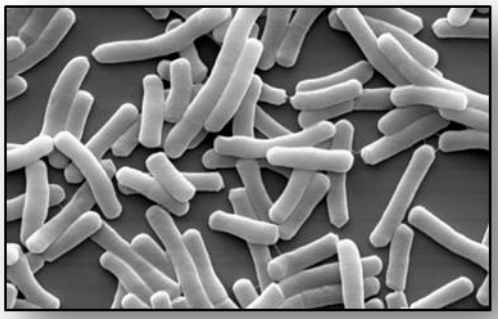
Microbial Reduction of Hexavalent Chromium - A Mechanistic and Batch Kinetic Approach



A thesis submitted by

Piku Sen

(Index no. 195/11/Engg, dated 12.07.2011)

Doctor of Philosophy (Engineering)

Department of Civil Engineering
Faculty Council of Engineering & Technology
Jadavpur University
Kolkata, India

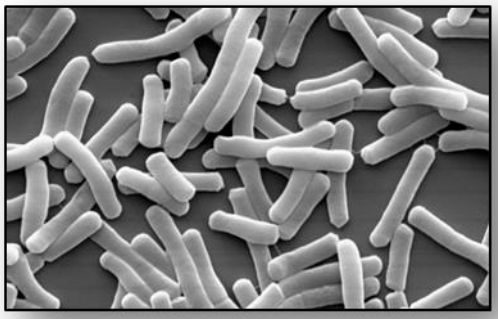
2018

Dedication

To

My Parents

Microbial Reduction of Hexavalent Chromium - A Mechanistic and Batch Kinetic Approach



A thesis submitted by

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For the Award of

Doctor of Philosophy (Engineering)

Under the guidance of

Dr. Anupam Debsarkar
Associate Professor
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Kolkata, India

2018

CERTIFICATE FROM SUPERVISORS

*This is to certify that, the thesis entitled "**Microbial Remediation of Hexavalent Chromium – A Mechanistic and Batch Kinetic Approach**", submitted by **Mrs. Piku Sen**, who got her name registered on dated **12.07.2011**, index no.**195/11/Engg**, for the award of **Ph.D (Engg.) degree** of **Jadavpur University** under **Faculty of Engineering & Technology**, is absolutely based upon her own work conducted under the supervision of the **Dr. Anupam Debsarkar** and **Dr. Debasish Pal** and that neither the thesis nor any part of it has been submitted for any degree/diploma or any other academic award anywhere before.*

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List of Publications

List of Journal Publications

1. **Piku Sen**, Debasish Pal and Anupam Debsarkar, ***"Isolation of Chromium Resistant Bacteria from Contaminated Soil and Its Performance Evaluation for Hexavalent Chromium Removal"***, International Journal of Engineering and Advanced Technology, ISSN: 2249-8958 (online), Volume 9, Issue 1, October 2019, p 6539-6546.

2. **Piku Sen**, Anupam Debsarkar and Debasish Pal, "***Hexavalent Chromium Tolerance of Bacterial Strains Isolated from Tannery Effluent Contaminated Soil***", International Journal of Innovative Research & Studies, 2018, Volume 8, Issue IV, DOI: 16.10086/IJIRS

3. **Piku Sen**, Anandita Pal, Budhhadeb Chattopadhyay, D. Pal, "***Chromium-tolerant bacteria in diversified soil microbial community in the bank of tannery waste water discharging canal of East Calcutta, West Bengal***", Journal of Biodiversity and Environmental Sciences (JBES) 2014, Vol. 4, No. 3, p. 233-238.

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List of Conference Presentations

1. **Oral** Presentation on ***"Remediation of Chromium: An Overview of Chemical and Microbial Processes and Mechanisms"***, Research Conclave, 2018, IIT-Guwahati.

2. **Poster** Presentation on ***"The quest for mechanistic insights for bioaccumulation of hexavalent chromium: a white route for bioremediation"*** at LERIG organized by CLRI in Chennai, 2017.

3. **Poster** Presentation on ***"Isolation of Chromium-tolerant bacteria in diversified soil microbial community" at "International Conference on Molecular Biology and Its Applications"*** organized by Department of Life Science and Biotechnology, Jadavpur University, February 14 & 15, 2014.

4. **Poster** Presentation on ***"Chromium-tolerant bacteria in diversified soil microbial community in the bank of tannery waste water discharging canal of East Calcutta, West Bengal"*** at National Conference on Biodiversity NCB – 2014 – Issues, Concern and Future Strategies, organized by West Bengal Biodiversity Board (Dept. of Environment, Govt. of West Bengal) 16-18 January 2014, Science City Mini Auditorium, Kolkata and TEQUIP Hall, Jadavpur University, Kolkata.

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.....

Jadavpur University (Piku Sen)

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List of Abbreviations

Abbreviated Form	Full Form
CRB	Chromium Resistant Bacteria
RNA	Ribonucleic Acid
SDS PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
SEM-EDX	Scanning Electron Microscopy–Energy Dispersive X-ray
FT IR	Fourier Transform-Infrared Spectroscopy
AAS	Atomic Absorption Spectrophotometry
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
CFE	Cell Free Extract
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
EPS	Extracellular Polysaccharide
SCOD	Soluble Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
MIC	Minimum Inhibitory Concentration
XRD	X-Ray Diffraction
SAED	Selected Area Electron Diffraction
LB	Luria Bertani
TEM	Transmission Electron Microscopy
ANN	Artificial Neural Network

Executive Summary

Chromium has long emerged as an essential component in the manufacturing processes of several products. With the advancement of technology, the uses of chromium have further proliferated into the fabric of various industries like steel, chemicals, plating, dyes and pigments, leather etc. The importance of chromium cannot be overstated. However, this heavy metal does leave a footprint in terms of its toxicity to the environment.

With globalization, as the consumption of products have spread across the world, so have the manufacturing industries - they have migrated to low cost, developing countries. Many of these countries do not have the required infrastructure to treat industrial waste effectively and such wastes are either discharged directly into the ecosystem or with nominal treatment – as a result, hazard of toxic chemicals like chromium has reached a significant scale. Thus, reducing chromium concentration down to acceptable limit is deemed to be enormously significant from sustainability standpoint.

Since, the most stable forms of chromium in environment are trivalent and hexavalent, mineralization of chromium waste is however not possible hence transformation is the solution to the problem of chromium related toxicity. Transformation of highly soluble Cr(VI) to less soluble Cr(III) restrict the toxicological effect of Cr(VI).

Studies on chromium toxicity and its remediation have also witnessed a lot of advances. Various processes and technologies have been introduced and adopted by industries. Most of the conventional methods for Cr (VI) remediation involve high energy inputs as well as chemicals which inevitably lead to the problem of secondary waste and are also costly to implement. Hence, it is necessary to develop new technologies which will be viable as well as scalable. This has led to the paradigm on bio-transformation, where biological species are used to remediate industrial wastes contaminated with chromium.

Reports of several researchers have been studied in detail during the review of literatures and the recurring observation was on the efficiency of bacterial strains in reducing chromium, or high chromium resistance of bacterial species over other microbes. A wide range of microbes, isolated either from contaminated or from chromium free soils, have revealed efficient reduction of Cr(VI) to Cr(III). Microbial tolerance limit of Cr(VI) concentration may play an important role in effective remediation of Cr(VI) from polluted environments. Underlying mechanism of chromium resistance by both aerobes and anaerobes were demonstrated by various researchers and the reduced product was further characterized to determine its immobility. Moreover, it was noted during the review of literatures that very few studies had been conducted for application-oriented studies on removal of chromium along with organic substrate, from real life wastewater.

Researchers in the recent years critically evaluated that pollution from leather processing industries has been reckoned to be one of the largest polluters in the world. Tannery

effluent is enriched with both organic and inorganic components. Since Cr(III) is used for processing of hides and skin, Cr(III) in the effluent is the most expected form. But due to redox reaction taking place in the sludge an increase in the formation of Cr(VI) may occur. This needs to be addressed for the sake of sustainable environment.

The present research investigation has been carried out with the objective to identify a potent biological species which can remove chromium; standardizing the optimum conditions for its growth and performance and investigate its mechanistic approach against chromium resistance. Further characterization of reduced product obtained from the biotransformation of chromium has also been carried out to specify the immobilization of the final product. The application of the same biological species in real life tannery wastewater has also been studied in a batch reactor for evaluating its performance under real life condition as well as its effectiveness for removal of chromium in situ environment.

In search for the chromium resistant bacteria from the bank soil sample contaminated with chromium (37085.82 ± 1124.183 mg/kg), twenty-four (24) bacteria were isolated with different types of colonies. Minimum inhibitory concentration of the isolates was observed within a range of 250 to 450 mg/L of Cr(VI) as $K_2Cr_2O_7$. Seven distinct microbial strains out of the twenty-four were selected based on the chromium removal efficiency and characterized morphologically. While further studying the growth pattern of the isolates under the stress of Cr(VI) one gram-positive chromium resistant bacteria CRB 1 showed least changes in doubling time on growth of biomass with a high removal efficiency and thus it was selected for further detailed studies.

The selected chromium resistant bacterium CRB 1 was identified as ***Bacillus mycoids*** based on biochemical and 16S rRNA gene sequencing. The optimum growth condition was standardized as pH 7.5, temperature 37 ˚C, salinity 1% and shaking velocity at 140 rpm. The total protein content and protein profile on SDS PAGE were investigated. Total protein content of the cell free extract of the bacterium was not changed so far, with or without treatment of Cr(VI). This observation referred that protein synthesis by the bacterium was least affected by the presence of Cr(VI) in cell metabolism. SDS PAGE profile of cytosolic proteins showed significant bands of two proteins of molecular weight around 72 kDa and 26 kDa for both samples treated with and without chromium.

The reduced product was characterized further to study the immobility of the final product. AAS analysis revealed that Cr(VI) is immobilized within the cell either its native oxidation state (Cr(VI)) or in reduced state. SEM-EDX study manifested the presence of chromium in the treated sample. In FT IR study, substantial changes in peak intensity and peak shift were observed for the bacterial sample treated with Cr(VI) and compared to the sample grown without Cr(VI).

Since, the properties of chromate resistance and reduction are not necessarily interrelated, before using a selected microorganism or an indigenous microbial mixed-culture for devising bioremediation strategies for Cr(VI) contaminated soils or wastewater, there is a need to understand how the Cr(VI) resistance mechanism takes place within the microbial system. When Cr(VI) is reduced to Cr(III) by a soluble

chromium reductase enzyme it produces intermediates viz. Cr(V) and/ or Cr(IV). These intermediates Cr(V) or Cr(IV) induce the formation of reactive oxygen species (ROS) due to its high reactivity and produce ROS generated oxidative stress inside the cell. It has been noted that microbes are able to activate several defense mechanisms such as releasing oxidative stress combating enzymes like superoxide dis-mutase (SOD), catalase and other enzymes which scavenge the ROS species inside the cell. Activity of chromium reductase enzyme was monitored in presence of electron donors like NADP and NADPH. The chromate reductase assay supported that the Cr(VI) get reduced by reductase enzyme and NADPH acted as electron donor for the aerobic direct Cr(VI) reduction. While on addition of NADPH to CFE the activity increased by 9.17 folds and 13.07 folds when Cr(VI) concentration was 0.32 mM and 0.67 mM respectively. It was observed that the chromate reductase activity in CFE was similar for the cells grown in medium in presence or absence of Cr(VI). This observation indicates that the chromate reductase property was not induced by Cr(VI). This was further substantiated by the protein profile obtained for the bacteria. From, Lineweaver–Burk double reciprocal plot k_m and V_{max} values were calculated to be 65.42 µg/mL and 9.514 µg/mL/h (R^2=0.989), respectively for removal of Cr(VI) from synthetic growth medium at 37 ˚C. The reduction of Cr(VI) to Cr(III) has been observed to follow first order reaction kinetics and rate constant was calculated as k = -1.09 X 10^{-4} / sec. The half-life (t_{half}) was calculated as 1.77 h. Activity of stress markers like SOD, Catalase, Glutathione reductase and peroxidase were studied. Notable increase in SOD activity, catalase activity and reduced glutathione reductase were found. These observations supported that Cr(VI) entered inside the cell and reduced to Cr(III) as ROS is induced by the intermediates like Cr(V) and/ or Cr(IV). Another stress marker peroxidase activity showed declining nature and it is indicative that presence of Cr(VI) may suppress the activity of peroxidase. *B. mycoids* also produced EPS and it may help to remove Cr(VI) in medium.

The performance of the bacterial species in a batch reactor using real life tannery waste water was studied. The average BOD_5:COD ratio obtained was 0.323 for primary treated composite tannery effluent. At the time of treatment of primary treated tannery effluent, the SCOD removal was found to vary 68-95% for a fixed Cr(VI) concentration and for Cr(VI) removal it was 45 -75 % for a fixed COD value over the retention period of 96 hours. Bio-kinetic coefficients for the pure culture have established satisfactory biodegradation of soluble organics present in real-life primary treated tannery effluent and their values are reasonably comparable with those observed for municipal wastewater. The marginal inhibitory effect of chromium uptake on substrate utilization is envisaged from decreasing values of Y and μ_{max} and increasing values of k_d with the increase in initial Cr(VI) concentration of 10 to 50 mg/L. The inhibitory effect of chromium uptake is also obvious from the increasing values of Chromium Inhibition Constants (K_i), which varied from 0.182 to 0.518 mg of Cr(VI)/L for the initial Cr(VI) concentration of 10 to 50 mg/L.

The present research work was conducted to develop an efficient strategy on chromium bioremediation using bacterial strain. This detailed understanding is necessary to enable

any lab scale biotechnological technique to evolve into large-scale reactor engineering, which can be a highly effective and proficient solution in this regard.

Introduction

1

1.1 General

Heavy metal contamination of environment has gradually become a challenge for life on earth. Anthropogenic activities from mining, processing and applications of heavy metals have amplified the problem of metal pollution to the environment over the past few decades. Consequently, the remediation of heavy metals from the contaminated environment has become a challenging task before the scientists and engineers. With time, the disposal of solid and (or) liquid waste comprising heavy metals from various industrial processes has been extensively discussed and studied. Subsequently, legislation for the environmental protection has gradually become more stringent throughout the globe demanding improved scientific understanding supported by continuous technological invention ***(Benedetti et al., 1998; Chen and Hao, 1998, Pablo et al., 2018).***

Chromium is one of the most extensively used metals in various industries like steel making, metal processing, electroplating, leather tanning, dyes and pigments, wood preservation etc. However, chromium is also a toxic element and thereby being treated as a hazardous contaminant. Chromium, according to United States Environmental Protection Agency (USEPA) is identified as one of the 17 chemicals causing the greatest threat to humans ***(Marsh et al., 2001, Pradhan et al., 2018).*** According to World Health Organization, the maximum allowable limit for total chromium in drinking water is 0.05 mg/L ***(WHO, Geneva, 2004)*** and we are at a risk of breaching this safety threshold due to extensive usage and discharge of chromium derivatives into the ecosystem.

Chromium is the seventh most naturally occurring abundant element in the earth's crust (0.1–0.3 mg/g) and is a 3d transition metal from group VI-B of the periodic table of atomic no 24 and atomic mass 51.9961 a.m.u ***(Molokwaneet al., 2008).*** This heavy metal is having a high melting point of 1907 °C. Chromium occurs in the environment in various oxidation states (Fig.1.1) which are from Cr(0) to Cr(VI) ***(Thacker et al., 2007).*** In soils the most common and stable forms are trivalent Cr(III) and hexavalent Cr(VI) species ***(Fendorf, 1995)*** which have distinct biological, geochemical and toxicological properties. In fact, in both its stable forms Cr(VI) and Cr(III) exposure can lead to allergic contact dermatitis.

Cr(VI) can get easily mixed when introduced into wastewater as it is readily soluble in all pH ranges. Cr(III) mainly spreads through adsorption on soil surface or precipitates as chromium hydroxide even in slight variations in pH from neutral range. The USEPA has categorized Cr(VI) as a Group `A' carcinogen for humans and flagged it as one of the main pollutants ***(USEPA, 2004).*** Due to its highly soluble nature Cr(VI) spreads fast from the source of contamination using aquatic systems and ground water as the medium

(Kamaludeen et al., 2003; Calder et al., 1988). CrO_4^{2-} and $HCrO_4^-$ ions are the primary mobile forms in which this contamination happens affecting the entire ecosystem.

1.2 Geochemistry of Chromium

Chromium is a lustrous, steel-grey, hard and brittle metal occurringin nature in the bound form. The several oxidation states of chromium compounds (stable or unstable) found in environment are: **0** [$Cr(CO)_6$] (metallic Cr); +**1** (unstable); +**2** (chromous) [$Cr(CH_3COO)_4$,CrO, $CrSO_4$] (unstable); +**3** (chromic) [$CrCl_3$, Cr_2O_3, $Cr_2(SO_4)_3$] (stable); +**4** [CrO_2] (unstable); +**5** [CrF_5] (unstable), and +**6** [$K_2Cr_2O_7$,$K_2Cr_2O_4$,CrO_3] (stable) (***Thacker et al., 2007)*** **(Fig-1.1)**

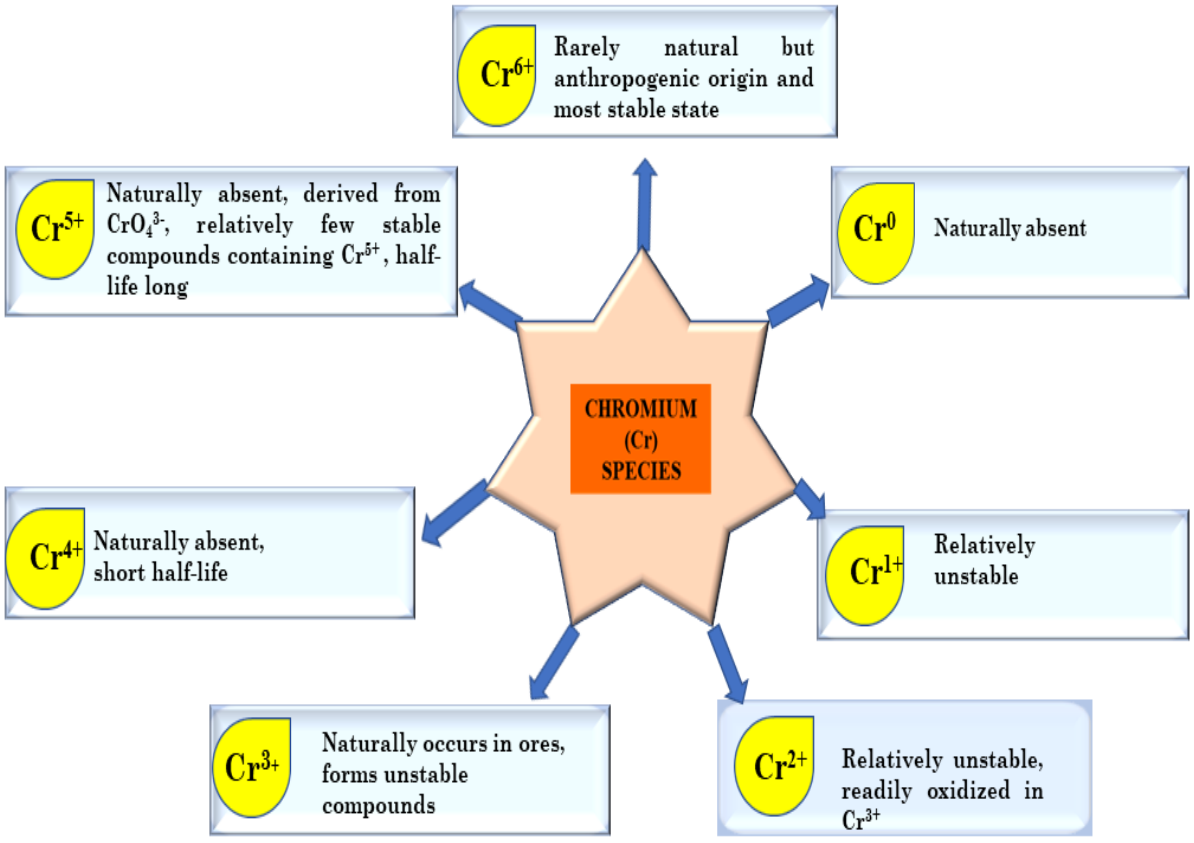


Fig- 1.1: Different oxidation states of chromium

As stated earlier, Cr(III) and Cr(VI) are most stable forms present in environment and have contradictory physical and chemical characteristics. The trivalent form is relatively immobile due to its strong affinity towards negatively charged ions and compounds in soil and water, producing sparingly soluble compounds like $Cr(OH)_3$. Such compounds dominate in the pH range of 4.0 to 8.0 ***(Fendorf, 1995)***. The characteristics of trivalent forms restrict the mobility as well as their bioavailability in soil and water. In equilibrium the concentrations of soluble Cr(III) with insoluble compounds are $<10^{-9}$ M in water at pH value 6.0 to less than 10^{-15} M at pH value 8.0 (***Richard and Bourg, 1991).***

Cr(VI) is the most reactive, oxidized, mobile and toxic form and it could have been the only existing state if chromium and the atmosphere were in thermodynamic equilibrium (***Berlette, 1991)*** Cr(VI) is more soluble and further bio-available than Cr(III) in water and soil. Under most environmental conditions, Cr(VI) exists as anion form. Depending on pH of the medium and total Cr(VI) concentration, Cr(VI) occurs as several species such as CrO_4^{2-},$Cr_2O_7^{2-}$ or $HCrO^{4-}$. H_2CrO_4 is a strong acid (***Sperling et al., 1992).*** Deprotonated form of Cr(VI), i.e. $HCrO^{4-}$, is predominant species in the pH range of 1.0 to 6.0. Above pH 7.0, CrO_4^{2-} ions only exist in solution for all concentration ranges ***(Cotton et al., 1980).***

$$H_2CrO_4 \rightarrow H^+ + HCrO^{4-} \qquad K_1 = 10^{-0.75}$$

$$HCrO_4 \rightarrow H^+ + CrO_4^{2-} \qquad K_2 = 10^{-6.45}$$

$$2HCrO_4 \longrightarrow Cr_2O_7^{2-} + H_2O \qquad K_3 = 10^{-2.2}$$

1.3 Toxicity of Chromium

The oxidation states of chromium are the primary determinants of the toxicity caused to biological species, in addition to its solubility, reactivity and absorption capacity ***(Daulton et al., 2007).*** Amongst different oxidation states, Cr(VI) species are more toxic i.e. comparatively, 1,000 folds more toxic than Cr(III) forms. Being mutagenic and carcinogenic in nature it causes oxidative damage ***(Chirwa and Molokwane. 2011)***. Cr(III) species are comparatively less toxic. They are unable to permeate cell membranes and enter into the cells due to their lower solubility and lower tendency of adsorption by organic carbon and mineral surfaces ***(Cervantes et al., 2001).*** Conversely, when Cr(VI) permeates the cell, it can subsequently react with reducing compounds present inside the cell (such as NAD(P)H, cysteines, $FADH_2$, pentoses and antioxidants such as glutathione and ascorbate or one-electron reducers like glutathione reductase) forming unstable intermediates such as Cr(V) and/or Cr(IV) as well as free radicals **(Fig 1.2).** These resultant intermediates can cause oxidative damage to DNA and proteins by forming DNA lesions, along with DNA-DNA crosslinks, Cr-DNA adducts, DNA-protein cross links ***(Cervantes and Campos-García, 2007; Nickens et al., 2010)***. Additionally, short-lived unstable intermediates Cr(V)/Cr(IV) and free radicals are the prime sources of hexavalent chromium mediated carcinogenesis ***(Salnikow et al., 2008)*** or apoptosis ***(Ye et al., 1999).***

Cr(V) in chemical solutions mostly generate free radicals like hydroxyl and superoxide which are collectively grouped as reactive oxygen species (ROS). During the redox cycle, the generated Cr(VI) is reduced again through one-electron reducers and cellular constituents, thus creating considerable ROS leading to substantial oxidative stress ***(Ackerley et al., 2004)***. It is also found that intracellular cationic Cr(III) and Cr(IV) complexes react electrostatically with negatively charged phosphate in DNA, inhibiting

DNA replication. This increases the errors during transcription of RNA ***(Cervantes and Campos-García, 2007, Ramírez-Díaz et al., 2008).*** Moreover, Cr(III) species react with the thiol and carboxyl groups in enzymes, affecting their enzymatic efficiency by changing the structure (***Cervantes and Campos-García, 2007).***

Cr(VI) is regarded as a carcinogen, based on the reports which attributed the cause of respiratory cancer in occupationally exposed groups to it ***(Langard, 1983)***. Over short-term, at exposure levels greater than the maximum contaminant level (MCL) of Cr(VI), irritation in the stomach or ulceration was observed. Long-term exposure to levels above the MCL caused damage to kidney, liver, nerve tissue and even death if present in larger doses ***(Katz and Salem, 1994; Kotas et al, 2000)***. Cr(VI) is also known to cause skin irritation and allergic contact dermatitis (***Nethercott et al., 1994).*** It can also cause skin ulcerations, referred to as chrome holes ***(Williams, 1997).*** Chromate is presently considered as one of the most frequently found contaminants at waste sites due to frequent improper disposal, poor storage and leakage, ***(Thacker et al., 2006).*** Besides being dangerously toxic, it is also extremely difficult to contain while spreading rapidly through subterranean aquatic systems and waterways ***(Gonzalez et al., 2003).*** Due to these nuances in containment and its hazardous nature, USEPA has declared chromium as a priority pollutant ***(Thacker et al., 2006).***

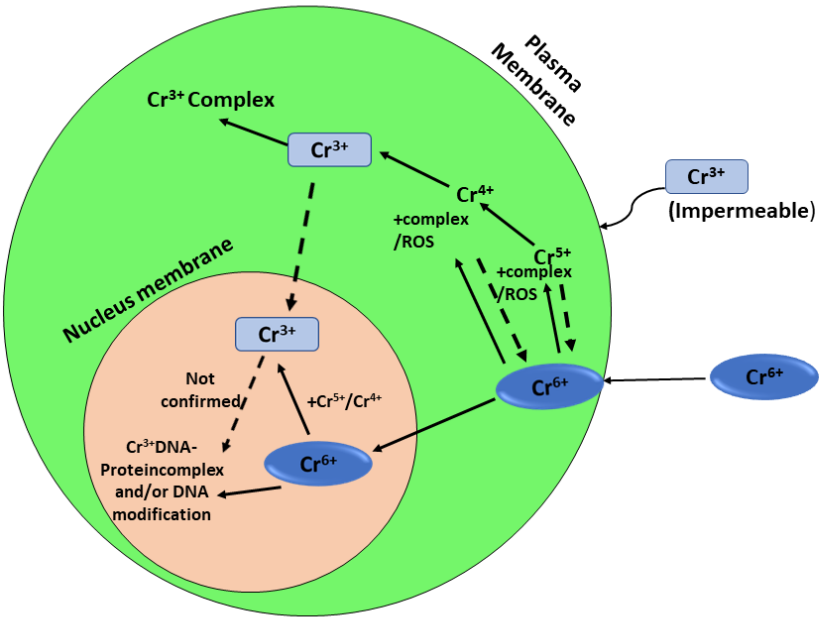


Fig-1.2: A schematic showing toxicity and mutagenicity of Cr(VI) (modified from *Cheung et al., 2007*)

1.4 Industrial Uses of Chromium

Usage of chromium is highly prevalent in industries. The various multifaced properties of chromium make it essential in using it in a wide range of industries. The hexavalent

chromium compounds are largely used in the metallurgical industries for chromium metal and chrome alloy production and in chrome plating. At room temperature chromium is resistant to corrosive agents, which accounts for its use in electroplating works to impart a protective layer. For production of ferrous alloys, mainly stainless steel, chromium compounds are used as they are both corrosion and oxidation resistant and provide a wide range of mechanical properties. Chromium is also added to cast iron (approx. 0.5% to 30%) providing properties like toughness, hardness, wear and corrosion resistance. In production of nonferrous alloys, chromium is also used in combination with nickel, aluminium, cobalt, copper, titanium and iron-nickel ***(Bielicka et al., 2005).***

In chemical industry, chromium is mainly used as an oxidizing agent and for other chromium compounds production. In the refractory industry, chromite is used for making bricks, mortar, ramming and gunning mixes. Chromite improves their slag resistance, thermal shock, strength and volume stability ***(Downing et al, 2000)***. Almost 80-90% of finished leather is tanned with chromium compounds as chromium provides better thermal stability ***(Paap, 2004).*** About 40% of used chromium in leather tanning industries is disposed of in the effluent as Cr(VI) and Cr(III) ***(Saha and Orvig, 2010).*** Largest application of chromium compounds is found in manufacturing of pigments used in paints and inks. Other applications of hexavalent chromium include metal corrosion inhibition, drilling mud, textile dyes, catalysts, wood and water treatment. Cr(III) salts are used to a lesser extent in textile dyeing, ceramics and glass industry and in photography.

1.5 Chromium Contamination of Soil and Water

Effects of chromium pollution caused by indiscriminate discharge of wastewater and solid waste have been detected in multiple sources and sites. Across the range of processes from chromium ore extraction to application in industries, it generates a variety of wastes. Residue from Chromite ore processing (COPR) is found in surface soils at many industrial sites in various countries. COPR is an industrial waste generated during manufacturing of chromate and bichromate compounds from the chromite ore. It is estimated that total amount of residue produced during processing is in the range 2.0 to 3.0 million tons. Electroplating and metal finishing industries produce large quantities of toxic sludge which contain chromium, lead, nickel and zinc. These wastes are mainly disposed on landfill sites, causing significant risk to the environment ***(Wang et al., 2001).*** The sludge from electroplating industry and specifically that from chrome plating units contain 7–11% Chromium. This sludge is highly complex as it contains metal ions and other inorganics, posing a serious problem during disposal. Solidification/ stabilization (S/S) has been extensively used to stabilize metals in solid waste. This metal containing waste from the metallurgical industries and by-products exist as slag, sludge, tailings and dust either in the final stages of the process or as an intermediate product ***(Lee and Pandey, 2012).***

Sludge and flue dust from blast furnaces and electric arc furnaces of Fe-Cr and alloying manufacturing units, contain high amount of several nonferrous metals.
Mining is also a process which can affect water and soil quality based on the chemical composition of the ore and dumped waste. Several reports have been published on the contamination by Cr(VI) around chromite mines around the world ***(Basu et al., 2010)***.

Municipal solid waste composts (MSWC) containing toxic heavy metals like chromium have raised serious concerns on the adverse environmental impact as a result of their excessive application on agricultural lands ***(Ayari et al., 2008).*** Heavy metals find their way into the environment mostly from municipal solid waste which is not separated at source. Excessive accumulation of heavy metals in higher quantities in soil and other media contaminate both the human and animal food chain (***Iwegbueet al., 2005).*** Assessment of some contaminated sites for the chromium content in waste dump created by industrial activities **(*Machender et al., 2011)*** showed very high accumulation of chromium.

1.6 Chromium Pollution in Leather Industry

Tanning is one of the oldest and fastest growing industries in India. There are about 2,000 tanneries located at different parts of India with a total processing capacity of 600,000 tons of hides and skins per year. The utilization of chromium in leather tanning accelerates the transport rates and mobility of chromium, which exceeds the rate of natural cycle processes. This has resulted in serious environmental issues in countries where leather industry has thrived ***(Taylor et al., 1990).*** Although leather tanning industry has environmental significance as the principal user of waste produced by meat industry, it is still considered as a consumer of resources and producing pollutants. One metric ton of raw hide produces 200 kg of final product (3 kg Cr) and generates 250 kg of solid non-tanned waste, 200 kg of solid tanned waste (3 kg Cr), and nearly 50,000 kg of contaminated wastewater (5 kg Cr). Thus, merely 20% of the raw material is finally converted into finished leather, and almost 60% of the chromium is discharged in the solid/liquid waste ***(Saha and Orvig, 2010).*** Basic chromium sulfate (BCS) as used in leather tanning processes is not wholly taken up by the hides and skins. Its uptake is limited to 55–70% and the remaining portion is discharged as waste. It is estimated that 40,000 tons of BCS is used by Indian tanneries per year and 15,000 tons of chromium in terms of BCS is discharged as waste in the effluent. Soil pollution caused by tannery effluent and sludge is quite different from air and water pollution as the presence of heavy metals in soil is found to be for much longer duration than in other components of the biosphere.

1.7 Pathways of Chromium Remediation

Multiple techniques are available for removal of chromium from wastewater and solid waste. These removal techniques can be classified into two major categories: physico-chemical and biological processes. Fig. 1.3 enlists the available remediation techniques. Physico-chemical processes involve different physical techniques like adsorption, filtration etc. along with different chemical techniques like ion exchange, electro-coagulation etc. The surface-based adsorption process is used broadly for removal of chromium from industrial wastewater. A wide range of both natural and synthetic materials as well as modified adsorbents have been used as Cr(VI) sorbents, including activated carbons, chitosan, zeolites, biological materials, agricultural by-products and industrial wastes ***(Nomanbhay, 2005; Mohantyet al., 2005; Sankara Ramakrishnan et al., 2008).*** Adsorption has considerable advantages like availability, low cost, profitability, efficiency and ease of operation compared to conventional methods like ion exchange or membrane filtration, particularly from economic and environmental viewpoints. Membrane filtration is one of the basic techniques for wastewater treatment. Membranes are capable of isolating heavy metals like Cr(VI) from wastewater. Several types of membranes like polymeric, inorganic and liquid are actively used for removal of Cr(VI)***(Aroua et al., 2007; Muthukrishnan et al., 2008).*** In recent years, many studies are reported using various types of ion exchange resins studying the uptake of hexavalent chromium from wastewater. Though this technique is energy efficient and has high removal efficiency, close monitoring of the solution concentration is always required to get the desired results. Electrochemical treatment techniques are another alternate wastewater treatment method since many industrial methods produce toxic wastewater, which are not easily biodegradable and involve costlier physical and/or physico-chemical treatments. The two familiar and effective electrochemical techniques for hexavalent chromium removal are Membrane Electrolysis and Electrochemical Precipitation ***(Yehia et al., 2015; Pan et al., 2016).***

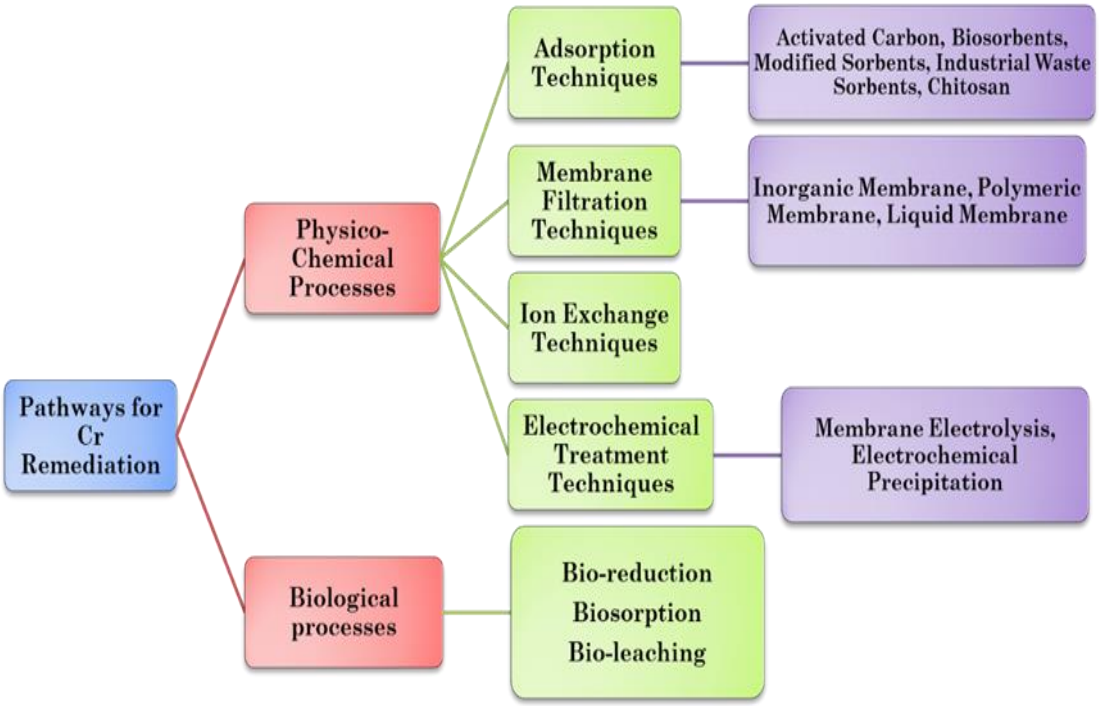


Fig- 1.3: Various pathways for chromium remediation

The biotransformation of Cr(VI) to Cr(III) has been considered as an alternative process for treating Cr(VI) contaminated wastes because the insolubility of Cr(III) facilitates its precipitation and removal ***(Williams et al., 1984; Ohtake and Silver, 1994).*** Bio-reduction has gained much interest due to its adaptability, efficiency and perceived sustainability. Biological systems engaging processes like bio-accumulation, biosorption or bio-leaching using living microbial cells have been extensively studied for their chromium removal abilities ***(Fude et al., 1993; Sharma and Forster 1993).*** These systems have a lot of potential to be developed as a viable and sustainable alternative for existing Cr(VI) removal technologies from wastewaters.

1.8 Biological Treatment of Chromium

Treating wastewater containing heavy metals involving microorganisms has gained greater focus as an area of research and lot of activities has been witnessed in recent years.In comparison to conventional physico-chemical methods, biological treatment has many advantages. These include low cost of operations, eco-friendliness in terms of effects, steady performance and ease of recovery of valuable metals present in the substrate. Biological processes are more suitable for secondary treatment after a primary treatment to remove major portion of nutrients and dissolved organics from wastewater.

A diverse genus of bacteria has been reported to reduce Cr(VI) including *Pseudomonas sp.****(Bopp and Ehrlich, 1988; Ishibashi et al., 1990, DeLeo and Ehrlich, 1994; Ganguli and Tripathi, 1999; Rajkumar et al., 2005)***, *Ochrobactrum sp.* ***(Francisco***

et al., 2002; Thacker and Madamwar, 2005), *Bacillus sp.***(Campos et al., 1995; Garbisu et al., 1998; Camargo et al., 2004; Faisal et al., 2006; Elangovan et al., 2006; Liuet al., 2006; Rehman et al., 2008; Dhal et al., 2010; Mangaiyarkarasi et al, 2011);** *Escherichia* **(Shen and Wang, 1993; Bae et al., 2000);** *Arthrobacter sp.***(Megharaj et al., 2003; Guti´errez et al., 2010),** *Microbacterium sp.***(Guti´errez et al., 2010; Pattanapipitpaisal et al., 2001)**, *Deinococcus sp.* **(Fredrickson et al., 2000)**, *Enterobacter sp.***(Wang et al., 1989; Komori et al., 1989; Wang et al., 1990; Komori et al., 1990a; Komori et al., 1990b; Clark,1994)**, *Agrobacterium sp.***(Llovera et al., 1993; Guti´errez et al., 2010)**, *Shewanella sp.***(Middleton et al., 2003; Myers et al, 2000; Viamajala et al., 2002; Viamajala et al., 2004),** *Desulfovibrio sp.***(Mabbett et al., 2001; Goulhen et al., 2006)** and many other species. These strains have been reported to reduce Cr(VI) from growth medium by to the extent of 70 – 100% under specific growth conditions both aerobically and anaerobically. However, the removal efficiency of these pure cultures in real life wastewater under ambient conditions has not been standardized yet. To devise a strategy for effective removal of Cr(VI) from real life wastewater and to develop a disposal method for reduced product, further studies need to be conducted with pure cultures. The possible advantages of a pure culture strain over mixed cultures are – control over the reaction mechanism and consistency of the reduced product. These two factors are considered to be critical for devising the afore-mentioned strategy for real life wastewater as they help us to bring in predictive analytics, standardization and subsequently the metrics for scalability.

Review of Literature

2

2.1 General

During the last few decades, it has become increasingly evident that a cost effective and scalable technology needs to be developed for Cr(VI) removal. The recent studies on this aspect have focused mainly on using biotransformation methodsas this is believed to be the way forward from viability and scalability point of view. In the present study, various literatures have been reviewed on isolation and characterization of Chromium resistant bacteria, characterization of the reduced product, understanding the mechanism of chromium resistance and batch kinetic studies with real life wastewater.

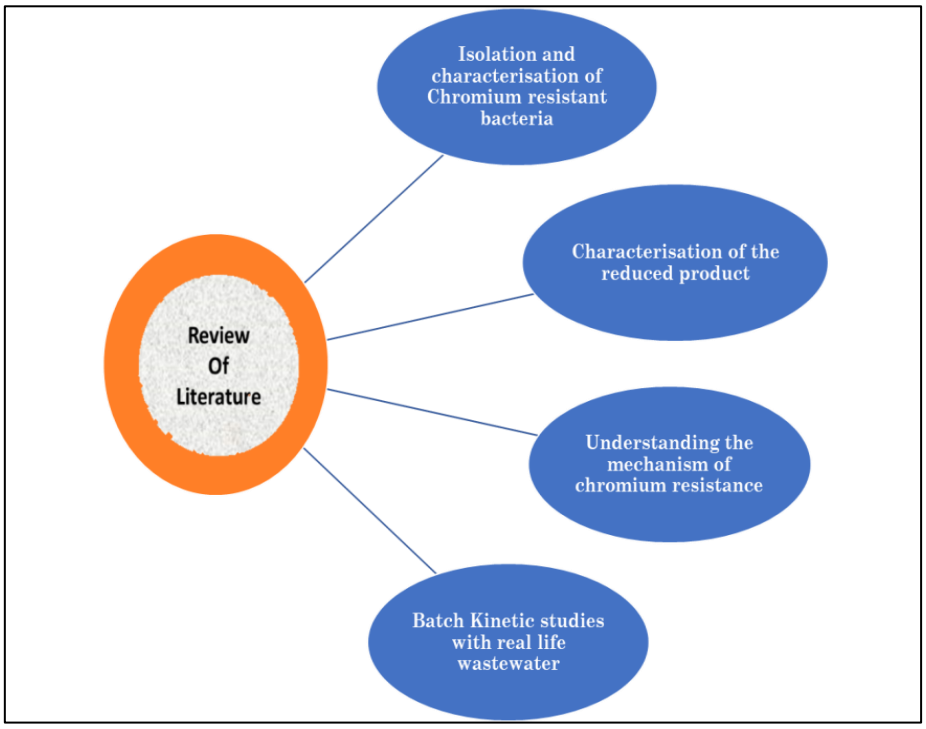


Fig- 2.1: A schematic representation of different aspects of review of literature

2.2 Isolation and Characterization of Chromium Resistant Bacteria

Various studies have been conducted to explore the chromium resistance properties of microorganisms, more specifically of the bacterial strains. The terms resistance and tolerance are often used interchangeably, but their significance is different.

Gadd (1992) defined "resistance" as "the ability of a microorganism to survive toxic effects of metal exposure by means of a detoxification mechanism produced in direct

response to the metal species concerned" and defined tolerance as "the ability of a microorganism to survive metal toxicity by means of intrinsic properties and or environmental modification of toxicity".

Romanenko and Korenkov (1977) isolated the first Cr(VI) reducing strain *Pseudomonas sp.* from industrial wastewater. Since then, several researchers have isolated various Cr(VI) resistant microorganisms from different sources. Numerous chromium-resistant bacteria are isolated and each species varies in their degree of resistance. This difference is due to the capability of a particular strain to resist the toxicity. Although, many microbes in the environment are sensitive to Cr(VI) with few exceptions, bacteria isolated from Cr(VI) contaminated sites are reported to be highly resistant. ***Mergeay, M (1995)*** described that the tolerance/resistance parameter is not absolute, but it depends on the medium used. The rate at which hexavalent chromium is reduced by microorganisms is influenced by the cell density, Cr(VI) concentration, salt concentrations, besides pH and temperature ***(Philip et al., 1998; Shen et al., 1994).*** Other factors such as presence of electron donors, oxidation-reduction potential, oxyanions, metabolic inhibitors, and presence of other heavy metals also have a significant influence on efficient Cr(VI) reduction.

Shakoori et al. (1999) isolated a gram-positive bacterium CMBL Crl3 from effluent of leather industry in Luria Bertani (LB) medium. It was found that the optimum pH and temperature for maximum removal of Cr(VI) by CMBL Crl3 was identified at 7.3 -7.5 and (35 -37) ºC respectively for removal of 45 g/L Cr(VI) from the medium.

Srinath et al. (2001) identified several facultative anaerobes having tolerance to high levels of Cr(VI) (>400 μg/ml). The isolated anaerobes from tannery effluents were identified from the genus of *Aero-coccus, Micrococcus and Aeromonas*.These isolates displayed efficient reduction of 70% Cr(VI) under anaerobic condition in agar medium. This study was the first report on *Aerococcus sp.* for its tolerance to heavy metals and removal of chromium.

Verma et al. **(2001)** investigated the organisms resistant to antibiotics in tannery effluent and described the occurrence of metal tolerance. Seventy seven isolates were selected for the detailed study which comprised heterotrophs and coliforms showing tolerance to chromate level of>50 µg/ml. The majority of the coliforms showed resistance to higher levels of chromate (200 µg/ml), while nearly 3% of the heterotrophs were resistant to Cr(VI) at a level of >150 µg/ml. All the chromate tolerant heterotrophs showed tolerance to Cu^{2+} (100%) whereas nearly 58.53% coliforms were tolerant to Cu^{2+}. Coliforms showed more resistance to cephaloridine ($P<0.001$) in comparison to heterotrophs. While a significantly higher number of heterotrophs showed resistance to carbencillin and streptomycin. All coliforms showed sensitivity to chloramphenicol. Coliforms and heterotrophs also exhibited sensitivity to the combination of antibiotics and metals (around 80% and 31.70%).

Humphries and Macaskie (2002) reported Cr(VI) removal by *Desulfovibrio vulgaris*, another anaerobe isolated from chromium contaminated sites. The optimum temperature and pH for maximum Cr(VI) removal was observed to be 37 ºC and 7.0 respectively.

