Isolation and Characterization of Carcinogenic Chromium reducing *Bacillus pumilus* from tannery effluent of Hazaribag area

A project submitted

by

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

Certification Statement

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

Signed

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Countersigned by the supervisor

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Abstract

Hexavalent Chromium, which is a heavy metal appears to be very toxic in nature. It also possesses the carcinogenic and mutagenic property and causes various serious diseases like skin cancer, lung cancer, pulmonary congestion etc. Due to its usefulness it has become a part of various industries like leather, tannery, dye, stainless steel etc. Therefore, they are being freed every day without any treatment and making the natural environment very hazardous for the human health. Chromium is available in two oxidative form from which its trivalent form is less toxic than its hexavalent form. It has become a necessity to reduce the hexavalent Chromium from the natural environment to preserve the earth. Through this study a strain was experimented to identify their ability to reduce hexavalent Chromium to its trivalent form as well as their tolerance to different concentration of Chromium. To conduct this research sample was collected from the Hazaribag tannery area which was then isolated and purified using different protocol. Then this purified strain was experimented using DPCZ based bioassay method to discover their reduction capacity which shows that the strain has good Chromium reduction capacity at 37˚C pH 5.5 and 7 as well as 42˚C, pH 7. Moreover, the strain has gone through the antibiotic resistant profile study to observe their resistance towards antibiotics. From this study it has found that Streptomycin and Kanamycin have a strong effect on this strain whereas some other antibiotics such as Penicillin, Cefixime, and Ceftriaxone do not have any effect on that strain. The tolerance level towards Chromium of the isolate was measured by Minimum Inhibitory Concentration method which reveals that the strain can tolerate up to 18Mm of Chromium concentration in its surroundings. In addition, to identify the strain, 16s rDNA sequencing was done and using the sequence data a phylogenetic tree was made using different software that shows resemblance between the experimental strain 4W100 and *Bacillus pumilus*.

Cr (VI) is increasing every single day in natural environment as they are not treated properly prior to its discharge. It has now become a necessity to reduce the hexavalent Chromium from the water and soil. This study design will aid in the identification of Chromium reducing bacteria thorough bioremediation method which may play a role in reducing hexavalent Chromium in future.

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List of Acronyms:

BLAST	Basic Local Alignment Search Tool
Cr	Chromium
DPCZ	Diphenyl Carbazide
EPA	Environmental Protection Agency
ETP	Effluent treatment plant
IDLH	Immediately Dangerous to Life and Health
Kg	Kilogram
MCL	Maximum contaminant level
Mg	Milligram
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
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

Chapter 1: Introduction

1.1 Background

Metals are found in nature like soil, rocks, ore, plants etc. at different concentrations. However, metals at high concentrations pose a great threat to the living organisms as they easily get included in the food chain. The actions towards nature by human has increased the amount of heavy metals on earth. Chromium is one kind of heavy metal whose increasing concentration is causing different types of cancer to the human (Bielicka, Bojanowska, & Wiśniewski, 2005).Chromium is broadly used in industries and a great source of water pollution. They show two oxidizing states in water based on their physic-chemical characteristics(Moore, 1991).Chromium is mainly utilized in metal factories ,textile production, pulp manufacturing etc.(Pattanapipitpaisal & Reakyai, 2013).

Cr (VI) seems to be highly toxic and soluble in water whereas Cr (III) is less toxic and insoluble in water (Yrjälä & Hasnain, 2012). In the leather industries, Cr (III) is transformed into Cr (VI) and is being freed into the nearby river water without any treatment (Kotaś & Stasicka, 2000; Sarangi & Krishnan, 2008). One step to reduce the amount of disposal from the water can be the bioremediation technique which utilizes the microbes (Safitri, Priadie, & Indah Permatasari, 2015).Heavy metals like Chromium cannot be totally removed using microorganisms in the bioremediation process. Besides microorganisms changes the oxidizing state of that heavy metal and changes their physicochemical properties(Garbisu & Alkorta, 2003; Mosa, Saadoun, Kumar, Helmy, & Dhankher, 2016).There are various other method to reduce the chromium toxicity from water like precipitation, ion exchange, adsorption etc. but these methods are costly than the bioremediation process(Yrjälä & Hasnain, 2012).

The purpose of the research is to evaluate the chromium resistant bacterial strain that are isolated in the laboratory to identify their Chromium reduction capacity along with their tolerance to the increasing Chromium concentration. This study also helps us to identify the chromium reductase enzyme which can be used in chemotherapy purpose.

1.2 Objectives

The purpose of this research is to find out whether the chromium resistance bacteria is capable of doing bioremediation of mutagenic and carcinogenic heavy metal like chromium and also to assess the predictable source of chromium reductase enzyme. For this purpose following experiments were done-

- ✓ Evaluation of the performance of the isolated bacteria in Chromium contaminated environment.
- ✓ Investigation of Minimum Inhibitory Concentration of Chromium to evaluate the tolerance of the isolated bacteria
- ✓ Investigation of the antibiotic resistance profile of the bacterial isolate.
- ✓ Construction of phylogenetic tree.

1.3 Literature Review

1.3.1 Introduction

Chromium is one kind of heavy metal which seems to be multiplying day by day. It produces different chemical states in the inorganic environment. Only trivalent and hexavalent forms are found abundantly in the nature. Trivalent chromium is an essential nutrient component while excess Chromium (VI) in biological systems has been implicated in specific forms of cancer. Cr (III) is required as a fundamental necessity of the living organisms whereas Cr (VI) has become the reason of various types of cancer. (Bielicka, Bojanowska, & Wisniewski, 2005).Humans when exposed to the chromium are supposed to develop toxicity despite of its being a necessary material of human body. In addition, it has been identified as pollutant for the nature, food, some industrial and consumer products. The potent of chromium to contaminate the nature has been observed from a long ago (Sellamuthu, Umbright, Chapman, Leonard, & Li, 2011). Various enzymatic activities related to starch and nitrogen metabolism are decreased by Cr toxicity either by direct interference with the enzymes or through the production of reactive oxygen species. Many metabolic processes related to nitrogen and starch get hampered by chromium toxicity either by immediate inclusion of the chromium or by the generation of reactive oxygen species .Cr initiates the

oxidative destruction by spoiling the lipid membrane and DNA(Ahmad, Khan, & Diwan, 2013).

1.3.2 Chemistry

The name Chromium originates from the Greek word 'chroma' which denotes color. Its color is silver with blue tinge and seems to be hard .Chromium is predominantly available in its chromate form. Being a transitional element it's trivalent and hexavalent form has only the biological importance. Cr (III) is mainly available in the environment but Cr (VI) is mainly utilized in the factories (Castro et al., 2014). Chromium materials are applied as industrial catalysts and pigments (bright green, yellow, red and orange). Chromium is useful for human in such a way that it assists in utilizing the glucose. However, it becomes toxic while exceeding the amount exposed to living organisms.

Table 1.1: Comprehensive data on the chemical element Chromium

Name, symbol, number	chromium, Cr, 24
Group, period, block	6, 4, d
Element category	Transition metal
Appearance silvery metallic	Silvery metallic
Standard atomic weight	51.9961(6)$g \cdot mol^{-1}$
Electron configuration	[Ar] $3d^5$ $4s^1$
Crystal structure cubic body centered	Cubic body centered
Oxidation states	6, 5, 4, **3**, 2, 1
Atomic radius	140 pm
Thermal conductivity	(300 K) 93.9 $W \cdot m^{-1} \cdot K^{-1}$

1.3.3 Sources of Chromium compounds

There are two main sources of chromium in the environment. In addition there are two ways by which chromium is generated in the nature-

1. The natural source i.e. Ferrochromite (Fe2Cr2O4)

2. Earth's crust (Augustynowicz et al., 2010).

Environmentally toxic metals like Cd, Cr, Ni, Hg enter into the environment using different pathways like metal smelters, manufactured wastage like utilizing fertilizers and pesticides (Davies, Puryear, Newton, Egilla, & Saraiva Grossi, 2001).It is carcinogenic in nature and accumulate in the soil after being discharged from the leather tanning, textile, carpet and electroplating industries as waste material (Vajpayee et al., 2001). It remains in soil as chromic (Cr3+) or chromate (Cr6+) ions. Chromium (VI) is kept unstable for a longer period of time in the soil without altering its oxidation state. Moreover the oxidative nature of chromium in soil has economical value whereas hexavalent form of it is poisonous for plants and animals (Rai, Vajpayee, Singh, & Mehrotra, 2004).

1.3.4 Chromium utilization

Many chromium compounds such as sodium chromate, ferrochromate, dichromates are utilized in industries widely .In addition, some of chromium compounds are implemented in stainless steel welding process, tanning leather, dyes and pigments, timber preservation. Chromium also has anti-corrosive property and used in boiler (Aslam & Yousafzai, 2017).

1.3.5 Regulation regarding level of Chromium

Chromium poses a great threat to the plants and animals when they are excessively present in environment. The concentration of chromium present the water of lakes and rivers ranges from 1 to 10 µg/L. However, the safe level of chromium in water and for the human health suggested by EPA is 50 to 100 µg Cr/L (Aslam & Yousafzai, 2017). An estimation by World Health Organization states the tolerable limit for Cr (VI) and Cr (III) in human to range from 0.05mg/L to 2mg/L (Gupta and Rastogi, 2009) and safe concentrations for chromium ingestion was shown to be at 0.1mg/L. Chromium is further found in leather factories, textile industries and in dyeing. The maximum Cr (VI) concentration permissible in color dyes is 56ppm and no more than 2.75% of its total weight in leather tanning. In addition the Occupational Safety and Health Administration (OSHA) also stated the maximum exposure limit or Permissible Exposure Limit (PEL) at 0.1 mg/m^3 and the National Institute for Occupational Safety and Health (NIOSH) specifies the amount to $15mg/m^3$.

1.3.6 Toxicity of Chromium and the effect of it towards health

1.3.6.1 Chromium toxicity

Cr (VI) has prominent toxicological features. Although it has specific role in metabolism of glucose, fats and proteins in animals and humans. In humans and animals high level of chromium (VI) in drinking water causes tumors in stomach(Paine, 2001).Reduction of Cr (VI) to Cr (III) can lessen the toxicity as in this form it cannot be transported inside the cells(Cohen, Kargacin, Klein, & Costa, 1993a). Interruption of cellular integrity and various functions may occur because of Cr (VI). The reason is, it can pass through different types of cells under various physiological conditions and produce reactive intermediates (Mattia et al., 2004).

1.3.6.2 Effects on human health

The epidemiological studies have reported frequent health problems in Cr (VI)-exposed population, such as cancer, dermatitis, asthma, chronic bronchitis, hypertension, chromosomal abrasions, back pains, metabolic syndrome, hemoglobin changes, and DNA detriment in lymphocytes (Junaid, Hashmi, Malik, & Pei, 2016).

