STUDIES ON HEXAVALENT CHROMIUM REDUCTION BY *Streptomyces* species: MOLECULAR, BIOCHEMICAL AND BIOTECHNOLOGICAL ASPECTS

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DEDICATION

I dedicate this thesis to my family without whose loving support I could never have made it this far. To my parents, who gave me the world, and taught me to go after my dreams, no matter how crazy they may seem. To my mother, for encouraging her daughter to be everything and anything she wanted, and then telling me to do it better than anyone else. To my father and to my brothers, thanks for all the love and laughs we have had through the years. Without that I couldn't have made it. Especially, I would like to give my special thanks to my husband whose patient love enabled me to complete this work and my darling daughter Avantika whose joyful ways saw me through the tough times.

SPECIALLY DEDICATED TO

MY MOTHER

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Ashwini C Poopal

CERTIFICATE

Certified that the work incorporated in the thesis

STUDIES ON HEXAVALENT CHROMIUM REDUCTION BY *Streptomyces* species: MOLECULAR, BIOCHEMICAL AND BIOTECHNOLOGICAL ASPECTS

Submitted by **Ms. Ashwini C Poopal** was carried out under my supervision at the Division of Biochemical Sciences, National Chemical Laboratory, Pune, India. Material obtained from other sources has been duly acknowledged in the thesis.

February 2010

Dr. (Mrs) Mala Rao
(Research Guide)

Dr. (Mrs) R Seeta Laxman
(Research Co-guide)

DECLARATION BY THE CANDIDATE

I declare that the thesis entitled **"STUDIES ON HEXAVALENT CHROMIUM REDUCTION BY S*treptomyces* species : MOLECULAR, BIOCHEMICAL AND BIOTECHNOLOGICAL ASPECTS"**, submitted by me for the degree of Doctor in Philosophy is the bonafide record of the work carried out by me in the Biochemical Sciences Division, National Chemical Laboratory, under the guidance of **Dr. (Mrs) Mala Rao** and **Dr (Mrs) R. Seeta Laxman** and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other university or other institute of higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

February 2010 **Ashwini C. Poopal**

ABBREVIATIONS

Cr(III)	Trivalent chromium
Cr(VI)	Hexavalent chromium
CFE	Cell Free Extract
DNA	deoxyribonucleic Acid
DDSA	Dodecynyl succinic anhydride
DEAE	Diethyl amino ethyl cellulose
DMP30	tridimethylaminomethyl phenol
DPC	Di-phenylcarbazide
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EPR	Electron Paramagnetic Reasonance
GLP	Giant Linear Plasmid
MNA	Methyl nadic anhydride
NADH	Nicotinamide adenine dinucloetide (reduced)
NADPH	Nicotinamide adenine dinucloetide- phosphate (reduced)
NCIM	National Collection of Industrial Microorganisms
ORP	Oxido-reduction potential
PAGE	Polyacrylamide gel electrophoresis
PFGE	Pulse Field Gel Electrophoresis
ROS	Reactive Oxygen species
SDS- PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SRB	Sulphate Reducing Bacteria
TEM	Transmission Electron Microscopy
ppm	Parts per million
ppb	Parts per billion
PVA	Polyvinyl Alcohol
USEPA	United States Environmental Protection Agency

ABSTRACT

Industrialization is a hallmark of civilization, however, industrial emanations have been adversely affecting the environment, leading to destruction of agricultural lands and water bodies, thus becoming a matter of great concern. Chromate (Cr) waste is generated from many industrial applications such as leather tanning, textile production, etc. The permissible concentration for discharge into water bodies is < 1ppm. Alternative methods for reducing Cr discharge are being explored because conventional methods are either expensive or unable to bring the metal concentration to permissible levels and may generate contaminated sludge. Biological methods like biosorption /metal transformation are attractive, less energy intensive and produce no secondary by-products.

Cr(VI) is mutagenic and carcinogenic, it can be reduced to Cr (III) which is less toxic and less soluble than Cr(VI). There are very few reports on chromate reduction by actinomycetes. Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* account for 90% of the total actinomycetes. Being natural inhabitants of soil, water and manure, actinomycetes are continuously exposed to different metals present in these habitats. In order to survive in the polluted environment, they have to adapt or acquire tolerance or resistance to these metals. Their metabolic diversity, mycelial growth characteristics, and their capability to rapidly colonize a variety of substrates make them the most suitable agents for bioremediation of metal and organic compounds. In view of the paucity of information on chromate tolerance/ reduction/resistance in actinomycetes, it was important to assess the predominance of chromate reductase activity among actinomycetes, knowledge regarding which will be useful in understanding its potential utility for bioremediation of Cr(VI) contaminated industrial effluents. After extensive screening of actinomycetes for chromium toxicity and reduction a strain of *S. griseus* (NCIM 2020) was selected which was tolerant to Cr(VI) and was found to be an efficient Cr(VI)-reducing organism.

Chapter 1. General Introduction

The ***first chapter*** is general introduction of the thesis and it gives brief review of literature on chromium history, distribution, pollution sources, chemistry, permissible levels, Indian and International perspective, chromium interactions as well as the research carried out on Cr(VI) resistance in bacteria, Cr(VI) reduction, its types, mechanisms, pathways of Cr(VI) reduction, and scope of Cr(VI) reduction in actinomycetes.

Chapter 2. Studies on chromate tolerance and reduction in actinomycetes

The ***second chapter*** deals with screening of actinomycetes and identification of potential chromate reducing strain. Among the actinomycetes tested for chromate tolerance and reduction, thirteen strains reduced Cr(VI) to Cr(III), of which *S. griseus* (NCIM 2020) was most efficient showing complete reduction of 25 mg/L of Cr(VI) within 24 h. Effect of initial chromium concentration indicated that the organism was tolerant up to 40 mg/L of Cr(VI) with no significant inhibition of growth. Reduction was nearly complete within 12 h when the initial Cr(VI) concentration ranged between 10-15 mg/L while it was slower and required longer incubation time of 36-48 h with higher initial Cr(VI) concentration. Rate of reduction (mg Cr(VI) reduced/L/h) by *S. griseus* increased linearly with increasing Cr(VI) concentration but decreased with incubation time and the highest rate of reduction was 2.85 mg Cr(VI) reduced/L/h during the initial period of 12 h. with initial chromate concentration of 89 mg/L. To the best of our knowledge, this is one of the highest rates reported so far among actinomycetes. The organism was able to use a number of carbon sources as electron donors. Maximum Cr(VI) reduction was observed in presence of glucose followed by glycerol and acetate. No reduction was observed in the absence of added electron donor or in presence of glycine indicating the requirement for an electron donor for growth and reduction Sulphate, nitrate, chloride and carbonate had no effect on chromate reduction while cations such as Cd(II), Ni(II), Co(II) and Cu(II) were inhibitory to varying degrees. Resting cells of *S. griseus* completely reduced 25 mg/L of Cr(VI) in presence of 0.4-1.0 mM Cu(II) in 3-6 h, thus greatly enhancing reduction compared to control without Cu(II) which required 24 h. The metabolic inhibitor sodium azide partially inhibited Cr(VI) reduction.

During growth of the organism Cr(VI) which was yellow in colour turned bluish green after conversion to Cr(III) and a fraction of it was taken up by the biomass giving it a bluish tinge. *S. griseus* cells treated with and without 50 mg/L of Cr(VI) for 48 h were used for transmission electron microscopy (TEM). TEM studies indicated presence of chromium in the biomass.

Chapter 3. Immobilization of *S. griseus* for chromate reduction

Reduction of toxic Cr(VI) to the less toxic Cr(III) by microorganisms is potentially a useful bioremediation process. ***Chapter third*** consists of batch and bioreactor studies using immobilized *S. griseus* cells for chromate removal by reduction.

Batch Studies

This section deals with chromate reduction by free and immobilized *S. griseus* cells in batch studies. As the Cr(VI) reduction was associated with the cell, Cr(VI) reduction by free as well as immobilized cells was studied. Performance of the various matrices with respect to bead integrity and reduction was studied. Free and PVA-alginate, agarose-immobilized cells completely reduced 25 mg/L of Cr(VI) in 18–24 h. But reduction did not go to completion even after 96 h by PVA-nitrate and PVA-borate-immobilized cells. Both these beads were unstable and disintegrated within 24 h. No metal reduction was observed in absence of cells. (beads without cells). Among the matrices tested PVA-alginate had the highest (100%) Cr(VI) removal efficiency in 24 h with reduction rates similar to free cells and hence used for further studies. Immobilized cells completely reduced 25 mg/L of Cr(VI) in 24 h. PVA-alginate immobilized cells could be reused four times to completely reduce 25 mg/L of Cr(VI) in 24 h each time. Chromate in a simulated effluent containing Cu(II), Mg(II), Mn(II) and Zn(II) was completely reduced by PVA-alginate immobilized cells within 9 h. Faster reduction compared to medium containing Cr(VI) alone could be due to stimulatory effect of Cu(II) ions.

Bioreactor Studies

This section deals with continuous removal of chromate by reduction using immobilized *S. griseus* cells in a bioreactor. As PVA-alginate proved to be most effective among the matrices tested during batch studies, PVA-alginate cells were used for

reduction of hexavalent chromium in a bioreactor. Effect of various parameters on reduction such as Cr(VI) and biomass concentrations, flow rate, influent composition etc. were investigated. Efficiency of Cr(VI) reduction decreased with increasing Cr(VI) concentration from 2 to 12 mg/L and increased with increase in biomass concentration. However, increasing the flow rate from 2 to 8 ml/h did not significantly affect Cr(VI) reduction. The reduction was faster in simulated effluent than in synthetic medium and complete removal of 8 mg/L of Cr(VI) from effluent and synthetic medium occurred in 2 and 12 h respectively. Our results indicate that immobilized *S. griseus* cells could have potential application in bioremediation of chromate containing effluents and wastewaters.

Chapter 4. Isolation, partial purification and characterization of chromate reductase

The ***fourth chapter*** deals with isolation, partial purification and characterization of partially purified chromate reductase from *S. griseus*. Chromate reduction was associated with the bacterial cells and sonication was found to be the best method of cell breakage to release the enzyme. The enzyme was constitutive and did not require presence of chromate during growth for expression of activity. Chromate reduction with cell free extract (CFE) was observed without added NADH. However, addition of NAD(P)H resulted in 2-3 fold increase in activity. The chromate reductase activity was heat labile and was lost completely by heating the enzyme at 100°C for 5 min. Fractional ammonium sulphate precipitation of the cell free enzyme showed that major portion of chromate reductase activity was found in 40-60% ammonium sulphate precipitate fraction while part of it was also detected in 20-40% ammonium sulphate precipitate. The 40-60% ammonium sulphate precipitate fraction was used for property studies. Optimum temperature and pH for chromate reductase activity were found to be 28°C and pH 7 respectively. Effect of metal ions on chromate reductase activity showed mercury to be highly inhibitory (relative activity of 21%) but copper enhanced the activity, while no inhibition was observed in presence of sodium azide, Ag(I), Mn(II) etc. Dialyzed 40-60% ammonium sulphate precipitate fraction was applied to DEAE cellulose column and eluted with NaCl gradient (0.1 to 0.5 M) in 25 mM Tris HCl pH 7.0. The enzyme was

eluted with 0.1 M NaCl. The molecular weight of the partially purified enzyme was around 46-48 kDa using 10% non-denaturing SDS PAGE.

Electron paramagnetic resonance (EPR) spectroscopic studies revealed that reduction of Cr(VI) by the cell free chromate reductase with NADH as a co-factor yielded Cr(V) as well as Cr(III) species. The presence of Cr(V) as a transient intermediate in the conversion of Cr(VI) to Cr(III) thus indicating a one-electron reduction as the first step.

The localization of chromate reductase activity was studied by fractionation of the cell free extract using ultracentrifugation. The chromate reductase activity was found to be associated with soluble fraction and no activity was found with the membrane fraction.

Chapter 5. Investigation of the genetic determinants of chromate resistance/ reduction

The ***fifth chapter*** deals with investigation of genetic determinants of chromate resistance/reduction in *S. griseus*. Plasmid DNA isolated from *S. griseus* did not migrate on 0.5% agarose gel electrophoresis. A survey of the total cellular DNA from Cr(VI) reducing *S. griseus* by pulsed field gel electrophoresis showed the presence of giant linear plasmid (200 kb). Transformation of *E. coli* JM109 with purified plasmid DNA resulted in simultaneous acquisition of resistance to chromate by the transformants. Most importantly, the plasmid transfer was found to confer chromate reduction ability on to the *E. coli* transformants. Hence we can conclude that chromate resistance and reduction found to be plasmid mediated in case of *S. griseus*.

Chapter 6. Summary and conclusions

The ***sixth chapter*** deals with the summary and conclusions and future scope of work.

LIST OF PUBLICATIONS

[1] **Ashwini C. Poopal** and R Seeta Laxman (2008) Hexavalent Cr(VI) reduction by immobilized *S. griseus. Biotech. Lett.* **30**, 1005-1010.

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[3] **Ashwini C. Poopal** and R Seeta Laxman. (2009) Studies on enzymatic reduction of chromate by *S. griseus. J. Hazardous Materials.* **169,** 539-545.

[4] Plasmid mediated chromate reduction and resistance in *S. griseus*. **Ashwini C. Poopal**[a], Deepak Patil[b], Yogesh Shouche[b], Mala Rao[a] and R Seeta Laxma[a]. National Chemical Laboratory, b National Centre for Cell Sciences, Pune, India. (Manuscript under preparation).

CONFERENCES/POSTERS/ABSTRACTS

- **Biosorption of heavy metals from aqueous solution by waste fungal biomass**. Presented at 74th Annual Meeting of Society of Biological Chemists, CDRI, Lucknow (INDIA). November 7-10, 2005.

- **Hexavalent chromium reduction by immobilized** *Streptomyces griseus.* Presented at 75th Annual Meeting of Society of Biological Chemists, JNU, New Delhi (INDIA). December 8-11, 2006.

- **Hexavalent chromate reduction by immobilized** *Streptomyces griseus* **in a bioreactor system.** Presented at International Conference on New Horizons in Biotechnology, NIIST, Trivandrum (INDIA). November 26-29, 2007.

- Presented Business plan on **Visible markers for GE foods - in Biotechnology Young Entrepreneurs Scheme (YES), DBT,** (INDIA). September 2007.

Workshop Attended:

- Atomic absorption spectroscopy training at Perkin Elmer Centre, Mumbai. February 16-18, 2005.

- UGC sponsored workshop on “Techniques in Cell Biology” at Ballygunge Science College, Department of Botany, University of Calcutta. March 30-April 5, 2007.

