Isolation and Characterization of Carcinogenic Chromium Reducing *Bacillus cereus* from Soil of Buriganga River

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A project submitted to the Department of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Honours)

Department of Pharmacy

Brac University
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Declaration

It is hereby declared that

1. The project submitted is my own original work while completing degree at Brac University.
2. The project does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The project does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The project titled "Isolation and Characterization of Carcinogenic Chromium Reducing *Bacillus cereus* from Soil of Buriganga River" submitted by
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of Summer,2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of pharmacy (Honours) on 19th August, 2019

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Abstract

Hexavalent Chromium has mutagenic and carcinogenic property and it causes different diseases. In many industries, it is used and often thrown to environment without proper treatment. To protect the environment and human health, a bacterial strain *Bacillus cereus* was identified which has the ability to reduce hexavalent chromium to trivalent chromium. Sample was collected from Buriganga River. Nutrient agar media with Potassium chromate (K_2CrO_4) solution was used for selection of desired strain. 16s rDNA sequencing method was followed for identification of bacterial strain. Diphenyl Carbazide base bioremediation assay was performed at 25ºC, 37ºC, 42ºC and pH 5.5, 7, and 8.5. Optimum temperature and pH for reduction of chromium for the bacterial strain was found 37ºC and pH 8.5. MIC of the bacterial strain was found 37mM at K_2CrO_4. *Bacillus cereus* shows resistance to 4 antibiotics, Cefexime (CFM5), Penicillin (P10), Sulphamethoxazole (STX25), and Amoxicillin (AML10).

Keywords: Chromium; Bioremediation; MIC; DPCZ; Cancer.

This work is dedicated to my parents for their unconditional love and continuous support.

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List of Acronyms

BLAST	Basic Local Alignment Search Tool
Cr	Chromium
Conc.	Concentration
Hrs	Hours
DPCZ	Diphenyl Carbazide
IARC	International Agency for Research on Cancer
Kg	Kilogram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
mL	Milliliter
mM	Millimolar
MOPS	3-(n-Morpholino)Propanesulfonic Acid
NA	Nutrient Agar
NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth
Nm	Nanometer
O.D.	Optical density
Ppm	Parts per million
ROS	Reactive oxygen species
RPM	Revolution per minute
rRNA	Ribosomal ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
WHO	World Health Organization
ZI	Zone of Inhibition
µM	micro molar
µg	Micro gram
PCR	Polymerase Chain Reaction

Chapter 1

Introduction

1.1 Background

The elements which have atomic number in between 63-200 and also have density 5 times greater than water are called heavy metal (M. F. Malik, Shah, & Azzam, 2017). Heavy metals are the most toxic inorganic element present in the environment. Harmful effects of some heavy metals are given below.

Table 1: Harmful effects of different metals

Metal	**Harmful Effects**
As	It interferes with some cellular processes like synthesis of ATP.
Hg	It causes autoimmune diseases, depression, condition of drowsiness, severe fatigue, loss of hair, insomnia, disturbed vision, memory loss, tremors, and loss of temper, damage to lungs, kidney failure, brain damage and restlessness.
Pb	Affects and damages our central nervous system, reproductive system, kidneys, process of circulation. In children, it causes impaired development, lower the intelligence, short-term

	loss of memory, decreases learning abilities and acts as a cardiovascular risk factor as well.
Cd	It causes cancer, mutations, disrupts endocrine functions, causes damage to lungs, weaken the bones and interferes with the regulation of Ca- ions within the biological systems.
Cr	Carcinogenic, short time exposure results in irritation, sickness, ulceration where as long term exposure can affect nervous system, circulatory system, liver and kidney. It causes hair loss as well.

Chromium is one of these heavy metals. It was discovered in 1797 by Vauquelin as a compound of lead ore (Baruthio, 1992). And after that, very soon, it was used for many industrial purposes like as dye, pigments, leather industries, Chromium plating etc. Not only that, chemical industries also used Chromium heavily as catalyst, wood preservative, corrosion inhibitor, fungicides and many others reasons. When the applications of Chromium was increasing, their effects on health was discovering. People knew about their toxic effect before the biological functions have been discovered. In 1957, Schwarz and Mertz discovered the biological function of Chromium (Baruthio, 1992). Hexavalent compounds are more toxic than di- and trivalent compounds of Chromium. Hexavalent compounds are corrosive and cause irritation. They can easily go through lungs, skin and gastro-intestinal track. Hexavalent Chromium also has mutagenic property and thus they cause DNA damage which results in cancer (M. F. Malik et al., 2017).

The term bioremediation is attributed to a natural process which involve biological agents, predominantly microorganisms (bacteria, yeast, and fungi), algae and plants to decrease metal contamination, for the elimination of toxic wastes and to reduce environmental pollution. Microorganisms are either present in the affected site or are isolated from elsewhere and injected in the site to reduce the toxicity of heavy metals (M. F. Malik et al., 2017).

The current research will give us an opportunity to find out a bacterium which will convert hexavalent Chromium into bivalent Chromium and thus will reduce the chances of occurrence of cancer as well as will reduce Chromium contamination from environment.

1.2 Methodology

For the identification of the isolated culture, biochemical tests were performed. Morphological imaging with microscope was performed during the study.

Most important task of the study was to search for a species of bacteria that has resistency to hexavalent (VI) Cr that is in the culture. To find out our desired species, we have conducted our experiments to find out chromium (VI) resistance bacteria in a controlled way. In these experiments, we have used nutrient broth media with which chromium solution was mixed. Isolation process was done repeatedly until pure and unadulterated colony of desired strain was found.

1.3 Aim of the Study

The main objective of the investigation is to find out a bacteria strain that is resistant to chromium. These bacteria will help us to reduce chromium pollution in environment also it will reduce the possibility of cancer. Moreover, to find out a source of reductase (Chromium) enzyme for further use. Tests that were performed are given bellow:

- Finding of bacteria strain in chromium containing medium
- Determination of MIC (Minimum inhibitory concentration) to identify how much chromium can be tolerate by bacteria.
- Finding antibiotic resistance of desired bacteria strain
- Buildup of phylogenic tree
- Evaluation of endo or exo enzyme

1.4 Literature Review

1.4.1 Introduction

Chromium is an element with atomic number 24 and symbol Cr. Color of chromium is silvery grey which is hard and brittle and it is also a transition metal. Its melting point is 1907ºC and boiling point is 2671ºC. It is 7^{th} most abundant element and 21^{st} most abundant metal of earth's crust (Sinha, Saxena, & Singh, 2005). It is one of the 18 core hazardous element of air pollution and ranked 7^{th} in 20 most hazardous substances by the agency for Toxic substances and Disease Registry (Oh, Song, Shin, Choi, & Kim, 2007). It is also categorized as number 1 carcinogenic agent by IARC (International Agency for Research on Cancer). Thus, this metal needs deep

monitoring about its harmful effects and necessary steps should be taken to reduce the loss. Because of many amount of natural and anthropogenic activity of chromium contamination in water and soil, it has now become a burning question. Chromium's role in environment pollution is now known to all. Chromium enters in human body through food and these foods are being contaminated throng soil contamination with chromium. These causes various health risk. There are many mechanism of entering chromium in plant body and many factors are controlling them (Broadway et al., 2010). Chromium was first invented in 1997 in mineral crocoite ($PbCrO_4$). It was then used as coloring agent because of its color. The name chromium has been derived from Greek word Chroma which means color. Chromium can be found naturally in the environment (rocks, soil, volcanic dust and gases, water). Naturally Cr is found as chromite ($FeCr_2O_4$) in serpentine or ultramafic rocks or as a constituent of vauquelinite ($CuPb_2CrO_4$-PO_4OH), tarapacaite (K_2CrO_4), bentorite ($Ca_6(CrAl)_2(SO_4)_3$) and crocoite ($PbCrO_4$).It can stay as original minerals\ metal or can also be stayed with formation of compound with manganese (Mn) , Aluminum(Al), Iron (Fe) oxides and hydroxides which makes compounds with soil particles or soil organic compounds and thus causes contamination (Hsu, Liu, & Tzou, 2015). Natural level of chromium in earth crust ranges between 0.1-0.3 mg\kg. Different studies have found different level of Chromium concentration in soil of different geographical regions. The studies that have been found is shown below:

Table 2: Natural and average concentration of chromium in soil

Serial	**Chromium concentration(mg\Kg)**	**Parameter**	**Place**
1	500-600	Background Concentration	-
2	5-3000	Background Concentration	India
3	2-60	Natural concentration	Turkey
4	10-50	Natural concentration	-
5	100	Average concentration	West indies
6	59.5	Average Concentration	Poland
7	22	Average Concentration	Sweden
8	58	Average Concentration	Japan
9	54	Average Concentration	USA
10	94.8	Average Concentration	Finland

Average Chromium level ranges from 50-100 mg\kg which varies from properties of soil like amount of clay and etc. Chromium concentration in fresh water ranges from 0.1-117 mg\kg. Sea water contains chromium in a level of 0.2-50 mg\L. amount of chromium in air sample in greater in urban area than remote area (Shahid et al., 2017)

1.4.2 Chemistry

Chromium is lustrous brittle and hard metal. It is highly polished. When chromium in burn in presence of air, it forms green color chromic oxides. Chromium often reacts with oxygen and

forms a layer of oxide which protects the metal inside. The name chromium was derived from Greek Chroma means color. Chromium compounds create color and that's why they are used in dye and painting.

Table 3: Properties of Chromium

Name	Chromium
Discovery Date	1797
Discovered by	Nicholas Louis Vauquelin
Origin of the Name	Greek word Chroma
Atomic Number	24
Valences	1-6
Group	6
Period	4
Block	d
Atomic number	24
State in room temperature	Solid
Electron configuration	$[Ar]3d^5 4s^1$
Melting point	1907ºC 3465ºF 2180 k

Boiling point	2671ºC 4840ºF 2944 k
Density	7.15 gcm^{-3}
Relative atomic mass	51.996
Key isotope	^{52}Cr

1.4.3 Common sources of Chromium compounds

Chromium is found in nature usually in 2 valence sates. Trivalent Chromium (Cr III) and hexavalent chromium (Cr VI). This exposure occur because of natural or industrial source of chromium. Chromium III is more toxic than Chromium VI. Chromium toxicity damages respiratory track mostly. Chromium III is an essential element for our body. However, our body can detoxify very little amount of hexavalent chromium to trivalent chromium (Environmental Protection Agency, 2000). Trivalent chromium is mostly found in nature. Though hexavalent chromium is found, small in amount. The only ore of chromium is Chromite ($FeOCr_2O_3$) which contains significant amount of Chromium. It is not found in pure form rather 55% chromic oxide. Ferrochromium is mainly produced in Finland, Italy, Norway, and France. Potassium Chromate is mainly produced in Italy, Switzerland, Germany, and United Kingdom. The most important chromium product now are sodium chromate and dichromate which are used to produce chromic acid, chromium pigment and tanning and corrosion control. Level of chromium in soil varies for the concentration of chromium in soil for anthropogenic sources. Tests have shown concentration of chromium in soil from 1 to 1000 mg\kg with an average of 14-70 mg\kg. Organic matter can transform Chromium (VI) to Chromium (III). In chromium mining countries, airborne chromium is common there and toxicity is found (World Health Organization, 2000).

1.4.4 Uses of Chromium

I. Chromium is used to prevent erosion in metal and also for glossy finish.

- Chromium plating
- Amalgam constitute in stainless steel. 18% chromium is used to produce stainless steel.

II. Chromium is used in paints and dyes

- Green rouge is a metal shine of chromium (III) oxide.
- Chromium salt is used to produce green shaded glass.
- Chromium is responsible for the red color of ruby. So it is used to prepare synthetic ruby.
- Makes yellow paints.