Pattanapipitpaisal P. et al. (2002) observed that Cr(VI) resistance is usually associated with the cellular exclusion. Thus, precluding the enrichment techniques for isolation of organisms which can accumulate Cr(VI) via bio-reduction to insoluble Cr(III). They illustrated the development of a technique to screen for potential Cr(VI) reducing strains from approximately 2000 strains isolated from a coastal environment. This was based on the non-specific reduction of tellurite and selenite to TeO and Se0 and the reduction of tetrazolium blue to insoluble blue formazan. The strains, which were perceived as high potential, were further screened in a liquid culture. Three strains were isolated, and identified by 16S rRNA sequence analysis as *Exiguobacterium aurantiacum*, *Bacillus pumilus* and *Pseudomonas synxantha*. All three strains were able to reduce 100 μM Cr(VI) anaerobically, without growth. The removal of Cr(VI) was observed to be 90% and 80%, respectively by *B. pumilus* and *E. aurantiacum* after 48 h and it was 80% and by *P. synxantha* after 192 h. In the gram-positive strains Cr(VI) effected loss of flagella and for *B. pumilus*, Cr(VI) was observed to cause lysis of some cells. Chromium was found to be deposited as an exocellular precipitate containing Cr and P, using energy dispersive X-ray microanalysis (EDX). The above observation prompted testing of *Citrobacter sp.* N14 (which was subsequently re-assigned from 16S rRNA sequence analysis and other biochemical studies as a strain of Serratia family) which bio-precipitated metal cation phosphates through enzymatically-liberated phosphates. This strain was observed to reduce Cr(VI) at a rate similar to that of *P. synxantha* but here Cr(III) was not bio-precipitated. However, Cr(III) was observed to be removed as $CrPO_4$ and a similar amount of phosphate was produced when Cr(III) was present. As *B. pumilus* removed a large portion of Cr(VI), with the formation of cell-bound $CrPO_4$, indicating that this strain could have future bio-removal potential.

Viti et al. (2003) described that bacterial strains which were previously isolated from a soil sample polluted by chromium, were identified using gram reaction and biochemical characteristics (Biolog system). Chromate MICs, chromate reduction capacity, multiple heavy metal tolerance and sensitivity to antibiotics were tested for each isolate. All the strains exceptonewas found to be gram-positive and they also showed resistance to high concentrations of chromate. *Corynebacterium hoagie* was found to be the most Cr (VI)-resistant isolate (22m M). All Cr (VI)-resistant strains were found capable of catalyzing Cr (VI) reduction to Cr (III) which is a less water-soluble and less toxic form of chromium, except the isolate ChrC20. The only isolate which was Cr(VI)-sensitive and also exhibited Cr(VI) reduction was found to belong to the genus *Pseudomonas*. Isolates were also screened to check the presence of plasmid DNA. The strains ChrB20 and ChrC20 showed the presence of one and two plasmids respectively, of higher molecular mass. This approach helped to validate that some bacterial strains can be used for bioremediation of environments polluted by Cr(VI).

Camargo et al. (2003) described that the extensive use of chromium in various industrial applications has led to substantial environmental contamination. Bacterial strains isolated from tannery effluent soils can offer a solution as they can remove toxic Cr(VI) from the contaminated environments. The objective of this study was to isolate

chromium–resistant bacteria from contaminated soils, using dichromate and to describe the effects of some environmental factors, i.e. pH, and explain the Cr(VI) reduction and resistance. They observed that chromium resistant bacteria were able to tolerate 2500 mg /L of Cr(VI). However, most of the isolates were able to tolerate and reduce Cr(VI) at concentrations lower than 1500 mg/L.

Faisal et al. (2004) described how CrT-1 and CrT-13, two chromium-resistant bacterial strains showed tolerance up to 40 mg K_2CrO_4 per ml on nutrient agar, up to 10 mg/ml in acetate-minimal media, up to 25 mg/ml in nutrient broth. The bacterial strains were identified using 16S rRNA gene sequencing, as *Ochrobactrum intermedium* and *Brevibacterium sp.,* respectively. Living cells showed a greater uptake of chromate when compared to heat-killed dried cells. CrT-1 was observed to reduce 82%, 28% and 16% of Cr(VI) at 100, 500, and 1000 µg/ml after 24h while CrT-13 was able to reduce 41%, 14% and 9%. Other heavy metals were not able to affect these reductions at low concentrations. At 150 and 300 µg/ml of an industrial effluent sample, CrT-1 reduced Cr(VI) by 87% and 71% respectively and CrT-13 was able to reduce by 68% and 47%.

Donmezet al. (2005) described that mixed cultures have been isolated from saline wastewater from industries contaminated with Cr(VI). Enrichment was done in the presence of 50 mg/L of Cr(VI) and 4% (w/v) of NaCl at pH 8.0. Molasses (M) medium was selected as a suitable medium to carry out chromium bioaccumulation by the mixed cultures, effectively. Eleven pure isolates were obtained from the mixed cultures. Some of them showed high bioaccumulation characteristic in the M media which contained about 100 mg/L Cr(VI) and 4% NaCl. Strains 8 (99.3%) and 10 (99.1%) were observed to be able to bioaccumulate more efficiently than the mixed culture (98.9%) in this media. However, the highest specific Cr uptake was observed for the mixed cultures followed by strain 8 and 10, the respective values were 56.71, 33.14 and 21.7 mg/g. Bioaccumulation of Cr(VI) ions by strain 8 grown in the media along with Cr(VI) and NaCl was then studied in a batch system to derive it as a function of concentrations of initial Cr(VI) (86.6–547.6 mg/L) and NaCl (0, 2, 4, 6% w/v). The uptake yield observed to be consistently high for all the experiments for strain 8; it was highly affected by NaCl concentration in the medium and for high initial Cr(VI) concentrations. However, at low Cr(VI) concentration, there was no effect of NaCl concentrations in the medium on strain 8. The maximum uptake yields obtained in the M media were 98.8% for 110.0 mg/L, 98.6% for 217.1 mg/L, 98.6% for 381.7 mg/L and 98.2% for 547.6 mg/L of initial Cr(VI) concentrations respectively, and with 2% NaCl. The strain 8 was able to tolerate a 6% (w/v) NaCl concentration and was able to bioaccumulate higher than 95% of the applied Cr(VI) when the initial Cr(VI) concentrations were 97.6–224.4 mg/L. The results presented in this paper have shown that the pure and mixed cultures were more effective for the bioaccumulation of Cr(VI) present in saline wastewater.

Hameed et al. (2005) described the isolation of multiple unicellular cyanobacteria from different sites: ponds, fields, polluted water and soils from Kasur and Muredkey tannery areas, near Lahore, Pakistan. Different media like Bold Basal medium, BG 11 medium, Chu's 10 medium and Gorham's medium, in their standard forms with only slight

variations of ingredients, different temperature, pH and light regimes were used to check the optimum growth of the isolates. This isolation procedure was repeated for different chromium concentrations, to select the resistant strains. These selected strains were grown on chromium to the range 100–200 µg/ml in the BG 11 medium. *Cyanobacteria* were maintained in both solid and liquid media with and without shaking. The *Cyanobacterial* strains which were collected from their natural habitats were also accompanied by diversified group of organisms which included bacteria, protozoan, and rotifers etc. To eliminate these agents which can be termed as contaminants, several methods were used, including phenol treatment, antibiotics and careful manual selection and picking of unicellular cyanobacteria. Resistance exhibited by these strains against different heavy metals present in $ZnSO_4$, $CoCl_2$, $MnSO_4$, $NiSO_4$, $Pb(NO_3)_2$, $CuSO_4$, $AgNO_3$, $HgCl_2$, and $CdCl_2$ and antibiotics like erythromycin, streptomycin, kanamycin, chloramphenicol and neomycin were evaluated. Variable pH and optimum temperature of 30°C was used for the reduction of Cr(VI) into Cr(III) in majority of the strains.

Thacker et al. (2006) isolated a gram-negative bacterium with chromate reducing properties (UTDM314) from the contaminated sites of chemical industries. This strain was identified as *Providencia* sp. with biochemical methods and 16SrRNA analysis. The bacterial isolate was able to grow and reduce chromate by 100% at concentrations ranging from 100–300 mg/L and by 99.31% at a concentration of 400 mg/L, temperature 37 °C and pH 7.0. It also exhibited tolerance to multiple heavy metals (Ni, Zn, Hg, Pb, Co). Assays conducted with permeabilized cells which were treated with toluene and Triton X100, resting cells and crude cell-free extracts demonstrated that soluble fraction of the cell is mainly associated with the reduction of hexavalent chromium. *Providencia* sp. has shown great potential for bioremediation of wastes containing Cr(VI). This approach permits the selection of bacterial strains, which could be effectively used for specific environmental cleanup processes.

Shakoori et al. (2006) was able to isolate a gram-positive, chromium resistant bacterial strain (ATCC 700729) from effluent of tanneries. The strain was grown in media containing potassium dichromate with concentration up to 80 mg/ml of the medium. To check the dichromate reducing capability of the bacterium, the amount of Cr(VI) in the medium was estimated before and after introducing the bacterial culture. The factors which influence the reduction, like pH, concentration of chromium and the amount of the inoculum was studied in detail to determine the capability of the bacterium to reduce Cr(VI) under various conditions. In a medium with dichromate concentration of 20 mg/ml, more than 87% dichromate ions were reduced within 72 h. The feasibility of using this bacterial strain to detoxify dichromate in the industrial wastewater sample has been assessed. It was observed that this isolated strain can be used for specific environmental clean-up operations.

Shukla et al. (2007) described the toxicity of tannery effluent discharged from Common Effluent Treatment Plant (CETP), Unnao (U.P, India), which had high levels of BOD, COD, TDS and Cr content (5.88 mg/L) and supported the growth of chromate tolerant bacteria. NBRIP-1, NBRIP-2, NBRIP-3 and NBRIP-4 were the four bacterial strains

isolated from these effluent samples which showed maximum chromate tolerance and were characterized in this study. It was observed that these strains demonstrated multiple metal and antibiotic resistances. The growth of these strains was observed to be reduced at higher Cr concentration with extension of the lag phase. Chromium accumulation observed for these isolates may have a great potential effective treatment of tannery effluent by recovery and detoxification of Cr.

Nakatsu et al. (2007) isolated chromium resistant strains from contaminated soils and described the ecology of heavy metal resistance in bacteria. Soil sample was collected from an old tannery site with chromium contamination. Metal tolerance of bacteria isolated from these soils was determined by calculating the minimum inhibitory concentrations of these metals for bacterial growth. Chromium resistant bacteria were observed to be able to grow in concentrations as high as 50 mM CrO_4^{-2}. The isolated Cr15 strain was studied in greater detail. In the absence of Cr, metal sensitive mutants of the Cr15 strain were obtained. The Cr resistance genes were observed to have a mobile element by which the trait could be transferred using conjugation experiments from the resistant to sensitive strain. Molecular genetic analysis of the strains revealed that plasmids were present in both the strains, however a deletion in the plasmid of the sensitive strain was observed. Nucleotide sequence analysis revealed the presence of chromium resistant genes with amino acids similar to ChrA and ChrB, in the deleted region.

Sultan and Hasnain (2007) observed that Cr(VI) reduction by the strain SDCr-5 was found to be highest in Dey-Engley (DE) medium (98%).This was followed by glucose supplemented M9 (52%), nutrient broth (39%), and the least in KSC (16%). This observation demonstrated that reduction of Cr(VI) can sometimes vary with the different isolation media used. Cr(VI) reduction by bacterial strains grown in rich medium were also reported to be more efficient than when the strains are grown in minimal medium by two separate studies.

Kaushik et al. **(2008)** observed that utilization of microorganisms for removal of heavy metals from industrial effluent can be an area of extensive research and development. Various attempts have been made on isolation and characterization of metal-resistant microorganisms during treatment of samples of oil mill industry effluent wastewater. The metal-resistant organisms which showed minimum inhibitory concentration for metals (Cd, Ni, Cr and Pb) in the range of 100 to 800 mg/L level were screened, Isolate BC15 was observed to be a potent metal-resistant organism from the wastewater samples, which was tentatively identified as *Pseudomonas sp*. A detailed biochemical, morphological and 16S rDNA sequence analysis of the isolate revealed that it was very similar to *Pseudomonas aeruginosa* (94%). *Pseudomonas* BC15 was observed to be capable of absorbing 65% Pb, 50% Cd, 93% Ni and 30% Cr within 48 h in the medium containing 100 mg of each of the mentioned heavy metals per liter. The multiple metal tolerances shown by this strain was also reported to be associated with resistance to antibiotics, e.g. tetracycline, ampicillin, chloramphenicol, erythromycin, kanamycin and streptomycin.

Parameswari et al. **(2009)** described that prolific use of hexavalent chromium in numerous industrial applications has resulted in substantial environmental hazard. It

was noted that utilization of Cr(VI) reducing microbes have enhanced the efficiency of the detoxification process for reduction of Cr(VI) to Cr(III). Chromium resistant bacteria like *Pseudomonas fluorescens* and *Bacillus* sp. were isolated from soil contaminated by heavy metal and examined for their tolerance property to Cr(VI) and their capability to reduce Cr(VI) to Cr(III). The detoxification process in cell extracts and cell suspensions was also studied. Influence of various factors like pH, initial metal concentrations and time intervalon the reduction of hexavalent chromium by the bacterial isolates was studied. Both the bacterial isolates were able to tolerate Cr(VI) at 100 mg/L in a minimal salt broth. At 25 mg/L, *Bacillus* sp and *Pseudomonas fluorescens* recorded the respective maximum accumulation rates of 87.8 % and 93 %. *Bacillus* sp. was observed to reduce 40%, 60%, 68%, 81% and 75% of Cr(VI) to Cr(III) and *Pseudomonas fluorescens* was noted to reduce 52%, 58%, 61%, 72% and 75% of Cr(VI) to Cr(III) at multiple levels of pH values such as 5.0, 6.0, 7.0, 8.0 and 9.0. Maximum Cr(VI) reduction was observed at pH 7.0 - 8.0. These results indicate that the microbial consortia for these isolates and the mono cultures can be useful for remediation of chromium contaminated environment.

Sundar et al. (2010) conducted the basic survey studies on tanneries and pollution from their discharges into the Palar river basin of Vellore District. They observed that the river has been contaminated with heavy metals, mainly chromium and salts. This study helps to improve the understanding on chromium contamination levels and the metal tolerance of the native bacterial flora and the ecology of our study area. Strains exhibiting chromium tolerance were isolated from the contaminated sediments, discharged wastewater and effluents of various tanneries. It was observed that the minimum and maximum concentrations of chromium sediments ranged from 47.4 to 682.4 mg/L, while the average value was 306.28 mg/L. After isolating sixty-eight chromium resistant bacterial strains, Maximum Tolerance Concentration (MTC) studies were conducted, which indicated tolerance concentrations of the isolates ranged from 100 to 3300 mg/L. The resistance to other heavy metals like Pb, Ni, Zn, Cd and Fe for these bacterial isolates was also checked. Eighty percent of the isolates demonstrated resistance to Ni, Pb, Zn and Fe at 100 mg/L level and at 45% they showed resistance to Cd. The isolates also demonstrated tolerance to salt (NaCl) up to 9%. Significant observations were made on the concentration of chromium and the chromium tolerance capability of the bacteria in the study area, which indicate that these chromium tolerant strains can be used as indicators of Cr contamination.

Abskharon et al. **(2010)** described the reduction of toxic Cr(VI) to Cr(III) by using four chromium resistant strains of *E.coli*, ASU 3,7,8 and 18 which were isolated from wastewater discharged into El Malah canal located in the Assuit city, Egypt. These strains demonstrated relatively high MICs and were found to be plasmid mediated, ASU 7 with 65 and 27 kb. *E.coli* represented the best performance with respect to resistance and reducing power for Cr(VI). This strain may be a suitable candidate for developing bioremediation processes. Alteration of protein profile was also carried out for the *E.coli* ASU 7 strain in SDS-PAGE under different concentrations of Chromium stress.

Masood and Malik (2011) studied the metal binding capacity of *Bacillus sp.* FM1 which was isolated from soil contaminated with tannery effluent, using synthetic metal solutions and wastewater discharged from tanneries. Biosorption of Cr(VI) and Cu(II) ions using *Bacillus* from aqueous solutions was investigated as a function of pH, contact time and initial metal ion concentration. The optimum adsorption pH value was observed to be 2.0 and 5.0 for Cr(VI) and Cu(II), respectively. Metal ion uptake was observed to be positively correlated with initial metal concentration but there was no significant difference when contact time was increased beyond 60 min. Maximum uptake capacity for chromium was estimated to be 64.102 mg/g, and for copper it was 78.125 mg/g. Equilibrium data for this experiment were well described using the Langmuir and Freundlich adsorption relations. Fourier Transform Infrared (FTIR) spectroscopy was used to confirm the fact that it was possible for functional groups present on cell wall surface of the biomass to interact with the metal ions. The use of *Bacillus* to remove Cr(VI) and Cu(II) revealed that the biomass may be capable of removing both the metals. However, it was observed that the biosorption efficiency was slightly lower when compared with that of synthetic metal solutions. Various factors can be responsible for this difference, however, it was suggested that the most important factor was probably the presence of other contaminants in the effluent such as organics, anions and other trace metals.

Ibrahim et al. (2012) isolated *Bacillus sp.* strain KSUCr9a from soil samples and water of soda lakes located in North Egypt using Nutrient agar medium, at initial Cr(VI) concentration of 300 mg /L. The optimum growth condition was observed at pH 10.5 and temperature of 30 ºC.

Soni et al. (2012) were able to isolate four efficient Cr(VI)-reducing bacterial strains from rhizospheric soil of plants which were irrigated with tannery effluent, which was then investigated for in vitro Cr(VI) reduction. Using 16S rRNA gene sequencing SUCR44, SUCR140, SUCR186, and SUCR188 were the isolated strains. These were identified as *Bacillus sp.* (JN674188), *Bacillus subtilis* (JN674195), *Microbacterium sp.* (JN674183) and *Bacillus thuringiensis* (JN674184), respectively. All four isolates were able to completely reduce Cr(VI) in a culture media at 0.2 mM concentration and within a period of 24–120 h; SUCR140 was able to completely reduce Cr(VI) within 24 h. Assay conducted with the permeabilized cells (treated with Tween 80 and Triton X-100) and cell-free assay showed that the Cr(VI) reduction activity was primarily associated with the soluble part of cells. As a major amount of chromium was observed to be reduced within 24–48 h, it was inferred that these fractions may have been released extracellularly too, during their growth. At the optimal temperature of 28°C and pH 7.0, Cr(VI) reduction activity was determined to be 0.32, 0.42, 0.34, and 0.28 µmol Cr(VI)/min/mg protein for isolates SUCR44, SUCR140, SUCR186, and SUCR188, respectively. It was observed that by adding 0.1 mM NADH Cr(VI) reduction in the cell free extracts of all four strains were enhanced. In the cell-free extracts, the Cr(VI) reduction activity for all the isolates was stable even in the presence of different metal ions tested with the exception of Hg^{2+}. In addition to this, urea and thiourea were also able to reduce the activity of chromate reduction by significant levels.

Dhal et al. (2013) studied Cr(VI) generated from natural oxidation of the chromite minerals found in chromite mine overburden (COB) dumps at Sukinda, India, and found that they were characterized by different physico-chemical methods. Here the Cr(VI) was observed to be associated with the goethite matrix at contamination level of 500 mg Cr(VI)/ kg of COB. The isolated *Bacillus sp.* from the overburden sample exhibited high tolerance to Cr(VI) and was used for the removal of Cr(VI) present in the overburden sample. This process was optimized by varying the parameters like pH (2.0–9.0), temperature (25–40 °C) and pulp density (10–60%). Optimal reduction of more than 98% of Cr(VI) was observed in the COB sample in 16 h at pH ~ 7.0 and 60% pulp density using the *Bacillus sp.* (4.05×10^7 cells/ mL) in absence of the media. The exponential rate equation had yielded a rate constant value of 2.14 × 10/ h at pulp density of 60%. FT-IR, XRD, EPMA and SEM-EDX studies were used to establish the mode of bio-reduction of Cr(VI) present in the overburden sample.

Murugavel H (2013) observed the bio reduction of Cr(VI) by using bacterial strains isolated from soil samples which were contaminated with tannery wastewater. This isolated species was found to be *Bacillus cereus.* Glucose was found to be a suitable electron donor for Cr(VI) reduction by using the free cells of the isolated culture. A maximum specific reduction rate of 0.83 mg/g/h was yielded by Glucose for an initial Cr(VI) concentration of 60 mg/L. The reported reduction of Cr(VI) was almost complete for initial Cr(VI) concentrations ranging from 10– 50 mg/L. The isolated culture was observed to be capable of reducing Cr(VI) by up to 96.7% and 72.1% when initial Cr(VI) concentration was 60 and 70 mg/L respectively. The optimum pH observed for the Cr(VI) reduction with free cells of Bacillus sp. was 6.0. A maximum of 96% reduction was reported for the cell free extracts (CFE) for initial Cr(VI) concentration of 60 mg/L. NADP and NADPH were reported to be the suitable electron donors for reduction of Cr(VI) with CFE. The optimum pH observed for reduction of Cr(VI) with CFE was 6.5. PCP, KCK, NaN3 and EDTA were the inhibitors used for Cr(VI) reduction with CFE.

Dey and Paul (2015) studied the chromate reducing actinomycetes, *Arthrobacter* sp. SUK 1205, which was isolated from chromite mine overburden in Odisha, India. This strain was observed to significantly reduce chromate during growth and the resultant insoluble precipitate was pale green in colour. Increase in inoculum density increased the reduction of chromate but decline in the reduction potential was observed as and when there was an increase in the Cr(VI) concentration in the medium. Chromate reducing efficiency was observed to increase when glucose and glycerol were used as the electron donors. The pH and temperature were maintained at 7.0 and 35°C, respectively. Several metal ions and metabolic inhibitors were observed to be able to inhibit the reduction process but Cu(II), Mn(II) and DNP were found to be unable to inhibit the reduction. Whole cells immobilized with Ca-alginate were found to be most effective among the matrices tested to examine whole cell immobilization. The efficiency was found comparable with non-immobilized cells. Minimal salts (MS) medium was observed to be the most effective base for studies on Cr(VI) reduction with immobilized cells. Under these conditions, the immobilized cells were able to retain their enzymatic activity for at least

for four consecutive cycles indicating that the high potential of *Arthrobacter* sp. SUK 1205 in development of bioremediation techniques for environmental chromium pollution.

Fernández et al. (2016) reported that chromate-resistant microorganisms to possess the ability of reducing toxic Cr(VI) to a less toxic Cr(III). An alternative culture medium using *Wickerhamomyces anomalus* M10 to reduce Cr(VI) was optimized. The Plackett-Burman design was used to determine that K_2HPO_4, sucrose and inoculum size had some significant effect on chromate removal at 24 h. A complete factorial design was used to adjust the concentrations of these significant factors. However, in this case, only the effect of K_2HPO_4 was significant at 12 h of culture and Cr(VI) removal was higher at low concentration (1.2 g/L). The validation of the optimum medium was done using the fermenter scale level. It was observed that optimal culture conditions for the complete removal of Cr(VI) (1 mM) were agitation at 400 rpm. Moreover *W. anomalus* M10 was able to completely remove consecutively added pulses of Cr(VI) (1 mM). The results showed interesting characteristics from biotechnology stand point with regard to future remediation processes using *W. anomalus* M10. It was inferred to be able to lead to an efficient and highly profitable technology for chromium remediation.

Karthik et al. (2017) identified a novel Cr(VI) tolerant bacterial strain AR6 which was isolated from soil sample contaminated with effluent from leather industry. It was identified as *Cellulosimicrobium funkei* using 16S rDNA gene sequencing. Effects of various physicochemical conditions on the Cr(VI) reducing performance of the strain was also studied. It was observed that *C. funkei* strain AR6 demonstrated tolerance to very high concentrations of Cr(VI) (1200 μ g/ml) and was still capable of reducing Cr(VI) over a wide range of pH (6.0-8.0) and temperature (30-40°C). The optimum performance was observed at pH7.0 and 35°C. Further studies on the strain verified that removal of Cr(VI) was primarily achieved by a bio reduction process dependent on metabolism. The *C. funkei* strain AR6 was observed to reduce and accumulate chromium both inside and around the cells, the cell walls were observed to act as major binding sites for chromium. It was further confirmed from Raman spectroscopy analysis that chromium immobilization by the cells was occurring in the Cr(III) state. The toxicity of the reduced product was investigated by toxicity study using embryos of zebrafish (*Danio rerio*) model. Interestingly, when the embryo was exposed to Cr(VI) survival rates (22.19%) and hatching rates (26.71%) were reduced significantly. However, the Cr(VI) intoxicated embryos were observed to significantly increase the cardiac functions (132 ± 2.65 beat/min) at 60 hpf. However, the reduced product exerted lesser teratogenic effects on zebrafish embryos. These observations suggested that transformation of Cr(VI) to Cr(III) by novel *C.funkei* can be considered as an effective and eco-friendly strategy for remediation of Cr(VI) contaminated sites.

Baldiris et al. (2018) worked with a gram-negative strain of *S. maltophilia*, which is indigenous to environments contaminated by Cr(VI). This strain was identified using biochemical methods and 16S rRNA gene analysis. It was able to reduce chromate by 100%, 98–99% and 92% at initial concentrations in the ranges 10–70, 80–300, and 500 mg/L, respectively with pH 7.0 and temperature 37°C. However, increased concentrations

of Cr(VI) in the medium was observed to lower the growth rate but this could not be directly correlated with the quantity of Cr(VI) reduced. This strain also exhibited multiple resistance towards antibiotics and tolerance and resistance to some heavy metals (Ni, Cu and Zn), with the exception of Hg. Hexavalent chromium reduction was primarily associated with the soluble fractions of the cell and were evaluated with crude cell-free extracts. The researchers were able to detect a protein of molecular weight around 25 kDa on SDS-PAGE gel which was dependant on the concentration of hexavalent chromium (0, 100 and 500 mg/L) in the medium. In silico analysis of this contribution, revealed that the presence of chromate reductase gene ChrR in *S. maltophilia*, which was evidenced through a fragment of 468 bp obtained experimentally. The high Cr(VI) resistance and high Cr(VI) reduction capability of this strain makes it a suitable candidate for bioremediation.

2.3 Characterization of Reduced Product

Mary Mangaiyarkarasi et al. **(2010)** observed that detoxification of Cr(VI) under alkaline pH requires attention due to the alkaline nature of many effluents. An alkaliphilic gram-positive *Bacillus subtilis* isolated from tannery effluent contaminated soil was found to grow and reduce Cr(VI) up to 100% at an alkaline pH 9.0. Decrease in pH to acidic range with growth of the bacterium signified the role played by metabolites (organic acids) in chromium resistance and reduction mechanism. The XPS and FT-IR spectra confirmed the reduction of Cr(VI) by bacteria into +3 oxidation state. Chromate reductase assay indicated that the reduction was mediated by constitutive membrane bound enzymes. The kinetics of Cr(VI) reduction activity derived using the monod equation proved (K_s = 0.00032) high affinity of the organism to the metal. This study thus helped to localize the reduction activity at sub-cellular level in a chromium resistant alkaliphilic *Bacillus sp.*

A strain CSB 9 isolated from chromite mine soil of Sukinda, India was identified as *Bacillus amyloliquefaciens* by ***Das et al. (2014)*** based on biochemical and 16S rRNA gene sequencing. The strain exhibited relatively high tolerance to Cr(VI) (6900 mg/L) and fast reduction rate of 2.22 mg Cr(VI)/ L/h, under optimized conditions of 100 mg/ L Cr(VI), pH 7.0 and temperature 35 °C within 45 h. Mechanism of Cr(VI) reduction as well as nature and fate of the reduced product were studied to determine the scope of removal of reduced Cr(III) end product. AAS analyses of the culture products treated with Cr(VI) for 45 h showed the distribution of Cr(III) in pellet and culture supernatant to be 37.4 ± 1.7 and 62.6 ± 3.4 mg/L respectively. In SEM images, the bacterial pellets with Cr(VI) treatment appeared coagulated, rough and porous whereas the pellets without Cr(VI) treatment appeared regular, smooth and non-porous in structure. SEM–EDX of the bacterial precipitates under Cr(VI) treatment revealed immobilization of Cr(III) species on the bacterial cell surface. Further Raman spectroscopy analysis confirmed the presence of Cr(III) species, with characteristic peak at around 600 cm^{-1}. TEM–EDX study of the

bacterial precipitates under Cr(VI) treatment showed intracellular deposition of Cr(III), which are in nanometric range. Further characterization of reduced product by XRD, FT-IR and SAED analyses suggested the formation of poorly crystalline end products. A Cr(VI) removal mechanism considering both the surface immobilization and intracellular accumulation of Cr(III) along with the formation of coagulated cell precipitate by living *B. myloliquefaciens* was suggested.

Singh et al., (2015) studied on iron reducing thermophilic microorganisms for their ability to reduce toxic metals in high temperature environments (up to 70 °C). In this study, an obligate thermophilic methanogen *Methanothermobacter thermautotrophicus*, was used to study the reduction of hexavalent chromium. A growth medium with H_2/CO_2 as substrate was used for conducting the experiments with various concentrations of Cr(VI) (0.2, 0.4, 1, 3, and 5 mM) as potassium dichromate ($K_2Cr_2O_7$). Complete reduction of the 0.2 and 0.4 mM Cr^{6+} solutions by this methanogen was observed in time-course study of Cr^{6+} concentrations in solutions using 1,5-diphenyl carbazide colorimetric method. However, considerably lower reduction levels of 43.6%, 13.0%, and 3.7% were detected at increasing Cr^{6+} concentrations of 1, 3 and 5 mM, respectively. These results suggested a toxic effect of Cr^{6+} in solution to cells at the higher concentration range. It was also observed that methanogenesis was inhibited at these higher concentrations of chromium and it was evident from decreased total cellular protein production and live/dead cell ratio that cell growth was impaired. Similarly, bio-reduction rates were decreasing on increasing initial concentrations of Cr(VI) from 13.3 to 1.9 mM/h. Reduction of soluble Cr(VI) to insoluble Cr(III) precipitates was confirmed by X-ray absorption near-edge structure (XANES) spectroscopy. The presence of amorphous chromium hydroxide in sample was revealed by selected area electron diffraction pattern. Though, a small fraction of reduced Cr was present as aqueous Cr(III). *M. thermautotrophicus* cells after Cr(VI) treatment was studied with scanning and transmission electron microscope. Observations suggested both intra- and extracellular chromium reduction mechanisms. Outcomes of this study demonstrated the ability of *M. thermautotrophicus* to reduce toxic Cr(VI) to less toxic C(III) and its possible application in chromium bioremediation, exclusively at subsurface radioactive waste disposal sites, at a temperature of ~70 °C.

2.4 Understanding the Mechanism of Chromium Resistance

The various modes of resistance in bacterial cells which overcome the Cr(VI) stress includes expulsion or reduced uptake of Cr(VI) compounds across the transmembrane sulphate shuttle present in membranes, biosorption and regulation of genes related to oxidative stress response ***(Cheungand Gu, 2007; Ramírez-Díaz MI et al., 2008). Ramírez-Díaz MI et al. (2008)*** observed that in chromium resistant bacteria Cr(VI) resistance was mostly plasmid borne, whereas Cr(VI) reductase genes are found both on plasmids and on the main chromosome. ***Thacker et al. (2006)*** found that among several mechanisms for bacterial resistance to chromate, the most characterized mechanisms are

demonstrated as efflux of chromate ions from the cell cytoplasm (plasmid borne) and reduction of Cr(VI) to Cr(III).

Juhnke et al. (2002) reported that the Chr protein family in bacteria was encoded by the plasmid vector and/or chromosomal DNA. Molecular analysis was carried out for the determinants of chromate resistance from plasmid pUM505 of *Pseudomonas Aeruginosa* and plasmid pMOL28 of *Alcaligeneseutrophus*, which revealed that ChrA gene, hydrophobic protein ChrA (416 and 401 amino acid residues) were responsible for the resistance phenotype. ***Alvarez et al. (1999)*** observed plasmid-determined resistance to hexavalent chromium ions in the genera Pseudomonas, Streptococcus and Alcaligenes. Tolerance to chromate conferred by the ChrA protein was attributed to reduced accumulation of CrO_4^{2-} in both *P. aeruginosa* and *A. eutrophus*. It was hypothesized that ChrA is involved in the separation of chromate ions.

Juhnke et al. (2002) reported that *Cupravidus metallidurans* and *P. aeruginosa* could be model organisms for chromate efflux which is occurring through the ChrA protein producing resistance levels of 4 and 0.3 mM, respectively. However, ***Branco et al. (2008)*** reported that chromate efflux was only a resistance mechanism in *Proteobacteria*. They further reported that a highly tolerant strain *Ochrobactrum tritici* 5bvl1 had survived chromate concentrations of >50 mM and had the transposon TnOtChr containing groups of chrB, chrA, chrC and chrF genes. The chrB and chrA genes were specifically essential for establishing higher resistance in chromium-sensitive *O. tritici*. They also reported that, chromate or dichromate strongly induced the Chr promoter, but showed no response to Cr(III), sulphates, oxidants or other oxyanions. ***Ramírez-Díaz MI et al. (2008)*** reported that the CHR protein family currently contains about 135 sequences from all three life domains and this protein family also includes putative ChrA orthologs. However, ***Díaz-Pérez C. (2007)*** revealed that there was considerable variation in the genomic context for ChrA orthologs.

The microbial reduction of Cr(VI) involves soluble and membrane-bound reductase enzymes along with compounds like glutathione, cysteine, sulphite and thiosulfates ***(Donati E, 2003). Ramírez-Díaz MI et al. (2008)*** explored that these soluble and membrane-bound reductases existed in a diverse range of aerobic, anaerobic and facultative bacteria. ***Cervantes C et al. (1992)*** found chromate reduction was not considered to be a typical resistance mechanism but they were deemed as independent processes.

Mechanism of bacterial Cr(VI) reduction is of biological and ecological significance as they transform toxic and mobile chromium derivatives into innocuous and immobile species by reducing them ***(Daulton et al, 2007; Soni et al, 2013). Opperman et al., (2008), He et al. (2009), Alam and Ahmad (2012), Batool et al. (2012) and Ge et al. (2012)*** have successfully isolated aerobic as well as anaerobic Cr(VI) reducing bacteria of diverse genera from diverse environments from Cr(VI) contaminated as well as non-contaminated water bodies, soil or sediments. ***Cervantes et al. (2007)*** reported that bacteria reduced Cr(VI) either aerobically or anaerobically depending on the microbial species while ***Lowe***

et al, (2003) and ***Alam et al, (2012)*** mentioned the presence of other factors affecting their reducing efficiency.

Suzuki et al. (1992), Park et al. (2000), Rida et al. (2012), Tripathi et al. (2013) have demonstrated chromate reductase activity in various cell-free extracts during Cr(VI) reduction in aerobic conditions. Electrons, NADH and NADPH from the existing reserve are identified as the electron donors in aerobic Cr(VI) reduction process. ***Qamar et al. (2011)*** reported that reductases viz., ChrR, YieF and Tkw3 reduced the Cr(VI) derivatives by shuttling electrons and forming Cr(III). ***Elangovan et al. (2006)*** observed that aerobic Cr(VI) reduction was generally prevalent in soluble proteins which utilize NADH as an electron donor, either to enhance activity or as a requirement. However, it is still unclear whether reduction of Cr(V) to Cr(IV) and that of Cr(IV) to Cr(III) is a spontaneous process or enzyme mediated ***(Cheung and Gu, 2007).***

Cheung and Gu (2007), Mangaiyarkarasi et al. (2011) explored that in anaerobic conditions, Cr(VI) acted as a terminal electron acceptor within the respiratory chain for a wide array of electron donors, including proteins, carbohydrates, fats, hydrogen, NAD(P)H and existing electron reserves. Both the soluble and membrane-associated enzymes had been able to mediated Cr(VI) reduction activity in anaerobic conditions. ***Mangaiyarkarasi et al. (2011)*** also reported that the Cr(VI)-reducing activities of anaerobes was different than in aerobes, as they were associated with the electron transfer systems which ubiquitously catalysed the electron shuttle through the respiratory chain. It is also observed that the cytochrome family (e.g., cytochrome b and c) is highly involved in anaerobic Cr(VI) reduction using enzymes.

Viti et al. (2007) investigated the relation between sulphate and iron-reducing bacteria (SRB and IRB) with the removal of chromium in the microbial community. They found that reduction of Cr(VI) by Fe(II) and sulphides which were generated by IRB and SRB is 100 times faster than if CRB was used alone. The proposed mechanism mentioned that the SRB produced H_2S, which acted as a Cr(VI) reductant involving three stages: (1) reduction of sulphates, (2) reduction of chromate by sulphides and (3) precipitation of Cr(VI) by sulphide. Reduction of Cr(VI) by Fe (II) happened when IRB reduced Fe(III) to Fe(II), this in turn reduced Cr(VI) to Cr(III).

Chirwa et al. (2011) showed two pathways for Cr(VI) reduction deduced for gram-negative bacteria. In the first mechanism, it was suggested that reduction of Cr(VI) was mediated by soluble reductase, and NADH served as the electron donor. This was either necessitated or for achieving maximum activity. The NADH dehydrogenase pathway was shown to be dominant under aerobic conditions. Soluble Cr(VI) reductases, or, Cr(VI) reducing enzymes that were produced by the cell were exported into the media. These reductases reduced Cr(VI) and a lot of focus was on their mechanism. ***Cheng and Gu (2007)*** observed as protein excretion process was energy intensive, most of the enzymes were produced constitutively, i.e., their production was triggered only when Cr(VI) was detected in the medium and therefore it was highly regulated. ***Harish et al. (2011)*** mentioned that extracellular Cr(VI) reduction was beneficial to the organism as the cell did not require additional transport mechanisms for carrying the chromate and

dichromate inside the cell and to release the Cr(III) into the medium later while ***Chen and Gu, (2007)*** stated if Cr(VI) was reduced internally, resultant Cr(III) tend to accumulate inside the cell, which made the recovery of reduced chromium and regeneration of cells difficult.

Ackerley et al. (2004) noted that several components of the cell protoplasm also reduced Cr(VI). Components like NADH (NADPH in some species), heme-proteins and other flavoproteins are reported to reduce Cr(VI) to Cr (III).

Chirwa et al. (2011) also reported an additional mechanism of chromate resistance. As the generation of ROS occurred during reduction of Cr(VI) to Cr(III), bacterial proteins participated in the defence against oxidative stress by activating ROS scavenging enzymes (e.g., catalase, superoxide dismutase). ***Ackerley et al. (2006)*** reported that E. coli activates enzymes such as superoxide dis-mutase (SOD) and catalase as chromate protective systems. Additionally, on chromate exposure the depletion of glutathione and other thiols in E. coli, suggested that they are important in detoxifying Cr(VI). ***Samuel et al. (2013)*** also analysed the stress responses against hexavalent chromium for *Bacillus sp* and *E. coli* after adaptation to the Cr-enriched environment.

Cervantes et al. (2007) and ***Hu et al. (2005)*** explored DNA repair by SOS response enzymes (RecA, RecG, RuvAB) countering DNA damage due to the presence of hexavalent chromium which was considered as another defensive mechanism against Cr toxicity. It had been established that inducing *E. coli* SOS repair system protected DNA from oxidative damage due to Cr(VI). ***Miranda et al. (2005)*** explored other components of recombinational DNA repair system, such as DNA helicases RecG and RuvB, participated in the response to DNA damage caused by chromate in *P. aeruginosa.*

2.5 Batch Kinetic Studies with Real Life Wastewater

The bio-transformation of Cr(VI) to Cr(III) was possible by both pure and enriched mixed cultures of microbial species under aerobic and anaerobic environments ***(Shen and Wang, 1993; Philip et al., 1998)***. ***Chirwa and Wang (2000), Smith et al. (2002)*** mentioned that most of the biological systems treating Cr(VI) bearing wastewater were operated in batch mode. The probable reason as mentioned was the eventual loss of active biomass due to metal toxicity. ***Cheng and Hao (1997)*** reported that continuous removal of Cr(VI) was almost impossible on a long-run without intermittently reseeding to the biological system. However, of late, fixed-film bioreactors and continuous-flow had been working for biological removal of Cr(VI). ***Shen and Wang (1995)*** studied removal of Cr(VI) in a two-stage, continuous-flow suspended growth bioreactor system. *Escherichia coli* cells selected for Cr(VI) removal and grown in the first-stage mixed reactor. Completely grown culture were pumped into the second-stage plug-flow reactor to reduce Cr(VI). The proficiency of a fixed-film bioreactor for Cr(VI) reduction was first reported by ***Chirwa and Wang (1997)***.

They reported biological mechanisms of Cr(VI) reduction in a continuous-flow lab-scale biofilm reactor. In these reactors, new Cr(VI) reducing microbial cells were not recurrently added but electron donors were externally supplied to the wastewater according to the requirement. ***Imai and Gloyna (1990)*** observed the performance of activated sludge process using mixed microbial consortia. 60% Cr(VI) removal and 90% Organic substrate removal were reported for the react period of 23 h. ***Shen and Wang (1995)*** studied the performance of *Pseudomonas* sp. in Bench-scale continuous stirred tank reactor and reported 88% Cr(VI) removal and 40% Organic substrate removal. ***Brunet et al. (2006)*** observed the performance of Sulfate-reducing organism, *desulfomicrobium norvegicum*in a pilot bioreactor and reported 99% Cr(VI) removal and 20% Organic substrate removal. 99.7% Cr(VI) removal was reported by ***Dermou et al. (2008)*** after studying performance of mixed culture in pilot -scale trickling filter. ***Tziotzios et al. (2008)*** reported 100% Cr(VI) and around 50% Organic substrate removal by mixed culture (aerobic) in pilot-scale packed-bed reactor. ***Cordoba et al, (2008)*** worked with *Arthobacter* sp. in packed-bed reactor under SBR recirculating mode and reported 100% Cr(VI) removal. ***Elangovan and Philip (2009)***, worked with *Arthobacter rhombi-RE* in aerobic system in both suspended growth system and packed bed reactor and reported 99.6%, 99.4% Cr(VI) removal and 90-95%, 92-96% Organic substrate removal respectively. ***Elangovan and Philip (2009)*** also reported 99.8% Cr(VI) removal and 50-55% organic substrate removal while working with *Arthobacter rhombi-RE* in anaerobic system in a packed bed reactor.

2.6 Artificial Neural Network in Wastewater Treatment

Biological wastewater treatment has been found to be an effective method for treating different types of wastewaters. Each treatment scheme is unique in characteristics due to the temporal variation in the properties of wastewater depending on its source. This makes replication of a treatment method from one system to another very difficult. Mathematical models have been developed to understand and regulate the systems effectively. However, these models have several drawbacks including the fact that they are site specific and require a large number of parameters to be studied and quantified to apply the model. Also, it is difficult to make these models adapt to fluctuations in one or more parameters ***(Baxter et al., 2002; Cinar, 2005).***

Artificial Neural Networks (ANN) are based on artificial intelligence modelling techniques because of their inherent capacity to identify patterns, learn from the data and make inferences on new data sets. ANN has been extensively used in the water treatment industry for water quality development, process models, model-based process-control and automation tools ***(Wahl et al., 2010).***

Fanaie et al. (2016) applied ANN to study biosorption of p-chlorophenol by dried activated sludge. A three-layered feed forward neural network was developed. The optimum number of neurons was obtained as 6 in the hidden layer. The input parameters

included adsorbent dosage, pH, contact time and initial concentration of the compound. The effects on the removal efficiency of the compound were studied.

Doust et al. (2015) studied COD and Biological Oxygen Demand (BOD) removal in an aerobic treatment process for refinery wastewater. Eight input variables were assigned into ANN and the effect on the BOD and COD removal was modelled. The optimum number of neurons in the hidden layer was found to be 27. ***Atasoy et al. (2013)*** also utilised Feed forward neural network to study the treatment of Acid Mine Drainage in Fluidised Bed reactors. They found an optimal neuron count of 24.

Another area where ANN has found extensive use is the study of large-scale wastewater treatment plants. Here. ANN models were used for prediction of various parameters like COD, BOD, and Total Suspended Solids (TSS) in large scale plants. The data were collected from the plant processes and predictions for the output parameters were made. ***Farouq et al. (2007)*** predicted various plant outputs for a large-scale plant in Doha. This study was based on Levenberg–Marquardt network training algorithms for all the runs. The researchers reported that this algorithm is faster in learning speed and performance level compared to other optimisation algorithms. ***Khallafallah et al. (2004)*** studies the same on large scale plants in a paper mill wastewater treatment.

Thus, ANN has developed as an important tool to study wastewater treatment systems. Several studies have been reported, aiming at better process control and prediction of wastewater treatment plants.

2.7 Critical Review

Various anthropogenic and industrials activities pollute the environment through their discharges - chromium compounds are one of these major pollutants. Cr(III) and Cr(VI), two of the most prevalent states of chromium, may exhibit different behaviours in the environment. The high mobility and solubility of Cr(VI) enhance the possibilities of its diffusion through cell membrane and thus Cr(VI) becomes a carcinogen, teratogen and mutagen. Cr(III) is rather less toxic and sparingly soluble in aqueous media. The exposure to this heavy metal is inducing a stress in the environment. Therefore, a versatile, eco-friendly and cost-effective method is necessary for removal of Cr(VI) from water and soil systems. Bioremediation is a green avenue to achieve this compared to the traditional physico-chemical methods. Microorganisms (bacteria, fungi, yeast and algae) offer greater capacity towards metal tolerance and this attributes to their ability to survive under extreme environmental conditions. The development of effective bioremediation processes (biosorption, biotransformation and bioaccumulation) suggest various opportunities for detoxification of Cr(VI). Biotransformation involves the enzymatic Cr(VI) reduction both in aerobic and anaerobic conditions and immobilizes Cr(VI) (Cr(III)-containing precipitates). Further, proteomic studies on microbial responses to Cr(VI) and microbial stress enzyme studies reveal the detailed mechanism behind their battling against Cr(VI). It is a promising approach and commercial solution for ex situ Cr(VI) remediation.

Cr(VI) bioremediation has been tested in various microbial systems based on their removal efficiency in synthetic media. It is also essential to assess their performance in

batch, continuous or fed-batch set-up according to the different operational settings in real life wastewater to ensure actual hydrodynamic and mass transfer requirements in treatment plant environment. The knowledge of reduction mechanism along with genetics and enzyme studies expand the prospects of these biological detoxification processes for substantial advancements. It is evident from this review study that an intense research should be made in this field to scale up these techniques to the large-scale industrial remediation processes.

Green Area, Objectives and Scopes of the Research

3

3.1 Green Area of the Research

Based on the available literatures on microbial remediation of hexavalent chromium, covered under Chapter-2, the following research gaps are identified.

- ✓ *Bioremediation of chromium-contaminated environment using microorganisms has enormous potential to be a sustainable and economically viable solution.*
- ✓ *Knowledge of the interaction of chromium with isolated bacterial strains, factors influencing the efficient removal of Cr(VI), resistance and reduction mechanism towards Cr(VI) remediation are to be inculcated to enable any lab-scale biotechnological technique to evolve into a large-scale reactor engineering, which can be a highly practical approach.*
- ✓ *In-depth study of real-life applicability of the isolated microorganism in tannery effluent has not so far been explored extensively.*

3.2 Objectives of the Research

The objectives of present research work are stated below.

- ➢ *To investigate into the chromium resistance mechanism of an isolate under standardized conditions for optimum removal of Chromium from wastewater*
- ➢ *To conduct batch kinetic study and examine the performance of the isolate while remediating real life tannery wastewater*

3.3 Scopes of the Research

To accomplish the research objectives the scopes of research have been set as stated underneath.

