pittman, 2006). To facilitate the expulsion of hexavalent Chromium, a GAC type of filtrate Aquatic Air Mud Pollute 400 functions efficiently when placed in an aqueous media of the heavy metal effluent (Demirbasa, Kobyab, Senturkb & Ozkana, 2004). The adsorption of carbon takes place optimally at a high temperature (Mohan & Pittman, 2006). In addition, variables such as pH and salt concentration of the solution is dependent on the levels of Chromium.

2.7.5 Membrane filtration

The process of filtration using a membrane has become an important contribution to the treatment of heavy metal contaminated water. Here, aquatic hydrolytic force is utilized to separate macro solids using a partially permeable film. Several types of membranes are used, including polymeric membranes, aquatic membranes, inorganic membranes, etc. for the separation of hexavalent Chromium (Pugazenthi et al, 2005). Other examples are non-inter penetrating radical filtration film made of carbon that utilizes a Nitrogenous based gas phase along with hydrazine hydrate. Therefore to separate hexavalent Chromium from its water effluent, the membrane was used. According to the results, the strategy of Chromium filtration was successful with 96 % Chromate removal using a basic membrane, 84% using a nitrogen based carbon membrane and 88 % using an aminated membrane. For nano-filtration, the membranes are based on diverse polyamide structures that alters the pH of the incoming waste effluent prior to the ejection of hexavalent Cr. (Muthukrishnan & Guha, 2008).

2.7.6 Bio mineralization

Bio mineralization is the process by which aqueous mineral ions, including Chromium are converted into crystalline, non-distinct precipitates using microorganisms. This method is seen to be prospective and financially feasible for remediation of Chromium. For example, precipitation of Arsenic was a potential mechanism for remediating natural sediments that contained arsenic (Focardi et al, 2010). Other practical methods of bioremediation are immobilization, naturally mediated alteration and mineralization of toxic metals (Cheng, Holman & Lin, 2012).

2.8 Influence of metals on microorganisms

2.8.1 Mechanism of metal resistance in bacteria

In the environment, the presence of a mixture of heavy metal amongst water bodies has led to development of significant resistance by the microbes residing in the water. From a biochemical perspective, a microorganism's mechanism is both diverse and complex, under the influence of its chromosomal and plasmid guidance. There are five mechanisms by which a microorganism develops resistance (Rouch et al, 1995) and are as follows:

1. Disposal of metal via membrane permeability inhibitors.
2. Eradication of metal from human cells via active transportation.
3. Isolation of heavy metal intracellularly by protein restriction which prevents it from cellular oxidation and ultimately organelles.
4. Extracellular expulsion of metals.
5. Decontamination of metal, where the metal itself is sufficiently restricted to the point where it utilizes only a minimal amount of energy, thus being harmless to cell damage.

2.8.2 Metal sensitive cellular components

Heavy metals are capable of decreasing the activities of catalysts, reassembling the sequence or mechanisms of the catalytic activity, modifying the active sites of the enzymes and even sealing off enzyme activities permanently in a discrete manner. The heavy metal ions can do lethal damage to human cells, especially in the DNA structure, by disrupting cross links in the DNA strands, influencing the base pair data of DNA via indirect mechanisms and even reducing the entire process of DNA synthesis (Beyersman, 1994).

Because of a massive diversity in cells, there are several major focal points for metal based destruction. And all these regions of the cells bear significant contribution to cellular performance. For example, DNA is crucial for replication and lysosomes function in macromolecular degradation. In specific optimal concentrations, metals are generally required for cell performance; however if metal concentrations goes beyond threshold to toxic levels, this not only leads to a stunted cellular metabolism but cell necrosis as a consequence.

Therefore, based on these facts, a cell must develop certain countermeasures in order to ensure protection and survival of many of its target organelles. The greater the diversity of heavy metals present, the more the cells require development of resistance. For instance, E. coli defends against heavy metals such including Cu^{2+} and Zn^{2+} by temporarily inhibiting the production of certain proteins; done by transformation in a single gene. This ultimately provides an extended resistance towards incoming metals (Lutkenhaus, 1997).

2.9 Metal as a biological requirement

There are several substantial metals necessary for maintaining the metabolic activities of a microbial cell. Most classes of bacteria require Iron, Chromium, and Nickel while certain other classes of species need Cobalt, Molybdenum and Tungsten. Due to presence of these heavy metals in a fundamental level, the metabolic functions of these cells appear significantly different as opposed to bacterial cells without metal; which demonstrates non-optimal metabolic capabilities. Therefore, heavy metals are needed in fundamental concentrations since they provide a metabolic frameworks to the cell that would enable it adjust and optimize its metabolic functions as efficiently as possible.

2.10 Mechanism of chromosome resistance in bacteria

In a wide range of bacteria, it is observed that the Chromate ions utilize the sulfate pathway to pass into the cytoplasm directly. The Cr^{3+} ions form several intermediate obscure compounds; the reason being its slow motility through the membrane barrier (Cary, 1982). There are numerous enzymatic and non-enzymatic activities that lead to the reduction of hexavalent Chromium to its trivalent counterpart; and these activities may show toxic effects in the cell's cytoplasm (Cervantes et al, 2001). The development of resistance towards Chromate ions by bacterial isolates is shown by the plasmids and/or chromosomal genes (Cervantes and Campos-Garica, 2007). In addition, several

methods including reduction of hexavalent Chromium ion removal of free radicles and toxic metabolites, replacement and maintenance of damaged DNA and its loci homeostasis of sulfur ions, etc contribute to the resistance mechanism, as guided by the chromosomes of the bacteria. Further researches on Chromate reducing reductases focuses on the endogenous and exogenous enzymes capable of efficient reducing activity.

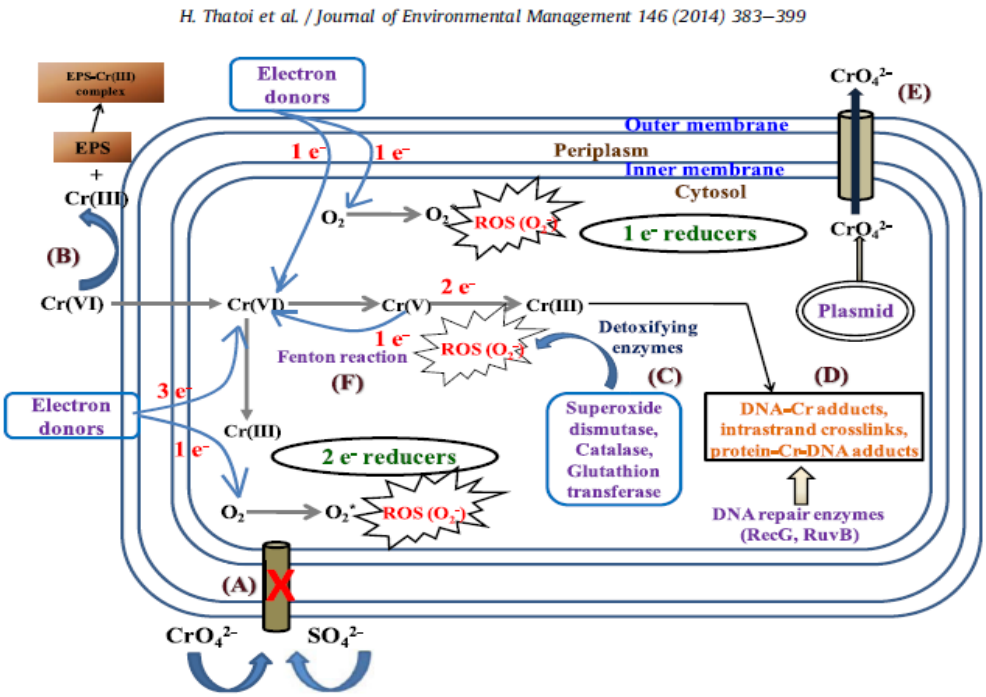


Fig 2.10: Mechanism of Chromate resistance in bacterial cells. A: Transformation in the chromosome-encrypted sulfate uptake pathway, B: Reduction of hexavalent Chromium to its trivalent state extracellularly, C: Reduction of hexavalent Chromium to its trivalent counterpart via chromate reductase enzyme, D: Utilizing the recovery technique of SOS in diminishing oxidative pressure, E: Efflux of Chromate from cytoplasm, F: Enzyme activity for scavenging ROS to diminish oxidative pressure (Thatoi et al., 2014).

A. Reduced uptake of Cr (VI)

Along with the reduction of hexavalent Chromium to its harmless trivalent state, a defense mechanism involves decreasing intake of Cr^{6+}. As CrO^{2-} ions are up taken through the cell membrane, it is exchange with tetra dimensional sulfate molecule, which is pumped out. This is the reason why Chromate ions can pass into a cell by utilizing the sulfate pathway, where PO_4^{3-} and SO_4^{2-} ions take role in being chief candidates for exchange with Cr ions (Ramirez-Diaz et al, 2008). Microorganisms that inhibit a metal

rich environment has demonstrated an increasing rate of developing resistance to Cr^{6+} via sulfate pathway. This is shown by modification in the bacteria's genomic sequence that alters its characteristic to developing resistance in the environment (Kummer, 2004).

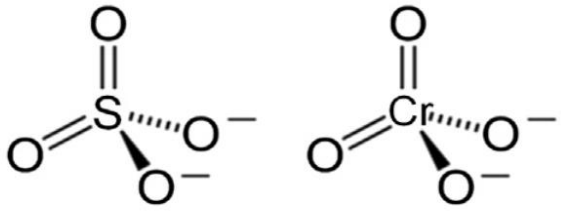


Fig 2.10 Resemblance among ions of sulfate and chromate.

B. Detoxifying enzymes of ROS

Reactive Oxygen Species or ROS are radicles formed during reduction of Cr^{6+} to Cr^{3+} organisms face oxidative stress due to formation of free oxygen radicles or ROS. In this way, the Chromate impels the bacterial proteins which gives protection against oxidative stress by prompting an extra defense mechanism of chromium resistance (Ramirez-Diaz et al, 2008).

C. DNA Repair Enzymes

Using DNA repair enzymes to damage DNA, Cr^{6+} ions are shielded against by bacterial organisms; and this serves as an extra layer of protection towards heavy metals. Various enzymes and non- enzymatic pathways convert Cr^{6+} to Cr^{3+} and this creates ROS, which leads to the degradation of proteins and DNA intracellularly. ROS alters structure of DNA by targeting base changes, breaking DNA strands into single strands, etc. These types of cellular injury can be impaired via maintenance of damaged DNA, through mechanisms such as involving SOS repercussion enzymes.