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

Background

Chromium (Cr) is found in air, soil, and water in small quantities. As a transition metal, Cr can occur under several oxidation states from -2 ($Na_2[Cr(CO)_5]$) to +6 (K_2CrO_4). In aqueous environments, however, Cr has two stable forms: hexavalent chromium [Cr(VI)] and trivalent chromium [Cr(III)]. Cr(VI) is usually associated with anthropogenic contamination, primarily from industrial and commercial processes, including electroplating, leather tanning, ore and petroleum refining, textile manufacturing, wood preservation, inorganic chemicals and pulp production and many metal finishing industries (Langard 1980; James 1996). Of these two valence states, Cr(VI) is toxic and carcinogenic (Roe and Carter 1969; Enterline 1974; Mertz 1974). The Maximum Contaminant Level (MCL) for chromium in drinking water has been established by the U.S. Environmental Protection Agency at 0.1 mg/L total chromium (USEPA 1995). The oxyanions of Cr(VI) (chromate, CrO_4^{2-}, and dichromate, $Cr_2O_7^{2-}$) are soluble and mobile in groundwater (Dragun 1988). Cr(VI) contamination is a major environmental problem. Cr(III), the other stable form of Cr, is naturally occurring. It is less toxic and less mobile (Cervantes et al. 2001). It adsorbs on most soils and has a relatively high soil partition coefficient (Barnhart 1997). Consequently, research has been focused on the reduction of Cr(VI) to Cr(III) for remediation of Cr(VI)-contaminated soil and groundwater.

The existing treatment methods for Cr(VI) contaminated soil or groundwater involve physical and chemical methods, e.g., excavation and off-site disposal or pumping and subsequent chemical or electrochemical reduction and precipitation or ion exchange (Patterson 1985; Nyer 1992). However, these methods are relatively expensive and sometimes generate secondary wastes that require subsequent disposal. Alternatively, *in-situ* bioremediation technology can be applied to circumvent the limitations of physical and/or chemical methods. Direct metabolic reduction of Cr(VI) by bacteria has been documented by several researchers and bioreduction of Cr(VI) appears to be ubiquitous since Cr(VI) reducing consortia were isolated from Cr(VI) contaminated sites as well as uncontaminated sites (Turick et al. 1996; Chen and Hao 1998; Schmieman et al. 1998; Sani et al. 2002; Camargo et al. 2003). Following microbial reduction, it is commonly

assumed that Cr(VI) species are transformed to insoluble and immobile chromium hydroxide. Hence, this technology has potential to be applied at field sites to immobilize Cr(VI) in the subsurface.

History of Chromium

Chromium was first discovered in Siberian red lead ore (crocoite) as a red pigment for oil paints) in 1798 by the French chemist Nicholas-Louis Vauquelin and was named as chrom from the Greek word khroma, owing to the brilliant hues of the compound. Since then, chromium has found a variety of uses in the industries that exploit these colors and other characteristics such as its strength, hardness, corrosion resistance, and the oxidizing capabilities of certain chromium species.

Chemical and Physical Characteristics

Chromium is steel-gray, lustrous, hard, metallic, and takes a high polish. Chromium is a transition metal belonging to group VI-B of the periodic table. Chromium can exist in several valence forms however, the two most stable states are Cr(III) and Cr(VI). Hexavalent Chromium (HC) is a strong oxidizing agent. The solubility of Cr (VI) can be very important but it depends on the cation with which it is associated. K_2CrO_4 shows a solubility of 38.96 g/L at 20°C while the complex ones $PbCrO_4$, $CaCrO_4$ and $BaCrO_4$ show very low solubilities of the order of 0.005×10^{-3} g/L, 0.2 g/L at 18°C and of 50×10^{-3} g/L at 25°C respectively. Trivalent Chromium (TC) has low mobility and bioavailability. Chromium, however, is largely substitution inert and serves poorly in enzyme catalysis. Chromium will replace other metals in biological systems with toxic effects. Cr(VI) is a known human carcinogen. In this context, most studies on prokaryotes and chromium involve reduction of Cr(VI) to less toxic and less mobile forms such as Cr(III). In the stable hydrate form, it is unable to cross biological membranes. Cr(VI) toxicity to fungi was found to be due to antagonism to sulfate uptake while Cr(III) toxicity was found to be due to conditional iron deficiency (Venkata Ramana and Sivarama Sastry 1994). In man it is recognized as an essential element and diabetes symptoms are reported to be associated with Cr dietary deficiency (Kaim and Schwederski 1994).

Sources of Chromium

Chromium is the seventh most abundant element on earth (McGrath and Smith 1990) which ranks 21st in crustal abundance. Cr abundance in Earth's crust ranges from 100 to 300 μg/g with an average concentration in the continental crust as 125 mg/kg (Cervantes et al. 2001). Chromium is not found as the free metal in nature but is extracted from chromite ($FeCr_2O_4$), the most important and abundant ore found in many parts of the world including India. Crocoite, $PbCrO_4$, is also a rare chromium mineral and is found in Russia, Brazil, USA, and Tasmania. The world production of Cr is in the order of 10^7 tons per year (McGrath and Smith 1990). Over half of the world's supply of chromite ore comes from South Africa, where 8.0 Mt were mined in 2005 (Papp 2005). The remaining four of the top five producers, listed in order of decreasing production, are as follows: Kazakhstan (3.2 Mt), India (2.3 Mt), Zimbabwe (0.73 Mt, 2003 total), and Finland (0.55 Mt, 2003 total). Since chromite ore prices are on the order of $100/Mt (Papp 2005), chromite mining is a very important economic activity.

Uses of Chromium

Chromium has many and varied applications. The vast majority of the world's chromite is utilized by the metallurgical industry, which in 2003 accounted for 91.2% of all chromate consumption. The remaining chromite that year went to the chemical industry (5.2%), the foundry industry (2.8%), and the refractory industry (0.8%). In metallurgy: chromium is used to impart corrosion resistance, create a shiny finish, or increase hardness. It is used as an alloy constituent in stainless steel. Ferrochromium is the intermediate between chromite ore and stainless steel, representing the most consumed chromium containing material. In 2003, 5.849 Mt of ferrochromium were produced worldwide. It is used by the aircraft and other industries for anodising aluminium (Papp 2004).

Chromium and its compounds are used as dyes and paints. It is responsible for the green colour of emeralds and the red colour of rubies and therefore is used in producing synthetic rubies. Chromium is used to give glass an emerald green colour. Cr(III) oxide is a metal polish known as green rouge. Cr(III) sulfate ($Cr_2(SO_4)_3$) is used as a green pigment in paints, in ceramic, varnishes and inks as well as in chrome plating. Lead

chromate is used as chrome yellow pigment. Chromium is widely used as a catalyst. Potassium dichromate is a chemical reagent and is used as oxidising agent and in quantitative analysis. It is also used in the textile industry as mordants (i.e., a fixing agent) for dyes in fabric. Chromium salts are used in the tanning of leather. Between 80-90% of tanned leather is tanned with chromium chemicals (Papp 2004). Owing to its high melting point, moderate thermal expansion, stable crystalline structure and chemical inertness, chromium has found use in the production of refractory bricks, mortars and metal castings. More recently, however, use of chromium in the refractory industry has been declining over environmental and technological issues (Papp 2004). Chromium hexacarbonyl ($Cr(CO)_6$) is used as a gasoline additive. Chromium boride (CrB) is used as a high-temperature electrical conductor. Cr(IV) oxide (CrO_2) is used to manufacture magnetic tape, where its higher coercivity than iron oxide tapes gives better performance. Cr(VI) is used in the post Ballard preparation of Gravure (rotogravure) printing Forme Cylinders. By electroplating the metal onto the second coat of copper (after the Ballard skin), the longevity of the printing cylinder is increased.

In medicine, as a dietary supplement or slimming aid, usually as Cr(III) chloride, Cr(III) picolinate, Cr(III) polynicotinate or as an amino acid chelate, such as chromium(III) D-phenylalanine.

Because of these important applications, chromium maintains a large and growing global market (Papp 1998, 2004, 2005). In 2004, the United States consumed 300,000 metric tons (Mt) of chromium, representing only 10% of the worldwide total (Papp 2005). Given its widespread application, it is to be expected that large amounts of chromium waste are annually released into the environment. According to the USEPA, 32,589.6 Mt of chromium and chromium compounds were released into the environment in 2003, about half of which was land-filled (USEPA 2005). Owing to its potential toxicity, chromium contamination resulting from land-filling is a major environmental and human health concern.

Indian Scenario

More than 1, 70,000 tons of Cr wastes are discharged to the environment annually as a consequence of industrial and manufacturing activities (Kamaludeen et al. 2003).

Some of the highly contaminated sites in India are listed below

1. Sukinda in Orissa state which contains one of the largest open cast chromite ore mines in the world is highly polluted with hexavalent chromium and affected 2,600,000 people. 60% of the drinking water contains hexavalent chromium at levels more than double international standards. An Indian health group estimated that 84.75% of deaths in the mining areas where regulations are nonexistent are due to chromite-related diseases. There has been virtually no attempt to clean up the contamination.
2. The Orissa State Pollution Control Board has conceded that the only source of water for the population of 26 lakh, the Brahmani river, is highly contaminated.
3. Noraiakheda (Kanpur) groundwater reported to be heavily contaminated with chromium and other toxicants. The groundwater of the area was reported to be laced with high concentration of chromium (6.2 mg/l), Iron (351.8 mg/l), fluorides (4.2 mg/l) and pesticides (Lindane 83.47 ng/l, DDT (192.36 ng/l) for the first time in 1997 by Central Pollution Control Board (CPCB). The concentrations of Chromium, Iron, Fluorides and Pesticides were 125 times, more than 1000 times, 4-5 times and more than hundred times respectively than the desirable limits for drinking water.
4. In Ludhiana and Chennai, chromium levels in underground water have been recorded at more than 12 mg/L and 550–1500 mg/L, respectively.
5. Nearly 80% of the tanneries in India are engaged in the chrome tanning processes. Most of them discharge untreated wastewater into the environment. In such aqueous waste, Cr(VI) is present as either dichromate ($Cr_2O_7^{2-}$) in acidic environments or as chromate (CrO_4^{2-}) in alkaline environments.
6. Chandra et al. (1997) estimated that in India alone about 2000 to 3200 tones of elemental Cr escape into the environment annually from the tanning industries, with chromium concentrations ranging between 2000 and 5000 mg/L in the effluent.

Nutritional and toxic effects of chromium

Nutritionally, Cr(III) is an essential component of a balanced human and animal diet for preventing adverse effects in the metabolism of glucose and lipids (e.g., impaired

glucose tolerance, elevated fasting insulin, elevated cholesterol and triglycerides, and hypoglycemic symptoms) (Anderson 1989, 1997). Cr(III) seems to exercise an important role in the metabolism of the plasmatic lipoproteins and phospholipids. In trace amounts, chromium is an essential component of human and animal nutrition (Jeejeebhoy et al. 1977; Mertz 1969). Cr(III) is associated with glucose metabolism (Mertz 1969) and has been shown to be an integral component of glucose tolerance factor (GTF), a factor required for maintaining normal glucose tolerance and its deficiency may cause a disease called chromium deficiency. Chromium functions by regulating and potentiating insulin action by increasing insulin binding to cell (Anderson et al. 1987). Chromium is also known to be of importance in fat metabolism in animals (Anderson 1989). In anything other than trace amounts, chromium compounds should be regarded as highly toxic. The intracellular Cr(VI) reductants naturally available are frequently obligatory one electron reducers, which generate Cr(V) and a large amount of ROS that causes the deleterious effects of Cr(VI) (Fig.1.1).

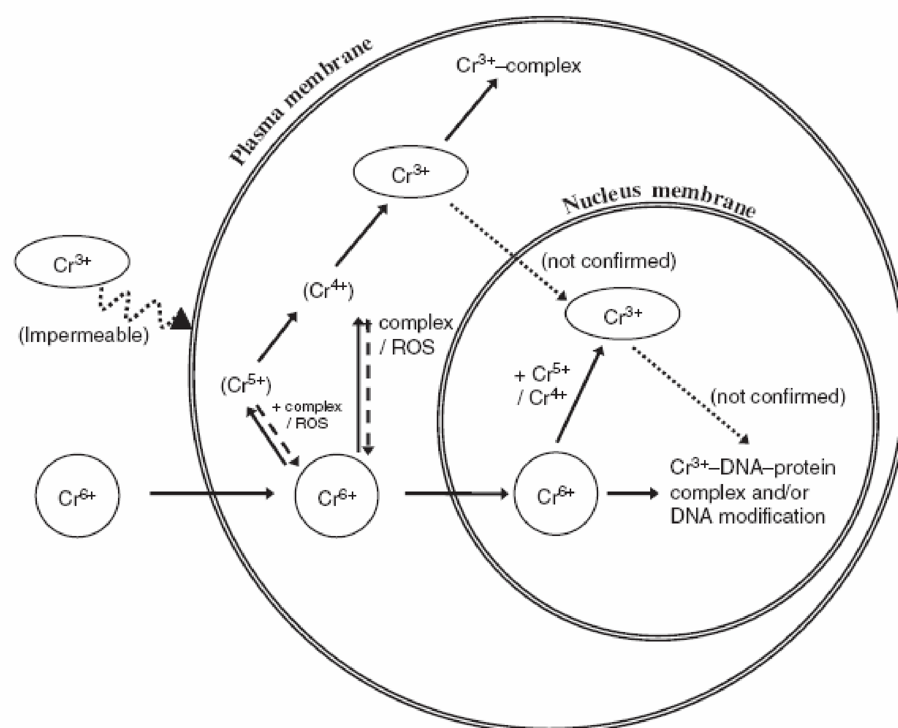

Fig.1.1: Schematic diagram of toxicity and mutagenicity of Cr(VI) (Cheung and Gu 2007)

In contrast, hexavalent chromium (Cr(VI) compounds have been estimated to be 10 – 100 times more toxic than Cr(III) compounds and considered to be mutagenic (Katz and Salem 1994). In relation to the issue of its carcinogenicity, Cr(VI) is also considered a mutagen, in light of its ability to interfere with DNA synthesis (Leonard and Lauwerys 1980), although certain metabolic pathways have been shown capable of deactivating Cr(VI) mutagenicity (Petrilli and DeFlora 1978). The biotoxicity of chromate is largely a function of its ability to cross biological membranes and its powerful oxidizing capabilities (NAS 1974). The toxico-kinetics of a given chromium compound depends on the valence state of the chromium compound (ATSDR 1999). Cr(VI) absorption is higher than Cr(III) because chromate anion $(CrO_4)^{2-}$ enters the cells by facilitated diffusion (ATSDR 1999). Humans can absorb Cr(VI) compounds through inhalation, dermal contact and ingestion. The organs where the concentration of chromium is obvious are liver, kidneys, spleen, ovaries, testicles and also the bones. In humans, several traumata are associated with Cr(VI) exposure, including respiratory irritation and ulceration, dermatitis, eardrum perforation and lung cancer, kidney and liver damage and damage to various proteins and nucleic acids, leading to mutation and carcinogenesis (Bianchi et al. 1984, Gibb et al. 2000 a, b). Furthermore, Cr(VI) can accumulate in the placenta, impairing fetal development in mammals (Saxena et al. 1990). Studies suggest that toxicity effects of Cr(VI) compounds result from the destruction of cellular components. Destruction of cells is caused by generation of free radicals.