- ✓ Isolation and screening of Cr(VI) resistant bacteria from soil contaminated with tannery discharge

- ✓ Morphological characterization and interaction of Cr(VI) with various isolates
- ✓ Identification of most efficient isolate and genomic and proteomics study of the isolate
- ✓ Optimization of different parameters of growth medium
- ✓ Characterization of Cr(VI) reduced products
- ✓ Investigation of the Cr(VI) resistance pathways i.e. reductase enzyme assay, oxidative stress analysis and identify extra cellular mechanism, if any
- ✓ Evaluation of the performance of the isolated bacteria in a laboratory-scale batch reactor for combined removal of biodegradable organics (COD) and chromium from real-life tannery effluent
- ✓ Batch kinetic study of isolated bacterial strain for combined removal of biodegradable organics (COD) and chromium from real-life tannery effluent
- ✓ Modeling of the performance of the isolated bacterial strain in batch system for removal of chromium(VI) from real life tannery wastewater by using Artificial Neural Network (ANN)

3.4 Comprehensive Study Plan

Based on the research objective and scope of the study the entire work has been segmented into six phases as depicted below:

Phase-1: ***Isolation and characterization of chromium resistant bacteria***
Phase-2: ***Optimization of parameters of growth medium and molecular characterization of the selected isolate***
Phase-3: ***Characterization of the reduced product***
Phase-4: ***Understanding mechanism behind chromium reduction***
Phase-5: ***Batch kinetic study of pure culture with tannery wastewater.***

Isolation and Characterization of Chromium Resistant Bacteria

4

4.1 Purpose of the Work

Developing a detailed understanding of microbe-metal interactions within the environment has attained considerable importance during the last two decades ***(Losi and Frankenberger, 1994; Marsh et al., 2001; Ackerley et al., 2004; Dhalet al., 2010; Beller et al., 2013; Rani Pratibha and Upadhaye Seema, 2016)***. While various aspects of these interactions have been studied, it has emerged that microorganisms can play a vital role in the remediation of contaminated water, sediments and soil ***(QuiIntana et al., 2001; Thacker et al., 2007)***. Microorganisms can affect parameters like solubility and toxicity of metals and effect in situ remediation of contaminated substrate. Field studies, conducted to explore the potential of microbes to alter or remove environmental contaminants by direct or indirect means, have established that stimulation of these indigenous microorganisms can be an effective remediation method to be followed.

A wide number of chromium resistant bacteria, both aerobes and anaerobes, were isolated from water and soil contaminated with chromium. The review of literature enumerated different types of microbial strains showing high tolerance to chromium. This phase of study aims at the isolation of few chromium resistant bacteria, and their characterization on the basis of their morphology, minimum inhibitory concentration, gram staining and growth kinetic study under the stress of chromium. This study helps to select one bacterial strain out of the total isolated strains for efficient removal of chromium.

4.2 Methodology

4.2.1 Sampling Site Selection

Untreated wastewater from different industrial units including tanneries, electroplating industries, battery industries etc. and municipal sewage of Calcutta City flow down through a web of canals into the **East Calcutta Wetlands (Lat 22°33' - 22°40' N; Long 88°25'- 88°35'E)**. The bank of such wastewater carrying canal was selected as the sample site for the reason that bank soil was continuously fed with high metal contaminants.

4.2.2 Sampling Site Description

The annual average maximum temperature of sample site was 31.26ºC and average minimum temperature was 22.23ºC till the year 2002 (Source: www.indiawaterportal.org). The annual average rainfall was recorded 1532 mm. The canal beside which the sampling was done passes through East Calcutta Wetlands: Ramsar site. The East Kolkata Wetlands is believed to host the largest sewage fed aquaculture in the world and acts as the natural sewage treatment plant for the city of Kolkata. The wetlands comprise of salt marshes, salt meadows, sewage farms and settling ponds which have played a vital role in development and sustenance of the ecosystem.

Fig.4.1: Satellite imagery of sampling site mentioning the sampling point in East Calcutta Wetland ((lat 22°33' - 22°40' N; long 88°25'- 88°35'E)

Fig-4.2: Sampling Site

Fig-4.3: Sampling Point

4.2.3 Collection of Sample

Bank soil samples from three different points of the selected sampling site at East Calcutta Wetland were collected under aseptic condition in sterilized glass containers. Samples were used for microbial analysis and therefore carried to the laboratory in refrigerated condition. As a precaution, soil sample was not collected during monsoon or just after rainfall. This is because rainwater might have washed out the bio-organisms of our interest from the soil.

4.2.4 Analysis of Soil Sample

The analysis of metal present in the soil sample was done by following the method stated by ***Isaac and Kerber, 1971***. Moist soil sample was dried at 75ºC for 48 h. 1 g of finely grinded, dry soil was digested in acid-digestion chamber with 1:1 HCl and $HClO_4$(Merck India Co. Ltd., AR Grade). All metals (Cr, Pb, Zn, Fe, Mn and Cu) were detected by Atomic Absorption Spectrophotometry (AAnalyst 100, PerkinElmer) with interfacing AA Win Lab Software using specific hollow cathode lamp in default condition by flame absorption mode. Standard recommended by Perkin-Elmer were used for checking the sensitivity of the instrument and also the calibration. Each time the measurement of concentration was replicated thrice at each of the 3.0s integration times. The mean concentration, standard deviation (SD) and relative standard deviation (RSD) displayed in result window of the component were printed ***(Chattopadhyay et al., 2002)***.

4.2.5 Preparation of Growth Media

In the course of the present phase of research, Luria Bertani (LB) agar medium (g/L): Tryptone (Merck India, AR Grade) 10.0; Yeast extract (Himedia, AR Grade) 5.0; NaCl (Merck India, AR Grade) 10.0; Agar (SRL India, AR Grade) 1.5%; Sugar (SRL India, AR Grade) 0.10) was used for growth of bacteria. Cr(VI) was supplemented to the sterile molten LB medium. A filter sterilized (0.22 µm Whatman filter) solution of $K_2Cr_2O_7$ (Merck India, AR Grade) was used as the source of hexavalent chromium and was added after autoclaving the LB medium at 15 psi for 20 mins. In the liquid medium agar was not added. Deionized water (DI) (18 MΩ) from a Millipore water purification unit (RiOsDI 3) was used for this purpose and in all the subsequent experiments during the entire course of this research.

4.2.6 Isolation of Chrome Resistant Bacteria

Cr(VI) resistant bacteria were isolated from the soil sample using dilution plate technique method ***(Das et al., 2012)***. 1 g of the soil sample was dissolved in 10 mL of sterile water (10^{-1} dilution). The suspension was then serially diluted to attain a dilution of 10^{-5} and

then first plated on LB-Agar (pH 7.0) medium. Agar plates were incubated at 37ºC for 96 h. Colonies obtained on these plates were picked and again plated on LB agar plates amended with 100 mg/LCr(VI) as $K_2Cr_2O_7$. From this initial screening, strains showing resistance to chromium were selected and then purified by many rounds of re-streaking. These isolates were used for further studies. Slants were made from these isolates and stored at 4ºC for future use. The full course of the experiment was done inside the laminar airflow chamber (ZHCHENG, ZHJH – C2112C) to maintain the aseptic condition.

4.2.7 MIC Assay for Chromium Resistance Isolates

Minimum inhibitory concentration (MIC) was assessed in LB medium amended with different concentration of Cr(VI)i.e. 0, 50, 100,150, 200, 250, 300, 350, 400, 450 and 500 mg/Las $K_2Cr_2O_7$. 500 µl of a fresh overnight culture (OD_{600}=0.8) was inoculated in 5 ml of LB medium. Tubes without chromium were used as control. At 37ºC all tubes were incubated with shaking at 140 rpm in an orbital motion shaker-incubator (ZHCHENG, ZHWY 100B). The growth of biomass was determined by measuring absorbance at λ_{600} against sterile LB blank (UV-VIS Spectrophotometer, Lambda 25 Perkin Elmer). The lowest concentration of metal that completely prevented the growth was determined as MIC ***(Yilmaz et al., 2003)***.

4.2.8 Gram Staining of Bacteria and Morphological Study

Cells were cultivated in LB medium overnight and then microscopically examined under (1000X) after gram staining ***(Bailey and Scott, 1996)***. Morphology of single colony of bacterium was examined under normal visual inspection.

4.2.9 Growth Curve of Bacteria

The growth of bacteria was observed in LB medium. The experimentation was continued until the stationary phase was reached. A control i.e. without Cr in medium was carried out concurrently. Sample was collected at an interval of 15 mins or 30 mins or 1 h depending on type of bacteria and growth of biomass was assessed by measuring absorbance at λ_{600} against a sterile LB blank sample. Absorbance obtained at different intervals were plotted on X axis against the time plotted along Y axis. This graph presents the growth of the biomass under stress of Chromium.

4.2.10 Determination of Cr(VI)removal ability

The chromate removal ability was determined in LB medium under aerobic condition. Fresh medium was inoculated with 500µl of overnight culture. Tubes were incubated with shaking at 150 rpm and 37ºC. Residual Cr(VI) in the supernatant was measured after 24

hrs. Cr(VI)was determined spectrophotometrically (Lambda 25 Perkin Elmer) by 1, 5-diphenyl carbazide method ***(APHA, 1998).*** The Cr(VI)content in supernatant was determined by adding 0.2ml orthophosphoric acid and 0.2 ml of acetone solution of 1, 5-diphenyl carbazide. The absorbance was measured at 540nm after incubation of sample for 10 mins at room temperature.

4.3 Results and Discussion

4.3.1 Analysis of soil sample

pH was observed to be slightly alkaline in water of sampling sites as well as of bank-soil samples. pH of the tannery effluent was dependent on the tannery unit operation and it could vary from 2.5 to 13.5. However, at sampling sites mean pH was noted to be 7.81± 0.63 and during our sampling period it varied between 6.1 and 8.6. Maximum and minimum temperature of both air and water were observed during April-May and December respectively. The mean air temperature of (32.39±2.69)°C and water temperature of (31.84 ± 2.97)°C were noted during sampling. The equilibrium solubility of O_2 in water at 25°C was 2.7 x 10^{-4} per mol (8.7 mg/L). Dissolved oxygen (DO) is essential for not only the survival of aquatic organism but also for the various oxidation processes in physico-chemical interaction ***(Chattopadhyay et al., 2002)***. Various metal contents in soil sample are enlisted in the Table 4.1.

Table-4.1: Physico-chemical Properties of Bank Soil Sample

Properties	Values
pH	7.81 ± 0.63
Temperature	(31.84 ± 2.97) °C
Colour	Blackish grey
Total Chromium	37085.82 ± 1124.183 mg/kg
Copper	141.99 ± 8.52 mg/kg
Zinc	623.67 ± 32.65 mg/kg
Iron	32834.34 ± 1448.93 mg/kg
Manganese	3423.19 ± 32.18 mg/kg
Lead	237.54 ± 11.17 mg/kg

4.3.2 Chromium resistant bacterial isolates

The detection of metal tolerant bacteria in a particular given environment can be an indication that such area has been contaminated by heavy metals. Consequently, such an area may be suitable for adaptation and selection of heavy metal resistant organisms ***(Clausen, 2000)***. Isolation of metal resistant bacteria from the environment

contaminated with metal can be deemed to be an appropriate practice for the selection of the most appropriate metal resistant strain, which can be used for heavy metal removal employing bioremediation ***(Malik, 2004)***.

In the present study, the total number of bacterial isolates showing Cr resistance with different types of colonies in the soil samples was 24. Out of them 3 isolates were grown in 96 h. 7 isolates took 72 h and rests were grown in 24 h. Few microbes showed higher growth on solid medium than in liquid medium, which might be attributed to the greater diffusivity of metal in liquid. Availability and complex formation of metals in liquid medium were different from those observed in solid medium. This observation is in agreement with ***Mergeay et al., (1995)***.

4.3.3 MIC of Bacteria

Over the years, numerous chromium-resistant bacteria have been isolated and each species vary in point of degree of resistance. This variation in properties is due to the inherent capability of the specific strains to resist the toxicity of chromium.MIC of all 24 Cr(VI) resistant isolates is given in Fig-4.4. MICs of 4 isolates were obtained as 250 mg/L of Cr(VI) as $K_2Cr_2O_7$, 5 isolates as 450 mg/L of Cr(VI) as $K_2Cr_2O_7$, another 4 isolates as 400 mg/L of Cr(VI) as $K_2Cr_2O_7$ and rest 11 ranging between 300 to 350 mg/L of Cr(VI) as $K_2Cr_2O_7$. CRB 33, CRB 3, CRB 2A, CRB 4A and CRB 5A showed maximum MIC out of these 24 isolates i.e. 450 mg/L of Cr(VI) as $K_2Cr_2O_7$. Many researchers have been able to isolate species of bacteria with high Cr(VI) resistance but these microorganisms failed to remediate Cr(VI) at high concentrations (equal to their MIC). Therefore, an appropriate strategy is to be adopted for selection of potential bacterial strains which can be employed in remediating Cr(VI)-contaminated environments. This strategy should be preferably based on the growth capability of a strain in presence of high levels of chromium as well as their respective chromium reduction ability ***(Viti et al., 2003)***.

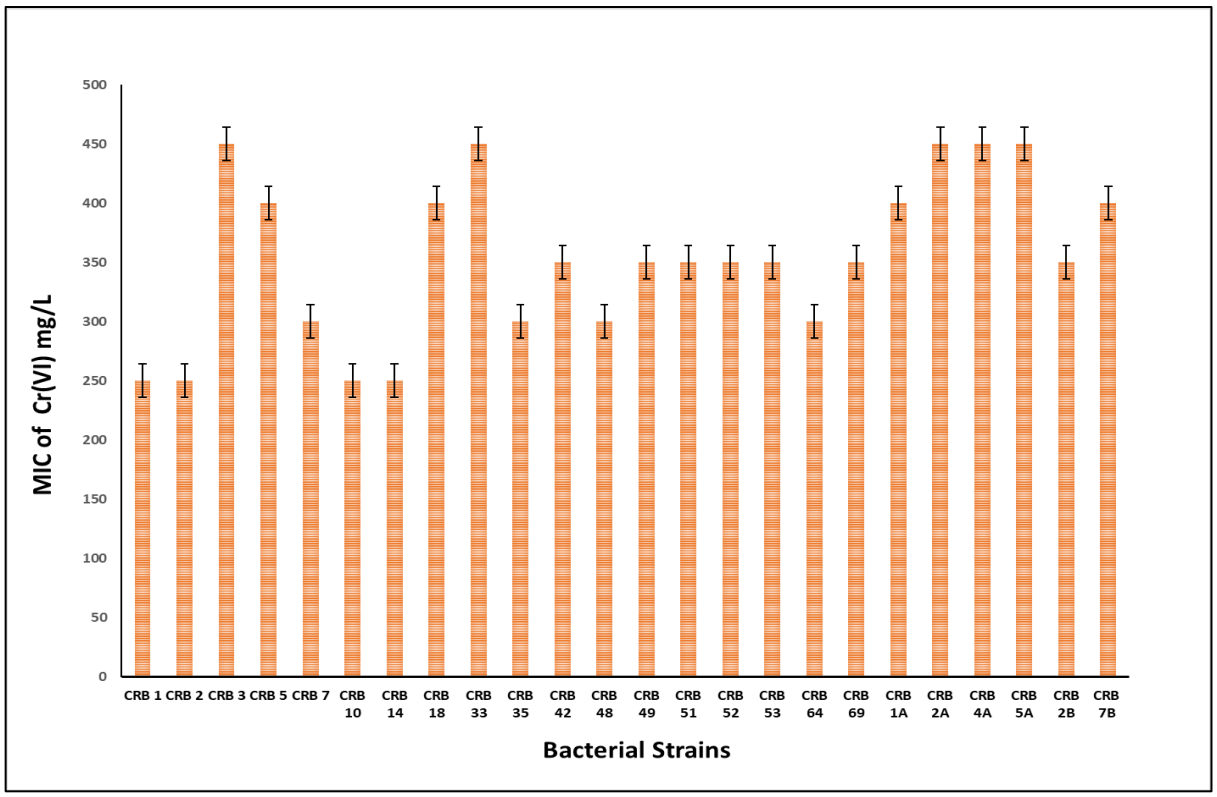


Fig-4.4: MIC of 24 Cr(VI) resistant bacteria (CRB)

4.3.4 Cr(VI) Removal Efficiency

Cr(VI) removal efficiency of the isolates was observed at 100 mg/L of Cr(VI) concentration after 24 h in LB medium. Seven strains showed Cr(VI) removal efficiency in the range of (88 – 98.5)%. ***Masood and Malik (2011)*** reported that 100% removal of 100 mg/L of Cr(VI) by *Bacillus sp.* can be achieved within 48 h. ***Kathiravan et al.,*****(2010)** also reported that 95.2% removal of 100 mg/L of Cr(VI) by *Bacillus sp.* can be achieved within 78 h in a minimal salt medium. ***Mary Mangaiyarkarasi et al., (2011)*** reported that within 144 h, *Bacillus sp* removed 71 % of 100 mg/L of Cr(VI). In our research, the seven isolates, i.e. CRB 2A, CRB 4A, CRB 5A, CRB 33, CRB 1A, CRB 2B and CRB 7B were further studied to check their growth responses in presence of Chromium. However, for experimental assistance all seven isolates were given a nomenclature of CRB 1-7 as given in Table-4.2.

4.3.5 Colony Morphology Study of Cr(VI) Resistant Bacterial Strains

In Table 4.2, we have presented a summary of the study of colony morphology of all the seven bacterial strains. Gram staining of these strains revealed that five out of the seven strains were gram positive. It is also suggested from different studies that *Bacillus* is the predominant genus in case of gram-positive bacteria ***(Narayani and Vidya Shetty,***

2013). Colonies of the isolated bacteria observed to be usually small to medium in size and round. With the exception of CRB 2 and CRB 3, other strains were observed to be in paired or long chain or clustered arrangement. The surface of the cream coloured colony of CRB 1 was rough and convex in elevation, while the surface of colonies for CRB-2, 3, 5, 6 were smooth, however the elevation and colour were different for these colonies. The colony of CRB 4 appeared to have wrinkles on the surface and umbonate. The colony of CRB 7 was examined to be flat and glossy on the surface and dull in colour. None of the bacterial strains have any observed pigmentation.

Table – 4.2: Morphological Characteristics of Chromium Resistant Isolates

Strain Name	Gram Reaction	Colony Shape/ Size	Cell Arrangement	Surface	Colony Elevation	Colony Colour	Pigmentation
CRB 1 (CRB 5A)	Gram +ve, Rod	Round, Medium	Single, paired, long chain	Rough	Convex	Cream	-
CRB 2 (CRB 1A)	Gram +ve, Rod	Irregular, small	Single	Smooth	Flat	Pale	-
CRB 3 (CRB 2A)	Gram +ve, Rod	Round, very Small	Single	Smooth	Flat	Pale	-
CRB 4 (CRB 7B)	Gram - ve, Circular	Circle, very Small	Single, paired	Wrinkled	Umbonate	White	-
CRB 5 (CRB 4A)	Gram +ve, Rod	Round, Medium	Single, long chain	Smooth	Convex	Cream	-
CRB 6 (CRB 33)	Gram -ve, Rod	Round, Small	Single, long chain	Smooth	Raised	White	-
CRB 7 (CRB 2B)	Gram +ve, Circular	Circle, Medium	Single, Clustered	Glossy	Flat	Dull	-

4.3.6 Growth Curve of Chromium Resistant Strains

Comparative growth patterns were observed for seven bacterial isolates (CRB 1-CRB 7) under the stress of Cr(VI) and without Cr(VI). The respective biomass growth data was collected as optical density (OD=600) and mentioned as LBC for sample treated with chromium and LB for sample treated without chromium. The results are showed in Fig-4.5 to Fig- 4.11. The results suggested that out of the seven bacterial isolates CRB 1, CRB 2, CRB 5 and CRB 7 showed least change in growth of the bacteria due to presence of Cr(VI). While chromium acted as stress for normal growth of bacteria for CRB 3, CRB 4 and CRB 6. The doubling time for all seven strains were calculated and given in table 4.3. The doubling time value increased for CRB 3, CRB 4, CRB 6 and CRB 7 when exposed to chromium. While, CRB 2and CRB 5 showed significant decrease in doubling time on treatment with chromium. However, CRB 1 showed least change in doubling time when exposed to Cr(VI) among all the seven strains and the value was lowest for this strain. This indicates that CRB 1 may have high resistivity towards the chromium contaminated environment and also there is no interference by chromium in the metabolism of the bacteria. Therefore, CRB 1 was selected for the detailed study.

Table-4.3: Doubling Time for Seven Bacterial Isolate Samples treated with and without Chromium

Bacterial Sample Name	Doubling Time (h) for sample without Cr(VI)	Doubling Time (h) for sample with Cr(VI)
CRB 1	**1.25**	**1.25**
CRB 2	**3**	**2.8**
CRB 3	**3**	**5.5**
CRB 4	**2**	**4.9**
CRB 5	**1.9**	**1.2**
CRB 6	**2**	**5**
CRB 7	**3.7**	**6**

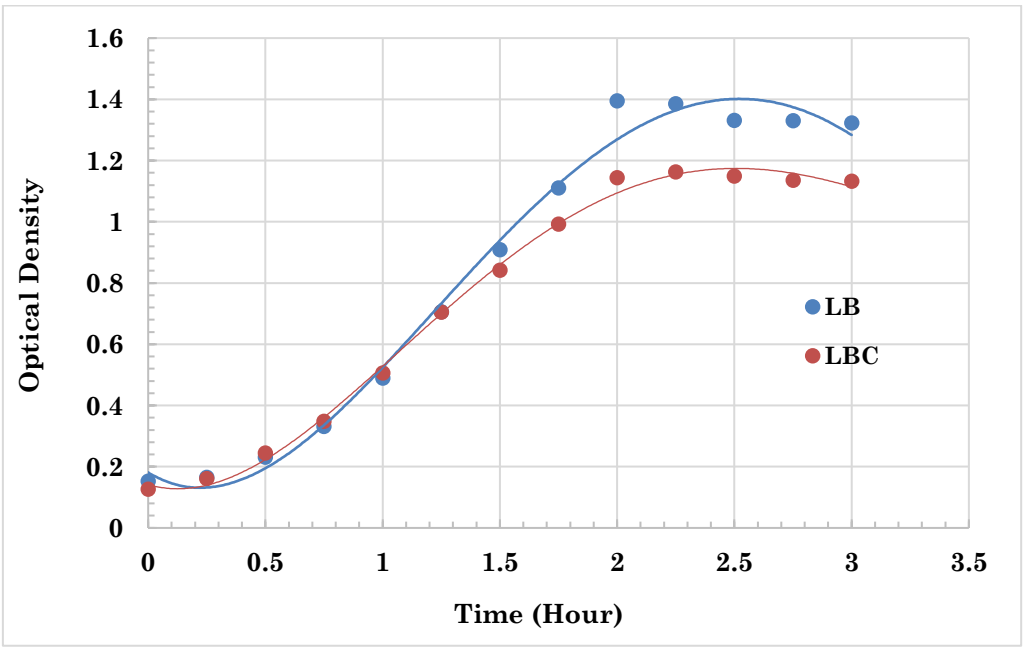


Fig- 4.5: Growth pattern of CRB 1 with (LBC) and without (LB) Cr(VI)

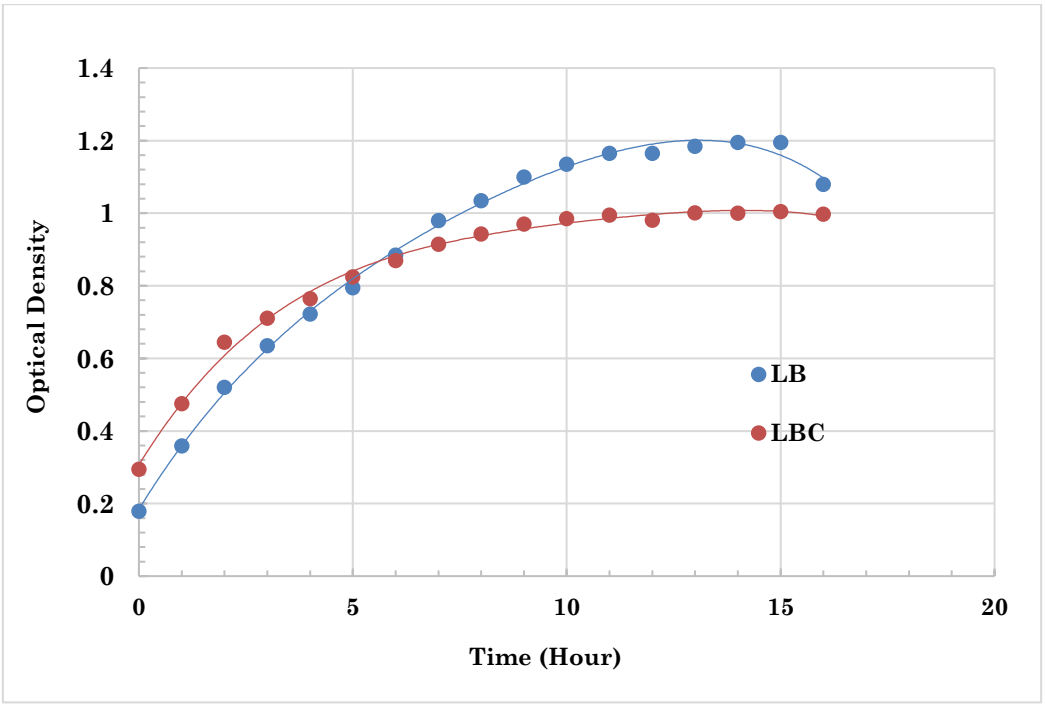


Fig- 4.6: Growth pattern of CRB 2 with (LBC) and without (LB) Cr(VI)

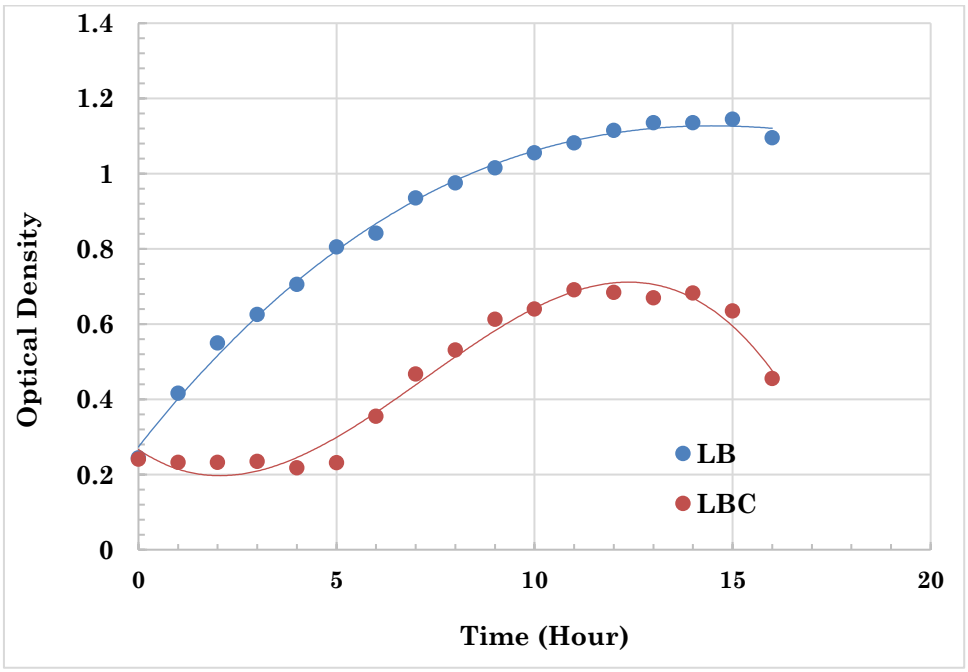


Fig- 4.7: Growth pattern of CRB 3 with (LBC) and without (LB) Cr(VI)

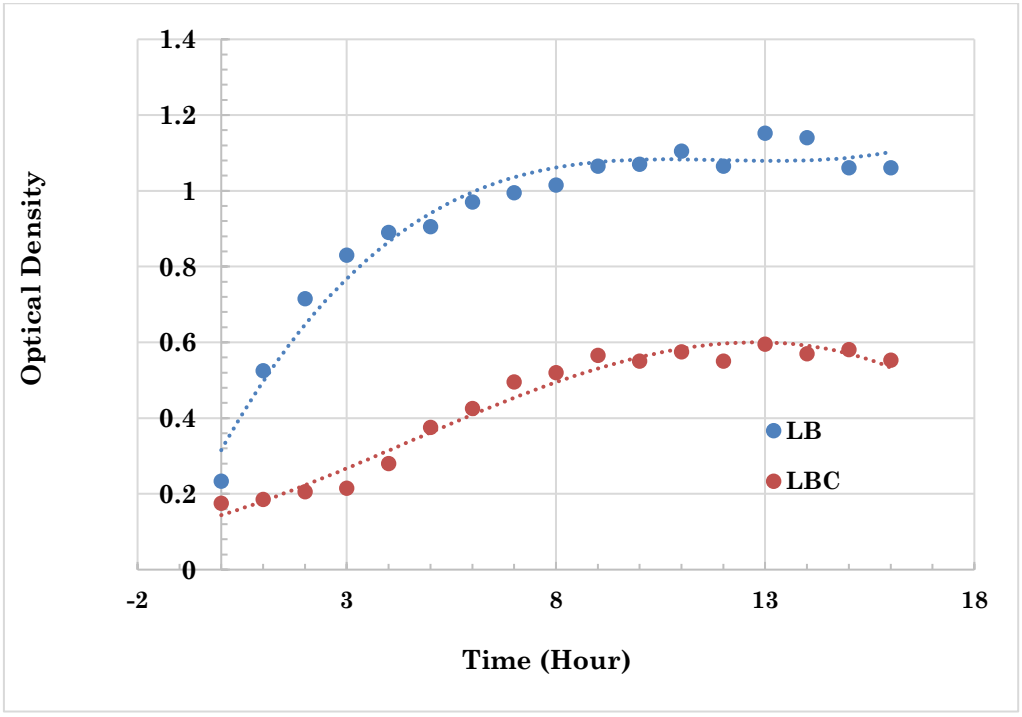


Fig- 4.8: Growth pattern of CRB 4 with (LBC) and without (LB) Cr(VI)

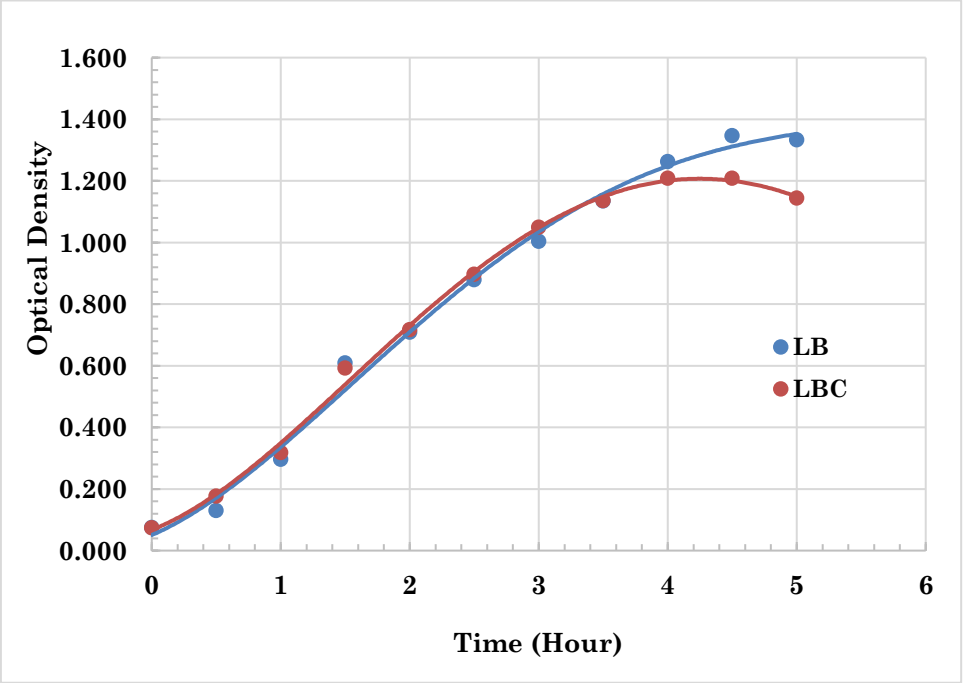


Fig- 4.9: Growth pattern of CRB 5 with (LBC) and without (LB) Cr(VI)

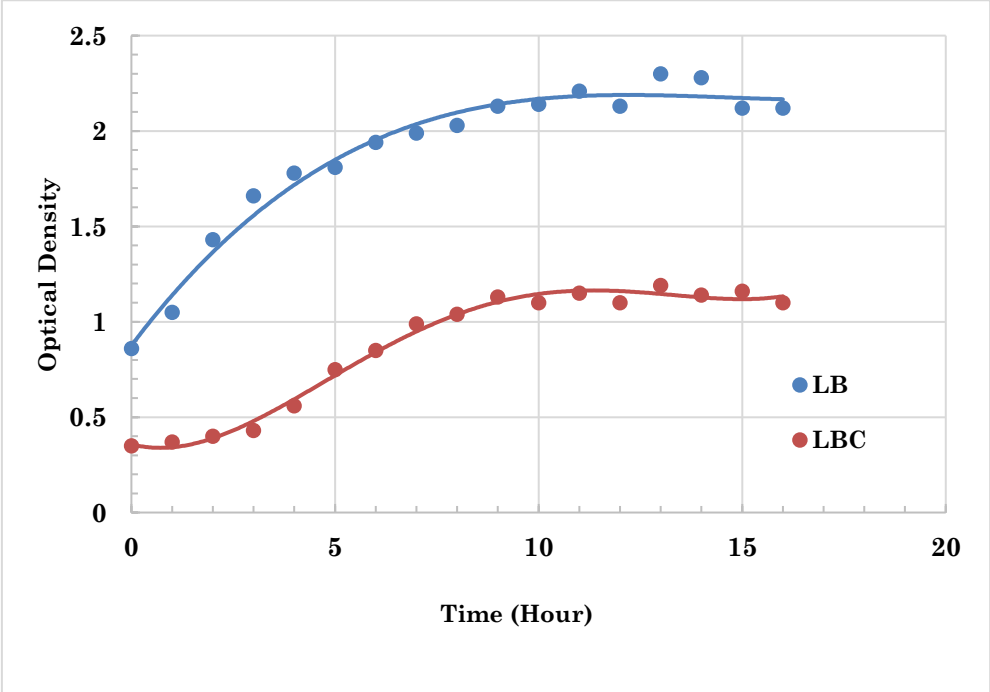


Fig- 4.10: Growth pattern of CRB 6 with (LBC) and without (LB) Cr(VI)

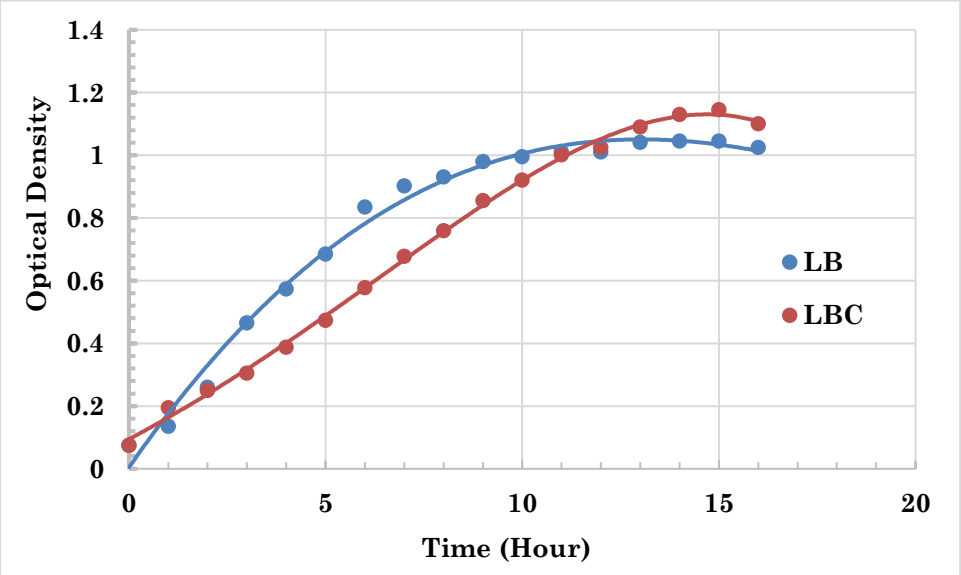


Fig- 4.11: Growth pattern of CRB 7 with (LBC) and without (LB) Cr(VI)

Optimization of Parameters of Growth Medium And Molecular Characterization of the Selected Isolate

5

5.1 Purpose of the Work

Bacterial growth is directly correlated with various abiotic factors of the growth medium. These factors can influence the efficiency of hexavalent chromium removal by bacteria significantly. The optimal conditions for parameters like pH, temperature, salinity, initial chromium concentration and shaking velocity need to be defined in order to improve the potential of removal of hexavalent chromium by a specific bacterial strain under stressful conditions as well as in contaminated environments. Since, enzymatic activity widely depends on some factors like pH, temperature, aerobic/anaerobic environments, optimization of these factors are therefore most crucial for achieving greater efficiency of the bacteria in the remediation process. This phase of work was conducted to optimize different abiotic factors of growth medium for the selected CRB obtained in the previous phase of the study.

The sequence of 16S rRNA genes (rDNA) is considered to be a valuable genetic marker to establish formation of various phylogenetic relationships between different organisms. The conserved characteristics of these molecules along with regions of higher variability, genetic stability, their ubiquitous distribution and functional constancy endows them to be a suitable candidate for these applications. The phylogenetic trees for cluster organisms are based on genetic makeup. The phenotypic characters do not need to be considered. Hence, 16S rDNA was used for this study to identify the isolated CRB.

The guanine and cytosine (G+C) content analyses and proteomic profile of the same isolate were studied. The shape and size of the CRB-1 was studied under Scanning Electron Microscope (SEM) and its changes under the stress of chromium.

The specific objectives of this study can be summarized as to (i) optimize the parameters of growth medium for effective removal of chromium by CRB-1, (ii) identify CRB-1 by 16S rRNA gene analyses, (iii) perform phylogenetic analysis of the CRB-1, (iv) study the G+C content and proteomic profile of the selected isolate, (v) conduct ultra-structural study of CRB-1.

5.2 Methodology

5.2.1 Preparation of Medium

LB media was used as growth medium for the bacteria. The detailed media preparation has been described in Chapter 4 under section 4.2.4.

5.2.2 Optimization of Parameters of Growth Medium for Cr(VI) Removal

Effects of temperature, pH, salinity, initial Cr(VI) concentration and shaking velocity on bacterial growth and Cr(VI) bio-reduction wereexamined using the LB medium amended with Cr(VI) (100 mg/L) for the purpose of optimization of the Cr(VI) reduction efficiency.Cultures were incubated at 25, 30, 37 and 42˚C to explore the effect of temperature. In the experiment that examined the influence of pH, sterilized LB medium was adjusted to pH 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 by addition of predetermined amounts of filter-sterilized 1 M NaOH or 1 M HCl solution. The effect of salt concentration was examined using the same LB medium to which varying concentration of NaCl (1%, 3%, 5%, 7%, 9% and 11%) were added. The effects of different concentrations of Cr(VI) were determined using the medium contaminated with 0, 50, 100, 150, 200, 250, 300, 350 and 400 mg/L of Cr(VI) using aliquots of filter-sterilized $K_2Cr_2O_7$ stock solution. To study the effects of variable shaking velocity, the bacterium was incubated and shaken at 100, 120, 140, 160 and 180 rpm in an orbital shaker-incubator.
Autoclaved LB medium were inoculated with 100 µL of cell suspension from log phase of bacteria culture (OD_{600}=0.87) and aerobically incubated for 24 h in orbital motion shaker (120 rpm) at 37˚C. Initial and final Cr(VI) concentration and biomass (OD_{600}) were determined for each set. All the experiments including controls were conducted in triplicates and average of all data set with standard deviations were presented.

5.2.3 Cr (VI) Analyses and Biomass Determination

Hexavalent chromium content in supernatant was measured by Diphenyl Carbazide method ***(APHA, 1998)*** and the biomass was assessed by measuring absorbance at λ_{600} against a sterile LB blank sample as discussed in the Chapter 4 under section 4.2.10.

5.2.4 Identification of Isolated Strain

DNA of the bacterial strain was extracted using standard methods ***(Sambrook and Russell, 2001)*** and subsequently run in 1% agarose gel (Sigma Aldrich) to check purity

of the extracted DNA. Quantification of the DNA was undertaken using Nanodrop. PCR amplification of the bacterial 16S rDNA region was undertaken using eubacterial primers namely, FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers. PCR product was gel purified and subsequently sequencing of the product was undertaken based on Big Dye Terminator Chemistry in an ABI 3730 Genetic Analyzer. Generated chromatogram was checked for any ambiguity or error before undertaking BLASTn validation (http://blast.ncbi.nlm.nih.gov/). Top ten hits (cultured bacterial 16S rDNA sequences from published sequence databases such as GenBank/EMBL/DDBJ) were noted to identity the bacterial strain.

5.2.5 Phylogenetic Analyses of the Isolates

The molecular phylogenetic tree was analyzed byNeighbor Joining method i.e. clustering the cultured bacterial 16S rDNA sequences with published 16S rDNA sequences of several strains.

5.2.6 Scanning Electron Microscopy (SEM)

Desiccator dried samples were subjected to SEM analysis for high resolution imaging of bacterial surface. A Zeiss EVO-MA 10 Scanning Electron Microscopy operating with a high voltage is used to study the surface morphology of the bacteria. Prior to investigation, the bacterial sample was coated by sputtering with platinum. The SEM images of bacterial isolate were obtained at 15000X magnification ***(Samuel et al., 2013)***.

5.2.7 Estimation of Total Protein by Lowry's Method

Total protein content of the isolated bacteria was analyzed by standard procedure of ***Lowry et al., (1951)***. The protein of the bacterial isolate was extracted from the cells by sonication in PBS buffer (pH 7.4). Amount of total protein was estimated from the standard curve of Bovine Serum Albumin (BSA).

5.2.8 Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis

Protein profile of bacterial cells grown with 100 mg/L Cr(VI) and without chromium was done to compare the inducibility of chromium reductase. Bacterial cells were separated by centrifugation of 1.5 mL overnight culture at 5000 g in a cold centrifuge. 8 μL of denatured bacterial protein were loaded onto an 8% SDS polyacrylamide gel in a Biotech Slab Gel System. The gels were run at 85 V continuously for 4 h at room temperature and

were stained overnight with Coomassie Brilliant Blue. Total proteins of the same samples were estimated according to ***Lowry et al., (1951)***.

5.3 Results and Discussion

5.3.1 Optimization of Different Physico-chemical Parameters

As reduction of Cr(VI) is notably influenced by various physico-chemical parameters, the reduction by CRB-1 was optimized under different parameters viz. pH, temperature, shaking velocity and initial Cr(VI) concentration.

5.3.1.1. Effect of Temperature

The variation in bio-reduction of CRB-1 with temperature has been furnished in the Fig-5.1 and Fig-5.2. It is obvious from these figures that the maximum removal efficiency (92%) was obtained at 37˚C, whereas at temperature higher or lower than 37˚C, it was less than 92%. It can be concluded that 37˚C is the optimum temperature for Cr(VI) reduction. The chromate reduction efficiency is found to be considerably influenced by the loss of viability or metabolic activity of cells at extreme temperature condition (***Mclean and Beveridge, 2000; Kathiravan et al, 2011***).

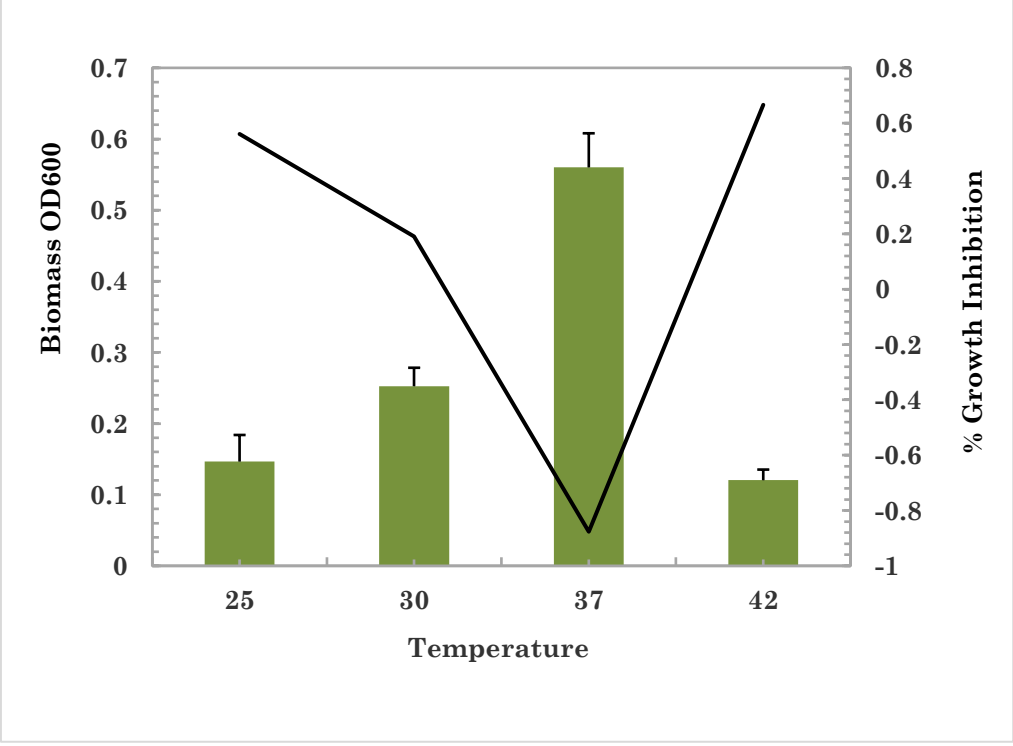


Fig- 5.1: Effect of temperature on growth of biomass of selected strain

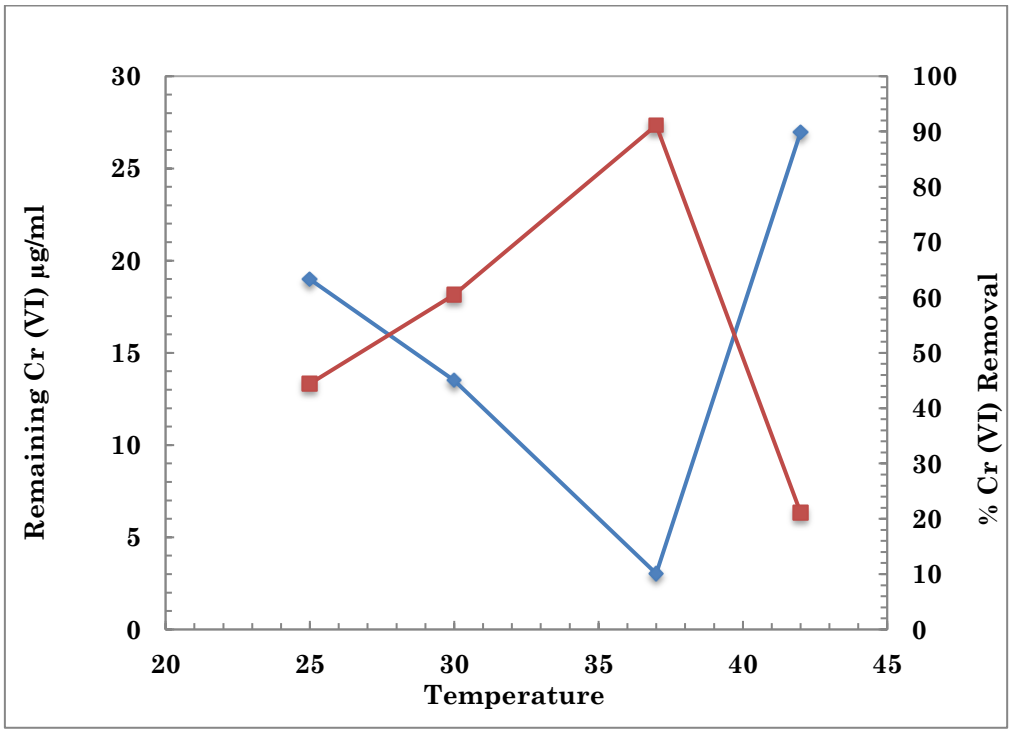


Fig- 5.2: Effect of temperature on removal efficiency of Cr(VI) by the selected bacterial strain

5.3.1.2. Effect of pH

The optimum Cr(VI) reduction by most of the bacterial strains was found in the pH range of 6-8.5 ***(Kathiravan et al., 2010)***. In current study, the bio-removal of Cr(VI) was studied under distinctive H^+ concentration i.e. pH 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 in LB medium containing 100 mg/L of Cr(VI) at 120 rpm and 37˚C. As per Fig-5.3 and Fig-5.4, while increasing the pH from 6 to 9, initially an increase in Cr(VI) removal was observed followed by a decreasing trend. At pH 7.5 the bacterium showed highest Cr(VI) removal. Growth of Cr(VI) resistant bacteria at optimum pH range of 7.0–7.8 was reported by ***Losi and Frankenberger (1994)***, while ***Shakoori et al. (2000)*** reported prevalence of optimum pH of 9 for Cr(VI) removal by gram-positive bacterium. Wide variation in the relationship between pH and Cr(VI) reduction is not unexpected as chromate (CrO_2^{-4}) is the predominant Cr(VI) species in an aqueous environment in the pH range of 6.5 to 9.0 ***(McLean and Beveridge, 2001)***.