III. It has catalytic ability

IV. In tannery industries, Chromium is used.

V. It is used for terminating of blocks.

VI. Potassium dichromate is used for washing of laboratory glassware.

VII. It is also used as an agent for titration.

VIII. For the production of magnetic tape.

IX. In pharmaceutical, Chromium (III) chloride is used as supplement

X. Chromium Hexacarbonyl is used with fuel.

XI. Cr is used as fixing agent.

1.4.5 Toxicity of chromium and its effects on health

Cr is necessary for both human and animal body. Due to deficiency of Chromium, many symptoms occur. WHO has been set up the scale of daily requirement of Chromium and that is 33µg\person (World Health Organization, 2000)

Table 4: Chromium toxicity around the world

Cr level in soil(mg/kg)	Studied site	Sources of Cr	Fold higher than MAL*
328,000	Soil near a tannery facility in Michigan	Tannery effluent	1307
44,615	Ranipet, Tamilnadu, India	Tamil Nadu Chromates and Chemicals Limited Factory	179
40,000	Sediments near a tannery facility in Michigan	Tannery effluent	159
16,291	Soil of Gujarat, India	Industrial landfill sites	65
5490	Ivano-Frankovsk, Ukraine	Leather tannery	22
5406	Faridabad, India	Industrial area	22
5000	Soil close to a wood preservation factory, Dartmouth, Canada	Industrial area	20
1509	Agricultural soil, India	Mining area	6
1501	Agricultural soil, Vietnam	Mining area	6
856	Mourikie Thivaarea, Greece	Parent material (carbonate rocks, ophiolites, shales, limestone)	3.4
850	Morón borough, Argentina	Industrial and urban area	3.4
692	Shenyang, China	Fertilizer plant site	2.8
630	Soil near a tannery, Pakistan	Tannery effluent	2.5
462.8	Soil near steel-alloy factory in Hunan, China	Steel-alloy factory	1.9
321	Baghejar Chromite Mine, Iran	Baghjar Chromite Mine	1.3
75,000 tons	Slags and sludge near Mexico City	industrial and urban waste dumping site	e
	* Maximum Allowable Level (MAL, 250 mg/kg)		

1.4.5.1 Effects on human

Subjects exposed to Chromium (VI) compounds showed different diseases like corrosive reaction non nasal septum, chrome ulcers and allergic dermatitis. Two third of subjects were affected by ulceration in nasal septum while exposed to chromium (VI) smoke. Effects of chromium compounds in different organs like kidneys, liver, airways of animal and human body has been studied systemically. In western world, the exposure of chromium is low. Thus the symptoms are not that much available. But these symptoms are still available in developing countries as workers are more exposed to chromium compounds. Ulceration in nasal septum is a mark of exposure to chromium smoke. Study has further found that, ingestion of chromium compound cause gastrointestinal bleeding from ulceration of intestinal mucosa which is associated with cardiovascular shock (mainly 70-80 years patients).if the patient survives, kidney or liver damage occurs. Systemic effects have been reported to occur in the airways. High amount of chromates cause necroses of proximal and distal tube. An experiment was done on mice to see the effect of chromium (VI) .The mice was injected with chromium (VI) and then glutathione was added. It was seen that, chromium did not harm the mice. A study was also conduced to find whether there is any relation between cancer and chromium. And the result is positive. This study was done on workers who are continuously exposed to chromium smoke. Then the data was collected which end up with the result of carcinogenicity of chromium (World Health Organization, 2000).

A. Effects on brain and respiratory track

Hyperplasia in bronchial epithelium and fibrosis are common to workers who are exposed to chromium smoke and chromate production because they are more prone to inhale the dust of chromium. The amount of the Chromium is delivered to brain very rapidly through olfactory and

terminal nerves (Salama, Hegazy, & Hassan, 2016). If chromium is inhaled, it causes different diseases like asthma, ulceration in nasal mucosa. (Services, 2012)

B. Effects of hexavalent Chromium on skin

As chromium has cytotoxic activity, it cause skin irritation to the people who are exposed to it (Bruynzeel, Hennipman, & van Ketel, 1988). In skin irritation and dermatitis, there are 2 mechanism involved:

-induction

-sensitization.

Penetration of Cr through skin occurs in induction. And immune response is involved in sensitization. A lot of symptoms are found. They are

- Cracking
- Swelling
- Rash (Environmental Protection Agency, 2000).

1.4.5.2 Effects on animal

As chromium exposure is increasing, the health of marine environment is at risk. Chromium enters in marine water by runoff of water and through the chromium contaminated river water also from deposition from the atmosphere. Cr (VI) shows acute toxicity in marine fish in the range of 20-90 mg\L. This toxicity depends on hardness of water, temperature, salinity of water. Cr (VI) also reduce disease resistance, altered enzyme effects and activity.

1.4.6 Carcinogenic property of Chromium

Chromium (VI) also acts as genotoxic compound. It damages DNA structure and causes mutation which results in cancer. However, Chromium (III) does not cause DNA damage and thus it is not mutagenic. Chromium (VI) binds with DNA, interfere with the base pair stacking and thus, cause mutation. The mechanism is given in following figure:

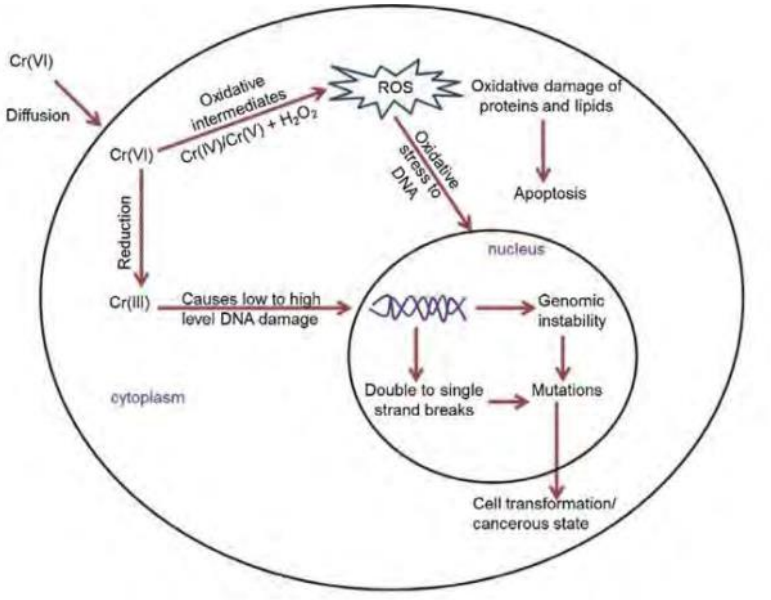

Fig 1: mechanism of chromium acts as genotoxic element (Ranasinghe et al., 2016)

1.4.7 Traditional methods for chromium reduction

Traditionally, different methods are used to reduce level of chromium in sample. They are:

- Molecule exchange
- Filtration
- Precipitation
- Adsorption
- Electro chemical treatment

- Chemical lessening
- Vanishing recovery
- Film developments

1.4.7.1 Electro chemical treatment

As this method extract metal which is present in water in ppm, this technique is famous.The inventors of this technique are Polprasert and Kongsricharroern. They invented it in 1995. The range of chromium that can be removed by this method is 0.2-3860 mg/L. this test requires development of various chemicals. Due to low pH and different salts, this method is impacted.

1.4.7.2 Exchange of Ion

Methodologies that are used to remove chromium from water, exchange of ion is one of them and it is very popular. Insoluble stock material make the particles unstock in this method. The mechanism of this method is, from one side, chromium is entered and then it passes resin bed and thus chromium is removed. For the exchange of ion, ion exchange resin is used. Resigns are brought out from the system in which chromium is complexed. Resin that are chosen must have the ability to supply sufficient ion to the metal which will be removed. Or, Cr will not be removed perfectly and for this reason, we have to run the test again which will increase cost. The main drawbacks of this method is, it cannot purify concentrated solution. Because, ion exchange resin is fragile to pH.

1.4.7.3 Biosorption

Another popular method of removal of chromium from waste water is biosorption. For the removal of chromium from water, adsorption tools were got farming waste. Metal biosorption is a complex

process and a number of variables can affect it. System required in the biosorption method fuse chemisorption, incorporate complexation, micro precipitation, ion exchange, adsorption–complexation on pores and 17 surface significant metal hydroxide development onto the surface adsorption and bio surface (Gardea-Torresdey, Peralta-Videa, De La Rosa, & Parsons, 2005).

1.4.7.4 Adsorption using activated carbon

There are a lot of things which can be used as the sources of activated carbon. They are

- Sawdust
- Nutshell
- Coconut shell

If we decrease the particle size of adsorbents, surface area will be increased and thus it will reduce more Cr (VI). It has also been found that, chromium adsorption is more in high temperature. This adsorption is dependent on 2 things, presence of salt and the pH of the solution. Nuts of *Terminalia arjuna* few activated carbons were found using Zn at pH 1. (Mohanty, Das, & Biswas, 2005).

1.4.7.5 Membrane filtration

A noteworthy consideration has been achieved by membrane filtration for the waste water treatment. Through this method, chromium is separated by a semipermeable membrane which is impermeable for chromium but permeable for water. Hydraulic pressure of water is used in this method for separation of chromium. Different membranes are used in this method. For example:

- Polymeric
- Fluid films
- Inorganic (Pugazhenthi & Kumar, 2005)

1.4.8 Mechanism of Metal resistance

Metals are very much available in environment and it causes metal resistance in microbes. Metal resistance of microorganism is heterogeneous. There are five mechanisms through which bacteria form heavy metal resistance. They are given below

- Segregation from the cell by active export
- Permeability barrier by segregation of metals
- Repossession of extracellular components
- Metal binding with protein and saves metal sensitive cellular organisms
- When amount of metal is less, resistance occurs through purification

1.4.9 Cellular components which are metal sensitive

Performance of enzymes are regulated by metal. They change enzyme specificity by making many changes, altering active site and transport structure. Metal ion can decrease the synthesis of DNA and also can hamper the structure of DNA. Many cellular components are potential targets of metal induced damage. Heavy metals can bind with proteins and inhibit their biological activities. For example, yeast L-glutamine inhibits by Methylmercury. The mechanism has not been discovered yet. Heavy metals are also responsible for misfolded protein. As the concentration of heavy metal increases, the more necessity of protection for cellular components is required. In E. coli, the production of major proteins can be stopped by increase the concentration of metal (Tamás, Sharma, Ibstedt, Jacobson, & Christen, 2014).

1.4.10 Uptake system of metal resistance

Resistance mechanism is affected by the metal uptake system in metal sensitive cells. Inorganic membrane contains lipid component which is impermeable to hydrophilic ions. Thorough the less resistant places, metal passes. For instance, Cobalt, arsenate enter through the phosphate transport system in E. coli (Thatoi, Das, Mishra, & Prasad, 2014).

1.4.11 Metal as biological requirement

To maintain the metabolic activity in bacteria, heavy metal is a must. Many of them require Copper, Iron and nickel. And many of them require Molybdenum, Tungsten. Bacteria nee it in a very less amount and that's why it is not that much toxic for them (Hughes & Poole, 2009).

1.4.12 Cr (VI) reduction in microbes

Hexavalent chromium is a contaminant of ground water, it is toxic, and also carcinogenic. The physical and chemical properties of Cr (VI) vary from acidic to basic chromite. By doing various treatment, Cr (VI) is converted to Cr(III) which is not harmful. It is reduced as following:

$$(CrO_4)^{2-} + 8H^+ + 3e^- = Cr3^+ + 4H_2O$$

This reduction depends on various properties like existence of proton and pH of waste water. Nernst equation is useful here to find out the concentration of reactants.

$$E' = E'_0 - \frac{RT}{nF} \times \ln \frac{[Cr^{3+}]}{[(CrO_4)^{2-}]\,[H^+]^8}$$

Here, E'_o is potential, T is temperature in kelvin (K), R is molar gas constant which is 8.31447 j mol^{-1} k^{-1} , n is the number of electron exchanged , F represents Faraday's constant. (96,485.3 C mol^{-1}). Thus, it is seen that, the lower the concentration of proton, the higher the pH and decreased potential of couple.

Chapter 2

Materials and method

2.1 Introduction

Here, the procedure of experiment, equipment that have been used throughout experiment will be discussed. The section will provide detailed explanation starting from collection of the sample, isolation of bacteria to different tests that were carried out and also the identification of the sample on which the paper is designed on. Water and soil samples are collected from different area of Dhaka city. Pargandaria, Swarighat, Faridabad. Several studies like antimicrobial resistance profiling, minimum inhibitory concentration (MIC) and DPCZ based Chromium reduction bioassay of the isolated bacteria from collected sample were carried out those of which are discussed in this section.

2.2 Chemicals

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

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

2.3. Glassware and instruments

The following tabulated instruments and glassware was used throughout the entire time of study:

Table 5: Instruments used along with their function

Name of instrument and glassware	Function	Origin	Model
Autoclave machine	Sterilization	Vision Scientific, Korea	VS-1221
Incubator	Incubation of solid culture mediums (agar plates)	Thermo Scientific, Germany	Heratherm IGS60
Digital Shaking incubator	Incubation of liquid culture mediums (broth)	OVAN, Spain	I10-OE+OL30-ME
Electric balance	Measurement of weight	CSC Balance, Japan	JT2003D
pH meter	Measurement of pH	Mettler Toledo, Switzerland	S220
Laminar Air Flow	Maintenance of aseptic environment	Labtech, Korea	LCB-1101VE
Water Distillation Apparatus	Preparation of culture stock	BOECO,Germany	BOE-8704000
UV-Vis Spectrophptometer	Measurement of absorbance pertaining to the bacterial growth and reduction of hexavalent Chromium	Shimadzu, Japan	UV- 1800

Electronic Centrifuge	Collection of supernatant	China	80-2
Micropipette	For withdrawing reagent and media in minute amount	Eppendorf	N32164B, M34137B
SIEMENS Up-ride Freezer	For storing bacterial culture stock	SIEMENS, Germany	GS36NVW30G
Vortex Mixer	For proper mixing during serial dilution in MIC	Hwashintech, Korea	260VM

2.4 Sample collection

Dhaka, located on the bank of river Buriganga which is considered as the most polluted river in the country. A lot of industries have been established around the city and the number is increasing day by day.This is the reason of discharge of untreated water into the river which is increasing in an alarming rate(Kawser Ahmed et al., 2016). This led to absolute deterioration of the quality of water of Buriganga River. The main problem is the dumping of enormous volume of toxic waste into the river on a continuous basis. This amount increases with increase in population each year. The contaminants include heavy metals, glass, textiles, polythene materials, cardboard etc. The elevated usage of heavy metals in industries has led to the elevated release of dangerous heavy metals into the river water. Metals and metalloids enter into the aquatic environment where they impose a grave threat to humans and ecological health, due to their toxicity, extensive persistence, bioaccumulation and biomagnifications in the food chain (Kawser Ahmed et al., 2016). In order to conduct experiment, we collected our samples from Buriganga River from three different locations: Showari ghat, Pargandaria and Faridabadh, GPS coordinates of those areas are listed in

the table 1.2 below. Water samples were collected from all three of the location and soil sample was collected from Showari ghat and Pargandaria. Isolate G2 was collected from Showari ghat with coordinates of 23°42’29.32” N 90°23, 31.65” E.