Chromium Interactions in Microorganisms

Cr(VI) is highly mobile in some soils, and contact with Cr(VI) may be inevitable for aquatic and terrestrial organisms, including humans. Chromium is not an essential metal for the growth of the microorganisms, however, certain authors asserted the opposite (Horitsu et al. 1987). To the best of our knowledge, there is no report of bacterium requiring chromium for its growth, however its presence can be tolerated by microorganisms. Certain bacteria can use chromium as a final electron acceptor. Chromium has a toxic effect on bacteria (saprophytes and nitrificants), filamentous mushrooms, seaweeds and phytoplankton. Cr(VI) is toxic and mutagenic to most bacteria. Cr(VI) concentrations of 10-12 mg/L were inhibitory to most soil bacteria in

liquid media and, in general, gram-negative bacteria were more sensitive to Cr(VI) than were gram-positive bacteria while the same concentration of Cr(III) had no effect (Ross et al. 1981).

Cr(VI) impairs cell equipment, the metabolism and the physiological reactions. Among the visible effects reported in bacteria are cell elongation, cell enlargement, and inhibited cell division, which eventually leads to cell growth inhibition (Coleman and Paran 1983; Theodotou et al. 1976). Changes in morphologies of gram-positive and gram-negative bacteria are also reported. Few colonies of bacterial species such as *Staphylococcus aureus, S. epidermidis, Bacillus cereus,* and *Bacillus subtilis* were formed with degenerate cells that were reduced in size (Bondarenko and Ctarodoobova 1981). Increased content of Cr(VI) in soil was toxic to saprophytic and nitrifying bacteria. Lowered microbial biomass in soil was observed in the presence of high Cr(VI) in soil when it was determined using adenosine triphosphate (ATP) method (Ajmal et al. 1984; Zibilske and Wanger 1982). Other bacteria such as *E coli, Serratia marcescens and Enterobacter aerogenes* were unable to grow in Cr(VI) concentrations of 1 mM (Arnold et al. 1988). Metabolic effects of Cr(VI) on bacteria were evident by the observed changes in electron transport systems (Quershi et al. 1984).

Cr(VI) has been shown to cause mutagenic effects in *E. coli, Bacillus subtilis,* and *Salmonella typhimurium* (Nishioka 1975; Petrilli and DeFlora 1977; Venitt and Levy 1974). The mutagenic effects of chromium are effective only when chromium crosses the cell membrane. Cr(VI) can easily diffuse across the cell membranes, unlike Cr(III) which can do so only under extreme conditions such as long incubations and high concentrations. Cell culture studies have shown that cellular uptake of chromate is at least 10 times greater than that of Cr(III) from equimolar solutions, (Levis et al. 1978). However, once inside the cell, most of Cr(VI) is reduced to Cr(III) by several reducing agents such as ascorbic acid, sodium sulfite, glutathione, NADPH and NADH (Petrifilli and DeFlora 1978). Based on several studies, it was concluded that Cr(VI) causes DNA-strand breaks (Bianchi and Levis 1984, Bianchi et al. 1984, DeFlora et al. 1984; Levis et al. 1978; Tsapakos et al. 1983; Tsapakos and Wetterhahn 1983; Tsuda and Kato 1977). Cr(VI) causes genotoxic effects on bacterial cells, including frameshift mutations and base pair substitutions (Petrilli and DeFlora 1977). DeFlora et al. (1984) reported a more

general effect of unbalanced nucleotide pools. These studies suggest that although Cr(III) form is the major agent responsible for molecular events leading to mutagenicity, it is Cr(VI) that poses the greater risk due to its ability to easily enter the cell.

Permissible Levels of Chromium

International Standards: The recommended guidelines for fresh water life are 1µg/L for Cr(VI) and 8 µg/L for Cr(III), for marine life 1 µg/L for Cr(VI) and 50 µg/L for Cr(III) for irrigation water 8 µg/L for Cr(VI) and 5 µg/L for Cr(III) and for drinking water is 50 µg/L for Cr(VI) (Krishnamurthy and Wilkens 1994; Pawlisz 1997). The potentially toxics effects of chromium exposure has led the USEPA to limit the amount of total chromium in drinking water to 100 parts per billion (ppb) (USEPA 2003). A recent report has demonstrated that this limit is sufficient in preventing Cr(VI) in tap water from becoming a threat to human health (Paustenbach 2003).

Indian Standards: The drinking water quality standards as recommended by IS-10500 (1991) the maximum allowable limit is 0.05 mg/L.

Chromium Remediation

The physical removal of chromium from contaminated soils is very costly and difficult (Lovley and Lloyd 2000). For this reason, chromium clean-up is almost exclusively associated with immobilization and detoxification rather than the complete removal of chromium species. In other words, reduction of Cr(VI) to Cr(III) is the fundamental goal of most chromium remediation strategies (Lovley and Coates 1997; Lovley and Lloyd 2000, Lovley and Coates 2000; Khan and Puls 2003; Alowitz and Scherer 2002; Melitas et al. 2001). Several strategies are being developed to enhance one or more of the naturally occurring biotic or abiotic Cr(VI) reduction processes in soils. Jardine et al. (1999) suggested amending contaminated soils with natural organic matter (NOM) to reduce and subsequently precipitate or adsorb hexavalent chromium. However, the reaction kinetics were only favorable in acidic soils (pH $\leq$ 4), thus limiting its applicability. Amendment using inorganic reducers as an in situ remediation technique has also received attention. In particular, permeable reactive barriers (PRBs) have been successfully implemented and are regarded by USEPA as a proven technology (Lovley

2001; Melitas et al. 2001, USEPA 2000). These barriers take advantage of iron's ability to reduce Cr(VI) by introducing zero valent iron metal into groundwater plumes (Alowitz and Scherer 2002; Melitas et al. 2001). Subsequent alkaline conditions lead to inefficient Cr(VI) reduction (Alowitz and Scherer 2002). PRBs are also limited in that they aren't very useful when it comes to source area remediation. Sodium dithionate ($Na_2S_2O_4$), a reducing agent often used by chromium consuming industries, was used for in situ treatment of a source Cr(VI) contamination site (Khan and Puls 2003). Over the course of 48 weeks, ground-water Cr(VI) concentrations were reduced below the detection limit of 10 mg/L. The reaction stoichiometry proposed by Khan and Puls (2003) suggests high pH values are favored by this reaction. Recent studies have dealt with the role of bacterial Cr(VI) reduction in soils, and their remedial potential (Fendorf et al. 2000; Tokunaga et al. 2003; Tseng and Bielefeldt 2002). Recognition of the importance of metal reducing bacteria, particularly in anaerobic subsurfaces, was only given attention in the last decade (Lovley 1997). Since then, metal reducing bacteria have been incorporated into many bioremediation schemes (Lloyd et al. 2000; Lovley and Lloyd 2000; Lovely 2001).

Mechanisms of chromate resistance in bacteria

The persistent nature of some metals in environment has led to considerable modifications of the microbial community and their activities. Heavy metals have been shown to inhibit microbial growth and other enzymatic activities by blocking essential functional groups, displacing essential metal ions and modifying the conformations of the biological molecules (Gadd 1993). In metal-contaminated environments, the responses of the microbial communities depend on the concentrations of the toxic agents they are exposed to among other factors such as nature of nutrients, chemical form of the toxic agent and so on. Persistence of metals in the environment leads to selection of strains either resistant or possessing the reduction capability. Organisms isolated from sediments of Cr(VI) contaminated metal-processing waste evaporation ponds were found to be more Cr-tolerant compared with those found outside the ponds (Losi and Frankenberger 1994).

Microorganisms may adopt several strategies to reduce metal sensitivity to cellular targets: (i) mutations to decrease the sensitivity to the metal (ii) increased production of damaged cell component, (iii) increased efficiency of repair of damaged

cell component, (iv) utilization of plasmid-encoded resistance mechanism. These mechanisms may either occur singly or in various combinations. The mechanisms proposed for heavy metal resistance in bacteria include extracellular sequestration, detoxification by chemical modification of the toxic to non-toxic form of the metal, exclusion by permeability barrier, exclusion by active transport and intracellular physical sequestration by the binding proteins of the cell. Mechanisms of chromate transport, toxicity and resistance in bacteria are presented in Fig. 1.1 and Table 1.1.

Increased polysaccharide production has been reported in *Pseudomonas* sp. (Aislabie and Loutit 1986). Studies with *Pseudomonas ambigua* and its Cr(VI) sensitive mutant S-1 led to the conclusion that the presence of thick membranes around the parent cell decreased the permeability of the cells to Cr(VI) and increased the resistance of the bacteria. Cr(VI) sensitive *Pseudomonas ambigua* strain accumulated six times more chromate than a resistant strain (Horitsu et al. 1983).

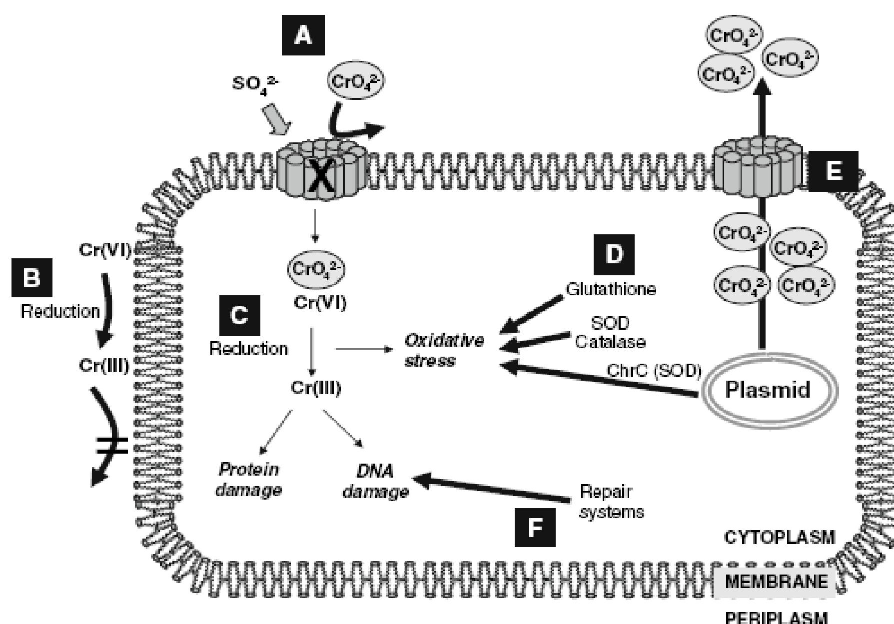

Fig. 1.2: Mechanisms of chromate transport, toxicity and resistance in bacteria
Mechanisms of damage (thin) and resistance (heavy) are indicated by arrows (Ramirez-Dıaz et al. 2008)

A: Reduced Cr(VI) uptake; B: Extracellular reduction of Cr(VI); C: Intracellular Cr(VI) reduction; D: Detoxifying enzymes in protection against oxidative stress; E: Efflux chromate; F DNA repair

Ohtake et al. (1987) reported that the chromate resistance in *P. fluorescens* LB300 (pLHB1) was related to the decreased uptake of chromate under aerobic conditions. Studies with radioactive Cr(VI) showed a clear difference in Cr(VI) accumulation between *E. cloacae* HO1 and IAM 1624 under aerobic conditions; the chromate-sensitive IAM 1624 cells accumulated about three times more Cr(VI) than did strain HO1 (Wang et al. 1989).

Among the various mechanisms of chromate resistance reported, the best characterized mechanisms comprise efflux of chromate ions from the cell cytoplasm and reduction of Cr(VI) to Cr(III). Chromate efflux by the ChrA transporter has been established in *Pseudomonas aeruginosa* and *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) and consists of an energy-dependent process driven by the membrane potential (Ramirez-Dıaz et al. 2008). The *chr*A gene of *Pseudomonas aeruginosa* plasmid pUM505 encodes the hydrophobic protein ChrA, which confers resistance to chromate by the energy-dependent efflux of chromate ions (Aguilera et al. 2004).

Chromate reduction is carried out by chromate reductases from diverse bacterial species generating Cr(III) that may be detoxified by other mechanisms. Several examples of bacterial systems protecting from the oxidative stress caused by chromate have been described. Other mechanisms of bacterial resistance to chromate involve the expression of components of the machinery for repair of DNA damage, and systems related to the homeostasis of iron and sulfur. The mechanisms of resistance to Cr(VI) may be encoded either by plasmids or by chromosomal genes (Nies et al. 1998; Cervantes and Campos-Garcı´a 2007). Plasmid-associated metal resistance in bacteria is reviewed by Silver and Phung (1996); Cervantes and Silver (1992). Usually, the genes located in plasmids encode membrane transporters, which directly mediate efflux of chromate ions from the cell's cytoplasm (Fig. 1.2). On the other hand, resistance systems encoded within bacterial chromosomes are generally related to strategies such as specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, repairing of DNA damage, and processes associated with sulfur or iron homeostasis (Fig. 1.2). Chromate resistant (mutant) and chromate sensitive (wild type) strains of *P. fluorescens* were capable of

Cr(VI) reduction and chromate reduction and resistance were not linked in this organism (Bopp and Ehrlich 1988).

Table 1.1: Mechanisms of chromate tolerance/ resistance in bacteria

Mechanism	Organism	Enzyme/system	Function	Reference
Transport	*Pseudomonas aeruginosa*	ChrA transporter	Efflux of cytoplasmic chromate	Alvarez et al. 1999
	Shewanella oneidensis	Cys operon products	Sulfate transport	Brown et al. 2006
		Ton B receptor, hemin transporter	Iron transport	
Reduction	*Arthrobacter* sp., *Bacillus* sp.	Chromate reductases	Reduction of Cr(VI) to Cr(III)	Cervantes et al. 2001
General and oxidative stress	*Escherichia coli*	Superoxide dismutase (SOD), catalase	Combat of oxidative stress	Ackerley et al. 2004
	Caulobacter crescentus	SOD, glutathione –S transferase	Combat of oxidative stress	Hu et al. 2005
	Caulobacter crescentus	Outer membrane proteins	General stress response	Hu et al. 2005
	Ralstonia metallidurans strain CH34 (formerly *Alcaligenes eutrophus* CH34) *Cupriavidus metallidurans*	Fe-SOD ChrC (a tetrameric, Fe containing superoxide dismutase)	Detoxification of free radicals	Juhnke et al. 2002
DNA repair	*Escherichia coli*	SOS response	Repair of DNA damage	Llagostera et al. 1986
	Pseudomonas aeruginosa	DNA helicases	Repair of DNA damage	Chourey et al. 2006
	Shewanella oneidensis	SO0368, *uvr*D and *hrp*A helicases	Repair of DNA damage	Chourey et al. 2006
Other mechanisms	*Shewanella oneidensis*	Adenyl sulfate kinase; Sulfite reductase	Sulfur metabolism	Brown et al. 2006
		Ferritin	Iron binding	

Exclusion by permeability barrier

Chromate is transported into the cytoplasm through nonspecific chromosome encoded sulfate transport system due to its structural similarity to sulfate. One of the protective systems of the Cr toxic effects are probably associated with a reduced uptake of Cr(VI) and with sulfur or iron homeostasis (Table 1.1). When it is mutated (X) the transport of chromate diminishes. *Caulobacter crescentus* seems not to have a chromate efflux system, but Cr stress down-regulates a sulfate transport system probably reducing chromate uptake (Hu et al. 2005).