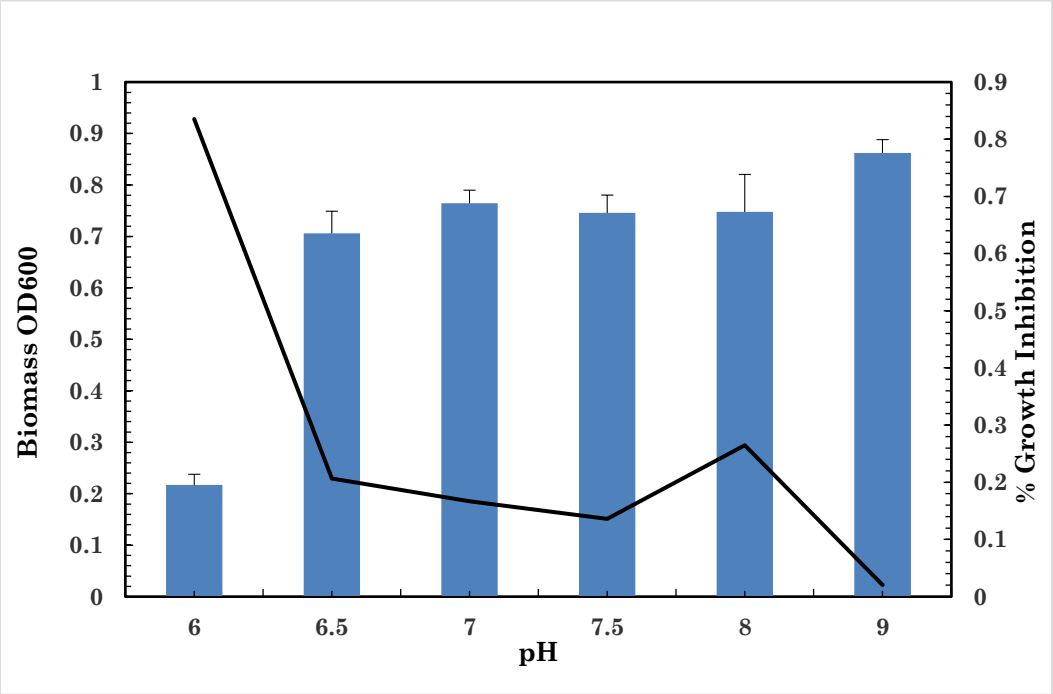


Fig- 5.3: Effect of pH on growth of biomass of selected strain

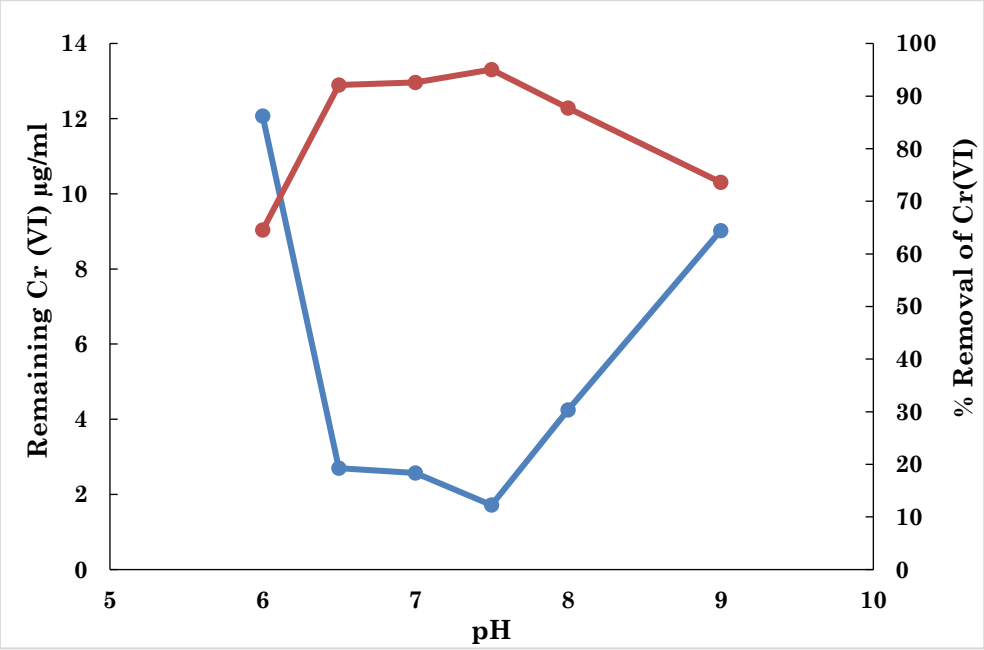


Fig- 5.4: Effect of pH on removal efficiency of Cr(VI) by the selected bacterial strain

5.3.1.3 Effect of Initial Cr(VI) Concentration

Bio-reduction of Cr(VI) by CRB-1 was investigated over theCr(VI) concentration range of 50 to 400 mg/ L. The growth of the bacteria at different initial Cr(VI) concentration is showed in Fig- 5.5. Effect of Cr(VI) concentration on Cr(VI) removal efficiency under constant biomass for a fixed time period is shown in the Fig-5.6.Substantial amount of Cr(VI) was reduced over the entire Cr(VI) concentration range (at 100 mg/L, 92% Cr(VI) reduction as high as 99% was observed at 50 mg/L. However, initial Cr(VI) concentration of 100 mg/L was selected for the studies on optimum removal efficiency.

5.3.1.4 Effect of Salt Concentration

Bacterial biomass development along with Cr(VI) bio-removal at various salt concentrations are shown in Fig- 5.7. With increasing salinity Cr(VI) bio-removal as well as bacterial growth were restricted. Even at 9% and 11% salinity, bacterial growth declined considerably and there was no removal of chromium. Substantial removal of Cr(VI) was noted at 0, 1 and 3 % concentrations of NaCl (Fig- 5.8). However, the isolated bacterium might be treated as facultative halobacterium.

5.3.1.5 Effect of Shaking Velocity

Cr(VI) bio-removal was observed at variable shaking velocity i.e. 100, 120, 140, 160, 180 rpm at pH 7.5 and initial Cr(VI) concentration of 100 mg/L. As per Fig- 5.9 and Fig- 5.10 the optimum shaker speed was obtained as 140 rpm at which 91% Cr(VI) removal was achieved. Cr(VI) removal varied insignificantly between 120 and 140 rpm. Atrelatively lower (100 rpm) or higher (180 rpm) shaker speed the removal efficiency decreased significantly. Similar trends have been reported by ***Das et al. (2014)*** and ***Dhal et al. (2010)*** for *Bacillus sp.*

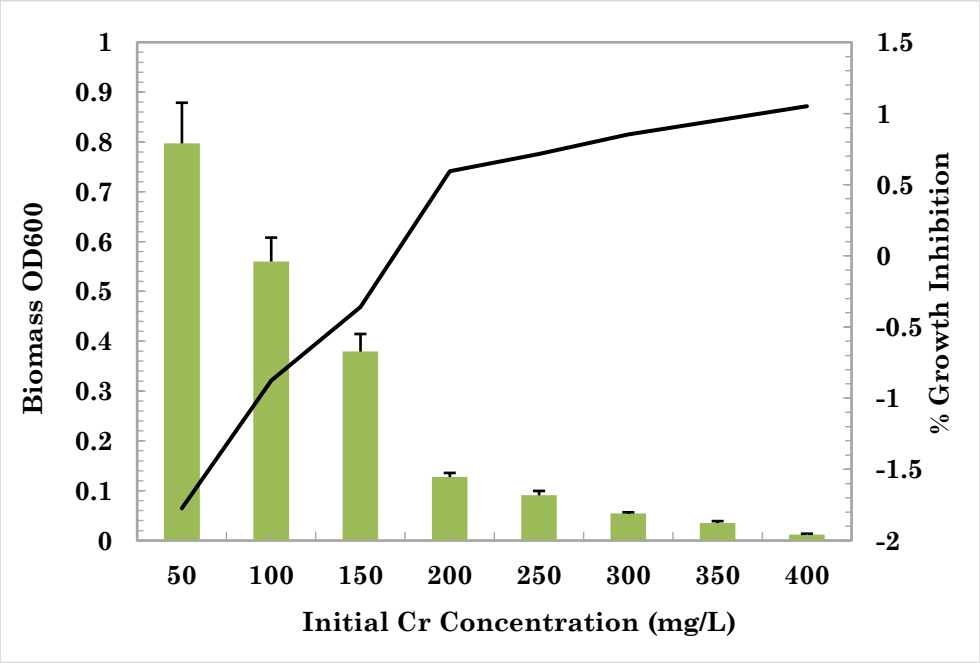


Fig- 5.5: Effect of initial Cr(VI) concentration on growth of biomass of selected strain

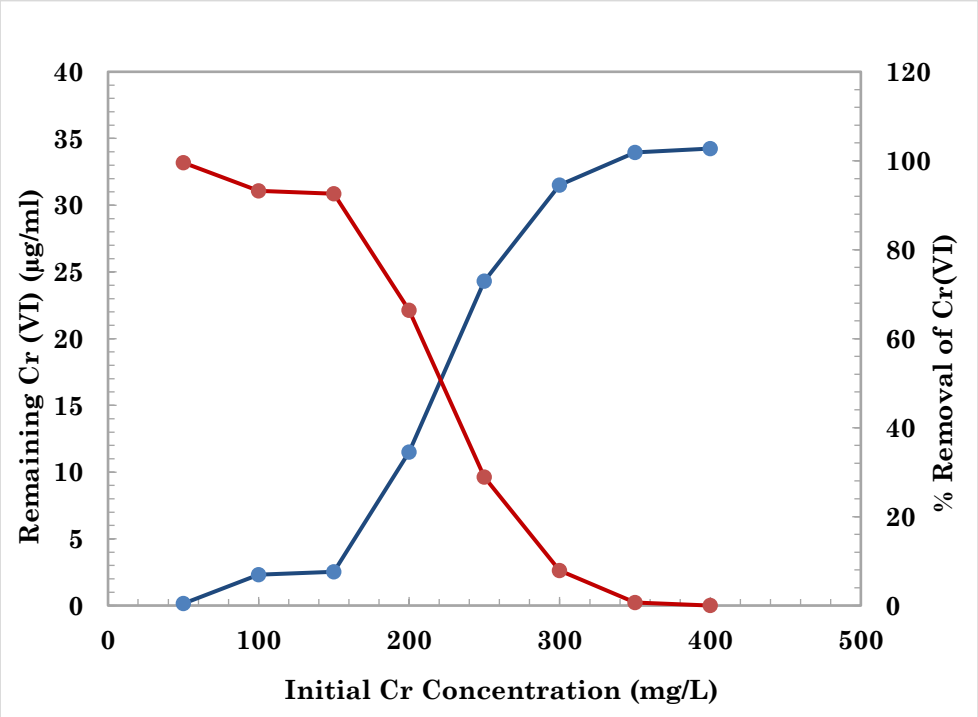


Fig- 5.6: Effect of initial Cr(VI) concentration on removal efficiency of Cr(VI) by the selected bacterial strain

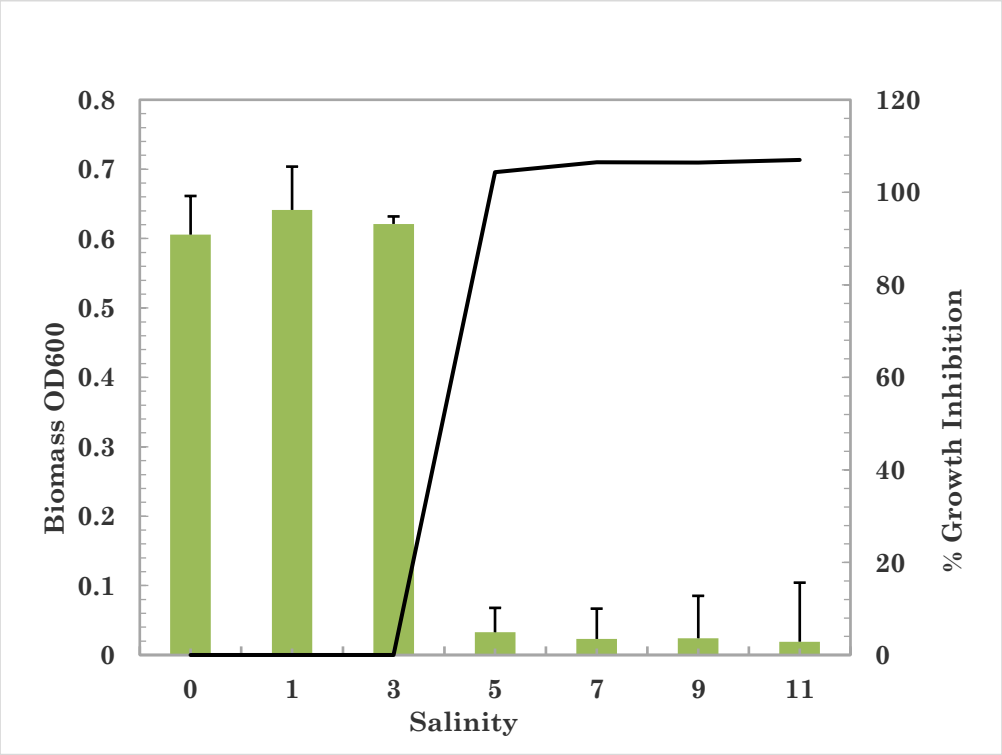


Fig- 5.7: Effect of salinity on growth of biomass of selected strain

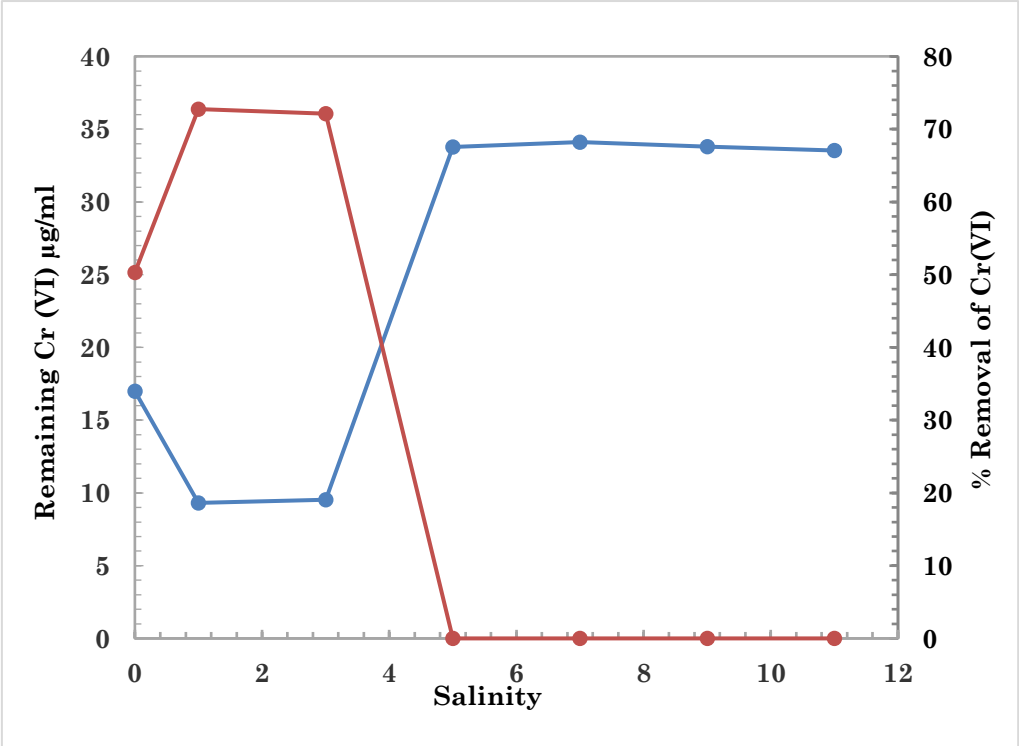


Fig- 5.8: Effect of salinity on removal efficiency of Cr(VI) by the selected bacterial strain

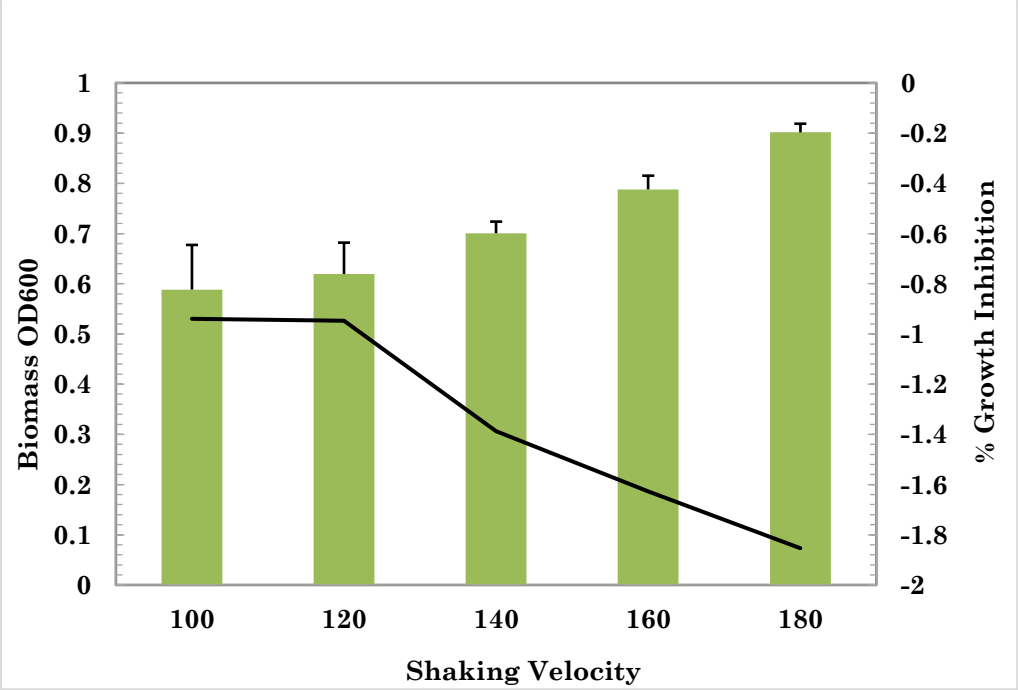


Fig- 5.9: Effect of shaking velocity on growth of biomass of selected strain

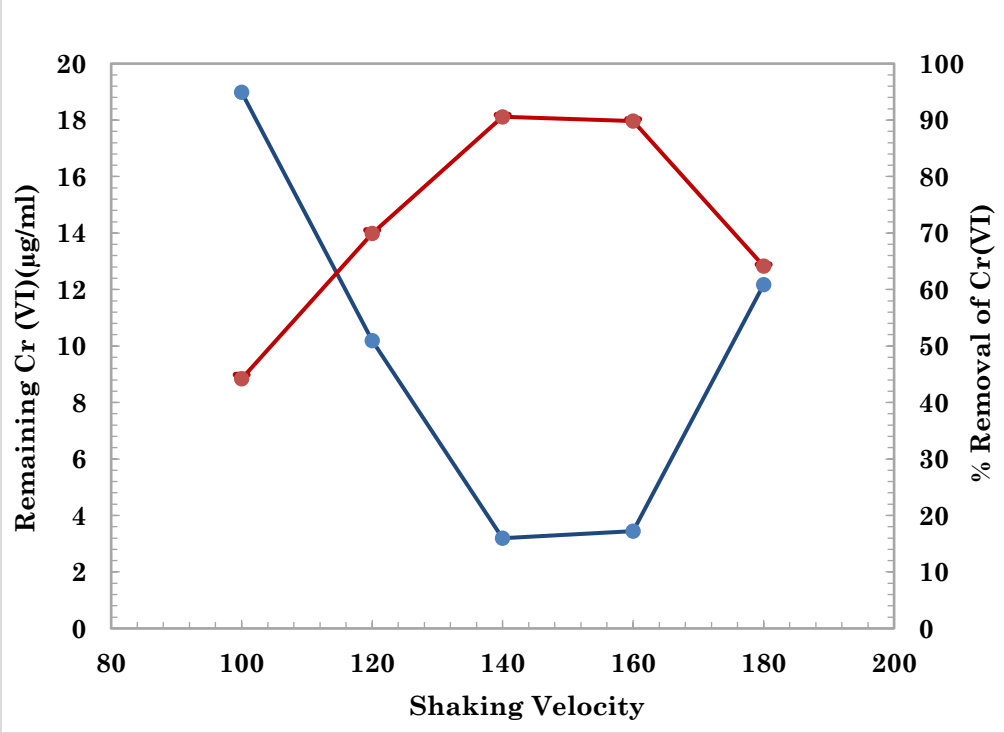


Fig- 5.10: Effect of shaking velocity on removal efficiency of Cr(VI) by the selected bacterial strain

5.3.2 Identification of Chromium Resistant Bacteria

CRB-1 was found to be gram positive, rod-shaped, non-motile and aerobic, heterotrophic soil bacterium. On the basis ofthe results obtained from 16S rRNA gene sequencing method and phylogenetic analysis of CRB-1 (based on the neighbor joining algorithm), the bacterial strain was identified as *Bacillus mycoides* (100% similarities) (Fig.5.11). Continuous search for metal bio-accumulator having efficiency over a wide range of environmental conditions motivates the isolation of CRB ***(M. Narayani & K. Vidya Shetty, 2013)***. Very few reports are available for *B. mycoides* with Cr(VI) removal efficiency. However, understanding the mechanisms behind the Cr(VI) resistance and reduction along with the batch kinetic study of *B. mycoids* may be a novel approach which is so far, rarely explored.

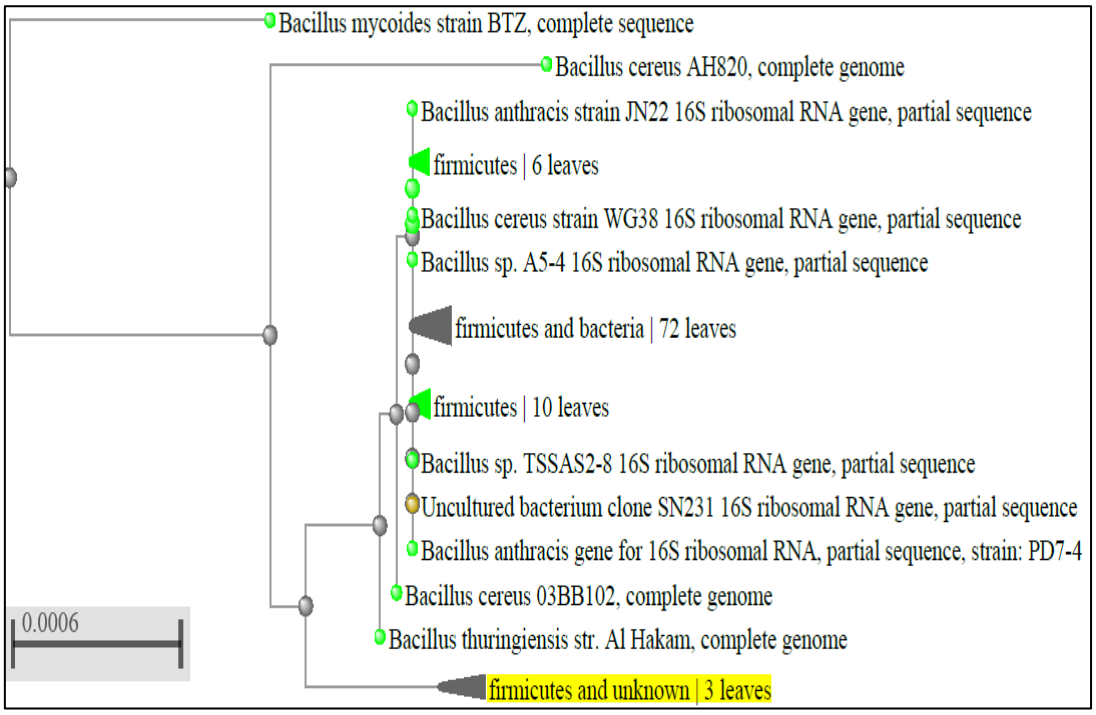


Fig-5.11: Phylogenetic analysis, based on the neighbour joining algorithm, identified the strain CRB 1 to be *Bacillus mycoids* with 100% similarities

5.3.3 Ultra Structure Study of *B. mycoids*

In the present work, the effect of Cr(VI) on morphology of the bacterium was perceived by the scanning electron microscopy. *B. mycoids* cells were grown on LB medium amended with 100 mg/ L of Cr(VI), while cells grown in the absence of Cr(VI) were treated as control.

In the SEM micrograph of *B. mycoids* as shown in Fig 5.12, the bacterium was observed as rod shaped non-motile bacteria while Cr(VI) was not treated(LB). The size of bacteria

was affected due to the stress of chromium. The bacterial cells un-affected by chromium showed plump and smooth bacterial surface. While scanning electron micrographs of Cr(VI) treated bacteria showed the cell surface as shrunk, apparently smooth but elongated cell length (LBC). Average cell length and diameter for the microbial species exposed to untreated sample was 3.28 µm and 0.868 µm respectively. It was observed to be increased to an average cell length of 5.02 µm and average diameter of 0.754 µm for the species exposed to treated sample. Similar observation regarding increase in cell length due to stress induced by chromium was reported for several bacterial species i.e. *E. coli* ***(Samuel et al., 2013)***, *Ochrobactrum tritici* 5bv11 ***(Francisco et al., 2010)***, *E. coli* K-12 ***(Ackereley et al., 2006)***. ***Srivastava and Thakur (2007)*** also stated the morphological differences of *Acinetobacter* sp. grown with or without Cr(VI). After 24 hrs of treatment with Cr(VI), *Acinetobacter* sp. had wrinkled appearance.The molecular characteristics of CRB-1 are listed in Table 5.1.

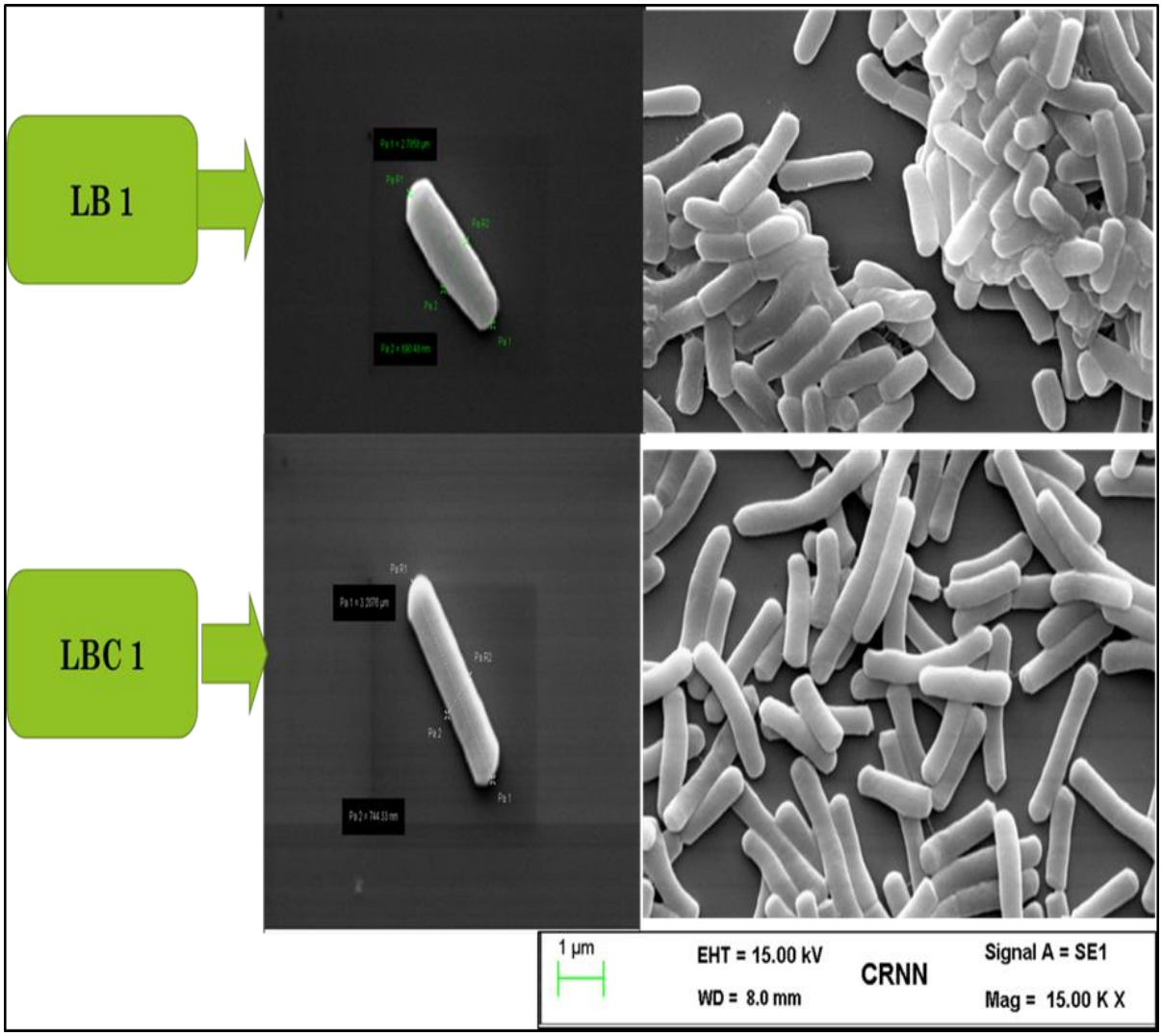


Fig-5.12: Scanning Electron Micrographs of CRB-1 with Chromium (LBC) and without Chromium (LB)

Table-5.1: Molecular Characteristics of CRB-1 (*B. mycoids*)

Organism	CRB 1
Genus	Bacillus
G+C mol content	53.08 % (Fig-5.13)
Size	Rod
Length of rod	Avg 3.28 µm by 0.868 nm (without Cr) Avg 5.02 µm by 0.754 nm (with Cr)
Motility	Non-motile
Halo-tolerant	Moderately Halophilic
Oxygen requirement	Aerobic
Carbon utilization	Heterotrophic

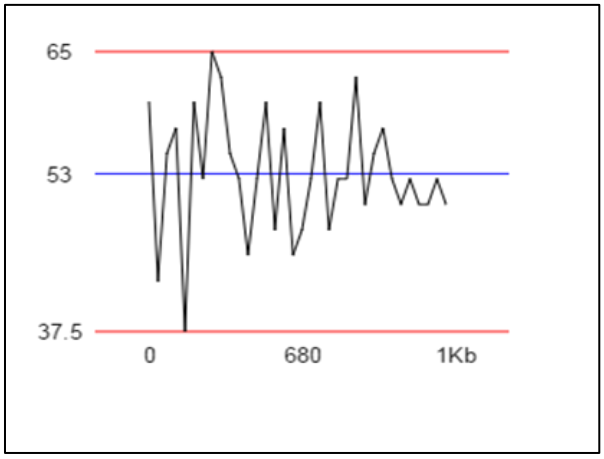


Fig. 5.13: Sequence GC richness: 53.08%

5.3.4 Protein Profile of CRB-1

The total protein of the strain was estimated by the method of ***Lowry et al. (1951)***. Total protein content was calculated for sample without chromium as 10.305 µg/ mg of wet bacterial pellet and as 10.055 µg/ mg of wet bacterial pellet for the sample exposed to 100 mg/L Cr(VI) concentration. It is therefore suggested that no notable variation occur in the process of protein synthesis in the bacterial cell. SDS PAGE profile of proteins of bacterial isolate is shown in Fig-5.14. The isolated bacteria were grown in LB medium with 100 mg/L of chromium and without chromium (as control). Comparison of the protein profiles with chromium and without chromium shows that two proteins of molecular weight around 72 kDa and 26 kDa were induced on SDS-PAGE gel in the presence of chromium in *Bacillus mycoids.*

Similar types of protein induction studies have been conducted by other researchers. A chromate reductase enzyme had been purified from *P. putida* MK1 having a molecular

weight of 20 kDa on SDS-PAGE by ***Park et al. (2000).*** In *P. ambigua* chromate reductase was shown to have a molecular weight of 25 kDa on SDS-PAGE but the native protein was 65 kDa. ***Ganguli and Tripathi (2002)*** reported that in *P. aeruginosa,* a 30 kDa protein was induced in the presence of chromium. ***Thacker and Madamwar (2005)*** also reported similar results in *Ochrobactrum sp.* in presence of chromium.

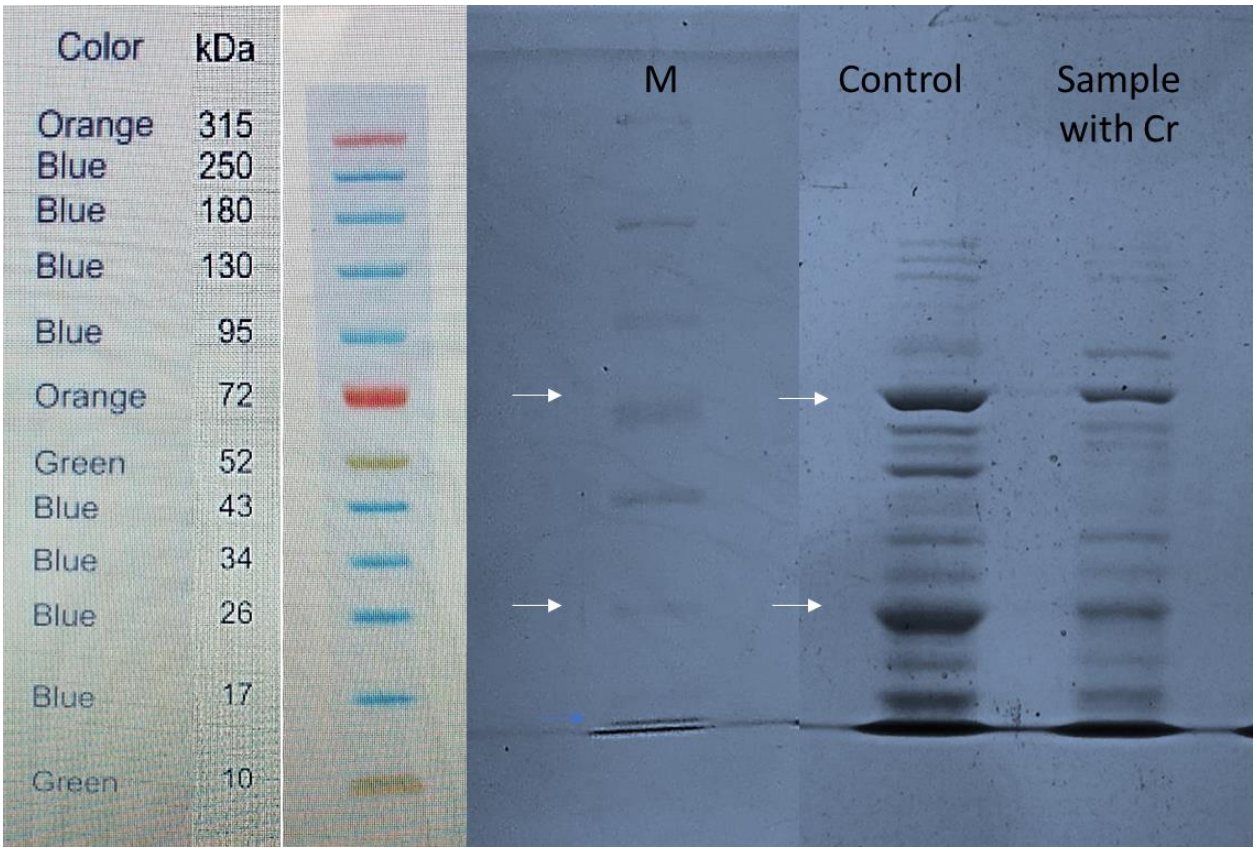


Fig- 5.14: SDS-PAGE gel prepared from cytosolic protein of *B. mycoids* (CRB 1): Lane 1: Protein molecular marker Pure Gene PMT 2922. Lane 2: Strainexposed to 0 mg/L of Cr(VI). Lane 3: Strain exposed to 100 mg/L of Cr(VI).

Characterization of Reduced Product

6.1 Purpose of the Work

This phase of the study elucidates the characterization of reduced product in *B. mycoids* in terms of localization of chromium, possible effect of chromium on surface architecture and interaction of chromium with functional groups present in bacteria.The cells of *B mycoids* associated with the reduced product were characterized by atomic absorption spectrophotometry (AAS), scanning electron microscopy–energy dispersive X-ray (SEM–EDX) and Fourier transform-infrared (FT-IR) spectroscopy.

6.2 Methodology

6.2.1 Preparation of Medium

B. mycoids was grown in LB medium (described under section 4.2.5) at the optimized condition i.e. at pH 7.5, with shaking velocity 140 rpm, salinity 1%, at initial Cr(VI) concentration of 100 mg/L as $K_2Cr_2O_7$ and incubated at temperature 37°C for characterization of reduced products. Bacteria grown without chromium were treated as control.

6.2.2 Atomic Absorption Spectroscopy (AAS) Analyses

The total chromium concentration was quantified by atomic absorption spectroscopy (AAS; A Analyst 100, Perkin Elmer). After incubation for 24 h, the bacterial pellets were separated at 10,000x g in a cold centrifuge (Thermo Sorval RC C Plus). Pellet and supernatant were separately digested with 1:1 HCl followed by filtration and washing with 5% (v/v) HCl (Merck India, AR Grade) ***(Das et al., 2014).*** The total chromium concentration in pellet as well as in the supernatant was detected by Atomic Absorption Spectrophotometry (A Analyst 100 Perkin Elmer) with interfacing AA Win Lab Software using specific hollow cathode lamp in default condition by flame absorption mode (discussed in details under section 4.2.4) ***(Chattopadhyay et al., 2002).***

6.2.3 Scanning Electron Microscopy – Energy Dispersion X-ray (SEM-EDX)

The change in surface elemental composition of the reduced product along with bacterial cells was analyzed using SEM–EDX. Cell suspensions with and without chromium were

collected at 20 h of incubation (OD_{600}= 0.9), washed and fixed on glass slides using 2.5 % glutaraldehyde (Merck India, AR Grade) for 1.5 h. Subsequently, it was washed by a series of dehydration with ethanol (Merck India, AR Grade) and then desiccator dried. With carbon tape, the glass slide was fixed on specimen mount. Platinum sputtering was carried out prior to the observation. The surface elemental composition was observed under SEM–EDX (ZEISS EVO-MA 10). The SEM images and EDX spectra of bacterial isolate was obtained at 15000X magnification ***(Samuel et al, 2013; Das et al, 2014).***

6.2.4 Fourier Transform-Infrared (FT-IR) Spectroscopy

For FT-IR analyses, the bacterial cells were exposed to 100 mg/L of Cr(VI) and incubated for 24 h under optimized conditions. The cells were then separated by centrifugation (Thermo Sorval RC C Plus) at 10,000x g at 4°C for 10 min. The supernatant was separated while the pellets were washed with 1X PBS buffer for several times and then lyophilized (Virtis Freezemobile 6EL). The bacterial cells grown under similar conditions but without Cr(VI), were also separated, lyophilized and kept as controls to be used for comparison. Sample was mixed with 100 mg KBr and was pressed to form a pellet. The tablets obtained were inserted in the instrument and the spectra is recorded with the range of 400 – 4000 cm^{-1} ***(Sannasi et al., 2009, Samuel et al., 2012, Das et al., 2014).*** The FT-IR spectra of dried cells associated with or without reduced product in KBr pellet were recorded using FT-IR (FT-IR 6300, JASCO) spectrophotometer. This study was carried out to reveal the change in the functionalities of the chromium reducing bacteria.

6.3 Results and Discussion

6.3.1 AAS Analyses

Total chromium content of pellet and supernatant of 24h incubated sample was measured in AAS (Fig. 6.1). It is obvious from the figure that the majority of chromium which was spiked in the medium was present in bacterial biomass after incubation. Total chromium of supernatant of incubated culture measured was very low compared to the chromium concentration in pellet. However, AAS measures the total Cr content of the sample which predominantly comprises Cr(III) and Cr(VI). Therefore, it can be assumed that Cr(VI) is immobilized within the cell either in its native oxidation state(Cr(VI)) or in the reduced state.

