ISOLATION AND CHARACTERIZATION OF CHROMIUM RESISTANT *Vibrio sinaloensis* FROM MARINE SOIL

A project submitted
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This work is dedicated to my parents for their love and constant support.

Certification Statement

This is to certify that this project titled “Isolation and Characterization of Chromium Reducing Bacteria From Marine Soil” 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 Mohammad 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 thesis itself.

Signed

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Countersigned by the supervisor

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ABSTRACT

Now-a-days, hexavalent chromium is one of the most toxic environmental pollutants which is very toxic transportable and also is used in almost every industries and released in the similar manner. Reduction of the hexavalent chromium to its water- insoluble trivalent chromium makes them less toxic as their bioavailability decreases. Recently, this particular matter has acknowledged specific attention for their effective use in bioremediation process. To isolate, characterize and identify the bacteria, samples were collected from the coastal regions of Bangladesh situated in Ship Breaking Yard of Sitakunda, which are capable of reducing hexavalent chromium to trivalent chromium. The isolated organism has been studied for chromium (VI) reduction activity for their dependence on growth. The isolate was resistant to hexavalent chromium and also had reducing activity. The isolate S_2 showed the optimum reduction activity at 37^0C temperature and both in pH 7 and 8.5. Finally, cultural and biochemical tests of the isolate were carried out for identification of bacteria. After analyzing the biochemical test data in Advanced Bacterial Identification System Version 6 (ABIS 6.0), the isolate was identified as *Vibrio sinaloensis*.

CONTENTS

CHAPTER THREE- MATERIALS AND METHODS

CHAPTER FOUR- RESULT and DISCUSSION

LIST OF TABLES

LIST OF FIGURES:

LIST OF ABBREVIATIONS

Cr	Chromium
DPCZ	Diphenyl Carbazide
ETP	Effluent treatment plant
IDLH	Immediately dangerous to life and health
Kg	Kilogram
MCL	Maximum contaminant level
mg	Milligram
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
µM	Micro molar

CHAPTER -1: INTRODUCTION

1.1 Background

Hexavalent chromium works as a very good mutagenic and carcinogenic agent. Regularly, They are acquainted with the environment by different commercial processes like electroplating, leather tanning, dying, pigment manufacturing etc. from the environment, this toxic metal enter in to our food chain and causes different human health hazards like cancer, dermatitis etc. Chromium can be found in the environment and they are mostly existing in rocks, animal, plants, soil, animal and rocks. Moreover, it is the most plentiful element ranking seventh on Earth's crust (McGrath and Smith, 1990 Shewary and Peterson, 1976). Chromium is disposed into the environment in an unregulated way which results in the contamination of soil and ground water environments.

Most of the contaminated sites around the world are treated using abiotic processes implemented with pump-and-treat or dig-and-treat methods that require follow up precipitation or immobilization steps (Cifuentes et al., 1996; Quintana et al., 2001). Biological reduction methods is one of the latest findings that has helped in the treatment of wastes which contain hexavalent chromium (Donat and Guruchet, 2003, Rege et al., 1997,Rajwade and Paknikar, 1997, Mel Lytle et al., 1998, Salunkhe et al., 1998).

Bioremediation is considered as an alternative to physical & chemical treatment technologies since they do not introduce any foreign material into the ecosystem which can be harmful for human. They also do not involve further transportation of toxic material which may lead more dangerous spillage in transit.

The current research gives us the opportunity to evaluate the unknown chromium resistant bacterial strains to be used as a potential remedy for chromium intoxication by preventing the hexavalent chromium by its reduction before coming in contact with human food chain and also they can be an important source of chromium reductase enzyme as they are working as a great chemotherapeutical agent now a days. (Park et al., 1999).

1.2 Methodology

The isolated cultures were characterized by biochemical tests as well as microscopic morphological imaging at the later part of the investigation.

The significant part of the current investigation was to find out a suitable microbial strain which shows resistance to the Chromium (VI) existing in the media in a remarkable fashion. The methods we have applied to evaluate the reduction process were conducted in a controlled way so that we can identify the specific chromium reducing species. The experiments were conducted in chromium containing NB media. A thorough isolation process was conducted before evaluating the chromium reduction profile of the isolated bacteria in repeated fashion until the pure colonies of bacteria were identified successfully.

1.3 Objectives

The main objective of this study was to identify that particular bacteria that is resistant in the chromium rich environment and also to find out that if they can decrease the level of chromium and to see that if they work as a possible source of chromium reductase enzyme which works like a chemotherapeutic agent.

1.4 Main Findings

From the evaluation of Chromium reduction process of the isolate S_2, we had seen that there was optimum lessening of Cr(VI) by the microorganism within 24 hours when they were incubated at 37^0C and the pH was 7 and they were resistant to Cr(VI). After that, biochemical tests were done and the strains exhibited resemblances with *Vibrio sinaloensis*.

CHAPTER -2: LITERATURE REVIEW

2.1 Introduction

Due to chromium's extensive application in a huge number of industrial activities like leather tanning, dyes and pigments, electroplating, manufacturing alloys and petroleum refining , it is an important industrial chemical (Poopal and Laxman 2009; Elangovan et al. 2010). The tri- and hexavalent oxidation states are the most stable and dominant species in natural systems. Dichromate $Cr_2O_7^{2-}$ (Das et al., 2008), is very soluble and also causes serious diseases to all kinds of living thing (plants, animal, etc.). On the other hand, as a requisite nutrient to animals and humans (Cervantes, 1991), trivalent chromium has lower toxicity, and has powerful sorptive affinity to solid surface making this specie more immobile in the environment (Das et al., 2008; Dogan et al., 2011). So, the reduction of hexavalent chromium to its trivalent form is an operational way for treatment of polluted wastes and environments (Chen et al., 2011).

Conventional physico-chemical technologies like chemical reduction with reducing agents such as sulfides (Hsiao et al., 2008) or sodium thiosulfate (Gagrai et al., 2013), ion exchange (Cheng et al., 2010) and absorption on clay minerals (Pal and Paul 2004) have been adopted to detoxify and remove chromate from Cr(VI)-contaminated environments. Nevertheless, these remediation methodologies require huge amount of chemical agents and generate pollution caused by sludge that contain metal (Ozturk et al., 2012; Ge et al., 2013) and make these treatment processes costly as well as unsustainable. Currently, attention has been given to the bioremediation of Cr(VI)-rich land with microorganisms like bacteria, fungi and alga, which have the ability to remove Cr(VI). In addition, using various strains of bacteria, the bioremediation of Cr(VI) to Cr(III) can be declared as an economical and eco-friendly alternative to traditional methods (Cheng et al., 2010).

There are various Bio reduction mechanisms of Cr(VI) which are also type-dependent (Das et al., 2014). Microbial reduction of Cr(VI) is catalyzed by enzymatic reaction and also it has attributed to soluble reductases or membrane-bound enzymes (Dogan et al., 2011). Because of the influence of environmental conditions where biological reduction of Cr(VI) happens, however, bacterial chromate reduction has become an intricacy (Liu et al., 2006). For this, determination of optimum conditions is a great matter of concern for enhancing the efficiency of bio reduction.

2.2 Chemistry

Chromium is a steel-gray, odorless, hard metal. They get solubilized in sulfuric and dilute hydrochloric acid but not in alkali and strong alkalis (Merck, 1989). Chromium metal is produced mainly from the mineral chromite which is known also as chromite ore and can't be found in nature. In relative abundance, Chromium, Cr, is the twenty-first element which also loosely called Chrome. As Cr is concentrated in the earth's core and mantle, it is the seventh most abundant element. It is in the group 6(VIB) of the Periodic Table and has atomic number 24. Chromium's highest oxidation state is 6+ and lowest 2- , 1- , 0 and 1+. Chromium (3+) is the most important and stable oxidation state of the element. Including many examples of isomerism, Chromium (III) complexes are extremely numerous and varied. Chromium's one of the valence states (+6) is the hexavalent chromium of the element. It is produced by an industrial or commercial process and can cause cancer. Moreover, it targets the eyes, kidneys, respiratory system, skin and liver.

Table 2.1: Chromium compounds and their oxidation states

Oxidation states	Compounds
-2	$Na_2[Cr(CO)_5]$
-1	$Na_2[Cr(CO)_{10}]$
0	$Cr(CO)_6$
+1	
+2	CrO, CrF_2, $CrCl_2$, CrS, $Cr_2(SO_4)_3$
+3	Cr_2O_3, CrF_3, $CrCl_3$,$[Cr(H2_O)_6]^{3+}$
+4	CrO_2, CrF_4
+5	CrF_5
+6	CrO_3, $Na_2Cr_2O_7$, CrO_4^{2-}, $CrOF_4$

Table 2.2: Physical characteristics of Chromium

Atomic Weight	51.966
Atomic Number	24
Valences	1-6
Boiling Point	2642^{o}C
Melting Point	1900^{o}C
Density	7.14

2.3 Sources and Occurrence of Chromium compounds

Chromium is naturally occurred as a trace material of most crude oils. The trivalent form is released from sewer sludge incineration, cement production, oil combustion, refractories and municipal waste (ARB, 1986d). It exists in a stable form and is an inorganic pigment which is used for rubber and plastic products and paints (Howard, 1990). Chromium has not quantified in motor vehicle exhaust by the Air Resources Board but it has been detected (ARB) (ARB, 1995c).Chromium is used for steel production as protective coating for automotive, corrosion resistance, equipment accessories.

Environmental occurrence

Chromium distribution and occurrence in the environment has been extensively reviewed. Although chromium is primarily discharged in the environment because of some anthropogenic activities, Naturally, Chromium (VI) can occur in the earth's crust. (Mukherjee, 1998; Kotaś & Stasicka, 2000)

Natural occurrence

Only potassium dichromate (as lopezite) and lead chromate (as crocoite) occur in nature (IARC, 1990). Lead chromate is the most commonly seen hexavalent chromium. The yellow paint in traffic lanes is Lead chromate.

Water

In water solution, hexavalent chromium is converted to the bright metallic chromium coating by electroplating which is seen on metal or plastic products such as shower heads or car bumpers. Chromium concentration in uncontaminated water is extremely low. Anthropogenic activities like leather tanning, electroplating, and wastewater discharge from sites such as landfills may cause contamination of the drinking-water (EVM, 2002).In the USA, Chromium (VI) has been identified in surface as well as in samples of groundwater collected from 120 harmful waste sites (ATSDR, 2000) and in California, USA, 38% of community sources of drinking-water have chromium (VI) levels greater than the detection limit of1 µg/L (Sedman et al., 2006).