Extracellular reduction of Cr(VI) to Cr(III)

Cr(VI) reduction outside the cell generates Cr(III) which cannot cross cellular membranes. Desjardin et al. (2003) reported that *Streptomyces thermocarboxydus* NH50, an aerobic bacterium produces reducing agents into the culture supernatants. Cr(VI) reduction by these substances is accelerated by the presence of small concentration of cupric ions. The reducing agent(s) can be easily recovered from the bacterial cultures and used as cell-free solution to treat contaminated soils by *in situ* or *ex situ* processes.

Intracellular Cr(VI) to Cr(III) reduction

Intracellular Cr(VI) to Cr(III) reduction may generate oxidative stress, as well as protein and DNA damage. Intracellular reduction of Cr(VI) to Cr(V) and Cr(III) may also be carried out by chemical reactions associated with compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione releasing free radicals. The Cr(III) formed may bind to phosphates in DNA. Oxidative damage to DNA is responsible for the toxic effect of chromate. For instance, ascorbate is capable of reducing Cr(VI), and riboflavin derivatives FAD and FMN which are essential coenzymes for chromate reducing flavoenzymes (Ramirez-Dıaz et al. 2008). The action of YieF from *E. coli* involves an obligatory four-electron reduction of Cr(VI) by the protein dimer (50 kDa), in which the enzyme simultaneously transfers three electrons to Cr(VI) to produce Cr(III) and one electron to molecular oxygen generating ROS; no flavin semiquinone is generated during this process (Ackerley et al. 2004). YieF is speculated to provide to *E.*

coli an effective protection mechanism against chromate toxicity by forming a lower amount of ROS.

Protection against oxidative stress

Since the generation of reactive oxygen species (ROS) occurs during Cr(VI) reduction to Cr(III) (Fig. 1.1), the participation of bacterial proteins in the defense against oxidative stress induced by chromate represents an additional mechanism of chromate resistance. Enzymes such as superoxide dismutase and catalase that participate in detoxification of ROS generated after Cr(VI) exposure and reduction may be involved in the protection against the deleterious effects of chromate (Ackerley et al. 2006; Ramirez-Dıaz et al. 2008).

Transmembrane efflux of chromate

Efflux pumps are the major currently known group of resistance systems, with both plasmid and chromosomal systems. Frequently though not always, the mechanisms are the same in all bacterial types. Chromate influx is known to occur via sulfate uptake systems in some microorganisms (reviewed by Nies 1999). Both ATPases and chemiosmotic efflux pumps are known (Silver and Phung 1996). The efflux of chromate is a resistance mechanism conferred by the ChrA protein (Table 1). ChrA is encoded by plasmids pUM505 of *Pseudomonas aeruginosa* and pMOL28 from *Cupriavidus metallidurans* (previously *Alcaligenes eutrophus* and *Ralstonia metallidurans*) (Cervantes et al. 1990; Nies et al. 1990). Cr(VI) resistance in pseudomonads has been thoroughly investigated. In *Pseudomonas aeruginosa*, Cr(VI) resistance is attributed to the decreased uptake and/or enhanced efflux of Cr(VI) by the cell membrane (Ohtake et al. 1987; Alvarez et al. 1999; Aguilera et al. 2004). A similar mechanism of resistance has been reported for *Alcaligenes eutrophus* CH34 (recently re-classified as *Waustersia eutropha*) (Nies and Silver 1989).

Bacterial Cr(VI) reduction

Microbial Cr(VI) reduction has been observed most often under aerobic conditions, but can also occur under anaerobic conditions (reviewed by Ohtake and Silver

1994). Cytochrome c_3 and *b* have been implicated in chromium reduction, but the mechanisms of chromium bioreduction have not been completely determined (Lovley and Phillips 1994). Cr(VI) reduction is likely a detoxification mechanism, but bacterial chromium resistance is also mediated by non-reductive plasmid-based systems that appear to exclude chromium (as chromate ions) from the cell (reviewed by Silver 1998). Chromium resistance has also been described in fungi and algae, and some of these organisms can immobilize soluble chromium via bioaccumulation and biosorption (reviewed by Cervantes et al. 2001).

Many bacterial isolates known to reduce Fe(III) have been used in Cr(VI) reduction experiments, particularly among them are *Cellulomonas* (Sani et al 2002), *Shewanella* and *Geobacter* (Liu et al. 2002) species. Sulfate reducing bacteria have demonstrated Cr(VI) reducing capability (Fude et al. 1994). Denitrifying bacteria of the genus *Pseudomonas* have also been used in many Cr(VI) reduction experiments (Konovalova et al. 2003). Consortia and novel species of Cr(VI) reducing bacteria have been isolated from a variety of Cr(VI) contaminated sites including soils (Badar et al, 2000; Camargo et al. 2003); Jeyasingh and Philip 2005), sludges (Komori et al. 1990), wastes (Pattanapipitpaisal 2001) and effluents (Shakoori et al. 2000).

Factors influencing reduction

Several experimental factors such as initial Cr(VI) concentration, cell density, inhibitors, activators, temperature, pH affect observed Cr(VI) reduction rates. One of the most crucial is the presence or absence of oxygen, since the toxic effects of Cr(VI) can vary between these two conditions. Since Cr(VI) is toxic, bacterial Cr(VI) tolerance is another important factor. Some of the more resistant bacterial strains have come from Cr(VI) contaminated sites. Camargo et al. (2003) found bacteria living in alkaline soil contaminated with dichromate able to withstand Cr(VI) concentrations as high as 0.048 M.

Pattanapipitpaisal et al. (2001) reported the effect of initial concentrations on Cr(VI) reduction rates. Although overall rates increased, specific rates decreased. This inverse relationship has been reported for a variety of species. Choice of substrate, or electron donor, is another important factor affecting not only Cr(VI) reduction rates.

While substrates such as tryptic soy broth (TSB) and lactate are among the more commonly used electron donors in Cr(VI) reduction studies, acetate has also received attention, as it is inexpensive and therefore a more economical alternative. Acetate, like lactate, also carries the benefit of being chemically defined. However, minimal media, such as media containing acetate, result in reduced reduction rates. In a study of Cr(VI) reduction by *Shewanella algy* BrY under different growth conditions, Guha et al. (2001) found a direct relationship between reduction rates and substrate richness. Cr(VI) containing media can be amended with Fe(III), which, upon bacterial reduction to Fe(II), an couple reoxidation to Fe(III) with Cr(VI) reduction to Cr(III) (Wielinga et al. 2001). The presence of Fe(III) or Fe(II) can therefore enhance Cr(VI) reduction capability. Anthroquinone-2,6-disulfonate (AQDS), a compound commonly used as a humic substance analogue, can also act as an electron shuttle and enhance Cr(VI) reduction rates (Fredrickson et al. 2000). Many examples of bacterial Cr(VI) reduction have been reported, although virtually all of them were conducted at near neutral pH values. Few reports of bacterial Cr(VI) reduction under alkaline conditions have been published (Ye et al. 2004).

Anaerobic Cr(VI) reduction

Cr(VI) reducing anaerobes

Early investigations on the biotransformation of Cr(VI) focused on the facultative anaerobes such as *Pseudomonas dechromaticans*, *Pseudomonas chromatophila* and *Aeromonas dechromatica.* A number of chromium-resistant microorganisms were subsequently isolated, such as *Enterobacter cloacae*, *Desulfovibrio desulfuricans* and *D. vulgaris*. Sulfate-reducing bacteria (SRB) have been extensively studied for reduction of metals, including Cr(VI). For instance, Cr(VI) reduction in *D. vulgaris* was found to involve a soluble c_3 cytochrome (Lovley and Phillips 1994). In *Desulfomicrobium norvegicum*, a hydrogenase and a c-type cytochrome catalyzed Cr(VI) reduction (Michel et al. 2001). *Desulfotomaculum reducens* MI-1 was capable of utilizing Cr(VI) as sole electron acceptor (Tebo and Obraztsova 1998), this capability was only reported in another SRB consortium (Cheung and Gu 2003). *E. cloacae* HO1 is another Cr(VI)

reducing facultative anaerobe that has been extensively investigated. In this species, Cr(VI) reduction is inhibited by oxygen and the concomitant presence of other metals that inhibits Cr(VI) reduction owing to the toxicity of the metals to the microorganism (Wang et al. 1989).

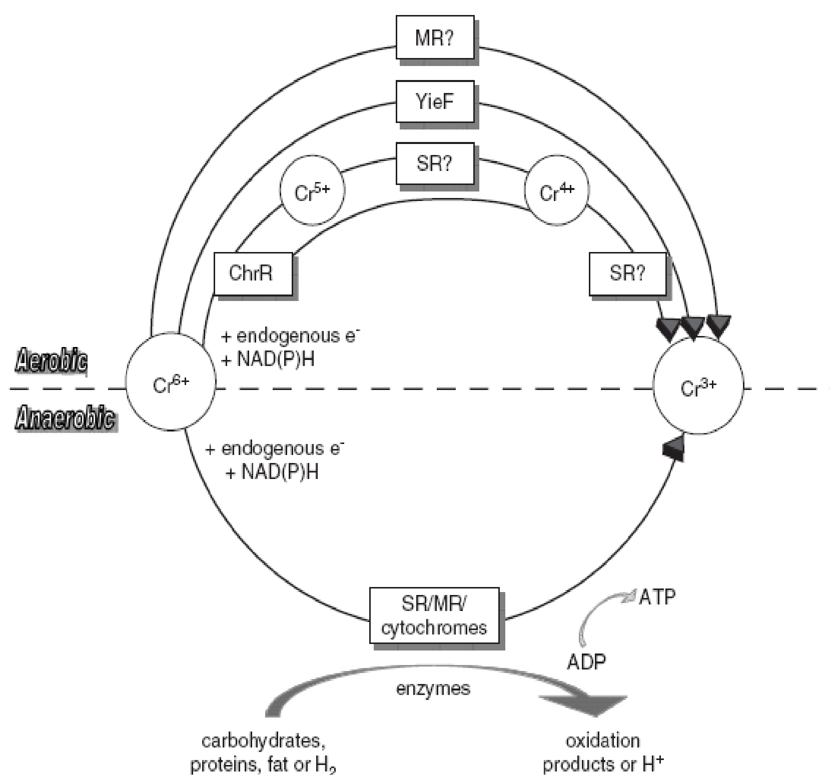

Fig.1.3. Plausible mechanisms of enzymatic Cr(VI) reduction (Cheung and Gu 2007)
Aerobic (upper); Anaerobic (lower) conditions

Other reported Cr(VI) reducing anaerobes included *Microbacterium* sp. MP30 (Pattanapipitpaisal et al. 2001), *Geobacter metallireducens* (Liu et al. 2002), *Shewanella putrefaciens* MR-1 (Myers et al. 2000), *Pantoea agglomerans* SP1 (Francis et al. 2000), *Agrobacterium radiobacter* EPS-916 (Llovera et al. 1993) and a consortium capable of simultaneously reducing Cr(VI) and degrading benzoate (Shen et al. 1996). A few species of extremophiles have been found to reduce Cr(VI). Among them are the radiation-resistant *Deinococcus radiodurans* R1 (Fredrickson et al. 2000), thermophilic bacterium *Thermoanaerobacter ethanolicus* isolated from deep subsurface sediments (Roh et al.

2002), and *Pyrobaculum islandicum* (Kashefi and Lovley, 2000) which reduced Cr(VI) at high temperatures.

Cr(VI) reductases of anaerobes

Both soluble and membrane-associated enzymes were found to mediate the process of Cr(VI) reduction under anaerobic conditions. Unlike the Cr(VI) reductases isolated from aerobes, the Cr(VI) reducing activities of anaerobes are associated with their electron transfer systems ubiquitously catalyzing the electron shuttle along the respiratory chains (Wang et al. 1989). The cytochrome families (e.g., cytochrome b and cytochrome c) were frequently shown to be involved in the enzymatic anaerobic Cr(VI) reduction. The widespread occurrence of anaerobes possessing Cr(VI) reducing activities offers great potential for in situ bioremediation of Cr(VI) contaminated sediments; which would only require the supplementation of nutrients and the modulation of physical conditions to facilitate the reaction (Turick et al. 1996).

Mechanism of anaerobic Cr(VI) reduction

Natural metabolites of anaerobes such as H_2S produced by SRB, are effective chemical Cr(VI) reductants under anoxic environment (Fude et al. 1994). In the past, the anaerobic reduction of Cr(VI) was considered as a fortuitous process that provides no energy for microbial growth. However, a SRB isolate was found to use for its growth the energy generated during anaerobic Cr(VI) reduction (Tebo and Obraztsova 1998). In the absence of oxygen, Cr(VI) can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors, including carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves (Wang et al. 1989).

An uncharacterized membrane-associated Cr(VI) reductase has been isolated from *B. megaterium* TKW3. Under anaerobic conditions, both soluble and membrane-associated Cr(VI) reducing enzymes, including cytochromes, associated with the electron transfer system have been reported. The typical anaerobic Cr(VI) reduction is shown in Fig. 1.3 (lower). Enzymes involved in the reduction of Cr(VI) are in boxes. SR and MR represent soluble and membrane-associated reductase, respectively (Cheung and Gu 2007).

Aerobic Cr(VI) reduction

Cr(VI) reducing aerobes

A number of chromium-resistant microorganisms such as *B. cereus*, *B. subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas ambigua*, *Pseudomonas fluorescens*, *E. coli*, *Achromobacter eurydice* (Gopalan and Veeramani, 1994) *Micrococcus roseus* are able to reduce Cr(VI) aerobically. Other pseudomonads capable of reducing Cr(VI) include unidentified species (McLean et al. 2000). A number of bacteria in other genera, viz. *Bacillus* spp., *E. coli* ATCC 33456, *Shewanella* alga BrY-MT and a few unidentified strains have also been shown to reduce Cr(VI) (Shen and Wang 1994; Wang and Xiao 1995; Shakoori et al. 2000; Camargo et al. 2003). It is noteworthy that, unlike most reported Cr(VI) reducing aerobes, which utilized reductases soluble in the cytosol, in *Pseudomonas maltophilia* O-2 and *Bacillus megaterium* TKW3 Cr(VI) reduction was associated with the membrane cell fractions (Blake II et al. 1993; Cheung et al. 2006).