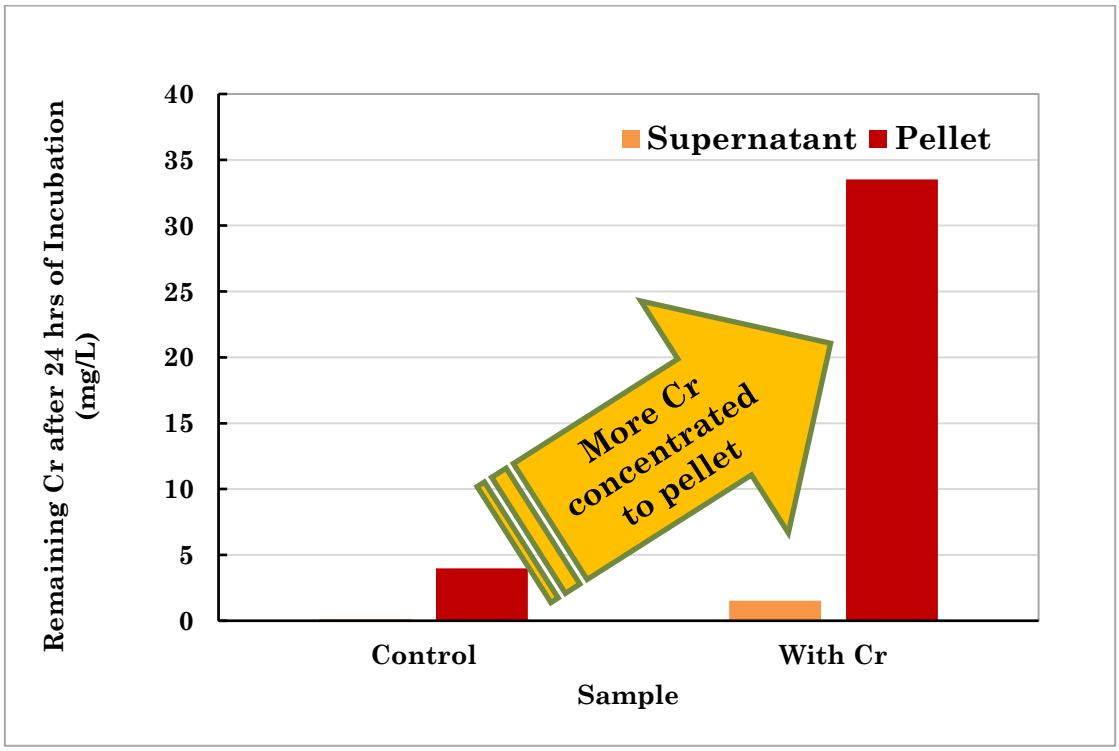
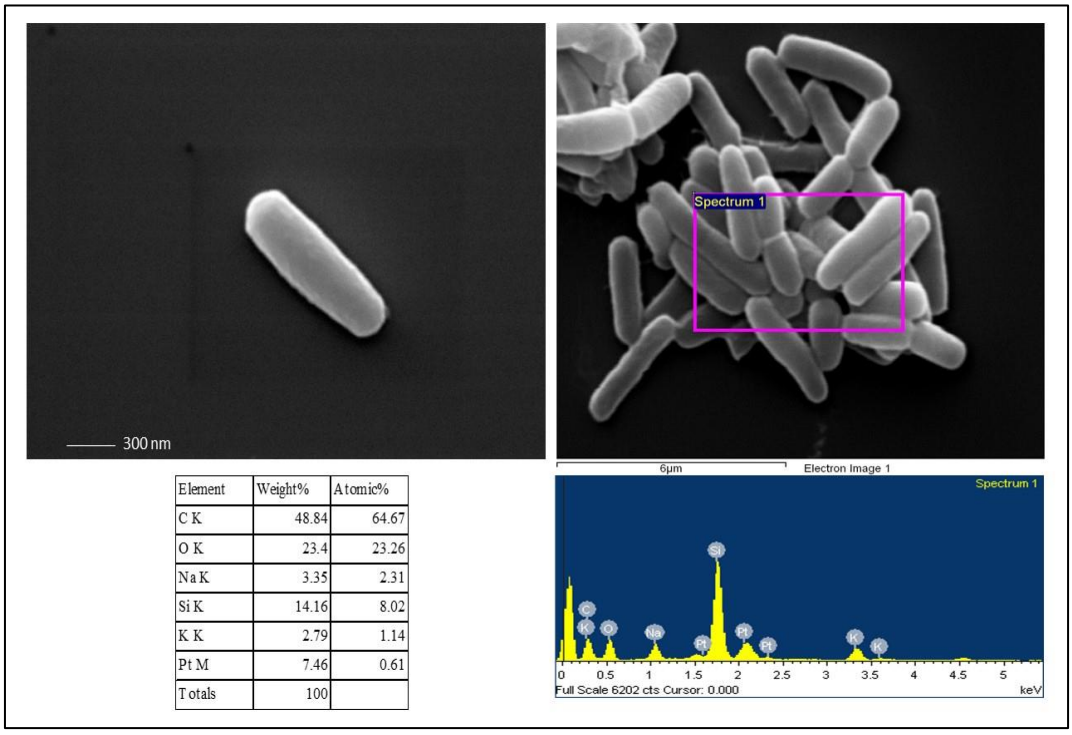


Fig 6.1: Distribution of chromium in pellet and supernatant of bacteria *B. mycoids* after incubation for 24 h at 100 mg/L Cr(VI) concentration

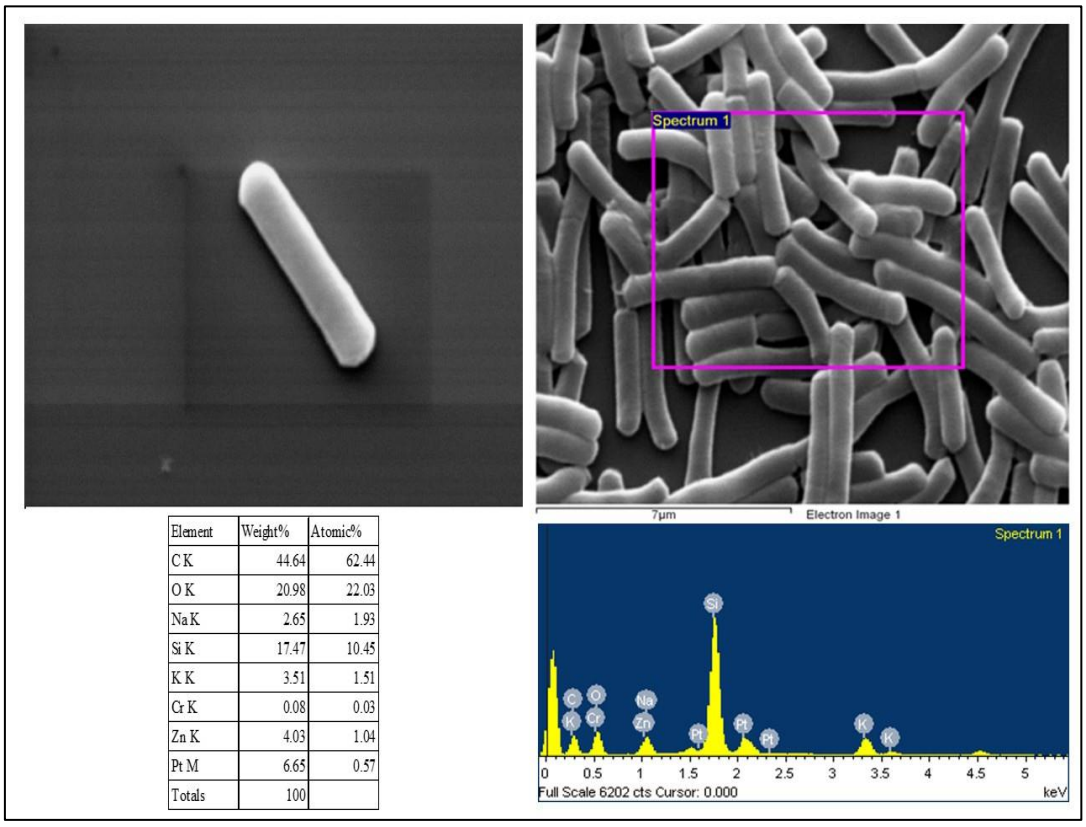
6.3.2 SEM-EDX Study

In order to get further insight into the nature of reduced chromium species, the SEM study of bacterial cell, grown with and without chromium, were undertaken and results obtained are present in Fig.6.2 and 6.3. The changes in shape of bacteria on interaction with chromium were discussed in details under section 5.3.3. EDX study of the treated sample showed presence of chromium in the surface architecture of the bacteria while treated with chromium. Similar investigation for the sample treated without chromium showed no presence of chromium on the surface of bacteria. It is also evident from the SEM investigation that treated sample with Cr(VI) showed long chain length as well as stickiness due to which cells were adjoined. EDX analysis also reveals presence of chromium in the treated sample. This could be due to deposition of Cr(III) hydroxide on the cell surface or due to formation of an intermediate complex of Cr(III) species. Another possible reason could be formation of extracellular polysaccharide to resist the exposure of chromium.



Element	Weight%	Atomic%
C K	48.84	64.67
O K	23.4	23.26
Na K	3.35	2.31
Si K	14.16	8.02
K K	2.79	1.14
Pt M	7.46	0.61
Totals	100	

Fig-6.2: Scanning Electron micrograph and EDX Analysis of *B. mycoids* after 24 h grown in LB medium



Element	Weight%	Atomic%
C K	44.64	62.44
O K	20.98	22.03
Na K	2.65	1.93
Si K	17.47	10.45
K K	3.51	1.51
Cr K	0.08	0.03
Zn K	4.03	1.04
Pt M	6.65	0.57
Totals	100	

Fig-6.3: Scanning Electron micrograph and EDX Analysis of *B. mycoids* after continuous exposure to Cr(VI) for 24 h grown in LB medium

6.3.3 FT-IR Study

The FTIR analysis was carried out to identify the likely interaction between the bacteria and Cr(VI). Bacteria sample grown in LB medium in presence of Cr(VI) was analyzed in the range of 400-4000 cm^{-1} onto KBr-pellet (Fig-6.4). After the interaction of the bacteria with Cr(VI), substantial changes in peak intensity and peak shift were observed and compared to the sample grown without Cr(VI). The observations are tabulated below.

Table-6.1: Peak Analysis of FTIR study for *B. mycoids* grown in LB medium with and without Cr(VI)

Functional Group	Wavelength range (cm^{-1})	Peak observed for bacteria sample		
		Control (without Cr(VI))	Grown with Cr(VI)	Differences
Surface OH stretching	3500- 3000	3309.24	3304.42	-5
Aliphatic CH stretching/ N-H stretch of amine	3000-2900	2955.37	2955.37	-
Aldehyde CH stretching	2830 - 2695	2927.41	2926.44	-1
C=O stretching of carboxylic acid	1740-1710	1730.79	1732.72	+2
Amide I	1685-1665	1651.73	1650.76	-1
Amide II	1560-1510	1540.84	1539.88	-1
COO- stretching	1470-1400	1398.13	1396.21	-2
PO_4- vibrations	1280-1120	1242.89	1249.64	+7
C-N stretching	1220-1020	-	1179.25	
Polysaccharides stretching	1220-950	108.2 1048.12	105.76 1053.90	-1
Cr-O stretching	850-600	668.21	669.17	+1
CH_2 vibrations of polysaccharides and glycogen units		519.72	517.79	-2

Infrared spectroscopy is broadly used for recognizing surface functional groups of microorganisms resulting from proteins, lipids, glycopeptides, polysaccharides, teichoic acid and polyphosphates. The objective of this spectroscopic study was to detect active

chemical groups (centres) of analyzed bacteria. Fig-6.4 demonstrates the peaks around 3309 cm^{-1} resulting from vibration of hydroxyl groups present in water ***(Lin et al., 2006)*** as well as for vibration of hydroxyl and amino groups of bacterial components (e.g. proteins, sugars). The peaks at 2955.37, 2927.41 and 2869 cm^{-1} corresponds to the N-H vibration. These are also characteristic region of vibration for aliphatic C-H stretching and aldehyde C-H stretching respectively ***(Naiya et al., 2015).*** Therefore, important range for evaluation of obtained spectra for microbial sample is 500 –2000 cm^{-1} ***(Ojeda et al., 2008).*** The peaks at 1730.79 -1732.72 cm^{-1} may be attributed to carbonyl group as observed for carboxyl groups in lipids and fatty acids ***(Nichols et al., 1985).*** Intensity and shape of these peak for sample with Cr(VI) corresponds to a change that may occur due to presence of the metal in medium. Intense peak areas at approximately 1650 – 1651.73 cm^{-1} and 1540 cm^{-1} are attributed to the amide groups in proteins, amide I and amide II respectively. Stretching vibrations of C=O in amide I is generated the first peak and bending vibrations of N−H and stretching vibrations of C=N in amide II is generated the second peak. Bending vibrations of N−H and $N-H_2$ from amines can also be attributed to both the peaks ***(Bogusław et al., 2015).*** A minor shift and decrease in the intensity of the amide peaks are noted for microbial sample interacted with chromium. For the same sample the appearance of a new band in this region (1544 cm^{-1}) may be attributed to changes in protein conformation. The peak, localized at 1455 cm^{-1} can be inferred to be due to aliphatic groups of the amide II present in peptide only can and phospholipid of bacterial cell wall ***(Bogusław et al., 2015).*** In this case the peak intensity also differs from control sample which indicates a change in protein conformation due to chromium. The peak noted at 1396-1398 cm^{-1} corresponds to stretching vibrations of COO- in bacterial peptides ***(Kavitha et al., 2011).*** Asymmetric stretching vibrations of phosphate groups were also observed at 1242.89 -1249.64 cm^{-1} corresponding to the phospholipid of cell wall ***(Kamnev et al., 2008).*** A new peak was observed for the sample treated with chromium at 1179.25 cm^{-1}. It may result from C – N stretching of protein. The absence of this peak in control indicates either formation of new protein or change in protein configuration due to chromium stress. There is a broad peak observed in both samples at 1057-1058.72 cm^{-1} corresponding to C−OH, C−C and C−O−C vibrations of polysaccharides ***(Harish et al., 2012).*** A sharp narrow band at 669.17 cm^{-1} was observed in sample treated with chromium, which confirmed the presence of Cr – O tension ***(Gomez-Cuaspud et al., 2010).*** A broad peak was noted for both samples at 517.79-519.72 cm^{-1}, which corresponds to CH_2 vibrations of polysaccharides and glycogen units at the finger print region ***(Samuel et al., 2013, Parikh et al., 2005).***

Thus, it is evident from the FTIR spectra of the microbial samples, that key functional groups are carboxyl, amino and phosphate which are major constituents of gram-positive bacterial cell as well as the main contributors to the formation of metal bonds, in this case Cr – O.

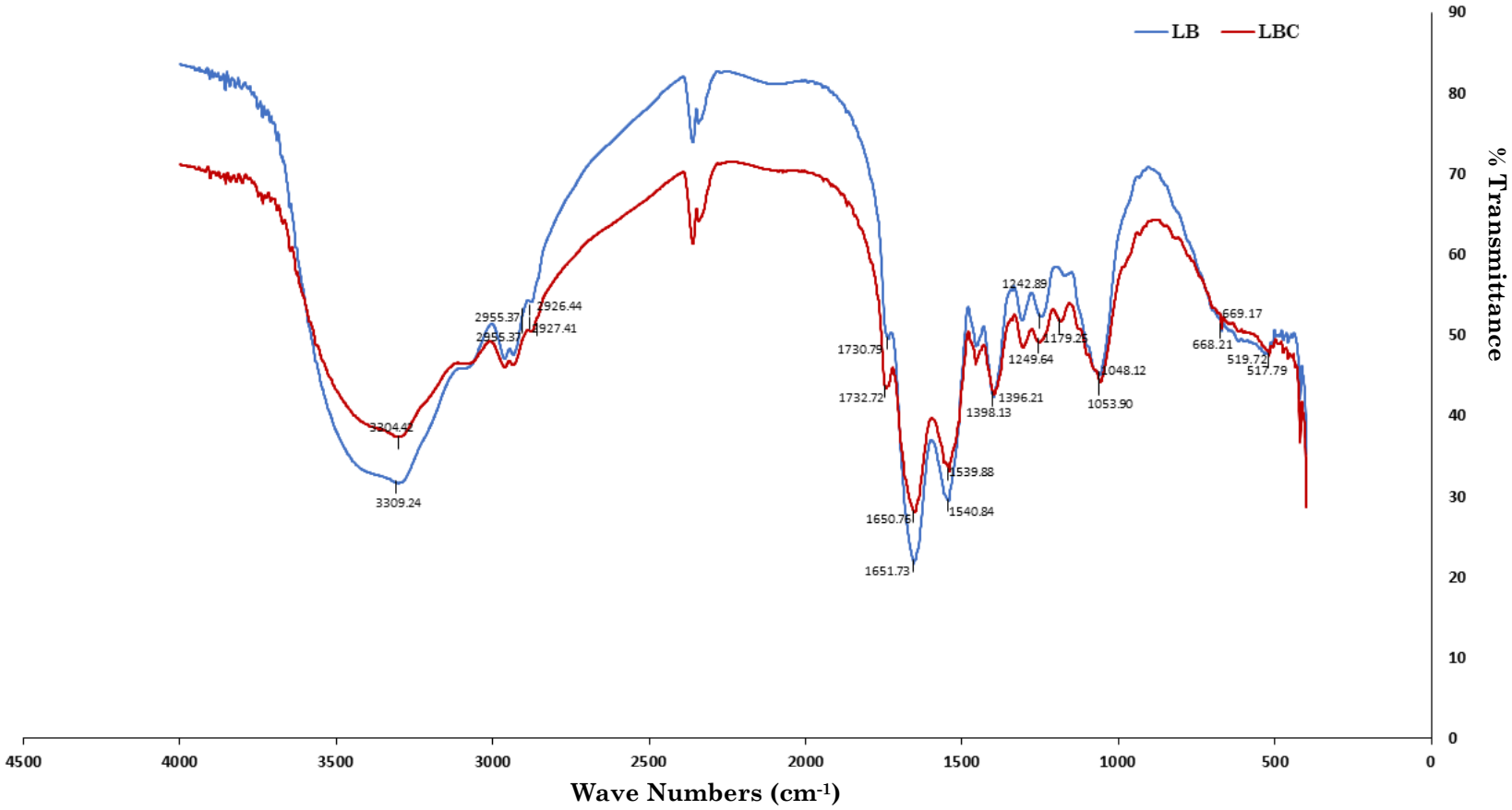


Fig- 6.4: FTIR spectra of reduced product formed by *B. mycoids* with Cr(VI) in LB medium

Understanding of Mechanism behind Chromium Resistance

7

7.1 Purpose of the Work

The properties of chromate resistance and reduction are not necessarily interrelated. Cr(VI) may be reduced by both Cr(VI)-sensitive and resistant bacterial strains.However, not all Cr(VI)-resistant bacteria can reduce Cr(VI) to Cr(III). It is clearly evident from the discussion in Chapter-6 that the isolated and identified bacteria, *B. mycoids* can remove Cr(VI) from the medium. Furthermore, the bacteria while accumulating Cr(VI) inside the cell may reduce it to Cr(III) state. This chapter aims to establish the oxidation states of Cr(VI) and to examine the possible pathways of chromium resistance. Before using a selected microorganism or an indigenous microbial mixed-culture for devising bioremediation strategies for Cr(VI) contaminated soils or wastewater, there is a need to understand how the Cr(VI) resistance mechanism takes place within the microbial system.

When Cr(VI) is reduced to Cr(III) by soluble chromium reductase enzyme through aerobic chromate reduction mechanisms, it produces intermediates like Cr(V) and/ or Cr(IV). These Cr(V) or Cr(IV) are highly reactive and induce the formation of reactive oxygen species (ROS), causing metal stress inside the cell. It has been noted that microbes are able to activate several protective systems such as superoxide dis-mutase (SOD), catalase enzymes and other oxidative stress release enzymes which accompany the metal stress on cell at higher concentration (***He et al., 2011; Ibrahim et al., 2011***). We have investigated the stress response of the isolated microorganisms in the presence of chromium, in this chapter. This study also contributes to the mechanism of chromium reduction of the isolated microbial species. Complex formation of dissolved metal ions with microbial products (exopolysaccharides, EPS) is an attractive and an alternative research in the field of remediation of heavy metals. As reports are available on remediation of subsurface chromium contamination using EPS and enhancement of chromium removal from soil in the presence of EPS, this phenomenon has also been investigated under the purview of the present phase of work.

Evaluation of chromium reduction kinetics refers to the process collectively. Kinetics of this selective phenomenon identifies the factors affecting the reduction process. The present study aims to explore a suitable fit of the reaction order kinetics in the present reduction process.

The summarized scopes of this chapter are– *i) determination of chromium reductase activity ii) determination of enzyme kinetic parameters as well as reaction order iii) determination of stress enzymes responses like SOD activity, catalase activity, reduced glutathione and peroxidase activity and iv) extraction and quantification of extracellular polysaccharide (EPS).*

7.2 Theoretical Consideration

7.2.1 Mechanism of Bacterial Chromium Resistance

The mechanism of bacterial reduction for Cr(VI) varies from strain to strain depending upon their bio-geochemical activities and nutrient utilization patterns which directly affect the resistance/tolerance to chromate ***(Chirwa and Wang, 2001; Zhang and Li, 2011)***. The various modes of resistance in bacterial cells which overcome the Cr(VI) stress includes expulsion or reduced uptake of Cr(VI) compounds across the transmembrane sulphate shuttle present in membranes, biosorption and regulation of genes related to oxidative stress response ***(Brown et al., 2006; Ramírez-Díaz et al., 2008).*** There are several mechanisms behind the bacterial resistance to chromate (Fig- 7.1).

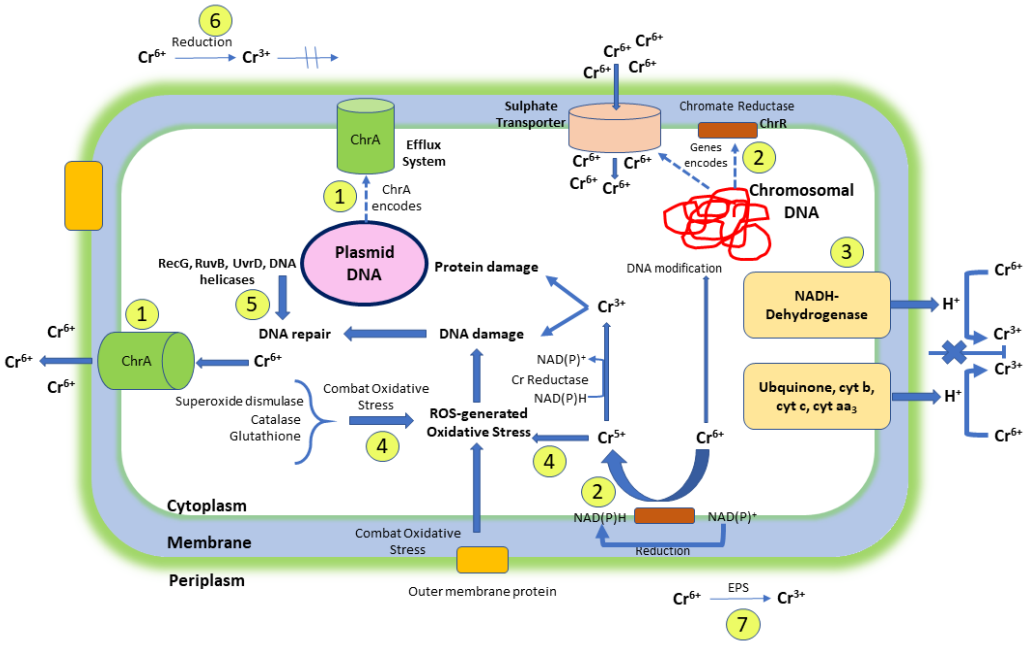


Fig.-7.1: A schematic representation of Cr(VI) resistance in bacterial cell: (1) Due to structural analogue with sulphate ions(SO_4^{2-}), chromate (CrO_4^{2-}) enters the cell through sulphate transporter. Plasmid DNA encode Chr proteins which activate chromate efflux pump system and intracellular chromate ions are expelled from cytoplasm; (2) Aerobic enzymatic reduction of intracellular Cr^{6+} to Cr^{3+} using NAD(P)H as electron donor; (3) Anaerobic enzymatic reduction of Cr^{6+} to Cr^{3+}mediated by soluble or membrane bound enzymes; (4) Protective mechanism by producing SOD, glutathione, catalase enzymes to combat ROS generated oxidative stress caused by Cr^{6+}; (5) DNA repair mechanism; (6) Extracellular reduction of Cr^{6+}; (7) Resistance of Cr^{6+} due to EPS.(modified from ***Martha et al., 2008; Ahmed M, 2014***).

7.2.1.1 Efflux System

Chromate enters cells as a structural analogue of sulphate (SO_4^{2-}) through sulphate uptake systems. If the bacteria contain intracellular chromate reductase, Cr(VI) is reduced to Cr(III). In absence of reductase, accumulated Cr(VI) inside the cell induces the Chr operon, activating the chromate efflux pump which is encoded by ChrA. Thus, the bacterial cell protects itself from Cr(VI) toxicity as it is repelled outside the cell. The Chr protein family in bacteria is encoded by the plasmid vector and/or chromosomal DNA ***(Juhnke et al., 2002).***

7.2.1.2 Reduction of Cr(VI)

Bacteria reduce Cr(VI) either aerobically or anaerobically depending on the microbial species ***(Cervantes et al., 2007).*** Microbial Cr(VI) reduction is divided into two categories: (i) Direct Cr(VI) reduction and (ii) indirect Cr(VI) reduction.

Direct Cr(VI) Reduction

Aerobic Cr(VI) Reduction

As shown in Fig- 7.2, aerobic bacterial Cr(VI) reduction occurs as a two or three stage process. Cr(VI) is initially reduced to the unstable intermediates like Cr(V) and/or Cr(IV) before they are further reduced to the eventual thermodynamically stable end product, Cr(III). Cr(V) goes through a one-electron redox cycle to generate Cr(VI) by transferring electron to oxygen.

$$Cr^{6+} + e^- \longrightarrow Cr^{5+}$$

$$Cr^{5+} + 2e^- \longrightarrow Cr^{3+}$$

This process produces ROS that combines readily with DNA–protein complexes. However, it is still unclear whether reduction of Cr(V) to Cr(IV) and that of Cr(IV) to Cr(III) is a spontaneous process or enzyme mediated ***(Cheung and Gu, 2007).*** Electrons, NADH and NADPH from the existing reserve are identified as the electron donors in aerobic Cr(VI) reduction process. Reductases (viz., ChrR, YieF and Tkw3) reduce the Cr(VI) derivatives by shuttling electrons and forming Cr(III) (***Qamar et al., 2011***). Aerobic Cr(VI) reduction is generally prevalent in soluble proteins which utilize NADH as an electron donor, either to enhance activity or as a requirement (***Elangovan et al., 2006; Singh et al., 2011***).

Anaerobic Cr(VI) Reduction

In anaerobic conditions, Cr(VI) can act as a terminal electron acceptor within the respiratory chain for a wide array of electron donors, including proteins, carbohydrates, fats, hydrogen, NAD(P)H and existing electron reserves (Fig- 7.2). Both the soluble and membrane-associated enzymes have been able to mediated Cr(VI) reduction activity in anaerobic conditions (***Cheung and Gu, 2007***).

$$CrO_4^{2-}(aq) + 8H^+(aq) + 3e^- \longrightarrow Cr^{3+}(aq) + H_2O$$
$$Cr^{3+}(aq) + 4\,H_2O \longrightarrow Cr(OH)_{3(S)} + 3H^+ + H_2O$$
$$C_6H_{12}O_6 + 8\,CrO_4^{2-}(aq) + 16\,H_2O \longrightarrow 8\,Cr(OH)_{3(S)} + 10\,OH^-(aq) + 6HCO^-(aq)$$

The Cr(VI)-reducing activities of anaerobes are different than in aerobes, as they are associated with the electron transfer systems which ubiquitously catalyse the electron shuttle through the respiratory chain. It is also observed that the cytochrome family (e.g., cytochrome b and c) is highly involved in anaerobic Cr(VI) reduction using enzymes (***Mangaiyarkarasi et al., 2011***).

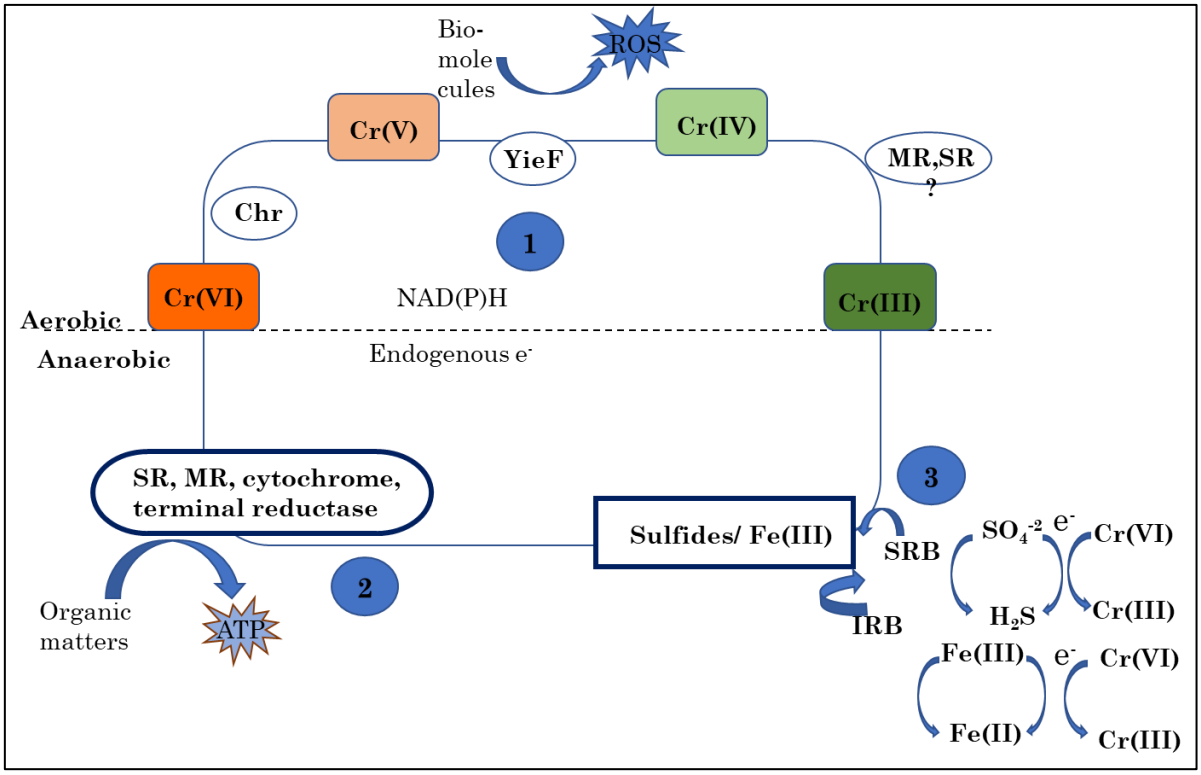


Fig- 7.2: A schematic on Mechanism of Chromate Reduction in bacteria cell: (1) Direct Aerobic Cr(VI) reduction by soluble enzymes using NAD(P)H as electron donor. (2) Direct Anaerobic Cr(VI) reduction mediated by both soluble and membrane associated enzymes. (3) Indirect Cr(VI) reduction by Sulphur reducing bacteria(SRB) or Iron reducing bacteria (IRB) (modified from ***Cheung and Gu, 2007***).

Indirect Cr(VI) Reduction via Iron- and Sulphate-Reducing Bacteria

In the microbial community, sulphate- and iron-reducing bacteria (SRB and IRB) are the prominent members. They have attracted considerable economic, environmental and biotechnological interest. Reduction of Cr(VI) by Fe(II) and sulphides which are generated by IRB and SRB is 100 times faster than if CRB is used alone. As shown in Fig- 7.2, SRB produces H_2S, which acts as a Cr(VI) reductant involving three stages: (1) reduction of sulphates, (2) reduction of chromate by sulphides and (3) precipitation of Cr(VI) by sulphide. Reduction of Cr(VI) by Fe (II) happens when IRB has reduced Fe(III) to Fe(II), this in turn reduces Cr(VI) to Cr(III) (Fig.7.2) (***Viti and Giovannetti, 2007***).

Extracellular Cr(VI) Reduction

Extracellular Cr(VI) reduction is another resistance mechanism (***Ngwenya and Chirwa***, ***2011***). Reduced Cr(III) binds to the functional groups on bacterial cell surface like peptidoglycan components for easy removal from the contamination (***Hoyle and Beveridge, 1983***). Some species of bacteria are able to absorb which help the elimination of metal ions from aquatic environment. Carboxyl, phosphate, amine, sulfhydryl and hydroxyl groups on the cell surface are mainly attributed these properties to the microbial cells (***Parmar et al., 2000***).

7.2.1.3 Protection against Oxidative Stress

When Cr(VI) reduce to Cr(III) highly reactive intermediate Cr(V) radical is produced as intermediates with a short life and generate redox cycles. In this process, Cr(V) is oxidized again to Cr(VI), releasing its electron to dioxygen and causing generation of reactive oxygen species (ROS).

$$\mathbf{Cr(V) + H_2O_2 \longrightarrow Cr(VI) + \cdot OH + OH^-}$$

ROS, thus generated, results in oxidative stress inside the bacterial cell. The defense mechanism against oxidative stress is an additional mechanism of chromate resistance (***Ramirez-Diaz et al., 2008***). However, the oxidative stress causing due to the ROS are scavenged to a large amount by detoxifying enzymes like super-oxide dismutase (SOD), catalase, glutathione transferase etc. (***Ackerley et al., 2004b***).

7.2.1.4 DNA Repair

When Cr(VI) enters into the bacterial cell and readily reduced to Cr(III) in presence of various enzymatic or non-enzymatic activities leads to generation of ROS. ROS species exerts very harmful effects on protein and DNA in both cytosol as well as nucleus. The ROS generated damages to DNA, like single-strand breaks, base modification, double-strand breaks can be repaired by SOS response enzymes (RecA, RecG, RuvB) which is a

superior DNA repair technique (***Hu et al., 2005, Zhitkovich, 2011***). This is another defence shield in bacterial cell against Cr(VI) toxicity.

7.2.2 Enzyme Kinetics

Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzymes. Enzymes are usually protein molecules that manipulate other molecules—the enzyme's substrates. These target molecules bind to an enzyme's active site and are transformed into products through a series of steps known as the enzymatic mechanism.

E + S ⇄ ES ⇄ ES* ⇄ EP ⇄ E + P

These mechanisms can be divided into single-substrate and multiple-substrate mechanisms.

Michaelis–Menten kinetics

Michaelis–Menten kinetics is one of the best-known models of for single substrate mechanism.In the chemical reaction mechanism with enzyme catalysis the enzyme (E) binds substrate (S) to produce product (P).

$$S \longrightarrow P \qquad \text{..... Spontaneous}$$

$$E + S \underset{\text{Binding}}{\rightleftharpoons} E\,S \xrightarrow[\text{Catalysis}]{} E + P \qquad \text{..... Catalysis}$$

Theequation describing the rate of enzymatic reactionsaccording to **Michaelis–Menten kinetics** is given by:

$$\boldsymbol{v} = \frac{\boldsymbol{d[P]}}{\boldsymbol{dt}} = \frac{\boldsymbol{V_{max}\,[S]}}{\boldsymbol{K_m + [S]}} \text{..... 7.1}$$

Where, v = reaction rate

[P] = rate of formation of product

[S] = concentration of substrate

V_{max} = *maximum rate achieved by the system*

K_m = Michaelis Constant orSubstrate concentration at which the reaction rate is half of the V_{max}

Equ. (7.1) can be rewrite as

$$\frac{\boldsymbol{1}}{\boldsymbol{v}} = \frac{\boldsymbol{K_m}}{\boldsymbol{v_{max}}}\frac{\boldsymbol{1}}{\boldsymbol{[S]}} + \frac{\boldsymbol{1}}{\boldsymbol{v_{max}}} \text{..... 7.2}$$

For determination of kinetic parameters of the above equation a series of enzyme is to be run at varying substrate concentration and the data generated may be used for estimating the initial reaction rate. 'Initial' here implies that the reaction rate is measured after a relatively short time period, during which it is assumed that the enzyme-substrate complex has been formed, but that the substrate concentration held approximately constant, and so the equilibrium or quasi-steady-state approximation remain valid.
By plotting rate (v) along the Y axis and concentration along the X-axis using nonlinear regression of Michaelis–Menten equation (equ. 7.2), the parameters can be calculated. A number of graphical systems relating to the linearization of the Michaelis–Menten equation is used which includes the Lineweaver–Burkplot, Hanes–Woolf plot and Eadie–Hofstee diagram.

7.2.3 Reaction Kinetics Order Model

The reaction order can be determined from the experimental data, using either the differential rate law or the integrated rate law. Often, the exponents in the rate law are the positive integers: 1 and 2 or even 0. Thus the reactions are zeroth, first, or second order in each reactant. The common patterns used to identify the reaction order are described underneath; where characteristic types of differential and integrated rate laws and method of determining the reaction order from experimental data has been focused.

Zero Order Reaction
For a zeroth-order reaction is rate is independent of concentration.

$$\mathbf{[A] = [A_0] - kt}$$

First Order Reaction
A first-order reaction is one where the reaction rate is directly proportional to the concentration of one of the reactants.

$[A] = [A_0]\, e^{-kt}$
$\mathbf{\ln [A] = \ln [A_0] - kt}$

Second Order Reaction
The second-order reaction is one whose rate is proportional to the square of the concentration of one reactant.

$$\frac{\mathbf{1}}{[\boldsymbol{A}]} = \frac{\mathbf{1}}{\boldsymbol{A_0}} + \boldsymbol{kt}$$

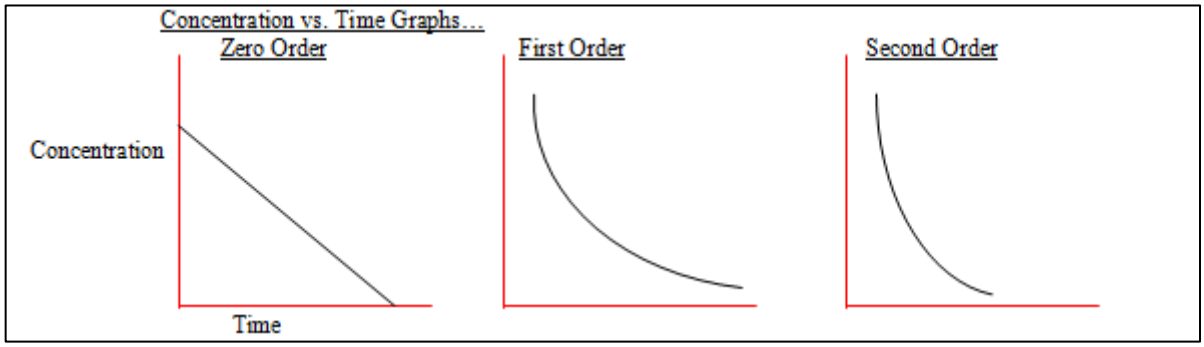


Fig- 7.3: Comparison between concentration vs. Time graphs of zero, first and second order reaction

7.3 Methodology

7.3.1 Cr(VI) Reduction

Sterile LB medium aseptically contaminated with Cr(VI) was inoculated with a cell suspension (OD=0.9) of *B. mycoids* and incubated for 18 h at optimum temperature 37°C, pH 7.5, salinity 1% and at 140 rpm in an orbital shaker incubator. Deionized water (18 MΩ) obtained from a Millipore water purification unit (RiOs DI 3) was used for media preparation and throughout the entire course of experiment this water was used for the preparation of the samples, reagents and for obtaining required dilution.

7.3.2 Cell-Free Extract (CFE) Preparation

Activity of chromate reductase in crude enzyme sample was measured as described by ***Sarangi et al, (2008)*** with modification. Cells were extracted from cultures grown for 18 h (OD=0.9) in LB medium containing $K_2Cr_2O_7$(0, 0.32 and 0.67 mM Cr(VI))and resuspended in 0.05 M potassium phosphate buffer at pH 7.4. The suspension was sonicated (B-Braun Melsungen AG Model Sonicator) for lysis of cells (40s on and 40s off for 5 mins, followed by centrifugation (Thermo Sorval RC C Plus) at 12000 rpm for 30 mins at 4˚C. The clear cell free extract was collected and used as crude enzyme sample for reductase assay.

7.3.3 Chromate Reductase Enzyme Assay

Chromate reductase activity was determined by measuring the decrease in hexavalent chromium concentration using DPC method in presence of an electron donor. NADH and NADPH both were used as electron donors. Each mL of the assay mixture contained 0.1 mL crude enzyme, 0.1 mM NADH/NADP(H) and 20 µM Cr(VI) in 50 mM potassium

phosphate buffer of pH 7.4. Reaction mixtures having similar composition mentioned above excepting enzyme or NADP/NADP(H) were used as respective controls. The assay mixtures were incubated at 37°C for 30 min. The residual Cr(VI) in the reaction mixture was estimated. One unit of chromate reductase activity is defined as the amount of enzyme that reduced one nanomole of Cr(VI) per min under the assay.

7.3.4 Estimation of Hexavalent Chromium

Hexavalent chromium content in aqueous solution was measured by Diphenyl Carbazide method (APHA, 1998) discussed in the Chapter-4 under section 4.2.10.

7.3.5 Time course of Hexavalent Chromium Bio-removal

A time course study for the bio-removal of hexavalent chromium from synthetic medium was conducted with LB media. Bacteria were grown in sterile LB medium as described in section 7.3.1. A final concentration of Cr(VI) was set to 3.5 µg/mL.Cultures were taken out at different time intervals (0, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 h) for estimation of remaining Cr(VI). Biomass concentration and final pH were also determined.

7.3.6 Determination of Kinetic Parameters

Data pertaining to the study of observing the effect of the substrate concentration on bio-reduction of Cr(VI) was used to determine the enzyme kinetic parameters. The sterile LB media was contaminated with a range of Cr(VI) concentration i.e. 5, 10, 15, 20, 25, 30, 35 and 40 mg/L as $K_2Cr_2O_7$. Sterile Cr(VI) contaminated LB medium with 100 µL of *B. mycoids* (OD_{600} = 0.81) was incubated aerobically at 37 °C for 18 h with orbital shaking (140 rpm). The maximum velocity (V_{max}) and the half saturation constant (K_m) were calculated from the available data.

7.3.7 Stress Enzyme Assay

Different stress enzymes assay was conducted for *B. mycoids* at Cr(VI) concentration of 0, 25, 50, 75, 100 mg/L as $K_2Cr_2O_7$ in LB medium. The methods for respective enzymes are stated below.

7.3.7.1 Estimation of SOD Activity

Estimation of SOD Activity was done by the method followed by ***Marklund and Marklund (1974).*** 0.1 mL of cell suspension was prepared with 50 µL of cold absolute ethanol and 30 µL of chloroform for complete lysis of bacterial cells. After 20 min of

continuous shaking, the suspension was centrifuged at 13,000 rpm and 4°C for 20 min. The cell free extract thus obtained was used for the assay. The reaction mixture for auto-oxidation comprised of 0.5 mL of 2 mM pyrogallol, 2 mL Tris–HCl buffer (pH 8.2), aliquots of the enzyme extract and water to give a final volume of 5 mL.With 2 ml of Tris–HCl buffer (pH=8.2) and 2.5 mL of deionized water the blank sample was prepared. The color developed in the sample was measured at 470nm in UV–VIS double beam spectrophotometer (Lamda-25, Perkin-Elmer, USA).The enzyme activity was expressed in terms of unit/ mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

7.3.7.2 Estimation of Catalase Activity

Bacteria pellets were homogenized by sonication in 0.05 M potassium phosphate buffer (pH=7.4) for complete lysis and to obtain a clear solution or extracts. Samples were subsequently centrifuged at 12,000 rpm for 30 min at 4°C. Enzyme assay was determined according to ***Aebi (1984)*** with the help of a UV-VIS spectrophotometer (Lamda-25, Perkin-Elmer, USA).

7.3.7.3 Estimation of Peroxidase Activity

The activity of peroxidase enzyme was determined as per the method followed by ***Chance and Maehly (1955)*** with slight modifications. The cell free extract was prepared following the method described under section 7.3.7.1. The enzyme activity was assayed spectrophotometrically (Lamda-25, Perkin-Elmer, USA). The absorbances were recorded at 420 nm at time intervals of 0, 30, 60, 90, 120, 150 and 180 s after incubation. Specific activity was expressed in terms of change in optical density at 420 nm per min per mg of protein.

7.3.7.4 Estimation of Glutathione Reductase

The assay of glutathione reductase was conducted by the method followed of ***David and Richard (1983).*** The cell free extract was prepared according to section 7.3.7.1. 1 ml of Potassium buffer (0.12M, pH 7.2), 0.1ml of Sodium azide, 0.1ml of EDTA and 0.1 ml of oxidized glutathione were added to 0.1 ml of crude enzyme extract. The final volume was made up to 2.0 ml with deionized water. Afterwards, the reaction mixture was kept at room temperature for three minutes. 0.1ml of NADPH was added subsequently and the absorbance was noted at 340nm at intervals of 15 seconds for 2 to 3 minutes.One unit of glutathione reductase is stated as μM of NADPH oxidized per minute per gram of protein.

7.3.7.5 Estimation of Total Protein (Lowry et al., 1951)

Total protein in cytosol of the bacteria was estimated by the method followed by ***Lowry et al. (1951)*** for protein estimation using folin-phenol solution discussed in details in chapter-5 under section 5.2.7.

7.3.8 Extraction and Quantification of Exopolysaccharides (EPS)

The isolate was inoculated into 100 ml of LB medium to identify the production of EPS and was allowed to grow for 24 h at 37°C with an orbital shaking at 180 rpm. The culture was then centrifuged at 10,000×g for 20 min and the supernatant was collected. An equal volume of ice-cold ethanol was added to the collected supernatant and was kept overnight at 4°C to precipitate the EPS. The mixture was subsequently centrifuged at 10,000×g for 30 min to obtain the pellet. The pellet was then washed with ethanol for several times and was dialyzed against de-ionized water.

7.3.9 Quantification of EPS at Different Chromium Concentration

The micobial species, *B. mycoids* was subjected to grow into 100 ml of LB medium at varying Cr(VI) concentrations (0, 50 and 100 mg/L). The EPS was then extracted after 24 h and quantified using phenol–sulphuric acid method ***(Dubois et al., 1956).***

7.3.10 FT-IR analysis

The pellets of EPS obtained from the process described in section 7.3.9 with and without Cr(VI) were analysed by FT-IR spectroscopy to identify the possible metal-EPS interactions. The sample for FT-IR was prepared according to the method discussed under section 6.2.4. However, in this case the sample was washed with DI water instead of 1X PBS buffer.

7.4 Results and Discussion

7.4.1 Enzymatic Cr(VI) reduction

Chromate reductase enzyme assay was conducted using clear CFE as the enzyme source. The strain showed reductase activity under variable chromium concentrations (0, 0.32 and 0.67 mM Cr(VI)). NADH and NADPH played the role of electron donors. The reductase activity was very low in absence of NADPH, while on addition of NADPH to CFE the activity increased by 9.17 folds and 13.07 folds when Cr(VI) concentration was 0.32 mM and 0.67 mM respectively. But, there was no influence on reductase activity with

NADH. ***Suzuki et al., (1992)*** observed that the reductase activity in *P. ambigua* G-1 was dependent on both NADP and NADPH. Studies on chromate reductases have also reported reduction activity of *E. coli, Leucobactorsp*, *P. putida*, and *Bacillus sp.* which were NADPH dependent (***Park et al., 2000; Gonzalez et al., 2003;Ackerley et al., 2004b; Gonzalez et al., 2005;Sarangi et al., 2008***). Therefore, it can be concluded from the result that the bio-removal of chromium by *Basillus mycoids* is an enzyme mediated reduction process. Further, it was observed that the chromate reductase activity in CFE was similar for the cells grown in medium, in presence or absence of Cr(VI). This indicates that the chromate reductase property was not induced by Cr(VI). This was further substantiated by the protein profile obtained for the bacteria.

7.4.2 Kinetic Parameters for Cr(VI) Bio-reduction

Data obtained from the studies on effect of Cr(VI) concentration on the sample were used to calculate K_m and V_{max}. K_m and V_{max} values were derived as 65.42 µg/ mL and 9.514 µg/mL/h (R^2=0.989) respectively from Lineweaver–Burk double reciprocal plot.

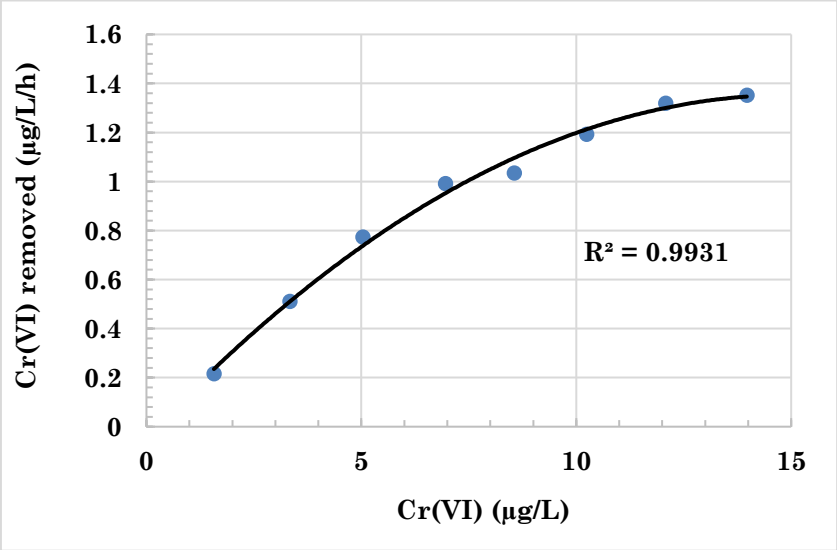


Fig- 7.4: Effect of Cr(VI) concentrationon rate of Cr(VI) bio-reduction.

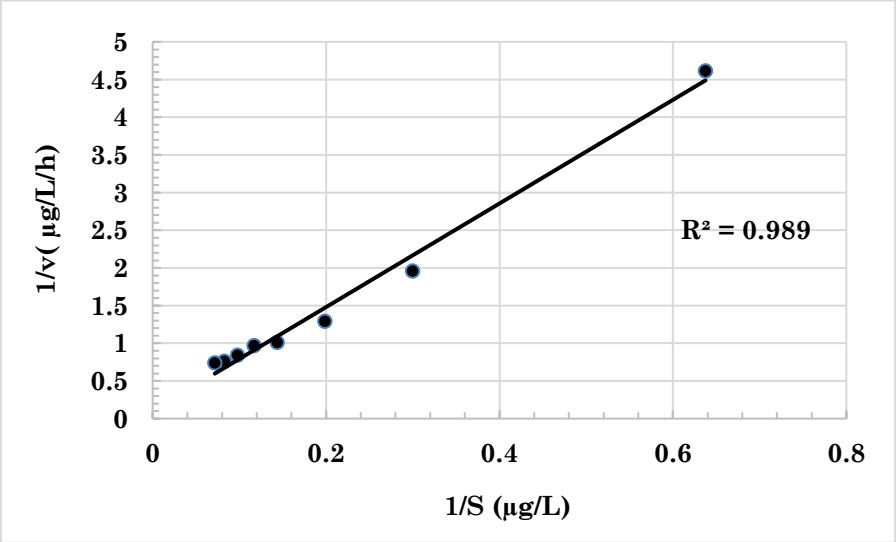


Fig- 7.5: The linearized Lineweaver-Burk double reciprocal plot for calculating K_m and v_{max}

Oh YS et al. (1997) reported higher values of K_m in the range of 13–1,730 mM at the time of bacterial removal of Cr(VI) in water. ***Camargo et al. (2003)*** reported five Cr(VI) reducing bacterial isolates which had displayed K_m values of 0.271 mg/ L (2.61 µM) to 1.51 mg/L (14.50 µM) and a V_{max} of 88.4 µg/L/h (14.17 nmol/min) to 489 µg/L/h (78.36 nmol/min). K_m and V_{max} values for bio-removal of Cr(VI) from water by *Exiguobacterium* sp.was reported to be 141.92 µg/mL and 13.32 µg/mL/h, respectively ***(Oteke, 2007).*** High K_m indicates a low affinity. Thus, with respect to affinity for the Cr(VI), the K_m values of *B. mycoids* in our present study, suggest that it may be a better organism for Cr(VI) reduction.

7.4.3 Kinetic Reaction Order for Cr(VI) Bio-reduction

B. mycoids, grown in LB medium at 10 mg/L of Cr(VI) as $K_2Cr_2O_7$ for 6 h was used for determining the reaction order for Cr(VI) bio-reduction from synthetic growth medium. The reduction rate constant calculated and R^2 value found from linear regression analysis of the data have been summarized in Table 7.1. The best linearity has been obtained for first order kinetic model. Hence, it may be concluded that the reaction rate is directly proportional to the concentration of any one of the reactants. The half life ($t_{1/2}$) was calculated as 1.77 h.

Table 7.1: Kinetic parameters from various kinetic models after fitting

Zero Order Model		First Order Model		Second Order Model	
R^2	k_0 (moles/L/sec)	R^2	k_1 (sec^{-1})	R^2	k_2 ($moles^{-1}sec^{-1}$)
0.9134	-0.75×10^{-6}	0.983	-1.09×10^{-4}	0.8666	28.333

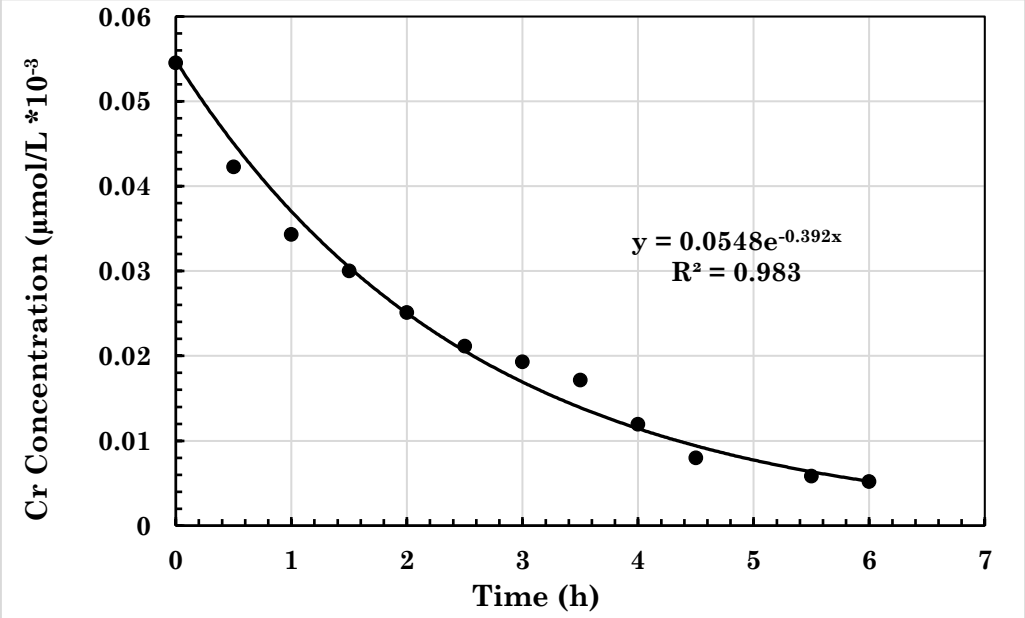


Fig-7.6: Curve for plot of Cr concentration vs. time for first order kinetic model

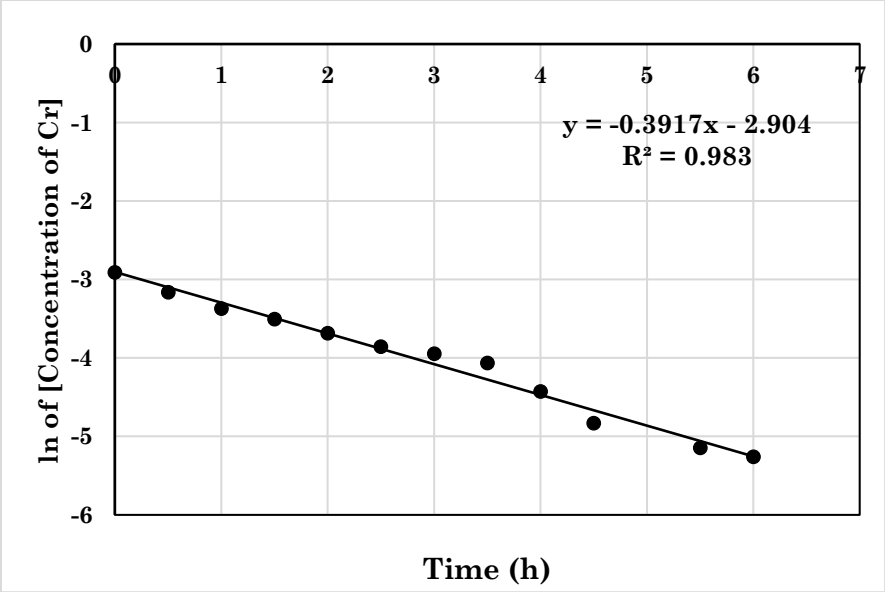


Fig- 7.7: Integrated Form of First Order Reaction

7.4.4 Stress Enzyme Assessment

Chromium resistant bacteria are provided with base line levels of antioxidant systems which are involved in multiple detoxification reactions, to maintain a balance between production and removal of endogenous reactive oxygen species (ROS) and other pro-oxidants produced due to presence of Cr(VI) inside the cell. Several existing literatures have established the role of oxidative stress on bacterial cells caused by ROS formation, in the presence of heavy metal in *E. coli* and *Salmonella typhimurium* ***(Lushchak 2001).*** ***Dimitrova et al. (1994)*** reported that when bio-organisms are exposed to pollutants a simultaneous induction response is observed in the activities of SOD and Catalase. In the present study a gradual increase in the activity of SOD and Catalase (Fig-7.8 and Fig-7.9) in bacterial cell was observed with an increase in concentrations of chromium. Increased activity of SOD is needed to protect against damage to cellular components and to minimize oxidative damage to host cells during immune defence ***(Chen et al., 2007).*** Similar stress responses were recorded for E. coli by ***Samuel et al. (2013).***

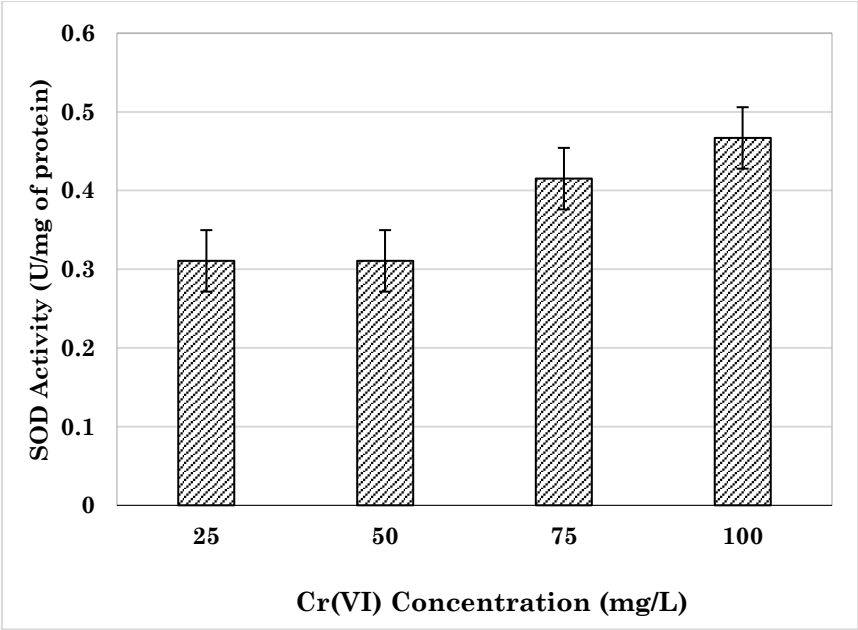


Fig-7.8: Variation of SOD activity at variable Cr(VI) concentration

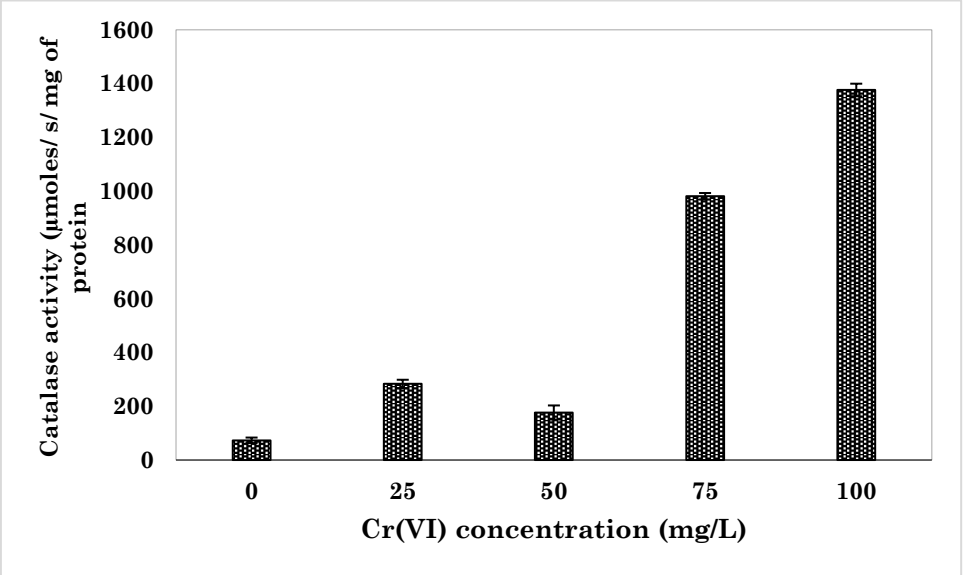


Fig- 7.9: Variation of Catalase activity at variable Cr(VI) concentration

A concentration dependent significant ($p>0.05$) increase in glutathione reductase level (Fig-7.10) was detected [25 < 50< 75 < 100 mg/L of Cr(VI)] for *B. mycoids*. The increase in reduced glutathione due to increase in ROS in the presence of Cr(VI) shows that the bacteria has the ability to scavenge ROS which is produced in response to Cr(VI) stress due to toxicity. A significant (25> 50> 75> 100) decrease in peroxidase activity (Fig- 7.11) was observed subsequently for *B. mycoids*. An overall decrease in peroxidase activity by an amount of 25% compared to the control was noted for the sample containing 100 mg/L Cr(VI). The observation may suggest that the activity of peroxidase was suppressed due to the toxicity of chromium. Similar observation was found by ***Sanaa et al., (2013).***

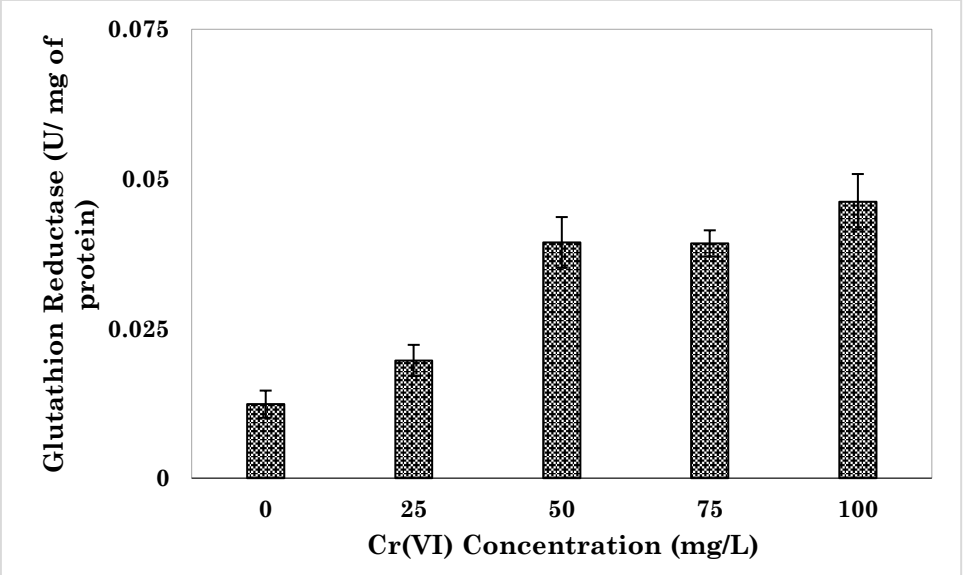


Fig- 7.10: Variation of Glutathione reductase at variable Cr(VI) concentration

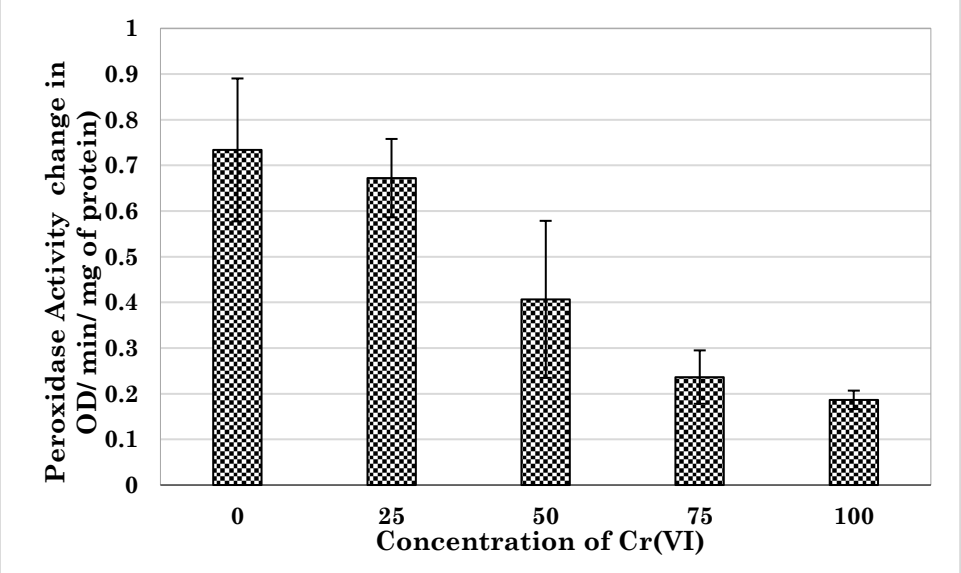


Fig- 7.11: Variation of Peroxidase activity at variable Cr(VI) concentration

7.4.5 Extraction of EPS at Different Chromium Concentration

The EPS extracted from 24 h grown culture medium of *B. mycoids* with a variable concentration of Cr(VI) viz, 0, 50 and 100 mg/L was quantified by using phenol-sulphuric acid. The yield of EPS was determined in the present study as 0.053 ±0.014, 0.066 ± 0.019 and 0.072 ± 0.022 mg/mL for Cr(VI) concentration of 0, 50 and 100 mg/L respectively. It is evident from the obtained data that, yield of EPS is significantly dependent on the concentration of chromium.Cr(VI) tolerant strains producing EPS of concentrations 356 and 548 mg/L by *Synechocystis sp*. BAS0672 and *Synechocystis sp*. BAS0670 have been reported by ***Ozturk et al. (2009). Quintelas et al. (2007)*** has also reported the polysaccharide production of three different strains namely *Bacillus coagulans* (CECT12), *Streptococcus equisimilis* (CECT 926) and *Escherichia coli* (CECT 515) to be 9.19, 7.24 and4.77 mg/g of biosorbent. ***Sundar et al. (2011)*** showed the production of EPS with concentrations ranging from 0.059 to 0.063 mg/ml by chromium resistant strains isolated from chromium polluted tannery site in Vellore, India. ***Harish et al. (2011)*** also reported the production of EPS by *Enterobacter cloacaea* quantified as 0.0683 ± 0.002 mg/ml.

7.4.6 FTIR Analysis of EPS

The extracted EPS from *B. mycoids* at variable concentration of Cr(VI) were subjected to FTIR analysis. The EPS samples were dried and analyzed in the range of 400-4000 cm^{-1} onto KBr-pellet (Fig- 7.12). The peaks obtained from the FTIR spectra analysis showed substantial changes while EPS was extracted from Cr(VI) containing medium. The changes in peak as well as peak density in the dotted area as shown for three samples

(Fig- 7.12) indicated the presence of more of polysaccharides while the concentration of chromium increased.

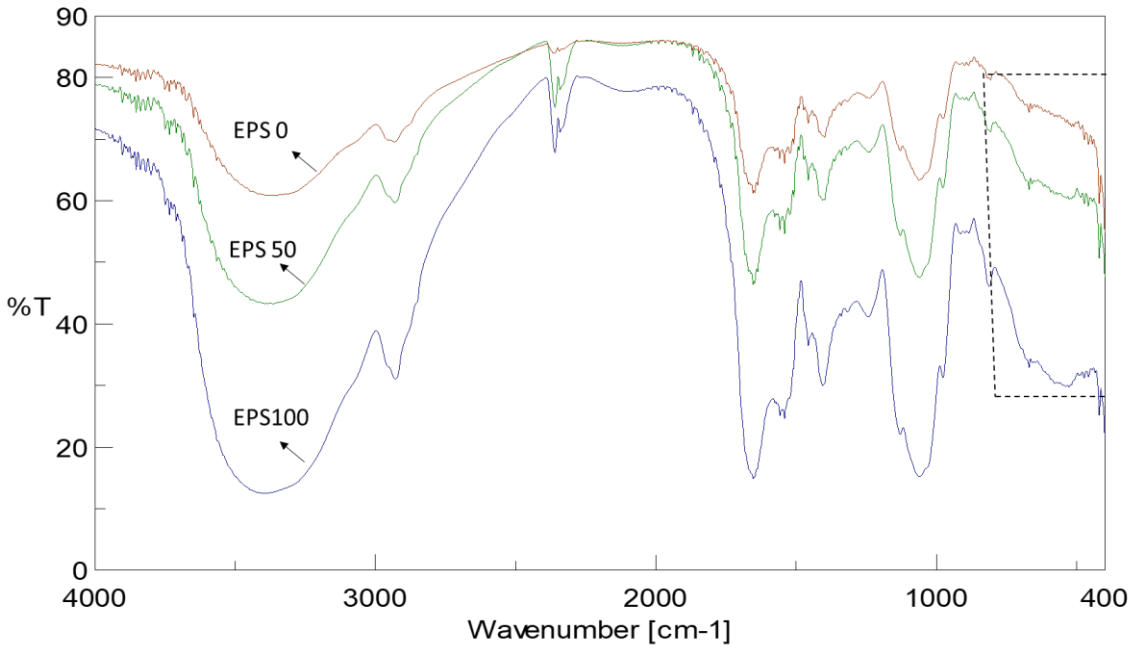


Fig-7.12: FTIR spectra of EPS formed by *B. mycoids* with variable Cr(VI) concentration of 0, 50 and 100 mg/L in LB medium mentioned as EPS 0, EPS 50 and EPS 100 respectively.

Batch Kinetic Study of Pure Culture with Tannery Wastewater

8

8.1 Purpose of Work

Bio-reduction of Cr(VI) to Cr(III) can be achieved by pure as well as enriched mixed cultures of microorganisms under both aerobic and anaerobic conditions ***(Shen and Wang, 1993; Philip et al, 1998; Schmieman et al, 1998).*** The biological systems commonly used for treatment of wastewater containing Cr(VI) are operated in batch mode ***(Chirwa and Wang, 2000; Smith et al, 2002; Wang and Xiao, 1995),*** the probable reason being eventual loss of active biomass in the system as a result of metal toxicity.
Industrial wastewaters, such as those discharged from tanneries, wood processing industries contain a high level of organic matter along with Cr(VI) **(*Khan and Mahmood, 2007).*** Thus, a biological system which can remove organic matter and also reduce Cr(VI) to Cr(III), can offer a viable solution for treating such wastewaters. However, very few cases have been reported on biological systems which can remove COD as well as reduce Cr(VI) to Cr(III). There have been very few studies conducted to evaluate the performance of biological systems in treatment of actual industrial wastewater. Hence, there is a strong merit behind development of a system for the effective treatment of high strength industrial effluent containing organic matter along with Cr(VI).
In the present phase of research, an attempt was made to evaluate the performance of the chromium reducing bacteria viz. *B. mycoids* in a batch reactor system for effective and simultaneous remediation of organic matter and Cr(VI) from tannery wastewater, under various operating conditions. Hence, the scopes of the present phase of study include (*i) characterization of primary treated tannery effluent (ii) design of batch reactor and fabrication (iii) acclimatization of the seed in synthetically simulated wastewater (iv) conduction of batch kinetic study with primary treated wastewater collected from tannery* and (*v) performance evaluation of the pure culture in treatment of primary treated tannery effluent.*

8.2 Theoretical Considerations

8.2.1 Kinetics of Carbon Oxidation and Nutrient Removal

Microorganisms act on organic substrate which leads to gradual conversion of the substrate into microbial cell and cellular energy until the substrate is exhausted. These new cells thus produced, will ultimately undergo endogenous respiration (i.e. the organic components in the cell break down to produce energy for sustenance of the remaining cell

mass) until finally a non-degradable cellular residue remains. In engineering terminology, the substrate is the wastewater and microbial cells constitute the sludge.
The basic equation relating to solids production and substrate removal is given by:

$$\frac{dX}{dt} = Y\left(\frac{dS_r}{dt}\right) \quad \text{..... 8.1}$$

where,
X = mass of microbial solids (cells) in the system i.e. the mixed liquor volatile suspended solids (MLVSS);
S_r = mass of soluble substrate removed
t = time
Y = yield co-efficient (mass/mass)
Since, the growth in biological system is proportional to the mass of cell present, the rate shouldbe studied in terms of growth rate per unit mass

i.e. $\frac{\frac{dX}{dt}}{X} = \mu$ 8.2

where, μ = specific growth rate constant, per unit time.
Experimentally, it has been found that the effect of a limiting substrate can often be definedadequately using the following expression proposed by **Monod (1949)**

$$\mu = \mu_m \frac{S}{K_S + S} \quad \text{..... 8.3}$$

where, μ = specific growth rate ($time^{-1}$)

μ_m = maximum specific growth rate ($time^{-1}$)
S = concentration of growth limiting substrate in solution (mass/unit volume)
K_S= half velocity constant, substrate concentration at one half the maximum growth rate (mass/unit volume)

Putting value of μ from equation (8.3) into equation (8.2)

$$\frac{dX}{dt} = \frac{\mu_m X S}{K_S + S} \quad \text{..... 8.4}$$

Also the rate of growth of bacterial cells can be expressed as

$$r_g = \mu X$$

where, r_g = rate of bacterial growth rate/unit volume. Time

$$\therefore \; r_g = \frac{\mu_m X S}{K_S + S} \quad \text{.....8.5}$$

It is observed that a portion of the substrate undergoes conversion to new cells and another portion is oxidized to organic and inorganic end products. Since the quantity of new cells produced has been observed to be reproducible for a given substrate, the

following relationship can be developed between the rate of substrate utilization and the rate of microbial growth

$\mathbf{r_g = -Y\, r_{su}}$.....8.6

where, r_g = rate of microbial growth, mass/unit volume. time

$\boldsymbol{Y}$ = maximum yield co-efficient (mg/mg) [defined as the ratio of the mass of cells formed to themass of substrate consumed, measured during any finite period of logarithmic growth]

$\mathbf{r_{su}}$= substrate utilization rate, mass/unit volume. time

from equation (8.5) and (8.6)

$$\mathbf{r_{su} = -\frac{\mu_m\, X\, S}{Y\,(K_S + S)}}$$

If $\frac{\mu_m}{Y}$= $\mathbf{k}$ = maximum rate of substrate utilization per unit mass of microorganism

$\therefore\ \mathbf{r_{su} = -\frac{k\, X\, S}{(K_S+S)}}$ 8.7

In bacterial systems used for wastewater treatment, it is observed that the distribution of cell ages is such that: not all the cells in the system are in log growth must be corrected to account for the energy required for cell maintenance. Other factors, such as death and perdition must be considered. It is assumed that the decrease in cell mass caused by them is proportional to the concentration of organism present. This decrease is often identified in the literature as the endogenous decay. The endogenous decay rate can be formulated as follows:

$\mathbf{r_d = -k_d X}$..... 8.8

where, k_d= endogenous decay co-efficient (time^{-1})

X= concentration of cells (mass/unit volume)

When equation (8.8) is combined with equation (8.6) and (8.7) the following expressions areobtained for the net rate of growth

$\mathbf{r'_g = \frac{\mu_m\, X\, S}{K_S+S} - k_d\, X}$.....8.9

$\mathbf{= -Y\, r_{su} - k_d\, X}$

r'_g = net rate of bacterial growth, mass/unit volume. time

The corresponding expression for the net specific growth rate is given by the following equation, which is the same as the expression proposed by **Vanuden.**

$\mathbf{\mu' = \mu_m \frac{S}{K_S+S} - k_d}$.....8.10

where, μ ′= net specific growth rate (time^{-1})

The effects of endogenous respiration on the net bacterial yields are accounted for by defining an observed yield as follows:

$$\mathbf{Y_{obs} = -\frac{r'_g}{r_{su}}}$$

In reviewing the kinetic expression used to describe the growth of microorganism and the removal of substrate it is very important to remember that the expression presented are empirical and were used for the purpose of illustration and that they are not the only expressions available. Other expressions which have been used to describe the rate of substrate utilization include the following:

$\mathbf{r_{su} = -k}$

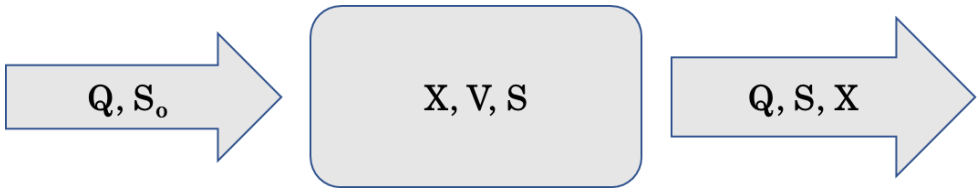
$\mathbf{r_{su} = -kS}$

$\mathbf{r_{su} = -kXS}$

$\boldsymbol{r_{su} = -k\,X\,\frac{S}{S_0}}$

Monod, Teissier, Contois and **Moser** were some of the researchers who have proposed the expression for specific growth rate. The fundamental aspect which determines the use of any rate expression is its applicability in mass balance analysis.

A mass balance in the complete mix reactor for the mass of organism is shown in the following figure



(1) General Word Statement

Rate of accumulation of microorganism within the system boundary	=	Rate of flow of microorganism in the system boundary	−	Rate of flow of microorganism out of the system boundary	+	Net growth of microorganism within the system boundary

(2) Simplified Word Statement

Accumulation = Inflow - Outflow + Net growth

(3) Symbolic Expression

$$\frac{dX}{dt}.V_r = QX_0 - QX + V_r r'_g \quad \text{.....8.11}$$

where, $\frac{dX}{dt}$ = rate of change of microorganism concentration in the reactor measured in terms of mass (volatile suspended solids) mass VSS/unit volume time

Q = flow rate, volume/time

X_0= concentration of microorganism in influent mass VSS/unit volume

X= concentration of microorganism in reactor, mass VSS/unit volume

r'_g = net rate of microorganism growth, mass VSS/unit volume. time

Putting the value of r'_g from equation (8.9)

$$\frac{dX}{dt}.V_r = QX_0 - QX + V_r\left(\frac{\mu_m X S}{K_S+S} - k_d X\right)$$

where, S = substrate concentration in effluent from reactor mg/L

if it is assumed that the concentration of microorganism in the influent can be neglected and steady state condition prevails, i.e. $\frac{dX}{dt} = 0$

then, $\frac{Q}{V_r} = \frac{1}{\theta} = \frac{\mu_m}{(K_S+S)} - k_d$

where, θ = hydraulic retention time $= \frac{Q}{V}$

1/θ corresponds to the net specific growth rate. The 1/θ also corresponds to $1/\theta_c$ where θ_c is the mean cell residence time. In the field of wastewater treatment θ_c may be defined as the mass of organism in the reactor divided by the mass of organism removed from the system in each day (a second commonly used definition). For the reactor θ_c is given by:

$\theta_c = Vr/Q$

Performing a substrate balance corresponding to the microorganism mass balance given by

$$\frac{dS}{dt}.V_r = QS_0 - QS + V_r\left(\frac{kX S}{K_S+S}\right)$$

At steady state $\frac{dS}{dt} = 0$

or, $S_0 - S - \theta_c\left(\frac{kX S}{K_S+S}\right) = 0$

or, $\theta_c\left(\frac{kX S}{K_S+S}\right) = (S_0 - S)$

or, $\frac{X\theta_c}{(S_0 - S)} = \frac{K_S}{k} \times \frac{1}{S} + \frac{1}{k}$

when COD is considered as substrate, the above equation can be written as

$$\frac{X\theta_c}{C_0 - C} = \frac{K_{sc}}{k_{sc}} \times \frac{1}{C} + \frac{1}{k_{sc}} \quad \text{.....8.12}$$

Where, $C_0 - C$= mass concentration of COD utilized (mass/volume)

θ_c= hydraulic retention time (time)

K_{sc} = COD concentration at half the maximum growth rate suspended solids (mass/volume)

k_{sc}= maximum rate of COD utilization per unit mass of suspended solids ($time^{-1}$)

The value of K_{sc} and k_{sc} for COD can be determined by plotting $\frac{X\theta_c}{C_0 - C}$ versus $\frac{1}{C}$.

Here for plotting value $\frac{X\theta_c}{C_0 - C}$ is taken as y-axis and value of $\frac{1}{C}$ is taken as x-axis.

We know, $\mathbf{r_{su} = -\frac{k\,X\,S}{K_S + S} = -\frac{S_0 - S}{\theta} = -\frac{C_0 - C}{\theta}}$

From equation (8.12) Y intercept is equal to $\frac{1}{k_{sc}}$

From the equation (8.11) or (8.12) Slope of the centre equals to $\frac{K_{sc}}{k_{sc}}$

For determining Y and k_d

We know $\mathbf{\frac{1}{\theta_c} = -Y\frac{r_{su}}{X} - k_d}$

or, $\mathbf{\frac{1}{\theta_c} = Y\frac{S_0 - S}{X\theta} - k_d}$

$\mathbf{= Y\frac{C_0 - C}{X\theta} - k_d}$

Here $\frac{1}{\theta_c}$ values are taken along Y-axis and $\frac{C_0 - C}{X\theta}$ is taken along X-axis. Y intercept equals ($-k_d$) and the value of the slope gives the value of (Y).Then we get the value of μ_m from equation

$\mathbf{\mu_m = kY = k_{sc}Y}$

Where, determination of value of k_{sc} has already been explained.

8.2.2 Kinetics of Substrate Inhibition Growth

A large set of widely published and different substrate inhibition models which analyze the inhibitory effects of heavy metals or other inhibitors on substrate utilization by the microorganisms are available. These models have either evolved from Monod's kinetics or are an extension of the same. The *Andrews* model helps to explain the inhibitory effects of substrate at higher concentrations, which however, reduces to the conventional Monod's equation when the inhibition constant becomes very high.

The Andrew's Model is given here for calculating the inhibition constant K_i due to chromium inhibition on conventional growth of the microorganism.

$$\mu = \left(\frac{\boldsymbol{\mu_m S}}{\boldsymbol{K_S + S}}\right)\left(\frac{\boldsymbol{K_i}}{\boldsymbol{K_i + \gamma_{Cr}}}\right)$$

where, K_i = the Cr(VI) inhibition constant (mg Cr(VI)/L)

γ_{Cr} = Cr(VI) concentration (mg/L)

8.3 Methodology

8.3.1 Selection of Sampling Point

Since, the wastewater is usually treated with microorganisms in the process of biological treatment; primary treated tannery effluent was subjected to performance evaluation and batch kinetic study with the isolated pure culture, *B. mycoids*, in the present research work. A typical primary treated tannery effluent was collected for this purpose from the Common Effluent Treatment Plant (CETP) of Calcutta Leather Complex (CLC), situated at Bantala, 24 Pgs (South). This CETP generally receives a composite tannery effluent.

8.3.2 Collection of Primary Treated Tannery Effluent

60.0 L of the primary treated effluent was collected at the point of its entry to secondary treatment unitand taken to the laboratory. Wastewater sample was collected in 10.0 L PVC containers. Temperature and pH were checked at the time of sampling with the help of portable pH meter. A portion of wastewater sample was transferred to a BOD bottle. DO of the sample was fixed by adding $MnSO_4$ and Alkali-Iodide-Azide solution at the time sampling. Samples were kept at deep freezer (-20°C) till further use.

8.3.3 Characterization of Primary Treated Tannery Effluent

The wastewater sample was first characterized and then subjected to the acclimatization and subsequent treatment with bacterial sample in laboratory scale batch reactor. Characterization of the tannery wastewater sample was performed on the basis of following parameters viz. COD, TDS, TSS, BOD_5 at 20°C, Chloride, Phosphate, Nitrate, Carbonate Hardness, Total Hardness, Acidity, Alkalinity, Sulphides, Iron and Total Chromium as per the standard methods prescribed by ***APHA, 1998***.

Table-8.1: Analytical Methods and Instruments used for Analysis of primary treated wastewater

Parameters	Analytical Methods	Name of the Instrument and make
COD	Closed Reflux Method	-
BOD_5 at 20°C	5 Day BOD Test	BOD Incubator (Sicco)
Total Dissolved Solid (TDS)	Gravimetric Method at (103-105)°C	Hot Air Oven
Total Suspended Solid (TSS)	Gravimetric Method at (103-105)°C	Hot Air Oven
Chloride	Argentometric Method	-
Phosphate	Stannus Chloride Method	-
Nitrite	Colorimetric Method	-
Carbonate Hardness	EDTA Titrimetric Method	-
Total Hardness	Titrimetric Method	-
Acidity	Titration Method	-
Alkalinity	Titration Method	-
Sulphides	Iodometric Method	-
Iron	By AAS	AAS (A Analyst), Perkin Elmer
Total Chromium	By AAS	AAS (A Analyst), Perkin Elmer

8.3.4 Reactor Design and Fabrication

Biodegradation studies for simultaneous removal of Cr(VI) and COD were carried out in a batch biological reactor. The reactor was made up of plexi-glass of 5.0mm thickness. The schematic diagram of the experimental set up is shown below:

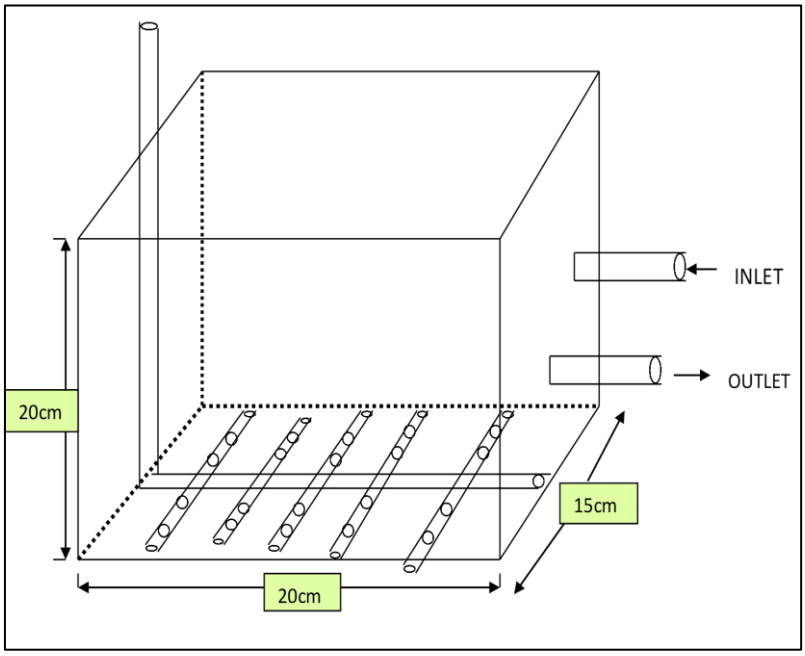


Fig. 8.1: A schematic diagram of the reactor

The capacity of the reactor is about 5.0 L, with a height of 20cm, length 20cm and breadth of 15cm. Two ports are connected to the reactor as inlet and outlet points. Aquarium compressor pumps (2 nos.) are used for supply of air within the reactor.

Fig.-8.2: Laboratory scale batch reactor set-up to study performance evaluation of primary treated tannery effluent with isolated pure culture

8.3.5 Seed Acclimatization

8.3.5.1 Seed Acclimatization for Carbon Oxidation

In the present study acclimatization of seeds was carried out under laboratory condition for carbon oxidation initially. The study was initiated in the batch reactor for 2.0L capacity as acclimatization unit. Since the type of microbial culture used in this study was isolated pure culture, the isolated bacteria i.e.*B. mycoids* was first grown in LB medium. About 1000 mL of bacterial culture was mixed with 1000mL double distilled deionized water to have a total volume of 2000 mL in the reactor. Aquarium pumps were used for aeration as the source of diffused air. At the very preliminary stage dextrose ($C_6H_{12}O_6$) was added as a sole source of carbon. The trace element and nutrients were added as per recommended dosage and their compositions are given in Table 8.1. The acclimatization process was continued with this combination for two weeks. The biomass growth was monitored as MLVSS concentration in the reactor. pH of the reactor content was kept in the range of 6.8-7.0 by adding required amount of sodium carbonate (Na_2CO_3) and phosphate buffer.The acclimatization was further carried out with a combination of 1000 mL acclimatized seed from the rector, 500 mL double distilled deionized water and 500 mL primary treated wastewater sample over a period of approximately three weeks.pH value in the range of 7.6-8.4 was adjusted by adding pre-fixed amount of sodium carbonate buffer solution. COD in the form of dextrose of strength 2,000 mg/L was added. Nutrients and trace materials were also added as per the suggested dosage. Carbon oxidation study was carried out by varying the volume of real-life tannery effluent from 500 mL to 1500 mL. The entire seed acclimatization for carbon oxidation was carried out over a time period of 45 days.

8.3.5.2 Seed Acclimatization for Combined Carbon Oxidation and Chromium Removal

In another part of the present study, combined carbon oxidation and chromium removal were carried out with acclimatized seeds developed as per the method discussed under the section 8.3.5.2. Since the hexavalent chromium concentration in the collected wastewater samples did not lie in the range of required concentration of the present experiment, hexavalent chromium was spiked further into the reactor as a calculated aliquot dosage from a filter sterilized (0.22 μm) $K_2Cr_2O_7$ solution. The concentration of Cr(VI) was gradually varied in the previously acclimatized system from 0 – 50 mg/L as $K_2Cr_2O_7$. pH value in the range of 7.2-8.0 was adjusted by adding sodium carbonate buffer solution. In this acclimatization study the sole source of the organic carbon was the organic substrate present in the tannery effluent. Dextrose was not added because additional carbon sources may influence the removal of chromium from the reactor

(Narayani and M Vidya Shetty, 2013). Nutrients and trace elements were added to the reactor in the recommended dosages as given in Table-8.2

Table-8.2: Composition of Nutrients and Trace Elements

Nutrients	**Volume to be added in 1000mL**
K_2HPO_4	60.0 mg
KH_2PO_4	40.0 mg
KNO_3	72.0 mg
Trace elements	**Volume to be added in 1000mL**
$MgSO_4.7H_2O$	500.0 mg
$FeCl_3.6H_2O$	710.0 mg
$ZnSO_4.7H_2O$	0.1mg
$CuSO_4.5H_2O$	0.1mg
$MnCl_2.2H_2O$	8.0 mg
$(NH_4)_6Mo_7O_{24}$	0.11mg
$CaCl_2.2H_2O$	100.0 mg
$CoCl_2.6H_2O$	200.0 mg
$Al_2(SO_4)_3.16H_2O$	55.0 mg
H_3BO_3	150 mg
EDTA	100 mg

8.3.6 Experimental Procedure

1000 mL of previously acclimatized bacterial culture of *B.mycoids* stored at -20ºC was mixed with 1000 mL of 1:1 diluted tannery effluent and was subsequently poured in the batch reactor. The aerobic batch reactor was operated initially at an HRT of 96 h spiked with 10 mg/l of Cr(VI) as $K_2Cr_2O_7$. Once the system was stabilized, the performance of the reactor was monitored in terms of COD and Cr(VI) reduction efficiency. Initially, the COD of reactor medium was adjusted to 1/4 of the COD of primary treated tannery effluent and gradually increased to higher concentrations of COD. The concentration of Cr(VI) was kept fixed at 20 mg/L. The Cr(VI) removal efficiency was observed at variable concentration of chromium ranging from 0 – 50 mg/L. The operational plan of the reactor is given in the table-8.3.

Table-8.3: Operational History for real-life tannery effluent (HRT=96 h for each set)

Initial Cr(VI) (mg/l)	**20**	**20**	**20**	**10**	**20**	**30**	**40**	**50**
COD (mg/l)	**560**	**1120**	**1458**	**1125**	**1120**	**1080**	**1180**	**1280**

8.3.7Analytical Methods followed for Monitoring of Different Parameters

During the entire course of study with the batch reactor fed with real-life primary treated tannery wastewater, 100mL of sample was collected from the reactor at 0, 2, 4, 6, 8, 24, 26, 28, 30, 48, 51, 54, 72, 75, 78 and 96 h. The samples were analyzed for the following parameters viz. pH, DO, temperature, MLSS, MLVSS, COD and Cr(VI)as per Standard Methods (APHA, AWWA, WPCF). The analytical methods and instruments used for the measurement of different parameters are given in the table 8.4.

Table-8.4: Analytical Methods and Instruments used for Different Parameters

Parameters	Analytical Methods	Name of the Instrument andmake
pH	Electrometric Method	Deluxe pH Meter, EI Make,Model-101E
Total Suspended Solids (TSS)/MLSS	Gravimetric Method at (103-105)°C	Hot Air Oven
Volatile Suspended Solids/MLVSS	Gravimetric Method at (550±50)°C	Muffle Furnace
Soluble COD (SCOD)	Closed Reflux Method	HACH USA make, DRB-200model
Dissolved Oxygen (DO)	Membrane Electrode Method	Digital DO meter, Model-811E,EI make
BOD_5 at 20°C	5-Day BOD Test	BOD Incubator (Sicco)
Cr(VI) concentration	Colorimetric method	UV – VIS Spectrophotometer (Lambda 25, Perkin Elmer)

8.3.7.1 Determination of the Chemical Oxygen Demand (COD) by Closed Reflux Method

Basic Principle

In the present study DRB 200 Digital Reactor Block was used for digestion of samples. Digestion was carried out for 2 hours at 150 °C, which was followed by titrimetric determination of SCOD. Potassium dichromate ($K_2Cr_2O_7$) was used as oxidizing agent, as it was capable of oxidizing a wide variety of organic substances and almost completely to CO_2 and H_2O. In this case,as a result of this elevated temperature significant loss of volatile organic compounds may take place. Certain organic compounds, particularly low-molecular fatty acids were oxidized by dichromate in presence of delivered ions as catalyst. However, the reaction involved may be represented in ageneral way as follows.

$$C_nH_aO_b + cCr_2O_7^{2-} + 8OH^- \rightarrow nCO_2 + (a+8c)/2H_2O + 2cCr^{3-}$$

Where **c= (2/3) *n + b/6 - b/3**

As a result of this reaction chromium is reduced from a state of valence state six to three. COD can be estimated from the expression mentioned underneath.

$$COD\ (mg/L) = \frac{8000^* (\text{Blank titration} - \text{Sample titration}) * \text{Strength of FAS solution}}{\text{Volume of the sample (mL)}}$$

Measurement of Excess Oxidizing Agent

It is necessary for the oxidizing agent ($K_2Cr_2O_7$) to be present in excess to ensure thecomplete oxidation of all organic matter. To measure the excess actual amount is reduced. It is therefore necessary to use a reducing reagent for dichromate. In our experiment we use 0.1 (N) ferrous ammonium sulphate $\{Fe(NH_4)_2(SO_4)_2,6H_2O\}$ as reducing agent which reacts with dichromate in the following way.

$$6Fe^{2+} + Cr_2O_7^{2-} + 14H^+ \rightarrow 6Fe^{3+} + 2Cr^{3+} + 7H_2O$$

Blank samples are required for this test in order to get of the interference of the organic matter, extraneous by nature.

8.3.7.2 Determination of Hexavalent Chromium

Hexavalent chromium in the sample was determined by standard method prescribed by APHA, 1998, which has been described in details under the section 4.2.10.

8.3.8 Artificial Neural Network (ANN) Approach

ANN is a mathematical technique which enables to understand any process that is difficult to define accurately or satisfactorily by classical mathematical models. This technique is exclusively helpful for complex processes or when number of variables is more. In an ANN model, data is read, information is processed by reading the data and by understanding the relationship between variables the output is generated.

There are different functions in ANN model and the ANN architecture is developed by combining these functions. The basic architecture of ANN involves three layers i.e. input layer, hidden layer and output layer. Each layer individually performs a definite function. All these three layers are interconnected by neurons which are the key functions in the processing of data. Each neuron in a layer is linked to other neurons in the subsequent layer by weights. This defines the strength of the linking. A propagation rule describes

the way by which these weights are varied to attain minimum error through prediction. The most commonly used rule is the back-propagation rule.

In the present study a neural network was developed using ANN architecture in MATLAB platform. The network architecture is as given in Fig.8.3A.

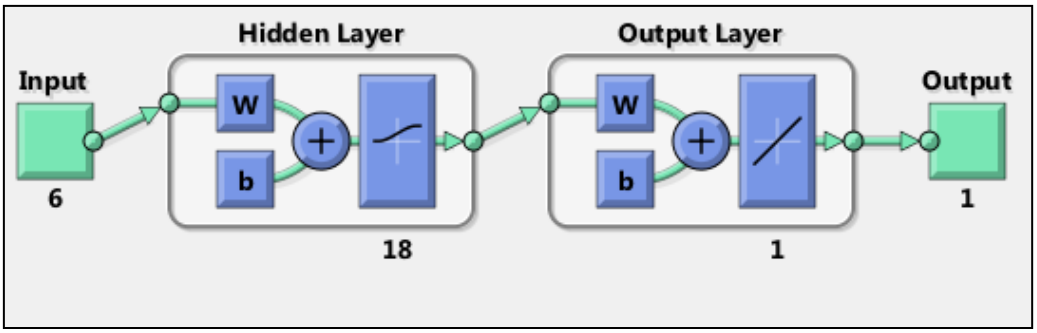


Fig-8.3A: The ANN Architecture

8.4 Results and Discussion

8.4.1 Characterization of Primary Treated Tannery Effluent

The composite tannery effluent sample, after primary treatment was collected from the inlet box prior to the secondary treatment plant of the CETP of Calcutta Leather Complex. The wastewater sample was collected three times over the entire course of the study. This collected wastewater sample was characterized in the laboratory with respect to the following parameters as shown in Table- 8.5.

Table-8.5: Characterization of the Primary Treated Composite Tannery Effluent

Parameters	Value
Odour	Obnoxious
pH	6.9 ± 0.152
Temperature (°C)	28.6 ± 0.75
DO (mg/L)	Below detectable limit
COD(mg/L)	2680 ±168.6
BOD_5 at 20°C(mg/L)	866.7±101.5
Total Dissolved Solid (TDS)(mg/L)	6420 ± 340.8
Total Suspended Solid (TSS)(mg/L)	100 ± 23.5
Chloride(mg/L)	6150 ± 563.7
Phosphate(mg/L)	0
Nitrate(mg/L)	51 ± 2.4
Carbonate Hardness(mg/L)	430.4 ± 58.9
Total Hardness(mg/L)	880 ± 65.2
Acidity (mmol/L)	1.8 ± 0.88
Alkalinity (mmol/L)	11.4 ± 0.95
Sulphides(mg/L)	19.163 ± 1.53
Iron(mg/L)	2.24 ± .077
Total Chromium(µg/L)	28.32 ± 1.66

The average BOD_5: COD ratio obtained was 0.323 for primary treated composite tannery effluent. Tannery effluent characteristically contains a composite mixture of both inorganic salts and soluble organic substances, hence the dissolved solid content in primary treated tannery effluent was observed to be in the higher side. Leather processing units generally use a significant amount of alkaline salts which cause the high alkalinity and hardness count in wastewater. The total hardness and carbonate hardness were determined as 880 mg/L and 430.4 mg/L respectively. The alkalinity was recorded as 11.4 mmol/L.

8.4.2 Kinetic Study for Organic Carbon and Chromium Removal from Primary Treated Tannery Wastewater in Batch Reactor

Reaction kinetics and reactor dynamics need to be evaluated for optimization and explanation of performance of a batch reactor. In the present study, based on the experimental results, different values for bio-kinetic coefficients for carbon oxidation are estimated using pseudo-steady state data under different initial chromium concentrations. The substrate inhibition constant due to the stress of chromium is also calculated for different initial chromium concentrations.

8.4.2.1 Carbon Oxidation Kinetics in Batch Reactor under different Chromium Concentration

In the initial stage of the present experimental work, batch studies were conducted to assess the potential for carbon oxidation utilizing acclimatized seed under different chromium concentrations in primary treated composite tannery wastewater. The method followed in the development of acclimatized seed has already been stated in details in this chapter under section 8.3.5.1 and 8.3.5.2. The operational conditions considered for batch kinetic studies are furnished in Table-8.3.

On the basis of the experimental data, regression lines are derived following the least square principle, which are shown in Fig 8.3 to Fig 8.14. The reciprocal of the specific substrate utilization rate (1/U) have been plotted against the reciprocal of effluent SCOD (1/S). Substrate removal kinetics are evaluated using the simple linear equation (Fig-8.3 to Fig-8.14). The slope of the straight line in graph was (Ks/k) and the intercept was (1/k). The values for the reciprocal of the mean cell residence time (1/θc) have been plotted against specific substrate utilization rate (U). The yield coefficient (Y) has been determined from the slope of the line while the endogenous decay coefficient (k_d) was obtained from intercept, $k_d = -C$. The values of the bio-kinetic coefficients (k, Ks, Y and k_d)for carbon-oxidation and combined carbon-oxidation and chromium removal are summarized and given in the Table 8.6.

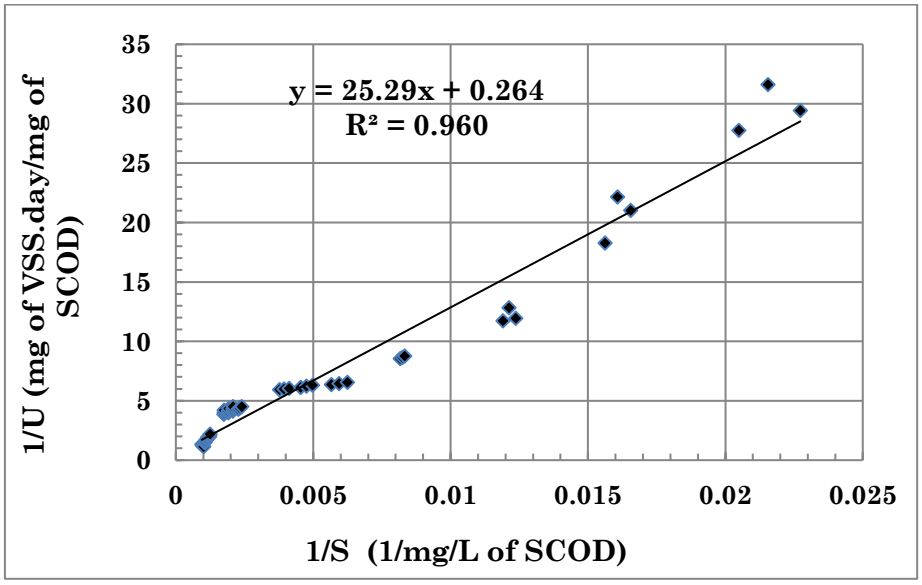


Fig-8.3: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr(VI) =0 mg/L)

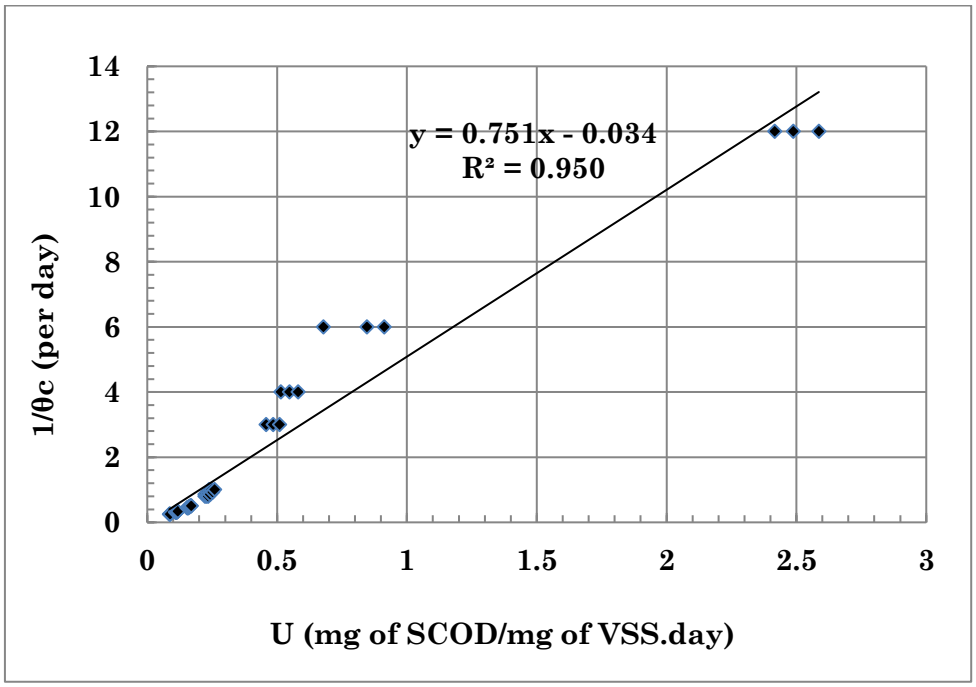


Fig-8.4: Microbial Growth Kinetics for Carbon Oxidation Study (Cr(VI)=0 mg/L)

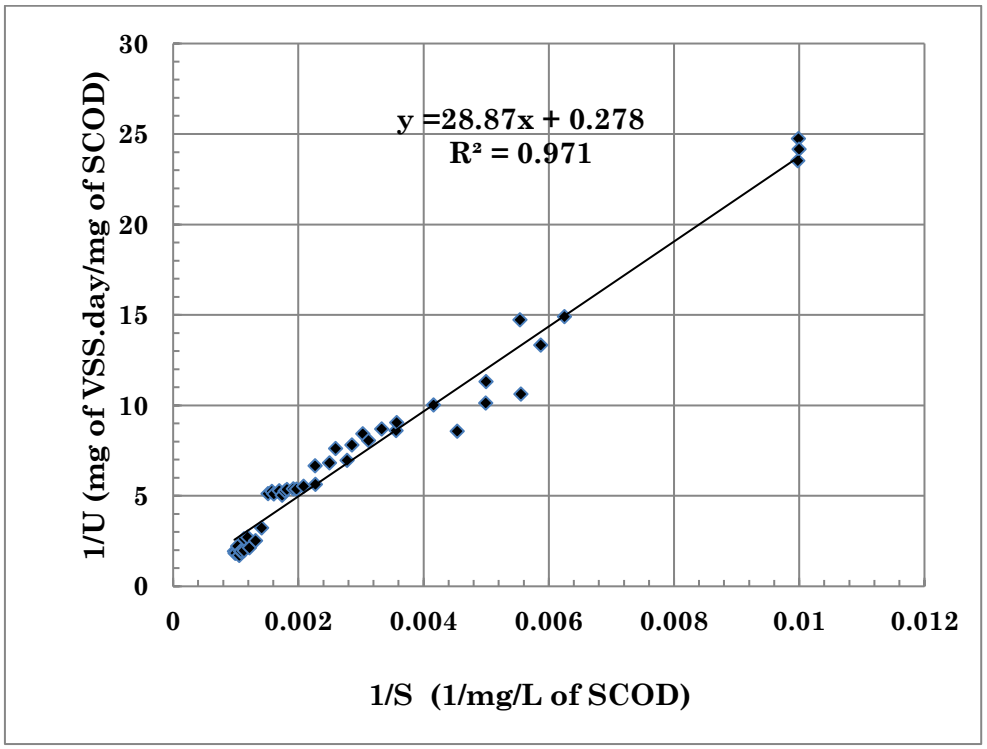


Fig-8.5: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr=10 mg/L)

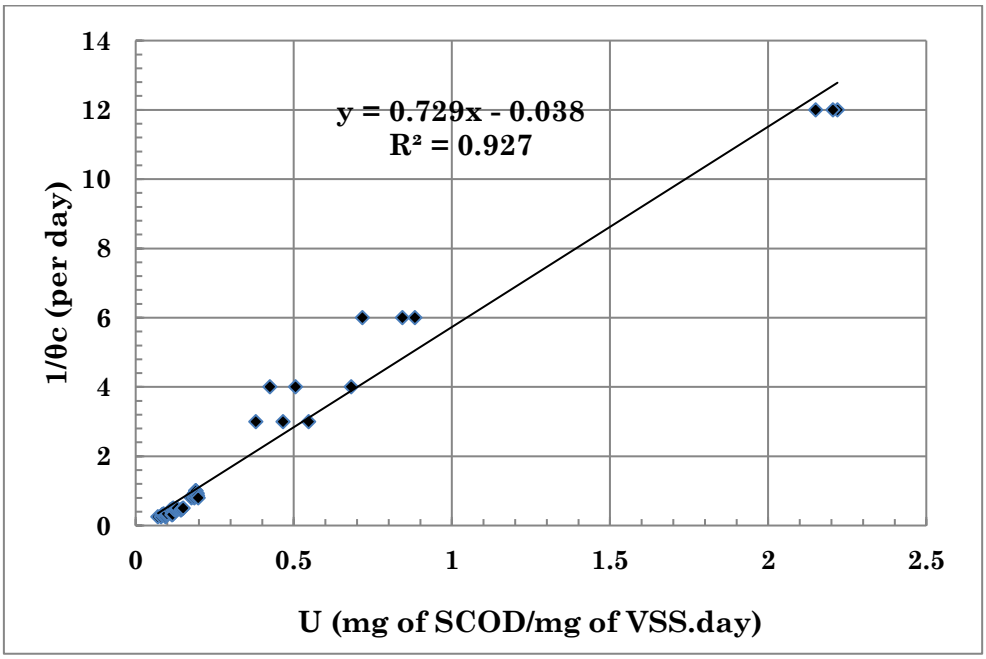


Fig-8.6: Microbial Growth Kinetics for Carbon Oxidation Study (Cr=10 mg/L)

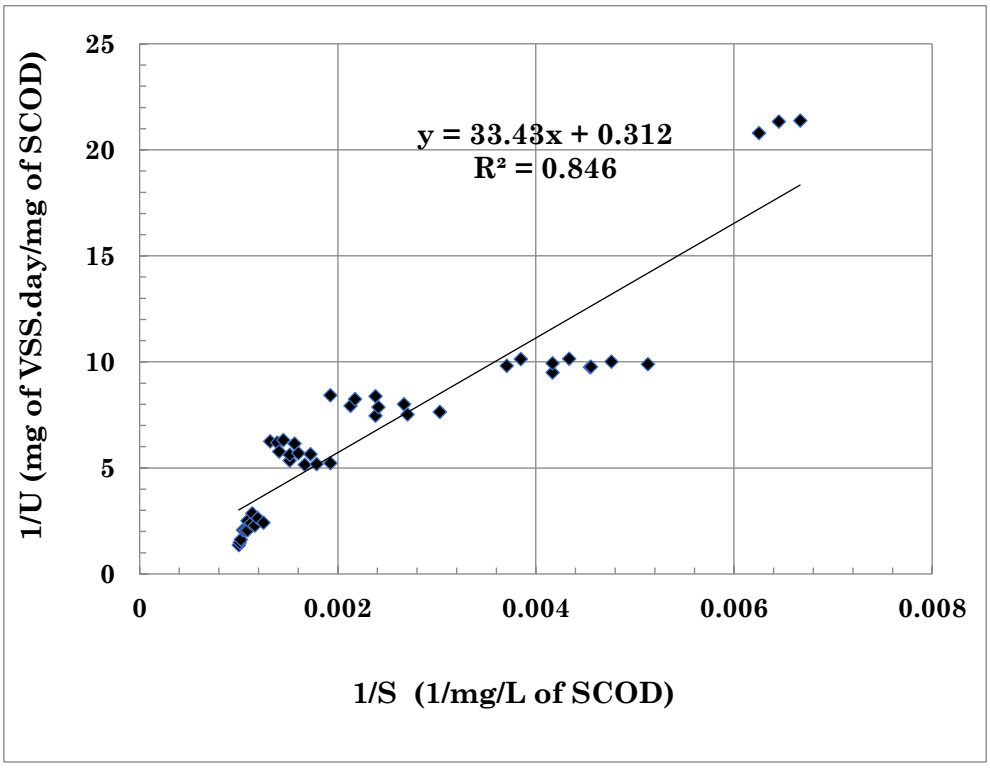


Fig-8.7: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr=20 mg/L)

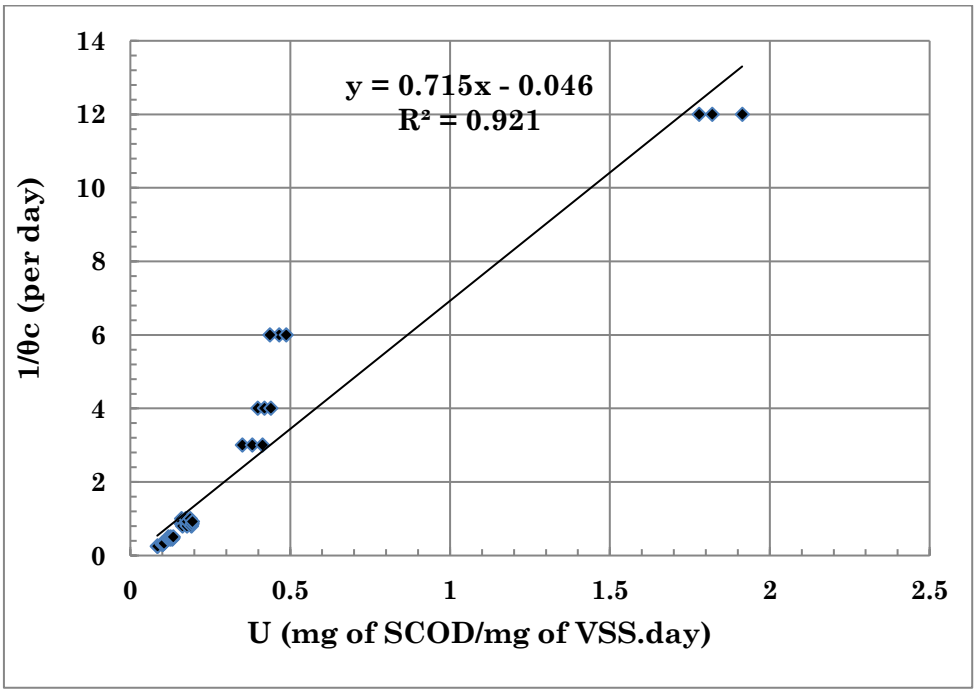


Fig-8.8: Microbial Growth Kinetics for Carbon Oxidation Study (Cr=20 mg/L)

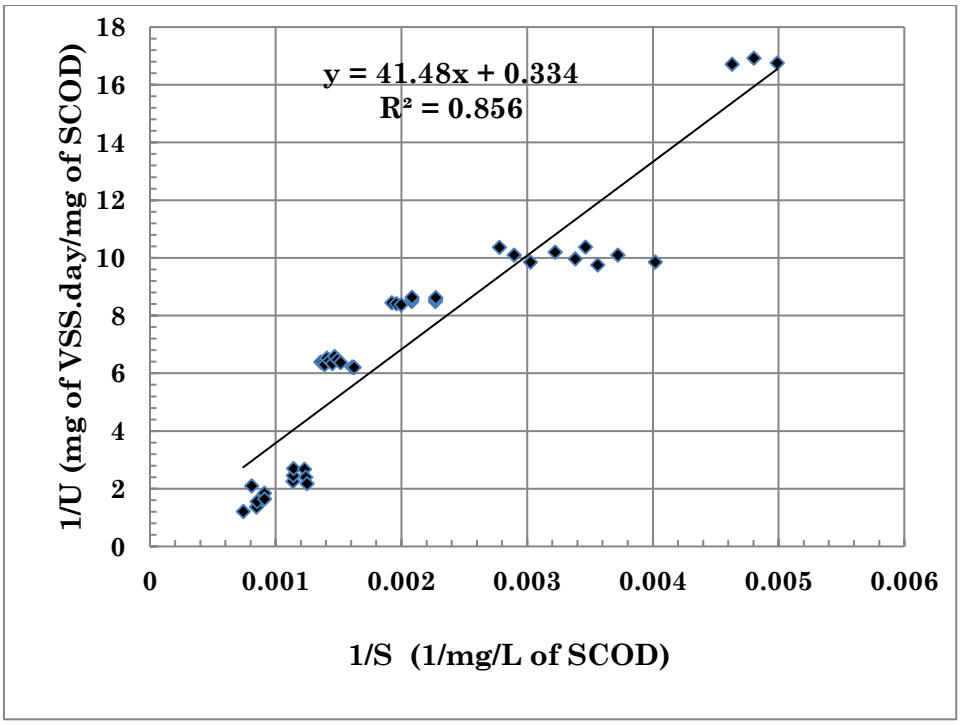


Fig-8.9: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr=30 mg/L)

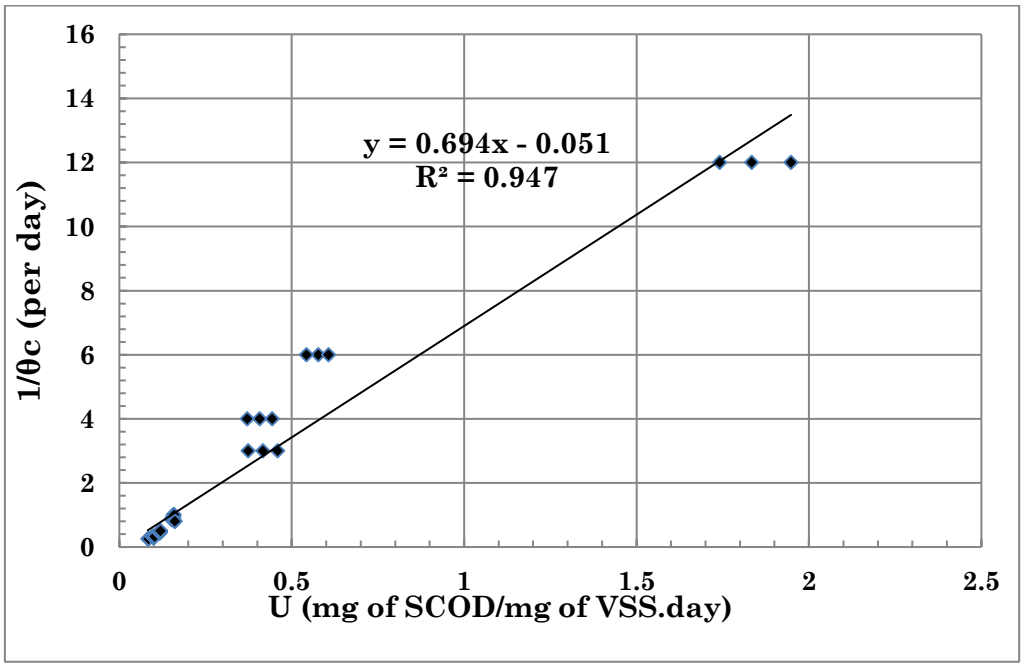


Fig-8.10: Microbial Growth Kinetics for Carbon Oxidation Study (Cr=30 mg/L)

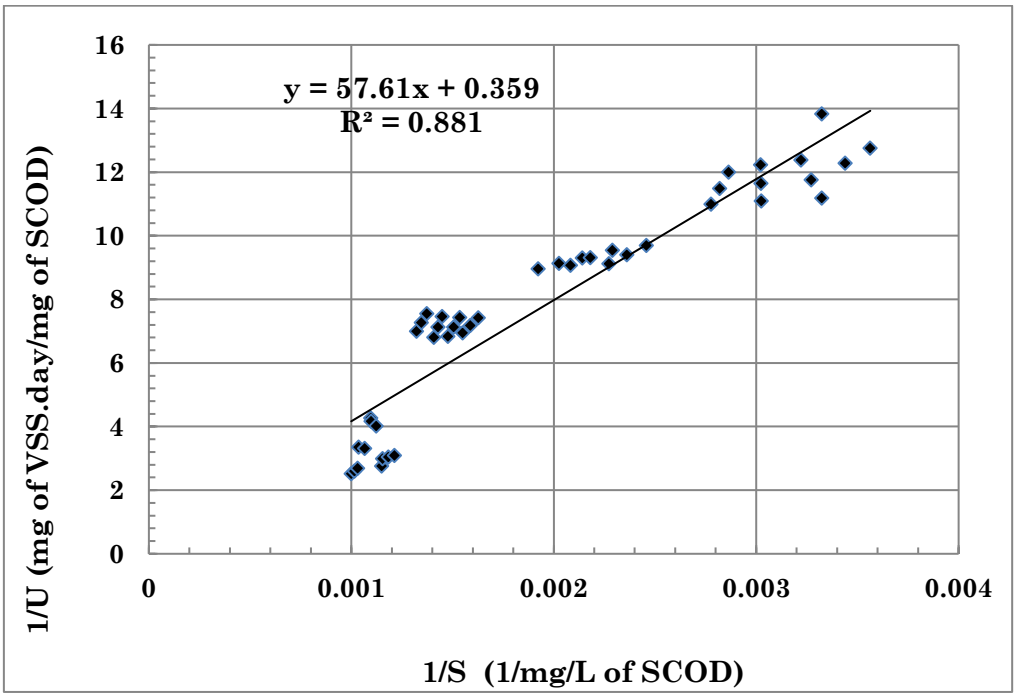


Fig-8.11: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr=40 mg/L)

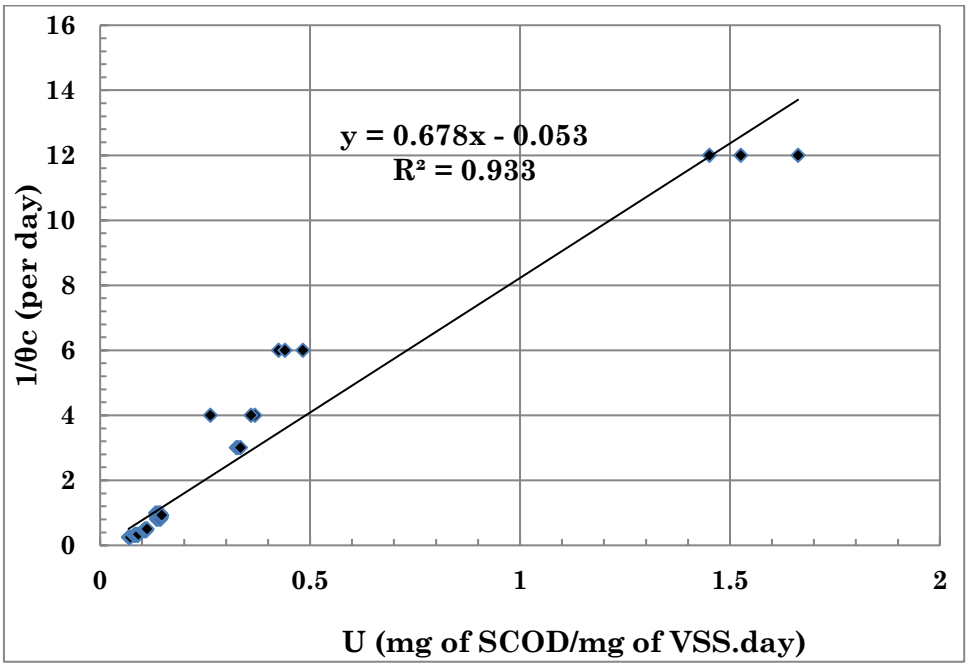


Fig-8.12: Microbial Growth Kinetics for Carbon Oxidation Study (Cr=40 mg/L)

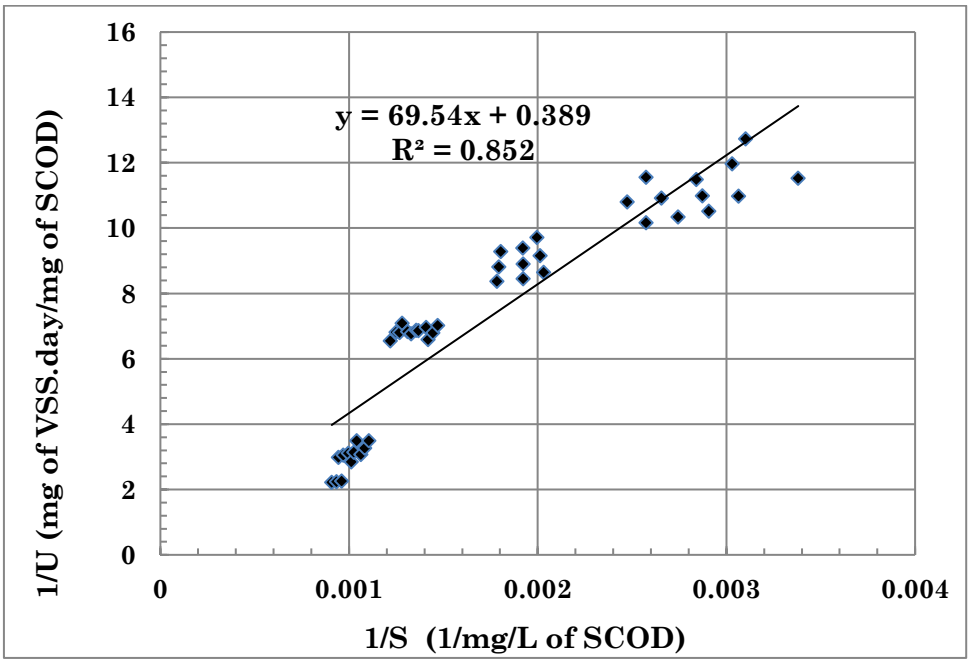


Fig-8.13: Substrate Utilization Kinetics for Batch Carbon Oxidation Study (Cr=50 mg/L)

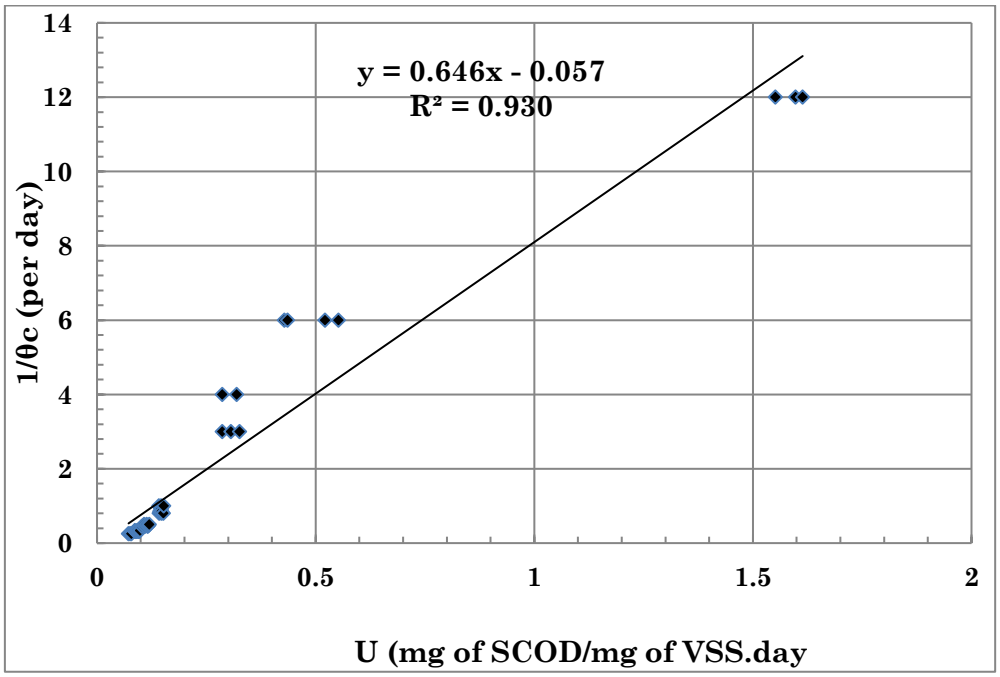


Fig-8.14: Microbial Growth Kinetics for Carbon Oxidation Study (Cr=50 mg/L)

Table- 8.6: Summarized Values of Bio-kinetic Coefficients for Batch Studies of Isolated Pure Culture under the stress of Chromium

Standard Values ***(Ref: Metcalf & Eddy, 1995)***	**Cr(VI) Conc. (mg/L)**	**Ks (mg/L)**	**k (per day)**	**Y (mg/ mg)**	**μ_m (per day)**	**k_d (per day)**	**Ref.**
K_s (mg/L of SCOD)= (15-70) **k (day^{-1})= (2-10)** **Y (mg VSS/mg SCOD)= (0.4-0.8)** **k_d (day^{-1}) = (0.025-0.075)**	0	95.59	3.78	0.751	2.84	0.034	Present Study
	10	103.84	3.597	0.729	2.62	0.038	
	20	107.14	3.205	0.715	2.291	0.045	
	30	124.19	2.994	0.694	2.077	0.051	
	40	160.44	2.785	0.678	1.888	0.053	
	50	178.72	2.570	0.646	1.660	0.057	
	-	190	-	0.377	2.34	0.05	*R. Elangovan, L. Philip, 2009*

It can be observed from the above table that the values of the bio-kinetic coefficients as obtained from the test results for carbon oxidation, i.e. k, Y and k_d (other than K_s), under different chromium concentration have a similar trend to their respective standard values depicted in Table-8.6. However, the K_s values observed for carbon-oxidation during the

treatment of primary treated tannery effluent are higher than the standard values. This is perhaps owing to higher initial concentrations of SCOD within the batch reactor. Further, a decrease in the value of maximum substrate utilization rate (k) (from3.78 to 2.570 per day) for carbon oxidation under different chromium concentration condition was observed, with an increase in chromium concentration from 0 to 50 mg/L. The possible reason for this decrease may be due to inhibitory effect of chromium during the combined substrate condition. This explanation is corroborated by the decrease in the value of yield coefficient (Y) (from 0.751 to 0.646 mg/mg) along with corresponding increase in endogenous decay rate constant (k_d) value (from 0.034 to 0.057 per day) with increasing chromium concentration. At the same time, the maximum specific growth rate μ_m also showed a declining trend with higher chromium concentration**.** This phenomenon demonstrates that chromium influences the growth rate of the pure culture bacterium *B. mycoids* in the present study. Theincrease in endogenous decay rate constant (k_d) for carbon-oxidation, from 0.034 /day to 0.057 /day indicates the presence of inhibitory effect of chromium toxicity on the pure culture, thoughits value lied within the standard range prescribed by ***Metcalf & Eddy (1995)*** for a typical municipal wastewater.

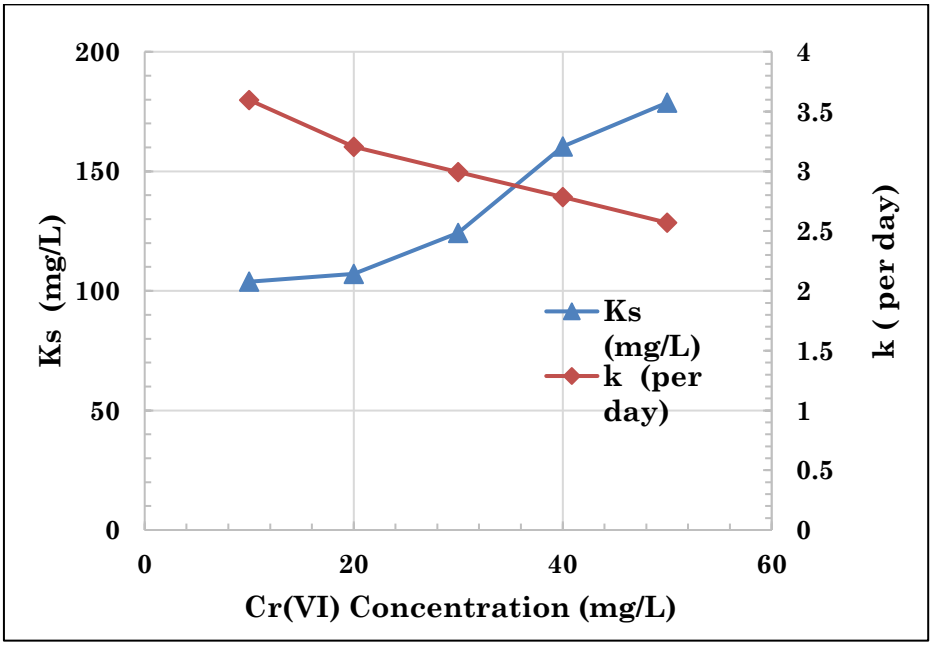


Fig- 8.15: Variation of K_s and k values with Cr(VI) concentration

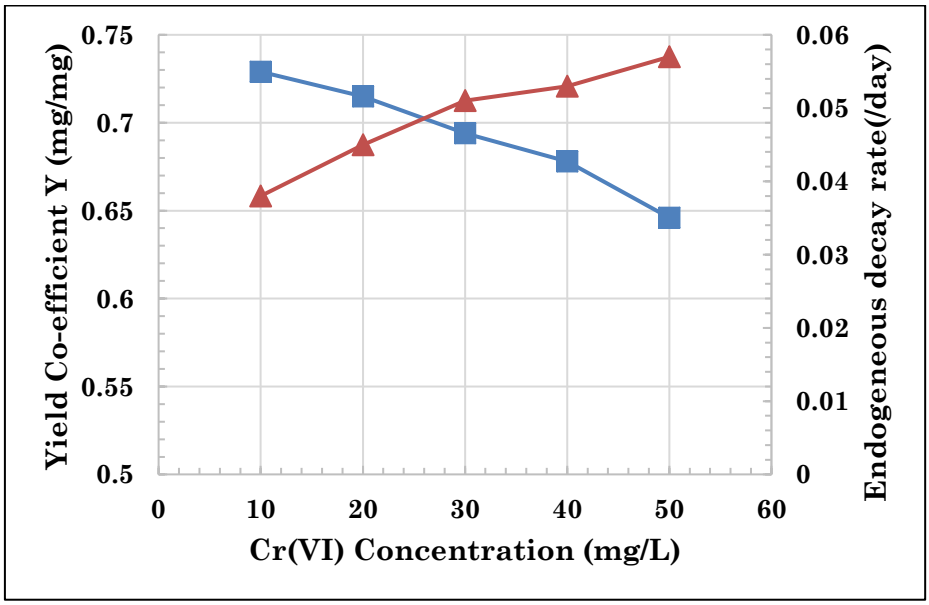


Fig- 8.16: Variation of Y and k_d values with Cr(VI) concentration

8.4.2.2 Variation in Substrate Inhibition Constant (K_i) under Different Chromium Concentration

In order to find out the inhibition constant (K_i), experiments were conducted by varying the Cr(VI) concentration from 0 to 50 mg/L in the reactor. K_i value indicates the point where growth rate of microbes starts decreasing as Cr(VI) concentration increases. The results of the present study for the real-life tannery effluent are presented in Table8.7.

Table-8.7: Substrate Inhibition Constant (K_i) under different chromium concentration

Cr(VI) concentration (mg/L)	Inhibition Constant (K_i)
10	0.1822
20	0.3336
30	0.4236
40	0.5464
50	0.5189

The inhibition constant (K_i) obtained for the aerobic system in nutrient medium for pure culture bacteria named *Arthobactor rhombi-RE* was reported 3.80 mg/L of Cr(VI) by ***R. Elangovan and L. Philip (2009). Espinosa et al. (2006)*** reported that Cr(VI) could inhibit the yeast growth and decrease the specific growth rate. The inhibition constant (K_i) was observed to be 5.8 mM. The results of inhibition constant in present study showed that the bacterium *B. mycoids* is highly prone to grow under the stress of chromium while

it is treated with real-life wastewater. In the present study, the value of the inhibition constant (K_i) increased steadily from 0.1822 to 0.5189 mg/L with the increase in Cr(VI) concentration from 10 to 50 mg/L.

8.4.3 Performance Evaluation for Combined Carbon Oxidation and Chromium Removalin Batch Reactor with Primary Treated Tannery Wastewater

Performance of a biological reactor mainly depends on the ability of the reactor to remove pollutants like total suspended solids (TSS), soluble chemical oxygen demand (SCOD), total kjeldahl nitrogen (TKN) etc. from the wastewater. In the present study, the performance of the laboratory-scale batch reactor system was investigated with a view to examine the efficacy of the treatment of medium to high-strength tannery effluent collected from common effluent treatment plant of Calcutta Leather Complex. It has been observed from different literatures and analysis-results of real-life tannery wastewater that the initial SCOD in the primary treated tannery wastewater, likely to be treated in the biological reactor was in the range of 2500- 9000 mg/L.Considering the high strength and complex nature of tannery effluent the performance of the present batch reactor was evaluated for the treatment of both diluted and undiluted tannery wastewater.
A dilution factor of 5.0 was adopted for initial SCOD concentration and gradually it was increased to higher range. The performance study was also conducted for variable chromium concentration.

8.4.3.1 Carbon Oxidation Profile in Batch Reactor

Organic carbon, which is the source of energy for microbial metabolism, has been estimated in terms of soluble chemical oxygen demand (SCOD) throughout the entire experimental work. Three sets of experiments each having different initial SCOD concentration were carried out to observe the performance of batch reactor in treating real life tannery wastewater in respect of both carbon oxidation with a constant chromium concentration (Cr(VI) = 20 mg/L). The time course of carbon oxidation is shown in following plots (Fig-8.17 to Fig-8.19). The tests were carried out for a detention period of 96 hours. In the case of present phase of experiment, carried out with an initial SCOD of 565.5 mg/L at 96 hours react period, it has been observed that the major fraction of SCOD removal (85.71%) took place within 48 hours of reaction time. In case of initial SCOD of around 1120 mg/L an almost similar trend of SCOD removal has been achieved but at 72 hours of the reaction period. When the react-period was 75.0 hours carbon oxidation was found to be maximum (more than 70%) for initial SCOD of around 1474.4 mg/L. However, in totality, at the end of 96.0 hour of total react period, about 85 ± 6% of SCOD removal has been observed under identical input substrate condition.

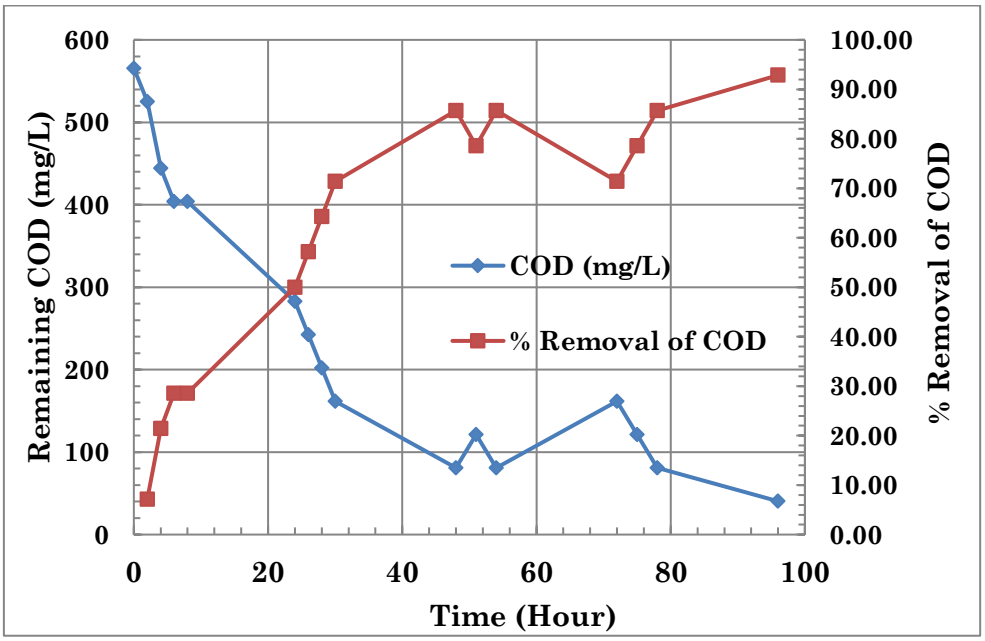


Fig.8.17: COD Removal Profile (SCOD = 565.6 mg/L, Cr(VI) = 20 mg/L)

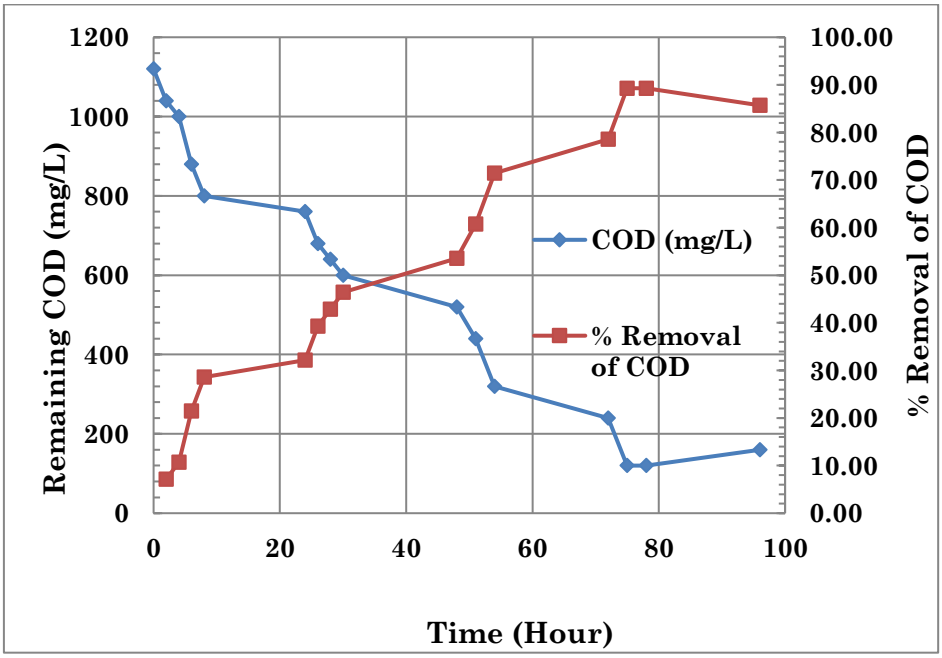


Fig.8.18: COD Removal Profile (SCOD = 1120 mg/L, Cr(VI) = 20 mg/L)

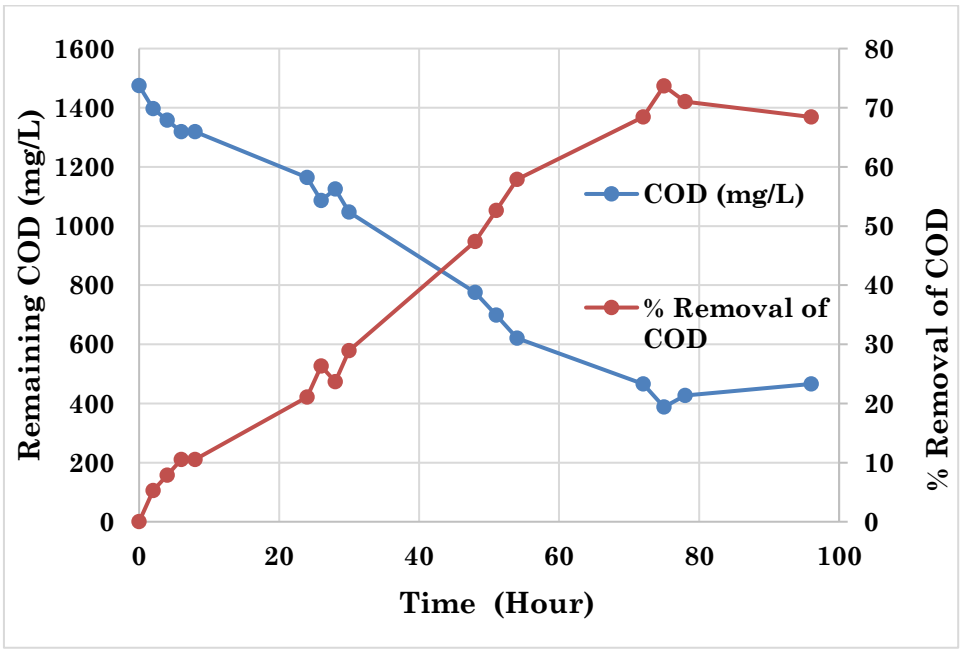


Fig.8.19: COD Removal Profile (SCOD = 1474.4 mg/L, Cr(VI) = 20 mg/L)

8.4.3.2 pH and MLVSS Profiles for Carbon Oxidation

The pH and MLVSS values of a biological system respond to microbial reactions and areconsidered to be significant aspects for microbial growth. Hence, the variation in pH and MLVSS often provides a good indication of the ongoing biological reactions. Rise and fall of pH in the reactor due to metabolic activities was monitored throughout the study period. The variation of pH and MLVSS of three sets of studies are showed in Fig 8.20 to Fig 8.22. It was observed that the pH was subsequently increased with time for all sets of experiments. It increased from average 7.5 to 9.2 at the end of the react period. Since the effluent was rich of carbonate salts (high carbonate hardness, Ref: Table 8.5) on aeration the wastewater underwent the reaction given below and pH of the medium thus increased.

$$(HCO_3)^- \longrightarrow CO_2(g) + (OH)^-$$

References showed chromium resistant bacteria can reduce Cr(VI) at pH from 7.5 to 9.0 ***(Mary Mangaiyarkarasi et al., 2011, Masood and Malik, 2011, Sau et al., 2008).*** The isolated bacteria of the present study *B. mycoids*also showed the similar observation discussed in chapter 5 under section 5.3.1.2. Hence, increase in pH has minimal effect on the growth of bacteria. A steady increase in MLVSS level showed in the following figures (Fig-8.20, Fig-8.21 and Fig-8.22) clearly demonstrate the continual microbial activity and resultant healthy microbial growth in the reactor. For all sets of study MLVSS were within the limit of (1800 – 3500) ±100 mg/L. However, the overall increase in MLVSS with increasing trend of pH, for all three sets of studies establish the effective existence of the microbial activity for organic carbon oxidation.