Soil:

Although chromium (VI) can occur under oxidizing conditions. In trivalent form, they can be present in most soils (ATSDR, 2008a). In the USA, 1319 samples were collected and the geometric mean concentration of total chromium was 37.0 mg/kg which ranged from 1.0–2000 mg/kg (ATSDR, 2000).

Food

In food, most of the ingested metal is chromium (III) (EVM, 2002).

Smoking

Smoke of tobacco has chromium (VI).Cigarette smoke can contain a huge amount of chromium (VI) found in outdoor air.

2.4 Uses of chromium

Chromium (VI) compounds are used extensively in applications like pigment for textile dyes and also for paints, plastics and inks, corrosion inhibitors for example zinc

chromate, wood preservatives like chromium trioxide and chrome plating like strontium chromate and leather tanning by ammonium dichromate. As impurity, Chromium (VI) can be present in Portland cement, and it can be generated and given off during cutting, welding, and casting operations in case of stainless steel (NTP, 2005; OHCOW, 2005).

2.4.1 Applications

- Chromium main uses are in chrome plating, alloys such as stainless steel and in metal ceramics. Chromium plating was used to give a silvery mirror coating to steels in metallurgy, chromium is used to give a shiny finish and corrosion resistance.
- Chromium can be used as dyes and paints and synthetic rubies are produced by them.
- Chromium is also used as a catalyst.
- Chromium is used for firing of bricks.
- In the leather tanning, chromium salts are used.
- In cleaning glassware and as titrating agent, they are used.
- They can be also used as a fixing agent
- Magnetic tapes are manufactured by using Chromium (VI) oxide (CrO_2)
- They are used in well drilling muds as an anti-corrosive.
- In medicinal products, they are used as a dietary supplement.
- Chromium hexacarbonyl is used as a gasoline additive.
- Chromium boride (CrB) is used as an electrical conductor.

2.5 Regulation regarding level of chromium

Thermodynamically, Cr(VI) can merely be found at moderately high pH. Depending on concentrations and pH, Cr(VI) varies from chromate (CrO_4^{2-}) having a pH of 6.5–14 through hydrogen chromate ($HCrO^{4-}$) and dichromate ($Cr_2O_7^{2-}$) which has a pH ranging from 0.7–6.5 to chromic acid (H_2CrO_4) having a pH $<$ 0.7. $HCrO^{4-}$ and CrO_4^{2-} can be easily converted to Cr(III) by using reducing agents like phosphate, Fe(II), sulfide, and other organic matter like humic acid. In association with different cations, Cr (VI) can

also be found in the form of solid minerals. These constitute different compounds like calcium, barium chromate and led which are the most insoluble. In contrast, dichromates are highly soluble. In soils, clay minerals that have a negative charge dominate and Cr (VI) anions are driven back by the soil particles that have negative charge and this incident makes hexavalent chromium relatively mobile. As a result, it can be concluded that Cr (VI) is very mobile and bioavailable in soil-water systems (N. Unceta, 23 January 2010).

Cr (VI) present in the water bodies more than the standard limit causes adverse effects to animals, human beings and also plants. Drinking water guidelines of World Health Organization (WHO) shows that the highest permissible limit for hexavalent chromium is .05mg/L and total chromium which include Cr (iii), Cr (vi) are 2mg/L (Gupta and Rastogi, 2009). From the data of Safe Drinking Water Act, Maximum Contaminant Level (MCL) is 0.1 mg/L. Maximum acceptable level of chromium in bottled water is 0.1 mg/L. Definite color additives have chromium less than 50 ppm. Occupational Safety and Health Administration (OSHA) recommends that based on chromic acid & chromates listing, for Cr (VI), 0.1 $mg/m^3$is the Permissible Exposure Limit. (National Institute for Occupational Safety and Health (NIOSH) specifies Immediately Dangerous to Life and Health (IDLH) limit as 15 mg/m^3. Suggested Exposure Limit is limited to 0.001 mg/m^3.

2.6 Chromium Toxicity and Health Effects

Hexavalent chromium compounds are class I human carcinogens and also the most common occupational chemical hazards. Inhalation of this insoluble chromate particles and inhalation of chromate fumes from stainless steel welding and electroplating create a high risk of respiratory cancers which are the most dangerous forms of Cr(VI) exposure. Micro molar concentrations of Cr(VI) in drinking water are not carcinogenic but they can alert the organism to other carcinogens such as UV radiation. Furthermore, recent studies shows that orally ingested of soluble chromates can lead to a variety of cancers. A recent finding include the ability of both water-soluble ($K_2Cr_2O_7$) and water-insoluble ($PbCrO_4$) chromates causing malignant transformation of noncancerous human lung epithelial cells in culture (Xie et al., 2007; Costa et al., 2010). Noteworthy progress has also been attained in understanding of the roles of disruptions of cell signaling in Cr(VI)-induced carcinogenicity and altered DNA repair mechanisms.

Cr(VI) compound's carcinogenic potential is determined by exposure routes and physical properties which are among the leading factors (Levina et al., 2003). For example, particles of insoluble Cr(VI) salts dissolve slowly when they adhere to the surface of bronchial epithelial cells and this leads to prolonged exposure of cells to small doses of Cr(VI) (Xie et al., 2007).

Chromium has no adverse effect as a pure metal but when they are present in a very large amount, they show little toxic effect. Hexavalent compounds are primarily responsible for acute and chronic toxicity of chromium .The most important toxic effects like after contact include dermatitis and after inhalation, or ingestion of hexavalent chromium compounds include allergic and eczematous skin reactions, skin and mucous membrane ulcerations, perforation of the nasal septum, allergic asthmatic reactions, bronchial carcinomas, gastro-enteritis, hepatocellular deficiency, and renal oligo anuric deficiency. (Baruthio, 1991)

A sequences of in vitro and in vivo studies have proven that chromium (VI) stimulates an oxidative stress which results in enhanced production of reactive oxygen species (ROS) and this leads to genomic DNA damage as well as oxidative deterioration of lipids and proteins. Studies show that, concentration-and time-dependent effects of sodium dichromate on enhanced production of superoxide anion and hydroxyl radicals change intracellular oxidized states. Fragmentation of DNA causes apoptotic cell death in human peripheral blood cells. Chromium (VI)-induced enhanced production of ROS, as well as oxidative tissue. DNA damage were observed in these cells. More effect was witnessed on chronic myelogenous leukemic K562 cells and J774A.1 murine macrophage cells. Furthermore, the effect of a single oral LD_{50} dose of chromium (VI) on female C57BLI6Ntac and p53-deficient C57BL/6TSG p53 mice on enhanced production of superoxide anion, lipid peroxidation and DNA fragmentation in the hepatic and brain tissues. Chromium (VI)-induced more oxidative damage in p53 deficient mice. This in vivo study highlighted that apoptotic regulatory protein p53 may play most important role in chromium (VI)-induced oxidative stress and toxicity. Oxidative stress, oxidative tissue damage, and a flow of cellular events including modulation of apoptotic regulatory gene p53 are involved in chromium (VI)-induced toxicity and carcinogenesis. (Mol Cell Biochem 222: 149-158, 2001)

2.6.1 Effects in animals

In summary, depending on the compound administered and the sex of the rat, acute oral median lethal doses in rats was exposed to hexavalent chromium compounds varied between 13 and 29 mg/kg bw (Gad et al., 1986). The main special effects observed in animals were decreasing body weight and fluctuations in hematological and immune factors.

Exposure of rats through inhalation resulted in pulmonary inflammation and neutrophil migration (Cohn et al., 1998).

Various studies showed that chromium compounds induced cancers in experimental animals following diverse exposure pathways including the, intratracheal, inhalation, intrapleural, oral route, intra muscular, intravenous, subcutaneous injections and intraperitoneal (ATSDR, 2008). Inhalation induced lung cancers in rats (Nettesheim et al., 1971) mice (Glaser et al., 1988). By depository injection, several chromium compounds like calcium chromate, zinc chromate and strontium chromate caused sarcomas. Orally given Potassium chromate enhanced UV-induced skin cancers indicating tumor systemic effects (Davidson et al., 2004).

2.6.2 Effects in humans

In humans maximum data on effects are derived from accidental exposure to very high doses and from occupational exposure by inhalation which include mainly the workers who are unprotected to chromium compounds.

Rashes and ulcers are caused by skin contact with compounds containing hexavalent chromium. Dermal exposure to chromium has also been linked to allergic dermatitis. A patch test was done which include 2 µg, prerequisite to evoke a skin reaction in hypersensitive subjects. The prevalence of chromium sensitivity in the general population has been estimated to be between 0.5% and 1.7% in studies in several European countries (Peltonen and Fräki, 1983;Hartwig, 2007; Hartwig, 2010). Inhalation in occupationally exposed workers induced effects in the airways like septal perforation and nasal mucosal ulceration. Variations in lung function parameters were also observed. Revelation was estimated based on the exposure period and on the mean and median annual chromium concentrations likely to be experienced in the job position detained when the symptoms first occurred (Gibb et al., 2000a;).

Hexavalent Chromium cause DNA damage by breaking DNA strand , cross linking of DNA–protein, chromosomal aberrations or exchanging of sister chromatids in the

lymphocytes of electroplaters, ferrochromium alloy foundry, welders who were unprotected by inhalation, as reviewed in WHO/IPCS, 2013.

Regarding the effect of chromium VI on nasal and nasal sinus cancers, the epidemiological evidence remains remindful but inconclusive (IARC, 2012).

An association between gastro-intestinal tract cancer and exposure to hexavalent chromium in drinking water has been reported at a contaminated location in China (Zhang and Li, 1997). A metadata analysis did not reveal any increase in cancers of the gastro-intestinal tract in workers who were unprotected by inhalation (Gatto et al., 2010), but the individual studies were small and interpretation was vulnerable by lack of adequate revelation measurements and lack of information on possible confounders such as smoking, alcohol consumption, dietary factors, and socioeconomic status.

2.7 Chromium induced Carcinogenesis

The National Academy of Science sand the National Institute of Occupational Safety and Health has reviewed the general toxicology of chromium compounds International Agency for Research on Cancer has specifically reviewed the carcinogenic properties of chromium compounds. At present, the most significant problems are about trivalent chromium which causes cancer and if there are differences between soluble and slightly soluble salts of hexavalent chromium in cancer causing effects. The specific compound or compounds which have caused cancer in chromate workers and the importance of smoking in chromium carcinogenesis is not known.