By inhalation, Chromium can enter into the human body most frequently. That is why the lungs are considered as the primary target organ for Chromium exposure. Skin penetration is another significant way by which human exposure to Chromium can take place (Annangi, Bonassi, Marcos, & Hernández, 2016). Chromium compounds cause several health risks and among these genotoxicity/carcinogenicity in humans has been widely studied (Christian, Oliver, Paustenbach, Kreider, & Finley, 2014). A number of epidemiological studies were carried out in chromium-exposed workers suggesting that occupational exposure to chromium increased carcinogenic risk in the respiratory tract (Cohen, Kargacin, Klein, & Costa, 1993b). In these studies, considering the genotoxic potential of Chromium (VI), different types of DNA damage have been observed both in vivo and in vitro. DNA strand breaks, inter-strand cross-links and Chromium-DNA adducts have been identified as potential mechanisms of Chromium (VI) genotoxicity (khorsandi & Rabbani-Chadegani, 2013; Salnikow & Zhitkovich, 2008). However, Chromium (III) has been largely considered non-genotoxic. In this regard, different experiments has carried out using cell-free systems which indicates that it binds to DNA, interfering with base-pair stacking and consequently, leading to mutations (Fang et al., 2014).

Fig.1.1 shows intracellular mechanisms explaining how chromium acts as a genotoxic compound.

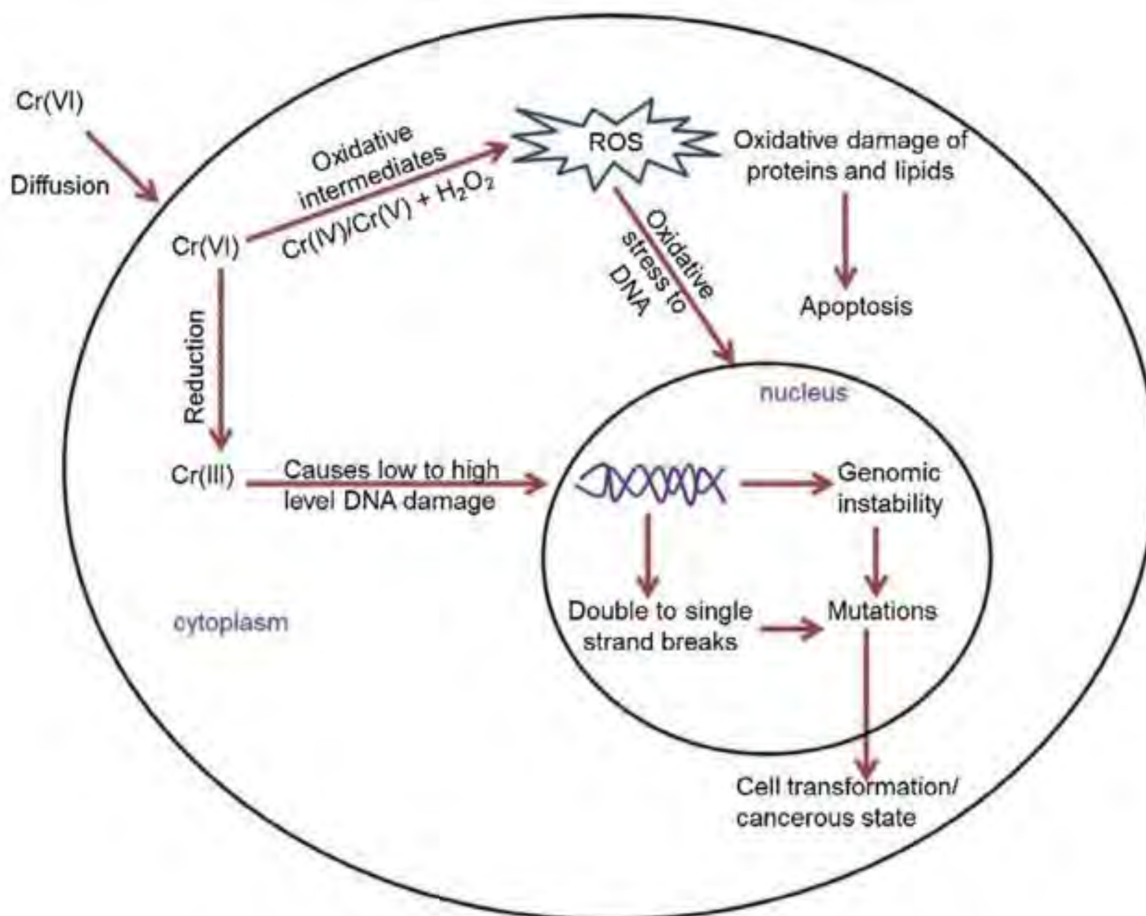

Figure 1.1: Intracellular mechanisms explaining how chromium acts as a genotoxic compound inducing micronuclei (Annangi et al., 2016).

1.3.6.3 Effects in animals

The health of the marine environment is in an alarming condition as exposure to Chromium is increasing. Chromium enters the marine environment primarily via surface water runoff from rivers and deposition from the atmosphere. (Goodale et al., 2008). In the range of 20.1–91 mg/l (96 h LC50), Cr (VI) shows acute toxicity to marine fish. Although, toxicity is highly dependent on temperature, salinity and water hardness. However, more recent studies have shown Cr (VI) to have a variety of sub lethal effects in fish, including altered enzyme activity, decreased disease resistance, oxidative stress responses and histological alterations to gill and liver tissue. (Goodale et al., 2008).

1.3.7 Carcinogenesis induced by Chromium

One of the prime cancer-causing agents is Cr (VI), known to alter human DNA. Unlike Cr (III), the hexavalent form stays as oxyanion and survives by accessing the sulfate anion pathway. The acceptance level of Cr (VI) within cell is reduced due to the redox framework and its subsequent reduction to tits trivalent form. The Cr (III) in contrary, attaches to DNA and proteins situated in cellular framework and further possess high amount of affinity for organic ligands. In the reduction process of Cr6+ Cr+ several intermediate valance species of Cr and dynamic oxygen species are made (Conett, 1983; Mattagajasingh, 1995; 1997), and these commence in the cancer-causing activity by altering the DNA molecule (Kawanishi, 1986). Hydroxyl ions are generated because of Cr reduction causes cross linkage with DNA-protein and expressed as agents for chromate carcinogenesis. Medium valance of Cr causes DNA damage as well. This DNA-protein crosslink upsets the regular DNA-protein incorporation, changes heredity outcomes and leads to interruption in gene expression. To understand the nature of Chromium and its toxic nature, it is essential to detect and analyze the proteins that are targeted in turn.

1.3.8 Traditional methods for reduction of chromium toxicity

Traditionally , various physico-chemical techniques were once applied effectively to reduce the Cr (VII) concentration in order to detoxify (K.Chung ,2007).To detoxify contaminated water , many physical and many mixed techniques includes molecular exchange , filtering , sedimentation , electrochemical treatment , film development are used (Ahluwalia & Goyal, 2007; Al-Sou'od,2012).

1.3.8.1 Electrochemical precipitation

The process utilizes electric potential to aid its heavy metal removal from contaminated water over traditional methods (Kurniawana, Chana, Loa, & Babelb,2006).This process has been found to be useful in treating both industrial water and portable water and by utilizing the same principle of an electrotype cell, the actions of a cathode, anode and a DC powered house were applied on to the contaminated electrolyte solution (Kongsricharoern and Polprasert, 1996).The Cr (VI) concentration can be reduced from 3.860 mg\L to 0.2 mg\L

with the ECP method . Not only has the method proven to be highly efficient, but also versatile, as various metals including ferric chloride, ferric hydroxide, arsenic and phosphate removal could be accomplished by the ECP process (Pinisakul, Polprasert et al, 2002).

1.3.8.2 Ion exchange

Although this method was applied by delay, the exchanging of ion has proven to be significantly effective, in chromium removal from waste water. During the course of action, any targeted distinct metal species are removed by insoluble exchange with a substitute species. Chromium upon entering, passes through side of a segment, crosses through a resin bed and is finally expelled. Once the amount of the resins decreases, all the accumulated solids are cleared.

Resins such as Dowex 2-X4 are utilized to filter Cr (VI) from waste water (Sapari, Idris & Hamid, 1996), with an anionic exchanger consisting of OH- ions. Even though this technique has proven beneficial, it is accompanied by certain limitations and obstacles. The exchange of fluid is highly specific for a heavy metal ion to be separated. The preferred resin must be capable of removing heavy metal completely. In addition, not only the ion-exchange equipment happens to be expensive, moreover the system is hampered by other organic sludge and diverse solid residues in the contaminated water.

1.3.9 Metal resistance mechanism into bacteria

There is a vital aspect of microorganism developing resistance towards metals especially in light that are abundant in nature. From a biochemical aspect, microbes have a heterogeneous approach towards metals. The bacteria demonstrates metal resistance in five segments (Rouch et al, 1995) as follows:-

1. Excluding metals via porousness hindrance.
2. Expulsion of metal through cellular active transport.
3. Extracellular separations.
4. Intracellular dissociation metal by inhabiting protein from harming suitable cell material.

1.3.9.1 Uptake system of metal resistance

Heavy metal ion's from numerous hazardous abilities of reducing catalyst activity altering the specific sites of enzymes by causative change and even sealing certain areas on enzymes. Metal ion's further pose a threat on the DNA structure by interfering with the cross-linking of the strands and even minimize the of DNA, thereby hampering the data of the DNA (Beyerman, 1994).

Due to the metal initiated destructive effects upon numerous focal points of a cell, serious issues comes into question regarding their protection especially the DNA of the cell. Cell advancement for resistance will take place because as metals are introduced in significant amounts, they are diminished through chain reaction mechanisms. This occurs until the concentration reaches to a crucial level where the cellular function is inactivated. Based on these facts, the cells must possess certain techniques which enables their target sites to be protected in order to survive. For example, protein on E.coli targeted by metals can be transformed in a single gene thereby bringing metal resistance (Lutkenhaus, 1977).

1.3.9.2 Metal as biological requirement

Not all metals has bear harm, in fact, different metals are have power to be a necessity by bacteria cells. A vast majority of bacterial species require iron, copper and nickel. Such metals have little to no detrimental effects within the cellular body.

1.3.10 Mechanism of Chromium resistance in bacteria

It has been observed in a wide range of bacterial strains that the chromate ion utilizes the sulfate uptake pathway to enter into cytoplasm (Cervantes and Campos- Garcıa 2007).The hexavalent toxic form of chromium is then reduced to its relatively trivalent form via numerous enzymatic and non-enzymatic activities (Cervantes, 2007). The plasmids or the chromosomal genes of the microbial species has been found to be responsible for exhibiting the procedures for resistance (Cervantes and Campos- Garcıa 2007). The genes in plasmids which conceal membrane transports initiate the expulsion of chromate ions from the cytoplasm. However, the other metabolic processes including hexavalent chromium reduction, free radical removal, repairing damaged DNA and various ion homeostasis have been regulated by the chromosomes in the bacteria.

A .Reduced uptake of Cr (VI)

The decreased uptake of chromate ion is related to the sulfur and iron uptake pathway. Both Cr2 O4- and dimensional sulfate molecule are ionized and re uptaken and this is the reason why chromate can access the sulfate ion pathway (Wenbo et al, 2000). A mutation occurring in the sulfate pathway also diminishes the chromium uptake as encoded in the chromosome (Ramirez et al, 2008). Microorganisms habituated heavy metal rich environments have demonstrated the activity to resist Cr (VI) thereby causing a low uptake of Cr (VI) through sulfate pathway.

Genetic information responsible for resistance or altering the microorganism's characteristics can influence the bacteria's resistance in any environment (Kummerer, 2004).

B. Detoxifying enzymes of ROS or Cr (VI) reduction intracellularly

The conversion of Cr (VI) to Cr (III) occurs via a redox reaction. The chromate ion is also obtained as an intermediate. Due to ROS formation of peroxide, superoxide and oxygen the organisms face oxidative stress. As a result chromate influences the bacterial proteins to protect themselves against such stress, thereby prompting chromate resistance (Ramirez-Diaz, 2005).

C. DNA repair enzymes

Entry of Cr (VI) into bacterial cell causes its immediate reduction to Cr (III) via numerous enzymatic and nonenzymatic processes. This leads to development of reactive oxygen species (ROS), which creates a destructive influence of the DNA and protein in the bacterial cell. DNA damage like base alteration, single strand or double strand splitting are the ultimate consequences due to ROS. Therefore, countermeasures are taken to mend the types of injury that occurs to DNA, for instance-utilizing the SOS enzymes (Rcc A, Rcc B, Rcc C, Hu et al, 2005). This has been observed in cases such as E.coli, where the presence of Cr (VI) was known, but the existence of a SOS separation framework to protect the DNA from oxidative degradation was unheard of (Llagostera et al, 1986).

D. Scavenging ROS

Once hexavalent Cr (VII) gain access into a cell, its valence is shortened to Cr (III). Electron helps enable the corrosion of toxic Cr (VII) into Cr (V). The pentavalent form although converts to its Cr (III), this is sometimes not so anomalously fast. As a result, a certain

portion of Cr (V) is reoxidized to back to Cr (VI), thereby generating a ROS system. Hydroxy radical (-OH) is one of the oxidative species formed in this process (Shi and Dalal, 1994) which is shown below:

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

This process leads to development of O_2- radicals which produces H_2O_2 dismutation.

1.3.11 Microbial reduction of hexavalent Chromium

The recent years has shown an increase in heavy metal contamination from an increasing global population, urbanization and industrialization and to respond to this the bioremediation process has provided an effective and innovative counter major for the wide variety of heavy metal contaminants (Akhtar, Chali, 2013). Heavy metals have proven to be hazardous to soil and aquatic biota and to protect themselves the microorganisms have shown activities of remediating such contaminants , which plays a key role for this technology(Obbard et al., 2001). From the perspective of Chromium, large amount of chemicals and energy are required in conventional methods, which proven to be unreliable (Ganguli and Tripthi, 2002) and since the microorganisms and their remedial ability to nullify Cr (VI) has shone light (Zhu et al, 2008), and further proven to be sustainable and economical (Ganguli and Tripthi, 2002), their purpose has been continuously after with various toxic heavy metals and various strains.

1.3.11.1 Bioremediation of chromium and its mechanisms

Various methods and physico chemical techniques have been employed for heavy metal removal including current methods of chemical precipitation , ion-exchange , filtration , coagulation - floculation , membrane-filtration , adsorption, electrodyalisis and photocatalysis (Wang & Fu,2010; Barakat, 2010). Although these techniques can be used to expel heavy metals from destination they poses their inherent advantages and limitation in application (Babel et al, 2016). Even though these techniques have proven to be effective, the drawback is, none of them are accepted as the best treatment option due to requirement of significant amount of chemical reagents or they provide a temporary solution which are too costly to implement in larger locations((Jeyasingh & Ligy, 2005; Komori, Rivas, Toda, & Ohtake, 1990). With the bioremediation of hexavalent chromium brings the prime focus of this

research, traditional methods have been put to effective use for its reduction i.e. adsorption, precipitation on ionexchange on enacted carbon, kaolinite, alum and slag and a vast majority of this techniques not only requires a high energy but also a lot of reagents as well (A. Ganguli & A. Tripathi, 2002). To respond to these short comings of inefficiency and wide expenses unconventional, inexpensive biomass have been used to test their chromium sequestering abilities including 1. Beet pulp 2. Saw dust 3. Pine bark 4. Shrimp chitin 5. Dead bacteria cells and 6. Fungus ((Kratochvil, Pimentel, & Volesky, 1998; Patterson, 1985), none of which have been proven to be effective in removal of tri and hexavalent chromium. Bioremediation paves a pathway where the toxic nature of heavy metals are nullified with the aid of the metal reduction capabilities of microorganisms. Microorganisms are able to tolerate and reduce hexavalent chromium in order to detoxify the contaminated environment (Frankenberger et al, 2003). In recent days the concept of bioremedial application are proving to be a prevailing strategy (Dey & Paul, 2013). Microbial and enzymatic methods have been proven to be not only effective but also further bring inexpensive and environmentally friendly and they do not produce waste metabolites in significant quantities (Hala & Laila, 2014). The feasible technique for detoxifying hexavalent chromium to trivalent chromium has been proven to have potential use in bioremediation (Jain et al, 2012).

1.3.11.1.1 Biosorption & Bioaccumulation

Bioremediation can be categorized into biosorption and bioaccumulation where bacteria, fungus, yeast and mold have been most widely used. Best on several factors such as pH ionic strain, biomass concentration and temperature, the microorganisms can detoxify metals through physico chemical retention between the metal and functional groups located on the surface of the cell (Luciene et al, 2015). A chief example of natural microorganisms is lactobacillus which has the ability to bind with metals including Cr (VI) to detoxify them in different regions of the human body (Monachese, Burton, &Reid, 2012). Only living biomass can contribute to bioaccumulation where as both living and dead biomass can participate in biosorption (Luciene et al, 2015).

1.3.11.1.2 Bio-mineralization

Bio-mineralization is the microbial process of accumulating and binding metals to form minerals. For example, the calcium ion is found in human cells as deposits which suggests a form of Bio-mineralization (Simkiss, 1977).

Another example of heavy metal precipitation is observed in the case of arsenic (Focardi et al, 2010).

1.3.11.1.3 Bio-augmentation-assisted phyto-extraction

The efficiency of bioremediation can be increased with the aid of bio-augmentation (Anna et al, 2016) and in combination with phyto-extraction. To work with plants it enables specific bacteria and /or fungus to accumulate metal and ultimately break down. The end goal of heavy metal attenuation to be executed by plants with the aid of bacteria is recommended (Lebeau, Braud & Jezequel, 2008).

Chapter 2: Materials & Method

2.1 Introduction

In this section the materials and ingredients that were used and the procedure that was followed to perform the study will be discussed. An overview of collection of sample, isolation steps will also be done. In addition, identification steps of the strain of Chromium resistant microorganism will also be discussed.

2.2 Chemicals

The list of the utilized chemicals and reagents are:

a. Extract of beef
b. Extract of yeast
c. Nutrient Agar
d. Nutrient Broth
e. Potassium Chromate
f. Diphenyl Carbazide
g. MOPS Buffer
h. Mueller Hinton Agar(MHA)

2.3 Glassware and Apparatus

Apparatus and glassware that were used are given here

Table 2.1: Instruments used for the purpose of study and their functions.

Instruments	Functions
Autoclave	Sterilization
Analytical Balance	Weight measurement
Laminar Airflow	Aseptic environment maintenance
pH Meter	pH measurement
BOD Incubator	Culture incubation
Water System	Preparation of stock culture

UV-Vis Spectrophotometer	Absorbance measurement
Centrifuge	Supernatant collection
Water Bath	Solubilization of media
Microscope	Observation of bacterial morphology
Shaking incubator	Culture of bacteria in liquid media
Micropipette	To collect chemical and media in minute volume

2.4 Collection of sample

The magnitude of Buriganga River is 17 km and it falls in the southern part of the North Central Region of Bangladesh. There is a meeting point of the Padma (Ganges) and upper Meghna rivers near Buriganga. The river flows through the south- western part of Dhaka city. (Rahman & Al Bakri, 2010). The River Buriganga is now counted as one of the most contaminated rivers in Bangladesh. Industrial areas are getting spreader around Dhaka city for couple of years and also number of industries are elevated. In most of the cases they do not treat their wastes before its exposure to the environment and directly throw it into water of Buriganga. As a result, wastes without treatment are shockingly increased in Buriganga and cause a threat to the living environment. (Ahmad, M. K., Islam, S., Rahman, M. S., Haque, M. R., and Islam, 2010). Moreover, the wastes of Hazaribag tannery are directly thrown to the Buriganga River. To conduct this study, the water sample was collected near the area of Hazaribag tannery.

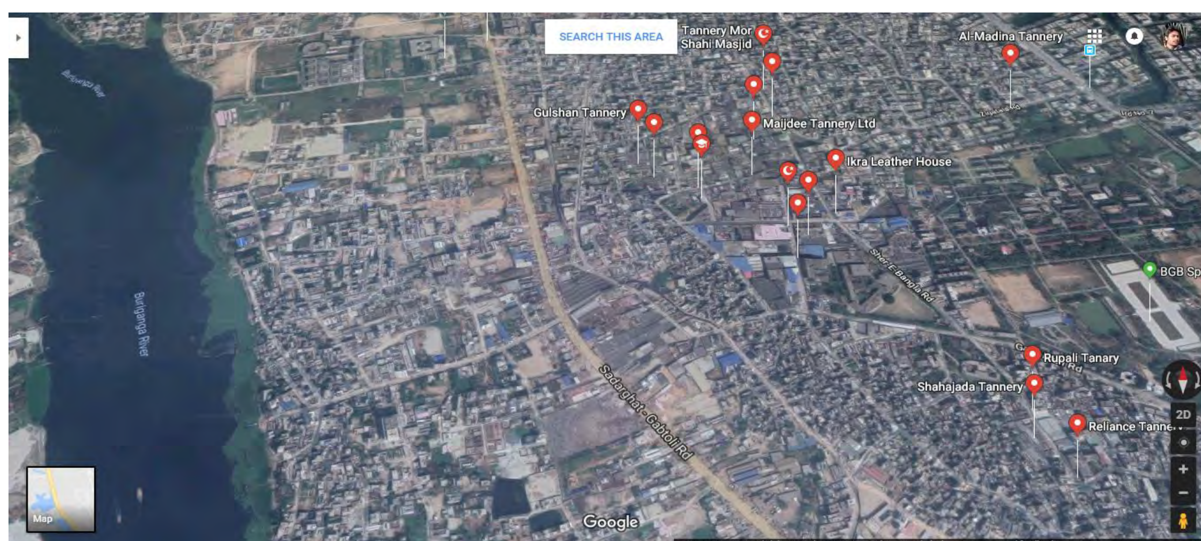

Fig 2.1: Hazaribag tannery area

2.5 Isolation and condition of culture

Isolation of the microorganisms from the collected sample was done by maintaining standard protocol. Chromium resistance microorganism was isolated by inoculation of sample into agar medium. 100 micro liter of water containing sample was inoculated into the nutrient agar dish where potassium chromate (K2CrO4) was added as complementary of 2mM of Cr (VI). Then the media was incubated for 24 hours at 37˚C and some bacterial colony was observed eventually. After that an agar medium was prepared containing 2.8 gm of agar in 100 ml of water. Then it was autoclaved for forty-five minutes at 121˚C under fifteen Lb pressure for sterilization. Followed by potassium chromate was incorporated into the media and transferred into the petri dishes for settlement. Isolated bacterial colony was then picked up with sterilized toothpick and streaked on agar medium comprising 2,3,4,5 mM of Cr (VI). Again the nutrient medium was incubated for 24 hours at 37˚C. It was repeatedly done with successively greater concentration of Cr (VI) like, six, seven, ten, fifteen, twenty, twenty-five, thirty, thirty-five, forty mM of hexavalent Chromium till the MIC (minimum inhibitory concentration) of the restrain of bacteria was achieved. Significant development and faster Cr (VI) degradation kinetics of the particular bacterial species was found in 40mM concentration of Cr (VI) during twenty-four hours of incubation at 37°C, were considered as Cr (VI) resistant. A specific strain fit for developing at this condition was chosen for further investigation. 8 single colonies were isolated from various nutrient agar plates containing Chromium resistance and fixation property after performing respective procedures and different name tags were given like 3W100, 4W100.

2.6Chromium reduction profile of chromium resistant bacteria

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

2.6.1. Chemical Preparation

2.6.1.1 10ml 3M H_2SO_4 preparation

At first, 8mL purified H_2O was poured into one falcon tube. Then 1670 µL of conc. H_2SO_4 was added dropwise into the falcon tube by using micropipette. Finally 330 µL H_2O was added to make the solution up to 10 ml.

2.6.1.2 Diphenyl Carbazide preparation

Firstly, 0.025 gm DPCZ was taken in a falcon tube. Then 9.67mL acetone and afterward 330µL of 3M H_2SO_4 was added to it. Mixing was done properly to ensure a uniform solution of DPCZ.

2.6.1.3 Preparation of MOPS buffer

At first, 0.1g sodium hydroxide has been taken into 50mL H_2O to prepare 50mL 1N sodium hydroxide solution. Then, to prepare 20mM buffer of MOPS, MOPS powder of 334.88mg has been putted with 80mL of purified H_2O. pH of MOPS buffer was adjusted to 7 through the addition of adequate quantity of 1N sodium hydroxide into the solution of MOPS buffer.

2.6.1.4 5mM 10ml K_2CrO_4 preparation

At first, for the preparation of 1M potassium chromate solution, 1.94g potassium chromate had been melted into 10mL purified H_2O. Then, filtration was done for this solution utilizing membrane sieve containing 0.45micron size of the pore. At last, dilution was done for this solution up to 5mM and it was preserved for further use.

2.6.2 Process of experiment

2.6.2.1 Standard curve preparation

2.6.2.1.1 Sample preparation for reaction

Several solutions of sample of subsequent concentrations have been managed. Absolute volume of every sample was one milliliter.

Table 2.2: Sample preparation for standard curve

Concentration Final	Quantity of 5mM K_2CrO_4 solution	Amount of NB added	Final volume to solution
50µM	10µ L	990µL	1ml
100µM	20µ L	980µL	1ml

150µM	30µ L	970µL	1ml
200µM	40µ L	960µL	1ml
300µM	60µ L	940µL	1ml
400µM	80µ L	920µL	1ml
500µM	100µ L	900µL	1ml
600µM	120µ L	880µL	1ml

2.6.2.1.2 Reaction protocol for standard curve

A falcon tube was taken which contains 600 microliter of sample and was mixed with 1.2ml 20Mm buffer of MOPS, 99 microliter 3M sulfuric acid, 981 microliter of purified water, lastly 120ml of DPCZ was added and shaken. After few moments it was observed that, the solution turns into purple color. After that the absorbance of that solution was obtained using UV-visible spectrophotometer at 540nm.

2.6.2.2 Evaluation of reduction profile of selected isolates

2.6.2.2.1 Procedure

Day 0

In the conical flask two nutrient broth medium was made at volume of 10 ml which was autoclaved for sterilization and put into the laminar airflow. From previously stored culture the sample was mixed with one nutrient medium and the other was left as untreated. Then the conical flask containing sample was kept inside the shaking incubator to culture bacteria at 37 degree Celsius for 24 hours and the other one was left in the laminar airflow.

Day 1

The next day two nutrient broth medium of 25ml was prepared in two conical flask and autoclaved. Then in the laminar airflow 15 microliter of potassium chromate was added in the conical flask to make the concentration 600 microliter. Then 2ml of sample was withdrawn into a falcon tube from the 10ml conical flask which was kept for the incubation of bacteria on previous day. Then the absorbance of sample was taken at 600nm to see the growth of culture. After that calculation was done to identify the amount to be added into the 25ml

culture from the 10ml culture to get 0.2 OD. Then the calculated amount was withdrawn from the 10ml culture into a falcon tube and centrifuged by which the cell got separated from the liquid. The liquid was withdrawn leaving the cell in the falcon tube. Again the same calculated amount also withdrawn from one 25 ml nutrient solution and mixed with the cell in the falcon tube and vortexed to mix them properly. Then they are transferred into the 25ml conical flask again from the falcon tube. The other 25ml was counted as the blank. 2ml solution from each 25ml conical flask was withdrawn in two falcon tubes and then the conical flasks were put into the shaking incubator to culture microorganisms in the hexavalent Chromium condition. Then the absorbance of the bacteria containing sample is taken at 600 nm. Like previously taken 2ml solution from each conical flask into falcon tube, after every 1.5 hours the sample and the blank were collected throughout the day and absorbance was taken to see the cell growth. Then it was centrifuged to collect the supernatant with which the reaction stated in 3.6.2.1.2 was performed to check the chromium level. This process was continued to next day by keeping the 25ml solution in incubator overnight to see its overnight activity towards chromium reduction.

2.7 Antibiotic resistance among Chromium resistant isolates

2.7.1 Strain culture preparation in nutrient broth (NB)

In a conical flask 20ml of nutrient broth was prepared in order to see the resistance of particular strain towards antibiotics. Then inoculation of bacteria culture was done and the conical flask was put in the shaking incubator at 37 degree Celsius for 24 hours.

2.7.2 Inoculation of the test plates

Mueller Hinton Agar was used to make culture plate. Using sterilized cotton swab which was dipped into the conical flask containing the overnight culture of strain, spreading over the MHA surface in the culture plate was done. This step was recurred for two more times by moving at 60° circle to assure the inoculum's uniformity.

Finally the agar's rim was mopped. The lid of the plate was opened for 3 to 5 minutes. It should not be uncovered for more than 15 minutes otherwise moisture will be absorbed over the plate.

2.7.3 Application of antibiotic discs

The following antibiotic discs were spread over the plate after inoculation of the strain to see the antibiotic resistance profile.

Gentamicin (CN: 10 mg)

Ofloxacin (OF: 5 mg)

Vancomycin (VA: 30mg)

Sulphametronazol / Trimethoprim (SXT: 25 mg)

Azithromycin (AZM: 15mg)

Neomycin (N: 30 mg)

Ceftriaxone (CRO: 30 mg)

Cefuroxime Sodium (CXM: 30 mg)

Penicillin-G (P: 10 mg)

Kanamycin (K: 30)

Cefixime (CFM)

Streptomycin(S: 10)

Amoxicillin (AML: 10)

Chloramphenicol(C: 30)

2.7.4 Incubation

As the discs were set over the plate, they were set in the incubator within 15 minutes at 37 degree Celsius for 24 hours.

2.8 Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the minimum antibacterial concentration which resists one particular bacterial strain's growth following incubation after 24 hours. MIC of chromate resistant bacteria was identified by counting the colony. Agar plate was prepared containing different concentrations of Potassium chromate from 2mM-30mM inoculated with 50 microliter nutrient broth culture which was kept in the incubator at 37 degree Celsius for 48 hours for colony growth.

2.9 Identification of isolate 4W100

Sequence of the bacterial strain was obtained by 16s rDNA sequencing. Purification of the sequence was done by Finch TV and saved as FASTA file. BLAST (Basic Local Alignment

Search Tool) was done in NCBI and the similar strains were collected on the basis of score. After that by using Bioedit and MEGA7 software phylogenetic tree was made to study the evolution and origin of 4W100.

Chapter 3: Result & Discussion

3.1 Isolation data of Chromium resistance bacteria

From the nutrient agar dishes containing different concentration of Chromium, Chromium resistant bacteria was isolated and was given different identical label, such as 4W100.

3.2 Chromium reduction profile of Chromium resistant bacteria

3.2.1 Standard curve

The standard curve was obtained using the method stated in 3.6.2.1.2. After that, utilizing the value of absorbance, plotting was done in a graph using Microsoft Excel Software. Results which have been obtained are given below:

Table 3.1: Data of standard curve of hexavalent Chromium:

Concentration(μM)	Absorbance
50	0.294
100	0.624
150	.907
200	1.214
300	1.675
400	2.117
500	2.587
600	2.875

From these data, one standard curve was found and that is given below:

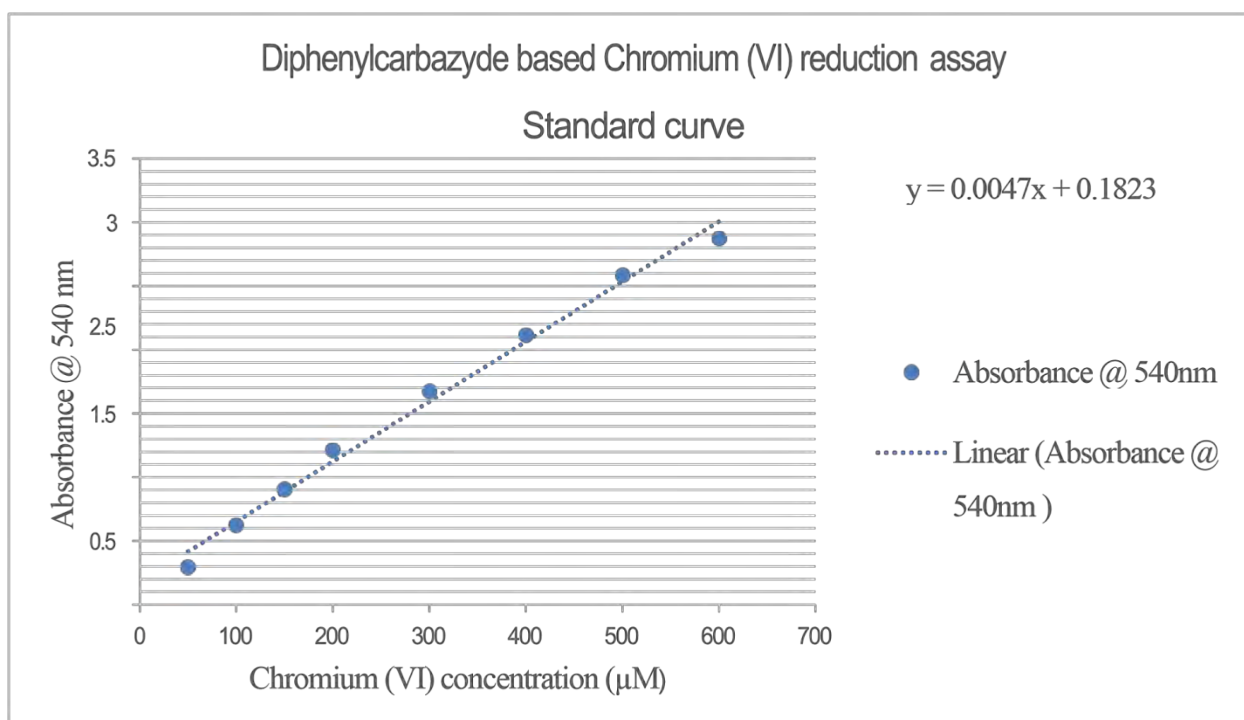

Figure 3.1: Standard curve of hexavalent Chromium

3.2.2 Reduction profile of isolate 4W100

The tests were undergone at different conditions like, altering the pH and temperature, and the outcomes of the tests are precisely described below.

Table 3.2: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 37ºC, pH 5.5

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	397.3120567	0.997	451.141844	0.014
1.5	379.5815603	1.384	449.2269504	0.019
3.0	155.0425532	1.794	440.858156	0.011

4.5	13.34042553	2.125	451.2836879	0.01
6.0	0	2.23	451.3546099	0.013
24	0	2.81	452.7021277	0.019

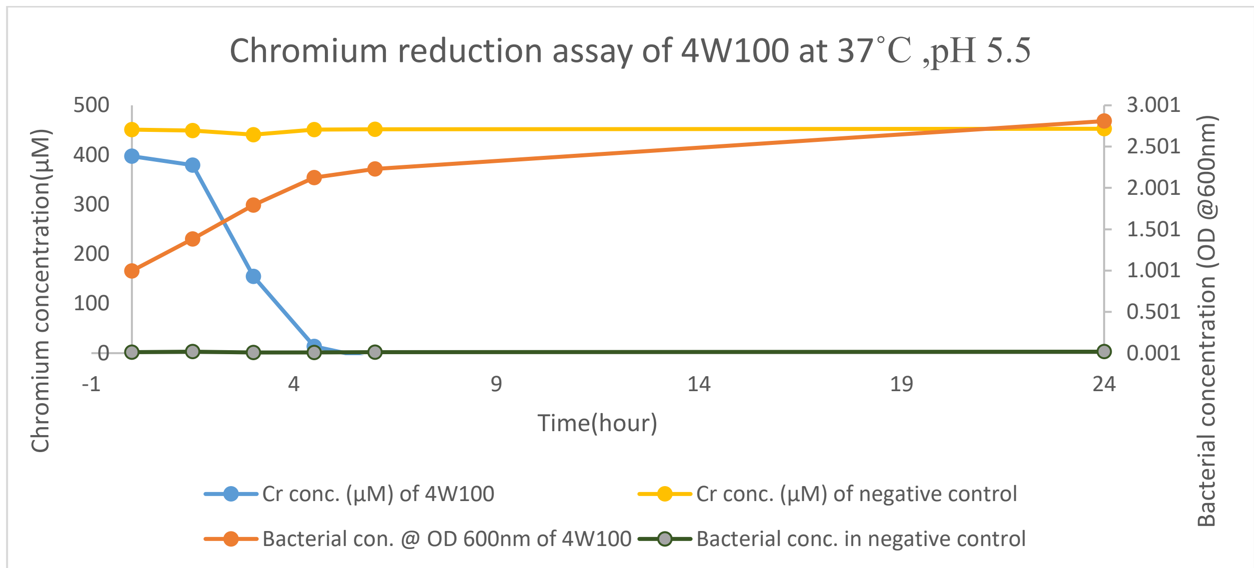

Figure 3.2: Chromium Reduction Vs Cell Growth in isolate 4W100 at 37˚C, pH 5.5

From the above figure 3.2, it is clear that, there was no change in chromium concentration at first 1.5 hours. Then after 3 hours concentration of Chromium changed drastically from 397.31µM to 155.04 µM. After 6 hours Chromium concentration was 0. On the other hand, bacterial concentration was getting increased. After 24 hours bacterial concentration was 2.81 and Chromium concentration was 0. In negative control no bacterial growth was observed. It indicates that at 37˚C, pH 5.5 bacteria show Chromium reduction property as well as bacterial growth is also noticeable.

Table 3.3: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 37ºC, pH 7

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	464.1914894	0.984	451.141844	0.014
1.5	442.7730496	1.019	449.2269504	0.019
3.0	171.4964539	1.386	440.858156	0.011
4.5	122.5602837	1.74	451.2836879	0.01
6.0	0	2.104	451.3546099	0.013
24	0	2.87	452.7021277	0.019

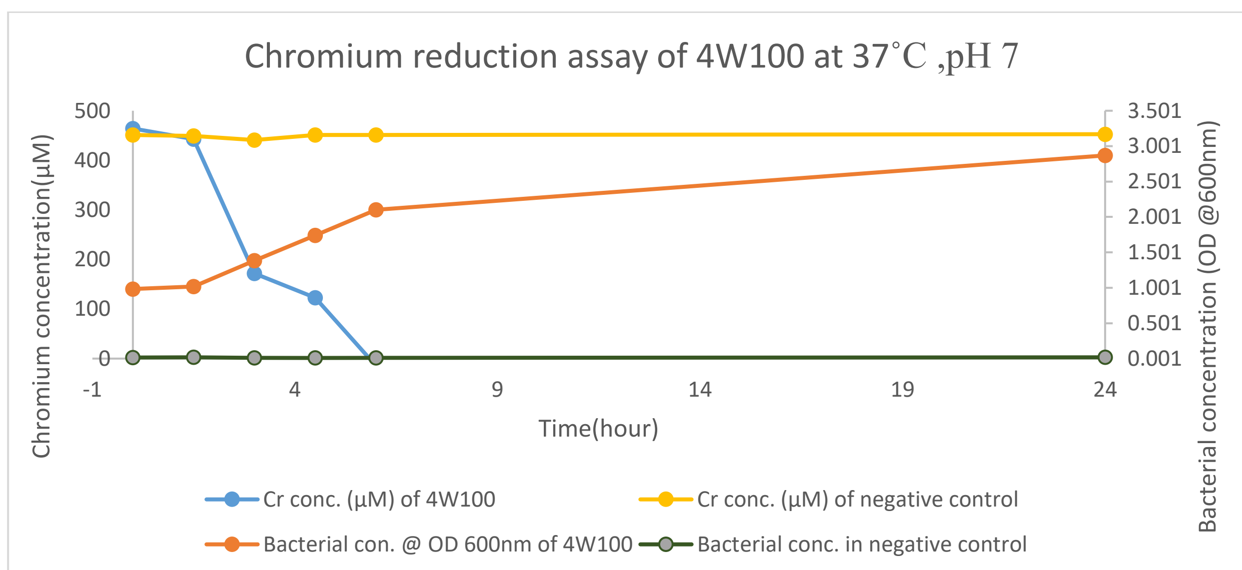

Figure 3.3: Chromium Reduction Vs Cell Growth in isolate 4W100 at 37˚C, pH 7

From the above figure 3.3 it is clear that, Chromium concentration reduced dramatically after 3 hours as it is seen, at 0 hour Chromium concentration was 464.19 µM and after 3 hours it

was 171.5 µM. After 6 hours Chromium concentration was 0. That indicates, bacteria completely reduced the Chromium after 6 hours. Additionally, bacterial concentration got increased after 24 hours and there was no bacterial growth in negative control.

So, at pH 7 and 37˚C temperature this strain shows good Chromium reduction activity.

Table 3.4: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 37ºC, pH 8.5

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	354.8297872	0.897	451.141844	0.014
1.5	354.7588652	1.18	449.2269504	0.019
3.0	161	1.426	440.858156	0.011
4.5	147.3829787	1.706	451.2836879	0.01
6.0	107.3120567	2.031	451.3546099	0.013
24	0	2.749	452.7021277	0.019

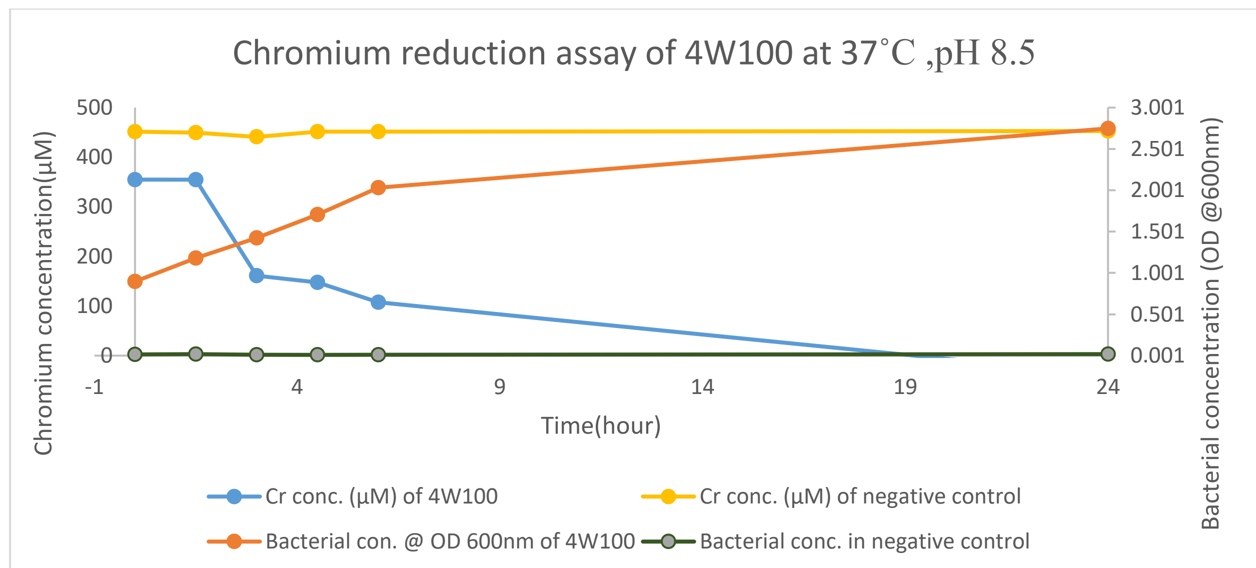

Figure 3.4: Chromium Reduction Vs Cell Growth in isolate 4W100 at 37˚C, pH 8.5

At pH 8.5 and temperature 37˚C, it is found from the above figure 3.4 that, Chromium concentration reduced from 354.83 µM to 161 µM after 3 hours. Bacterial concentration also

increased successively. Chromium concentration got reduced throughout the day and after 24 hours it was 0. No bacterial growth was observed in negative control. Significant bacterial growth was observed after 24 hours like, from 0.897 to 2.749. It shows that, this bacteria is Chromium resistant as well as have good chromium reduction capability.

Table 3.5: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 25ºC, pH 5.5

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentratio n at 600nm
0	366.177305	0.868	451.4255319	0.01
1.5	364.4042553	0.698	450.787234	0.006
3.0	346.6028369	0.692	449.7234043	0.005
4.5	338.1631206	0.555	449.0851064	0.009
6.0	300.929078	0.748	450.0780142	0.004
24	0	2.396	444.9716312	0.007

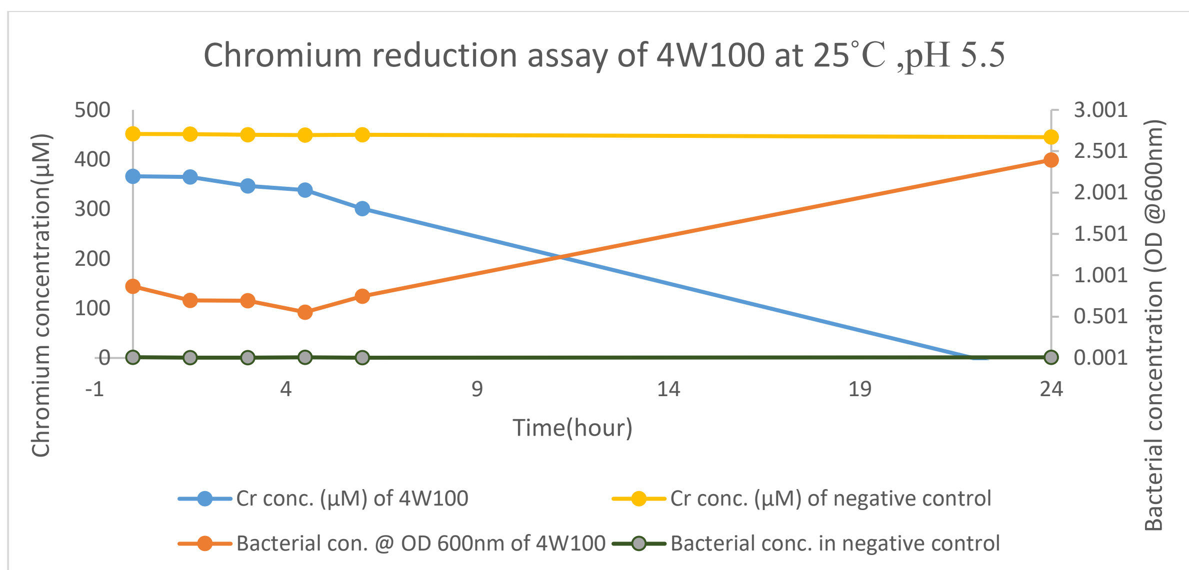

Figure 3.5: Chromium Reduction Vs Cell Growth in isolate 4W100 at 25˚C, pH 5.5

From the above figure 3.5 it is clear that, at 0 hour Chromium concentration was 366.18 µM and it got slightly decreased in rest of the day. A marked reduction of Chromium concentration was observed after 24 hours which was 0 µM. Initial bacterial concentration was 0.868 which got decreased up to 4.5 hours. Then after 6 hours it started to increase and finally after 24 hours it increased in a large number which was 2.396. There was no bacterial growth in negative control. So, it showed that, sample 4W100 has Chromium reducing capability at pH 5.5, 25˚C.

Table 3.6: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 25ºC, pH 7

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	333.8368794	0.858	451.4255319	0.01
1.5	328.3049645	0.744	450.787234	0.006
3.0	324.2624113	0.619	449.7234043	0.005

4.5	310.0780142	0.556	449.0851064	0.009
6.0	296.3900709	0.626	450.0780142	0.004
24	0	2.11	444.9716312	0.007

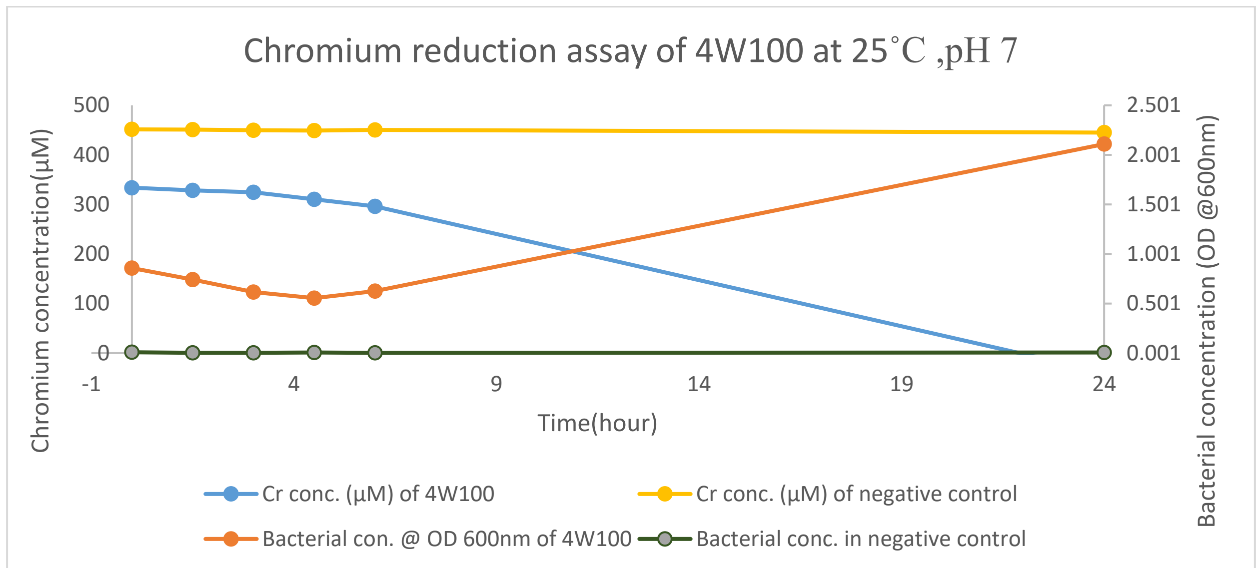

Figure 3.6: Chromium Reduction Vs Cell Growth in isolate 4W100 at 25˚C, pH 7

In this figure 3.6 it is cleared that, at pH 7, temperature 25˚C, Chromium concentration was 333.84 µM at 0 hour. Then it got decreased at a slow pace. After 6 hours Chromium concentration was 296.4 µM. Finally, a marked reduction of the concentration of Chromium was found after 24 hours which was 0. However, growth of bacterial concentration was observed from 0.858 to 2.11 during 24 hours at 600 nm. No bacterial growth or Chromium reduction was found in negative control. Therefore, it can said that, sample 4W100 can reduce chromium concentration and at the same also Chromium resistant.

Table 3.7: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 25ºC, pH 8.5

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	412.2056738	0.927	451.4255319	0.01
1.5	409.6524823	0.931	450.787234	0.006
3.0	403.5531915	0.936	449.7234043	0.005
4.5	404.1205674	1.117	449.0851064	0.009
6.0	374.3333333	1.21	450.0780142	0.004
24	0	2.443	444.9716312	0.007

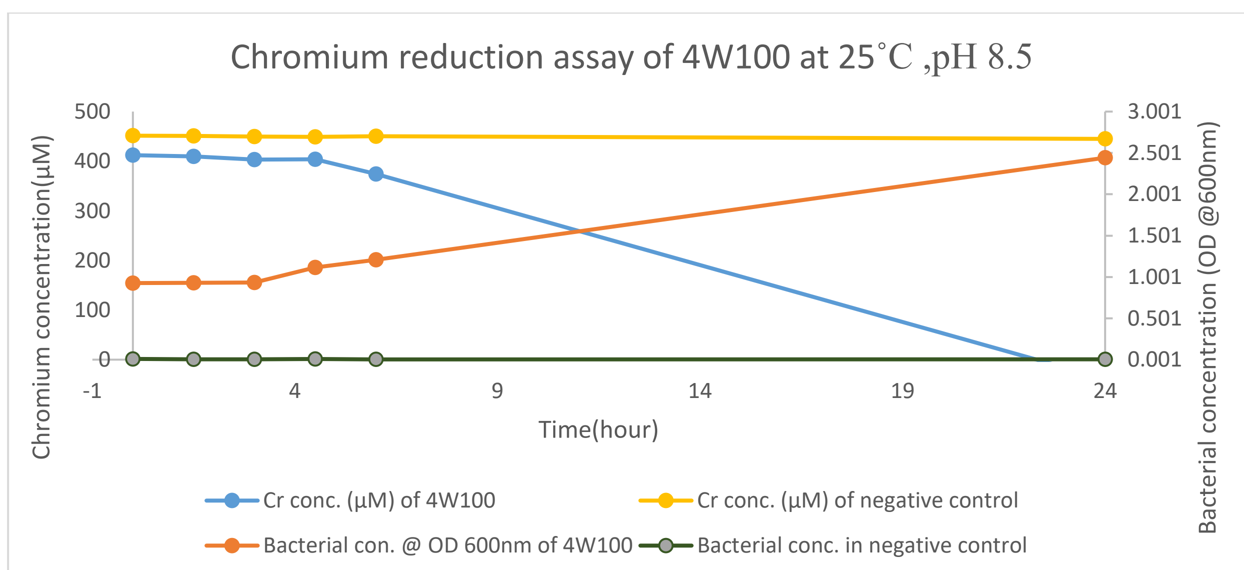

Figure 3.7: Chromium Reduction Vs Cell Growth in isolate 4W100 at 25˚C, pH 8.5

From the above figure 3.7, it is observed that, at 540nm, initially Chromium concentration was 412.21µM and remained almost same up to 4 hours. Then it started to reduce after 6 hours when Chromium concentration was 374.33 µM and eventually after 24 hours concentration was 0. Bacterial concentration was 0.927 initially which remained constant up to 3 hours. Then after 4.5 hours bacterial growth was increased. During this 24 hours no bacterial growth was found in negative control. So, sample 4W100 shows Chromium reduction activity at 25ºC temperature and pH 5.5.

Table 3.8: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 42ºC, pH 5.5

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentrati on at 600nm
0	439.5815603	0.815	435.2553191	0.002
1.5	438.6595745	0.658	439.3687943	0.003
3.0	373.9078014	0.647	430.787234	0.001
4.5	330.2907801	1.08	443.9078014	0.003
6.0	22.34751773	1.513	437.7375887	0.003
24	0	2.051	439.9361702	0.011

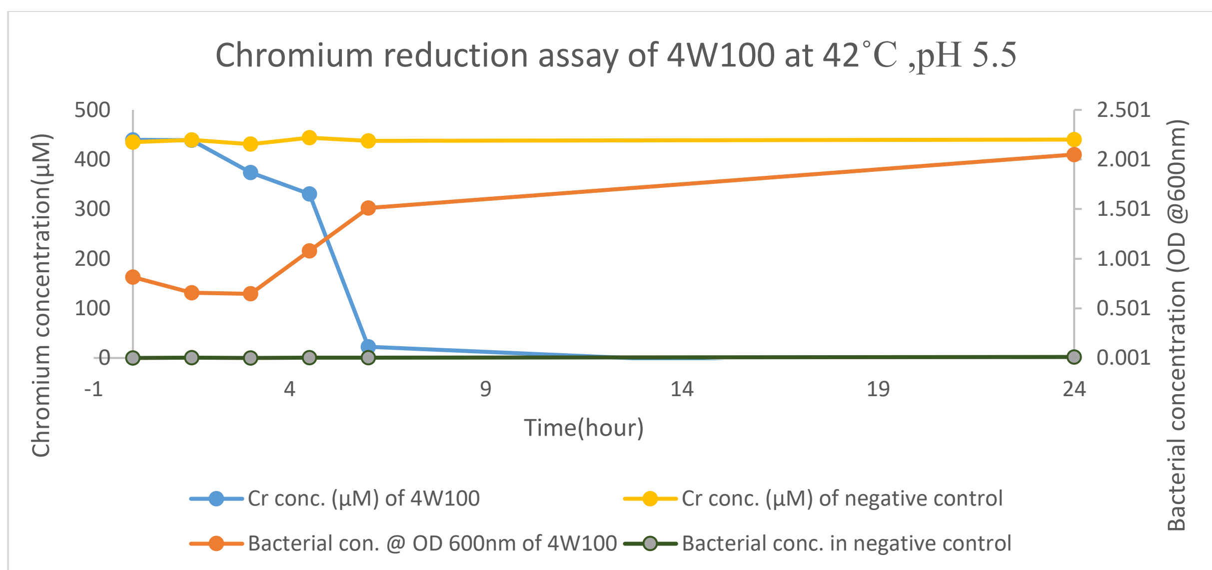

Figure 3.8: Chromium Reduction Vs Cell Growth in isolate 4W100 at 42˚C, pH 5.5

It is clear from the above figure 3.8 that, 4W100 reduced Chromium gradually as initial concentration of Chromium was 439.6 µM and after 4.5 hours it was 330.3 µM. Then after 6 hours it fell drastically as concentration found was 22.34 µM and finally after 24 hours Chromium concentration was 0 at 540 nm. Bacterial concentration got decreased up to 3 hours and then started to increase after 4.5 hours which continued up to 24 hours. No changes in Chromium concentration or growth of bacteria was found in negative control. Lastly, it is confirmed that, 4W100 is Chromium resistant and has Chromium reducing capability.

Table 3.9: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 42ºC, pH 7

Sample			Negative Control	
Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	456.6028369	0.91	435.2553191	0.002
1.5	452.7730496	0.737	439.3687943	0.003
3.0	396.106383	0.818	430.787234	0.001
4.5	204.9007092	1.511	443.9078014	0.003

6.0	0	1.75	437.7375887	0.003
24	0	2.079	439.9361702	0.011

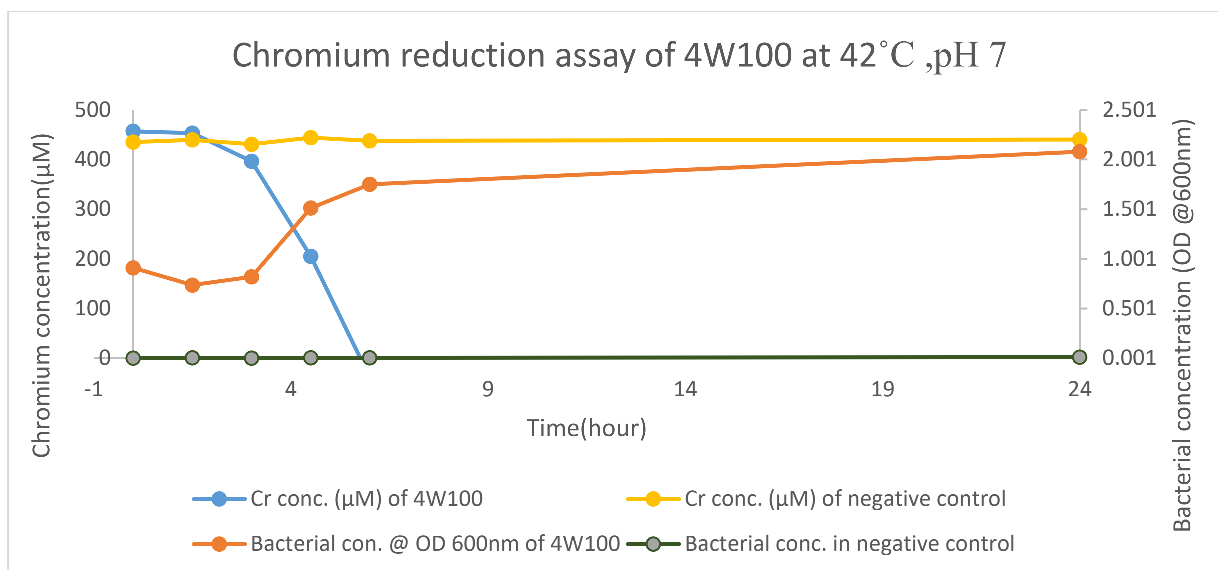

Figure 3.9: Chromium Reduction Vs Cell Growth in isolate 4W100 at 42˚C, pH 7

From the above figure it is observed that, in sample 4W100 at first Chromium concentration was 456.60 µM and it slowly declined. Then after 4.5 hours concentration of Chromium was 204.9 µM and after 6 hours it rapidly got decreased and concentration was nearly 0. Bacterial growth was observed from 0.91 to 2.079 during 24 hours where at first up to 3 hours bacterial concentration got decreased and then started to increase. On contrary, no reduction in the concentration of Chromium of bacterial growth was obtained in negative control. As a result, it can be said that sample 4W100 is both Chromium resistant and has Chromium reducing property at 42˚C temperature and 7 pH.

Table 3.10: Isolate 4W100: Chromium reduction profile Vs. Cell Growth at 42ºC, pH 8.5

Time (Hours)	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm	Chromium concentration on (µM) at 540 nm	Bacterial concentration at 600nm
0	429.2978723	0.856	435.2553191	0.002
1.5	429.6524823	0.765	439.3687943	0.003
3.0	382.9858156	0.845	430.787234	0.001
4.5	312.7730496	1.466	443.9078014	0.003
6.0	15.18439716	1.689	437.7375887	0.003
24	0	1.846	439.9361702	0.011

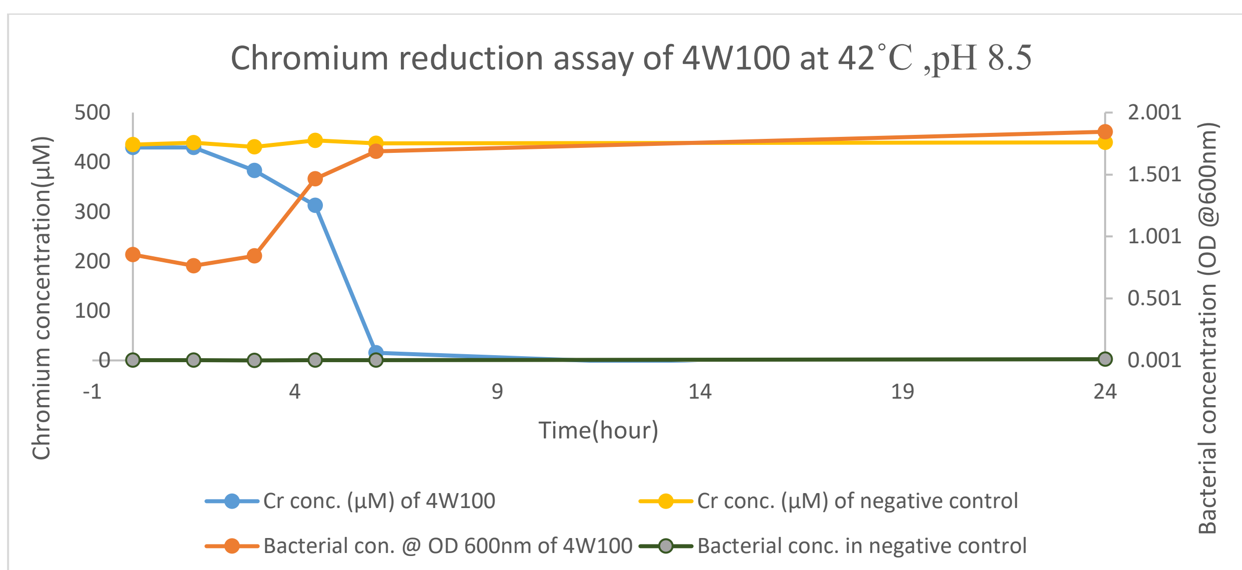

Figure 3.10: Chromium Reduction Vs Cell Growth in isolate 4W100 at 42˚C, pH 8.5

From the above figure, it is clear that, at 42˚C temperature and pH 8.5, sample 4W100 shows Chromium reducing property as initial concentration of Chromium was 429.3 µM which

gradually got decreased up to 4.5 hours. Then after 6 hours concentration got decreased drastically which was 15.18 µM. After 24 hours Chromium concentration was 0. Bacterial growth was also observed at 600 nm. On the other hand, no bacterial growth or changes in Chromium concentration was found in negative control. So, 4W100 is a Chromium resistant strain which also contain Chromium reducing property.

3.3 Antibiotic resistant among Chromium resistant isolate 4W100

This test was done by distributing and fixing 14 discs of Antibiotic on the surface of MHA plates which have been gone through inoculation and then incubation was done for 24 hours. After incubation, the activities of antibiotic discs against 4W100 isolate were determined by measuring the diameter of zone of inhibition in millimeter with the help of a transparent scale. The outcomes which has been obtained are given below:

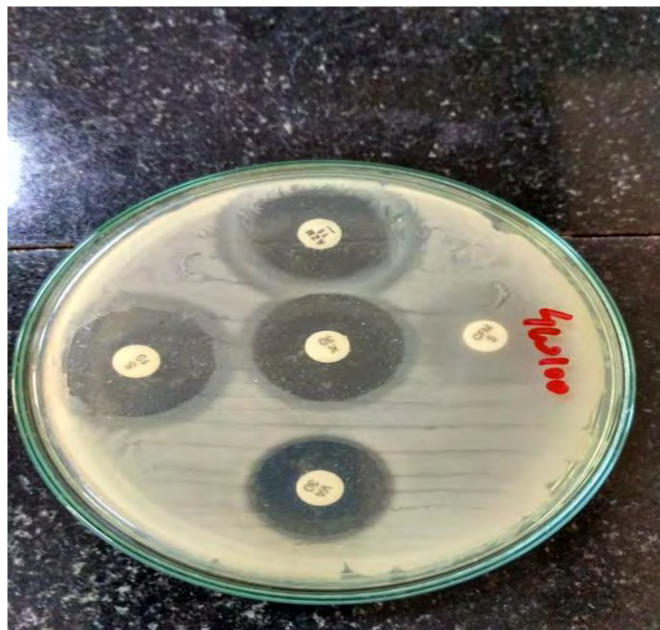

Figure 3.11: Zone of inhibition of antibiotics disc in isolate 4W100

Table 3.11: Antibiotic resistance profile

Name of antibiotic disc	Diameter of zone of inhibition (ZI) of 4W100 on mm
1. Chloramphenicol (C:30mg)	20
2. Kanamycin(K:30)	22
3. Gentamicin (CN:10mg)	20
4. Ofloxacin (OF:5mg)	17
5. Vancomycin (VA:30mg)	19
6. Sulphametronazol/Trimethoprim(SXT:25mg)	0
7.Ceftriaxone(CRO:30)	14
8.Amoxicillin(AMX)	0
9.Penicillin-G(P:10)	0
10.Neomycin(N:30)	19
11.Cefuroxime Sodium(CXM)	0
12.Azithromycin(AZM:15)	20
13.Cefixime(CFM)	0
14.Streptomycin(S:10)	22

It is clear from the above table that, highest zone of inhibition for sample 4W100 was recorded for Kanamycin and Streptomycin where the diameter of zone of inhibition for both was 22 millimeter. This 2 discs killed most of the 4W100 strain surrounding them compare to other discs and the sample showed no resistance towards Kanamycin and Streptomycin. The

zones of the inhibition for the discs of Gentamicin, Vancomycin, Neomycin, Ofloxacin, Ceftriaxone, and Azithromycin were in the range of 14-20 millimeter. This indicates that, sample 4W100 is less susceptible to them. However, Sulphametronazol/Trimethoprim, Amoxicilin, Penicillin-G, Cefuroxime Sodium, Cefixime showed no zone of inhibition. That means the sample 4W100 is resistant towards them.

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

3.4.1 MIC of isolate 4W100

In different concentrations of Chromium, different numbers of colonies have been found and they are tabulated below:

Table 3.12: MIC of isolate 4W100

Chromium concentration	Number of colonies
2mM	36
4mM	24
6mM	18
8mM	13
10mM	11
12mM	8
14mM	5
16mM	3
18mM	2
20mM	0
22mM	0
24mM	0

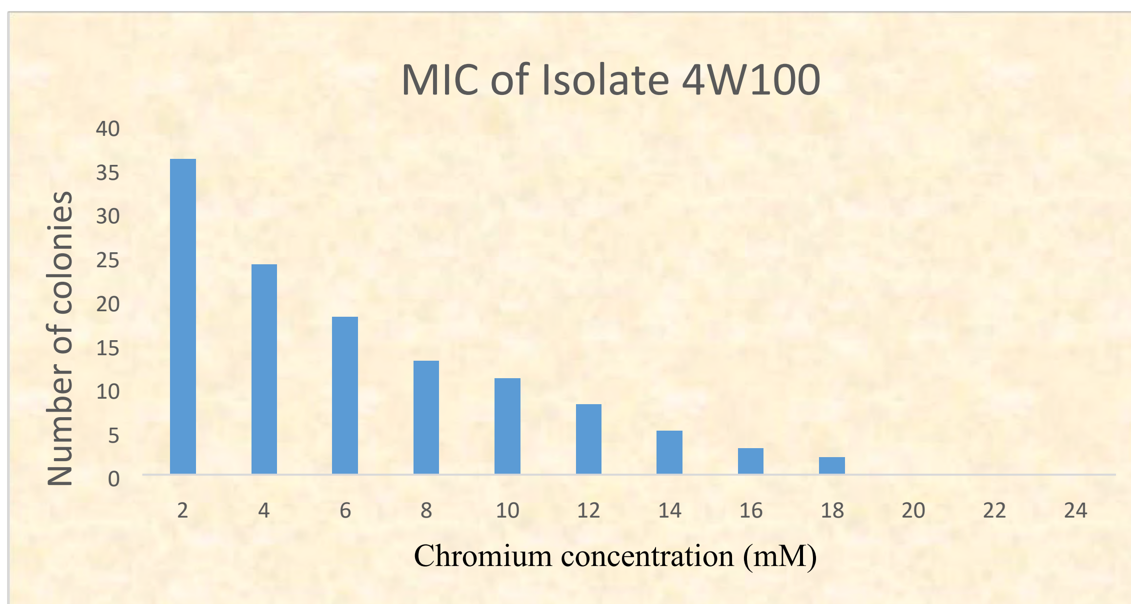

Figure 3.12: MIC of isolate 4W100

From the **Table 3.12** it is seen that, the isolate 4W100 tolerates the Chromium concentration up to 18mM, which means, it was resistant toward the Chromium concentration till 18mM. On the contrary, isolate 4W100 was totally susceptible from the Chromium concentration of 20mM because no colony was found in 20mM Chromium plate and also in the Chromium plate of more higher concentration. So, 20mM was the Minimum Inhibitory Concentration for 4W100 isolate.

3.5 Identification of Isolate 4W100

For the identification of the bacterial isolate, 16s rDNA sequencing was used. After that, BLAST of the sequence was done. Then using different software like Finch TV, BioEdit, MEGA7, data purification and phylogenetic tree of the sample was prepared. From the phylogenetic tree we got to know that the sample 4W100 is 100% similar to the strain *Bacillus pumilus* and origin of the sample is also *Bacillus pumilus*. So, identification of the sample is achieved.

Figure of the phylogenetic tree is given here

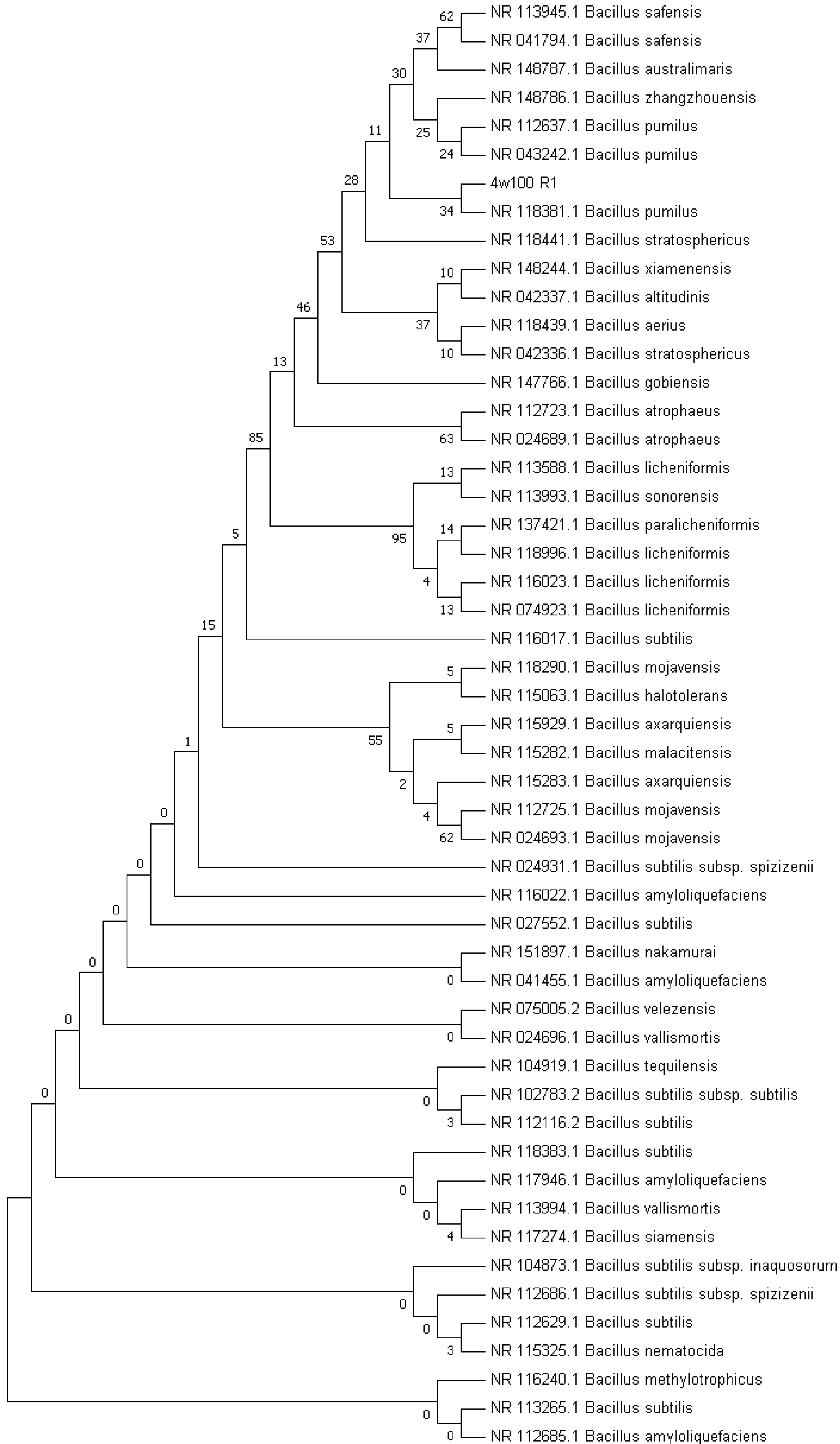

Figure 3.13: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985).The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura & Nei, 2004) 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 820 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

3.5 Discussion

Chromium is a toxic heavy metal which is exposed to environment in several ways like tannery industry, dyeing industry and has become a threat to human life and other livestock. Especially Cr (VI) is carcinogenic in nature. So, it has become a great concern to find out the solution to this life threatening issue. The purpose of this study was to find out any biological strain which will be capable of reducing toxic Chromium from the environment especially from water.

The sample was collected from the specified polluted area of Hazaribag tannery. The wastages of Hazaribag tannery are directly thrown into the nearby areas which ultimately goes into Buriganga River and causes several metallic toxicity along with Chromium. That is why this area was targeted for collection of sample so that a remediation can be achieved biologically from the polluted area itself. After collection, isolation and purification of the sample was obtained. Then following the standard protocol, a standard curve was prepared. Using the standard curve the absorbance of Chromium and bacterial solution was recorded at different temperature and pH. For instance, at 37˚C temperature and pH 5.5 and 7 it was found that, Chromium got completely reduced after 6 hours and at pH 8 it got reduced after 24 hours. The study was conducted in two more temperature condition like, at 25˚C and 42˚C. At 25˚C temperature in all three pH, Chromium got reduced after 24 hours. On the

other hand, at 42˚C most efficient reduction property was found at pH 7. So, considering all the data, it can be said that the isolate 4W100 shows its Chromium reduction property at 37˚C temperature and pH 5.5 and 7 and also at 42˚C and pH 7 most prominently over rest of the pH level of different temperature. At the above mentioned temperature and pH, almost 100% chromium was reduced after 6 hours. So, it can be counted as an efficient condition.

From the antibiotic resistance profile, it was found that the isolate 4W100 is resistant to Sulphametronazol/Trimethoprim, Amoxicilin, Penicillin-G, Cefuroxime Sodium, Cefixime antibiotics which means these antibiotics cannot destroy 4W100 isolate. On the other hand, two antibiotics named Kanamycin and Streptomycin were found to work effectively against the isolate 4W100. These two antibiotics had created a highest range of Zone of Inhibition around them which defies that the strain is not resistant against these two antibiotics. However, the strain has become resistant against most of the antibiotics which was proved by absence of Zone of inhibition around them. This strain also showed moderate susceptibility towards some other antibiotics like Gentamicin, Vancomycin, Neomycin, Ofloxacin, Ceftriaxone, and Azithromycin.

In addition, during the study of Minimum Inhibitory Concentration (MIC) it was found that, isolate 4W100 was capable of standing with up to 20 mM concentration of Chromium as no colony was found after that concentration. This information explains that this strain can cope up with the higher concentration of hexavalent Chromium. It can be related with the bacterial chromium reduction capacity in a way that they might be able to reduce higher concentration of Chromium from the environment as their chromium tolerance level is high.

Finally, 16s rDNA sequencing was done in the purpose of identification of the isolate. After the sequencing data were purified and a phylogenetic tree was made using various types of software which gives us the information about the predecessor of that strain which was *Bacillus pumilus*. Moreover, the experimental strain also showed similarities with the same strain as they were situated in the same branch on the phylogenetic tree. The origin of the strain was found also *Bacillus pumilus.* So, this finding can lead towards to more specific identification of the strain as well as the further investigation for finding the Chromium reductase enzyme.

Chapter 4: Conclusion

4.1Conclusion

From the study it is clear that, the isolated strain of 4W100 has the capability of reducing Chromium. It has the potential to reduce chromium from water biologically. For this Chromium reduction property of the bacteria, Chromium reductase enzyme in the strain can be utilized in chemotherapy in future. Moreover, the information that is revealed in this study indicates that, this strain is Chromium resistant. So it will be useful to find out a better way in treating the toxic effect of Chromium.

4.2 Further Direction

4W100 can be a source of Chromium reductase enzyme. Further study can be done to find out the correlation between antibiotic resistance profile and Chromium reduction assay. As the phylogenetic tree reveals the similarity of the strain with *Bacillus pumilus* so further investigation can be done to find out the respective enzyme responsible for Chromium reduction.

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