D. Scavenging of ROS

Following entry into a cell, Cr (VI) is reduced to a Cr (V) form, aided by the electron helpers. Although the Cr (V) is subsequently reduced to Cr (III) by a two electron interchange mechanism, sometimes this does not happen fast. Hence a proportion of Cr (V) ions are converted to Cr (VI) and this generates ROS. Through this process, the hydroxyl radical (-OH) are formed in microbial cells (Shi and Dalal, 1994) as shown in the following reaction:

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

According to the reaction, uni-atomic oxygen is converted to diatomic oxygen radicals, which further converts into H_2O_2 and reacts with Cr (V) to release hydroxyl ions by a Fenton like response.

Table 2.10: Mechanism of bacterial species for developing resistance to Chromate.

Enzyme/System	**Species**	**Function**	**Reference**
Transport			
ChrA transporter	*Pseudomonas Aeruginosa*	Efflux of cytoplasmic chromate	Alvarez et al, 1999
Cys operon products	*Shewanella Oneidensis*	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 et al, 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

2.11 Bioremediation of Chromium

There are several techniques for expelling heavy metals from contaminated sites such as compound precipitation, ion-exchange, evaporation, and filtration, adsorption on activated charcoal, kaolin, alum and cinder (Barceloux and Pardix, 1971). A major limitation is that these techniques require a significant variety and quantity of chemical agents, with an acceptance of creating secondary pollutant (Jeyasingh & Liggy, 2005). Moreover, from an aspect of efficacy, these conventional processes require a regular cleanup and of accumulated heavy metal & debris. In addition to the high cost for Cr (VI) reduction, the expulsion of Cr (VI) through these methods are not beneficial (Kratochvil, Pimentel and Volesky, 1998). Hence, bioremediation paves a new path for purifying waste effluents where the metabolic capabilities of microorganisms is put to effective use. Bioremediation techniques can be considered as in situ or ex situ, depending on whether the remediation is performed with the appropriate bacteria at the entire site of contamination or at segments of the site of interest, such as water, dregs, or soil, which would be transported to appropriate facilities for heavy metal treatment and expulsion (Pattan & Reakyai, 2013). Cr (VI) resistant microorganisms influence the reduction of Cr (VI) to Cr (III) thereby aiding in beneficial bioremediation applications (Raspor er al, 2000).

2.12 Microbial remediation of hexavalent Chromium

Initially, the work involving bioremediation was limited to research in a laboratory. As of recent events, most industrialized countries have adopted bioremediation as a potential scope for reducing contamination. Microorganisms are simple and sensitive markers towards abnormal constituents or pollutants in their environment; hence can adapt to becoming genetically resistant and even countering the degrading effects of heavy metals like Chromium. Ever since the discovery of microbes capable of decreasing Cr (VI) in 1970 (Zhu et al, 2008), the search for Cr^{6+} reducing biota has been regionally sought after.

Chapter 3: Materials and Methods

3.1 Chemicals

All chemical used for the experiment were unadulterated and of analytical grade. They were applied into every field of wet lab work, from preparing nutrient media to commencement of all chemical reactions. The following chemicals have been utilized for this investigation:

1) Glycerol
2) Ethanol (70 percent)
3) Nutrient Agar
4) Nutrient Broth
5) MOPS buffer
6) Diphenyl Carbazide powder
7) Potassium chromate (K_2CrO_4)
8) Mueller Hinton Agar (MHA)

3.2 Glassware and Apparatus

The devices and tools applied during the course of work are categorized into Table 2.3.

Table 3.2: Total number of tools utilized throughout the research and their role.

Instrument	**Function**
Autoclave	Sterilization of chemicals and apparatus
Analytical Balance	Measurement of weight
Laminar Airflow Hood	Creating an aseptic atmosphere
pH meter	Adjustment of media pH
Incubator	Incubation of Culture
Water Distillation Unit	Preparation of solution and media
UV-vis spectrophotometer	Cellular growth measurement and degradation of Cr (VI)
Centrifuge machine	Supernatant deposition and measurement of Cr (VI)
Digital Shaker	Incubation of bacterial culture at specific temperatures
Micro pipette	For withdrawing chemical and media at trace quantities

3.3 Collection of sample

Collection of soil samples

Slag samples of approximately 500 g were collected in plastic pocket sized containers. The location from which the sample slag was extracted from is located in Showarighat region of the Buriganga River, Dhaka, Bangladesh at 23˚ 42' 41.28" North latitude and 90˚ 23' 27.42" East longitude. The location of the site was determined using the Android GPS application UTM Geo Map, a reliable GPS (Global Positioning Software). The soil sample was collected from 7-10 inches below the surface from 3 different locations.

Collection of water samples

Water samples of approximately 500 milliliters were collected in fresh mineral water bottles. The location for the water samples was also from Showarighat region of the Buriganga River, Dhaka, Bangladesh at 23˚ 40' 0" North latitude and 86˚ 55' 60" East longitude. The location of the site was determined using the Android GPS application UTM Geo Map, a reliable GPS (Global Positioning Software). Water was collected from 9-10 inches below the water surface from 3 different locations.

Fig 3.3: GPS Coordinates for isolate C_2 (UTM Geo Map for Android, 2018).

3.4 Isolation and condition of the culture

Isolation of microorganisms from samples of the soil and water was conducted following standard framework procedures. Both water and soil samples were stabilized overnight in normal refrigerated temperature 4°C. The water was then filtered from accumulated waste debris using a filter paper. To withdraw of microorganism from the water that would show imperviousness to Chromium, 100 µL of the water specimen was inoculated. Also for the soil sample, 100 µL specimen of soil was taken directly from the sample container and mixed with 9 mL of the liquid of saline for crushing the soil to create an isotonic environment for the soil bacteria. Once purified, the liquid sample was streaked uniformly onto agar dish of nutrient towards media, to which 2 mM concentration of hexavalent Chromium in the form of as potassium chromate (K_2CrO_4) was added and then incubation was allowed at 37°C overnight, in an incubator. This led to development of bacterial colonies. The agar media was prepared by mixing 2.8 gram of nutrient agar powder into 100 ml of H_2O. The liquid media was then sterilized in an autoclave machine at 121^0C for 45 minutes with keeping 15 Lb. pressure. Once sterilized, potassium chromate (K_2CrO_4) was incorporated into molten media which was finally poured into Miramax petri dishes and left to settle, thereby creating a solidified agar media. The bacterial colonies that had been segregated on the previous day was contracted with toothpick which were sterilized and streaked upon the agar dish. Once again, incubation was done for 24 hours at 37°C. This procedure of sub culture was repeated for one week, until the final bacterial colonies were obtained; once which developed resistance towards chromium. To test its resistance further, the sub cultured specie was continuously exposed to greater strengths of Chromate of 2, 5, 7, 10, 15, 20, 25, 30, 35 and 45 mM concentration of hexavalent Chromium till the Minimum Inhibitory Concentration (MIC) of the bacteria was procured.

3.5 Reduction profile of Chromium resistant bacteria

To investigate H_2O and effluent H_2O, test of Diphenyl Carbazide complex formation assay was done to estimate hexavalent Chromium was attuned through standard procedures (Greenberg et al., 1992) and likewise these approaches were documented as per protocol (Turick et al., 1996). Arrangement was done for one curve that is standard for institutionalization of depletion chart of the microorganism that show imperviousness to Chromium.

3.5.1 Chemical preparation

3.5.1.1 Preparation of 10 ml 3M H_2SO_4

First, 8 mL purified H_2O was poured into a falcon tube. Then, 1670 µL conc. H_2SO_4 was added carefully drop by drop, which comprised of 8mL purified H_2O. Next, the solution's capacity was prepared to be equal to 10 mL though addition of 330 µL purified H_2O.

3.5.1.2 Preparation of Diphenyl Carbazide

At first, 0.025 Diphenyl Carbazide powder was added into a falcon tube. Then into the tube, 9.67 mL acetone was added followed by 3 M Sulphuric acid. 330 µL solution was taken consisted of the Diphenyl Carbazide powder. The falcon tube was finely swirled for mixing, to prepare uniform solution of DPCZ.

3.5.1.3 Preparation of MOPS buffer

At first, into 50 mL H_2O, 0.1 g Sodium Hydroxide was taken to prepare 50 mL 1 N sodium hydroxide. To prepare 20 mM buffer of MOPS, MOPS powder of 334.88 mg has been mixed with purified H_2O of 80 mL. The pH of the MOPS buffer was attuned to 7 through the addition of adequate quantity of 1 N Sodium Hydroxide in the buffer solution.

3.5.1.4 Preparation of 5 mM 10 mL K_2CrO_4

To prepare a solution of 1 M Potassium Chromate, 19.4 g Potassium Chromate was dissolved into 10 mL purified H_2O. Then, filtration was done for this solution utilizing a membrane sieve containing 0.45 micron size of the pore. Finally, dilution was done for this solution up to 5 mM and it was preserved so that it can be utilized in future.

3.5.2 Processes of experiment

3.5.2.1 Standard curve preparation

Sample preparation for reaction

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

Table 3.5: Sample preparation for Standard curve.

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

Reaction protocol for standard curve

Initially 600 µL of sample was added into one falcon tube. Then, 1.2 ml 20 mM buffer of MOPS, 99 µL 3M Sulfuric acid, 981 µL purified H_2O and 120 µL of Diphenyl Carbazide were taken progressively and thus and uniform blend of mixture was carefully prepared. Presence of free Cr (VI) ions in the solution will lead to its complex formation with diphenyl carbazide, and this in turn renders the entire solution into purple color due to

the complex formed. Finally, the response solution's absorbance has been measured through UV-Visible spectrophotometer at 540 nm.

3.5.2.2 Evaluation of reduction profile of bacterial isolate C_2 at 25°C, 37°C and 42°C and pH 5.5, 7 and 8.5

Procedure

Day 0

To nutrient broth was prepared in four distinct conical flasks in each of which, 10 ml volume of broth was added to. Following then, bacterial culture of 500 micro liter was taken from the Glycerol stock culture, and added into all of the freshly prepared nutrient medium. The flasks were tightly sealed with aluminum foil and labelled pH 5.5, 7 and 8.5 and Blank. This concluded the process of inoculation. The blank media served as a control and reflected whether or not that broth medium of nutrient where inoculation was properly done or not. Finally, all the flasks were secured in the rotating incubator, and left for incubation for 24 hours.