2.7.1 Biological Interactions

When animals are exposed to hexavalent chromium, the hexavalent form exists for some time indicated by a different distribution pattern than after exposure to the trivalent form. The hexavalent form of chromium is always connected to oxygen and thus also a strong oxidizing agent. Hexavalent chromium freely passes the cell membrane and in the cell, it is reduced to the trivalent. The site in the cell for this conversion are the plasma membrane, the endoplasmic reticulum, the mitochondria or the cell nucleus. Chromium carbonyl causes cancer in a transplantation animal technique.

2.7.2 Environmental Exposure

Because of chromium's skin sensitizing effect, exposure to the general public has become important health consequences. Chromium is among the most common sensitizers in allergic eczema. The daily consumption from food has been expected to be in the range of 0.03-0.1 mg. The chromium content in municipal drinking water has been estimated to be in the same range as in rivers and lakes (1-10 pimg/l.).Urban air concentrations have been reported from less than 10 mg/m^3 to about 50 mg/m and for rural stations seldom above 10 ng/m^3. A possible source of chromium exposure to the general public is waste dumps which causes water pollution. Tobacco has been reported to contain up to about30 mg/kg of chromium. No association has yet been made between chromium exposure to the general public and human cancer.

2.7.3Occupational Exposure

Potentially hazardous exposure to chromium compounds may take place in the chromate production industry, in the metallurgic industry and in the refractory brick industry. Hazardous chromium exposure is also reported in industries which are secondary users of chromium chemicals in pigments production and plating. Welders and grinders in the secondary metal industry (steel welding and grinding, anticorrosive paints) are also exposed to chromium compounds, and chromium may be found in small amounts in a variety of industrial settings. Chromium is extensively used in tanning. Mancuso and Hueper reported exposure levels of up to 1 mg/m^3 in a chromate producing plant, but most values were in the range of 0.26-0.52 mg/M3. A five-shift mean value of 1.35 mg/m^3 for a sack filling operation was reported by Langard and Norseth in an old chromate pigment plant, while in another modern plant levels were mostly below 0.2 mg/m^3. In a ferrochromium plant most average shift values(personal sampling) were below 0.05 mg/m^3, but occasional values up to 1.3 mg/m^3 (total chromium) were recorded . In a recent review a chromium exposure up to 5 mg/m^3 in the plating industry was mentioned, but most values were in the range 0.1-0.2 mg/m^3. Similar values for chromium exposure in different industries have been indicated in a review by Hayes. The exposure levels seem to have been fairly constant during the last 20years in older plants, but lower values are found in new plants (Norseth, 1981).

2.8 Conventional methods used for remediation of Chromium toxicity

Many kinds of physical and chemical methods are available for the reduction of Cr (VI) from the waste water steam. These methods are also called conventional methods. There are some common conventional methods that are usually used to reduce Cr (VI) level in water, like adsorption, membrane technologies, chemical reduction, filtration, precipitation, evaporation recovery, filtration etc. (Rama Krishna et al., 2005, Ahluwalia and Goyal 2007, Sikaily et al, 2007).

2.8.1 Electro Chemical Precipitation

Cr (VI) concentration could be removed from 3,860 mg/l to 0.2 mg/l by using ECP process from an electroplating wastewater, according to Kongsricharoern and Polprasert. To minimize the level of heavy metal in the contaminated water this is the most common method which uses an electrical potential and this method remove heavy metals over the conventional chemical precipitation method (Kurniawan et al., 2006).

pH and salts (ion) can affect the efficacy of the process. High water content sludge is produced due to addition of chemicals and the disposal of which is cost intensive. This method is cost effective but due to precipitation with lime, disulphide or iron exchange, it lacks specificity and ineffective in removing metal ions at this low concentration.

2.8.2 Ion Exchange

In recent years, ion exchange resin had become a popular method for the removal of chromium from water. In this method, solution containing chromium passes through a column under pressure through resin bed where chromium ion is displaced from an insoluble exchange material by ion of different species in solution. Synthetic organic ion exchange resins are mainly used for this method.

To investigate the uptake of Cr (VI) a specific ion exchange resin called synthetic Dowex 2-X4 was used and a strongly basic anion resin in hydroxide form was also used as column as anionic exchanger which was 100% success for removing Cr(VI) (Sapari et al., 1996).

Ion exchange resins are very specific which comes out rather as a drawback for chromium removal. A resin should be selected as such so that it particularly removes the metal contaminant of concern. Moreover, ion exchange material can be pricey and there can be partial removal of the chromium from the salt solution. On the other hand, it

cannot manage concentrated metal solution since the matrix gets effortlessly impure by organics and other elements in the wastewater. Ion exchange is nonspecific and is highly delicate to pH of the solution.

2.9 Metals and Microorganisms

2.9.1 Metal resistance mechanism in Bacteria

The universal nature of metals in the environment has resulted in the widespread presence of resistance in metals in microbes. Microbial resistances in metals are heterogeneous. There are five mechanisms that include resistance in heavy metals in Bacteria (Rouch et at., 1995) are as follows:

1. Segregation of metals by a permeability barrier.
2. Active export of metal by segregation from the cell.
3. Intracellular somatic appropriation of the metal by binding of proteins which prevent it from damaging metal- sensitive cell components.
4. Repossession of extracellular components.
5. Purification when metal is chemically modified to less active.

2.9.2 Metal sensitivity of cellular components

Metal ions increase or decrease enzyme doings, change enzyme specificity by bringing different changes, by locking them in specific conformations; or by forming firm bonds with active sites in enzymes and transport structures and thus inhibiting their role (Kronmannand Batcher, 1984).

Metal ion can damage the structure of DNA directly, for example by producing strand cross links (Yamane and Davodson, 1962), or they can affect the information content of DNA indirectly by decreasing the fidelity of synthesis of DNA (Beyersman, 1994).

Although for metal- induced damage, a wide range of cellular components are potential targets, a subject of these components are needed for important cell function like DNA for replication. Cell death will result from inactivation of metals so that the concentration of a particular metal rises and functions are inactivated when critical concentration is reached. Therefore, the cell should have some ways of safety depending on the metal concentration to survive one or more target sites. The higher the concentration of metal the larger the number of necessary components that needs protection. For instance,

production of the major proteins by E. coli bacteria can be stopped by mutation in a single gene which will result in increased resistance of metal (Lutkenhaus, 1977).

2.9.3 Metal uptake system and resistance

Cells that are highly sensitive to metals and necessary cell components are situated in the cytoplasm, the number of uptake process for the access of metal into cell affect the choice of resistance mechanism. The lipid component present inorganic membrane is highly not permeable to hydrophilic ions (Anderson, 1978). Metal, therefore, passes through the less resistant places in the membranes. For example, in case of E. coli Arsenate, Cobalt enter through phosphate transportation system of phosphate and Transport system of magnesium. (Silver et al., 1975).

2.9.4Biological requirement of metals

Heavy metals are essential in maintaining metabolic activities of bacteria. Most of the bacterial species require Copper, iron and nickel and on the other hand, cobalt, and Molybdenum, Tungsten are required in some bacteria (Frausto and Williams, 1993; Hughes and Poole, 1989). Associated with metal with no metabolic roles, they will be less toxic to the cell because the cell will exhibit mechanisms that will come up with the small activities in limited concentration.

2.9.5 Chromosome-mutation-determined resistance versus gene cassette

The genetic origin of metal resistant in resistant bacteria can be determined by the availability of a performed gene- cassette(s) that requires a dedicated mechanism of resistance. These are supposed to be adopted to give effective resistance by previous long term process of selection. The last two aspirants can help in the transfer of the linked resistance cassette between bacteria. Resistance may give higher levels of resistance than that of available chromosomal mutations allow. The larger a population, the greater is the probability of a relevant cassette bearing genetic element being present.

2.10 Mechanism of Chromium resistance in Bacteria

Metals that are much of the time released in modern effluents are of grave concern as a result of their poisonous impacts. Chromium mixes have boundless mechanical applications in electroplating, wood conservation, leather tanning and steel fabricating segments and thus end up as contaminants.

Transport and toxicity

Cr(III) is a vital supplement in the eating routine and they helps in the digestion of glucose and lipids (Anderson, 1997). Ingesting a lot of it bring about issues like lung disease (Katz and Salem, 1994). Cr(VI) is a cancer-causing agent and 10–100 times more harmful than Cr(III) (Katz and Salem, 1994). This might be because of Cr(VI) enters mammalian cells more promptly than Cr(III). The United States Environmental Protection Agency (USEPA) decided its reference measurement to be 1 mg per kg per day (Katz and Salem, 1994).

Cr(VI) enters the cell through the film sulfate transport channels (Ohtake and Silver, 1994) and act as intracellular reductants.Cr(V) experiences a one-electron redox cycle to recover Cr(VI) by exchanging the electron to oxygen. This procedure include receptive oxygen species (ROS) which joins with DNA–protein (Fig: 2.1). Cr(IV) likewise tries to hinder their ordinary physiological capacities (Pesti et al., 2000; Cervantes et al., 2000). Therefore, it expels chromium contaminants, particularly Cr(VI) from the earth.

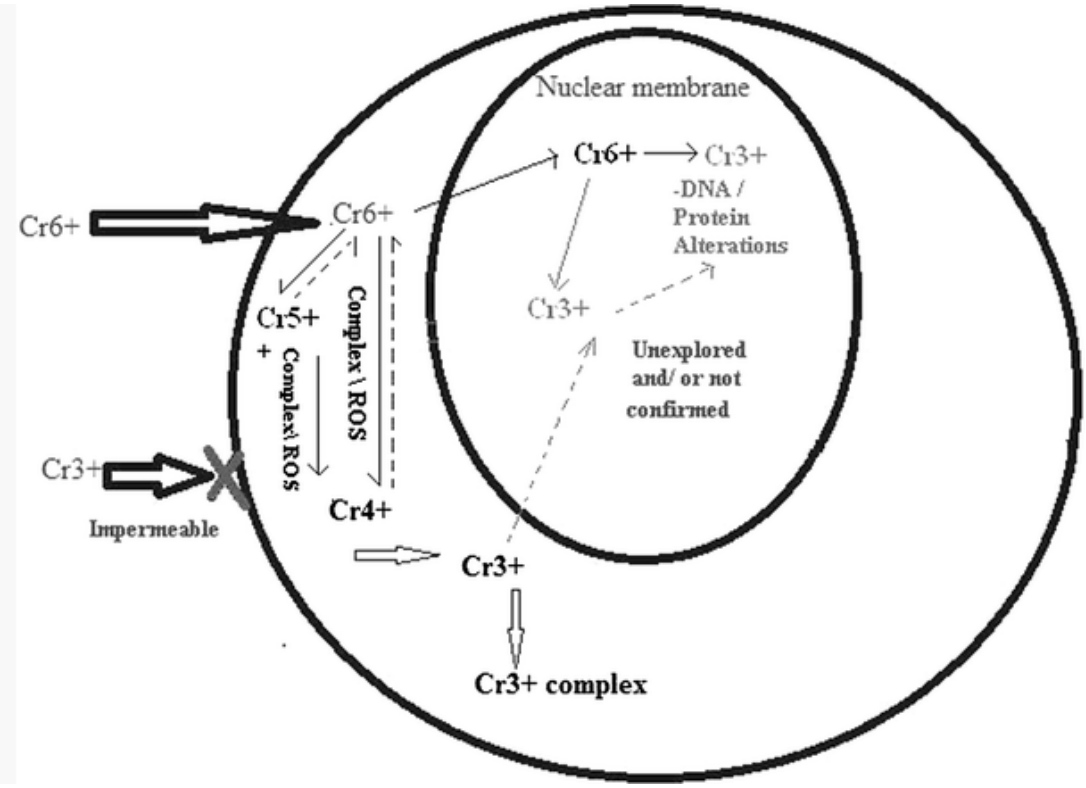

Fig 2.1: Schematic diagram of chromium uptake into the cell and its fate

The majority of the intracellular Cr(VI) reductants are one electron reducers producing Cr(V) and it results in the development of ROS that are transcendently in charge of the impacts of Cr(VI) (Cheung and Gu, 2007).