Table 6: GPS coordinates of the sample collection area

	Area	GPS Coordinates	Sample collected
1.	Showari Ghat	23°42’29.32”N 90°23,31.65” E	soil & water
2.	Faridabad	23°41’44.19”N 90°25’17.63”E	water
3.	Par Gandaria	23°41’48.01”N 90°25’11.67”E	soil & water

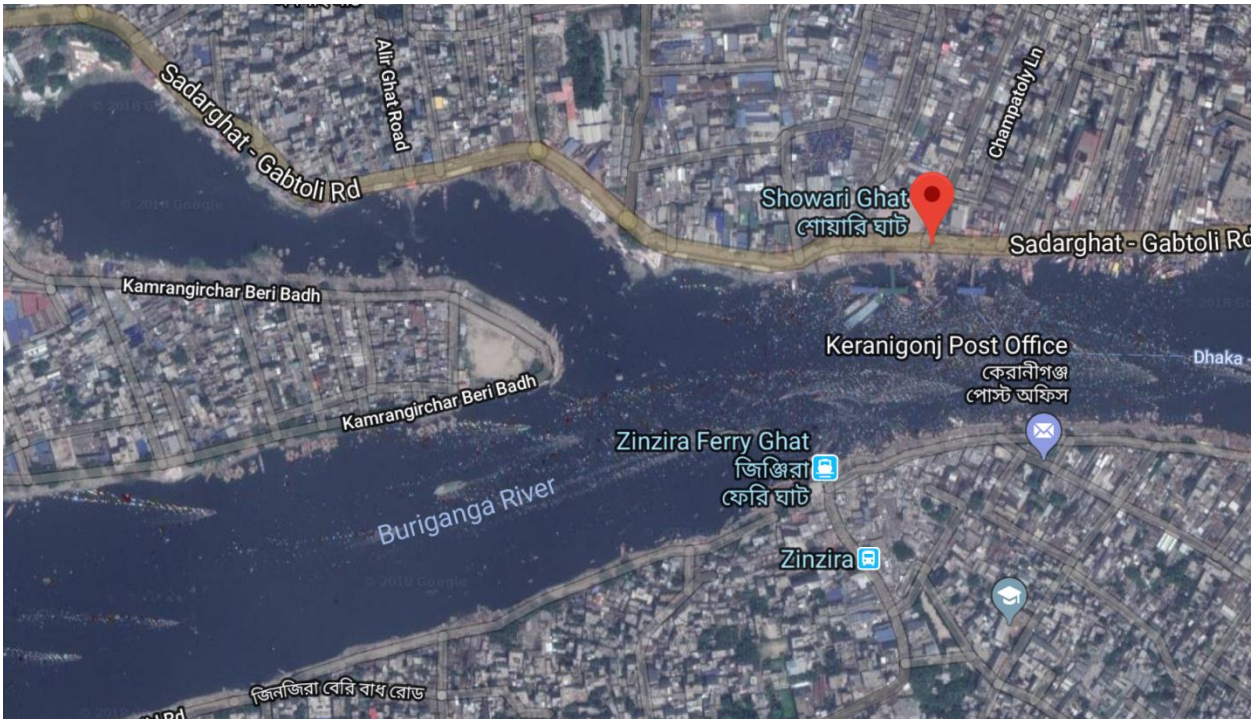

Figure 2: Showari Ghat area

2.5 Isolation and subculture of sample

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

24hours later, agar medium was prepared by mixing 2.8 gm NA in 100 ml water. Autoclave was run for 48 minutes and the temperature was 121°C and pressure was 15Lb. then, potassium chromate was added to the plates. Through sterilized toothpick, bacterial colony was transferred to a media with hexavalent chromium. Then, the media was incubated for 24 hours and the temperature was 37°C. Strains that survived were chosen for further experiments. After the investigation, 14 colonies were found which have chromium reductase enzyme. Isolated names were given like A, B, C, D etc.

2.6 Stock Culture Preparation

50% glycerol was produced by adding 50 mL of water in 50mL of glycerin. It was then autoclaved at 121°C and for 45 minutes pressure was 15 Lb. This ensured no place for cross contamination.

The eppendorff tubes in which the stock was to be stored were also autoclaved to ensure no presence of microbes in them.

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

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

2.7 Reduction profile of Chromium resistant bacteria

For the identification of amount of Cr (VI), DPCZ was used. (Greenberg, et al., 1992) and results were kept. A standard curve was prepared to compare the performance of microbes.

2.7.1 Chemicals preparation

2.7.1.1 10mL 3M H_2SO_4 preparation

In autoclaved falcon tube, 8mL distilled water and 1670µL conc. H_2SO_4 was mixed dropwise to prepare 10mL 3M H_2SO_4. The transfer was done by making use of a micropipette. Then, 330µL distilled water was added to make the volume of 10mL.

2.7.1.2 Diphenyl Carbazide Preparation

Diphenyl Carbazide was prepared by addition of 0.025gm of DPCZ and 9.67 ml of distilled water. Then, 9.67mL of acetone is added to it followed by 330µL of 3M sulfuric acid. The test tube was

shaken to give a uniform solution of DPCZ. Since DPCZ is light sensitive, it was wrapped in aluminum foil to protect it from exposure to light.

2.7.1.3 Preparation of MOPS buffer

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

2.7.1.4 10mL 5mM Potassium Chromate preparation

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

2.7.2 Experiment Procedure

2.7.2.1 Standard Curve preparation

A. Preparation of sample for reaction

Solution of different concentrations were prepared and the volume was milliliter.

Table 7: Preparation of sample for standard curve

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

B. Reaction protocol for standard curve

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

2.7.2.2 Reduction profile

A. Process

Day 0:

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

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

Day 1:

To prepare 600µL potassium chromate (K_2CrO_4), 15µL K_2CrO_4 was pipetted all conical flasks containing 25mL of nutrient broth. At first, 2mL of sample was withdrawn into a falcon tube from the 10mL conical flask which was kept for the incubation of bacteria the previous day. Absorbance of the 2mL sample was taken in UV-spectrometer at wavelength of 600nm. From the absorbance value, the amount of culture that has to be added to 25mL conical flasks containing nutrient broth

to get 0.2 OD (optical density) were calculated. Then the calculated amount was withdrawn from the 10ml culture into three falcon tubes and centrifuged for five minutes. This caused the bacteria to get settled at the bottom of the falcon tube leaving the supernatant at the top. The supernatant was withdrawn leaving the bacterial cells in the falcon tube. Again the same calculated amount of nutrient broth was withdrawn from one 25 ml nutrient solution and mixed with the cell in the falcon tube and vortexed to mix them properly. Then they are transferred into the three 25ml conical flask marked pH 5.5, 7 and 8.5 from the falcon tube. Rest 25 mL was blank. In 4 falcon tubes, 2 mL solution from each conical flask was withdrawn and kept. At 540 nm, UV reading was taken which means absorbance. This procedure is repeated throughout the day after every 1.5 hours for both the sample and the blank to see the bacterial growth for the positive control (sample) and negative control (without sample). After that, centrifugation was performed to collect supernatant like 2.7.2.1.2. 7 such readings were taken in every 90 minutes interval. Next day, the process was continued and solution was kept in shaking incubator. The whole procedure was repeated at three different temperatures: 25°C, 37°C and 42°C under three different pH conditions of 5.5, 7 and 8.5

2.8 Antibiotic resistance profiling of Chromium resistant bacteria

2.8.1 Strain culture preparation in nutrient broth (NB)

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

Murdoch, 2018) This ensures the amount of bacteria in each disc that were to be swapped by the suspension.

2.8.2 Test plates incubation

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

2.8.3 Application of antibiotic discs

A total of 14 antibiotic discs were applied on the inoculated plates; each plate containing 4 to 5 discs. Antibiotics discs that were used in this experiment are given below:

Table 8: Antibiotics used in antibiotic resistance profiling

Serial number	Antibiotics	Symbol	Strength
1.	Penicillin	(P10)	10mg/ml
2.	Kanamycin	(K30)	30 mg/ml
3.	Neomycin	(N30)	30mg/ml
4.	Vancomycin	(VA30)	30mg/ml
5.	Gentamycin	(CN10)	10mg/ml
6.	Cefixime	(CFM5)	5mg/ml
7.	Chloramphenicol	(C30)	30mg/ml
8.	Ceftriaxone	(CRO30)	30mg/ml
9.	Sulphamethoxazole	(STX25)	25mg/ml
10.	Cipropfloxacin	(CIP5)	5mg/ml
11.	Streptomycin	(S10)	10mg/ml
12.	Azithromycin	(AZM30)	30mg/ml

13.	Amoxycillin	(AML10)	10mg/ml
14.	Cefuroxime Sodium	(CXM30)	30mg/ml

2.8.4 Incubation

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

2.9 Minimum Inhibitory Concentration (MIC) determination

Minimum inhibitory concentration or MIC is the lowest concentration at which visible bacterial growth is inhibited. The test was carried out in nutrient broth media where different concentration of chromium solution was used (5-30mM). Before that, serial dilution of sample G2 was done in saline water. 50µL diluted bacteria was added to nutrient broth which was kept in shaking incubator for overnight. Next day, optical density @600nm of each test tube was measured. The lowest concentration in which no growth was found, was the minimum inhibitory concentration.

2.10 Method for 16s rDNA sequencing

First of all, DNA was extracted from sample G2. After that, DNA quantification was performed of the Nano drop of DNA. PCR was performed through gel electrophoresis and a fine band was found at 1500 base pair. After that, the PCR product was purified and sequencing was performed. Further analysis was performed to find the exact strain of G2 sample.

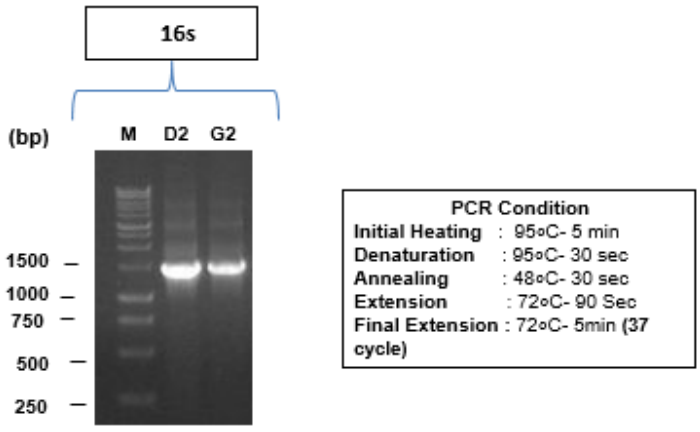

Figure 3: 16s profiles of 27 F and 1492 R primers generated from Bacteria

2.11 16S rDNA sequencing of sample G2

16s rDNA sequencing was performed to identify the bacterial strain that was isolated. The obtained sequence data was purified and saved as FASTA file. FinchTV was used to read chromatogram files. The file containing the sequence was simply draged and dropped in the interface. BLAST (Basic Local Alignment Search Tool) (Altschup, Gish, Pennsylvania, & Park, 1990) was performed of the purified sequence. Similar strains were identified and downloaded on account of similarity. The sequences were compiled in a single file in FASTA format and checked for any deletion using the software BioEdit. So, basically BioEdit is used for editing the sequences before construction of the phylogenetic tree. To study the evaluation of the strain, phylogenic tree was constructed. (Kumar, Stecher, & Tamura, 2016).

Chapter 3

Result and Discussion

3.1 Isolation data of Chromium resistant bacteria

Isolation was carried out for the individual colonies from the nutrient agar plates with Chromium. The isolate was labeled as G2 for our convenience.

3.2 Profile of Chromium reduction

3.2.1 Standard Curve

Method said in 2.7.2.1.1 was followed to construct the standard curve. Microsoft Excel Software was used to create the graph where values of absorbance were plotted against concentration of Chromium in micro mole. The results obtained are tabulated in Table 11 below:

Table 9: Concentration of Chromium with corresponding absorbance

Concentration µM	Absorbance @ 540nm
50	0.294
100	0.624
150	0.907
200	1.214
300	1.675
400	2.117
500	2.587
600	2.875

Standard curve was constructed from the data above.