Day 1

On the next day, four flasks 25mL nutrient broth was prepared, each labeled blank, pH 5.5, 7 and 8.5. Next, 2mL solution of bacterial culture was withdrawn from the overnight conical flasks to take measurements for its Optical Density. This ensured a positive control for the bacterial isolate C_2 and negative control, i.e. the blank nutrient broth. The bacterial isolate's OD (optical density) was recorded with the help of a UV-visible spectrophotometer at the wavelength of 600nm to observe the growth and development of bacterial colony overnight. Next, a calculation was done for the quantity that would be needed from the overnight culture in order to obtain 0.2 optical density into test culture. The bacterial sample was transferred into a falcon tube during measurement of Optical Density. Once performed, these tubes were next vortexed for five minutes, to enable centrifugation of the blank and samples, at the rate of 4000 rotations per minute. Once centrifuged, the desired amount of bacterial isolate that was calculated was then extracted from the falcon tubes using a micropipette and added to each 25 ml nutrient broth containing flasks respectively. The flasks were organized according to an increase in pH strength and all the conical flasks were introduced to 15 µL potassium chromate which contained hexavalent Chromium. Once all 25 mL flasks were inoculated at their

respective blank and pH values, they were replaced with the overnight conical flasks in the digital incubator for a day long period of incubation. Subsequent bioassay readings were taken at every 1.5 hour intervals, from 25ml conical flask of sample, positive and negative control, 2ml test cultures were withdrawn and optical density has been recorder at the wavelength of 600nm for observing the cell development. Next, these cultures of 2ml were centrifuged once again for five minutes at 4000 rpm and this led to bacteria precipitate at the bottom of falcon tube. Next, supernatant was collected from top of falcon tube at 600 µL into three test tubes and the bioassay was carried on by adding the respective reagents, i.e. MOPS, H_2SO_4, water and DPCZ according to the reaction procedure as described in the section 3.6.2.1.2. Optical density was then measured at the wavelength of 540 nm to investigate the amount of hexavalent chromium reduced. This entire process was reiterated following 0 hour, 1.5^{th} hour, 3^{rd} hour, 4.5^{th} hour, 6^{th} hour and finally reduction of hexavalent chromium after the growth of bacteria after 24 hours (overnight). For every interval triplicate reading were taken the final measurement was recorded. To assemble and interpret the data, Microsoft office excel 2016 were used to obtaining the reduction profile.

3.6 Antibiotic resistance among Chromium resistant bacterial isolates

3.6.1 Preparation Culture Strain in Nutrient broth (NB)

To investigate the sensitivity of the antibiotics, nutrient broth was prepared for culture strains. Conical flasks were taken and to each, 20 ml of nutrient broth was added and inoculation was done for the subsequent strains in the individual conical flasks comprising 20 ml nutrient broth and incubation was done for overnight at 37ºC. All conical flasks were labeled as C_2.

3.6.2 Inoculation of test plates

To prepare the test plates, Mueller Hinton Agar (MHA) was used as the culture media. The bacteria flasks which were left for incubation overnight were withdrawn. From each flask, bacteria was taken using sterile cotton buds and swabbed repeatedly in semi circles on the plates in clockwise rotation, to inoculate the plates of MHA. By swabbing the MHA plates for 2 more periods, and rotating the plate at about 60ºC for each swab, this ensures the inoculum's uniform spread. Finally, the agar plates were sealed and each

plate was marked equidistantly into five portions, each portion denoted the location a bacterial disc.

3.6.3 Application of Antibiotic discs

Once properly swabbed, labeled and marked, bacterial discs were introduced to each MHA plate. The discs were individually picked up using forceps and gently placed on the surface of the MHA. Then the individual discs were pushed down onto the surface of the agar to ensure a thorough contact within the surface of agar. The following antibiotic discs were utilized in this test:

- ✓ Chloramphenicol (C:30 mg)
- ✓ Ciprofloxacin (Cip:5 mg)
- ✓ Gentamicin (CN: 10 mg)
- ✓ Ofloxacin (OF: 5 mg)
- ✓ Kanamycin (KA : 10 mg)
- ✓ Vancomycin (VA: 30mg)
- ✓ Sulphametronazol / Trimethoprim (SXT: 25 mg)
- ✓ Azithromycin (AZM:15mg)
- ✓ Neomycin (N: 30 mg)
- ✓ Cefixime (C :20 mg)
- ✓ Ceftriaxone (CRO: 30 mg)
- ✓ Streptomycin (SM: 20mg)
- ✓ Cefuroxime Sodium (CXM: 30 mg)
- ✓ Penicillin-G (P: 10 mg)
- ✓ Trimethoprim (TP: 10 mg)

3.6.4 Incubation

Once the discs were assembled onto the plates, the test plates were kept in the incubator at 37ºC for 24 hours to enable growth of bacteria and subsequently allow the discs to develop zones.

3.7 Determination of Minimum Inhibitory Concentration (MIC)

In microbiology, MIC is defined as a minimum concentration of an anti- bacterial substance at which, a bacteria's observable development and metabolic functions will become stunted and permanently lost, after incubation of 24 hours. For this experiment, the minimum inhibitory concentration of the hexavalent Chromium for the bacterial specie of interest was determined by counting the number of bacterial colonies developed. Hence 50 µL of bacteria was inoculated on petri dishes that contained nutrient agar media. Then each plate was complemented with potassium chromate (K_2CrO_4) of several concentrations from 2mM – 30mM. Then all petri dishes were given for incubation at the temperature 37ºC for forty-eight hours. Finally, the bacterial development was measured through counting of the colony using digital colony counter.

3.8 Identification of the isolate C_2

Once the sequence data file for C_2 isolate was obtained by 16s rDNA sequencing, it was observed using the Finch TV tool. Using this software, the isolate's genetic sequence was purified and the file was saved as a FASTA format. Hence, this purified sequence served as a QUERY sequence. Next, nucleotide BLAST (Basic Local Alignment Search Tool) was performed upon the query sequence with existing database of NCBI (National Center for Biotechnology Information). Then the possible bacterial strains were identified on the basis of maximum similarity score, and 50 sequences were downloaded from the database, which were further aligned in the Bio Edit tool and the conserved region for all 50 sequences was obtained. Finally, the software- Mega 7 was used to develop a Phylogeny Tree for all the sequences, including the query sequence, to trace the origin of the isolate C_2.

Chapter 4: Results

4.1 Isolation data of Chromium resistance bacteria

Isolation was done for the individual colonies of two from the Agar dishes of nutrient with various concentrations of Chromate.

4.2 Chromium reduction profile of Chromium resistant bacteria

4.2.1 Standard Curve

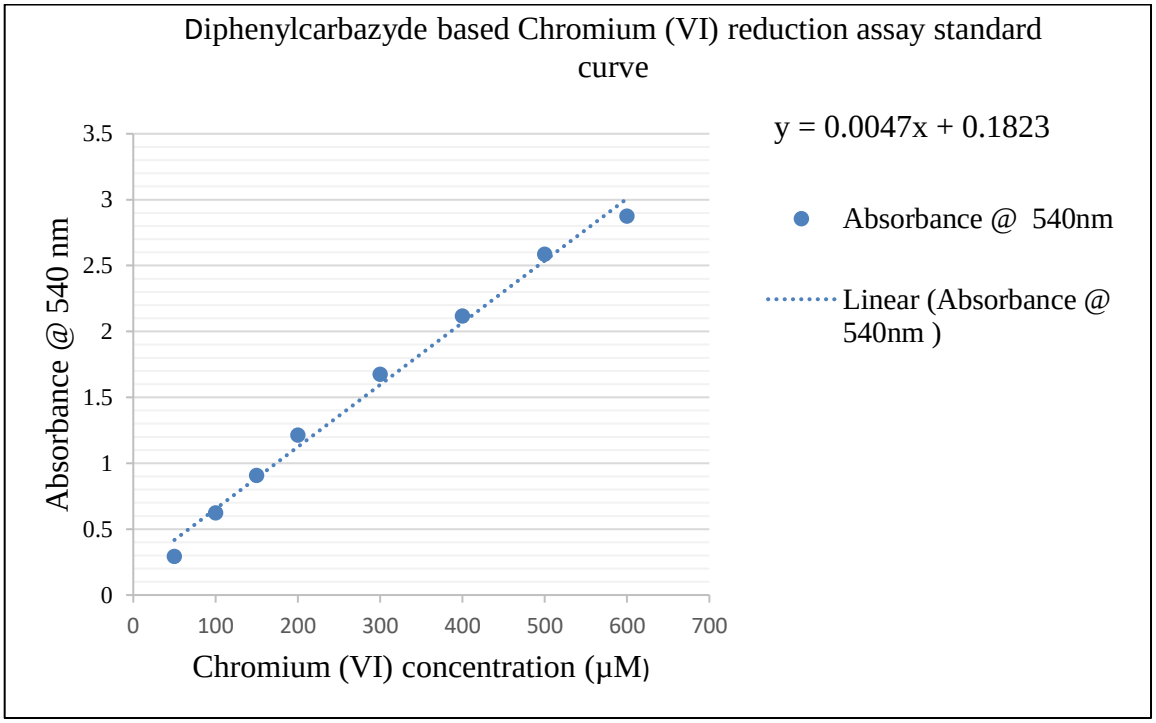
The method stated in section 2.6.2.1.2 was utilized to obtain the standard curve. Following on, the value of absorbance was used to plot a graph using Microsoft Excel 2013. The results obtained are shown below:

Table 4.2.1: Data of standard curve of hexavalent Chromium.

Concentration (µM)	**Absorbance (540 nm)**
50	0.293
100	0.623
150	0.906
200	1.213
300	1.676
400	2.118
500	2.585
600	2.876

From these data, a standard curve was found, as shown below:

Figure 4.2.1: Standard curve of Chromium (VI) at 540 nm wavelength.