Remediation of Cr contaminants: Physico-chemical versus biological methods

Chromium expulsion comprises of decreased precipitation or utilization of particle trade and adsorption. Biotransformation and biosorption use the capability of microorganisms or plants to adsorb substantial metals. Of these, biotransformation of Cr(VI) is less harmful, less portable. In biosorption, the contaminant gets collected in the biomass which again must be discarded, while in biotransformation, it is changed over to a harmless structure. Under vigorous conditions, microbial chromate lessening is achieved by dissolvable reductases. Without oxygen, both solvent and layer bound reductases including cytochromes have been embroiled. A huge materials including starches, proteins, fats, hydrogen, NAD(P)H and endogenous electron stores can serve as electron contributors in the diminishment procedure (Wang, 2000).

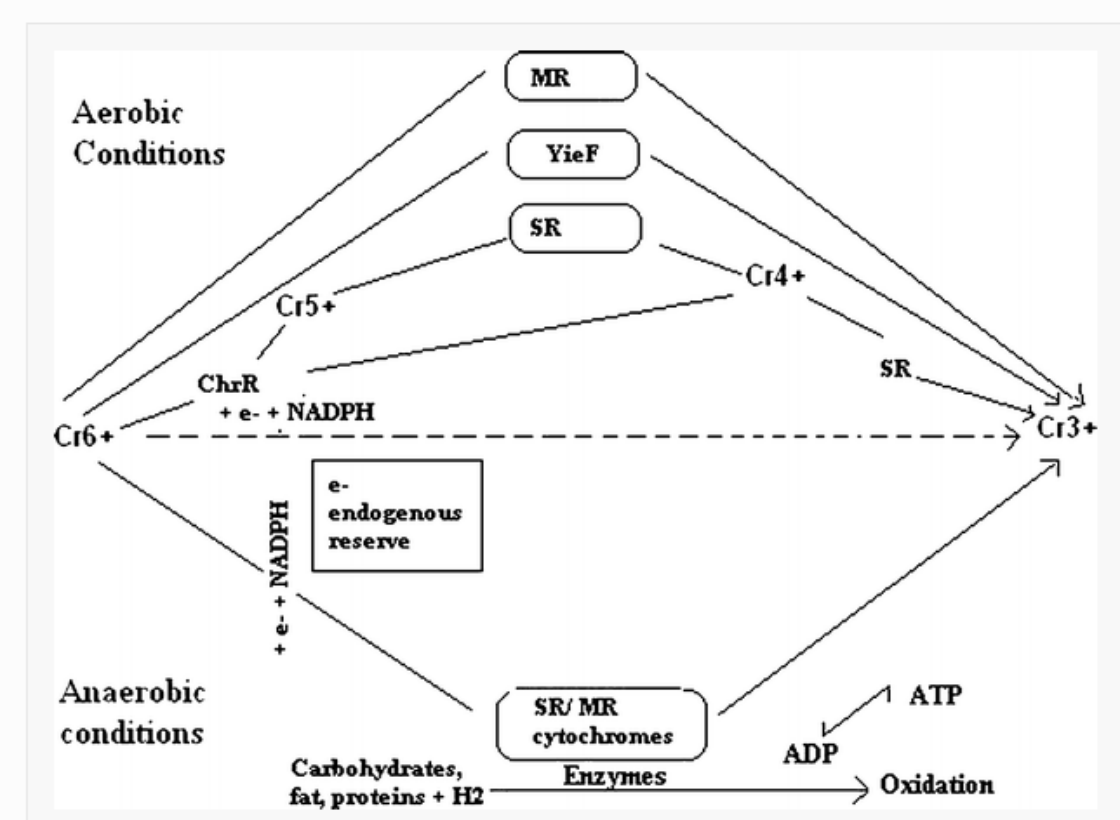

Fig 2.2: Mechanism likely to be involved in enzymatic reduction of chromium under aerobic and anaerobic condition.

Under vigorous conditions, ChrR of Pseudomonas putida MK1 catalyzes one and also two electron which changes to Cr(VI) with the transient development of Cr(V), while YieF of Escherichia coli realizes a four electron move bringing about the immediate decrease of Cr(VI) to Cr(III), with the rest of the electron getting exchanged to oxygen.

Under anaerobic conditions, both dissolvable and film related Cr(VI) decrease chemicals which include cytochromes connected with the electron exchange framework (Cheung and Gu ,2007).

Bacterial Cr(VI) reduction:

Since the revelation of the primary microorganism P. dechromaticans fit for lessening Cr(VI) in the 1970s (Romanenko and Korenkov 1977), the quest for chromate decreasing microorganisms has proceeded and much data has been accumulated on microbial-intervened. Cr(VI)- decreasing organisms have been disengaged from an assortment of situations which shows that such potential is far reaching in nature and can be utilized to encourage remediation of Cr polluted wastes.

Microbacterium sp. MP30 was supplemented with 15 mM chromate however did not lessen chromium concentration. Under anaerobic conditions, it diminished 100 µM sodium chromate within 30 h to the detriment of acetic acid derivation as electron giver (Pattanapipitpaisal et al., 2001). Respectably halophilic Nesterenkonia sp. MF2 was tolerant to a much higher grouping of 600 mM chromate and totally decreased 200 µM Cr(VI) in 24 h under high-impact condition. Starting Cr(VI) fixation up to 400 µM did not significantly affect Cr(VI) diminishment. There was decrease of chromate within different centralizations of salts (Amoozegar et al., 2007). A strain of B. circulans showed similarly far superior movement and diminished as much as 190 mM chromate both under anaerobic and oxygen consuming conditions, most extreme decrease being seen at 28 h of hatching (Chaturvedi et al., 2007). Exiguobacterium sp. ZM-2 indicated imperviousness to 12.37 mM potassium chromate and lessened 500 µM Cr(VI) inside 56 h. The nearness of metabolic inhibitors sodium azide and sodium cyanide extremely influenced chromate lessening, while 2, 4-dinitrophenol, and an uncoupling operator, fortified chromate diminishment (Alam and Malik, 2008).

In one study utilizing landfill separates, Bacillus sp. XW-4 was tolerant to and diminished 1.9 mM Cr(VI). Chromate diminishment by the strain was altogether improved by the nearness of glucose (Liu et al., 2006). In another study, suspended societies of B. cereus GIDM20, B. fusiformis GIDM22 and B. sphearicus GIDM64 showed more than 85% decrease of 1 mM Cr(VI) inside 30 h. The movement was observed to be chiefly connected with the solvent portion of cells, communicated constitutively and unaffected by nearness of various metal particles aside from Hg^{2+} and Ag^{+}. Expansion of NADH upgraded Cr(VI) diminishment (Desai et al., 2008). Another

bacterium, Ochrobactrum sp. demonstrated Cr(VI) lessening capacity under basic conditions. This seclude was tolerant to and lessened as much as 15.4 mM Cr(VI). For this situation as well, the expansion of glucose brought on an emotional increment in Cr(VI)- lessening, while the nearness of sulfate or nitrate had no impact (He et al., 2009). In another study, filamentous bacterium Sphaerotilusnatans CSCr-3 segregated from actuated slime frameworks vigorously diminished up to 1.5 mM Cr(VI) within the sight of a carbon source (Caravelli et al., 2008). This diminishment potential can be viewed as critical as S. natans is already known just for its biosorption capacity.

Bacillus sp. KCH2 and KCH3, Leucobacter KCH4 and Exiguobacterium KCH5 were disengaged from Cr defiled soil. Of these, KCH3 and KCH4 demonstrated higher Cr(VI) resistance (2 mM) and lessening (1.5 mM) than KCH5 (1.5 and 0.75 mM, separately). Cr(VI) decrease was hindered by Hg2+ and upgraded by Cu^{2+} (Sarangi and Krishnan 2008). Improved societies of indigenous microorganisms detached from a stream silt tainted by a color assembling industry lessened 31% of the 250 µM Cr(VI), which is proportional to the replaceable Cr(VI) fixation in the residue. The expulsion effectiveness was expanded with outer supply of electron giver as glucose, lactate or acetic acid derivation (Lee et al., 2008).

Staphylococcus epidermidis L-02 separated from a bacterial consortium utilized for the remediation of a chromate-defiled built wetland framework lessened Cr(VI) by utilizing pyruvate as an electron giver under anaerobic conditions. The nearness of nitrate expanded the particular decrease rate. Under denitrifying conditions, Cr(VI) lessening was not hindered by nitrite. The most extreme particular lessening rate was as high as 8.8–9.8 µM Cr 1010/cells/h (Vatsouria et al. 2005). Intrasporangium sp. Q5-1 separated from a Cr debased site demonstrated 17 mM/L least inhibitory focus (MIC) for Cr(VI). Resting cells likewise lessened chromate. At the point when immobilized with intensifying dabs containing 4% Poly Vinyl Alcohol (PVA), 3% sodium alginate, 1.5% dynamic carbon and 3% diatomite, the decrease rate stayed unaltered (Yang et al., 2009). These immobilized cells have the benefit of strength, reusability and less stopping up in nonstop frameworks.