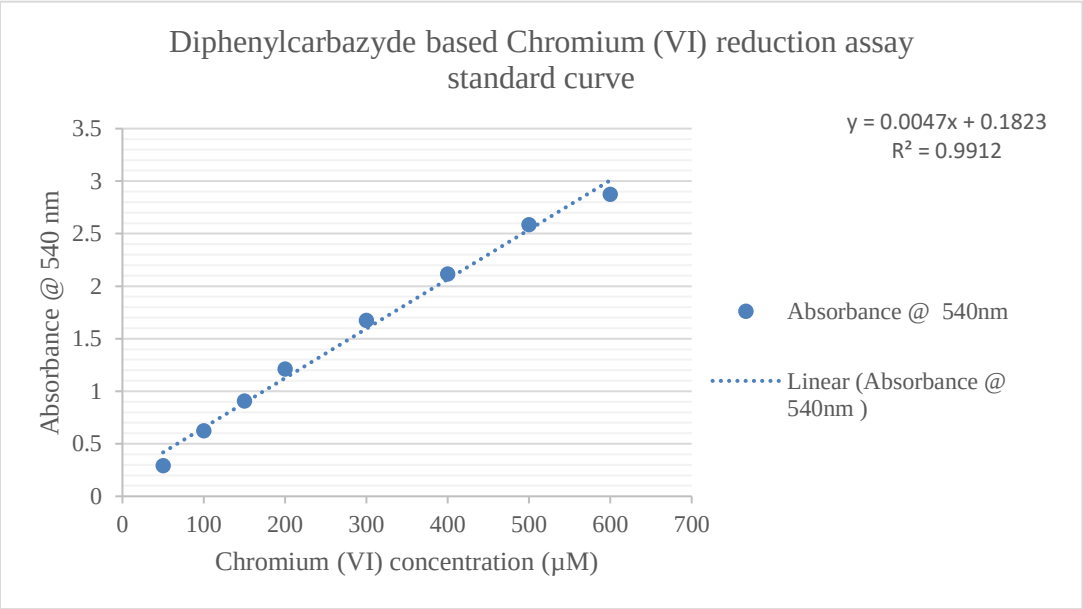

Figure 4: Standard curve of hexavalent Chromium with R^2 of 0.9912 showing high confidence level.

3.2.2 Chromium Reduction profile of G2

Three different temperature and pH was chosen for bioassay. The results of the experiment along with necessary graphs and tables are listed below in the subsections below.

3.2.2.1 Reduction profile at 25°C, pH 5.5

Table 10: Chromium reduction and cell growth at 25°C and pH 5.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	509.3687943	0.308	508.730496	0.007
1.5	533.6950355	0.278	532.2056738	0.003
3	495.3972	0.336	503.269504	0.003
4.5	506.7447	0.325	530.219858	0.001
6	491.9929	0.364	527.099291	0
7.5	483.3404	0.358	530.148936	0.002
24	419.5816	0.352	519.58156	0.006

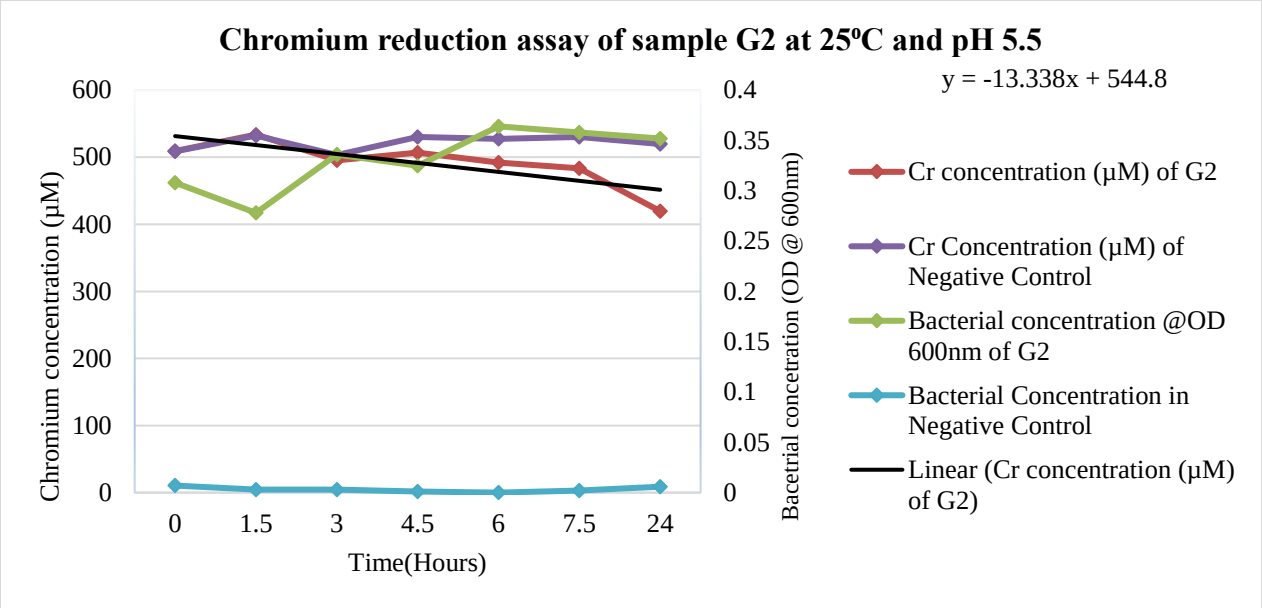

Figure 5: Bioremediation assay curve of sample G2 at 25 ºC & pH 5.5

From the figure above, it can be seen that only 17% Cr has been reduced and optical density of bacteria was 0.352 after 24 hours. It can be said that, in this temperature and pH, sample G2 could not survive much. The optical density had not raised after 24 hours. Thus, reduction of chromium was not satisfactory. No bacterial growth was observed in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.2 Reduction profile at 25°C, pH 7

Table 11: Chromium reduction and cell growth at 25°C and pH 7

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	529.0141844	0.527	508.730496	0.007
1.5	533.1985816	0.442	532.2056738	0.003
3	438.5177305	0.071	503.269504	0.003
4.5	380.0780142	1.04	530.219858	0.001
6	303.6950355	1.296	527.099291	0
7.5	230.787234	1.327	530.148936	0.002
24	77.45390071	1.554	519.58156	0.006

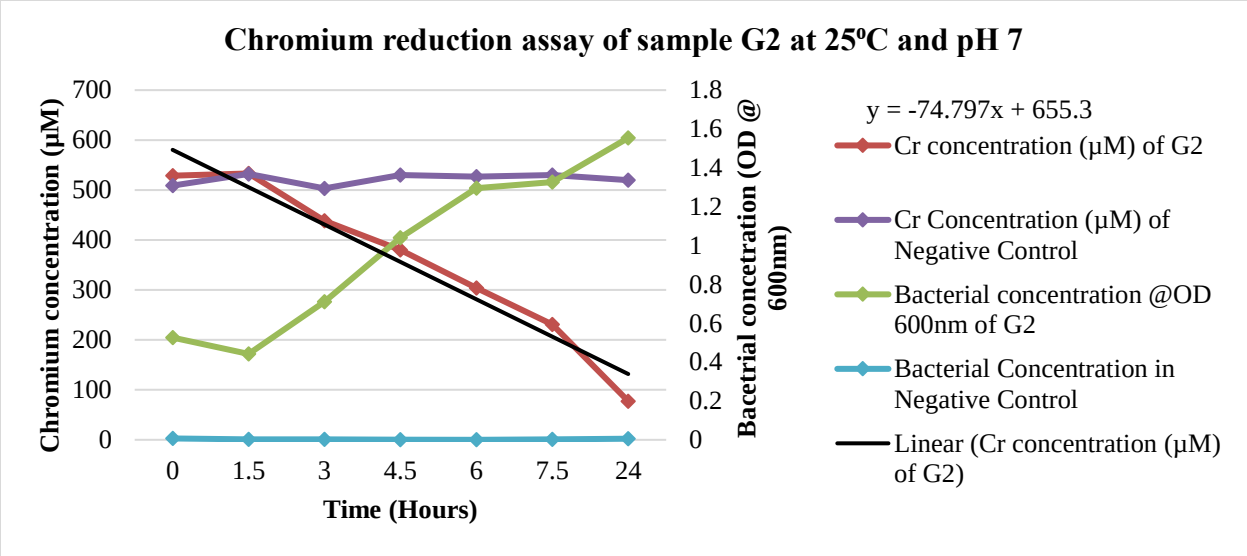

Figure 6: Bioremediation assay curve of sample G2 at 25 ºC & pH 7

From the figure above, it can be said that, the reduction of chromium at 25ºC and pH7 was satisfactory. Total 86% chromium was reduced. Optical density of G2 sample increased from 0.527-1.554. 57% chromium was reduced within 7.5 hours. No bacterial growth was observed in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.3 Reduction profile at 25°C, pH 8.5

Table 12: Chromium reduction and cell growth at 25°C and pH 8.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	465.964539	0.464	508.730496	0.007
1.5	481.141844	0.465	532.2056738	0.003
3	418.375887	0.837	503.269504	0.003
4.5	345.964539	1.297	530.219858	0.001
6	238.375887	1.772	527.099291	0
7.5	155.539007	1.982	530.148936	0.002
24	1.56737589	2.183	519.58156	0.006

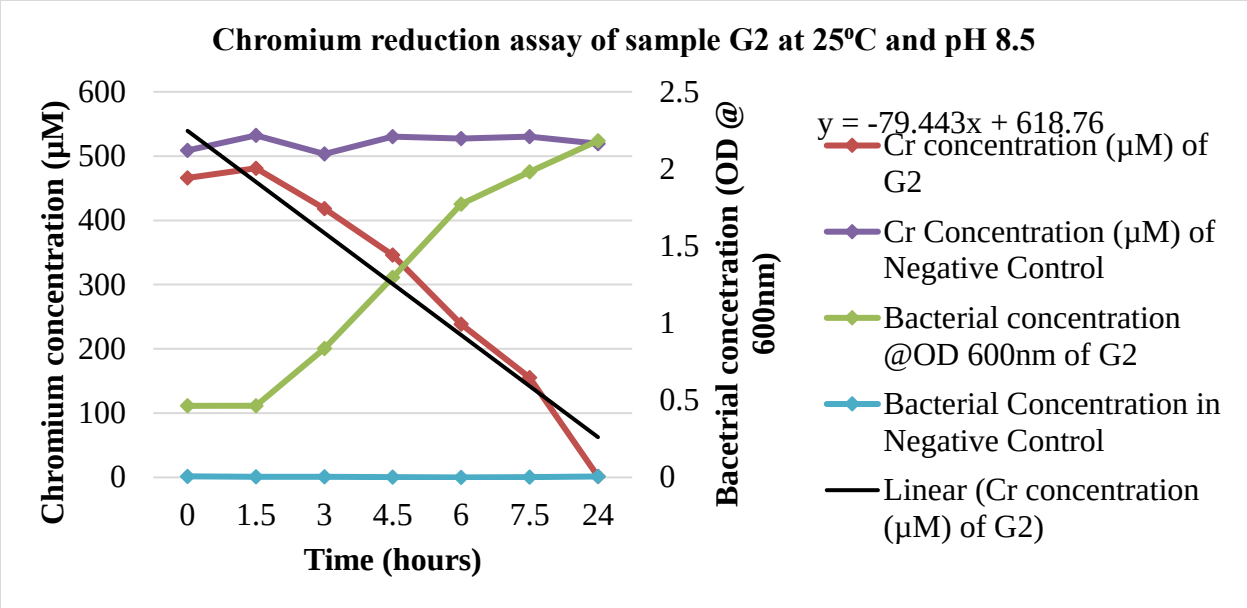

Figure 7: Bioremediation assay curve of sample G2 at 25 ºC & pH 8.5

From the figure, it can be said that 100% chromium was reduced after 24 hours. Optical density range was 0.464-2.183. 77% chromium was reduced after 7.5 hours. Thus it can be said that at 25^0C and pH 8.5, sample G2 gave the best reduction profile which is 100% reduction. No bacterial growth was observed in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.4 Reduction profile at 37°C, pH 5.5

Table 13: Chromium reduction and cell growth at 37°C, pH 5.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	566.9574468	0.43	541.212766	0.006
1.5	533.1276596	0.317	531.4964539	0.003
3	542.3475177	0.263	538.8014184	0.004
4.5	546.8865248	0.217	528.5177305	0.002
6	539.4397163	0.205	534.9716312	0.004
7.5	510.2907801	0.183	533.6950355	0.003
24	481.212766	0.156	531.6382979	0.002