Cr(VI) reductases of aerobes

Several earlier attempts have been reported of Cr(VI) reductase purification from pseudomonads. Ishibashi et al. (1990) partially purified a soluble Cr(VI) reductase from *Pseudomonas putida* PRS2000. Suzuki et al. (1992) reported a 38-fold purification of a soluble Cr(VI) reductase from *Pseudomonas ambigua* G-1. In a more recent investigation, the gene encoding this reductase was found to exhibit a high nucleotide sequence homology (58%) to a nitroreductase of *Vibrio harveyi* KCTC 2720 that was also endowed with Cr(VI) reducing activities (Kwak et al. 2003). Park et al. (2000) purified 600-fold a soluble Cr(VI) reductase, ChrR, from *Pseudomonas putida* MK1. The ChrR-coding gene, *chr*R, was identified from the genomic sequence of *P. putida* MK1, based on the known amino acid sequences of the N-terminal and internal amino acid segments of the pure enzyme (Park et al. 2002). Ackerley et al. (2004) described ChrR as a dimeric flavoprotein catalyzing the reduction of Cr(VI) optimally at 70ºC. An open reading frame, yieF, on the *E. coli* chromosome with no assigned function was found to have a high homology to *chr*R. This gene was cloned and the encoded protein, YieF, showed maximum reduction of Cr(VI) at 35ºC (Park et al. 2002). Recently, a membrane-

associated Cr(VI) reductase was identified from the proteome of *B. megaterium* TKW3 detected on a two-dimensional electrophoresis gel (Cheung et al. 2006).

Mechanism of aerobic Cr(VI) reduction

In the presence of oxygen, bacterial Cr(VI) reduction commonly occurs as a two- or three-step process with Cr(VI) initially reduced to the short-lived intermediates Cr(V) and/ or Cr(IV) before further reduction to the thermodynamically stable end product, Cr(III). Nevertheless, it is at present unclear as to whether the reduction of Cr(V) to Cr(IV) and Cr(IV) to Cr(III) was spontaneous or enzyme mediated. NADH, NADPH and electron from the endogenous reserve are implicated as electron donors in the Cr(VI) reduction process (Horitsu et al. 1987). Under aerobic conditions, the Cr(VI) reductase ChrR transiently reduces Cr(VI) with a one-electron shuttle to form Cr(V), followed by a two-electron transfer to generate Cr(III). Although a proportion of the Cr(V) intermediate is spontaneously reoxidized to generate ROS, its reduction through two electron transfer catalyzed by ChrR reduces the opportunity to produce harmful radicals (Ackerley et al. 2004).

Enzyme YieF is unique in that it catalyzes the direct reduction of Cr(VI) to Cr(III) through a four-electron transfer, in which three electrons are consumed in reducing Cr(VI) and the other is transferred to oxygen. Since the quantity of ROS generated by YieF in Cr(VI) reduction is minimal, it is regarded as a more effective reductase than ChrR for Cr(VI) reduction. The membrane-associated Cr(VI) reductase recently isolated from *B. megaterium* TKW3 utilized NADH as an electron donor, but the kinetics of Cr(VI) reduction is as yet uncharacterized. A diagram illustrating the mechanism of enzymatic Cr(VI) reduction under aerobic conditions is shown in upper portion of the Fig. 1.3 (Cheung and Gu 2007).

Direct Enzymatic Reduction of Cr(VI)

Although Chromate reducing bacteria (CRB) have been studied for many years now, little is known about the biochemistry and mechanism of Cr(VI) reduction. It still remains unclear if Cr(VI) is taken up by the cell and reduced in the cytoplasm or the periplasm or the electron are transferred to the outside of the cells or both. Enzymatic

reduction of Cr(VI) has been observed in some CRB (Bae et al. 2005; Clark 1994; Ganguli and Tripathi 2001; Oh and Choi 1997; Suzuki et al. 1992) . The CRB are able to reduce Cr(VI) by either soluble enzyme systems or the membrane-bound system. Membrane-associated chromate reductase activity was first observed in *Enterobacter cloacae* HO1 where the insoluble form of reduced chromate precipitates was seen on the cell surface. In the presence of ascorbate reduced phenazine methosulfate (PMS) as electron donor, high chromate reduction was shown by right-side-out membrane vesicles of *E. cloacae* HO1 (Wang et al. 1990). Membrane-associated constitutive enzyme that mediated the transfer of electrons from NADH to chromate was later elucidated by Bopp et al. (1988). In case of *Shewanella putrefaciens* MR-1 chromate reductase activity was associated with the cytoplasmic membrane of anaerobically grown cells (Myers et al. 2000). Formate and NADH served as electron donors for the reductase. No activity was observed when NADPH or L-lactate was provided as the electron donors. However, in *Pseudomonas putida*, unlike in *Shewanella putrefaciens*, both NADH and NADPH served as an electron donor for reduction (Park et al. 2000).

Studies conducted by Shen and Wang (1993) on *E. coli* suggested the presence of soluble chromate reductase. Cr(VI) reduction in another gram negative bacteria, *Pseudomonas* sp CRB5, was found to be mediated by a soluble enzyme contained in cytoplasm (McClean and Beveridge 2001). In addition to gram-negative bacteria, soluble chromate reductases have also been observed in gram-positive strains. NADPH was the preferred electron donor for the reduction of chromate by the soluble enzyme in *Bacillus coagulans* (Philip et al. 1998).

Bacterially mediated indirect reduction of Cr(VI)

Redox potential-pH

Changes in pH and redox conditions are known to occur in medium during growth of bacterial cultures due to various biochemical reactions and the metabolites formed. These changes may indirectly affect the reduction of Cr(VI) in the medium. Cr(VI) reduction occurs in a wide range of redox potentials. The optimum redox potential range has not been well established as yet. Reduction of Cr(VI) has been reported in

redox conditions as high as +300 mV (Wang and Xiao. 1995). In the same culture, after 48 hours, Cr(VI) reduction was observed even when the redox potential dropped to –500 mV. A higher rate of Cr(VI) reduction by *Agrobacterium radiobacter* was observed at –240 mV compared with –198 mV (Llovera et al. 1993). In contrary, no reduction of Cr(VI) was observed with redox potential of –140 mV for the first hour of incubation in cultures of *Escherichia coli* (Wang and Xiao. 1995).

Fe(III)-mediated reduction of Cr(VI)

Fe(III) is the most abundant electron acceptor for anaerobic respiration in many sedimentary environments due to its ability to act as terminal electron acceptor for many organisms. Microbial reduction of Fe(III) significantly affects Cr(VI) biogeochemistry as reduced iron in sediments is one of the most significant electron donors for the reduction of Cr(VI). Therefore, Fe(III) reducing bacteria that are unable to support their growth on reduction of Fe(III) can indirectly reduce Cr(VI) via Fe(III) reduction. Reduction of chromate by dissimilatory iron-reducing bacteria was reported by Wielinga et al. (2001). They elucidated the reduction of Cr(VI) to Cr(III) via a closely coupled biotic-abiotic pathway under iron-reducing conditions.

Quinone mediated reduction of Cr(VI)

Humic substances are ubiquitous in the environment. They are heterogeneous organic high-molecular-weight macromolecules that are composed of many potentially reactive moieties. Humics function as primary electron acceptors for iron-reducing bacteria. Quinones serve as the primary electron-accepting moiety in the humic acids when they are reduced to hydroquinones by accepting two electrons. Although humic-mediated Cr(VI) reduction has not been reported so far thermodynamically, transfer of electron from humics (E = 0.2 mV) to Cr(VI) (E = 1.23 mV) is plausible.

Reduction by the organic matter

The natural organic matter Humic acid (HA) or fulvic acid (FA) contained in the grounds or in waters are equally likely to reduce the Cr(VI) (Bartlett and Kimble 1976; James and Bartlett 1983). Due to their chelating power and oxido-reduction property, humic acid constitute an active system for oxido- reduction (ah_{ox}/ah_{red} E_0 = 0.7 mV). The

fulvic acids are better reducers than humic acids because they are less sensitive to inhibition by Cr(III) and they have a standard potential (0.5 V) weaker than that of humic acids (Palmer and Wittbrodt 1991).

Photochemical Reduction of Cr(VI)

Studies (Kleber and Helz 1992) on the photo reduction of the Cr(VI) in the natural environments showed that the mechanism was indirect. The couple Fe(II)/Fe(III) transfers the electrons towards organic ligands. The complex Fe (III) -organic ligands absorb the light producing Fe(II). Fe(II) reduces Cr(VI) to Cr(V) then to Cr(IV) and finally to Cr(III). At every step, the Fe(II) is reoxidised to Fe(III) that can again form a complex with organic ligands and resume the cycle.

Actinomycetes

Actinomycetes are a large group of gram-positive microorganisms. They are mostly non-motile and common inhabitants of soil, water and manure. They can be unicellular that grow in the form of coccoidal forms such as corneyform actinomyetes or have slender branched mycelia, which are narrower than fungal mycelium. They resemble bacteria in their diameter and are called thread bacteria. Actinomycetes are prokaryotic organisms with G+C content is greater than 70%. Like fungi, actinomycetes display extensive mycelial branching and sporulation. Growth of actinomycetes in liquid media tends to produce pellets similar to fungi rather than turbidity produced by bacteria. Genus *Streptomyces* dominates the group and represents nearly 90% of the total actinomycetes population. Life cycle of *Streptomyces* is shown in Fig. 1.4.

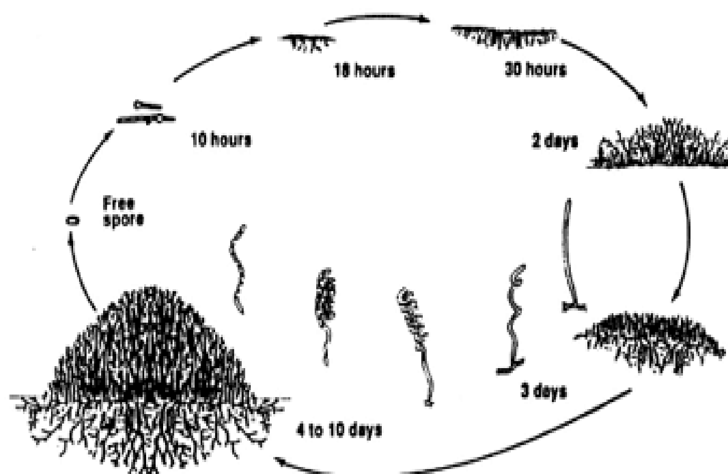

Fig 1.4: Life cycle of Streptomyces

Classification of *Streptomyces*

Streptomyces griseus is a member of the class of bacteria called the Actinobacteria (older name: Actinomycetes). A taxonomic classification is given below

Kingdom: Bacteria
Phylum: Actinobacteria
Class: Actinobacteria
Order: Actinomycetales
Family: Streptomycetaceae
Genus: *Streptomyces*
Species: *Streptomyces griseus*

Scope of chromate reduction by actinomycetes

Vast literature is available on chromate reduction by bacteria relating to physiological, biochemical and genetic aspects of chromate toxicity/resistance and reduction. Though actinomycetes constitute a significant component of microbial population there is no systematic study on chromate tolerance, toxicity/resistance or reduction by mycelium forming actinomycetes. There are only few reports available in literature on chromate reduction by actinomycetes. Das and Chandra (1990) partially purified a membrane associated chromate reductase from *Streptomyces* sp. The crude cell extracts from this organism reduced Cr(VI) to Cr(III) in the presence of NAD(P)H.

Amoroso et al. (2001) reported chromium accumulation in two *Streptomyces* spp. isolated from river sediments. Polti et al. (2007) reported qualitative and semi-quantitative screening of Cr(VI) resistance in actinomycete isolates. Chromate reduction during growth and by resting cells of *S. griseus* was reported by Laxman and More (2002). *Arthrobacter crystallopoietes* produced a periplasmic chromate reductase that was stimulated by NADH (Camargo et al. 2004). Desjardin et al. (2003) reported non-enzymatic chromate reduction by *Streptomyces thermocarboxydus* NH50. They isolated a small molecular weight (<1 kDa) reducing agent from culture filtrate but no identity of the reducing agent is reported. Chromate reduction was carried out by resting cells of *Achromobacter* sp. Ch-1 with lactate as electron donor under aerobic conditions (Ma et al. 2007). Mabrouk (2008) investigated the efficiency of the halophilic bacterium *Streptomyces* sp. MS-2 to reduce Cr(VI) under aerobic conditions; and optimized the culture conditions by using Plackett–Burman experimental design. Removal of hexavalent chromium by Cr(VI) tolerant *Streptomyces* sp.CG252 is reported by Morales et al. (2007).

As evident from literature, not much information pertaining to chromate reduction by actinomycetes is available in contrast to the extensive studies carried out in bacteria. Therefore, it is important to assess the predominance of chromate reductase activity among actinomycetes, knowledge regarding which will be useful in understanding its potential utility for bioremediation of Cr(VI) contaminated industrial effluents.

The present thesis deals with screening of actinomycete strains available in our culture collection for growth as well as chromate reduction and selection of a promising strain *S. griseus* (NCIM 2020) for detailed investigation on enzymatic reduction of Cr(VI) including the factors influencing chromate reduction, Cr(VI) reduction by immobilized *S. griseus* with batch and bioreactor studies, property studies of partially purified enzyme, purification of enzyme, investigation the genetic determinants of chromate reduction/resistance.

OBJECTIVES OF THE PRESENT INVESTIGATION

Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* account for 90% of the total actinomycetes. Being natural inhabitants of soil, water and manure, actinomycetes are continuously exposed to different metals present in these habitats and acquire tolerance or resistance to these metals. Their metabolic diversity, mycelial growth characteristics, and their capability to rapidly colonize a variety of substrates make them the most suitable agents for bioremediation of metal and organic compounds. Though they have been extensively studied for production of industrial enzymes and antibiotics, their use for bioremediation is limited. There are very few reports on metal tolerance/resistance/transformation in actinomycetes except mercury resistance which is investigated in detail. Paucity of information on chromate tolerance/ reduction/resistance in actinomycetes coupled with an earlier report from our laboratory on preliminary studies on chromate tolerance and reduction in *Streptomyces griseus*, encouraged us to take up this work. Present study was undertaken to screen actinomycetes for chromate tolerance and reduction, understand physiological, biochemical and genetic aspects of chromate toxicity/resistance and reduction in *S. griseus.*

The highlights of the work done are as follows:

1. Screening of actinomycetes for chromate tolerance and reduction

2. Immobilization of *S. griseus* for chromate reduction

A Batch Studies: Using free and immobilized cells

B Bioreactor Studies: Using immobilized cells

3. Isolation, partial purification and property study of chromate reductase

4. Investigation of the genetic determinants of chromate resistance/ reduction

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CHAPTER II

STUDIES ON CHROMATE TOLERANCE AND REDUCTION IN ACTINOMYCETES

ABSTRACT

Chromium is a toxic heavy metal used in various industries and leads to environmental pollution due to improper handling. The most toxic form of chromium Cr(VI) can be converted to less toxic Cr(III) by reduction. Around nineteen actinomycetes mainly belonging to genus *Streptomyces* were screened for Cr(VI) tolerance and reduction during growth on Broth-II medium containing 25 mg/L of Cr(VI). Among the actinomycetes tested for chromate reduction, thirteen strains reduced Cr(VI) to Cr(III). *Streptomyces griseus* strain NCIM 2020 was found to be most efficient showing complete reduction within 24 h. The organism was able to use a number of carbon sources as electron donors. Sulphate, nitrate, chloride and carbonate had no effect while cations such as Cd(II), Ni(II), Co(II) and Cu(II) were inhibitory to varying degrees on chromate reduction during growth. Copper enhanced chromate reduction by resting cells. Transmission Electron Microscopic studies indicated the presence of chromium in the biomass.