While trying to realize Cr(VI) remediation utilizing psychrophiles, center specimens acquired from a Cr(VI) tainted aquifer were improved in Vogel Bonner medium

supplemented with Cr(VI). The separate Arthrobacteraurescens P4 was impervious to 19.2 mM Cr(VI) however diminishment was moderate or not saw at or more 1.9 mM Cr(VI) (Horton et al. 2006). This species has beforehand not appeared to be able to do low temperature (10°C) Cr(VI) lessening.

Aside from such contamination by anthropogenic exercises, chromate safe microscopic organisms have been detached from regular habitants. B. sphearicus from actually happening chromium permeated soil of Andaman Islands was tolerant to 15.4 mM Cr (VI) and lessened >80% of it amid development. Solvent portion of the cell was in charge of vigorous lessening of chromate by this living being (Pal and Paul 2004). Burkholderiacepacia MCMB-821 separated from the soluble pit pool of Lonar was impervious to 19.2 mM Cr(VI) and lessened 98% of the 1.4 mM Cr(VI) inside 36 h within the sight of 2% salt and lactose as the electron benefactor (P. Kanmani, 2012).

Table 2.3: Bacterial Mechanisms of Chromate resistance

Enzyme/ system	Species	Function	Reference
Transport			
ChrA transporter	*Pseudomonas Aeruginosa*	Efflux of cytoplasmic Chromate	Alvarez et al. 1999
Cys operon products	*Shewanella Oneidensis*	Sulfate transport	Brown et al. 2006
TonB receptor, hemin Transporter	*S.oneidensis*	Iron transport	Brown et al. 2006
Reduction			
Chromate reductases	Diverse species	Reduction of Cr(VI) to Cr(III)	Cervantes et al. 2001
SOD, catalase	*Escherichia coli*	Combat of oxidative Stress	Ackerley et al. 2004

Outer membrane Proteins	*Caulobacter Crescentus*	General stress Response	Hu et al. 2005
DNA repair			
RecG and RuvB DNA Helicases	*Pseudomonas Aeruginosa*	Repair of DNA Damage	Miranda et al. 2005
SO0368, UvrD, and HrpA helicases	*Shewanella Oneidensis*	Repair of DNA Damage	Chourey et al. 2006
Other Mechanisms			
Cys operon products	*S. oneidensis*	Sulfur metabolism	Brown et al. 2006
Adenylyl sulfate Kinase	*S. oneidensis*	Sulfur metabolism	Brown et al. 2006
Ferritin	*S. oneidensis*	Iron binding	Brown et al. 2006

2.11 Hexavalent chromium reduction by microbes

It is known that Hexavalent chromium is the plentiful contaminants present in groundwater. It has mutagenic, toxic and carcinogenic effects on the living system (U.S.E.P.A., 2000). The physical and chemical characteristics of Cr(VI) vary from strongly acidic (Li et al., 2008) to the alkaline chromite (Stewart et al., 2007).Among various treatments,Cr(VI) reduction to less toxic trivalent form involves a great remediation (Brandhuber et al., 2004). According to Eq. 1:

$$\mathrm{CrO_4^{2-} + 8H^+ + 3e^- = Cr^{3+} + 4H_2O}$$

Though Cr(VI) remediation is an effective method, the reduction depends on the existence of protons as well as on the pH of the wastewater. Nernst equation relates the reactant's concentration with the redox potential of the Cr(VI)/Cr(III) redox couple:

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

Here, $E'_0$is denoted as the potential, R is the molar gas constant (8.31447 J mol−1 K−1), T is the temperature (K), n is the number of electrons exchanged (3), and F is Faraday's constant (96,485.3 C mol−1). From the equation it can be said that the lower the [H+] concentration the higher the pH and also the lower the redox potential of the Cr(VI),Cr(III) couple. So, the lower the tendency of Cr(VI) reduction will also be lower.

Technology like Microbial fuel cells (MFCs) is new for Cr(VI) reduction process. Principle of this process is shown in the figure. In the anode, organic compounds release electrons and they are transferred to the cathode by bacteria which act as "catalysts". The cathode can also use bacterial catalysts. Cathode receives electrons by Cr(VI) which is the last step of this process. Externally, a resistor is applied to the system and because of the movement of electrons which takes place from anode to cathode, electrical power is produced. Thus, it improves the sustainability of the remediation process. Protons are transferred to the cathode by a proton or a cation exchange membrane in order to close the electrical circuit and to run eq. 1.

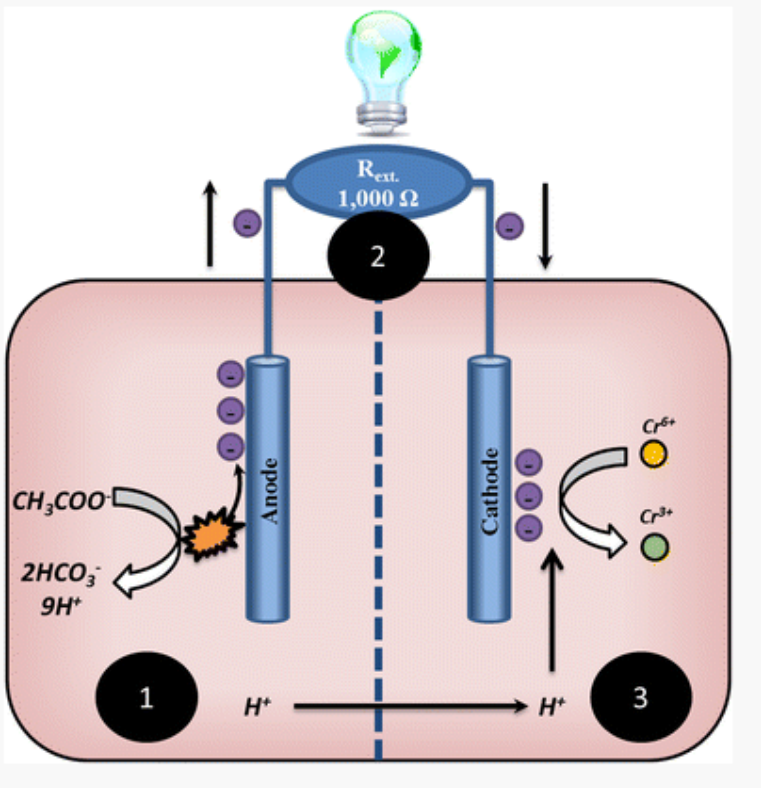

Fig 2.3: Microbial fuel cell

The theory of Cr(VI) remediation process in MFCs: 1. organic substrates are broken by microbes in the anode and thus they release protons and electrons; 2.electricity is

produced when protons and electrons are passing to the cathode through a proton-permeable membrane and also an external resistor, respectively;3. Cr(VI) is converted to Cr(III) in the cathode depending on the pH.Cr(VI) reduction takes place very fast because of the abundance of protons and the final product is mainly soluble which is Cr^{3+}. The process isslow down as protons are not rich in number at neutral or alkaline pH. As a result, the charge of the final product of the reduction process will vary with varying pH and thus positively charged Cr(III) compounds will be deposited on the negatively charged MFC cathode (Clark and McCreery 2002; Hurley and McCreery 2003). Cr(VI) remediation in MFCs depends on electrochemical Cr(VI) reduction. Therefore, deposition of electron will result in a self-inhibitory behavior of cathode. Finally, Cr(VI)'s poor ratio will be decreased per electrode surface area.

Another crucial part of functioning Cr(VI)-reducing MFCs is the toxicity of Cr(VI) and it might exist in the anode. Cr(VI) could be moved to anode by reactor failure. It could also be moved to anode by selecting an anion exchange membrane which would permit the negatively charged Cr(VI) oxides to be transferred from the cathode to the anode (Pandit et al., 2011). So it can be said that evaluation of Cr(VI) toxicity in MFC anodes is important.

CHAPTER -3: MATERIALS AND METHODS

3.1 Introduction

This chapter involves the materials that has been used throughout the process and describes the experimental procedure for biodegradation of hexavalent chromium. It also provides a summary of the process of isolation and categorization of bacterial strains that have been collected from Aquatic sample. Samples of water and soil were obtained from Sitakunda Ship Breaking Yard which were characterized and well documented. Different studies like Optimization of culture and process parameters for the cell growth and Cr (VI) degradation kinetics is also included in this chapter.

3.2 Chemicals

Analytical grade and pure chemicals were used in all experiments including media preparation for growth. The chemicals are:

- Nutrient agar
- DPCZ
- Nutrient broth
- Hydrochloric acid
- Potassium di chromate
- Sodium hydroxide
- MOPS buffer etc.

3.3 Glassware and Apparatus:

The instruments and apparatus used throughout the experiment are listed below in Table 3.1

Table 3.1: List of Instruments used during the whole experiment and function

Instruments	**Functions**
Vertical Autoclave	Sterilization
Analytical Balance	Weight Measurement
Laminar airflow	Aseptic Environment
pH	Measurement of pH
BOD Incubator	Incubation of cultures
Ultra Low Temperature	Preservation of cultures

freezer (-80^{o}C)	
water system	Preparation of the stock solution, throughout the experiment etc.
Spectrophotometer(UV/Vis)	Estimation of cell growth and Cr (VI) degradation
Centrifuge	For the separation of microorganisms
Water Bath	Media Solubulization
Microscope	Microbial morphology Observation
Micro Pipetter	To collect drop amount of chemicals.

3.4 Sample Collection:

In the coastal regions of Bangladesh, Ship breaking industry is one of the most important and growing industries and they have a lot of environmental drawbacks. This industry is situated in Sitakunda at the Fauzdarhat beach shown in Fig- 3.1. it covers 16 km area near the Bay of Bengal and almost 20-km southwest of Chittagong(DNV, 2001). Ship breaking is done in order to recycle the scrap metals. Bangladesh being the leader in breaking down of large ships, all the ships are brought in here (Ahmed et al., 2013). It breaks mainly large toxic ships like container ships (Frey, 2013). Maximum ships that are toxic, outdated ships age around 20-30 years, are brought back to Bangladesh (Sarraf et al., 2010). Generally, a ship consists of 95% steel and 5% hazardous materials (Khan et al., 2011). Since ship dismantling occurs in beach area, tidal zone, and sub-tidal zone, deep sea gradually becomes host to the different kind of wastes. These wastes contain heavy metals and bacterial pollutants (Reddy et al., 2005).