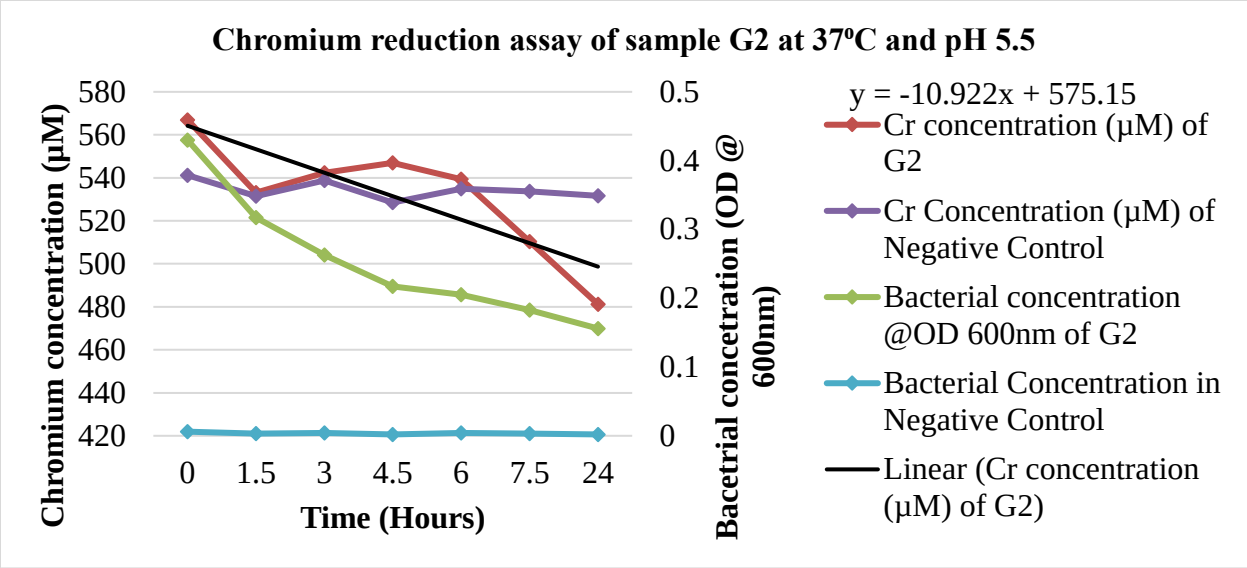

Figure 8: Bioremediation assay curve of sample G2 at 37 ºC & pH 5.5

From the figure above, it can be seen that, reduction of chromium was not satisfactory. Optical density of bacteria was reducing by the flow of time which means bacteria could not survive on this temperature and pH. For this reason only 16% chromium was reduced in 24 hours. No bacterial growth was observed in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.5 Reduction profile at 37°C, pH 7

Table 14: Chromium reduction and cell growth at 37°C, pH 7

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	572.9148936	0.404	541.212766	0.006
1.5	542.9858156	0.361	531.4964539	0.003
3	464.6170213	0.689	538.8014184	0.004
4.5	356.6028369	1.053	528.5177305	0.002
6	260.787234	1.22	534.9716312	0.004
7.5	154.4751773	1.537	533.6950355	0.003
24	-12.5460993	1.892	531.6382979	0.002

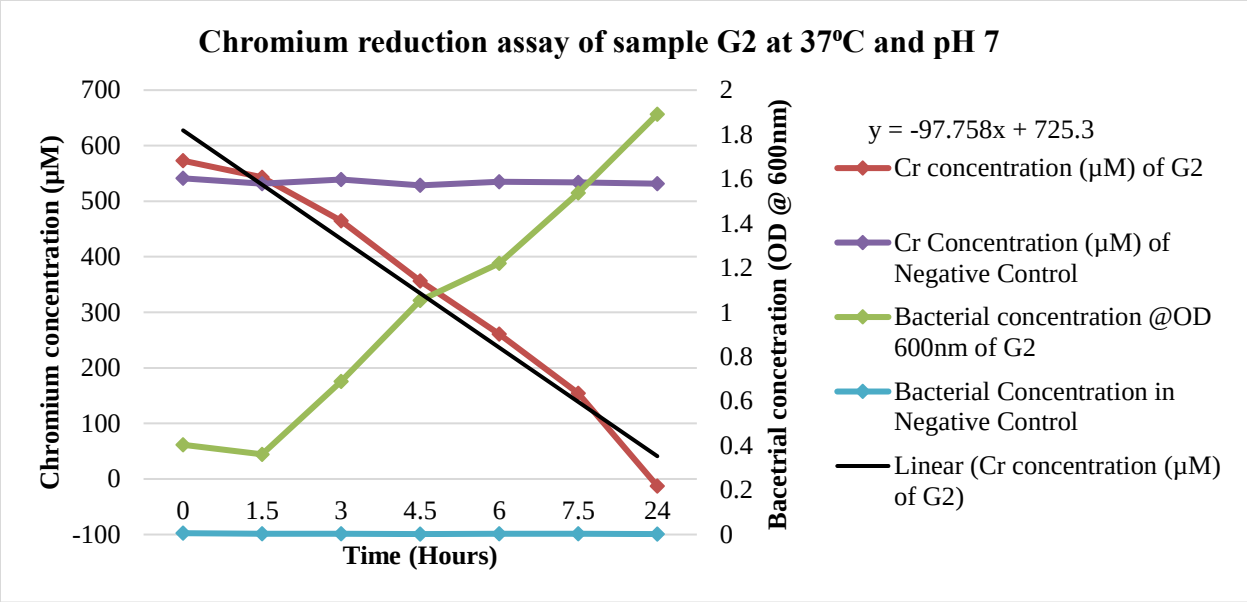

Figure 9: Bioremediation assay curve of sample G2 at 37 ºC & pH 7

From the above. It can be said that 100% Cr was reduced within 24hours. Chromium concentration at 0 hour was 572.9148936 µM which was 100% reduced. Also the optical density at 0 hour was 0.404 which increased to 1.892 after 24 hours. No bacterial growth was found in the negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.6 Reduction profile at 37°C, pH 8.5

Table 15: Chromium reduction and cell growth at 37°C, pH 8.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	562.56028	0.386	541.212766	0.006
1.5	518.30496	0.35	531.4964539	0.003
3	466.95745	0.553	538.8014184	0.004
4.5	297.52482	1.044	528.5177305	0.002
6	181.99291	1.412	534.9716312	0.004
7.5	152.63121	1.539	533.6950355	0.003
24	-14.46099	1.77	531.6382979	0.002

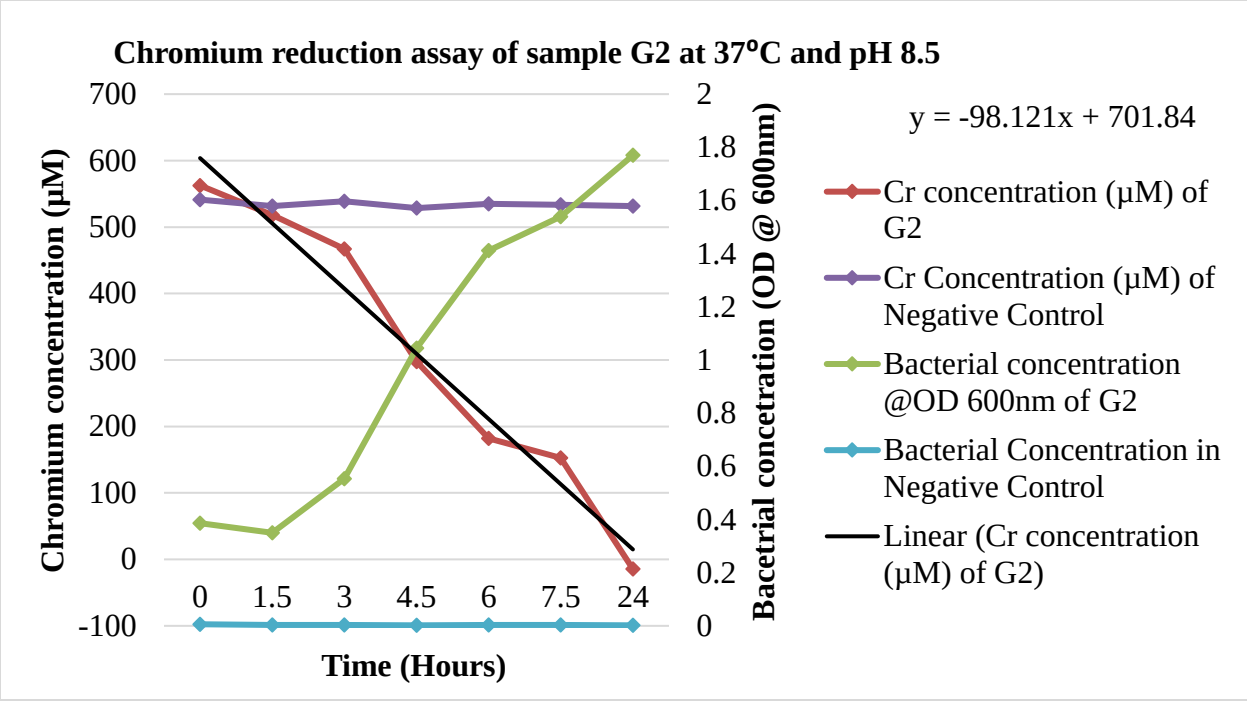

Figure 10: Bioremediation assay curve of sample G2 at 37 ℃ & pH 8.5

100% reduction was achieved after 24 hours, which is clear in figure 3.7. After 7.5 hours, 73% Chromium was reduced. Optical density at 0 hour was 0.386 which increased to 1.77 at 24 hours. So, it can be said that, the reduction profile at 37ºC and pH 8.5 was satisfactory. No bacterial growth was observed in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.7 Reduction profile at 42°C, pH 5.5

Table 16: Chromium reduction and cell growth at 42°C, pH 5.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	599.7234043	0.514	602.7021277	0.005
1.5	594.1205674	0.307	609.0851064	0.005
3	579.0141844	0.249	591.141844	0.01
4.5	582.9148936	0.212	593.2695035	0.006
6	552.0638298	0.192	596.6737589	0.003
7.5	539.0141844	0.174	590.7163121	0.002
24	532.1347518	0.159	598.9432624	0.002

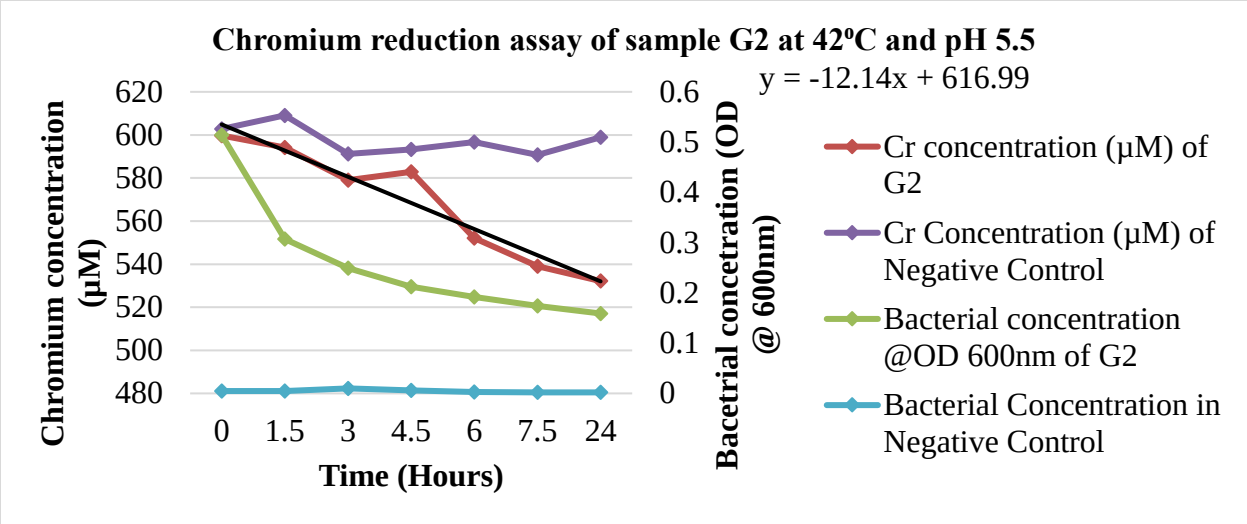

Figure 11: Bioremediation assay curve of sample G2 at 42 ℃ & pH 5.5

From the figure above, it is found that, reduction was not satisfactory at all. Optical density of bacteria was reducing by the flow of time which means bacteria could not survive on this temperature and pH. For this reason only 1% chromium was reduced in 24 hours. No bacterial growth was found in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.8 Reduction profile at 42°C, pH 7

Table 17: Chromium reduction and cell growth at 42°C, pH 7

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	592.1347518	0.501	602.7021277	0.005
1.5	595.3262411	0.375	609.0851064	0.005
3	518.3049645	0.485	591.141844	0.01
4.5	447.5957447	0.597	593.2695035	0.006
6	393.4822695	0.882	596.6737589	0.003
7.5	300.4326241	1.034	590.7163121	0.002
24	178.2340426	1.356	598.9432624	0.002