4.2.2 Reduction profile of Isolate C_2

At various constraints, this test was done such as, altering the pH and temperature and the outcomes which was attained precise underneath:

Table 4.2.2: C_2 Chromium reduction profile vs. Cell Growth at 25º C, pH 5.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	339.15	0.489	395.11	0.009
1.5	365.04	0.617	379.51	0
3.0	335.97	0.987	381.78	0.015
4.5	309.51	1.533	392.70	0.012
6.0	283.83	1.711	377.95	0.080
7.5	249.72	1.900	380.36	0.016
24	250.43	2.064	347.82	0

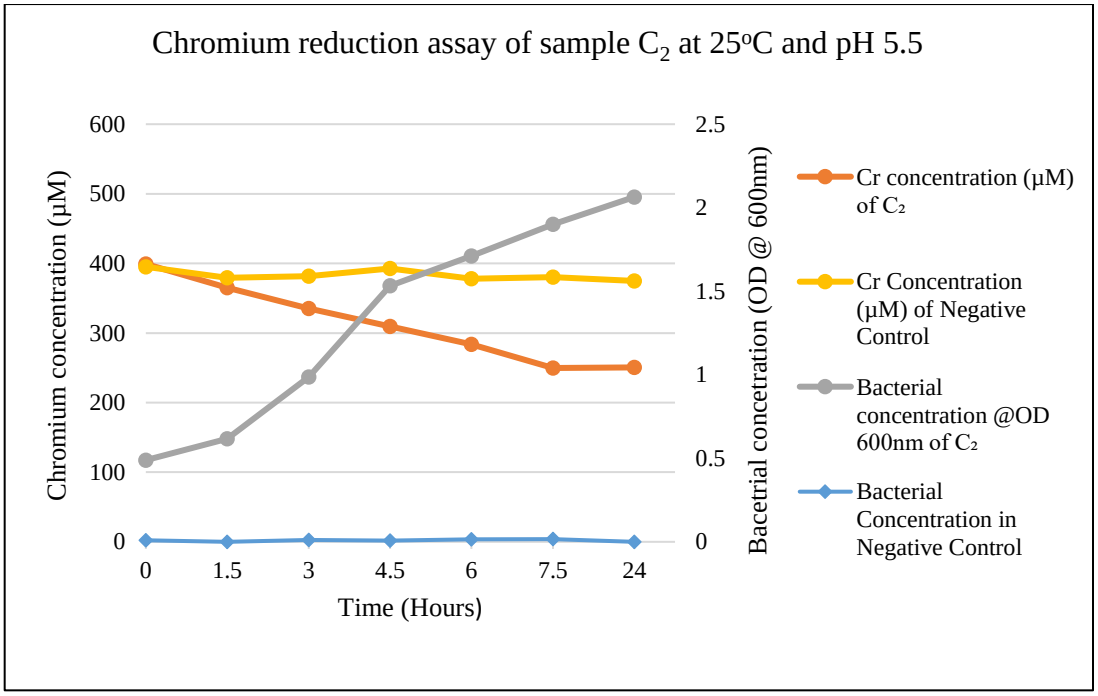


Figure 4.2.2: Chromium reduction vs. Cell Growth in C_2 isolate at 25^0C, pH 5.5.

From figure 4.2.2, it was observed that the initial concentration of Chromium was 399.15 µM at 540 nm. For the next 6 intervals, the concentration of Chromium steadily decreased from 399.15 µM to 249.72 µM in the 7^{th} hour. Eventually, the Chromium concentration became constant in the 24^{th} hour, at 250.43 µM. Moreover, the bacterial concentration at OD 600 nm showed an initial accelerated growth from the 0 hour till the 24^{th} hour, i.e. from 0.49 to 1.53. Eventually, the concentration rose constantly for the next three time intervals, i.e. from 1.53 up to 2.06. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent chromium by 37.43 %.

Table 4.2.3: C_2 Chromium reduction profile vs. Cell Growth at 25ºC, pH 7.0.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	449.51	0.627	395.11	0.009
1.5	370.85	0.833	379.51	0
3.0	337.95	1.349	381.78	0.015
4.5	329.08	1.882	392.70	0.012
6.0	296.03	2.041	377.95	0.080
7.5	257.52	2.901	380.36	0.016
24	224.26	3.262	347.82	0

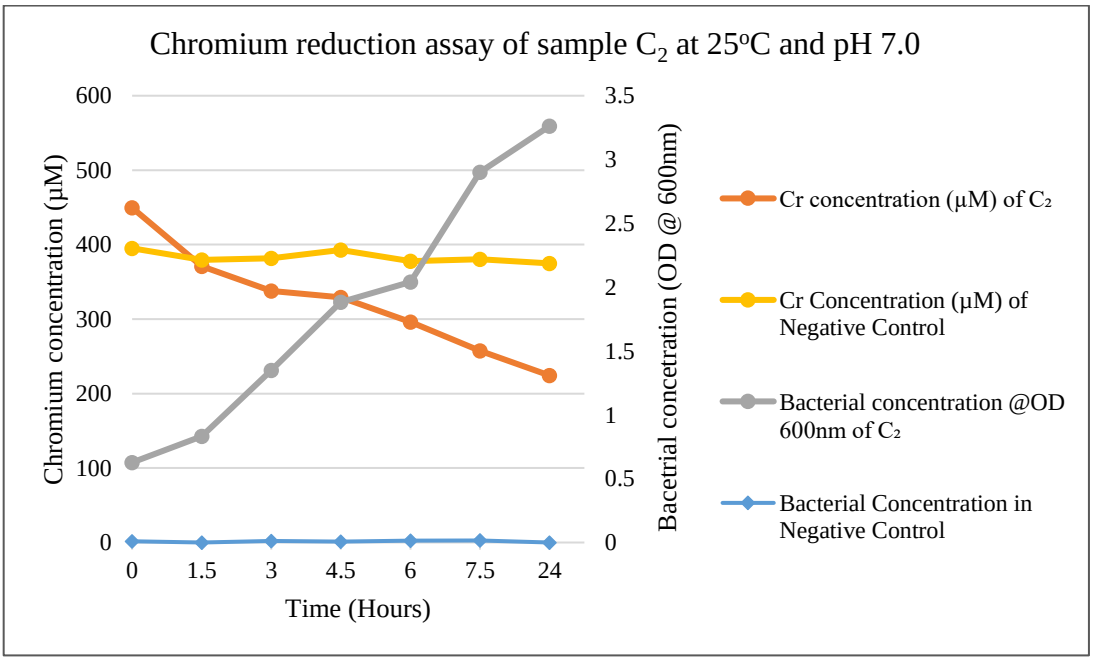


Figure 4.2.3: Chromium reduction vs. Cell Growth in C_2 isolate at 25ºC, pH 7.0

From figure 4.2.3, it was observed that the initial concentration of Chromium was 449.51 µM at 540 nm. For the next 3 hours, the concentration of Chromium showed a sharp decline to 329.08 µM. Eventually, the Chromium concentration decreased steadily from the 4.5th hour up to the 24th hour, i.e. from 329.08 µM to 224.26 µM. Moreover, the bacterial concentration at OD 600 nm showed a near constant increase in growth from 0 hour till the 24th hour, i.e. from 0.627 to 3.26. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent chromium by 50.11 %.

Table 4.2.4: C_2 Chromium reduction profile vs. Cell Growth at 25ºC, pH 8.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	387.45	0.671	395.11	0.009
1.5	336.03	0.849	379.51	0
3.0	299.22	1.400	381.78	0.015
4.5	187.31	1.857	392.70	0.012
6.0	161.28	2.209	377.95	0.080
7.5	114.04	2.216	380.36	0.016
24	81.70	2.626	347.82	0

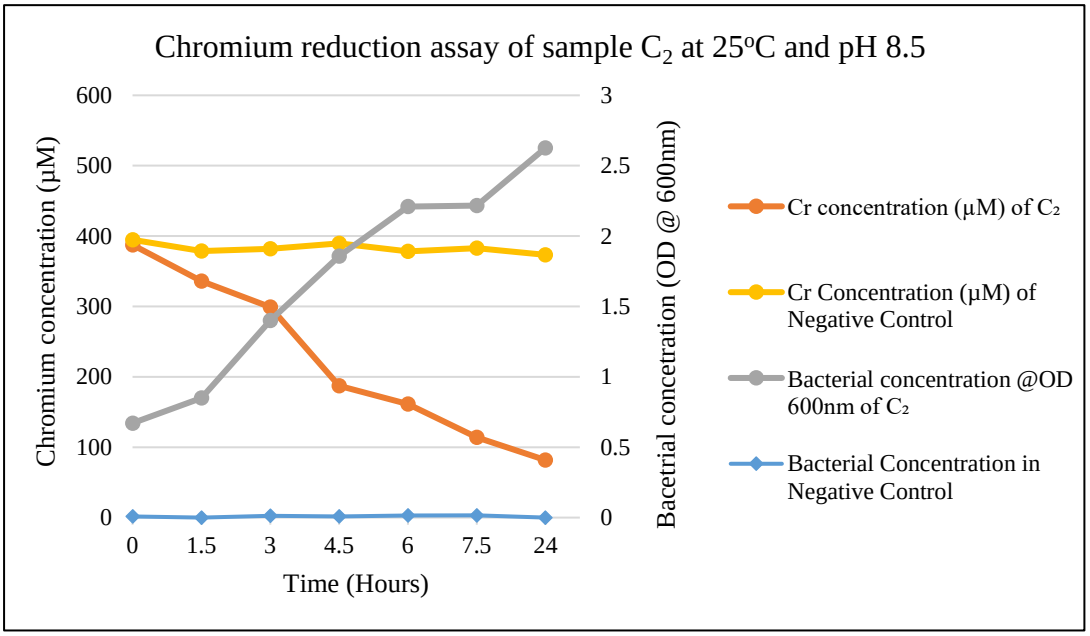


Figure 4.2.4: Chromium reduction vs. Cell Growth in C_2 isolate at 25ºC, pH 8.5.

From figure 4.2.4, it was observed that the initial concentration of Chromium was 387.45.15 µM at 540 nm. For the next 3 hours, the concentration of Chromium showed a sharp decrease from 387.15 µM to 187.31 µM in the 4.5th hour. Eventually, the concentration of Chromium constantly decreased up to the 24th hour, i.e. from 187.31 µM to 81.71 µM. Moreover, the bacterial concentration at OD 600 nm showed a constant growth from the 0 hour till the 24th hour, i.e. from 0.67 to 2.62. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent chromium by 78.91 %.

Table 4.2.5: C_2 Chromium reduction profile vs. Cell Growth at 37ºC, pH 5.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	404.33	0.488	395.11	0.009
1.5	393.19	0.530	379.51	0
3.0	365.89	0.570	381.78	0.015
4.5	283.69	0.543	392.70	0.012
6.0	213.83	0.560	377.95	0.080
7.5	38.51	0.582	380.36	0.016
24	-36.02	1.244	347.82	0

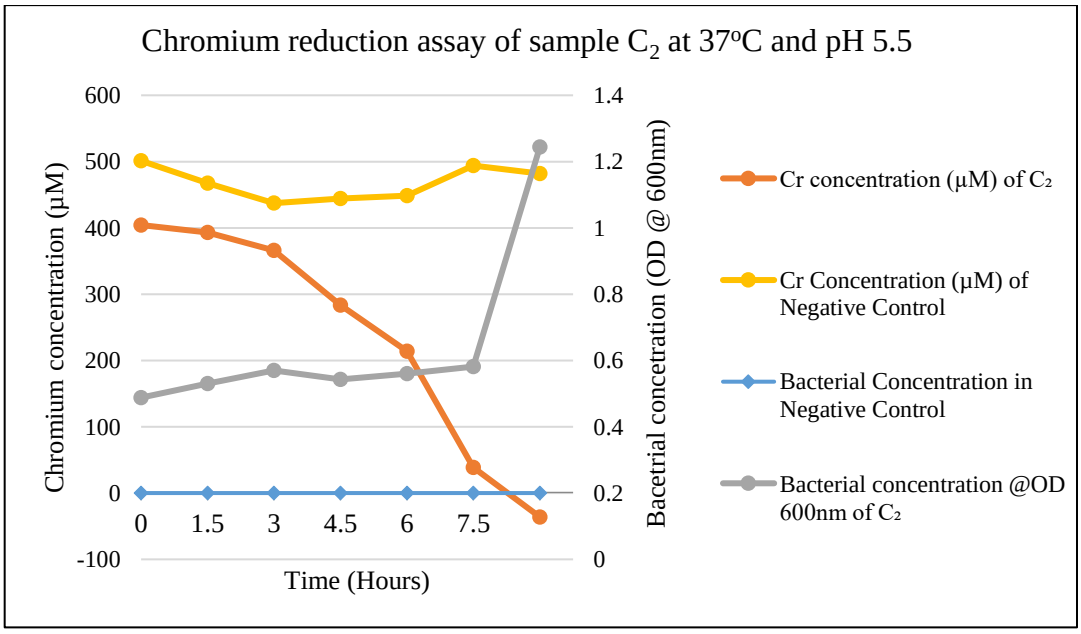


Figure 4.2.5: Chromium reduction vs. Cell Growth in C_2 isolate at 37ºC, pH 5.5.