INTRODUCTION

Industrialization is a hallmark of civilization, however, the fact remains that industrial emissions have been adversely affecting the environment leading to destruction of many agricultural lands and water bodies, thus becoming a matter of great concern. Hexavalent chromium (which exists as chromate) containing waste is generated from many industrial applications such as textile production, electroplating, metallurgy, and petroleum refining. At high concentrations, Cr(VI) is toxic, mutagenic, carcinogenic and teratogenic (Komori et al. 1990). According to the United States Environmental Protection Agency (USEPA), industries in the USA use more than 50,000 tons of Cr(VI) every year and release 5,000 kg/d into the environment (Chen and Hao 1997). Nearly 80% of the tanneries in India are engaged in the chrome tanning processes. Chandra et al. (1997) estimated that in India alone about 2000 to 3200 tones of elemental Cr escape into the environment annually from the tanning industries. Cr(VI) undergoes reduction to less toxic Cr(III) in presence of reducing agents and precipitates as hydroxides. Biological Cr(VI) reduction to Cr(III) has great potential for removing it from contaminated waters and waste streams.

Biological reduction of chromate is studied mainly for the purpose of bioremediation, which is based on the concept that Cr(III) has minimal solubility and negligible toxicity compared to Cr(VI). Chromate reducing ability has been demonstrated in a wide range of microbes, including bacteria, actinomycetes and fungi (Chen and Hao 1998; Das and Chandra 1990; Paknikar and Bhide 1993). A variety of Gram-negative (Bae et al. 2000; Bopp and Ehrlich 1988; Horitsu et al. 1987; Ishibashi et al. 1990; Park et al. 2000) as well as Gram-positive bacteria (Camargo et al. 2003; Camargo et al. 2004; Das and Chandra 1990; Campos-Garcia et al. 1997; Horton et al. 2006; Laxman and More 2002; Pal and Paul 2004; Wang and Xiao 1995) are known to reduce Cr(VI). Both aerobic (Das and Chandra 1990; Camargo et al. 2003; Laxman and More 2002; Pal and Paul 2004) as well as anaerobic (Komori et al. 1990; McLean and Beveridge 2001) chromate reduction are reported in literature.

Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* account for 90% of the total actinomycete population. Being natural inhabitants of soil, water and manure, actinomycetes are continuously exposed to

different metals present in these habitats. In order to survive in the polluted environment, they have to adapt or acquire tolerance or resistance to these metals. Their metabolic diversity, mycelial growth characteristics, and their capability to rapidly colonize a variety of substrates make them the most suitable agents for bioremediation of metal and organic compounds.

Reports on chromate reduction by actinomycetes are very few in literature. Das and Chandra (1990) reported reduction of Cr(VI) to Cr(III) by the crude cell extracts of *Streptomyces* sp. Amoroso et al. (2001) reported chromium accumulation in two *Streptomyces* spp. isolated from river sediments. Polti et al. (2007) reported qualitative and semi-quantitative screening of Cr(VI) resistance in actinomycete isolates. Morales et al. (2007) reported chromate reduction by a chromium tolerant *Streptomyces* sp. CG252 isolated from contaminated soils. Chromate reduction during growth and by resting cells of *S. griseus* was reported by Laxman and More (2002). Megharaj et al. (2003) reported hexavalent chromate reduction by *Arthrobacter* sp. isolated from soil contaminated with tannery waste. Desjardin et al. (2003) reported non-enzymatic chromate reduction by *Streptomyces thermocarboxydus* NH50. They isolated a small molecular weight (<1 kDa) reducing agent from culture filtrate but the identity of the reducing agent was not reported.

As evident from literature, not much information pertaining to chromate reduction by actinomycetes is available in contrast to the extensive studies carried out in bacteria. Therefore, actinomycetes with their useful characteristics as mentioned above need to be explored and assessed for predominance of chromate reduction, knowledge regarding which will be useful in understanding its potential utility for bioremediation of Cr(VI) contaminated soils and industrial effluents. In view of this, the study was undertaken and the present chapter deals with screening of the actinomycete strains for growth as well as chromate reduction. A promising strain was selected for detailed investigation on reduction of Cr(VI) including the factors influencing chromate reduction.

MATERIALS AND METHODS

Chemicals

Peptone, yeast extract and malt extract, were supplied by **M/s Hi Media chemicals, India**. Di-phenylcarbazide was procured from **Sigma, Chemicals Co, USA**. Potassium chromate and dichromate were obtained from **Sara Merck, India**. All other chemicals used were of analytical grade.

Microorganisms and culturing conditions

The following growth media adjusted to pH 7 were routinely used during the investigation:

1. Broth-II (g/L): glucose 10; yeast extract 0.2; peptone 2.5; ammonium nitrate 1.0; magnesium sulphate 0.5; calcium chloride 0.5.
2. MGYP (g/L): glucose 10; malt extract 3; yeast extract 3; peptone 5.

Actinomycete strains were routinely sub-cultured once in four weeks on Broth-II agar slants and preserved at 4°C after growth and sporulation. Growth and Cr(VI) reduction studies were carried out in submerged culture in 250 ml Erlenmeyer flasks containing 50 ml Broth-II medium containing potassium dichromate as source of Cr(VI). Stock hexavalent chromium solutions ($K_2Cr_2O_7$ / K_2CrO_4) were prepared in double distilled water (DDW), sterilized and added to the medium before inoculation to get the desired effective concentrations.

Inoculum was developed by inoculating spores from 7 to 10 days old slant. Ten percent (v/v) vegetative inoculum was used to inoculate the experimental flasks and incubated in a rotary incubator shaker (200 rpm) at 28°C for 2-5 days. Uninoculated controls incubated under identical conditions were included for each experiment. Growth of the organism was determined as mycelial dry weight from 50 ml medium. Samples were withdrawn at regular intervals and residual Cr(VI) in the culture supernatant was checked and the difference in initial and residual Cr(VI) was taken as Cr(VI) reduced. Residual Cr(VI) was expressed as percentage of initial Cr(VI). Data presented are mean of two independent experiments conducted in duplicates. The error bars in figures indicate standard error of mean.

Chromium (VI) analysis

Cr(VI) was determined spectrophotometrically using diphenylcarbazide (DPC) (Urone and Anders 1950). To 4.5 ml of appropriately diluted sample, 0.25 ml of 25% H_2SO_4 was added, followed by addition of 0.25 ml of freshly prepared DPC in 50% acetone. The absorbance of violet colored complex formed was measured at 540 nm. Chromium content in the sample was calculated from the standard graph (1-10 μg of Cr).

Transmission Electron Microscopic (TEM) studies

(a) Thin sections of cells for TEM studies were prepared as follows: Bits of *S. griseus* biomass grown in Broth-II medium with and without Cr(VI) was fixed in 2.5% aqueous gluteraldehyde solution at room temperature for 2 h. After fixation, the cells were sedimented (1500 rpm, 10 min) and washed thrice with double distilled water. Without post fixation, the pellet was subjected to dehydration with 30, 50, 70 and 90% ethanol for 15 min at each concentration followed by two changes in absolute ethanol. Since ethanol does not possess good miscibility with epoxy resins, propylene oxide was used as a linking agent.

(b) Dehydrated pellet was kept in propylene oxide for 15 min. Infiltration of the resin was done by placing the pellet overnight at room temperature in a 1 : 1 mixture of propylene oxide and the resin (Epon 812). Embedding was carried out using a mixture of Epon 812 and hardeners DDSA (dodecynyl succinic anhydride) and MNA (methyl nadic anhydride) in the ratio 1:1.5. Two drops of tridimethylaminomethyl phenol (DMP30) was added to accelerate the polymerization process.

(c) Polymerization was carried out using this mixture at 60°C for three days.

(d) Ultrathin sections were cut using an ultramicrotome (Leica Ultracut UCT) and were taken on copper TEM grids (40 mm x 40 mm mesh size).

(e) The sections were slightly stained with uranyl acetate and lead citrate prior to TEM analysis.

(f) TEM measurements were carried out on a JEOL Model 1200EX instrument operated at an accelerating voltage of 60 kV. A low operating voltage was used to minimize damage to the thin sections by electron beam heating.

RESULTS AND DISCUSSION

Screening of actinomycetes for chromium tolerance and reduction

Nineteen actinomycete strains (16 strains from National Collection of Industrial Microorganisms, Pune, India and 3 new isolates) mainly belonging to the genus *Streptomyces* were screened for Cr(VI) tolerance and reduction during growth on Broth-II medium containing 25 mg/L of Cr(VI). Extent of growth in 48 h varied with strain and ranged from 13-90 mg (Fig. 2.1). Thirteen strains among the nineteen actinomycetes tested showed good growth and the medium turned slightly bluish green due to reduction of Cr(VI) to Cr(III) while the remaining 6 strains did not grow in presence of Cr(VI). These thirteen actinomycetes were investigated for Cr(VI) reduction. Cr(VI) concentration in the culture supernatant decreased from initial concentration of 25 mg/L to less than 18 mg/L in 48 h depending on the strain while it was undetectable within 24 h for *S. griseus* (NCIM 2020). Residual Cr(VI) concentrations after 24 h ranged from 0 for NCIM 2020 (highest reduction) to 74.42% for NCIM 2624 (lowest reduction) corresponding to complete and 25.58% decrease respectively. After 48 h, residual concentration in case of NCIM 2624 decreased to 72.99% corresponding to 27% removal/reduction. Eight actinomycetes among 13 strains showed more than 40% reduction i.e. less than 60% residual Cr(VI) within 24 h. NCIM 2020 showed no residual Cr(VI) while NCIM 2623 which is the next best showed 25% residual chromium corresponding to 100% and 75% reduction in 24 h respectively. NCIM 2623 completely reduced 25 mg/L of Cr(VI) in 48 h. Quantitative recoveries of chromium in filtrates were obtained after acid digestion and oxidation by perchloric acid, indicating the reduction of Cr(VI) to Cr(III) (Laxman and More 2002). Concentration of Cr(VI) remained practically unchanged in an un-inoculated control (Table 2.1). Eleven strains showing decrease in Cr(VI) concentration belonged to *S. griseus* while the other two are yet to be identified. Das and Chandra (1990) reported a decrease of 25–30% of Cr(VI) in the culture filtrate in 72 h when *Streptomyces* sp. 3M was grown in peptone yeast extract medium containing 100 mg/L $K_2Cr_2O_7$ [36 mg/L of Cr(VI)] under aerobic conditions. Polti et al. (2007) reported 40-50% removal from 1 mM (52 mg/L) Cr(VI) after 72 h of incubation in minimal medium by *Streptomyces* M46 and strains isolated from wastewaters of

sugarcane plant. *Arthrobacter* sp. reduced 60% of 50 mg/L of Cr(VI) in 46 h under aerobic conditions when grown in minimal salts medium (Polti et al. 2007).

Bacillus subtilis decreased the chromate concentration from 25 mg/L to 2 mg/L after 24 h while 1 mM chromate slightly inhibited the growth and no significant Cr(VI) reduction was observed (Garbisu et al. 1998). *Bacillus* sp. completely reduced 25 mg/L of Cr(VI) in 96 h (Wang and Xiao 1995). The suspended cultures of all strains of *Bacillus* sp. exhibited more than 85% reduction of 1 mM Cr(VI) within 30 h (Desai et al. 2008).

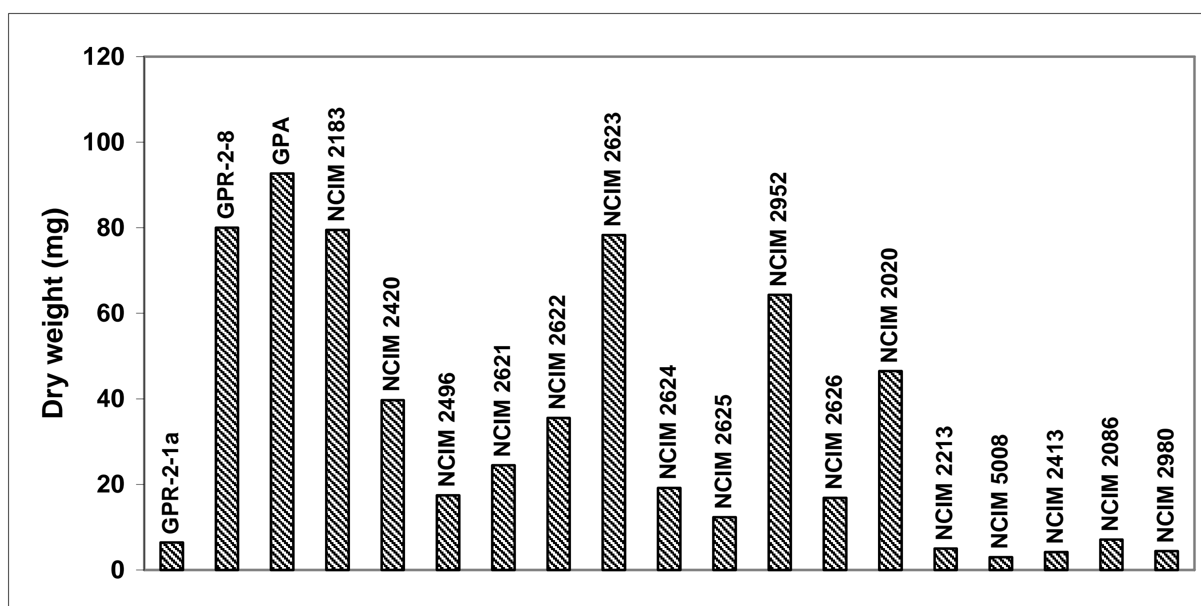


Fig. 2.1: Growth of actinomycetes in Broth-II + 25 mg/L of Cr(VI)

Rate of reduction (mg chromate reduced/L/h) among the actinomycetes tested varied for each strain and at the end of 24 h, it ranged between 0.3013 to 1.042 mg Cr(VI) reduced /L/h. Beyond 24 h, reduction was slower and rate of reduction ranged between 0.1591 to 0.5866 mg Cr(VI) reduced /L/h. *S. griseus* NCIM 2020 which showed highest rate of reduction in 24 h was found to be most efficient and selected for further studies (Table 2.1).