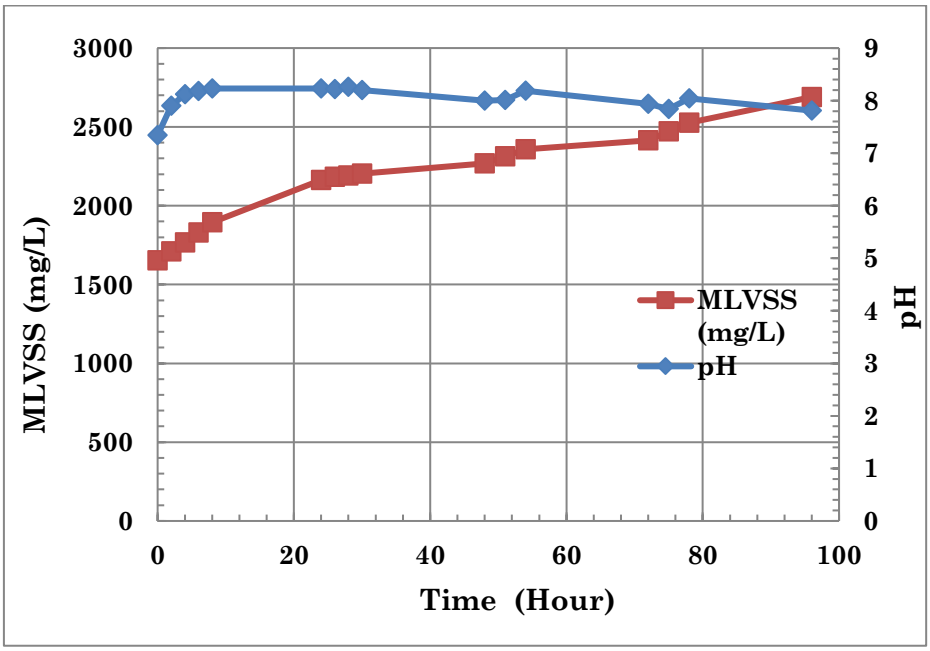


Fig. 8.20: pH and MLVSS Profile (SCOD = 565.6 mg/L, Cr(VI) = 20 mg/L)

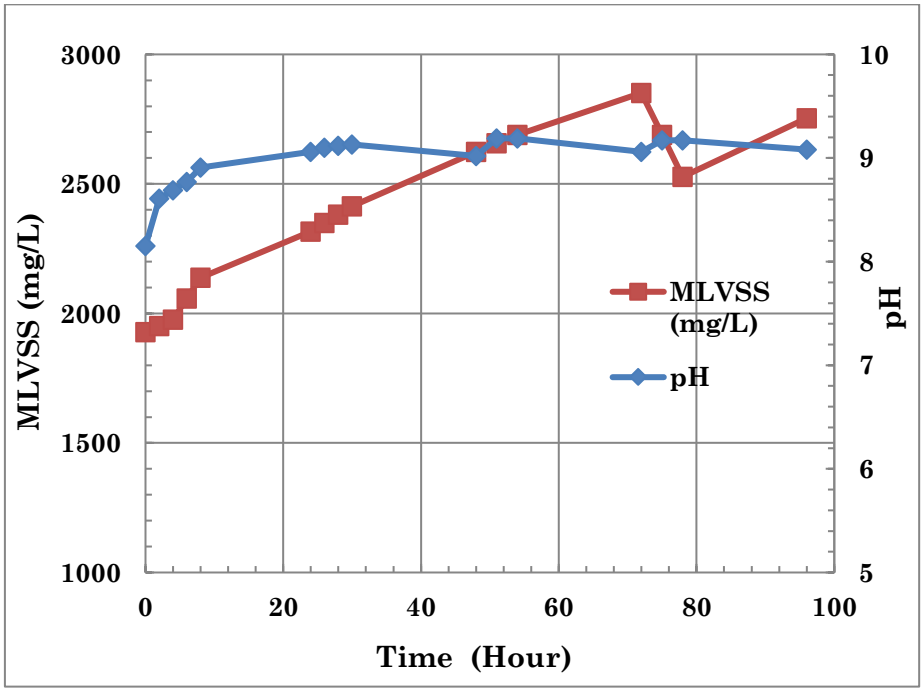


Fig.8.21: pH and MLVSS Profile (SCOD = 1120 mg/L, Cr(VI) = 20 mg/L)

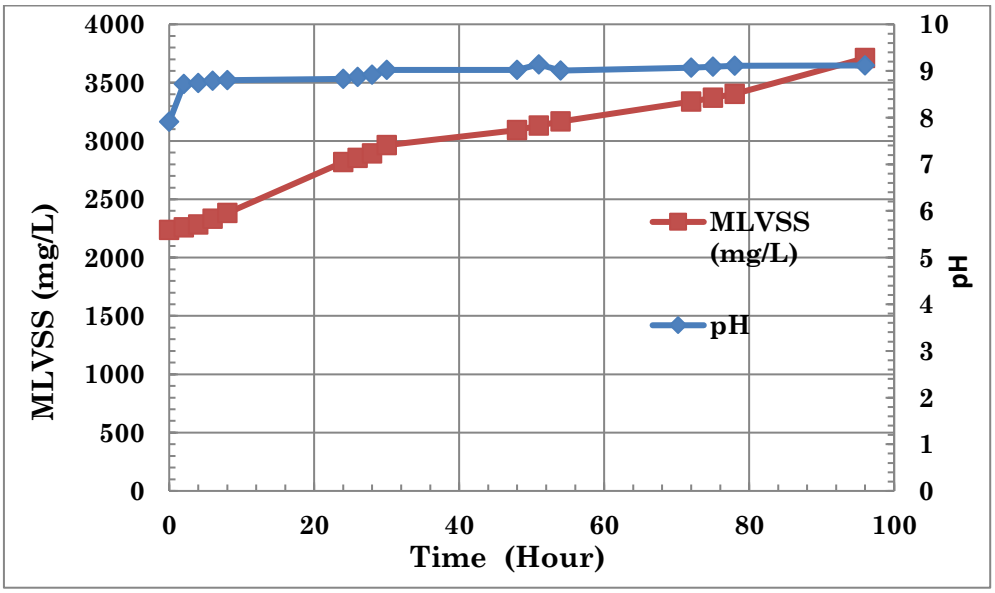


Fig. 8.22: pH and MLVSS Profile (SCOD = 1474.4 mg/L, Cr(VI) = 20 mg/L)

8.4.3.3 Dissolved Oxygen (DO) Profile for Carbon Oxidation

The DO has been found to be a very useful parameter for monitoring any aerobic biological system. In the present study, the DO varies throughout the entire reaction eriod for all three SCOD concentrations. Initially, DO gets reduced for higher SCOD values (Fig-8.23) which may also be utilized by the different inorganic substances present in the composite tannery effluent. However, it is observed that, in all three sets after 78 hours of the contact period, DO remains constant.

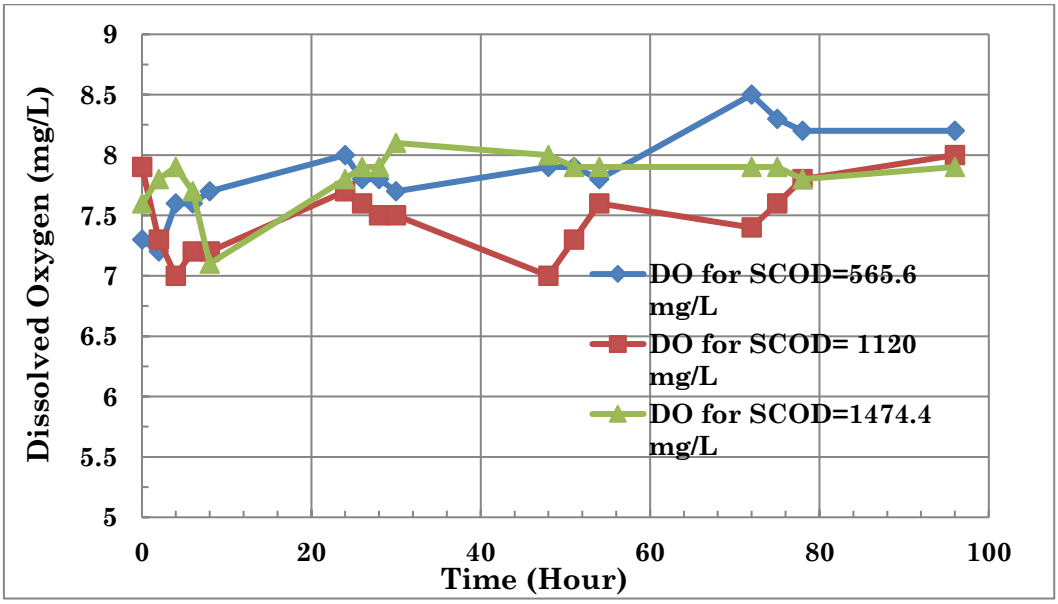


Fig. 8.23: DO Profile for Variable SCOD

8.4.3.4 Chromium Removal Profile for Batch Reactor

The performance study for chromium removal in batch reactor was carried out with primary treated tannery wastewater with variable Cr(VI) concentration but at fixed SCOD value. Cr(VI) concentration was varied from 10 – 50 mg/L while the SCOD was kept constant at around 1100 mg/L. Experiments were conducted for a reaction period of 96 h at 28 ± 2 °C. The chromium removal profiles are presented in Fig- 8.24 and Fig- 8.25. Results showed that the pure culture microbial population was able to reduce chromium in the composite tannery effluent. The maximum removal efficiency was obtained as 75.52% for Cr(VI) = 10 mg/L and minimum 45.5% for Cr(VI) = 50 mg/L. A gradual decrease is noted while chromium concentration is increased. It is already discussed in chapter 5 under section 5.3.1.1 that the bacteria *B. mycoids* can reduce more than 92% of 100mg/L Cr(VI) in synthetic medium at optimum temperature condition of 37 °C. It is also discussed in chapter-7 under section 7.4.1 that the removal of Cr(VI) is typically enzyme mediated. However, reactivity of an enzyme is highly influenced by temperature of the medium. Therefore, the results obtained in this phase of study may be due to prevalence of low temperature during the experiments.

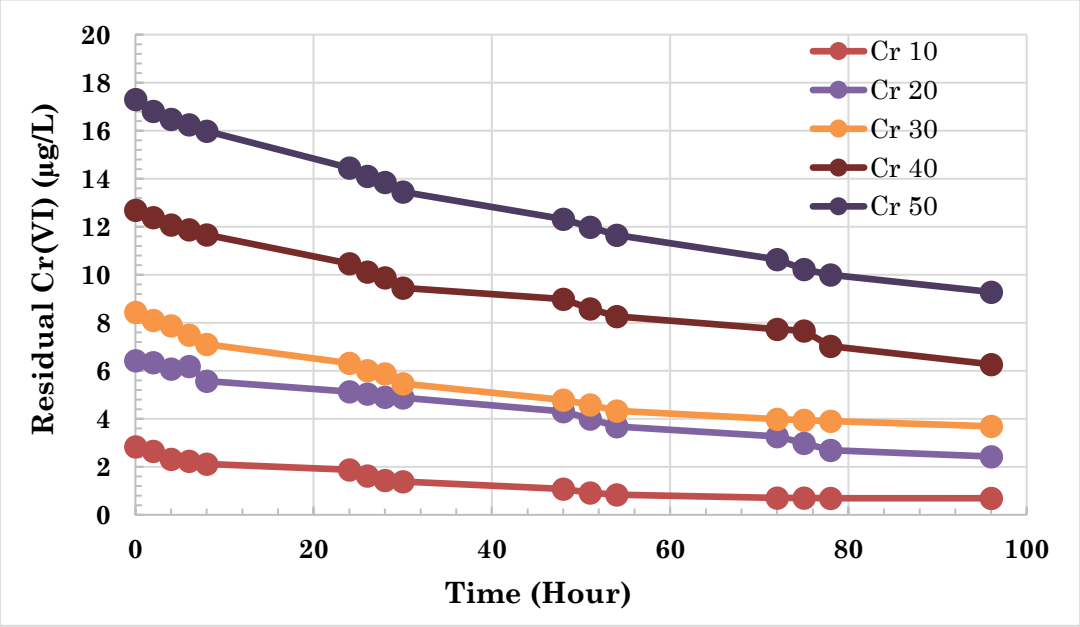


Fig- 8.24: Variation in Chromium Concentration with time at ambient temperature of 28 °C

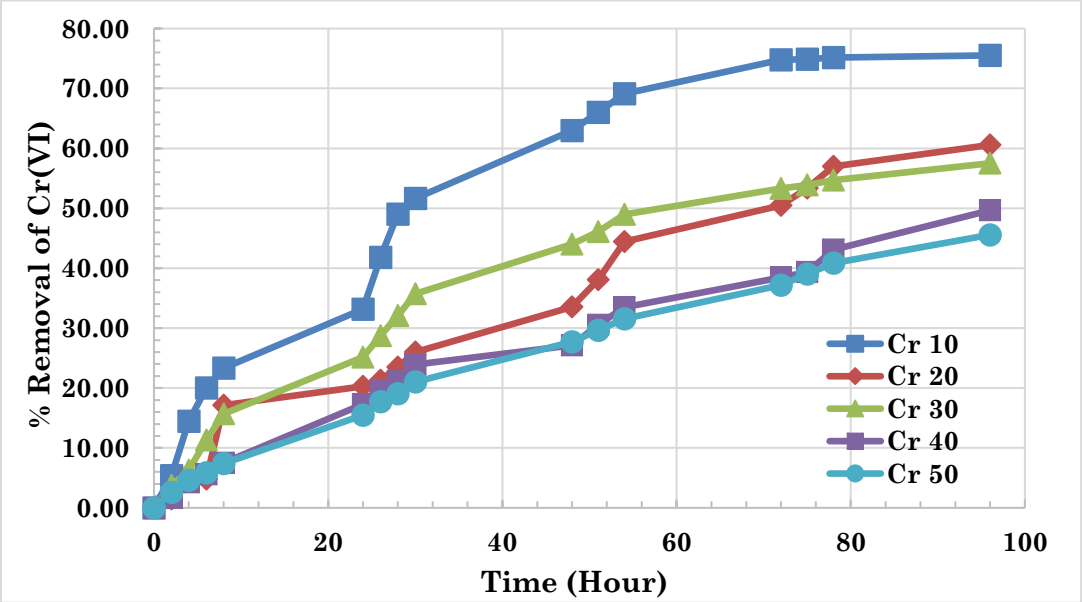


Fig-8.25: Variation in % Chromium Removal with time at ambient temperature of 28 °C

8.4.3.5 pH and MLVSS Profile for Chromium Removal

The pH and MLVSS are important parameters for bio-removal of chromium as well as for chromium reduction. The variation of MLVSS and pH for five sets of studies are showed in Fig 8.26 to Fig 8.30. pH of the reactor content subsequently increased from 7.2 to 9.0 at the end of the reaction period for all five sets of experiment. As it is already discussed that *B. mycoids* can grow even at higher pH and also able to reduce Cr(VI) at higher range of pH (Chapter 5 section 5.3.1.2), the variation in pH has least effect on Cr(VI) removal efficiency of the bacteria in the reactor system. The MLVSS levels steadily increased in all sets of experiments within the limit of (1650 – 3100) ±100 mg/L. However, the overall increase in MLVSS with increasing pH for all sets of studies establishes the effective occurrence of the microbial activity for bio-reduction of chromium.

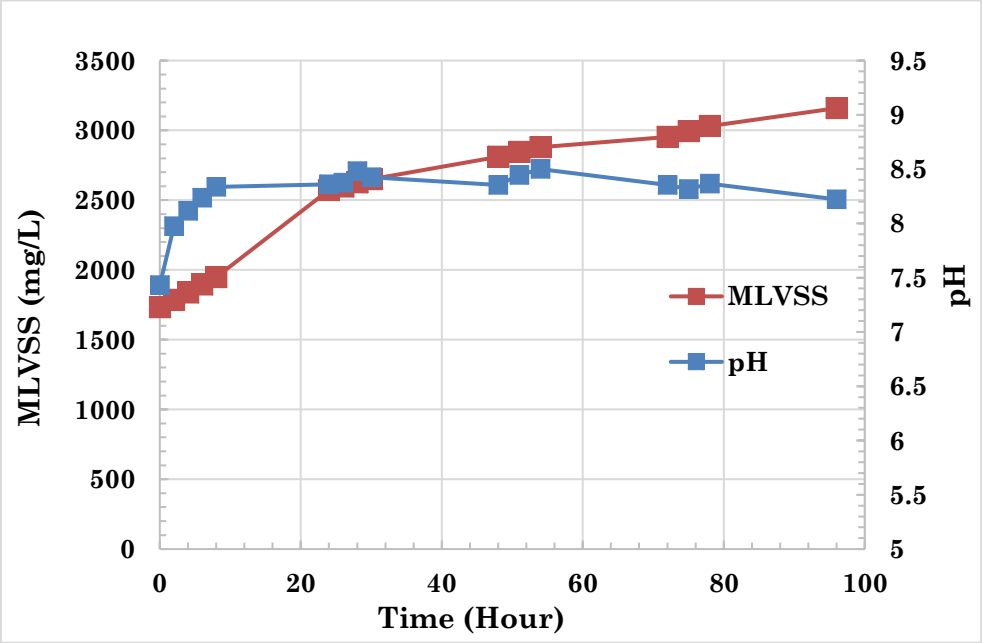


Fig- 8.26: pH and MLVSS Profile (SCOD = 1120 mg/L, Cr(VI) = 10 mg/L)

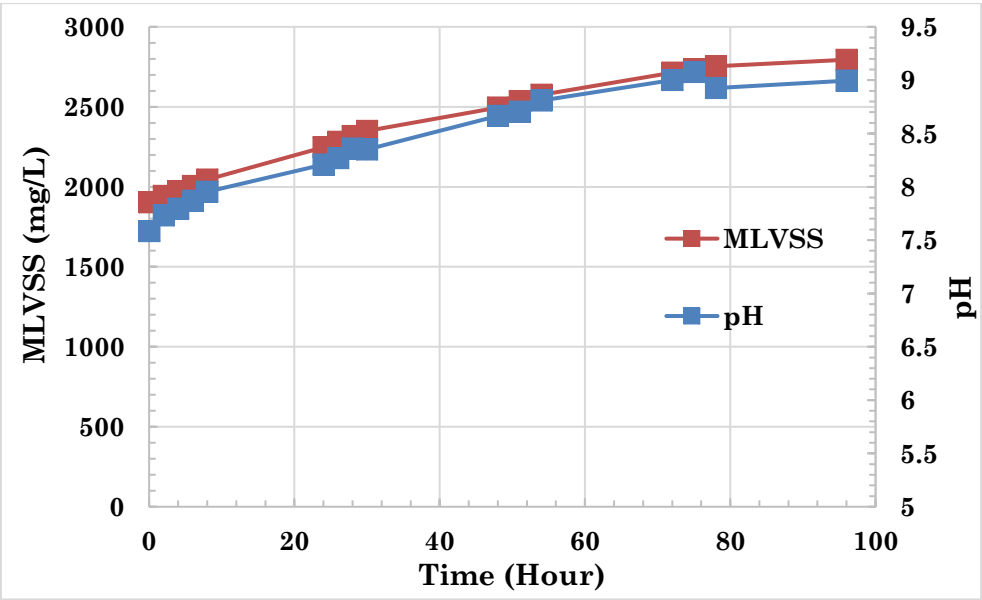


Fig. 8.27: pH and MLVSS Profile (SCOD = 1120 mg/L, Cr(VI) = 20 mg/L)

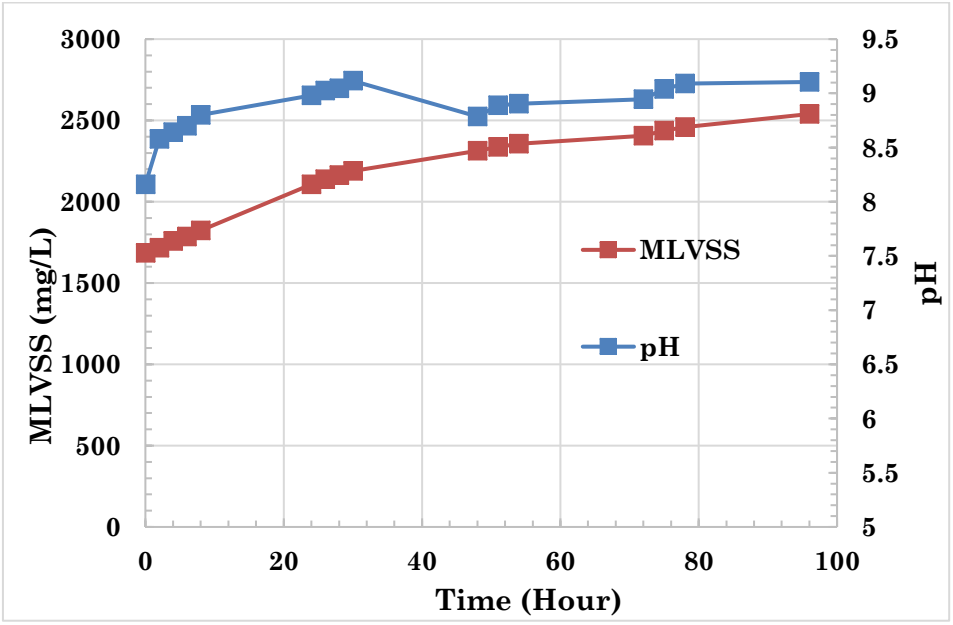


Fig. 8.28: pH and MLVSS Profile (SCOD = 1080 mg/L, Cr(VI) = 30 mg/L)

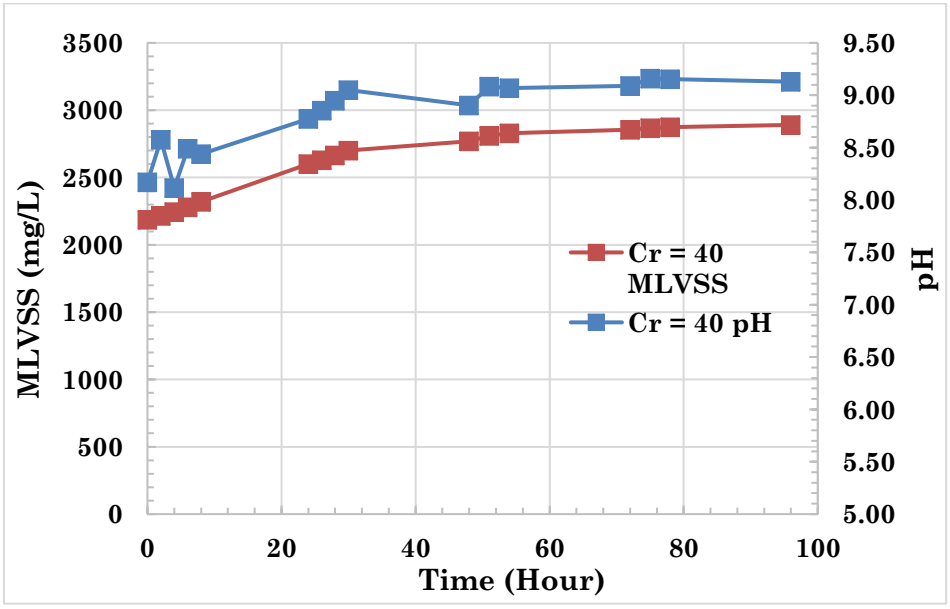


Fig. 8.29: pH and MLVSS Profile (SCOD = 1080 mg/L, Cr(VI) = 40 mg/L)

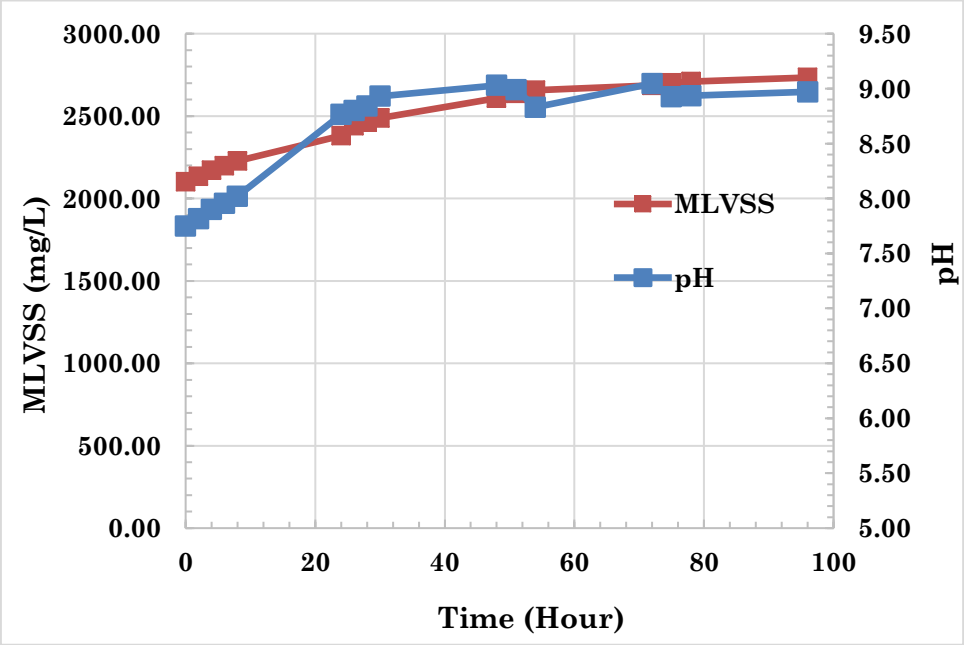


Fig. 8.30: pH and MLVSS Profile (SCOD = 1180 mg/L, Cr(VI) = 50 mg/L)

8.4.4 Artificial Neural Network (ANN) Analysis

The ANN approach of a multilayer network was established to predict the Cr(VI) removal in the treatment of tannery wastewater in a batch reactor using a pure culture bacterial isolate, *B. mycoids*. ANN modelling was done for the data obtained from performance evaluation of chromium removal in the batch reactor. The network consisted of six parameters viz. pH, MLVSS, initial Cr concentration, temperature, COD and retention time. The output of the system was the removal of chromium. The training phase of the network was done with Levenberg-Marquardt back propagation method using the trainlm function. 80% of data was taken into the training phase and 20% was used in the test phase. The aim was to obtain the minimum "Sum of Square Error (SSE)". The activation function from input layer to hidden layer was varied by using tangent sigmoid function (tansig) function and logarithmic sigmoid function (logsig). The activation function from hidden layer to output layer was taken as linear function (purelin) for all cases. The predicted result was found optimum when logsig-pureline combination was used and number of neurons was 18. The performance of the proposed model was evaluated by analysing the ANN predicted value of Co-efficient of determination (R^2), which should be closest to 1 among all the iterations performed for the number of neurons, shown in Fig-8.31 and 8.32. The graph shows that an R^2 value of 0.9683 was obtained for testing phase and that for training phase of 0.992.

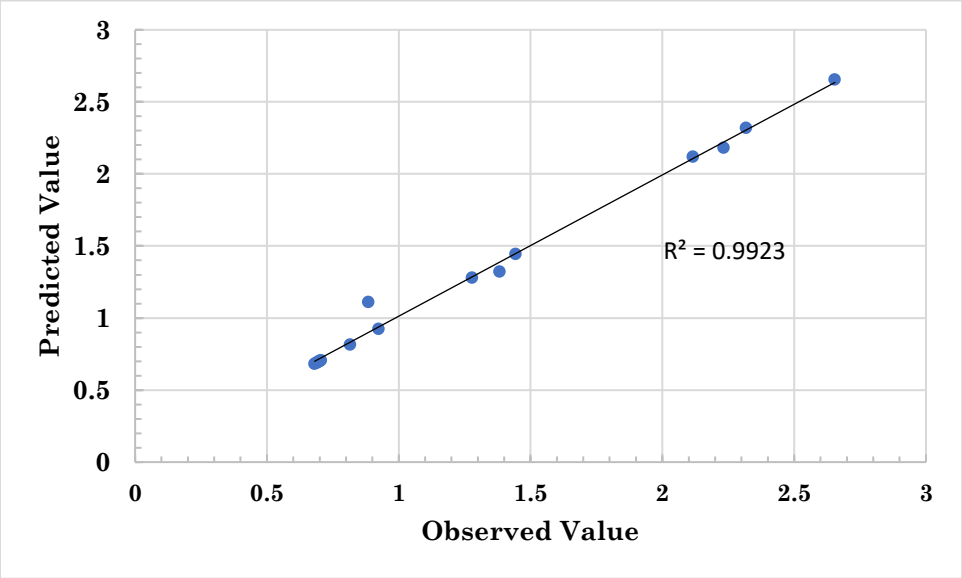


Fig.8.31. Predicted vs. Observed values using ANN (for Training Phase)

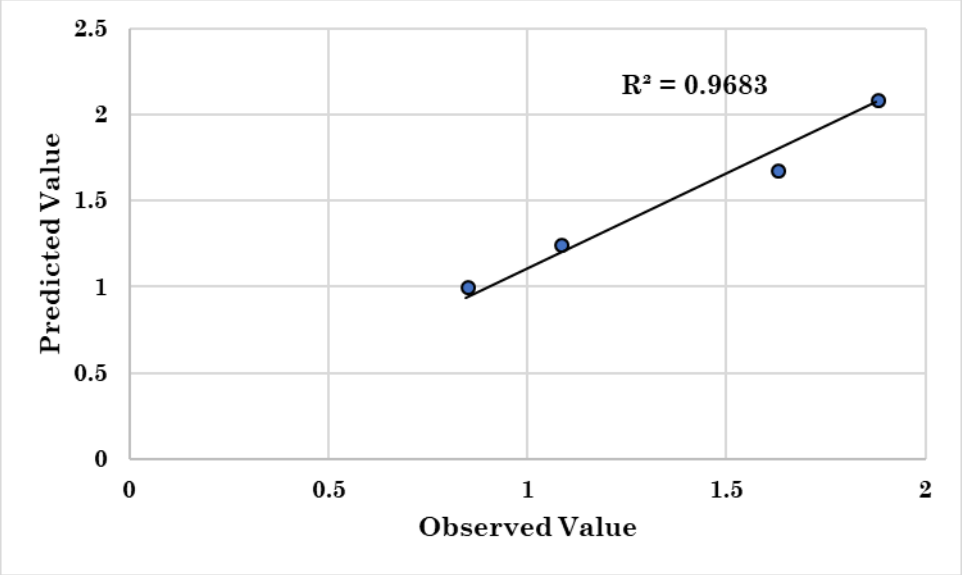


Fig.8.32. Predicted vs. Observed values using ANN (for Testing Phase)

Conclusion & Future Scope of Work

9

9.1 Conclusion

The present research investigation has been conducted with the objective to isolate appropriate chromium resistant microbial strains and to identify its resistance mechanism under optimized condition. The performance of the isolated bacterial strain was assessed in a lab-scale batch reactor for combined removal of biodegradable organics (COD) and chromium from tannery wastewater.

The following conclusions can be drawn on the basis of the outcomes of different phases of experimental works and analysis of results.

- ***Isolation and characterization of chromium resistant bacteria***

  - ✓ The total 24 bacterial isolates showing Cr resistance with different types of colonies in the bank soil samples enriched with chromium (37085.82 ± 1124.183 mg/kg) were identified.
  - ✓ MIC of the isolates was observed within a range of 250 to 450 mg/L of Cr(VI) as $K_2Cr_2O_7$.
  - ✓ Based on the chromium removal efficiency 7 distinct microbial strains are selected and characterized morphologically.
  - ✓ A gram-positive chromium resistant bacteria strain CRB 1 was selected for further detail study since it showed no significant changes on growth with and without chromium.

- ***Optimization of parameters of growth medium and molecular characterisation of the selected isolate***

  - ✓ The selected chromium resistant bacterium was identified as ***B. mycoids*** based on 16S rRNA gene sequencing.
  - ✓ The optimum growth condition can be summarized as pH 7.5, temperature 37 ˚C, salinity 1% and shaking velocity at 140 rpm. It has been observed that biomass growth and chromium reduction ability was not substantially changed at higher pH range. Therefore, this strain is to be applied for removal of Cr(VI) from wastewater of alkaline range.

- ✓ The total protein content was not changed so far, with or without treatment of Cr(VI). This observation referred that protein synthesis by the bacterium was least effected due the presence of Cr(VI).
- ✓ SDS PAGE profile of cytosolic proteins showed significant bands of two proteins of molecular weight around 72 kDa and 26 kDa for both samples treated with and without chromium.

- ➢ ***Characterization of the reduced product***

- ✓ The reduced product was characterized by AAS, SEM-EDX and FTIR. AAS analysis revealed that Cr(VI) is immobilized within the cell either its native oxidation state(Cr(VI)) or in reduced state.
- ✓ SEM-EDX study manifested the presence of chromium in the treated sample. This could be due to deposition of Cr(III) hydroxide on the cell surface or due to formation of an intermediate complex of Cr(III) species. Another possible reason could be formation of extracellular polysaccharide to prevent the exposure of chromium.
- ✓ In FT-IR study, substantial changes in peak intensity and peak shift were observed for the bacterial sample treated with Cr(VI) and compared to the sample grown without Cr(VI).
- ✓ It is evident from the FTIR spectra of the microbial samples, that key functional groups are carboxyl, amino and phosphate which are major constituents of gram-positive bacterial cell as well as the main contributors to the formation of metal bonds, in this case Cr – O.

- ➢ ***Understanding mechanism behind chromium mechanism***

- ✓ The chromate reductase assay supported that the Cr(VI) get reduced by reductase enzyme and NADPH acted as electron donor for the aerobic direct Cr(VI) reduction.
- ✓ While on addition of NADPH to CFE the activity increased by 9.17 folds and 13.07 folds when Cr(VI) concentration was 0.32 mM and 0.67 mM respectively.
- ✓ It was observed that the chromate reductase activity in CFE was similar for the cells grown in medium in presence or absence of Cr(VI). This observation indicated that the chromate reductase property was not induced by Cr(VI). This was further substantiated by the protein profile obtained for the bacteria.
- ✓ From, Lineweaver–Burk double reciprocal plot k_m and V_{max} values were calculated to be 65.42 and 9.514 (R^2=0.989), respectively for removal of Cr(VI) from synthetic growth medium at 37 ˚C.

- ✓ The reduction of Cr(VI) to Cr(III) has been observed to follow first order reaction kinetics and rate constant was calculated as k= - 1.09 X 10-4 / sec. The half-life (t_{half}) was calculated as 1.77 h.
- ✓ SOD activity, catalase activity, glutathione reductase and peroxidase activity were assessed.
- ✓ Notable increase in SOD activity, catalase activity and reduced glutathione reductase were found. These observations supported that Cr(VI) entered inside the cell and reduced to Cr(III) as ROS is induced by the intermediates like Cr(V) and/ or Cr(IV).
- ✓ Another stress marker peroxidase activity showed declining nature and it is indicative that presence of Cr(VI) may suppress the activity of peroxidase.
- ✓ *B. mycoids* also produced EPS and it may help to remove Cr(VI) in medium.

- ➢ ***Batch kinetic study of pure culture with tannery wastewater***

- ✓ The average BOD_5: COD ratio obtained was 0.323 for primary treated composite tannery effluent.
- ✓ At the time of treatment of primary treated tannery effluent, the SCOD removal was found to vary 68-95% for a fixed Cr(VI) concentration and for Cr(VI) removal it was 45 -75 % for a fixed COD value over the retention period of 96 hours.
- ✓ Bio-kinetic coefficients for the pure culture have established satisfactory biodegradation of soluble organics present in real-life primary treated tannery effluent and their values are reasonably comparable with those observed for municipal wastewater.
- ✓ The marginal inhibitory effect of chromium uptake on substrate utilization is envisaged from decreasing values of Y and μ_{max} and increasing values of k_d with the increase in initial Cr(VI) concentration of 10 to 50 mg/L.
- ✓ The inhibitory effect of chromium uptake is also obvious from the increasing values of Chromium Inhibition Constants (Ki), which varied from 0.182 to 0.518 mg of Cr(VI)/L for the initial Cr(VI) concentration of 10 to 50 mg/L.
- ✓ ANN model was developed with the observed data from experiments. The predictive statistic (R^2) generated by the selected iteration of the model was satisfactory.

In a nutshell the present study can be summerised as *Bacillus mycoids*, a Cr(VI) resistant bacteria, isolated from tannery effluent feeded soil sample was capable of removing 98.4% of Cr(VI) in 24 h at optimum growth conditions of pH 7.5, temperature 37 ˚C, 140 rpm and at initial Cr(VI) concentration of 100 mg/L. The mechanism behind thia removal was identified as bio-reduction of Cr(VI) to Cr(III) by chromium reducing enzyme and NADPH was the electron donor in this reduching pathways of Cr(VI) to Cr(III). The reducing

enzyme was not induced in the metabolism of the strain due to the stress of Cr(VI). This was evivent clearly from protein profile of the bacterium. It was also observed that the reduction process followed first order reaction kinetics. The stress enzyme analysis supported the presence Cr species inside the cell which caused stress in the cytosolic system. The SEM-EDX study showed the changes on the perifery of the cell occurred due to presence of Cr(VI) and the FTIR study also supported these observations. During the performance study of this resistant strain with real life wastewater i.e. primary treated tannery effluent, the bacterium showed satisfactory results and statistically approved.
To develop an efficient strategy on chromium bioremediation, a better understanding of allthe above factors is required. Further research is recommended at the lab scale, bench scale, at pilot level and in situ studies. Detailed study of the reduction pathways, treatment methods for toxic intermediates if any, and the possibility of formation of more stable products need to be explored. The scope for application ofprotein engineering and genetic engineering to further augment this research is there, which has the potential to take this technology to surge ahead of other developments in this field.

9.2 Future Scope of Work

The present research work can be explored in future in greater depth as suggested below.

- Study on purification and modification of chromate reductase enzyme which certainly provide new potentialities into biochemical industry.
- Study on development of the isolate as bio-indicator of chromium polluted environment.
- Study on the interaction of exopolysaccharide (EPS) with heavy metals.
- Study on the performance evaluation of this bacterial strain with other chromium contaminated industrial effluents.
- Study on continuous mode operation of bioreactors with the isolated bacteria.

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Annexure - I

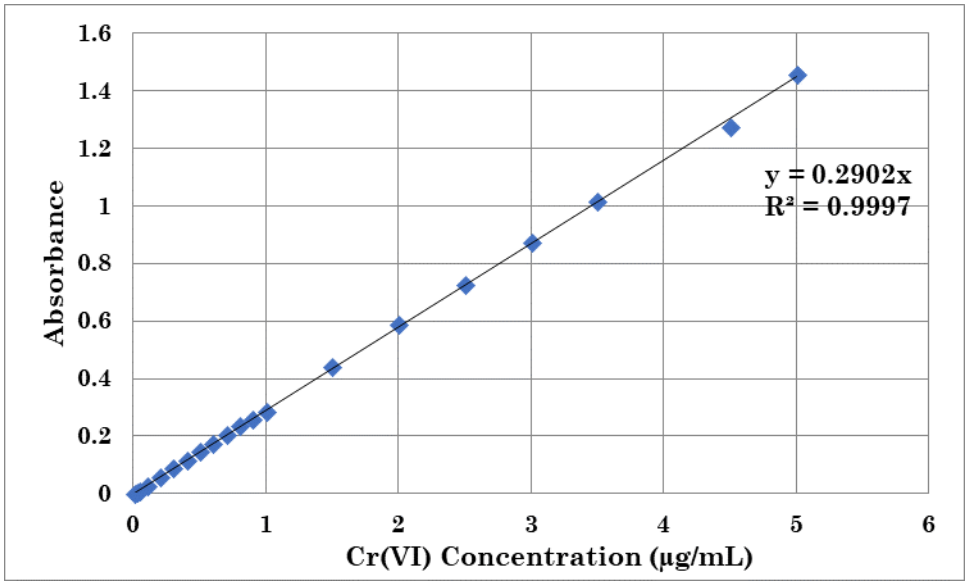

Fig- A: Standard Curve of Cr(VI) Determination

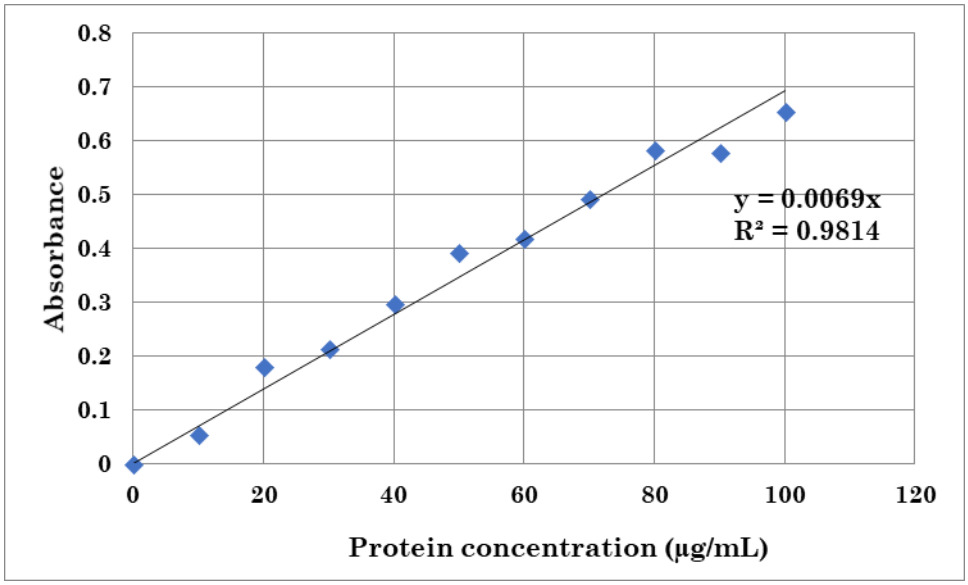

Fig- B: Standard Curve for Total Protein Estimation (*Lowry et al., 1951*)

ANNEXURE - II

ANSWER TO THE REVIEWERS' COMMENTS ON THE THESIS SUBMITTED

A) Objective and Scope

Reviewer 1: The scope of work is complying with the requirements, but should have been detailed in few cases especially for the modeling with ANN.

- The modeling with ANN in the scope of work is modified.

Reviewer 2: No such modification has been suggested.

B) Originality of the Problem

Reviewer 1: No comment

Reviewer 2: The originality of the problem can be presented clearly in separate paragraph.

- The originality of the problem has been discussed in "Green Area of Research". It is further modified as advised.

C) Literature Research and Understanding of the Prior Art

Reviewer 1: Literatures from journal/book sources have been understood by the candidate, but those are demonstrated discretely in some cases. It would have been better, if there are some "critical review" sections on different subject matters.

- Literature review is revised and rearranged accordingly. A separate paragraph on "critical review" has been included.

Reviewer 2: Include some more recent work reference.

- Recent work references are included in chapter-2 (review of literature), chapters 4, 5, 6, 7 and 8.

D) Materials and Methods

Reviewer 1: There is no dedicated sections entitled "Materials and Methods" and therefore a separate discussion on the "material components" is missing.

- The entire work has been segmented in six phases as the work deals with both biological as well as engineering aspects. The first four phases mostly covered the biological work and the last phase is focused on its engineering aspect. It has been done to avoid unnecessary repetitions of methods in biological work and at the same time to understand the engineering work that has entirely different set-up. This is the reason behind not incorporating a dedicated chapter on "Materials and Methods".

Reviewer 2: No comment

E) Results and Discussion

Reviewer 1: Sometimes with lack of information like legend and notifications.

- All graphs and figures are corrected with proper legends and notifications.

Reviewer 2:

i) The quality of most figures can be improved.

- All figures are freshly printed on glossy paper to improve its quality.

ii) The size of the equations in some pages are not uniform.

- The size of equations is made uniform throughout the entire thesis.

iii) Replace * in page 3, 122 (for multiplication sign)

- It is replaced.

iv) Some typological mistakes also need corrections.

- Typological mistakes are corrected accordingly.

F) Conclusions and new findings

Reviewer 1: New outcomes should be separately highlighted. Moreover, some conclusions could be drawn on the basis of overall perspective of the present study.
Reviewer 2: The new findings can be presented clearly in separate paragraph.

- A separate paragraph with new outcomes has been added in the "Conclusion Chapter" i.e. Chapter-9.