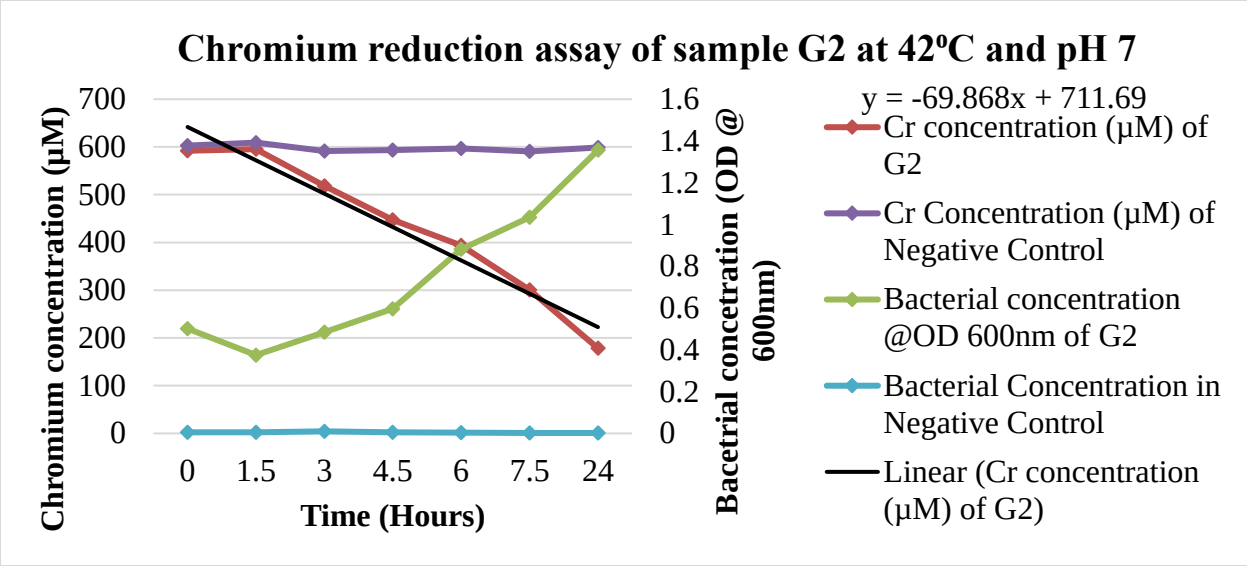

Figure 12: Bioremediation assay curve of sample G2 at 42 ºC & pH 7

From the figure above, it can be said that only 70% Chromium was reduced after 24 hours. 50% Chromium was reduced after 7.5 hours. Optical density of the sample was 0.501 at 0 hour which increased to 1.356 after 24 hours. No bacterial growth was found in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.2.2.9 Reduction profile at 42°C, pH 8.5

Table 18: Chromium reduction and cell growth at 42°C, pH 8.5

	Sample		**Negative Control**	
Time (Hrs)	Cr conc. (µM) with G2	Bacterial OD @ 600nm of G2	Cr Conc. (µM) of Negative Control	Bacterial OD in Negative Control
0	593.6950355	0.542	602.7021277	0.005
1.5	597.2411348	0.394	609.0851064	0.005
3	534.7588652	0.408	591.141844	0.01
4.5	442.9148936	0.619	593.2695035	0.006
6	376.3900709	0.823	596.6737589	0.003
7.5	294.7588652	1.281	590.7163121	0.002
24	145.2553191	1.774	598.9432624	0.002

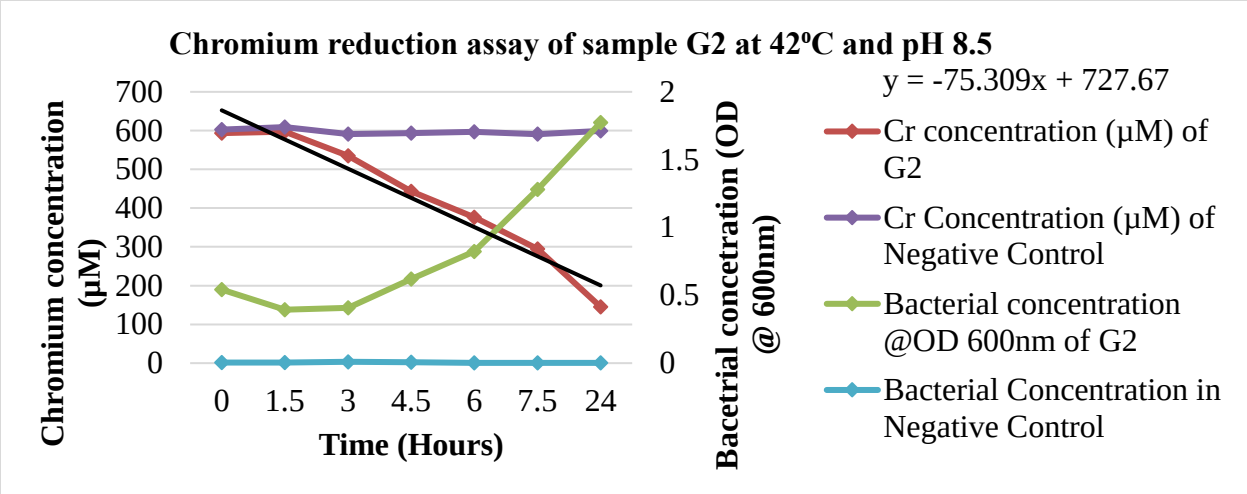

Figure 13: Bioremediation assay curve of sample G2 at 42 ℃ & pH 8.5

From the figure above, it can be said that 76% Chromium was reduced after 24 hours. 50% Chromium was reduced after 7.5 hours. Optical density of the sample was 0.542 at 0 hour which increased to 1.774 after 24 hours. No bacterial growth was found in negative control and the Chromium concentration was seen to be almost constant showing that no reduction took place without the presence of the bacteria under consideration.

3.3 Antibiotic resistant among Chromium resistant isolate G2

Antibiotic resistance method was discussed earlier. Results are given below:

Table 19: zone of inhibition of antibiotics

Name of antibiotics	ZI/mm	ZI/mm	ZI/mm	Average	Standard deviation	Inference
Penicillin (P10)	0	0	0	0	0	Resistant
Kanamycin (K30)	23	22	22	22.333	±0.5773502	Susceptible
Neomycin (N30)	24	24	23	23.667	±0.5773502	Susceptible
Vancomycin (VA30)	16	15	15	15.333	±0.5773502	Susceptible
Gentamycin (CN10)	22	23	24	23	±1	Susceptible
Cefixime (CFM5)	0	0	0	0	0	Resistant
Chloramphenicol (C30)	24	23	23	23.333	±0.5773502	Susceptible
Ceftriaxone (CRO30)	12	12	13	12.333	±0.5773502	Susceptible
Sulphamethoxazole (STX25)	0	0	0	0	0	Resistant
Cipropfloxacin (CIP5)	27	28	27	27.333	±0.5773502	Susceptible
Streptomycin (S10)	25	25	26	25.333	±0.5773502	Susceptible
Azithromycin(AZM30)	18	20	19	18.667	±0.5773502	Susceptible
Amoxycillin (AML10)	0	0	0	0	0	Resistant
Cefuroxime Sodium (CXM30)	9	8	9	8.667	±0.5773502	Susceptible

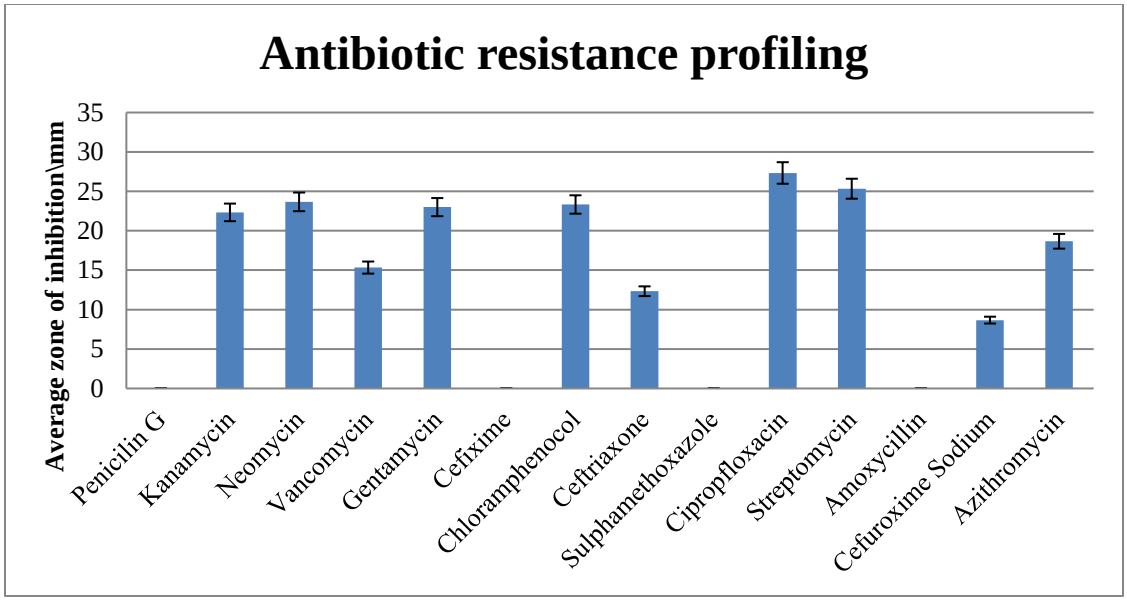

Figure 14: Graph showing the zone of inhibition against antibiotic discs.

From the figure, it can be seen that, the highest zone of inhibition was found in Cipropfloxacin (CIP5) which is 27.333 mm. So, it can be said the, Cipropfloxacin (CIP5) was more powerful than any other antibiotic for isolate G2. Thus, G2 is more susceptible against Cipropfloxacin (CIP5). The lowest zone of inhibition was found in Cefuroxime Sodium (CXM30) which is 8.667 mm. It is because, Cefuroxime Sodium (CXM30) could not stop the growth of sample G2 significantly. However, Sample G2 was resistant to some antibiotics. They are

- Penicillin (P10)
- Cefixime (CFM5)
- Sulphamethoxazole (STX25)
- Amoxycillin (AML10)

Only one antibiotic showed zone of inhibition below 10 mm which is Cefuroxime Sodium (CXM30) and rest of the antibiotic showed zone of inhibition above 10mm.

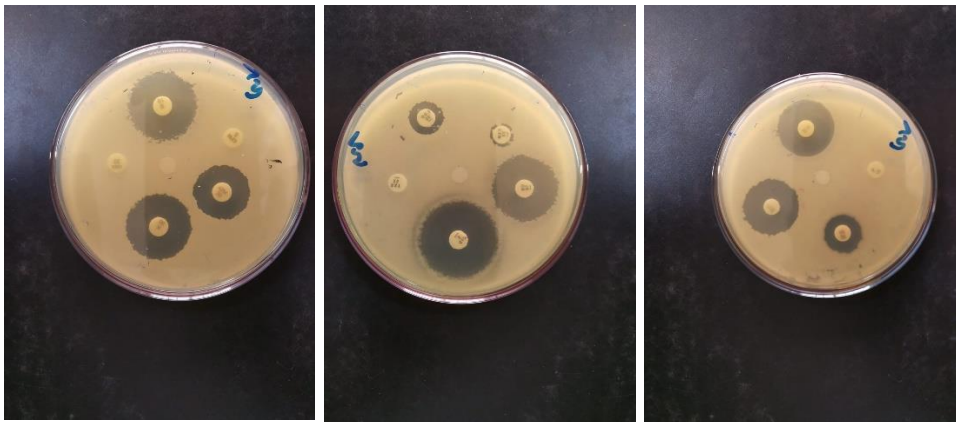

Figure 15: Zone of inhibition of the antibiotic discs in G2 swabbed HMA plate.