From figure 4.2.5, it was observed that the initial concentration of Chromium was 404.33 µM at 540 nm. For the next 4 hours, the concentration of Chromium gradually decreased from 404.33 µM to 213.83 µM in the 6th hour. Eventually, the Chromium concentration showed a sharp decrease up to the 24th hour, at -36.021 µM. Moreover, the bacterial concentration at OD 600 nm showed a near constant growth from the 0 hour till the 7.5th hour, i.e. from 0.48 to 0.58. Then the Bacterial growth rose sharply till the 7.5th hour i.e. from 0.58 to 1.24. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 100% within 24 hrs.

Table 4.2.6: C_2 Chromium reduction profile vs. Cell Growth at 37ºC, pH 7.0.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	323.83	0.596	395.11	0.009
1.5	250.14	1.003	379.51	0
3.0	314.47	1.535	381.78	0.015
4.5	263.26	1.793	392.70	0.012
6.0	56.74	1.879	377.95	0.080
7.5	2.06	1.982	380.36	0.016
24	23.76	2.477	347.82	0

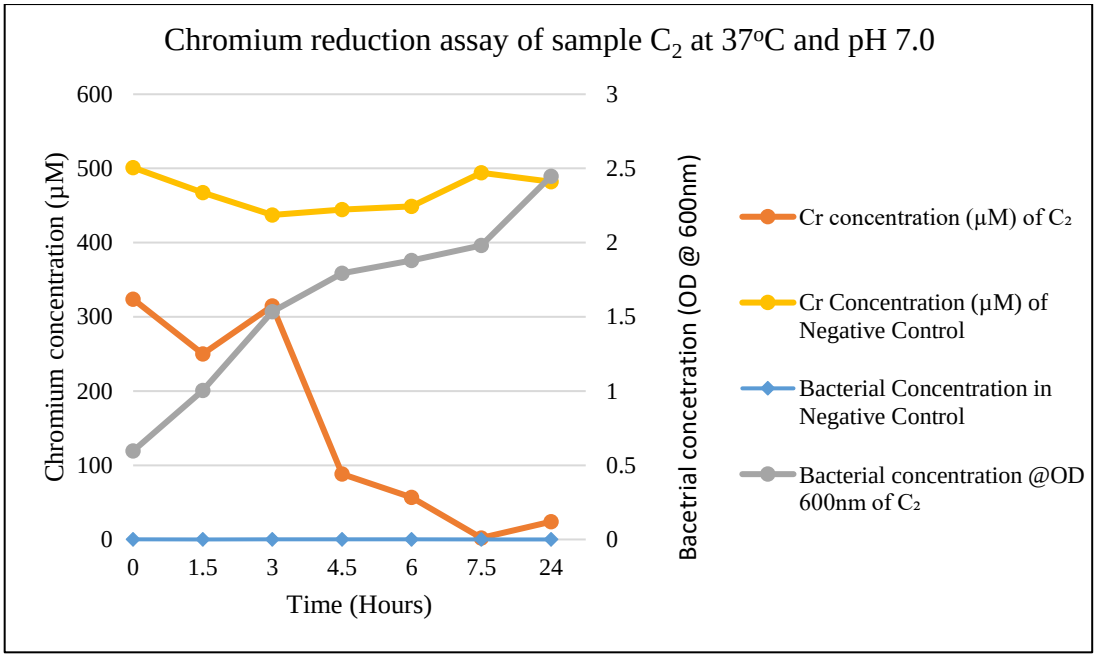


Figure 4.2.6: Chromium reduction vs. Cell Growth in C_2 isolate at 37ºC, pH 7.0.

From figure 4.2.6, it was observed that the initial concentration of Chromium was 323.84 µM at 540 nm. The concentration of Chromium had a sharp decline followed by an increase in the 1.5th and 3rd intervals, i.e. up to 314.47 µM in the 3rd hour. Eventually, the Chromium concentration showed a sharp decrease up to the 4.5th hour, at 81.85 µM and finally decreased steadily for the next two intervals, until all hexavalent Chromium was reduced. Moreover, the bacterial concentration at OD 600 nm showed a steady growth from the 1st hour till the 6th hour, i.e. from 0.59 to 2.44. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 100% within the 7.5th hour.

Table 4.2.7: C_2 Chromium reduction profile vs. Cell Growth at 37° C, pH 8.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	337.87	0.862	395.11	0.009
1.5	172.06	1.206	379.51	0
3.0	106.39	1.575	381.78	0.015
4.5	-8.64	1.850	392.70	0.012
6.0	-9.92	2.001	377.95	0.080
7.5	38.51	2.079	380.36	0.016
24	28.73	2.510	347.82	0

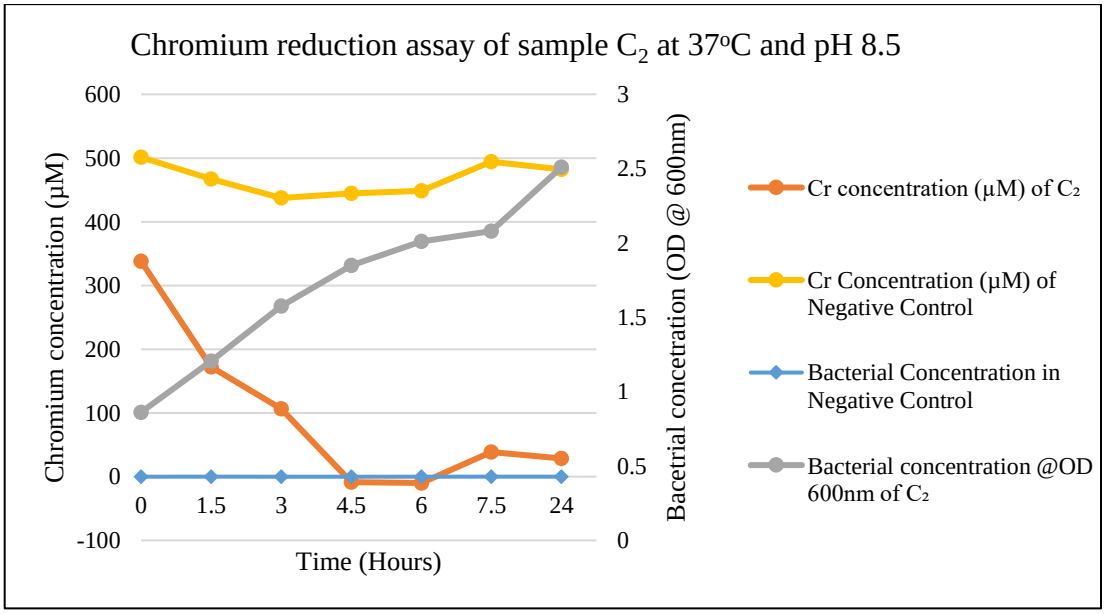


Figure 4.2.7: Chromium reduction vs. Cell Growth in C_2 isolate at 37°C, pH 8.5.

From figure 4.2.7, it was observed that the initial concentration of Chromium was 337.88 µM at 540 nm. The concentration of Chromium had a sharp decline from 0 hour to the 4.5th hour, i.e. from 337.88 µM to -9.92 µM and remained constant till the 6th hour. Eventually, the Chromium concentration slowly increased till the 24th hour, i.e. up to 28.7 µM. Moreover, the bacterial concentration at OD 600 nm showed a steady growth from the 1st hour till the 24th hour, i.e. from 0.86 to 2.51. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 100% within the 4.5th hour.

Table 4.2.8: C_2 Chromium reduction profile vs. Cell Growth at 42ºC, pH 5.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	361.99	0.204	395.11	0.009
1.5	327.66	0.332	379.51	0
3.0	280.21	0.531	381.78	0.015
4.5	193.83	0.691	392.70	0.012
6.0	144.75	0.968	377.95	0.080
7.5	101.0	1.212	380.36	0.016
24	41.63	2.094	347.82	0

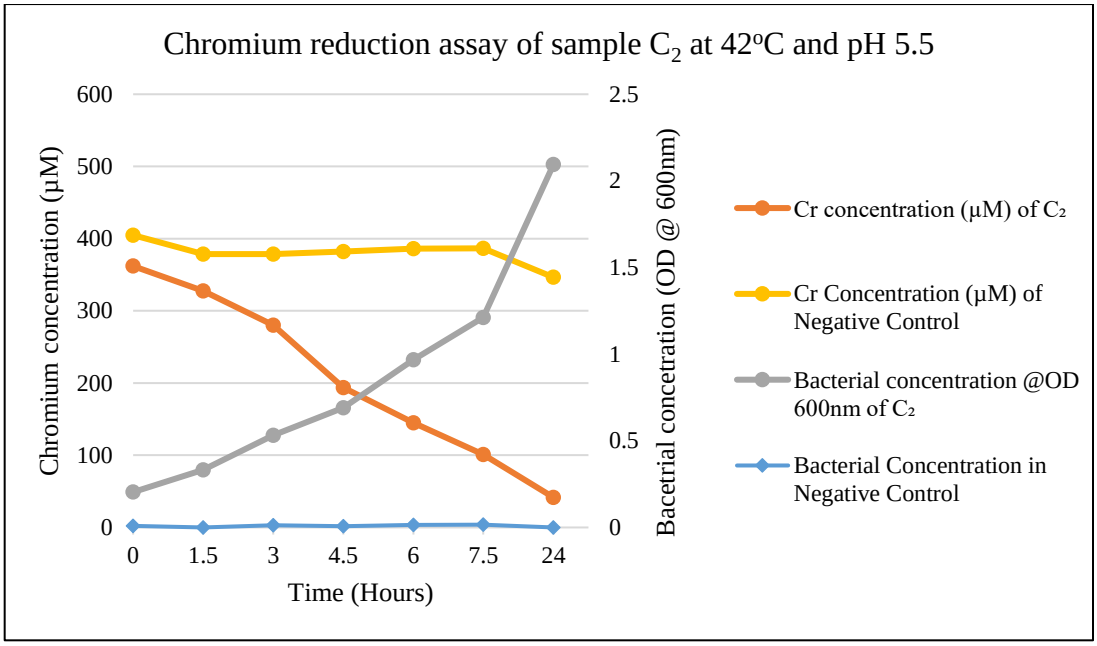


Figure 4.2.8: Chromium reduction vs. Cell Growth in C_2 isolate at 42ºC, pH 5.5.