Table 2.1: Cr(VI) reduction by actinomycetes during growth in Broth-II+25 mg/L of Cr(VI)

Name of Organism	Code	Residual Cr(VI) (%)		Rate of Chromate Reduction (mg Cr(VI) /L/ h)	
		24 h	48h	24 h	48h
S. griseus	NCIM 2183	56.25	36.71	0.5157±0.011	0.3731±0.040
S. griseus	NCIM 2020	0	-	1.0420±0.042	-
S. griseus	NCIM 2420	63.26	45.03	0.4335±0.033	0.3248±0.016
S. griseus	NCIM 2496	56.59	55.74	0.5122±0.019	0.2612±0.028
S. griseus	NCIM 2621	63.34	62.23	0.4325±0.016	0.2228±0.024
S. griseus	NCIM 2622	61.29	43.64	0.4560±0.042	0.3320±0.023
S. griseus	NCIM 2623	25.44	0.38	0.8781±0.014	0.5866±0.011
S. griseus	NCIM 2624	74.42	72.99	0.3013±0.012	0.1591±0.029
S. griseus	NCIM 2625	68.04	58.30	0.3772±0.022	0.2461±0.024
S. griseus	NCIM 2626	59.70	58.11	0.4746±0.011	0.2466±0.022
S. griseus	NCIM 2952	50.49	37.13	0.5842±0.039	0.3706±0.026
Isolate -2	GPR-2-8	55.85	33.48	0.5205±0.018	0.3922±0.033
Isolate -3	GPA	56.35	33.81	0.5145±0.032	0.3900±0.015
Uninoculated control	-	99.65	99.21	-	-

Rates of chromate reduction presented with standard error.

Reduction of chromate and dichromate

Though chromium in chromate and dichromate exists in hexavalent form, dichromate is reported to be chemically more reactive than chromate. Therefore, Cr(VI) reduction studies were carried out in Broth-II medium containing either potassium dichromate ($K_2Cr_2O_7$) or potassium chromate (K_2CrO_4) as source of Cr(VI). It was observed that *S. griseus* NCIM 2020 reduced Cr(VI) in both the forms at similar rates during growth. Slight increase in pH from the initial value of 6.97 to 7.02 and 7.04 was noticed after reduction of chromate and dichromate respectively (Fig. 2.2).

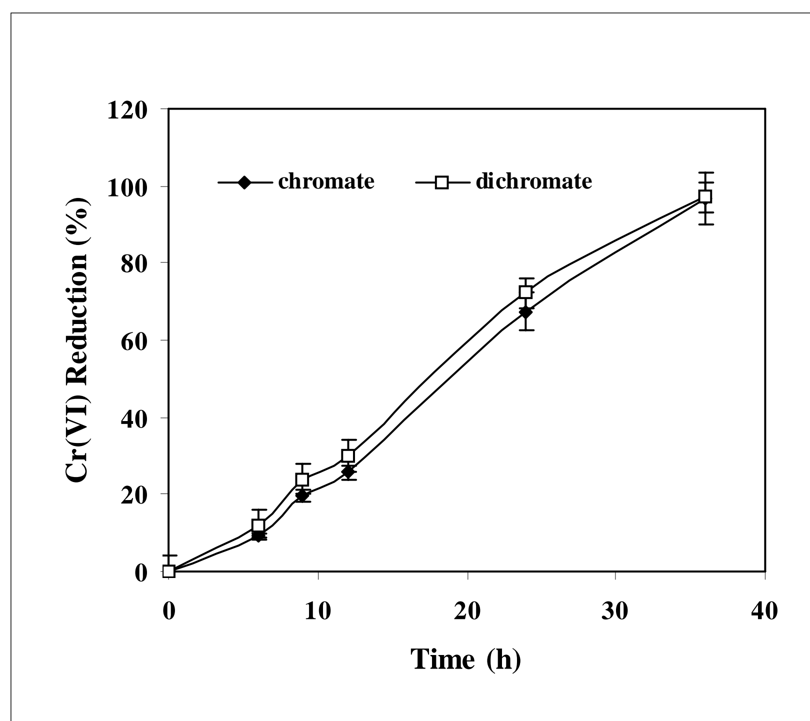


Fig. 2.2: Reduction of chromate and dichromate

Effect of chromium concentration

Effect of chromium concentration on growth and reduction by *S. griseus* NCIM 2020 was studied by growing the organism in Broth-II medium containing 0 to 89 mg/L of Cr(VI). Residual Cr(VI) concentration was monitored during growth and expressed as percentage of initial Cr(VI) values. Good growth with no significant inhibition was seen up to 40 mg/L of Cr(VI). Increasing the chromium concentration increased inhibition of

growth but visible growth (28% relative growth) was still observed in media containing 89 mg/L of Cr(VI) clearly indicating that increased Cr(VI) concentration inhibited cell growth (Table 2.2). However, specific reduction rate i.e. mg Cr reduced per gram of biomass increased from 11.45 to 182.31 with increase in initial Cr(VI) concentration from 15 to 89 mg/L. These rates are similar to those reported by Philip et al. (1998) where *B.coagulans*, *B. circulans* and *Pseudomonas aeruginosa* isolated from Cr(VI) contaminated soil reduced Cr(VI) with the specific reduction rates ranging from 33 to 79 mg Cr reduced/g biomass from initial Cr(VI) of 104 mg/L. Morales et al (2007) reported that *Streptomyces* sp. CG252 tolerated elevated levels of chromium despite its negative effect on growth and development, and was efficient at removing Cr(VI) by promoting reduction to Cr(III). They found that chromium removal was more efficient at lower concentrations.

Table 2.2: Effect of initial Cr(VI) concentration on growth and reduction (36 h)

Initial Cr(VI) (mg/L)	Residual Cr(VI) (mg/L)	Mycelial dry weight (mg/50ml)	Relative growth (%)	Mg Cr reduced by the biomass	Specific rate of reduction (mg Cr reduced/g biomass)
0	-	72.5	100.00	-	-
15	0.568	65.0	89.66	0.744	11.45
30	0.775	61.0	84.14	1.478	24.23
43	0.874	55.5	76.55	2.134	38.45
53	1.338	48.0	66.21	2.575	53.65
64	3.540	40.0	55.17	2.997	74.92
71	7.364	30.0	41.38	3.168	105.60
89	14.543	20.3	28.00	3.701	182.31

Reduction was nearly complete within 12 h when the initial Cr(VI) concentration was between 10-15 mg/L while it was slower and required longer incubation time with higher initial Cr(VI) concentration (Fig. 2.3a).

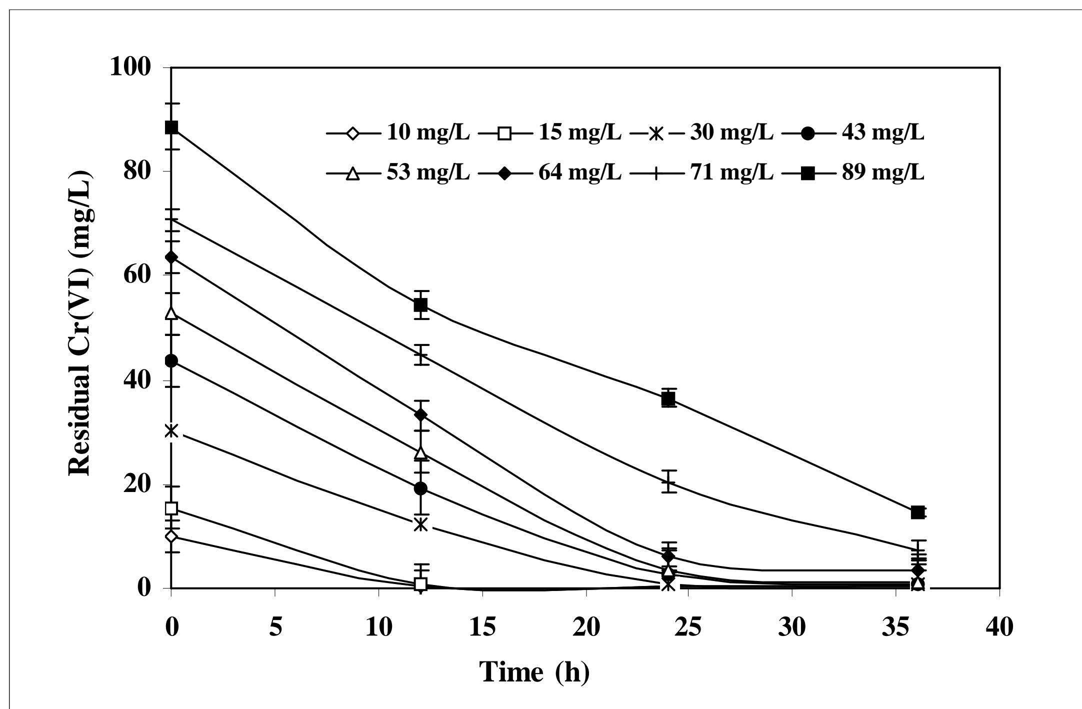


Fig. 2.3a: Effect of initial Cr(VI) concentration on chromate reduction

Rate of reduction for each concentration was calculated from mg of chromate reduced during the incubation period. Rate of reduction increased linearly with increasing Cr(VI) concentration but decreased with incubation time (Fig. 2.3b). Rate of reduction at 12 h increased from 0.788 mg/L/h for 10 mg/L of Cr(VI) to 2.85 mg/L/h for 89 mg/L of Cr(VI). However at a fixed initial Cr(VI) of 30 mg/L, rate decreased from 1.487 mg/L/h in 12 h to 0.815 mg/L/h in 36 h. High initial rates of reduction are also reported for bacterial cultures (Pal and Paul 2004). The highest rate of reduction with initial chromate concentration of 89 mg/L for *S. griseus* was 2.85 mg Cr(VI) reduced/L/h during the initial period of 12 h which is the one of the highest rate reported so far among gram positive bacteria (Table 2.3).

Table 2.3: Rate of chromate reduction by Gram-positive bacteria and actinomycetes

Name of organism	Initial Cr (mg/L)	Mg Cr(VI) reduced during the incubation period	Rate of reduction (mg Cr /L/h)	Reference
***Bacillus* sp.**	20	10.8 mg** in 24 h	0.45*	Wang and Xiao 1995
	30	11.04 mg** in 24 h	0.46*	
	50	11.52 mg** in 24 h	0.48*	
	70	10.56 mg** in 24 h	0.44*	
	90	6.96 mg** in 24 h	0.29*	
***Bacillus* sp.**	10	10 mg* in 12 h	0.833**	Liu et al. 2006
	20	20 mg* in 18 h	1.111**	
	40	33 mg** in 24 h	1.375**	
***Bacillus* sp.**	25	10 mg** in 24 h	0.417**	Wang and Shen 1997
B. subtilis	26	23.92mg** in 24 h	0.997**	Garbisu et al. 1998
***B. sphaericus* AND303**	20	9.6mg** in 24 h	0.400*	Pal and Paul 2004
	40	14.4 mg** in 24h	0.600*	
	60	21.6 mg** in 24 h	0.900*	
	80	28.8 mg** in 24 h	1.200*	
	100	34.08 mg** in 24 h	1.420*	
B. megaterium TKW3	23.4*	16.64 mg** in 360h	0.046**	Cheung and Gu 2005
***Bacillus* sp.**	19.76	18.48 mg** in 48h	0.385**	Sarangi and Krishnan. 2008
***Bacillus* sp.**	20	12 mg** in 24 h	0.500**	Elangovan et al. 2006
***Cellulomonas* sp. (strains ES6 And WS01)**	2.08	1.98 mg** in 96 h	0.020**	Viamajala et al. 2007
***Nesterenkonia* sp.**	10.4	10.4 mg* in 24 h	0.433**	Amoozegar et al. 2007
***Leucobacter* sp.**	19.76	13.77 mg** in 48 h	0.287**	Sarangi and Krishnan 2008
***Exiguobacterium* sp.**	19.76	13.20 mg** in 48 h	0.275**	Sarangi and Krishnan 2008
***Arthrobacter* sp.**	50	30 mg* in 46 h	0.652**	Megharaj et al. 2003
***Streptomyces* sp.**	≈ 36	35.6 mg** in 72 h	0.494**	Das and Chandra 1990
***Streptomyces* M46**	52	20.8-26 mg** in 72 h	0.289-0.361**	Polti et al. 2007
S. griseus	15	15 mg* in 12 h	1.250*	**Present work**
	30	29.4 mg* in 24 h	1.225*	
	43	40 mg* in 24 h	1.697*	
	64	54 mg* in 24 h	2.250*	
	89	34.2 mg* in 12 h	2.850*	

***Actual values. ** Values are calculated from the data given in the reference.**

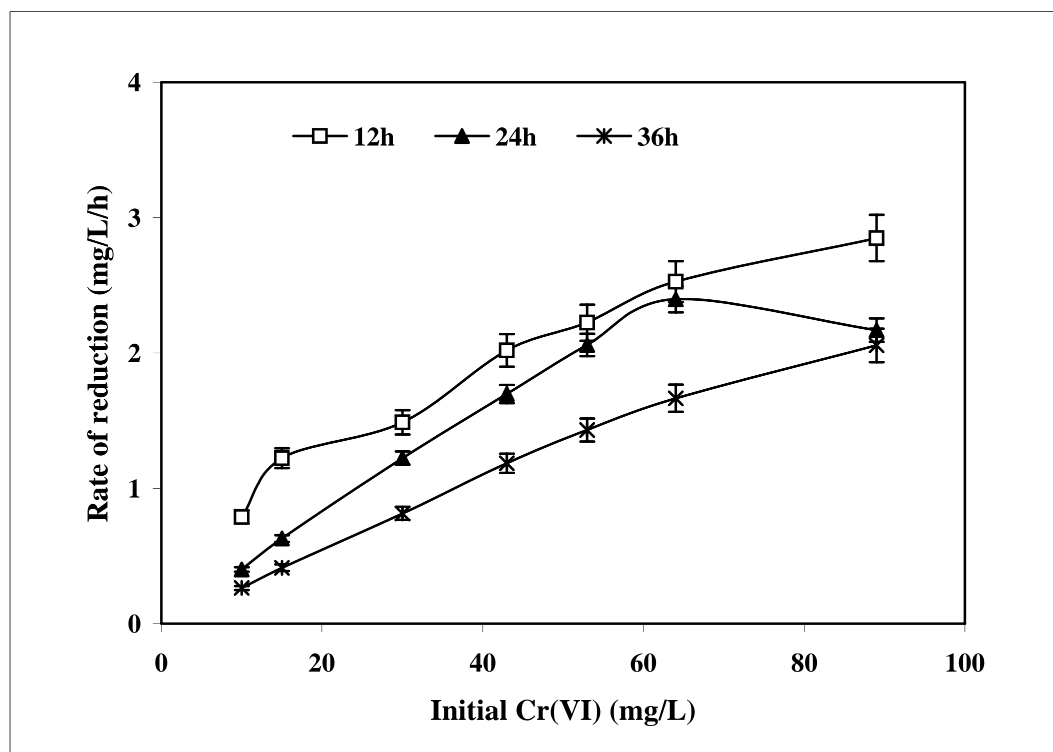


Fig. 2.3b: Rate of chromate reduction by *S. griseus* NCIM 2020

Effect of electron donors

Effect of electron donors on chromate reduction during growth was investigated in Broth-II medium containing 25 mg/L of Cr(VI) and one of the following substrates (0.2%): glucose, sucrose, glycerol, ethanol, glycine, sodium salts of acetic acid, citric acid and tartaric acid.