3.4 Determination of MIC

MIC was determined as the procedure discussed in 3.9. The result is given below:

Table 20: Data of MIC determination

Cr concentration	Absorbance at 600nm Test tube 1	Absorbance at 600nm Test tube 2	Absorbance at 600nm Test tube 3	Average	Standard Deviation
5mM	2.137	2.037	2.113	2.09566667	0.05220473
10mM	1.997	1.987	1.956	1.98	0.02137756
15mM	1.987	1.858	1.865	1.90333333	0.07254194
20mM	1.896	1.789	1.589	1.758	0.15583004
25mM	1.565	1.525	1.325	1.47166667	0.12858201
30mM	1.325	1.289	1.656	1.42333333	0.20229764
35mM	1.478	1.569	1.569	1.53866667	0.05253887
36mM	1.369	1.236	1.458	1.40733333	0.11172436
37mM	1.356	1.569	1.456	1.46033333	0.1065661
38mM	0	0	0	0	0
39mM	0	0	0	0	0
40mM	0	0	0	0	0

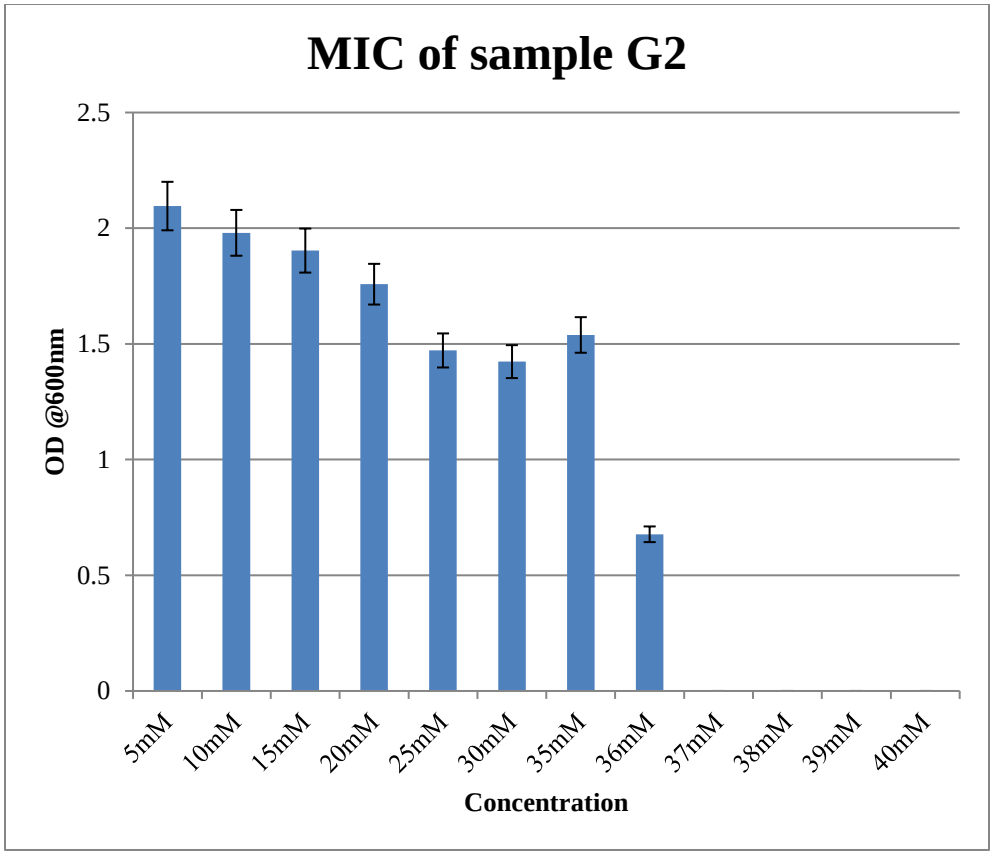

Figure 16: MIC of sample G2

From above, it can be seen that, in 5mM chromium optical density of the sample @600nm was highest which is 2.10 and the last growth of sample G2 was found in 36mM. After that, no bacteria growth was found. Thus, it can be said that the minimum inhibitory concentration for sample G2 is 37mM.

3.5 Optimum temperature and pH determination

It was done by plotting the percentile reduction of sample G2 in different temperature and pH. After that, a graph was constructed where in x-axis, there was percentile reduction and in y-axis, time was plotted. The most reduction in lowest time was chosen as optimum temperature and pH.

Table 21: optimum temperature and pH determination

Percentile reduction 42C pH8.5	Percentile reduction 42C pH7	Percentile reduction 42C pH5.5	Percentile reduction 37C pH5.5	Percentile reduction 37C pH7	Percentile reduction 37C pH8.5	Percentile reduction 25C pH5.5	Percentile reduction 25C pH7	Percentile reduction 25C pH8.5
100	100	100	100	100	100	100	100	100
100.5973	100.539	99.06576	94.0331	94.776	92.13323	104.7758	100.791	103.2572
90.07299	87.53159	96.54687	95.6593	81.09704	83.00576	97.25707	82.89338	89.78707
74.6031	75.59018	97.19729	96.45989	62.2436	52.88763	99.48483	71.84647	74.24697
63.39788	66.45147	92.05307	95.14642	45.51937	32.35083	96.58874	57.40773	51.15752
49.64819	50.73721	89.87713	90.00513	26.96302	27.13153	94.89007	43.62591	33.38001
24.46632	30.10025	88.73003	84.87635	-2.18987	-2.57057	82.37284	14.64118	0.336372

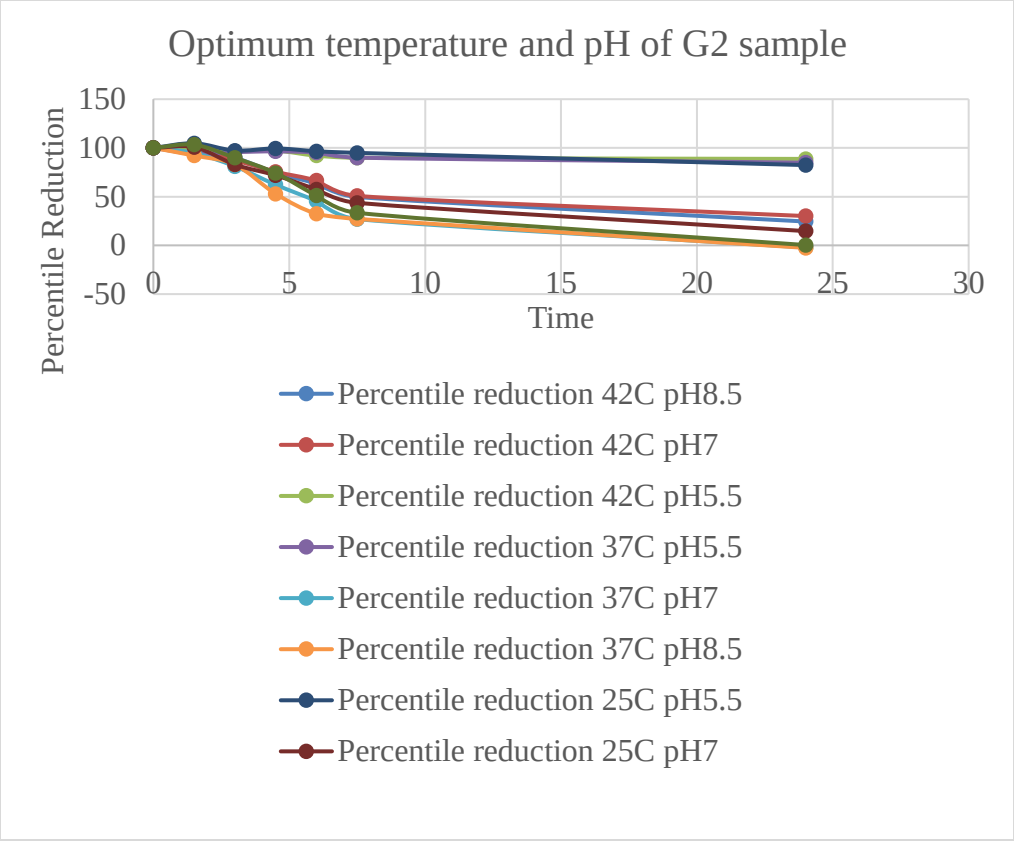

Figure 17: optimum temperature and pH for sample G2

From the figure above, it can be said that, the most reduction which take place in the lowest time is in 37ºC and pH 8.5. Thus, it is the optimum temperature for G2 sample.

3.6 Determination of Exo or Endo enzyme:

Here, concentration of chromium was measured over time in optimum temperature and pH which is 37ºC and 8.5

Table 22: Concentration of chromium in Sample G2 with time.

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

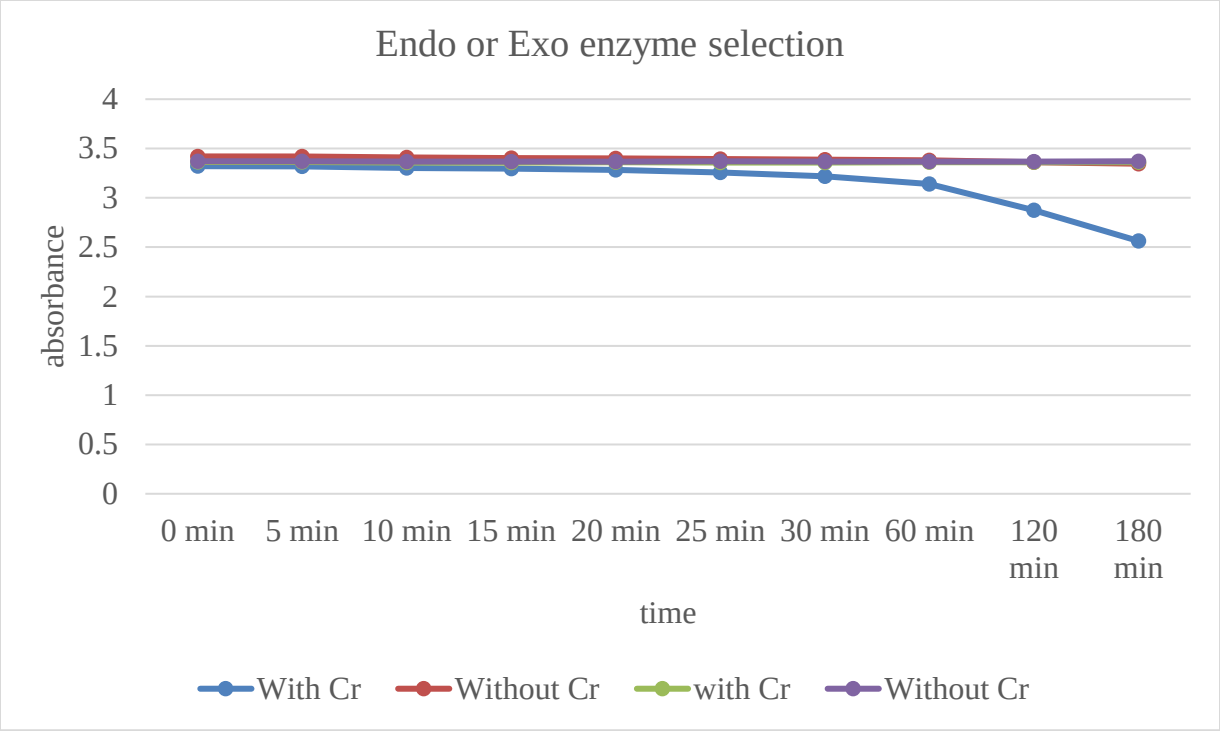

Figure 18: Determination of Exo or Endo enzyme

From the figure, it is sure that, the enzyme is exo enzyme because when we put the bacteria in broth, it started growing and concentration of chromium started lowering. Thus it can be said that, chromium reductase enzyme in sample G2 is an exo-enzyme.

3.7 Identification of G2

3.7.1 Obtained sequence by Sanger sequencing

The DNA sequence of the isolate was found out by Sanger sequencing. The sequence of the isolate G2 is shown in Appendix-A and B.

3.7.2 BLAST analysis of G2

The table below, table 25 contains the BLAST result:

Table 23: BLAST result of isolate G2

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus paramycoides strain MCCC 1A04098 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_157734.1
Bacillus albus strain MCCC 1A02146 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_157729.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA), partial sequence	1631	1631	100%	0.0	99.89%	NR_074540.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_113266.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_115714.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_112630.1
Bacillus cereus ATCC 14579 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_114582.1
Bacillus cereus strain IAM 12605 16S ribosomal RNA, partial sequence	1631	1631	100%	0.0	99.89%	NR_115526.1
Bacillus wiedmannii strain FSL W8-0169 16S ribosomal RNA, partial sequence	1626	1626	100%	0.0	99.77%	NR_152692.1
Bacillus proteolyticus strain MCCC 1A00365 16S ribosomal RNA, partial sequence	1626	1626	100%	0.0	99.77%	NR_157735.1

Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA, partial sequence	1483	1483	100%	0.0	99.75%	NR_114581.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA, partial sequence	1483	1483	100%	0.0	99.75%	NR_043403.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA, partial sequence	1480	1480	100%	0.0	99.63%	NR_112780.1
Bacillus mobilis strain MCCC 1A05942 16S ribosomal RNA, partial sequence	1478	1478	100%	0.0	99.63%	NR_157731.1
Bacillus pseudomycoides strain NBRC 101232 16S ribosomal RNA, partial sequence	1472	1472	100%	0.0	99.51%	NR_113991.1