From figure 4.2.8, it was observed that the initial concentration of Chromium was 361.99 µM at 540 nm. The concentration of Chromium showed a steady decrease up to the 24th hour, i.e. from 361.99 to 41.63 µM. Moreover, the bacterial concentration at OD 600 nm showed a steady growth from the 1st hour till the 24th hour, i.e. from 0.20 to 2.09. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 88.5 % within 24 hours.

Table 4.2.9: C_2 Chromium reduction profile vs. Cell growth at 42ºC, pH 7.0.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	307.80	0.207	395.11	0.009
1.5	343.30	0.491	379.51	0
3.0	238.65	1.118	381.78	0.015
4.5	169.51	1.562	392.70	0.012
6.0	60.07	1.864	377.95	0.080
7.5	39.01	1.756	380.36	0.016
24	32.91	2.208	347.82	0

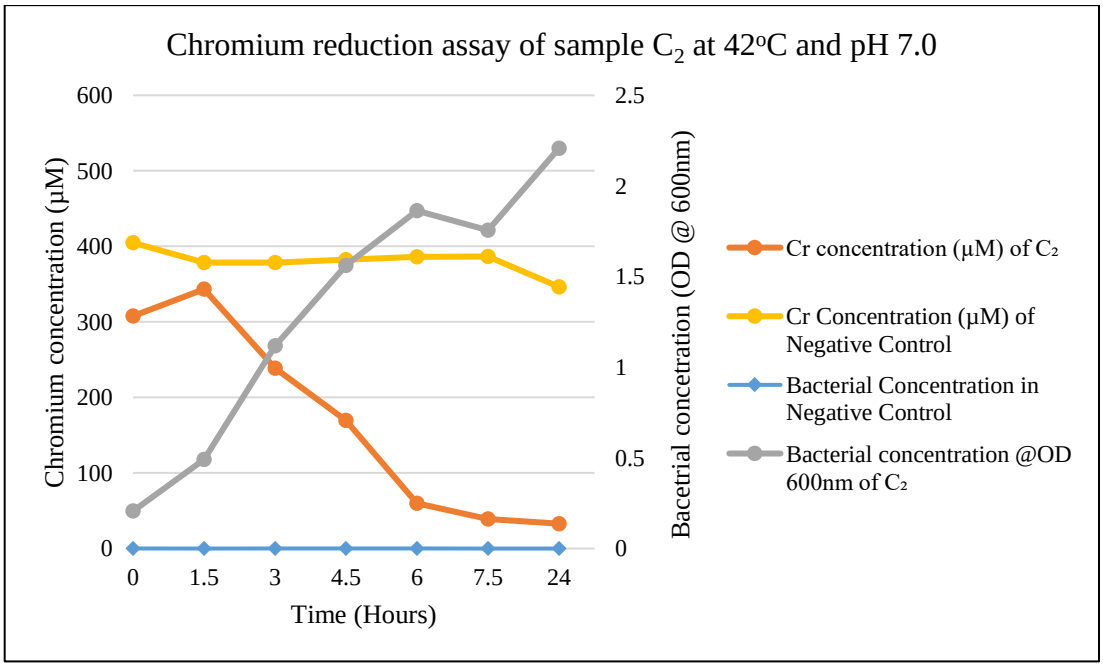


Figure 4.2.9: Chromium reduction vs. Cell Growth at 42ºC, pH 7.0.

From figure 4.2.9, it was observed that the initial concentration of Chromium was 307.81 µM at 540 nm. The concentration of Chromium increased at 1.5 hour, i.e. 343.34 µM then showed a steady decrease up to the 24th hour, i.e. up to 32.91 µM. Moreover, the bacterial concentration at OD 600 nm showed a steady growth from the 1st hour till the 6th hour, i.e. from 0.21 to 1.86, followed by a decrease to 1.76, and finally rose up to 2.21 in the 24th hour. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 90.4 % within 24 hours.

Table 4.2.10: C_2 Chromium reduction profile vs. Cell growth at 42ºC, pH 8.5.

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration in (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration in (µM) at 540 nm**	**Bacterial Concentration at 600 nm**
0	307.80	0.207	395.11	0.009
1.5	343.34	0.491	379.51	0
3.0	238.65	1.118	381.78	0.015
4.5	169.51	1.562	392.70	0.012
6.0	60.07	1.864	377.95	0.080
7.5	39.01	1.756	380.36	0.016
24	32.91	2.208	347.82	0

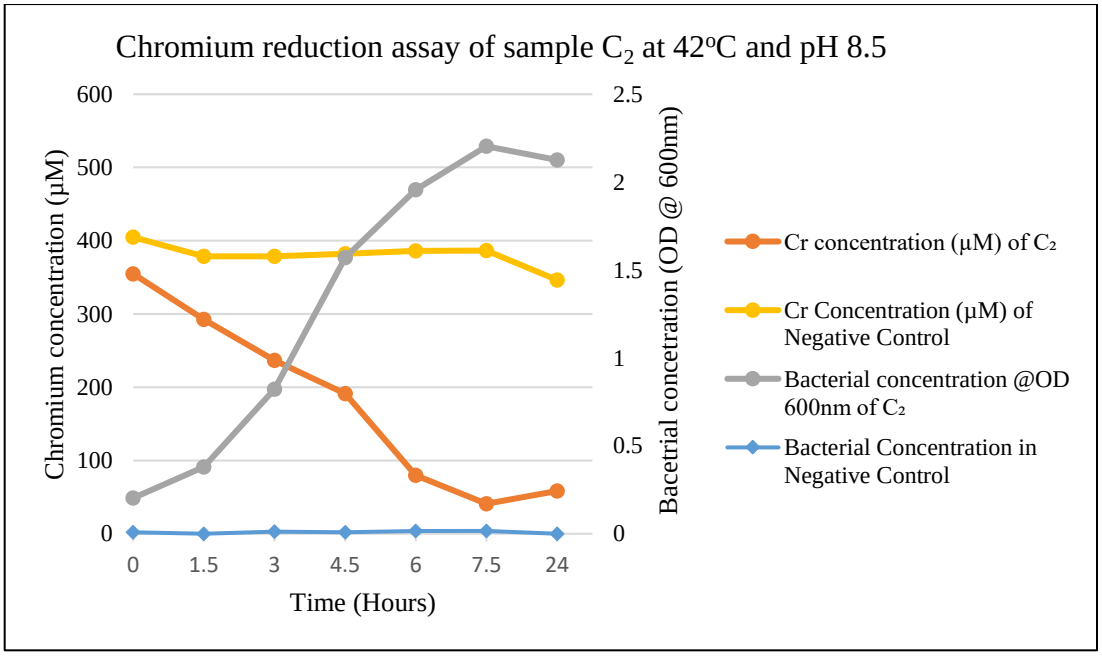


Figure 4.2.10: Chromium reduction vs. Cell Growth in C_2 isolate at 42ºC, pH 8.5.

From figure 4.2.10, it was observed that the initial concentration of Chromium was 354.90 µM at 540 nm. The concentration of Chromium decreased steadily till the 4.5th hour, i.e. up to 191.07 µM then showed a sharp decrease from the 4.5th to 7.5th hour, i.e. up to 40.92 µM. Moreover, the bacterial concentration at OD 600 nm showed a steady growth from the 1st hour till the 24th hour, i.e. from 0.21 to 2.12. However, the Chromium concentration at 540 nm and Bacterial concentration at 600 nm remained constant, hence showing no signs of contamination in the negative control. Therefore, it can be deduced that the isolate C_2 was capable of reducing hexavalent Chromium by 88.5 % within 7.5 hours.

4.3 Antibiotic resistance among Chromium resistant isolate C_2

This test was conducted by evenly setting 14 Antibiotic discs on the surface of MHA (Mueller-Hinton Agar) plates. The plates were initially inoculated and then incubated for 24 hours. Following incubation, the effect of antibiotic discs against isolate C_2 were determined by measuring the diameter of zone of inhibition (ZI) in millimeter with the help of a transparent ruler. The outcome of the investigation are shown as follows:

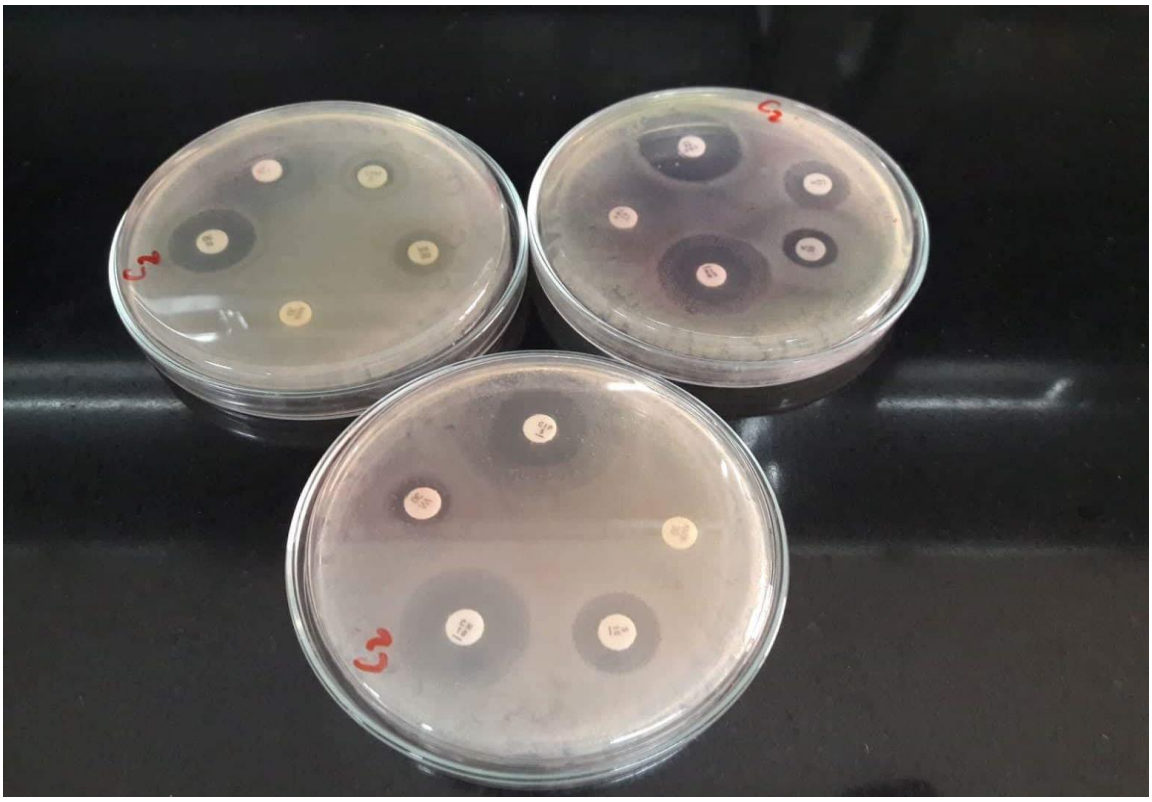


Figure 4.3: Zone of Inhibition of Antibiotic discs in C_2 isolate.