Heavy metals that are deposited in the marine soil, are associated with particulates. They can be suspended or create complex that are soluble to chromium biological accumulation (Reddy et al., 2005). Generally, a ship weighing 5000 ton to 40000 ton, is coated with 10- 100 ton of paint and they contain heavy metal (Khan et al., 2011). Entire heavy metals containing in soil and water sample is above the background level.

The samples of water and soil were collected from different locations where chromium gets deposited and also from the contaminated site of Sitakunda Ship Breaking Yard.

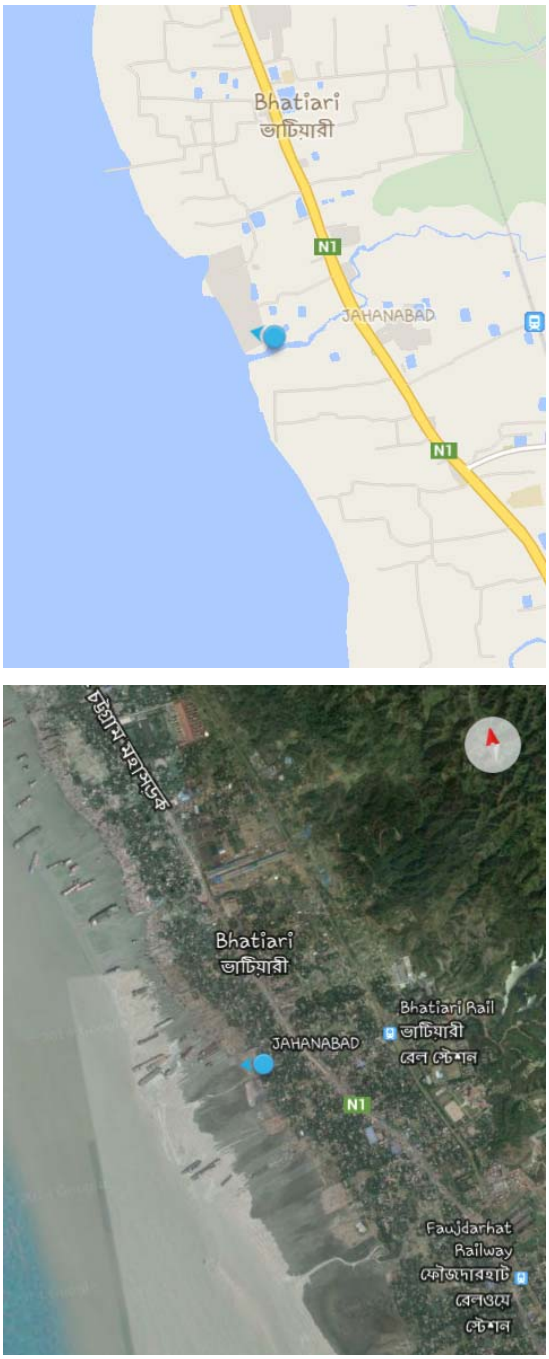

Figure 3.1: Ship Breaking Industry area in Bangladesh (Google Map, 2014)

3.5 Isolation and Culture Condition:

Bacterial isolation process was done according to standard techniques. First of all, inoculation of 100 µL sample of waste water and 100 µL fresh sample of soil was done by spreading technique on Agar Plate which contained 2mM of Cr^{6+} added with $K_2Cr_2O_7$ to the media and the colonies were incubated at 37^0C. After 24 hours, observations of the colonies were done.

Preparation of nutrient agar media was done when 2.8g of Nutrient Agar powder was dissolved in 100 ml water. Autoclaving was done at 121^oC for 45 min. after that, $K_2Cr_2O_7$ was added to the media and it was emptied into the plate to prepare the Nutrient Agar plate. Colonies that were isolated, were picked up with sterilized tooth pick. Isolates were lined on nutrient agar medium plate which contained 2mM, 3mM, 4mM, and 5mM Cr^{6+} respectively. Again, it was incubated at 37^oC for 24 hours. This procedure was repeatedly done with continuously greater concentrations of Cr^{6+} until the minimum inhibitory concentration of bacterial strain was found. Noteworthy growth as well as speedy Cr (VI) degradation kinetics of the specific bacterial species were noticed in the occurrence of 40mM Chromium (VI) for about 24 hours of incubation at 37°C, were considered as Cr (VI) resistant. . A particular strain which was able to grow at this condition was selected. At the end of the isolation process, two colonies from different Nutrient Agar plates and different chromium concentrations were isolated. Their names were given matching to their Chromium concentration.

3.6 Chromium reduction profile of Chromium resistant bacteria:

From Standard Methods, Diphenyl carbazide assay for measurement of Cr6+ was adapted for the Investigation of Wastewater (Greenberg et al., 1992) as well as the methods listed in Turick et al., 1996. A standard curve was prepared to standardize the reduction outline of the chromium reducing bacteria.

3.6.1 Chemicals Preparation:

3.6.1.1 10ml 3M H_2SO_4 Preparation:

First of all, 8ml of distilled water was placed in a Falcon tube. After that, 1670 µl of concn. H_2S0_4 was added by dropper into 8ml of distilled water. Finally, 330 µl of distilled water was added to make the volume up to 10ml.

3.6.1.2 Diphenyl Carbazide Preparation:

DPCZ powder weighing .025g was placed in falcon Tube and 1 ml of Acetone was added in it. Later on, 330 µl of 3M H_2SO_4 was also added in falcon tube and the tube was shaken very well to make consistent solution of Diphenyl Carbazide.

3.6.1.3 MOPS buffer Preparation:

At first, by adding 0.1 g NaOH in 50ml of water, 50 ml of 1N NaOH was made. Later on, 334.88mg MOPS was added with 80ml of distilled water and 20mM MOPS buffer was ready. The MOPS buffer pH was attuned to 7 by adding 1N NaOH drop by drop in the buffer solution.

3.6.1.4 5mM 10ml $K_2Cr_2O_7$ Preparation:

19.4 g of $K_2Cr_2O_7$ was melted with 10ml Distilled water to make 1M $K_2Cr_2O_7$ solution. After that, the solution was sieved by using filtered membrane. Next, the solution was diluted to 5mM concentration and was kept for the use in future.

3.6.2 Experimental procedures:

3.6.2.1 Standard Curve preparation:

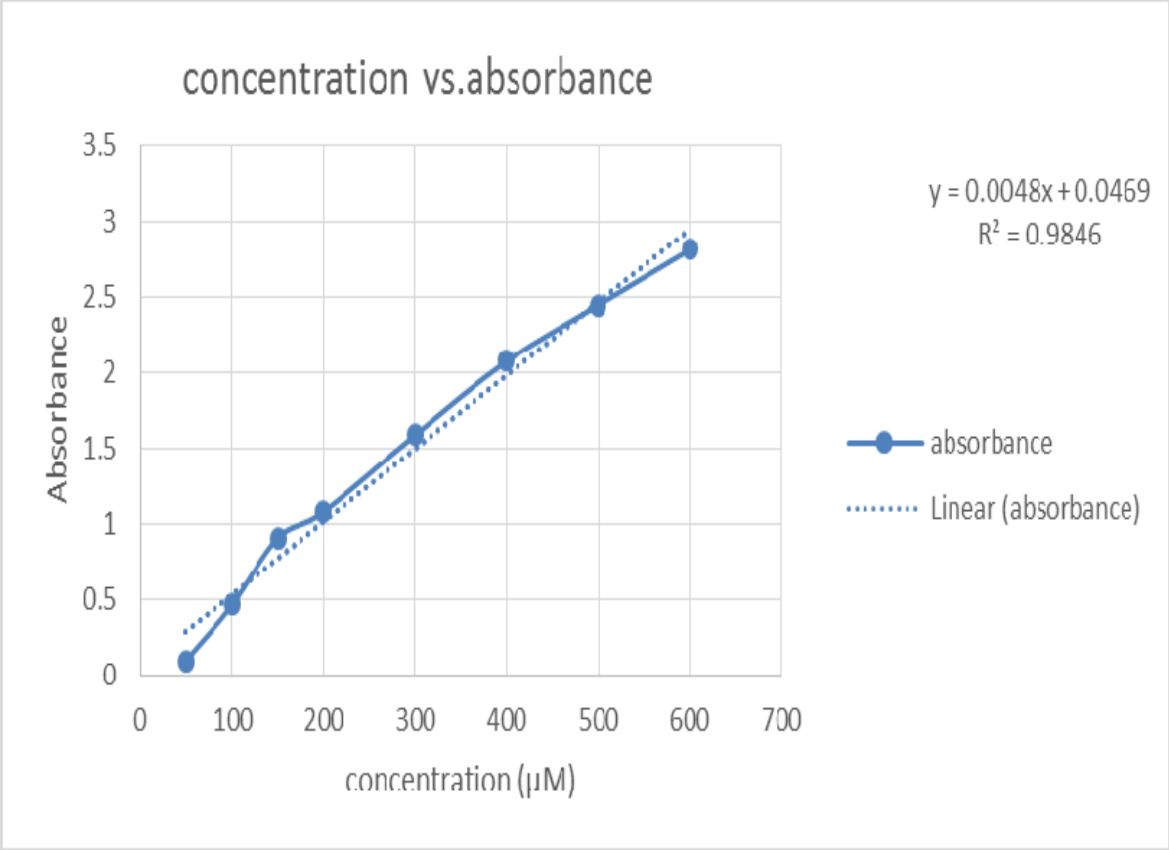

Fig 3.2: Standard curve of chromium (VI)

3.6.2.1.1 Preparation of sample for reaction:

Different strength of following sample solutions of were made and the final volume of each sample was 1ml.

Table 3.2: Standard curve data of chromium (VI)

Final Concentration	**5mM $K_2Cr_2O_7$ solution**	**Added NB**	**Final Volume**
50 µM	10 Ml	990 µL	1 ml
100 µM	20 Ml	980 µL	1 ml
150 µM	30 µL	970 µL	1 ml
200 µM	40 Ml	960 µL	1 ml
300 µM	60 µL	940 µL	1 ml
400 µM	80 µL	920 µL	1 ml
500 µM	100 µL	900 µL	1 ml
600 µM	120 µL	880 µL	1 ml
700 µM	140 µL	860 µL	1 ml
800 µM	160 µL	840 µL	1 ml
900 µM	180 µL	820 µL	1 ml
1000 µM or 1mM	200 µL	800 µL	1 ml

3.6.2.1.2 Reaction protocol for preparing standard curve:

At first, 600 µL sample solution was placed in a falcon tube. After that, 1.2 ml of 20mM MOPS buffer, 99 µL of 3M H_2SO_4, 981 µL of distilled water and 120 µL of DPCZ were added gradually and respectively to the sample solution. Then, the solution was shaken very well. The reaction took place and the color of the solution was changed from white to purple. Finally, the reaction solution was measured at 540 nm wavelength and absorbance was recorded.it was done by a UV- Visible spectrophotometer.