3.7.3 Phylogenetic tree of sample G2

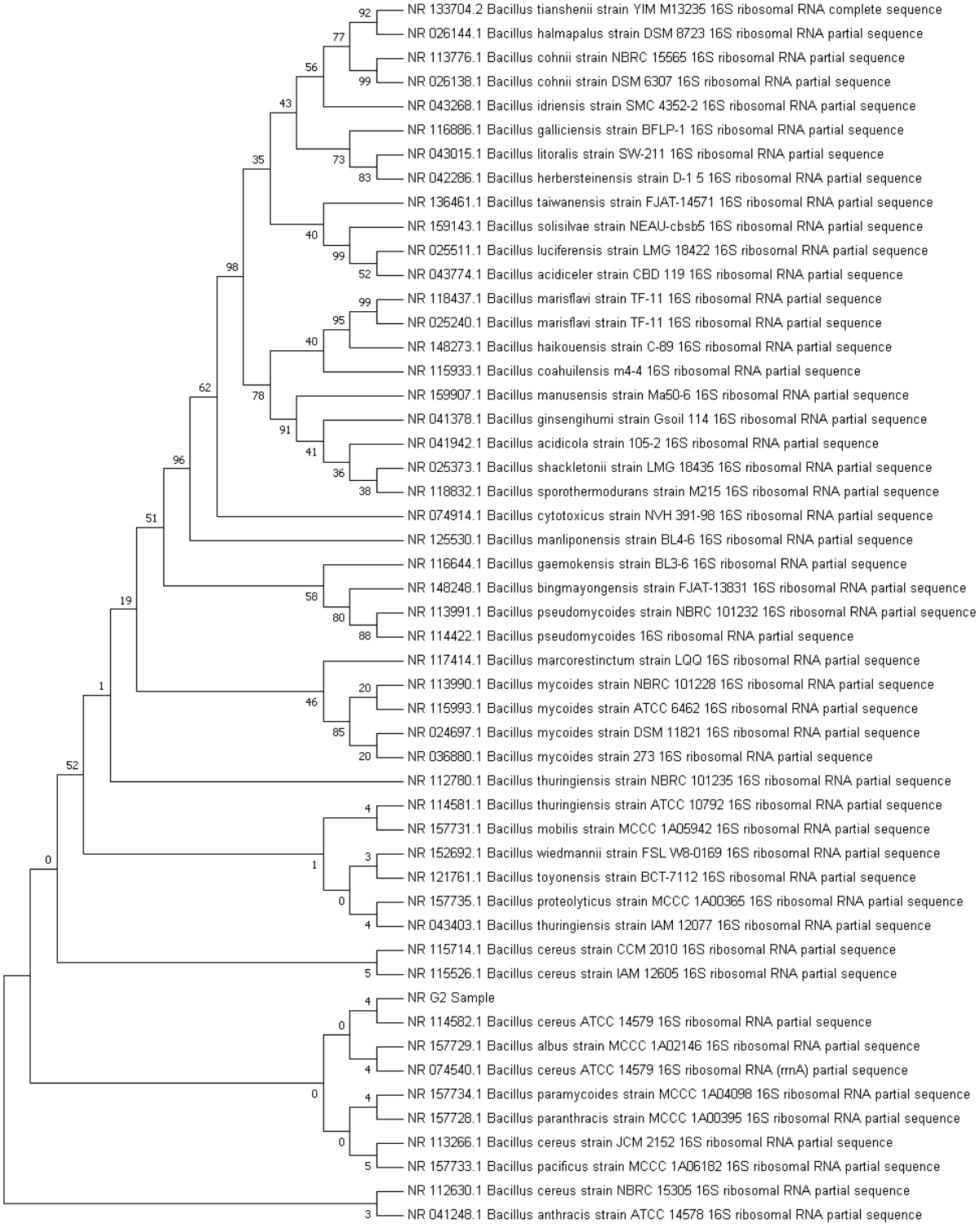

Figure 19: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.20873487 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 51 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 794 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

From the phylogenic tree that we can see above, it can be said that there are many clusters of microorganism from where identification of sample G2 has been confirmed. From the very first, we can see a cluster of *Bacillus tianshenii* , *Bacillus halmapalus*, *Bacillus cohnii*, *Bacillus idriensis*, *Bacillus galliciensis*, *Bacillus litoralis* and *Bacillus herbersteinensis*. This cluster have some common properties. They are given below:

- Gram Positive bacteria
- Rod shape bacteria
- Aerobes

Another cluster is found which is made up with *Bacillus marisflavi*, *Bacillus haikouensis*, *Bacillus coahuilensis*, *Bacillus manusensis*, *Bacillus ginsengihumi*, *Bacillus acidicola*, *Bacillus shackletonii*, and *Bacillus sporothermodurans* this cluster also holds some common properties like pathogenicity, source etc.

Another fine cluster is seen which is made up of G2, *Bacillus cereus, Bacillus albus, Bacillus paramycoids, Bacillus paranthracis, Bacillus pacificus* this cluster holds some common properties. They are:

- Facultative aerobic
- Forms Spores
- Can easily spread

3.8 Discussion

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

The analysis was conducted with G2 with various temperature and pH. The obtained data was tabulated and graph generated accordingly. 9 different graphs were generated (three different temperatures and for every temperature, experiment was carried out in three pH values) were and results were examined. There was also a blank (Negative control) prepared for better conduct of the experiment. After studying the graphs, it was evident that the isolate possessed Chromium reducing capacity. It was also seen that the reduction capacity of isolate G2 increased with increasing temperature. The quickest reduction was seen after 24 hours which was seen at 25°C, pH of 8.5 and also at 37°C, pH 7.

In antibiotic resistance, the highest zone of inhibition was found in Cipropfloxacin (CIP5) which is 27.333 mm. So, it can be said the, Cipropfloxacin (CIP5) was more powerful than any other antibiotic for isolate G2. Thus, G2 is more susceptible against Cipropfloxacin (CIP5). The lowest

zone of inhibition was found in Cefuroxime Sodium (CXM30) which is 8.667 mm. It is because, Cefuroxime Sodium (CXM30) could not stop the growth of sample G2 significantly. However, Sample G2 was resistant to some antibiotics. They are

- Penicillin (P10)
- Cefixime (CFM5)
- Sulphamethoxazole (STX25)
- Amoxycillin (AML10)

Only one antibiotic showed zone of inhibition below 10 mm which is Cefuroxime Sodium (CXM30) and rest of the antibiotic showed zone of inhibition above 10mm.

After the formation of phylogenic tree, it was found that G2 sample was *Bacillus cereus* ATCC 14579. Though many other bacteria had value near it, after constricting the neighbor joining tree, it showed the best result with *Bacillus cereus* ATCC 14579 since it has a common node. So, it can be concluded that, DNA G is most similar to *Bacillus cereus* than all the other DNA sequence that we had downloaded from NCBI BLAST. Thus, G2 sample is identified as *Bacillus cereus*.

Chapter 4

Conclusion

4.1 conclusion

After multiple studies that were carried out, it is assertive that the isolate G2, which was later identified as *Bacillus cereus*, possesses hexavalent Chromium reducing capabilities. The microorganism carries an astonishing potential to biologically reduce Chromium. It was observed that, the bacterial strain was able to reduce Chromium if provided its optimum condition for reduction. The rate of reduction was seen to increase in most cases with increase in time. Moreover, it was seen after study that the bacteria under consideration can withstand up to high concentration of Chromium which demonstrates its capacity to survive in very adverse condition. It must have some defense mechanism with which it survives such high concentration of Chromium. Information gathered from this project confirms for sure that the bacteria is capable of reducing hexavalent Chromium and that it can survive up to quite high concentration of Chromium.

4.2 Future works

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

Research can be further conducted to gather more knowledge about the following:

1. Isolate G2 can be a potential source for Chromium reductase enzyme. Comparative genomic studies might be carried out to find out best candidate for Chromium reduction activity.
2. To find out more about this bacteria, plasmid analysis can be performed.

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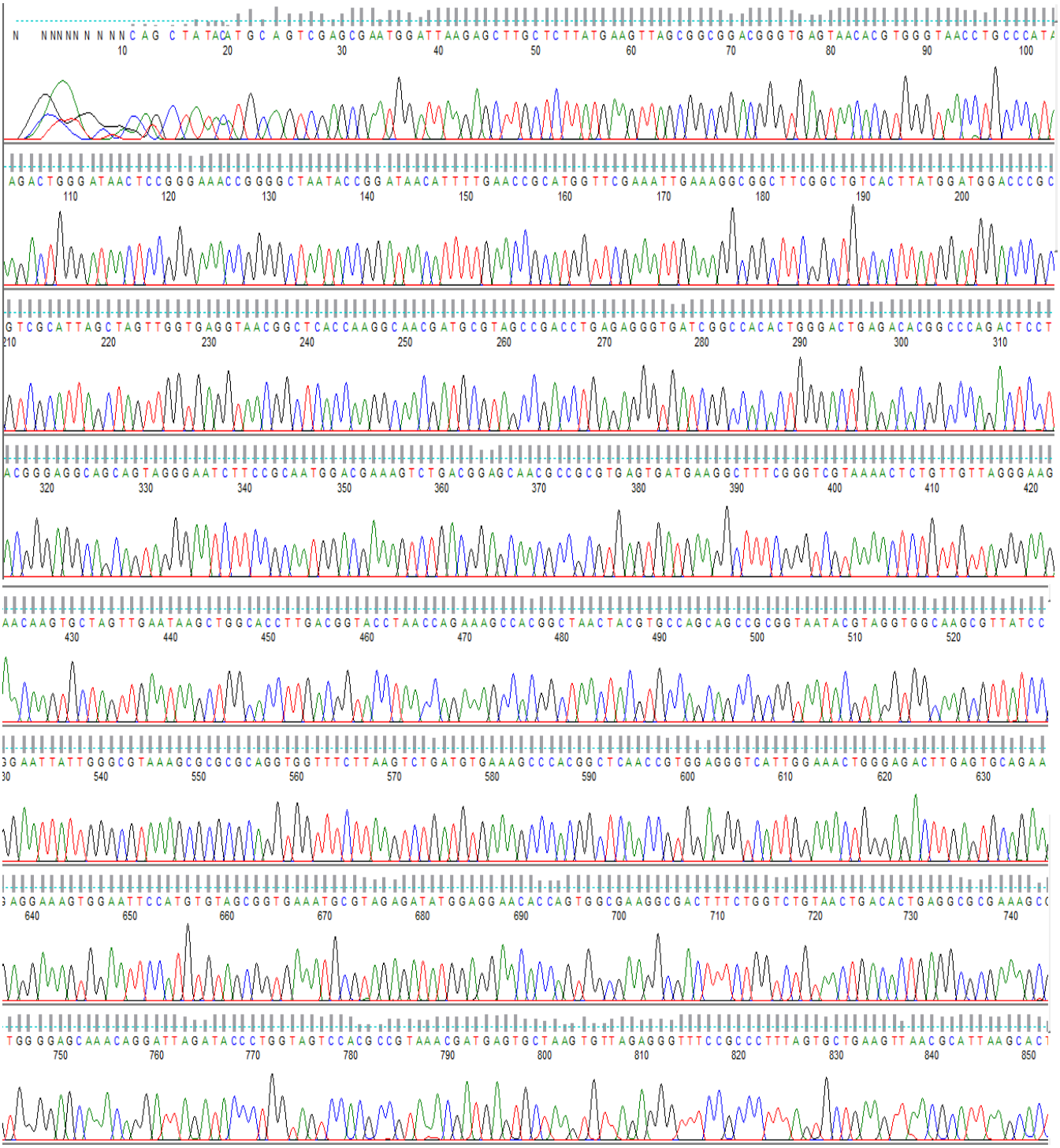
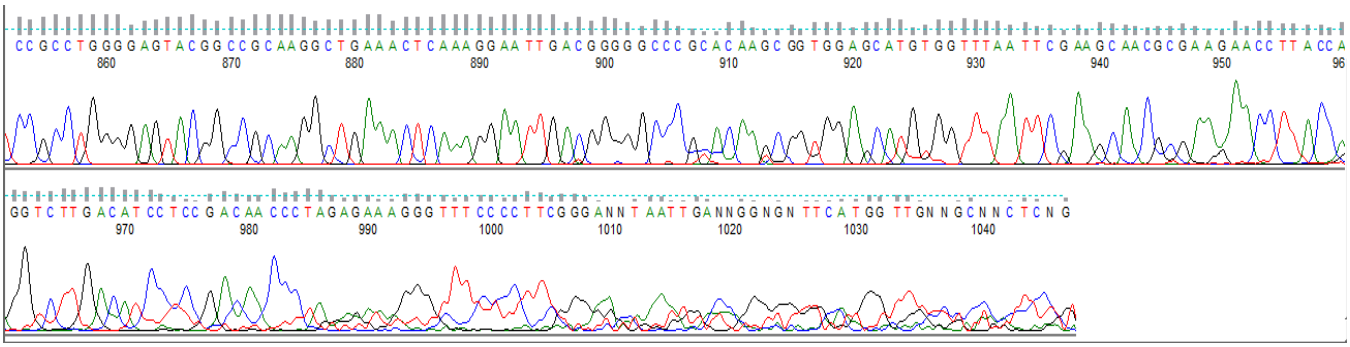
Appendix- A

Chromatogram of 16s rDNA sequencing of G2 (*Bacillus cereus*):

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860
870
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950
96
GGTCTTGACATCCTCCGACAACCCTAGAGAAAGGGTTTCCCCTTCGGGANNTAATTGANNGGNGNTTCATGGTTGNNGCNNCTCNG
970
980
990
1000
1010
1020
1030
1040

Appendix- B

Obtained partial sequence of G2 after performing 16s rDNA sequencing (using Sanger sequencing)

CTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAG
TGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAG
TGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGG
TGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGT
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
TGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCG
GATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTT
GACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCA
GCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTG
AGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGA
TATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT