Isolation and Characterization of Carcinogenic Chromium reducing *Staphylococcus sciuri* from tannery effluent of Hazaribag area

A project submitted

by

Tanjina Afrin Hira

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

Certification Statement

This is to certify that this project titled "Isolation and Characterization of Carcinogenic Chromium reducing *Staphylococcus sciuri* 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 is referred as a highly poisonous metal for the natural environment which plays a role of cancer causing agent for the human. It causes different types of cancer and toxicity like skin cancer, lung cancer, pulmonary congestion etc. Moreover, its concentration is increasing day by day due to the untreated effluent from different industries. The purpose of this study was to identify the bacterial strain that are resistant to hexavalent chromium and has the capacity to convert the hexavalent chromium into trivalent chromium. To accomplish this purpose, sample was collected from the Buriganga River near the Hazaribag tannery and was purified and isolated using different parameters and protocol. Then the isolated strain was studied to explore their capacity to reduce the hexavalent chromium. The outcome was that the strain is resistant to hexavalent chromium as well as it has chromium reduction capacity which varies depending on the temperature and pH. That strain has worked efficiently at 37℃, pH 5.5 rather than the other temperature and pH. In addition, Antibiotic resistance profile was also examined and it is found that they cannot resist the effect of certain antibiotics. However, they became resistant to penicillin, azithromycin and some other antibiotics. Minimum Inhibitory Concentration was also examined to see their tolerance level at different chromium concentrations which shows that it can tolerate up to 5Mm of chromium concentration. Moreover, BLAST was performed for the strain which shows maximum similarity with the strain *Staphylococcus sciuri.* To ensure the findings, a phylogenetic tree was created which also shows resemblance with *Staphylococcus sciuri* with the experimental strain.

Cr (VI) is a hazardous metal for the environment which is polluting water and soil as they are discharged untreated to the environment. It becomes a necessity to abolish them to preserve the natural environment. Through this study, bacterial strain which has chromium reduction capacity will be identified with their chromium tolerance level so that they can play a role in the reduction of poisonous hexavalent chromium from the earth.

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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

1. Introduction

1.1.Background:

Chromium is known as a transitional metal which is found in environment in its hexavalent and trivalent form. Between them, hexavalent Chromium appears to have mutagenic and carcinogenic property due to its toxicity(LEIVE, 1965).It is also the reason of pulmonary congestion, pneumonia .bronchitis, nasal itching, skin irritation leading to ulcer (Saha, Nandi, & Saha, 2011).Chromium is mainly utilized in tannery industries, paints and pigments, wood preservation etc(Baldi et al., 1990).Therefore, Chromium(VI) is being discharged in water and making obstacles in control of polluted water (Mabrouk, Arayes, & Sabry, 2014).However, Cr(III) shows less toxicity and easily produces insoluble oxides and hydroxides above pH 5(Park, Keyhan, Wielinga, Fendorf, & Matin, 2000). In addition, trivalent Chromium is a requirement for natural growth of human and other animals (Bielicka, Bojanowska, & Wi??niewski, 2005). Thus transfer of Chromium (VI) to Chromium (VIII) has become more efficient method to reduce Chromium toxicity from the water (Ramírez-Díaz et al., 2008).

There are various methods which have been established to reduce Chromium toxicity from water. Precipitation, ion exchange, adsorption on charcoal, alum, kaolinite, ash, membrane filtration, liquid-liquid extraction etc. are the few example of them. However, those methods are not cost effective, has low efficiency at low concentration as well as lead to the production of toxic sludge which requires extra cost to be removed(Komori, Rivas, Toda, & Ohtake, 1990; Nandi, Laskar, & Saha, 2017).On the other hand bioremediation is an updated biotechnology which utilizes the microorganisms showing its potential through efficiency and reasonable costing. Microorganisms used in bioremediation process does not demolish the metal rather they change their oxidizing state through a cascade of mechanisms.

Bioremediation process which is an environmental friendly method works by the binding capacity of heavy metal to microorganisms even at dilute concentration (Riggle & Kumamoto, 2000).Thus bioremediation process is more advantageous than the others(Coelho et al., 2015).In this chapter, Chromium resistant bacteria strains will be assessed to identify their Cr(VI) to Cr(III) biotransformation capacity so that they can be used in the waste water

detoxification process along with their Minimum Inhibitory Concentration(MIC) to increasing Cr concentration so that we can get concerned about our future hazardous consequences.

1.2.Methodology

The cultured bacterial strain is recognized by rDNA sequencing. The important portion of the current study was to identify the amount of bacterial strain that will show resistant to chromium which was appeared in an adequate amount in the culture medium. The process was designed in such a way so that we can examine the chromium reduction profile of the specific bacteria. Chromium supplement nutrient broth media were used to run the laboratory experiment. The isolation process was done repeatedly until the pure bacterial strain is identified morphologically and then, Chromium reduction profile of the isolated strains were examined.

1.3.Aims and Objectives

The motive of this study is to assess the availability of Chromium resistant bacteria which has the ability to bio remediate the heavy carcinogenic metal Chromium as well as to identify the possible source of chromium reductase enzyme. The experiments were carried out to reach the following objectives-

1. Evaluation of the performance of the isolated bacteria in chromium contaminated environment.
2. Identification of Minimum Inhibitory Concentration (MIC) to assess the tolerance of the isolated bacteria.
3. Investigation of the antibiotic resistance profile of the cultured bacterial strain.

1.4 Literature Review

1.4.1 Introduction

Ever since the industrial Revolution, the use of metals have been integral on a daily basis. Metals are utilized everyday on numerous purposes; metals including Arsenic, Copper, Nickel, Iron, Zinc, Aluminum and Chromium. But using much heavy metals on a daily basis bears hazardous consequences. Chromium is an example of such metal; which is used in large quantities in the production of alloys (Rifikin, Gwinn & Bower, 2004). Both Nickel and Chromium are used in equal ratios in making alloys (McGrath & Smith, 1990).

Chromium establishes itself as a transition metal, located between group II and III in the Periodic Table. Both Cr 3+ and Cr 6+ forms are readily available in the earth naturally, therefore explaining its location in the 21st position (Chandra & Khulshreshta, 2016). Excluding its most common valence states of +3 and +6, Chromium can adopt several oxidation states form Cr2+ to Cr6+ (Chandra & Khulshreshta, 2016). Chromium is also applied in dyes and pigments, leather tanning and electroplating. Chromium is used as rust protectants, clothes and high thermal heaters (Rifikin et al, 2004). Utilizing Chromium in these process leads to severe toxic drainage along with other heavy metals, which deposits and mixes along with water, air and soil. These scenario is commonly observed not only in underdeveloped but also in developed countries as well (Thacker and Madamwar, 2005). And even though chromium is considered as an essential micronutrient in the living body to aid glucose digestion, catalytic activities and in nucleic acid development, it also comes with hazardous consequences. The hexavalent form of chromium is known to be toxic and mutagenic to all cellular life forms (Thacker and Madamwar, 2005). When in human body, it travels through the cell membrane and oxidizes any intracellular component it comes into contact with. Aside ingestion, dermal contact of chromium also creates a systemic impact causing dermatitis (Thacker and Madamwar, 2005). On the contrary the trivalent form of chromium is deemed less harmful since its oxidation potential varies from Cr (VI) and it is further unable to cross through the plasma membrane. Chromium exists as Ferric Chromate in many mining sources in South Africa along with mineralized forms in several other countries including Finland, Philippines, Zimbabwe, India and Brazil.

1.4.2 Chemistry:

Chromium originates as a hardened, gray metal bearing no odor .In dilute forms of Hydrochloric and Sulfuric acids they solubilize readily whereas they fail to do so in dilute and concentrated alkali (Merk, 1989). In the periodic table it bears the symbol Cr with an atomic number of 24 and a boiling and melting point of 2672° C and 1907° C ("Chemical properties of Chromium - fitness effects of Chromium -Environmental results of Chromium," 1998-2016). Even though mentioned silvery grey, Chromium can be obtained in different colors ; thereby adopting the Greek name "Chroma"(Horn, 2013).The physical application of chromium is seen in stainless steel production and other alloys due to its resistant nature towards corrosion and erosion thereby making it a valuable element (Rifikin et al, 2004).

Comprehensive data on the chemical element Chromium is provided in Table 1.1.

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·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·m^{-1}·K^{-1}

1.4.3 Occurrences and sources of Chromium compounds:

Chromium is typically found in plant vegetation, rocks, soil and in animal organisms. Despite their trivalent and hexavalent forms existing predominantly, Chromium can attain various oxidation states. In aquatic and geologic conditions, oxidation is observed for both tri and hexavalent forms of Chromium. Reduction of Cr6+ to Cr3+ may occur upon the former Chromium m's contact with dust particles (Chandra & Khulshreshta, 2016).

1.4.4 Chromium Utilization

Despite its toxic nature Cr6+ comes with various uses.Cr6+ is applied in tanning leathers, pigment, wood, electroplating and manufacturing of stainless steel commonly (EPA, 2010). Pigments containing chromium are further seen in cosmetic products and as direction lines on the roads.

1.4.5 Regulation regarding level of Chromium

A level of hexavalent Chromium beyond a certain threshold level is seen to have detrimental effects on people, living organisms and microbial organisms. To determine and suppress the Cr6+ from achieving this level numerous study and work has been contributed. An estimation by World Health Organization states the tolerable limit for Cr6+ and Cr3+ 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 Cr6+ 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.

1.4.6 Toxicity of chromium and effect of it towards health

1.4.6.1 Effects on Health

The effects of toxic Cr6+ on human health are numerous. From dermis, infection, ulcer, skin infection, nasal infection, lung carcinogenic infection affect people who are exposed to Cr6+. Cr6+ is also found in the placenta, thereby hampering fetal growth. Chromium also affects nature; affecting microbes by altering their structure and hampering their metabolism, growth and development, and since a large variety of microbes are symbiotic in nature, the

contamination ultimately delays the development of organic soil matter. Within cells, Cr6+ induces oxidative stress upon the contamination and further deposits a hard film on the cellular membrane. This creates a barrier in all forms of transport in and out of the cell. On the contrary, the trivalent form of chromium is less permeable in the membrane, thereby being unable to deposit within the cell and cause any harm. All in all, Cr6+ is the most lethal form of chromium that has drastic biological impacts upon most life forms.

1.4.6.1.1 Kinetics

Data from various literature and studies on the aggregation of chromium (VI) were used to inquire into the metal's toxicokinetic nature.

1.4.6.1.2 Mode of Action

Studies validate that Cr6+ undertake both genotoxic and mutagenic pathways. Upon transferring over to the plasma layer via sulfate pathway, cases induced by Chromium include formation of DNA complexes, complexes between DNA, complexes between chromium and DNA, complexes between protein, chromium and DNA, DNA strand uncoiled and oxidation of bases (Wise, Holmes & Wise, 2008). Chromium binds to DNA inducing transmutation and genomic variation, according to studies conducted in vitro on bacterial and human cells (Quiveryn et al, 2003). Chromium further damages DNA using reactive oxygen species. Chromium (III) on the other hand, do not transport as adequately as Cr (VI) does, therefore poses less harm. However, if developed within the body itself by reduction of Cr (VI), it functions buy cross- linking with the DNA. By reduction of Cr (VI), ROS is formed along with Cr (III); the latter aids the oxygen species to inhabit inside cells and create 8-hydroxyguanosine in the DNA. Therefore, trivalent chromium serves as an agent for all reactive species to penetrate into a cell and cause damage (Hdjiliadis, 2012). Attempts on fixing chromium in vitro leads to origination of mitogen- activated kinases of proteins, including JNK, ERK-1, ERK-2, and P38(Chuang and Yang, 2001 ; Kim and Yurkow,1996) and mutagenic elucidation modules including ATF-2, C-JUN and NFkB's phosphorylation (Samet et al,1998; Ye et al,1995). Since these kinases and modules are crucial negotiators in tumor genesis and inflammatory mechanisms, they initiate cell transduction and further lose control of any cell advancement, regardless of the genotoxic framework available (Hartwig, 2007, 2010). Genotoxicity was demonstrated in vivo by chromium in rodents, which were given large amount of Chromium (ASTDR, 2000b; ASTDR, 2008; OEHHA, 20110).

1.4.6.2 Effects in animals

Several studies have been conducted to investigate the lethality of Cr (VI). Lethal doses, ranging from 13mg/Kg to 29mg/Kg bw were administered orally on rats, keeping record of the nature of compound administered and the gender of the subject. The effects showed no many major changes, which were loss of body weight, decreased immunity, and alteration in haemato-biological parameters. Further effects showed irritation in the respiratory tract and change in neutrophil count. Studies also showed sickness in rats and guiena pigs when given a mixture of Chromium through several other pathways, including intra-pleural, intra- tracheal, intravenous infusion. Intra-peritoneal, intra-muscular and subcutaneous routes (ASTDR, 2008). Cancerous growth were observed and a possibility of lung carcinoma was suggested in rats and mice (Glacier et al, 1986; Glacier et al, 1988; Nettescheim et al, 1971). In addition, orally administered potassium chromate led to UV-initiated skin cancer, characterized by successive tumor growth (Davidson et al, 2004).

1.4.6.3 Human Effects

According to reports, impacts of chromium is observed from coincidental existence of the metal in the area and through inhalation by industrial workers who exposed themselves in Chromate region, i.e., electroplating areas, dyes and pigments. Contact with Cr (VI) and Chromium related compounds lead to ulcers, rashes and dermatitis. A path test showed 2µg Chromium could induce a sensitive hypertensive skin reaction. Investigations in a series of European countries indicated Chromium levels in the range of 0.5% to 1.7% to demonstrate its toxic effects (Peltonen & Franki, 1983; Hartwig 2007; Hartwig, 2010). Inhalation led to detrimental effects in nasal routes leading to ulceration of the nasal mucosal orifice. Further damage is seen to be incited in DNA (Chromosomal abnormalities, DNA complexation, DNA protein crosslinks, and DNA strand breakdown). In lymphocytes (WHO/IPCS, 2013). These effects is observed and investigated upon, but 4e full extent of its toxic effects remain unverifiable on an epidemiological scale (IARC, 2012). This arise due to numerous irregularities in the course of study especially while estimating exposures (Brandt- Rauf, 2006; Beaumont et al, 2008 and follow-up correspondence; Smith, 2008).

1.4.7 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.

1.4.8 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).

1.4.8.1 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).

1.4.8.2 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.

1.4.9 Metal Resistance mechanism into Bacteria

1.4.9.1 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 demonstrate 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

1.4.9.1.1 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 come 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 enable 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).

1.4.9.1.2 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.

1.4.10 Mechanism of chromosome 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 reuptaken 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 non-enzymatic 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.

1.4.11 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.

1.4.12 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 ion-exchange 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 tobe 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).

1.4.12.1 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).

1.4.12.2 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).

1.4.12.3 Bio-augmentation-assisted phylo-extraction

The efficiency of bioremediation can be increased with the aid of bio-augmentation (Anna et al, 2016) and in combination with phylo-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

2. Materials and Methods

2.1 Introduction

This part gives an overview of the process and the ingredients ingredients used in this study. It also explains the microorganism's strains collected from the river. In addition to that culture method was also improved to let the microorganisms grow properly on the media.

2.2 Chemicals

Pure Chemicals and analytical marks were utilized to run this experiment. The chemicals that are used in this experimented are listed below:

- Extract of beef
- Extract of yeast
- Nutrient Agar
- Nutrient Broth
- Potassium Chromate
- Diphenyl Carbazide
- MOPS Buffer
- Mueller Hinton Agar(MHA)

2.3 Glassware and Apparatus

The apparatuses that were used are listed below:

Table2.1: Apparatus and Glassware

Apparatus	Purpose
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 Centrifuge	Absorbance measurement 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

According to a research, Buriganga is listed as the most contaminated river than the other rivers in Bangladesh. Tannery industry, dying industry are mainly playing the role in that case of pollution(Kibria, Hossain, Mallick, Lau, & Wu, 2016).Chromium is present in higher amount in the Buriganga river from the effluent from various industries than the other heavy metal(Asaduzzaman, Hasan, Rajia, Khan, & Kabir, 2016; Islam, Khan, Hoque, & Jolly, 2014).To conduct our study regarding the chromium reduction capacity of bacterial isolate the sample was collected from the Hazaribag tannery area where industrial effluent are discharged into the Buriganga River.

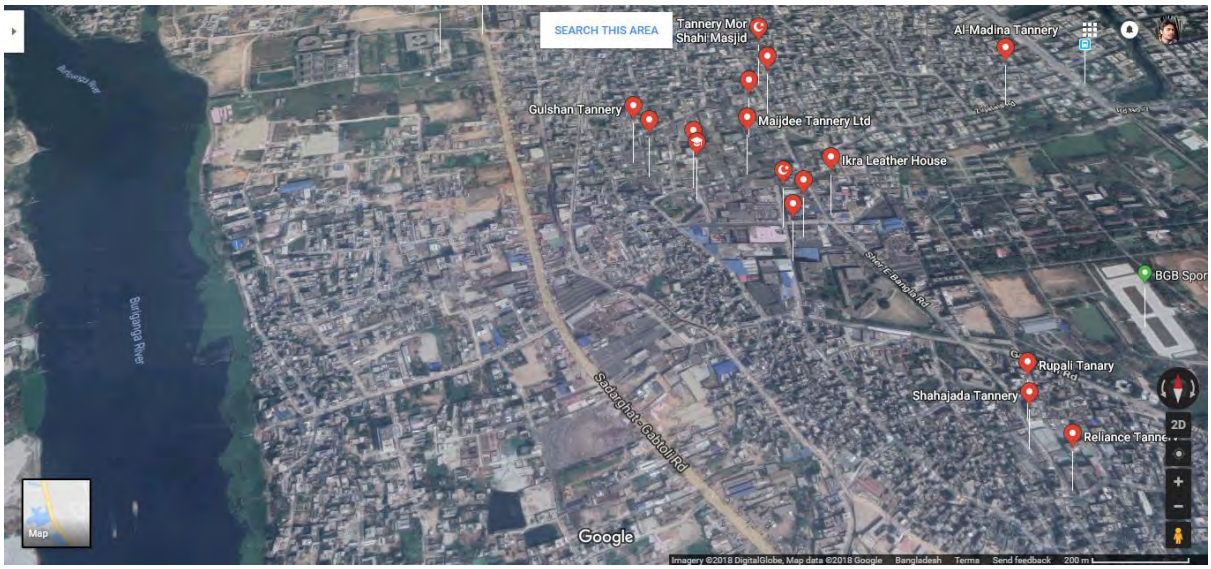

Fig 2.1: Hazaribag tannery area

2.5 Isolation and condition of the culture

Following a standard protocol detachment of the sample from soil was done. Microorganisms that are resistant to Chromium were separated by inoculation for one hundred microliter of sample of effluent liquid and one hundred microliter of soil collected from unaltered soil (one gram of soil was mixed with nine ml of liquid of saline by grinding the soil in a tube) by a series of process into the agar medium which contains potassium chromate. Then incubation was performed at 37 degree Celsius. Agar media was prepared by mixing 2.8g of nutrient agar into 100ml of water and then it was sterilized utilizing the autoclave machine at 121^{o}C.After that potassium chromate was a added to that media and poured into the dish and was let to cool and solidify. Following inoculation several colony was found on that medium and the desired bacteria was withdrawn by a toothpick which was sterilized using fire and that was touched slightly on another agar dish medium that had the concentration of 2,3,4,5 Milimolar of Cr (VI).Then incubation was performed for another time. This process has been repeated for several times at 6,7,10,15,20,25,30,35,40 milliliter of Cr (VI) till MIC.

2.6 Chromium reduction profile of chromium resistant bacteria

A chemical named Diphenyl Carbazide was utilized to identify the amount of Cr (VI) in the effluent water (Greenberg, et al., 1992) and the tasks were recorded. A standard curve was established with which the microorganism's performance was compared.

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. After that, into falcon tube, 1670 µL conc. H_2SO_4 has been putted on droplet through droplet comprising 8mL purified H_2O. Next, solution's capacity was prepared to the equal of 10mL though putting on 330µ L purified H_2O.

2.6.1.2 Diphenyl Carbazide preparation

0.025g diphenyl carbazide powder was added into one falcon tube. Then into falcon tube, 9.67mL acetone and afterward 3M sulphuric acid of 330µ L were taken comprising diphenyl carbazaide powder. Mixing was finely done of that falcon tube to prepare uniform solution of DPCZ.

2.6.1.3 MOPS buffer preparation

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

2.6.1.4 5mM 10mL K_2CrO_4 preparation

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

2.7 Process of experiment

2.7.1 Sample Preparation for reaction

Several solutions of sample of subsequent concentrations have been managed. Every sample's was absolute volume was one milliliter

Table 2.2 Sample preparation for standard curve

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

2.7.1.1 Reaction protocol for standard curve

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

2.7.1.2 Evaluation of reduction profile of selected isolates

2.7.1.2.1 Procedure

- Day 0

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

- Day 1

  The next day two nutrient broth medium of 25ml was prepared in two conical flask and sterilized. 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 from the 10ml conical flask that was inoculated by microorganisms and put in the shaking incubator for 24 hours and its absorbance 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 from each 25ml conical flask was withdrawn in two falcon tube and then the conical flasks were put into the shaking incubator to culture microorganisms in the hexavalent chromium condition. 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.

2.8 Standard curve preparation

2.8.1Antibiotic resistance among Chromium resistant isolates

2.8.1.1 Strain culture preparation in nutrient broth (NB)

Nutrient broth of 20ml was prepared to see the resistance of particular strain towards antibiotics in the conical flask. Then inoculation was performed and the conical flask was put in the shaking incubator at 37 degree Celsius overnight.

2.8.1.2 Inoculation of the test plates

Mueller Hinton Agar was used in this purpose to make culture plate. Using sterilized cotton swab the overnight culture of strain in the conical flask was spread over the MHA surface in the culture plate. This step was recurred for two more times by moving at 60^{o} 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.

2.8.1.3 Application of antibiotic discs

The following antibiotic discs were spread over the plate in antibiotic resistance profile following inoculation of the strain-

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)

2.8.1.4 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.

2.9 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-30Mminoculated with 50 microliter nutrient broth culture which was kept in the incubator at 37 degree Celsius for 48 hours for colony growth.

2.10 Identification of isolate 3W100

The bacterial strain was sequenced by 16s rDNA sequencing. That sequence was purified by Finchtv and saved as FASTA file. BLAST(Basic Local Alignment Search Tool) was done in NCBI and the similar strain were collected on the basis of score and using Bioedit and MEGA7 software phylogenetic tree was made to study the evolution of 3W100.

Chapter-3

3 Results

3.1 Isolation data of chromium resistance bacteria

The isolation process has been performed for the two separate colonies from different concentration of Cr in nutrient agar medium plates. Labelling was done to distinguish various concentration of Cr.

3.2 Chromium reduction profile of Chromium resistant bacteria

3.2.1 Standard Curve

Steps described in 2.6.2.1.2 was performed in order to make a standard curve which was plotted in the graph by using the value of absorbance with the help of Microsoft word excel Software of 2016.

Table 3.1: Data of standard curve of hexavalent Chromium

Concentration(µm)	Absorbance
50	0.294
100	0.624
150	0.907
200	1.214
300	1.675
400	2.117
500	2.587

600	2.875

Using these data a standard curve was prepared which is given below:

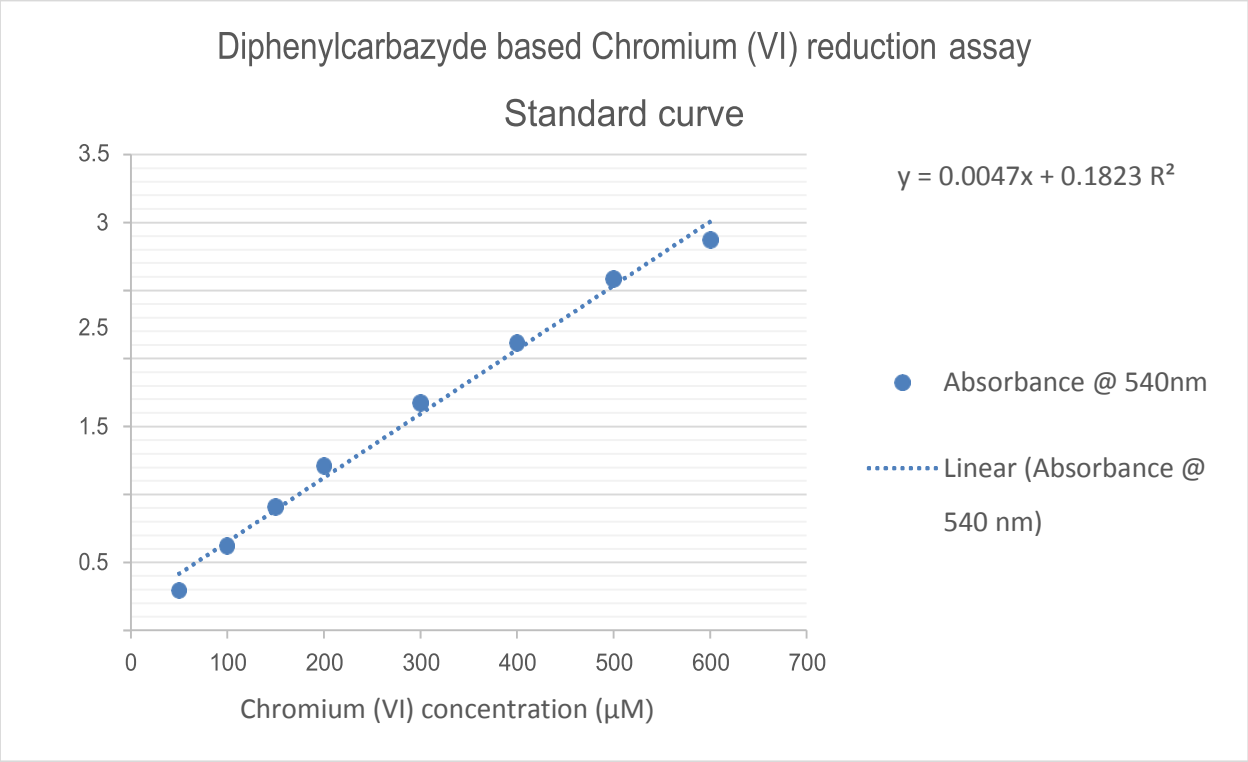

Fig 3.1: Diphenyl Carbazide based Cr (VI) reduction assay standard curve

3.3 Reduction profile of 3W100

To have a precise outcome from this experiment temperature and pH were altered.

Table 3.2: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	516.4609929	0.906	518.7304965	0.001
1.5	516.9574468	0..782	518.0212766	0.001
3.0	515.751773	0.768	519.4397163	0.003
4.5	513.9787234	0.728	520.3617021	0.003
6.0	511.070922	0.645	519.7234043	0.001
24	0	2.442	520.0070922	0.004

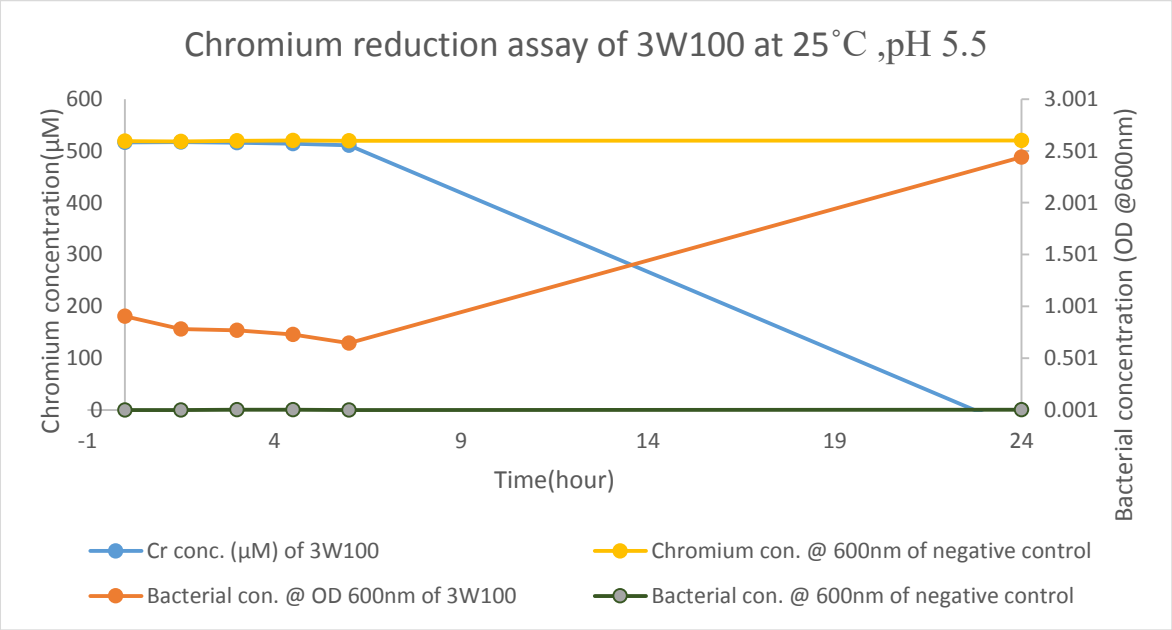

Fig3.2: Chromium reduction profile of 3W100 at 25℃ and pH 5.5

From the above figure it seems that the chromium concentration was nearly 516 µM at initial hour which remained almost same to the 6th hour of the first day. Then the concentration fell

drastically after keeping that culture overnight and makes the concentration 0µM. In addition to that, the bacterial concentration was high at starting point of the day which suddenly slightly decreased at the 6th hour of the day. However, that concentration drastically increased after overnight and showed a great reduction of chromium in the solution. The constant straight line in the figure also denotes that negative control was bacteria free.

Table 3.3: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	518.0921986	0.922	518.7304965	0.001
1.5	517.0992908	0..811	518.0212766	0.001
3.0	515.1134752	0.798	519.4397163	0.003
4.5	516.177305	0.794	520.3617021	0.003
6.0	516.6028369	0.734	519.7234043	0.001
24	0	2.365	520.0070922	0.004

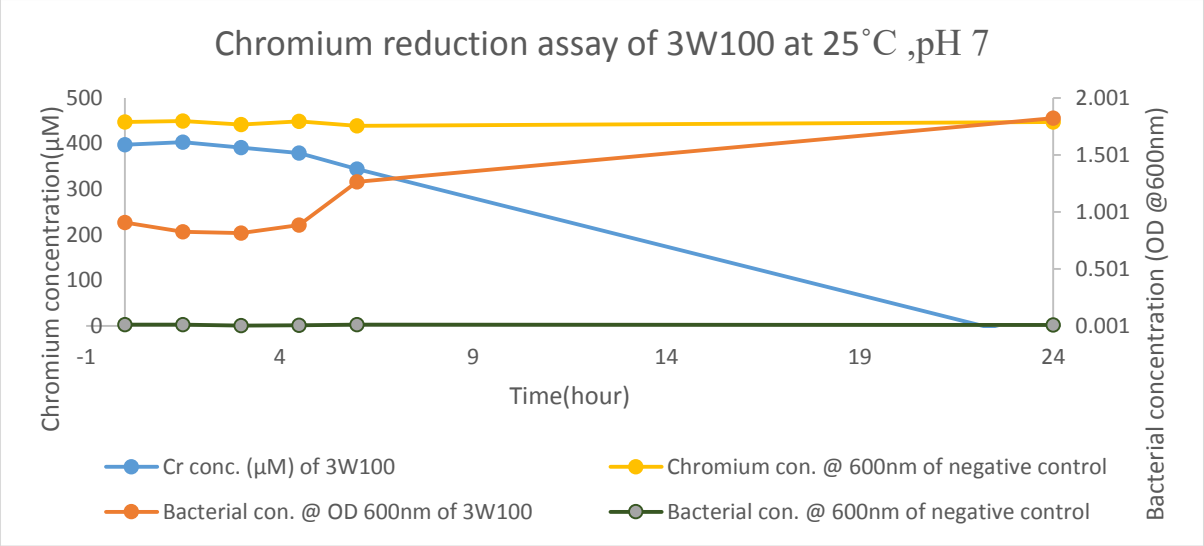

Fig3.3: Chromium reduction profile of 3W100 at 25℃ and pH 7

From the above showed figure it is obvious that at pH 7 the chromium concentration was almost same up to the 6th hours of the day which is nearly 516µm to 518µm.A great decrease in chromium reduction which is nearly 100% is found after overnight culture of the strain in the chromium environment. However, there was fluctuation in the bacterial culture concentration. Firstly the concentration were decreasing from their initial point but the concentration got doubled of initial concentration after overnight which causes a great reduction of chromium in the solution and there was no culture in the negative control as it made a straight line in the graph.

Table 3.4: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	495.822695	0.953	518.7304965	0.001
1.5	497.1702128	0.914	518.0212766	0.001
3.0	493.6241135	0.884	519.4397163	0.003
4.5	494.8297872	0.861	520.3617021	0.003

6.0	493.9078014	0.826	519.7234043	0.001
24	0	2.098	520.0070922	0.004

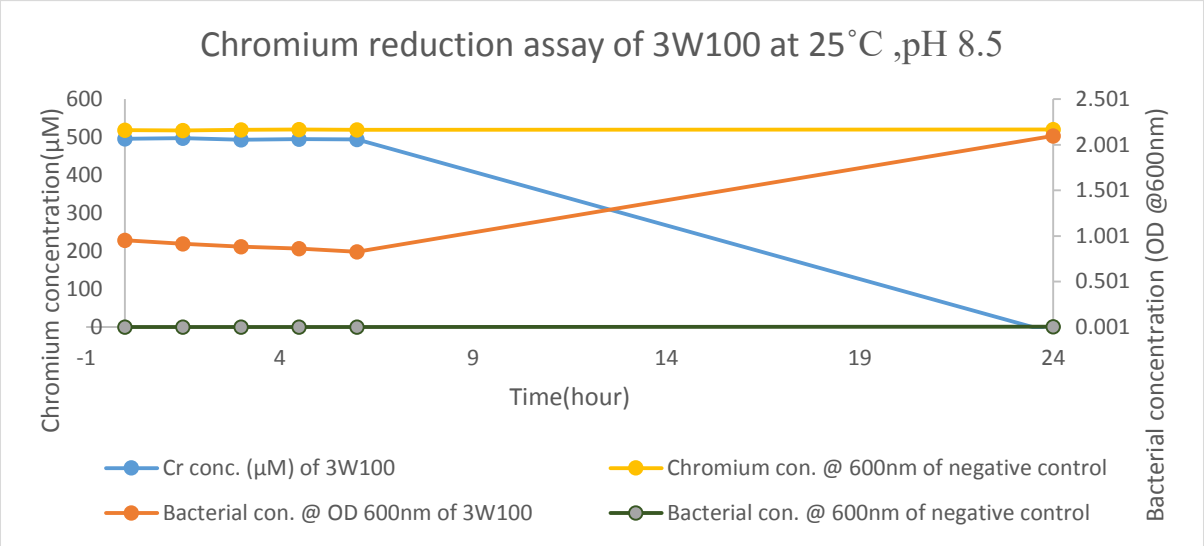

Fig3.4: Chromium reduction profile of 3W100 at 25℃ and pH 8.5

This figure shows the similarities with the result of previously two pH at same temperature 25°C. At the starting point the chromium concentration was around 495µM which remains almost same throughout the first day but a great decreased was found which was almost 100% reduction after overnight. Moreover , the bacterial concentration was decreasing initially from 0.953to 0.826 up to the 6th hours but it also got increased after keeping that culture overnight which became the reason of the great reduction of the chromium. However, no culture was found in the negative control during the whole process.

Table3.5: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	340.5744681	0.898	335.0425532	0.015

1.5	303.0567376	0.901	339.7943262	0.015
3.0	245.893617	0.927	327.4539007	0.019
4.5	213.2695035	1.155	338.6595745	0.021
6.0	0	1.585	339.8652482	0.016
24	0	2.495	353.1985816	0.013

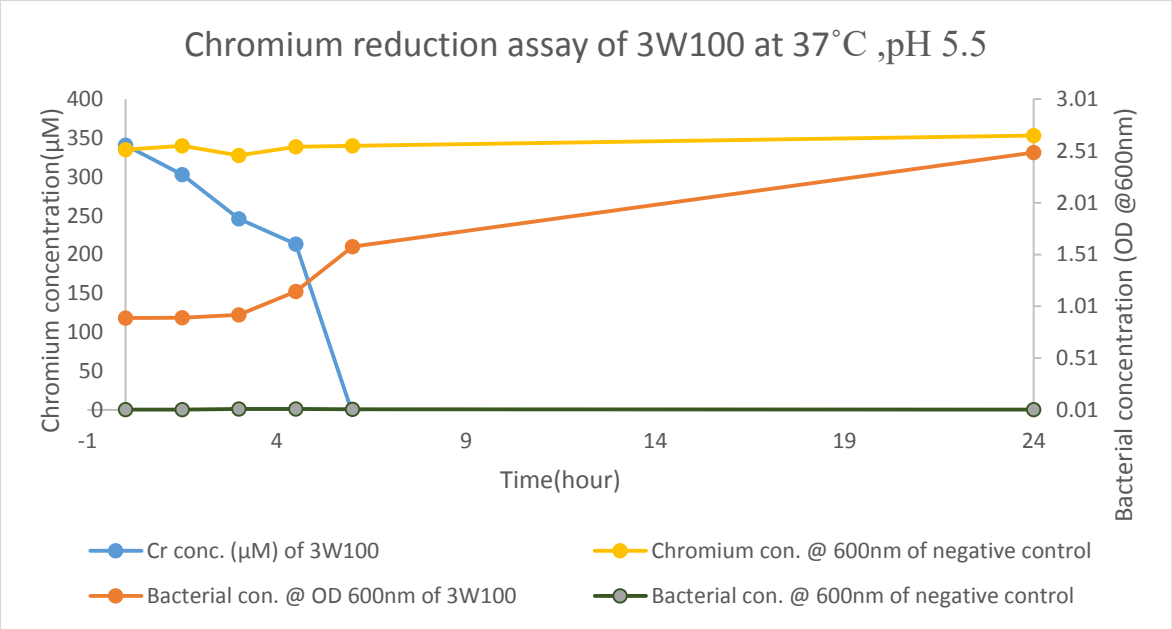

Fig3.5: Chromium reduction profile of 3W100 at 37℃ and pH 5.5

From the above showed figure it is clear that, at 0hour of the day the chromium concentration was nearly 340µM which was reduced to a certain amount after 1.5hours.This reduction was continued and at the 6th hour of the day and chromium was reduced by nearly 100% and the concentration becomes 0µM after keeping it overnight. In addition, this figure shows a sharp increase in the bacterial concentration and the concentration becomes more than the doubled value of the initial value which plays a great role in the reduction of chromium. However, there was no culture in the negative control. Hence it can be said that 3W100 at 37°C and pH 5.5 shows a great activity in chromium reduction.

Table3.6: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	315.822695	0.916	335.0425532	0.015
1.5	305.4680851	0.934	339.7943262	0.015
3.0	242.7730496	1.002	327.4539007	0.019
4.5	219.0141844	1.327	338.6595745	0.021
6.0	31.70921986	1.613	339.8652482	0.016
24	0	2.401	356.51074281	0.013

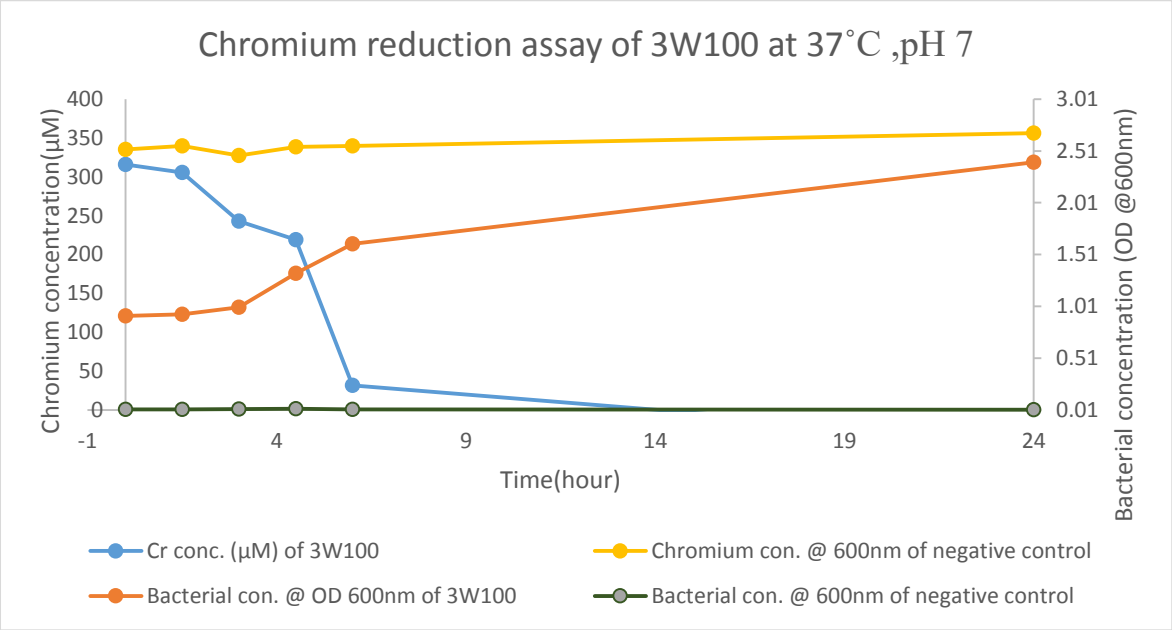

Fig3.6: Chromium reduction profile of 3W100 at 37˚C and pH 7

From the above figure it is found that at the 0 hour of the day the chromium concentration was around 315µM which started to decrease at 1.5th hour of that day. Then the reduction continued

and at the 6th hour of the day almost 90% reduction was complete. After that after keeping the culture overnight the chromium concentration was 0µM. Moreover, there was a great increase in the bacterial concentration which played an important role to reduce the chromium concentration but there was no bacterial culture in the negative control which shows a straight line in the graph. Therefore it can be said that strain 3W100 has a potent chromium reduction ability too at 37°C and at pH 7.

Table3.7: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	304.8297872	0.981	335.0425532	0.015
1.5	289.4397163	0.996	339.7943262	0.015
3.0	239.5815603	1.094	327.4539007	0.019
4.5	240.2198582	1.364	338.6595745	0.021
6.0	134.6170213	1.697	339.8652482	0.016
24	0	2.503	356.51074281	0.013

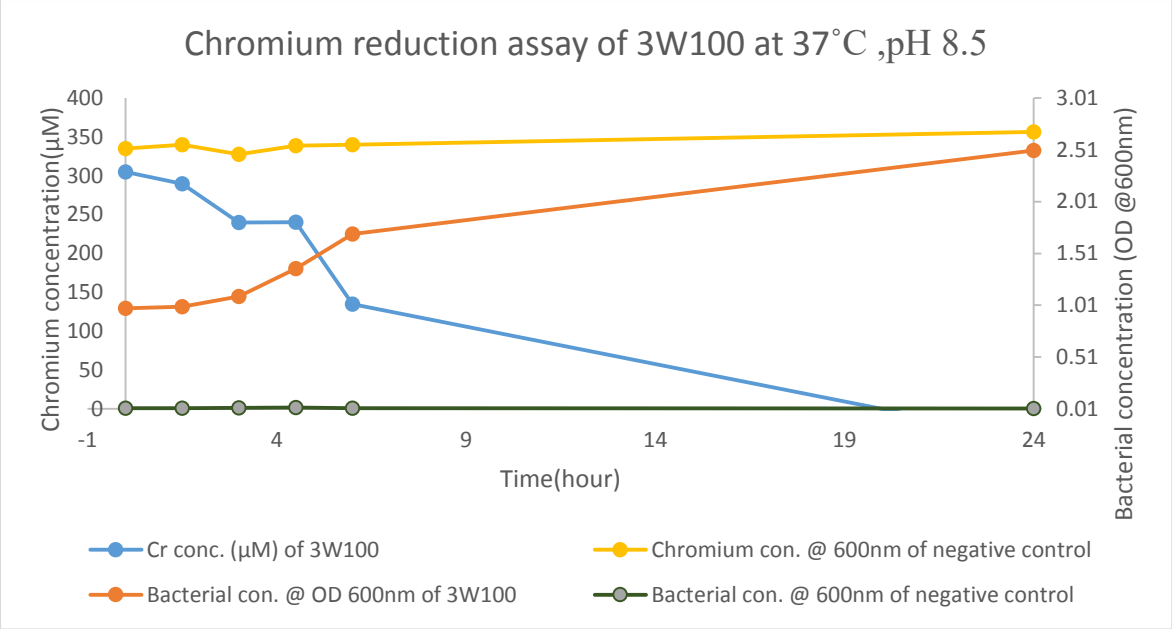

Fig3.7: Chromium reduction profile of 3W100 at 37°C and pH 8.5

The above figure shows that the initial concentration was around 304µM at 540nm which dramatically decreased at the 1.5th hours of the day. Then the reduction was continued and more than 50% was reduced by the 6th hours of the day to nearly 134µM.The uprising line of bacterial concentration in the graph denotes that there was a sharp increase in the bacterial concentration for which they were working potentially to reduce chromium from that solution. The straight line of negative control shows that there was no culture in it. Hence it can said that 3W100 shows good reduction at 37°C and pH 8.5.

Table 3.8: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	435.1843972	0.934	447.5248227	0.013
1.5	424.5460993	0.847	449.3687943	0.012
3.0	385.3971631	0.683	441.9219858	0.004

4.5	370.858156	0.771	448.5886525	0.006
6.0	334.0496454	0.935	438.9432624	0.013
24	0	1.825	447.1702128	0.01

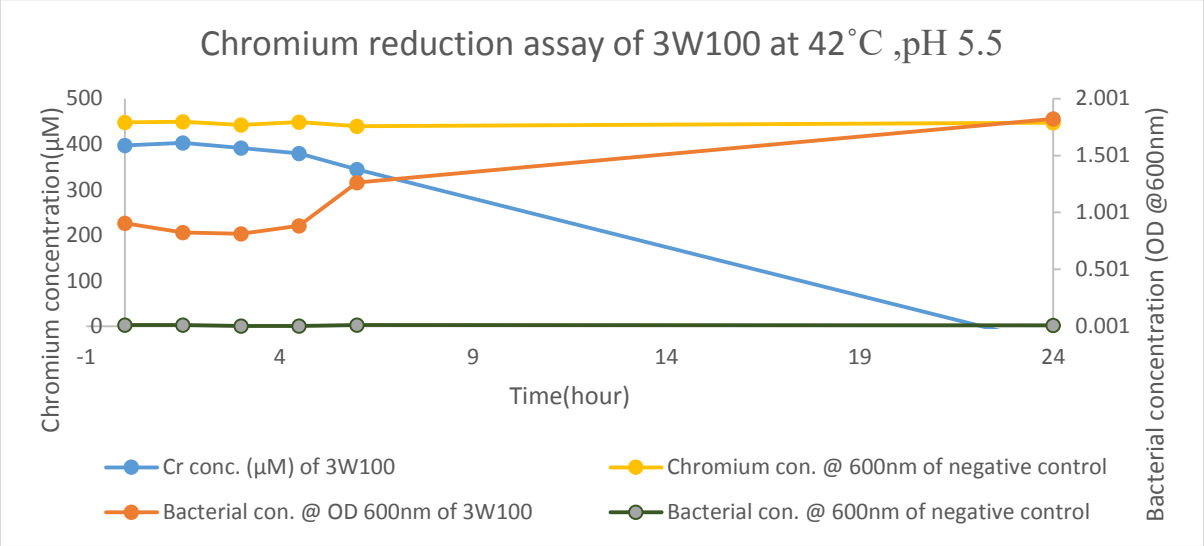

Fig3.8: Chromium reduction profile of 3W100 at 42℃ and pH 5.5

This figure reveals that the chromium concentration which was around 435µM at the initial point of the experiment started to decrease in a slow pace in every 1.5 hours of that day and at the 6th hour of that day it only decreased by . However, after keeping that culture overnight the chromium concentration was decreased by 100%.In addition the bacterial concentration was fluctuating during the whole process .It seems that bacterial concentration was decreasing up to 1.5th hour of that day but it was suddenly increasing dramatically after that and it was continued to overnight .However there was no bacterial culture in the negative culture. So it can be said that 3W100 at 42°C and at pH 5.5 does not show significant reduction up to overnight

Table3.9: Isolate 3W100: 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 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	409.7234043	0.934	447.5248227	0.013
1.5	424.8297872	0.797	449.3687943	0.012
3.0	388.5886525	0.821	441.9219858	0.004
4.5	358.7304965	1.051	448.5886525	0.006
6.0	138.1631206	1.584	438.9432624	0.013
24	0	1.763	447.1702128	0.01

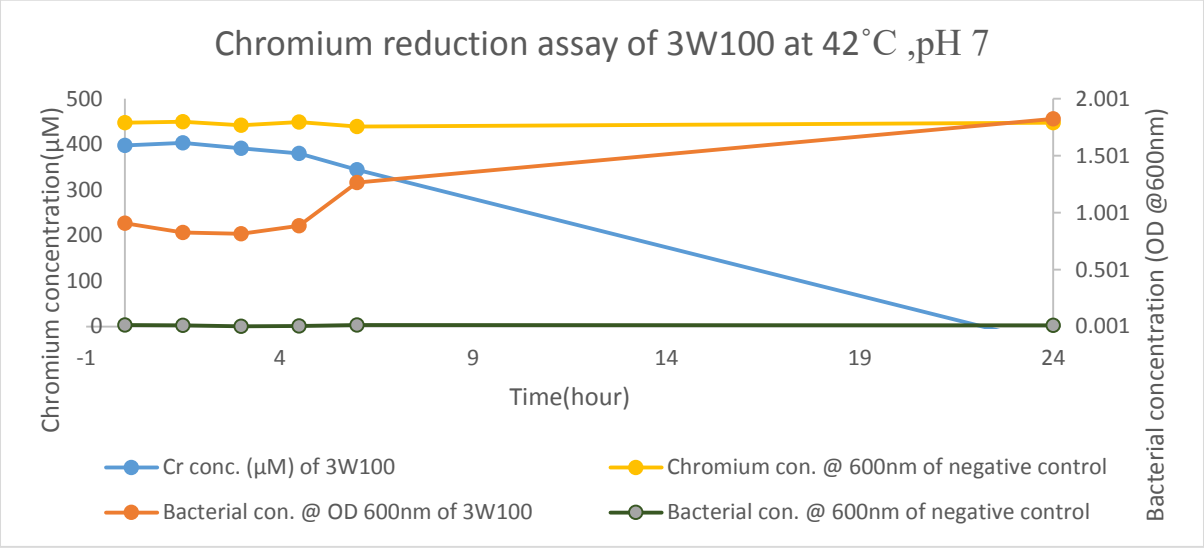

Fig3.9: Chromium reduction profile of 3W100 at 42℃ and pH 7

This figure explains that the initial concentration of the chromium was nearly 409µM which started decrease after 3hours.Then the reduction by the bacteria continued and it was reduced more than 50% after 6hours.Moreover, after overnight culture of that bacteria the chromium

concentration was decreased by 100%.In addition there was an ups and downs in the bacterial culture. Initially the bacterial concentration was decreasing but after 1.5 hours it was suddenly increasing which lead to a great reduction of the hexavalent chromium in that solution along with no culture in the negative control. Hence it is obvious that 3W100 at 42ºC and pH 7 shows a significant activity to chromium reduction.

Table3.10: Isolate 3W100: Chromium reduction profile Vs. Cell Growth at 42ºC, pH 8.5

Sample			**Negative Control**	
Time (Hours)	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**	**Chromium concentration on (µM) at 540 nm**	**Bacterial concentration at 600 nm**
0	397.3829787	0.908	447.5248227	0.013
1.5	403.0567376	0.826	449.3687943	0.012
3.0	391.212766	0.815	441.9219858	0.004
4.5	379.5106383	0.885	448.5886525	0.006
6.0	344.3333333	1.264	438.9432624	0.013
24	0	1.824	447.1702128	0.01

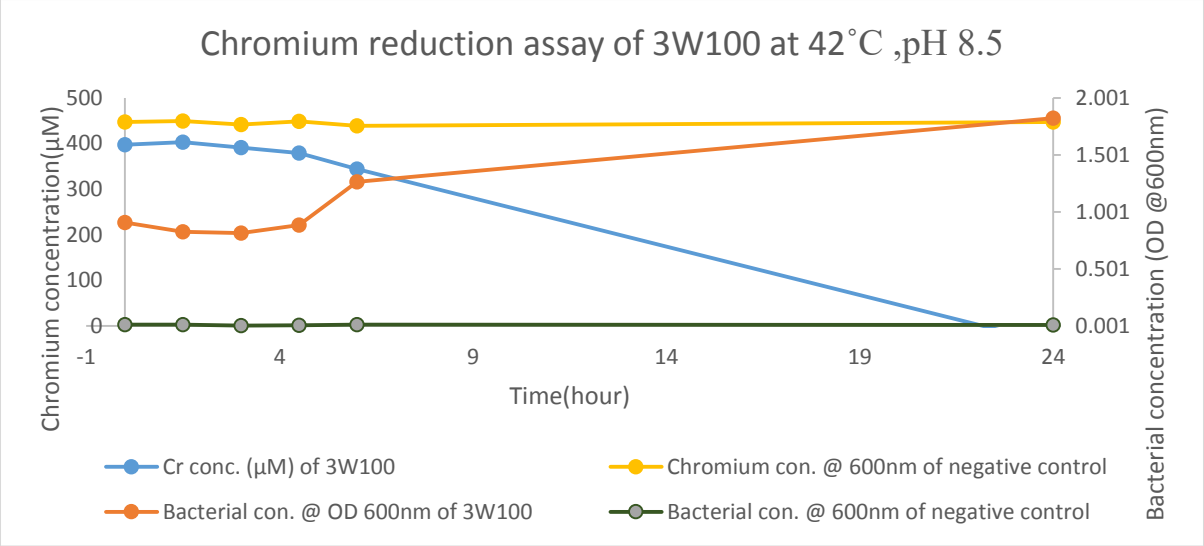

Fig3.10: Chromium reduction profile of 3W100 at 42℃ and pH 8.5

This above figure shows that the chromium concentration which was nearly 397µM remained almost same up to 3rd hour of the day. However it started to decrease after 3hours in a slow pace till 6th hour of that day. This reduction continued and after overnight it became 0µM of chromium concentration. In addition the bacterial concentration was decreasing from the initial value till 4.5th hour of the day. Then suddenly there was a dramatically increase in bacterial concentration which sustained after keeping that culture overnight but there was no bacteria found in the negative control. So it can be said that 3W100 at 42ºC, pH 8.5 does not show significant reduction of chromium up to overnight culture.

3.4 Antibiotic resistance among Chromium resistant isolate 3W100

13 antibiotic discs were distributed over the MHA plate which was inoculated by 3W100 and incubated for 24hours. After that the antibiotic resistance of that bacteria towards the antibiotic discs were counted by measuring the diameter of the zone of inhibition with the aid of a cm scale. The findings of antibiotic resistance are given below:

Table 3.11: Antibiotic resistance profile of isolate 3W100

Name of Antibiotic disc	Diameter of Zone of Inhibition (ZOI) of 3W100 on (mm)
1. Chloramphenicol (C:30mg)	20
2. Kanamycin(K:30)	23
3. Gentamicin (CN:10mg)	21
4. Ofloxacin (OF:5mg)	20
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)	20
11.Cefuroxime Sodium(CXM)	0
12.Azithromycin(AZM:15)	22
13.Cefixime(CFM)	0
14.Streptomycin(S:10)	23

From the above table it is obvious that Kanamycin, Streptomycin and Azithromycin has the highest zone of inhibition which means that they both can kill most of the bacteria of that strain than the other discs of antibiotics. This also denotes that 3W100 is not resistant to the antibiotic Kanamycin, Streptomycin and Azithromycin as they could not prevent the effect of Kanamycin. On the contrary Ceftriaxone has the lowest zone of inhibition which explains that it can't kill that much bacteria of this strain as well as could not stop the growth of bacteria surrounding it. In addition to that some antibiotic discs like Penicillin-G, Amoxicillin, Cefixime, Cefuroxime Sodium has value of 0 in zone of inhibition which means 3W100 is resistant towards them as they could not be able to kill any bacterial cell on the culture.

3.5 Minimum Inhibitory Concentration

The outcome of the minimum inhibitory concentration are listed below:

Table 3.12: MIC of isolate 3W100

Concentration	Number of colonies
1mM	115
2mM	62
3mM	29
4mM	14
5mM	6
6mM	0
7mM	0
8mM	0

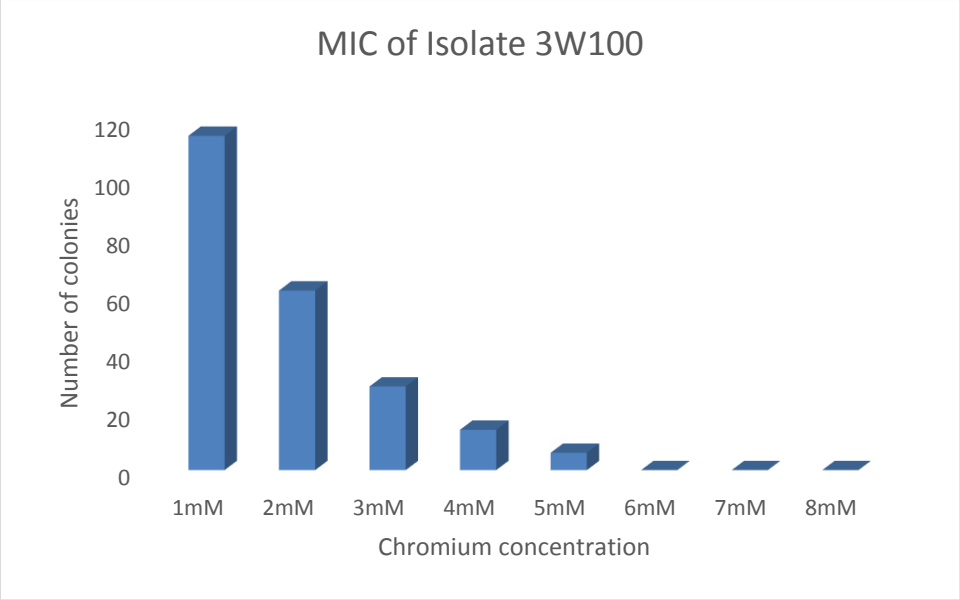

Fig 3.11: MIC of isolate 3W100

From the above figure it was obvious that the isolate 3W100 can tolerate up to 5mM chromium concentration in its surrounding environment which means it is able to show its resistance till 5Mm.After that when the concentration was rising, there was no colonies found on the medium which explains that the isolate 3W100 cannot tolerate the concentration more than 5mM.

3.5 Identification of 3W100

The sample for this experiment was collected from Buriganga River near the tannery industries. The sample's identity was unknown. To know which strain it is 16s rDNA Sequencing was done. Using different software the sequence were purified and BLAST was done where 100% similar result with the strain was *Staphylococcus sciuri*. To confirm this result a phylogenetic tree was made using MEGA7 software where the ancestor of this strain was also found which was *Staphylococcus stepanovicii* from where the other organism of this tree may have originated. The figure of the phylogenetic tree is given below:

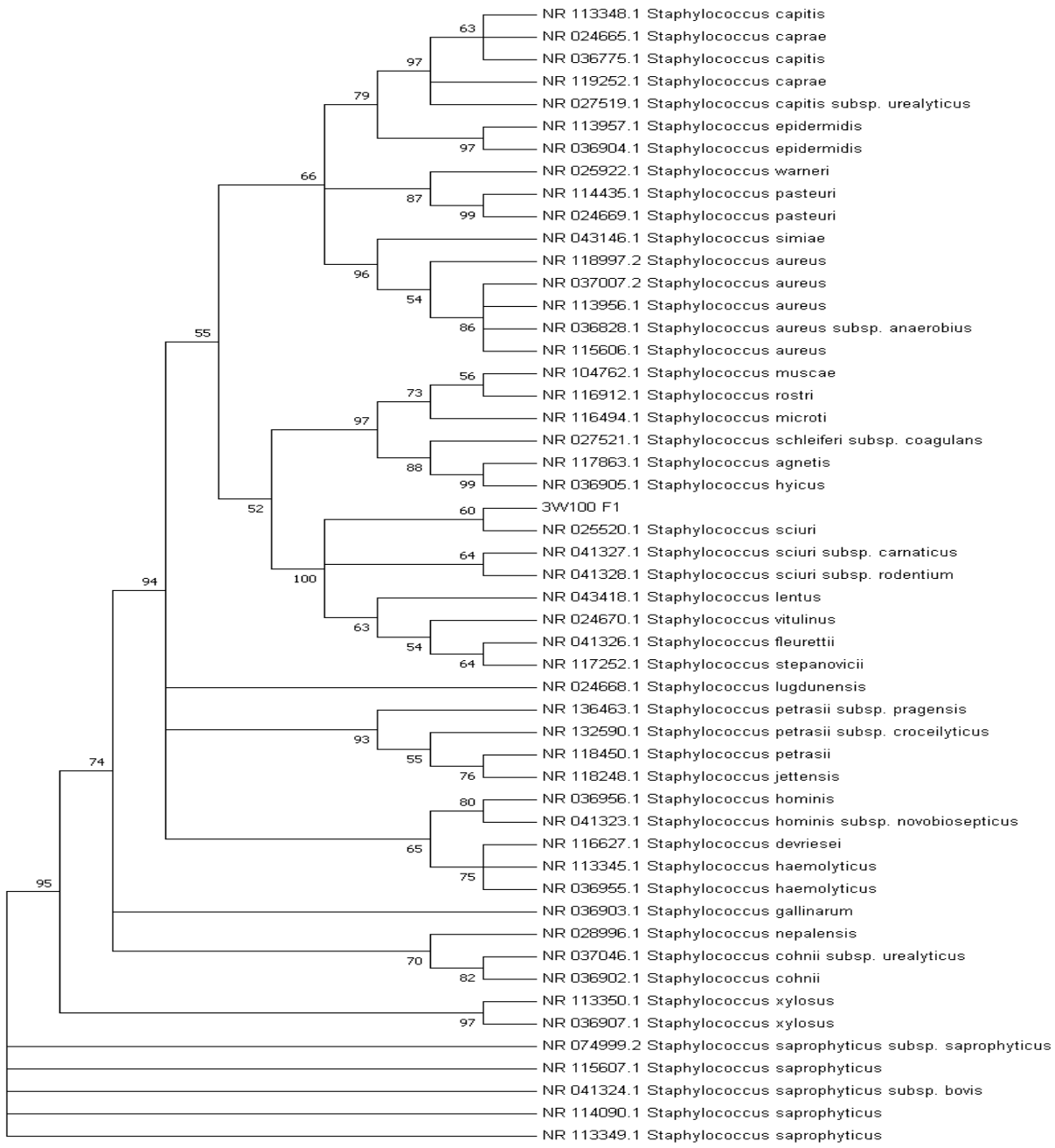

Fig3.12: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 0.15520780 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 (Felsenstein, 1985). 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 (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 894 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

3.6 Discussion

The environment is getting polluted everyday by discharge of various type of heavy metal like hexavalent Chromium. They are being freed every day in the water from numerous dying and tannery industries. They are becoming very hazardous to the health of human as they are carcinogenic and mutagenic. The main focus of this study was to identify the organism that will aid in reducing the toxicity of hexavalent chromium from water.

In this purpose the sample was isolated and purified using different parameter after collecting them from the toxic area. After making a standard curve the chromium reduction capacity of the bacteria was analyzed altering the temperature (25˚C, 37˚C, 42˚C) and the pH (5.5, 7, 8.5).Though the strain has showed reduction capacity at every temperature, the most prominent result for reducing chromium from the water sample was found for 37˚C.At 25℃ the isolate has reduced the amount of chromium in a longer period of time in every pH. The isolate has showed the same result and took overnight period of time to reduce Cr (VI) whereas it took only 6 hours to reduce chromium at 37℃, pH 5.5.Among the three pH at this temperature the sample strain was most efficient at pH 5.5 as almost 100% chromium was reduced at the 6^{th} hour of the experiment which is more faster process than the other temperature and pH.

In addition, the antibiotic resistance profile of that strain was also made which shows that isolate 3W100 shows resistant against Penicillin, Cefuroxime, Cefixime, and Amoxicillin which means these antibiotic cannot kill this isolate. However, three antibiotics named Streptomycin, Kanamycin, and Azithromycin were found by this profile which are able to exert their effect on this isolate and kill this isolate. From the findings of the Antibiotic resistance profile, we can draw the picture of antibiotics classes which can resist the effect of that isolate. Streptomycin and Kanamycin belong to the aminoglycoside class of antibiotics and Azithromycin fall under macrolide class and they are the protein synthesis inhibitor of bacterial isolate. So, we can assume that the strain 3W100 is not resistant to these two classes of antibiotics.

In the Minimum Inhibitory Concentration the maximum concentration of the chromium is identified which can be tolerated by the isolate 3W100 and the concentration was 5Mm.After

that value of concentration 3W100 cannot sustain which was confirmed by no colonies on the culture medium. This findings explain a correlation between MIC and the reduction capacity of that strain which is after 5Mm concentration the strain will not show any reduction capacity as they cannot tolerate and was killed. Therefore, it can be assumed that at higher concentration it will not show any reduction activity.

To identify the strain, the isolate was sequenced by 16s rDNA sequencing and purified. Then the strain 3W100 was uploaded on NCBI BLAST and the BLAST result reveals the 100% similarity with strain *Staphylococcus sciuri*. To confirm this result, a phylogenetic tree was created which discovers the root of this strain which is *Staphylococcus stepanovicci* from where the isolate might have expressed. This tree also confirmed the BLAST result of similarity by putting the strain 3W100 and *Staphylococcus sciuri* on the same branch.

Chapter-4

4 Conclusion

4.1 Conclusion

The study reveals that the bacterial isolate hat have been used to perform the experiment shows s great resistant to the chromium which means that they can sustain in the chromium rich environment up to a certain amount of chromium concentration. Moreover, it has been identified that this strain has the capability of reducing hexavalent chromium from the water. Their speed on performance depends on the pH and incubation time of the culture solution. Compiling these information obtained from the experiment the strain can be used to reduce the chromium toxicity from the environment which will lessen the health risk of the human and other living object.

4.2 Future direction

Isolate 3W100 can be further analyzed to identify the interrelation between antibiotic resistance and the chromium reduction capacity of that strain. This isolate can be a potential source of chromium reductase enzyme by further investigating their enzyme's mechanism. This can be done by finding whether they are exo-enzyme or endo-enzyme containing bacteria. Comparative genomic studies can be carried out to identify the best strain in chromium reduction activity.

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