3.6.2.2 Evaluation of selected isolates and their reduction profile at room temperature:

3.6.2.2.1 Procedure:

Day 0:

Preparation of nutrient broth was done by preparing for two for 10ml, two for 25ml and was kept as a blank solution. From stock culture, sample was taken and with the help of a loop it was inoculated in NB media. The 10ml flasks were incubated overnight at 37^0C.

Day 1:

The next day, 15 µL of $K_2Cr_2O_7$ was added to NB media and culture solution of 3ml was withdrawn from the culture flasks that were incubated at the earlier day. After that, using UV-Visible Spectrophotometer, the O.D. of both the sample and control were taken at 600nm wavelength and the growth of the cell was observed. The necessary amount of culture to obtain O.D. of 0.2 was calculated depending on that data. Then, from the overnight culture which include both the sample and control, necessary amount of solution was placed in falcon tubes that were already sterile. At the speed 4000 rpm, the tubes were centrifuged for 7 minutes. After that, the culture were taken according to the required amount and transferred into the 25ml NB which contained 1mM Cr (VI). Then, it was vortexed to obtain 0.2 optical density of the culture. The culture was incubated at room temperature. After one and half an hour, 3 ml cultures were taken from both sample and control and O.D. were measured at 600 nm wavelength. This time the cell growth was determined. Then, these cultures were centrifuged for about 7 minutes and the supernatants were taken. After that the reaction procedure stated in section 3.6.2.1.2 was led and the O.D. were recorded to take the absorbance of chromium at 540 nm wavelength. After 4^{th}, 6^{th}, 8^{th} hour, the process was repeated and the final reading was taken after 24 hours of incubation. To get the reduction outline, all the data were put in OriginPro v8.0 software.

3.7 Tests for characterization of isolates:

3.7.1 Study of the Colonial morphology:

A loop of overnight grown cultures were streaked on nutrient agar plate. Colonial growth was observes after incubation at 37^oC for 24 hours.

3.7.2 Study of the cellular morphology:

Cellular morphology was studied by picking by Gram staining. In this method, slides were prepared by spreading a loop full of cells onto it followed by heat fixing. After that, primary Stain or Crystal violet was added on the slide placed on a staining rack. The slide was left for one minute. Next, the crystal violet stain were removed by gentle flow of distilled water for 5 seconds. Later on, Grams iodine was applied on the slide for 1 minute to fix the crystal violet stain. Then, the slide was rinsed with 95% ethanol for 3 seconds followed by gentle rinsing with distilled water. Finally, Safranin (the secondary staining agent) was applied to the slide and kept for 1 minute and followed by rinsing with distilled water. At the end of the experiment the slides were allowed to dry and the cell morphology was observed under microscope in Oil immersion (1000X) magnification.

3.7.3 Biochemical Characteristics of the selected strain:

The following Biochemical test were conducted to characterize the isolated bacteria-

1. Selective Growth on McKonkey Agar.
2. Triple Sugar Phosphate (TSI) test.
3. Sugar Utilization Test. (Glucose, Sucrose, Lactose).
4. Simons Citrate test.
5. Sulfide-Indole-Motility (SIM) test.
6. Indole formation test.
7. Methyl Red (MR) test.
8. Voges Proskauer (VP) test.

Later on these biochemical test data were put in Advanced Bacterial Identification system (ABIS) 6 software to have some idea about the possible bacterial strain.

CHAPTER - 4: RESULTS AND DISCUSSION

4.1 Chromium resistant microorganism and their Isolation data:

At the end of the isolation process, one particular colony was secluded from Agar plates which contained different concentrations of chromium. The isolated strain was given a name corresponding to their original chromium concentration which was S_2.

4.2 Chromium resistant microorganism and their reduction profile:

4.2.1 Standard Curve:

The values of mean absorbance was designed by using Microsoft Excel 2010 software in order to obtain the standard curve. The results were as follows:

Table 4.1 Standard curve data of chromium (VI)

Concentration	Mean Absorbance
50µM	0.1
100 µM	0.464
150 µM	0.91
200 µM	1.08
300 µM	1.589
400 µM	2.075
500 µM	2.446
600 µM	2.811
700 µM	2.851
800 µM	3.02
900 µM	3.114
1000 µM	3.128

From above data, the following standard was obtained:

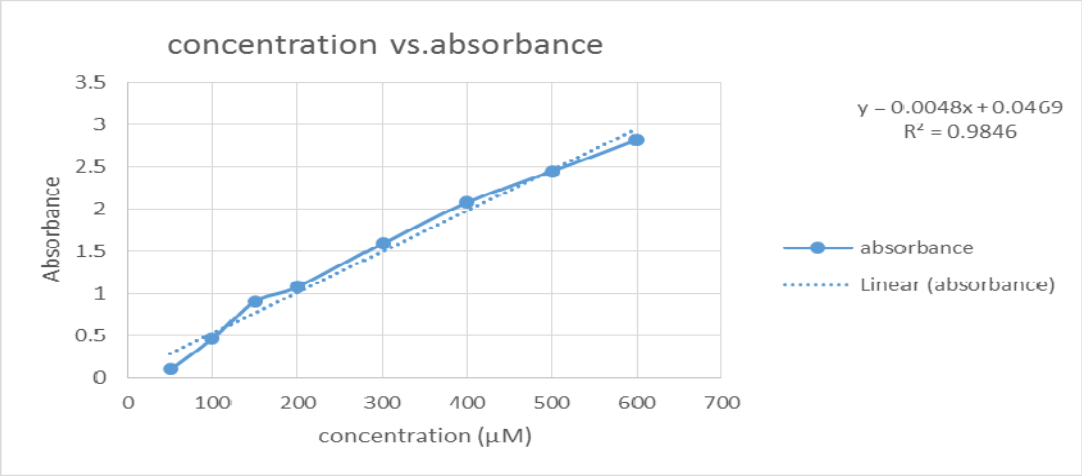

Figure 4.1: Standard curve of Chromium (VI)

4.2.2 Reduction outline of Isolate: S_2

The results obtained during the experiment were summarized in Table 4.2 and figure 4.2

Table 4.2: Isolate- S_2 at 37^0C, pH-7: Chromium reduction profile vs. Cell Growth

Time	Absorbance of chromium	Conc. of chromium at 540nm	Conc. of bacteria at 600nm
0	2.905	361.25	.159
1.5	1.846	346.5	.283
3	1.77	327.5	.34
4.5	1.0455	146.375	.421
6	0.9885	132.125	.644
24	.5635	25.875	1.012

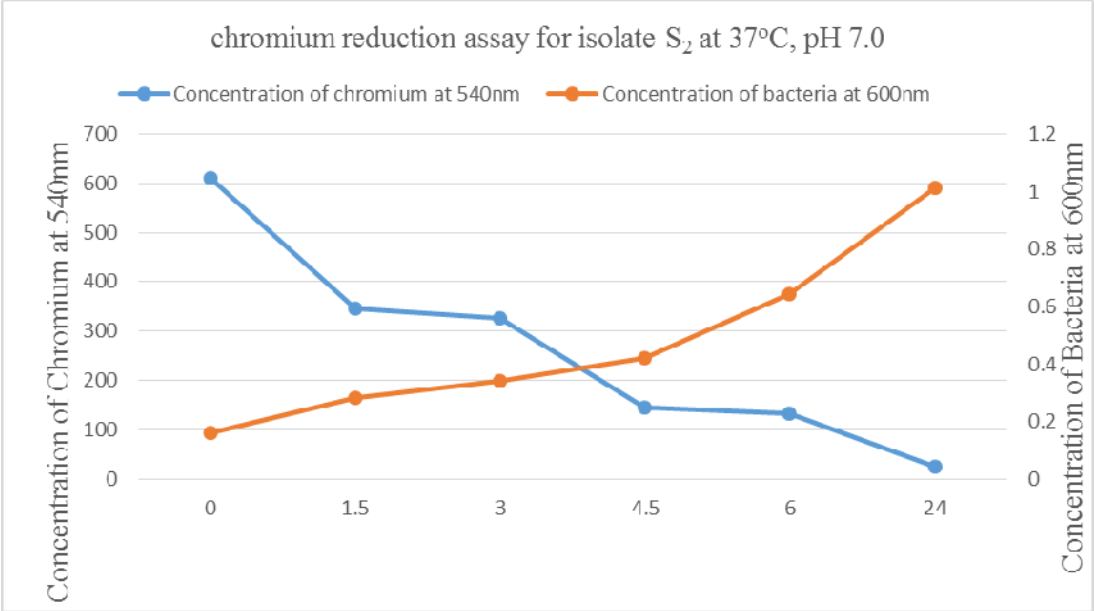

Figure 4.2: Chromium reduction vs. Cell Growth in S_2 isolate

Significant reduction was found in sample S_2 at 37^0C and pH 7

From this **Figure 4.2** it was seen that, there was significant reduction of conc. of chromium at 540 nm and there was a significant growth of O.D.at 600 nm which denoted that the isolated strain named S_2 was resistant to chromium (Figure 4.1) and also it was able to reduce the conc. of chromium. Therefore, Chromium reduction capacity of this strain was considered.

4.2.3 Reduction outline of Isolate: S_2 at 37^0C, pH-5.5

The results obtained during the experiment were summarized in Table 4.3 and Figure 4.3

Table 4.3: isolate- S_2: Chromium reduction profile vs. Cell Growth

Time	Absorbance of chromium	Conc. of chromium	Conc. of bacteria
0	2.4905	611.125	.227
1.5	1.9735	481.875	.333
3	1.852	451.5	.396
4.5	1.692	411.5	.432
6	1.563	379.25	.479
24	.8745	207.125	1.523

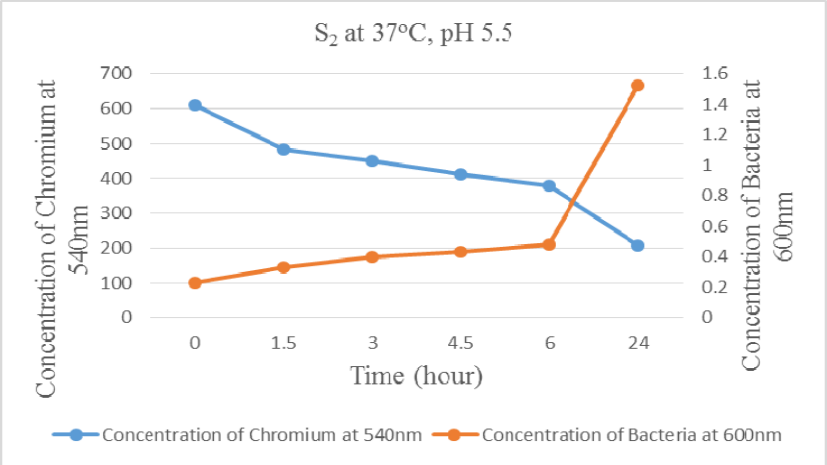

Figure 4.3: Chromium reduction vs. Cell Growth in S_2 isolate

Significant reduction was found in S_2 isolate at 37^0C, pH-5.5.

From the **Figure 4.3** ,we can say that there was significant reduction of conc. of chromium at 540 nm and also there was a significant growth of bacteria at 600 nm which denoted that the isolated strain named S_2 was not only resistant to chromium (Figure 4.3) but also had the capacity to reduce the conc. of chromium. Therefore, Chromium reduction capacity of this strain was considered.

4.2.4 Reduction outline of Isolate: S_2 at 37^0C, pH-8.5

The results obtained during the experiment were summarized in Table 4.4 and Figure 4.4:

Table 4.4: isolate- $_{S2}$: Chromium reduction profile vs. Cell Growth

Time	Absorbance of chromium	Conc. of chromium	Conc. of bacteria
0	2.264	554.5	.179
1.5	1.516	367.5	.307
3	1.3705	331.125	.443
4.5	1.023	244.25	.508
6	.958	228.0	.591
24	.2655	54.875	1.218

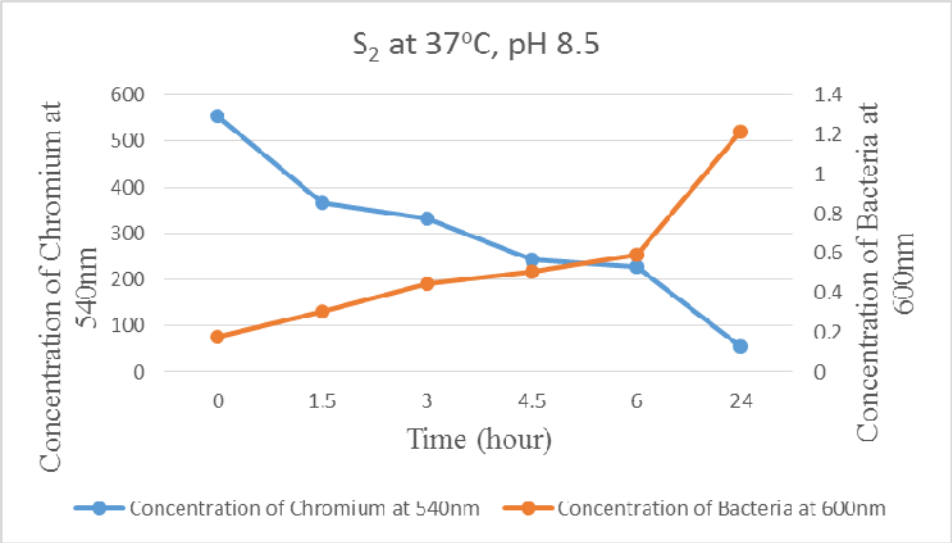

Figure 4.4: Chromium reduction vs. Cell Growth in S_2 isolate

Significant reduction was found in isolate S_2 at 37^0C, pH-8.5.

From the Figure 4.4 it was seen that, there was substantial reduction of conc. of chromium at 540 nm and also there was a significant growth of bacteria at 600 nm which denoted that the isolated strain named S_2 was not only resistant to chromium (Figure 4.3) but also had the capacity to reduce the conc. of chromium. Therefore, Chromium reduction capacity of this strain was considered.

4.2.5 Reduction outline of Isolate: S_2 at 42^0C, pH-7.0

The results obtained during the experiment were summarized in Table 4.5 and Figure 4.5:

Table 4.5: isolate- S_2: Chromium reduction profile vs. Cell Growth

Time	Absorbance of chromium	Conc. of chromium	Conc. of bacteria
0	**2.2385**	**548.125**	**.261**
1.5	**2.1475**	**525.375**	**.490**
3	**2.0515**	**501.375**	**.618**
4.5	**1.968**	**480.5**	**.705**
6	**1.794**	**437.0**	**.795**
24	**.315**	**67.25**	**1.418**

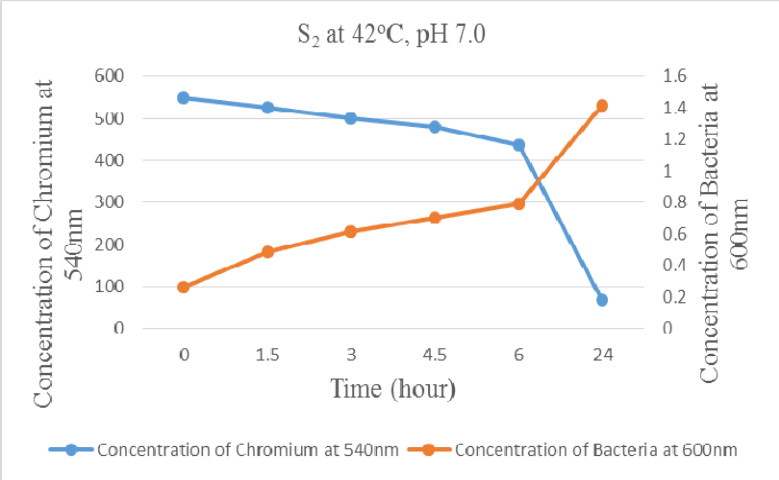

Figure 4.5: Chromium reduction vs. Cell Growth in S_2 isolate

Significant reduction was found in isolate S_2 at 42^0C, pH-7.0.

From the Figure 4.5 it had been seen that, there was major reduction of conc. of chromium at 540 nm and there was a significant growth of O.D.at 600 nm which denoted that the isolated strain named S_2 was resistant to chromium (Figure 4.5) and also it was able to reduce the conc. of chromium. Therefore, Chromium reduction capacity of this strain was considered.

4.2.6 Reduction outline of Isolate: S_2 at 25^0C, pH-7.0

The results obtained during the experiment were summarized in Table 4.5 and Figure 4.5:

Table 4.6: isolate- S_2: Chromium reduction profile vs. Cell Growth

Time	Absorbance of chromium	Conc. of chromium	Conc. of bacteria
0	**2.877**	**707.75**	**.169**
1.5	**2.711**	**666.25**	**.2**
3	**2.238**	**548.0**	**.329**
4.5	**2.2045**	**539.625**	**.36**
6	**2.0485**	**500.625**	**.446**
24	**1.2845**	**309.625**	**1.241**

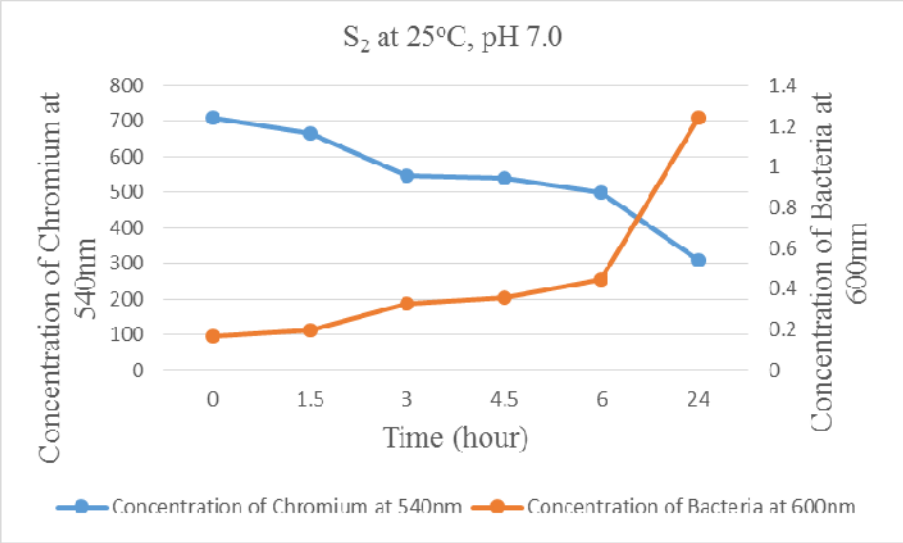

Figure 4.6: Chromium reduction vs. Cell Growth in S_2 isolate

Significant reduction was found in isolate S_2 at 25^0C, pH-7.0.

From the Figure 4.6 it can be said that, there was significant reduction of conc. of chromium at 540 nm and also there was a noteworthy growth of bacteria at 600 nm which denoted that the isolated strain named S_2 was resistant to chromium and chromium rich environment and also they were capable of reducing the conc. of chromium. Therefore, Chromium reduction capacity of this strain was considered.

The strain showed its optimum activity when they were incubated at 37^0C and at pH 7 condition.

4.3 Future works:

Further work such as 16s rRNA sequencing o the isolates is needed to confirm their identity. Chromium reducers isolated and characterized in this study could have a great activity for the bioreduction of chromium. Further studies like antibiotic resistance tests and Correlation of antibiotic resistance and heavy metal reducing capacity can be established.

CHAPTER- 5: CONCLUSION

5.1 Conclusion :

Cr (VI) formed from hexavalent chromium is very toxic and show carcinogenic effects on the biological systems as they are strong oxidizing agent in nature. The wide spread use of chromium oxyions in the commercial processes like leather tanning, textile , dyeing manufacturing and pulp production has resulted in huge amount of chromium being discharged in the environment. Microorganisms have evolved diverse resistance as well as reduction mechanisms to cope with chromate toxicity.

The present study concluded that indigenous bacterial species from polluted samples and effluents have their naturally existing machinery to degrade pollutants (Chromium), which is cost effective as compared to conventional methods. In this study, chromium resistance microorganisms have been isolated and they were found to reduce carcinogenic Cr very effectively. After 24hours of observation, the isolate showed significant reduction of chromium when they were incubated at 37ºC and the pH was 7. Again, they showed significant reduction when they were incubated at 37ºC and the pH was 8.5.These findings are potentially useful because the species can possibly be harnessed to detoxify chromium contamination sites and further optimization studies are required to optimize characters of the bacteria that can reduce high chromium concentration.

Finally, we moved for the identification of those bacterial strains, and we found that based on the biochemical test showed that. These results were based on the biochemical characterization.

Chapter 6- : References

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