Table 4.3.1: Isolate C_2: Antibiotic resistance profile.

Antibiotic	**ZI/mm**	**ZI/mm**	**ZI/mm**	**Average**	**Standard Deviation**
Penicillin (P10)	0	0	0	0	0
Kanamycin (K30)	12	15	14	13.67	1.52
Neomycin (N30)	20	19	18	19	1
Vancomycin (VA30)	8	8	9	8.33	0.57
Gentamycin (CN10)	20	19	17	18.67	1.52
Cefixime (CFM5)	10	10	9	9.67	0.57
Chloramphenicol (C30)	14	10	12	12	2
Ceftriaxone (CRO30)	21	21.5	18	20.17	1.89
Trimethoprim (STX25)	12	10	10	10.67	1.15
Cipropfloxacin (CIP5)	18	18	19.5	18.5	0.87
Streptomycin (S10)	14	15	13	14	1
Ofloxacin (OF5)	10	12	11	11	1
Amoxycillin (AML10)	10	10	10	10	0
Cefuroxime Sodium (CXM30)	10	10	12	10.67	1.15

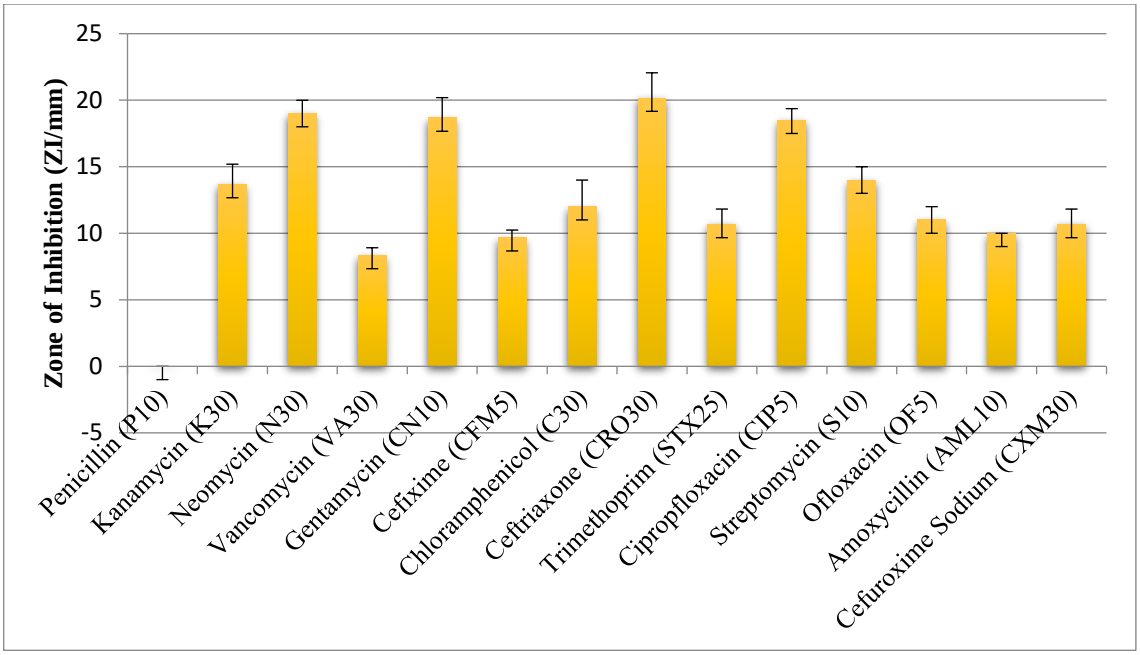


Figure 4.3.1: Isolate C_2: Antibiotic resistance profile.

From Table 4.3.1, it was observed that the greatest zone of inhibition was shown by Ceftriaxone (CRO30) which was 20.2 mm. Hence, Ceftriaxone was the most potent antibiotic for isolate C_2, due to its capability of killing most of the isolates in the colony. In addition, the smallest zone of inhibition was shown by Vancomycin (VA30) which was 8.33 mm. Therefore, the isolate C_2 developed significant resistance towards it. Overall, the isolate was more or less susceptible towards the remaining antibiotics.

For instance, Neomycin, Gentamycin and Ciprofloxacin showed greater zones of inhibition at near same magnitudes (19mm, 18.67mm, and 18.5mm) in contrast to Kanamycin, Trimethoprim and Amoxycillin (13.6 mm, 10.6 mm, and 10 mm).

4.4 Minimum Inhibitory Concentration of Chromium to inhibit the growth of bacteria

4.4.1 MIC of isolate: C_2

In different concentrations of Chromium, different number of colonies have been found and they are tabulated as follows:

Table 4.4.1: MIC of isolate C_2.

Concentration of Chromium (mM)	**No. of Colony Forming Unit (CFU)**
2	32
3	30
4	28
6	21
7	19
8	15
9	14
10	12
11	10
13	6
14	3
15	3
16	1
17	0

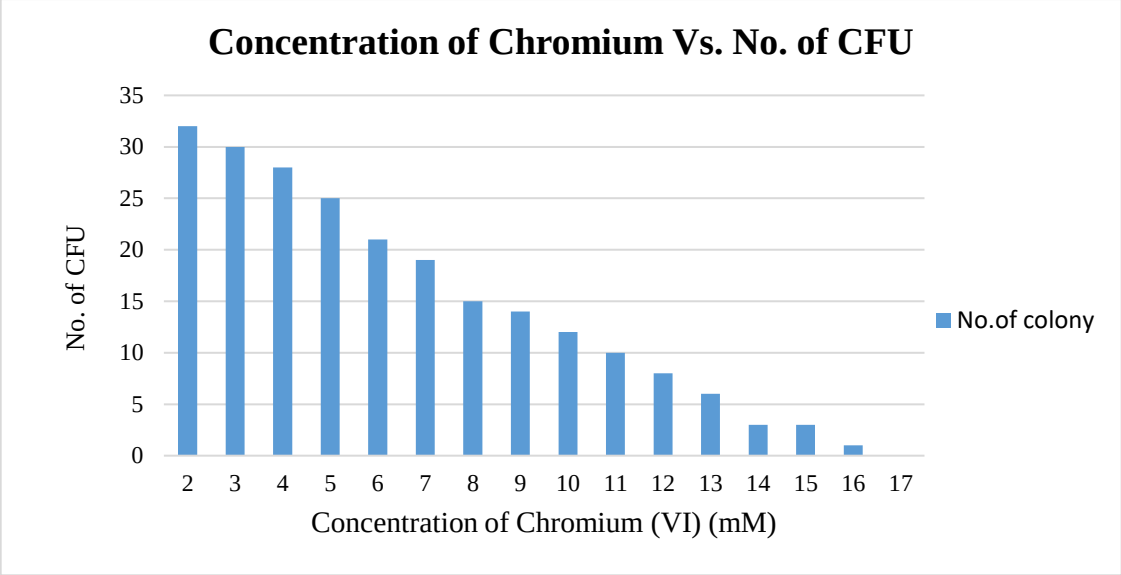


Figure 4.4.1: MIC of isolate C_2.

From Figure 4.4.1 it was observed that the isolate C_2 developed colonies in an enviromnent of hexavalent Chromium to a maximum concentration of 16 mM. This meant that the isolate was capable of showing resistance up to 16 mM. But isolate C_2 was totally failed to demonstate any growth of colony at 17 mM concentration of Chromium, meaning that it was susceptible to Chromium at that particular concentration and further above. Therefore, 17 mM was the Minimum Inhibitory Concentration for the C_2 isolate.

4.5 Identification of isolate C_2

16s rDNA sequencing was obtained using “Sanger sequencing method”. The sequence is shown in Appendix A. To indentify the bacterial isolate, the genetic sequence of the isolate was purified to remove all anomalous sequences at the beginning and the end of the sequence. Finally nucleotide BLAST was performed with the purified sequence. The result is tabulated in Table 4.5.

Table 4.5: BLAST result summary of C_2.

Sl.	Description	Max Score	Total Score	Query cover	E value	Identity	Accession number
1	*Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA gene, partial sequence*	1343	1343	100%	0.0	100%	NR 117678.1
2	*Pseudomonas aeruginosa strain NBRC 12689 16S ribosomal RNA gene, partial sequence*	1343	1343	100%	0.0	100%	NR 113599.1
3	*Pseudomonas aeruginosa strain ATCC 10145 16S ribosomal RNA gene, partial sequence*	1343	1343	100%	0.0	100%	NR 114471.1
4	*Pseudomonas guezennei strain RA26 16S ribosomal RNA, partial sequence*	1343	1343	100%	0.0	98%	NR 104957.1
5	*Pseudomonas otitidis strain MCC10330 16S ribosomal RNA, partial sequence*	1343	1343	100%	0.0	98%	NR 043289.1

6	*Pseudomonas resinovorans strain ATCC 14235 16S ribosomal RNA, partial sequence*	1343	1343	100%	0.0	98%	NR 112062.1
7	*Pseudomonas aeruginosa strain DSM 50071 16S ribosomal RNA, complete sequence*	1343	1343	100%	0.0	98%	NR 026078.1
8	*Pseudomonas alcaligenes strain ATCC 14909 16S ribosomal RNA gene, partial sequence*	1343	5368	100%	0.0	97%	NR 113646.1
9	*Pseudomonas alcaligenes strain NBRC 14159 16S ribosomal RNA gene, partial sequence*	1343	1343	100%	0.0	97%	NR 117827.1
10	*Pseudomonas indica strain NBRC 103045 16S ribosomal RNA gene, partial sequence*	1343	5359	100%	0.0	97%	NR 114196.1
11	*Pseudomonas alcaligenes 16S ribosomal RNA gene, partial sequence*	1343	5374	100%	0.0	97%	NR 114472.1

4.6 Phylogenetic Tree for Isolate C_2

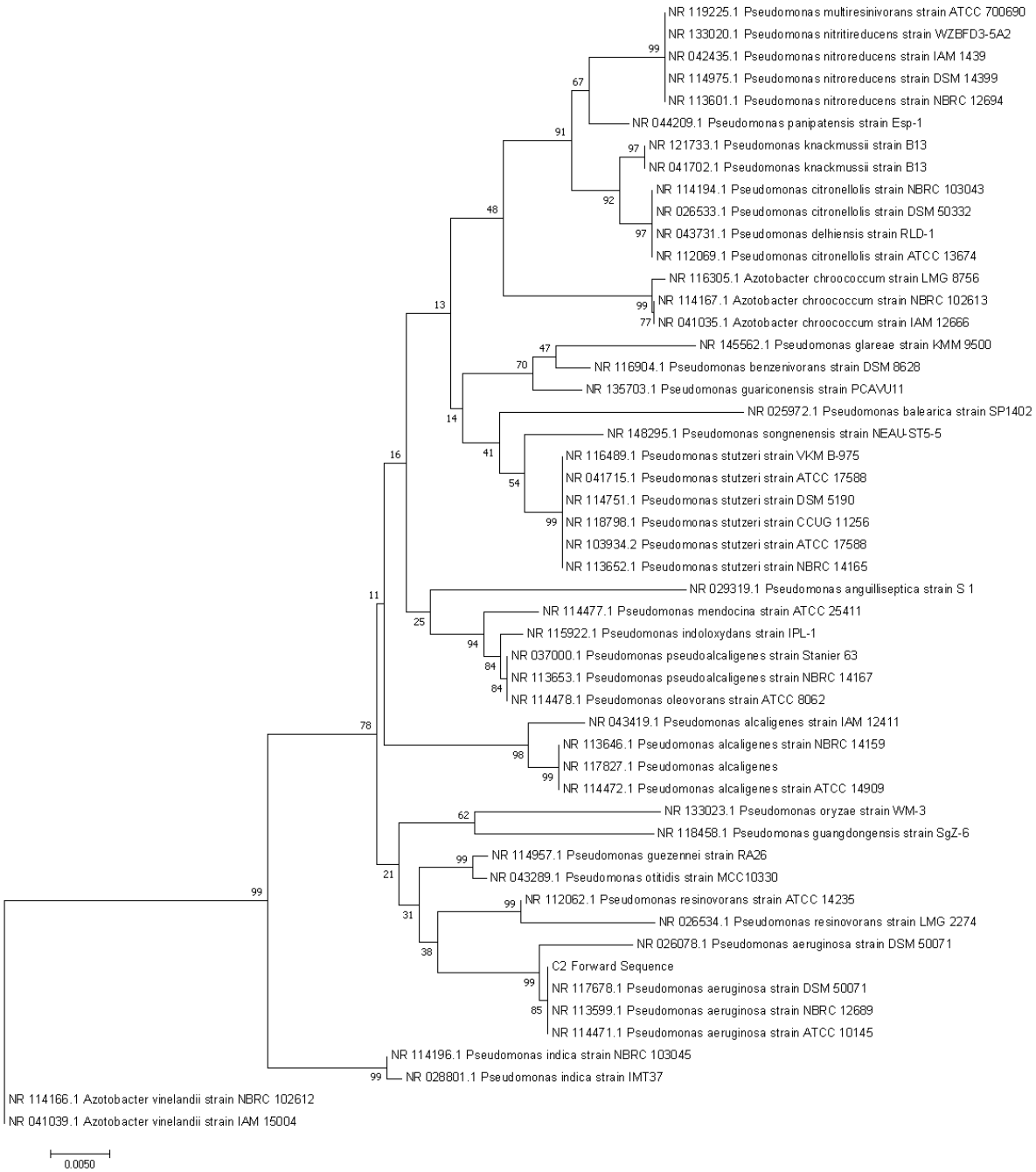


Figure 4.6: The evolutionary history of the sample was deduced by means of the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to characterize the evolutionary history of the taxa analyzed. Branches correlating to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum

Composite Likelihood method (Tamura & Nei, 2004) and are in the units of the number of base substitutions per site. The analysis involved a total of 51 nucleotide sequences. All positions which contained gaps and missing data were eliminated. There were a total of 801 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

4.7 Discussion

With progress of time, huge quantities of Chromium is being discharged into water bodies due to its passive utilization in industries such as, leather tanneries, textile, generation of metallic based bodies, production of colored dyes and so forth. As stated earlier, Cr (VI) is to a great extent a carcinogen and further exhibits mutagenic activities on any organic and cellular framework due to its nature of oxidizing molecules upon contact. Therefore, bacteria, in its natural ability to develop resistance to foreign particles, have developed and demonstrated different mechanisms of diminishment and resistance abilities in order to tolerate and survive the toxic nature of chromate.

This researched focused on isolation of unique strains of bacteria which were capable of showing resistance to Chromium and further possessed the ability to reduce the lethal effects of the cancer-causing Chromium effectively.

Analysis was carried on for the isolate C_2 at various parameters. Graphs were plotted upon obtaining data to utilize the outcomes. Upon investigation of all data, it was observed that the isolate C_2 developed resistance and was further capable at reducing Chromium concentration at numerous values of temperature and pH. The isolate showed complete reduction of hexavalent Chromium at 37ºC and pH 5.5, 7.0 and 8.5 followed on by a 90.4 % reduction at 42ºC in the pH of 7. Therefore, amongst all proposed values of temperature and pH, the isolate C_2 showed optimal activity at 37ºC, pH 5.5, 7.0 and 8.5 due to its ability to reduce Chromium by 100%.

For analyzing the antibiotic resistance capabilities of the isolate, it was observed that C_2 developed significant resistance against to Kanamycin, Trimethoprim, Amoxycillin and Cefuroxime Sodium. Ofloxacin, Neomycin, Ceftriaxone, Azithromycin and Cefuroxime Sodium. However, the greatest resistance by the isolate was shown against Penicillin and Vancomycin. On the contrary, the isolate demonstrated greatest susceptibility towards

Ceftriaxone. It also moderately susceptible towards Neomycin, Gentamycin and Ciprofloxacin.

Following on, the Minimum Inhibitory Concentration (MIC) test for the isolate C_2 against Chromium was analyzed. It was observed that the isolate tolerated the concentration of Chromium up to a maximum value of 16 mM. But no colony was found in the plates containing concentrations beyond 17mM. Therefore, the Minimum Inhibitory Concentration for this isolate was 17 mM.

Therefore, it can be said that microorganisms which show resistance towards Chromium are indeed reside in Chromium contaminated regions; and not only are they persistent in their survival under such circumstances; they have further developed the ability to reduce chromate itself in the aerobic situation they habituate in. This methodology enables genetically modified bacterial strains as such to develop biotechnological and environmental significance and become the ultimate solution to having an environment-one that is free from the burden of contaminated Chromium and other heavy metal waste effluents.

The phylogenetic tree in Fig 4.7 consists of 51 taxa, including the isolate C_2 and a total of 10 clades. In the tree, there are several outgroups, i.e. taxon outside the group of interest, for example, *Pseudomonas indica strain* and *Pseudomonas oleovorans strain,* since their lineage clade is located distantly from the clade containing the isolate C_2. The ancestor to all current bacterial generation is the *Azotobacter vinelandii strain*. The most recent generation of bacterial specie is the 10^{th} speciation, consisting of *Pseudomonas multiresinovorans strain*, *Pseudomonas nitroreducens strain* and *Pseudomonas stutzeri strain.*

The isolate C_2 is of the 8^{th} speciation and has a sister taxon of *Pseudomonas Mendocina strain*. However, C_2 has an average nucleotide substitution of 0.046 bases in its DNA sequence while *Pseudomonas mendocina* have 0.045 nucleotide substitutions in its genome, which led to their unique diversification. Over time, the isolate C_2 has evolved from *Azotobacter vinelandii strain* by an average substitution of 0.054 nucleotide in its DNA; and this rendered its tolerance and reduction capacity towards Cr^{6+}.

Since C_2 and *Pseudomonas aeruginosa* have a common node, it can be deduced that the genome of C_2 is most similar to *Pseudomonas aeruginosa*, amongst all other sequences. Therefore, C_2 was identified as *Pseudomonas aeruginosa*.

Finally, 16s rDNA sequencing was done to identify the isolated strains of bacteria.

Chapter 5: Conclusion

5.1 Conclusion

From the results of this investigation, we can assert that microorganisms habituating in a secluded manner amongst a heavy metal contaminated environment, standout as the potential bearers, capable of being persistent in surviving the toxic nature of hexavalent Chromium. These secluded microorganisms further possess a remarkable potential of reducing Chromium biologically. The rate at which Chromium concentration is reduced relies upon the time of incubation of the bacterial isolate of interest and further depends on the temperature and pH at which it functions optimally. As a consequence, the Chromium reductase enzyme released by the isolate can be utilized as a potential source for chemotherapy, upon conduction of research in the future. In addition, the bacteria would be the solution to reducing and ultimately cleansing the water body of the Buriganga River plagued with accumulated Chromate and other associated heavy metals. The data gathered through this research revealed that, the secluded strain C_2 was ready to be put to use; in association with all different microbes that were discovered by other distinct scientists and research works for relieving the toxic effects of Chromium from contaminated water.

5.2 Future Directions

In the future, investigation can be conducted to study the correlation between resistance profile of antibiotics and Chromium reduction assay. Additional studies include:

1) Investigation on cell free extract to help to interpret the mechanisms responsible for the reduction of Chromium.
2) Analyze whether the isolate C_2 can be a potential source for Chromium reductase enzyme. Upon investigating the synthesis of Chromium reductase enzyme, the exogenous or endogenous enzymes responsible for the reduction can be determined, examined further upon and ultimately isolated to enable its potential activities for heavy metal reduction.
3) Utilize the reductive capabilities of *Pseudomonas aeruginosa* technological and healthcare sectors.

Chapter Six: Reference

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Chapter Six: Appendix

A. 16S rDNA sequence of sample C_2:

CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGA
AACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCG
GACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAA
AGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
ATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAG
GTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATA
CCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCA
GCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAAC
CTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGG
AATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGC
GAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAG
CCGTTGG

B. 16S rDNA sequence of Isolate C_2 from Finch TV