S. griseus was capable of utilizing a variety of substrates as sole sources of carbon and energy for chromate reduction. Growth as mycelial dry weight ranged between 40-60 mg in all the substrates tested except in two cases, one where there was no addition and other where glycine was used as carbon source. Maximum Cr(VI) reduction was observed in presence of glucose followed by glycerol and acetate. No reduction was observed in the absence of added electron donor or in presence of glycine indicating the requirement for an electron donor for growth and reduction (Fig. 2.4).

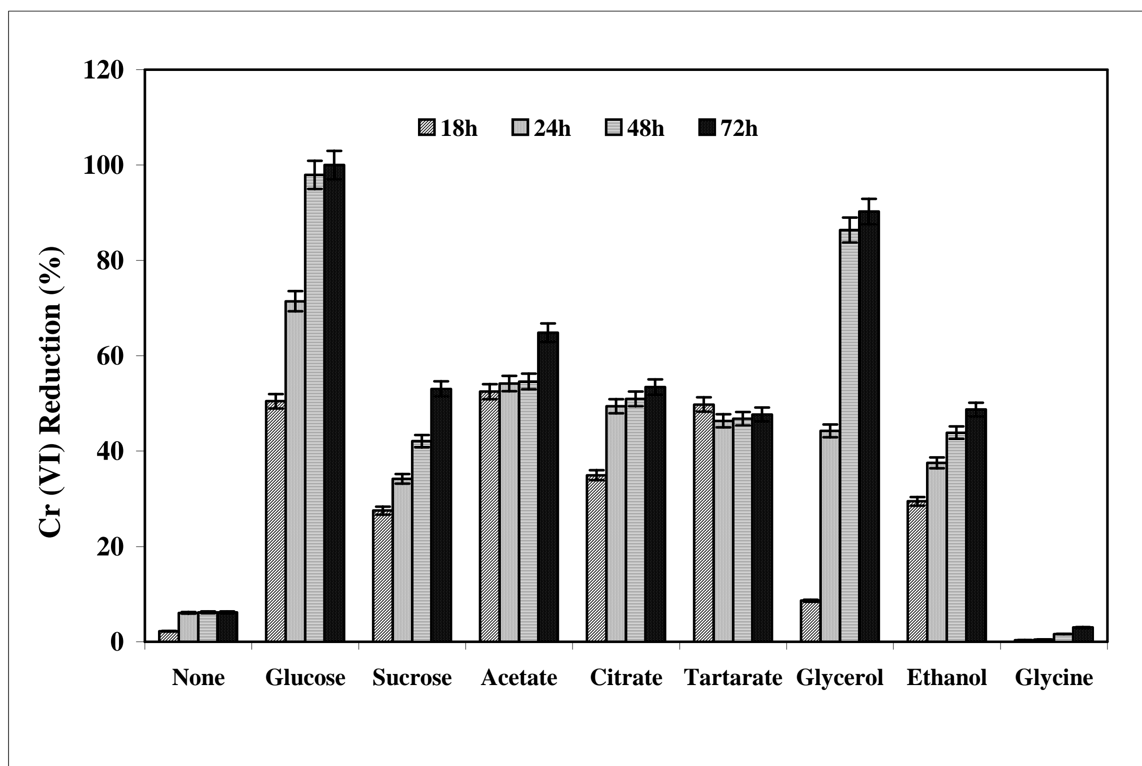


Fig. 2.4: Effect of carbon source on chromate reduction

These results are in agreement with other reports of requirement of glucose or acetate for Cr(VI) reduction. Das and Chandra (1990) reported 25-30% Cr(VI) reduction by *Streptomyces* sp. after 72 h of growth which increased to 97% in presence of 0.5% glucose. Though *B. sphaericus* used variety of organic compounds like amino acids, organic acids and nitrogenous substances as electron donors, pronounced reduction occurred only when glucose or yeast extract were used (Pal and Paul 2004). Cr(VI) reduction was enhanced significantly by the addition of glucose in case of *Bacillus* sp. (Liu et al. 2006) and *Ochrobactrum* sp. strain CSCr-3 (He et al. 2009). Rate of reduction increased 6 fold by media optimization and complete reduction of 75 mg/L of Cr(VI) by a halophilic *Streptomyces* sp. MS-2 within 12 h is reported by Mabrouk (2008). Glucose, peptone, yeast extract, inoculum size, and volume of the medium had a significant effect on Cr(VI) reduction.

Effect of anions and cations

Effect of anions and cations on chromate reduction during growth was investigated by growing the organism in Broth-II medium containing 25 mg/L of Cr(VI) supplemented with anions such as carbonate, nitrate, chloride, sulphate and cations such as cadmium, cobalt, copper and nickel at equimolar concentrations (0.5 mM). Cr(VI) removal from industrial effluents by biological reduction can be problematic as anions or cations or other heavy metals present in the wastewaters can influence chromate reduction**.** Effect of ions on Cr(VI) reduction during growth of *S. griseus* revealed that none of the anions tested had any significant effect while cations inhibited Cr(VI) reduction to varying degrees. Inhibition of growth was in the following order: Cd(II)>Cu(II)>Co(II)>Ni(II). Inhibition of Cr(VI) reduction also followed similar trend with the exception of Cu(II) which showed least inhibition (Fig. 2.5).

It is surprising to see that sulphate a known competitive inhibitor of chromate transport (Cervantes et al. 2001), did not inhibit Cr(VI) reduction in the present study. Neither sulphate nor nitrate affected chromate reduction in *P. putida* (Ishibashi et al. 1990), *Bacillus* sp. (Liu et al. 2006) and *Ochrobactrum* sp. strain CSCr-3 (He et al. 2009). Garbisu et al. (1998) reported chromate reduction by *B. subtilis* to be independent of nitrate. Ni(II) and Co(II) inhibited chromate reduction by *B. sphaericus*, while Pb(II) and Cd(II) had no effect (Pal and Paul 2004). The presence of metals, such as Cu(II), Co(II), Mn(II), etc., significantly stimulated Cr(VI) reduction by *Ochrobactrum* sp. CSCr-3 (He et al. 2009). Chromate reduction by culture supernatants of *Streptomyces thermocarboxydus* NH50 was enhanced by 0.1 mM Cu(II) whereas Ni(II) and Cd(II) had no significant effect (Desjardin et al. 2003).

Effect of copper on chromate reduction during growth of S. griseus

Since copper showed least inhibition, effect of copper on chromate reduction during growth was investigated in Broth-II medium containing 25 mg/L of Cr(VI) and 1 mM Cu(II). Samples were removed at regular intervals and residual Cr(VI) was determined. During the initial period of 12 h, chromate reduction was faster in media supplemented with 1 mM Cu(II) compared to media devoid of it after which it slowed

down and around 20% Cr(VI) was present after 36 h while in control, complete reduction was observed (Table 2.4).

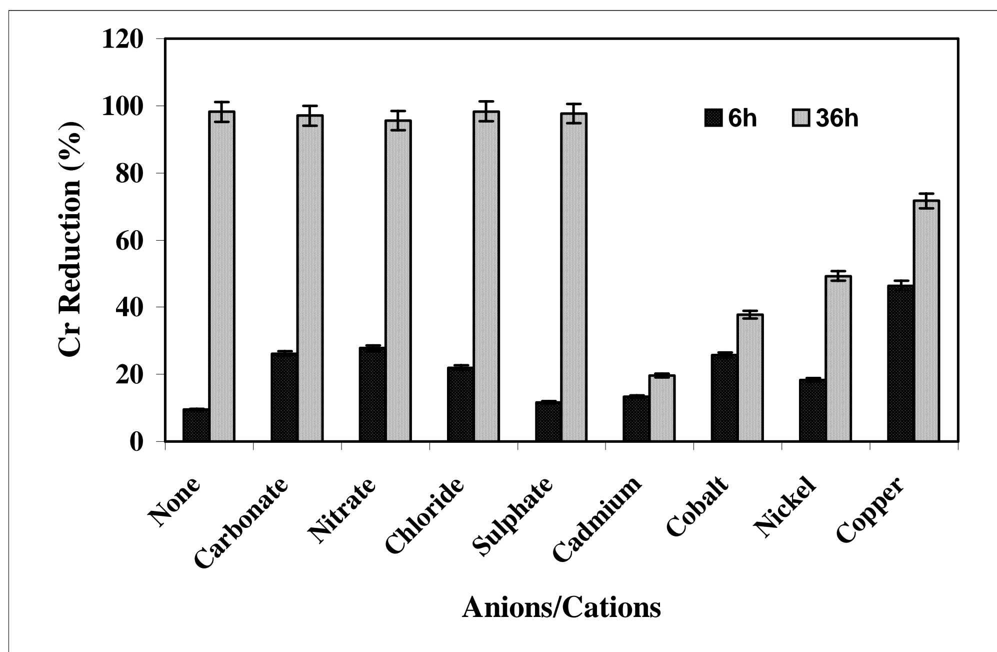


Fig. 2.5: Effect of anions/cations on chromate reduction

Table 2.4: Effect of copper on chromate reduction during growth of *S.griseus*

Time (h)	Without copper		1mM Copper	
	Residual Cr(VI) (%)	Cr(VI) reduction (%)	Residual Cr(VI) (%)	Cr(VI) reduction (%)
0	100	0	100	0
6	81.97	18.03	57.87	42.13
9	74.11	25.89	51.36	48.64
12	69.75	30.25	46.32	53.68
24	21.01	78.99	42.43	57.57
36	1.33	98.67	20.55	79.45

Effect of copper on chromate reduction by resting cells of S. griseus

Inhibition of growth was observed in Broth-II media containing 25 mg/L of Cr(VI) and 0.5-1 mM Cu(II). Therefore resting cells were used to investigate the effect of Cu(II) on Cr(VI) reduction. Reduction was studied with 2 g wet cells (100 mg dry weight) in Broth-II medium containing 25 mg/L of Cr(VI) and Cu(II) concentrations

ranging from 0.4 to 1.0 mM. Addition of 0.4 mM Cu(II) resulted in complete reduction of 25 mg/L of Cr(VI) in 6 h, while only 40% reduction was observed in its absence (Table 2.5). Increasing the Cu(II) concentration to 0.6 mM resulted in 90% reduction in 3 h but further increase had no significant effect. Cu(II) at 0.6 mM resulted nearly in 3-4 fold increase in reduction in 3 h and reduction was nearly complete. Even after 6 h, reduction in presence of 0.4-0.6 mM Cu(II) is 2.4 fold higher compared to control devoid of Cu(II). Probably there was enhancement of Cr(VI) reduction till the concentration of Cu(II) reached 0.5 mM which is equimolar to 25 mg/L i.e. 0.5 mM Cr(VI) beyond which there is no effect.

The stimulatory mechanism of Cr(VI) reduction by Cu(II) and other metals is not clear. However, Cu(II) is a prosthetic group for many reductases. The main function of Cu(II) has been reported to be related to electron transport protection or acting as electron redox center and, in some cases, as a shuttle for electrons between protein subunits (Camargo et al. 2003).

Table 2.5: Effect of copper on chromate reduction by the resting cells of *S. griseus*

Copper (mM)	Residual Cr(VI) (%)			Cr(VI) Reduced (%)			Increase in reduction (fold over control)	
	0 h	3 h	6 h	0 h	3 h	6 h	3 h	6 h
Nil	100	75.83	59.36	0	24.17	40.64	-	-
0.4	100	11.98	1.35	0	88.02	98.65	3.64	2.43
0.6	100	7.31	0.39	0	92.69	99.61	3.83	2.45
0.8	100	5.36	0.43	0	94.64	99.57	3.91	2.45
1.0	100	1.54	0.42	0	98.46	99.58	4.07	2.45
Uninoculated control	100	99.90	96.40	0	0.10	3.6	-	-

Changes in oxido-reduction potential (ORP)

Changes in pH and redox conditions are known to occur in medium during growth of bacterial cultures due to various biochemical reactions and the metabolites formed. These changes may indirectly affect the reduction of Cr(VI) in the medium. Cr(VI) reduction occurs in a wide range of redox potentials. The optimum redox potential

range has not been well established as yet. Lower redox and pH has been shown to favor reduction of Cr(VI) (Daulton et al. 2001). Changes in the oxido-reduction potential (ORP) during the growth and chromate reduction in Broth-II containing 25 mg/L of Cr(VI) was monitored electrometically by using Global redox electrodes. Cr(VI) reduction began immediately after inoculation and continued till 36-48 h (Fig. 2.6). The oxidation-reduction potential which was around +20 mV in the beginning dropped to –20 mV in the first 24 h of growth after which there was steady increase to reach the maximum of +60 mV at the end of 48 h. Cr(VI) reduction was observed over the ORP range of – 40 to +50 mV. Though changes in ORP were not very drastic nor a definite correlation was found between Cr(VI) reduction and ORP values of the culture medium, reduction occurred even with positive ORP of +20 mV. Our ORP results are in agreement with an earlier report where a rapid drop of ORP from +300 mV to about –500 mV was noticed in 48 h during Cr(VI) reduction by *Bacillus* sp. (Wang and Xiao 1995). They reported that Cr(VI) reduction by *Bacillus* sp. began almost immediately and continued throughout incubation while ORP and cell density remained constant during first 48 h after which there was sudden drop to –500 mV. However, others have demonstrated the requirement of a low ORP for Cr(VI) reduction. For example, the rate of Cr(VI) reduction by washed and resting cells of *Agrobacterium radiobacter* pre-grown under different carbon and energy sources was shown to be greater in cell suspensions with ORP values of –240 mV than with –198 mV (Chen and Hao 1998). Similarly, when Cr(VI) was introduced to *Escherichia coli* culture with ORP > –140 mV, no reduction of Cr(VI) was observed within the first 1 h. The trend of ORP changes in the present study indicates that low ORP is not necessary for Cr(VI) reduction by *S. griseus*.

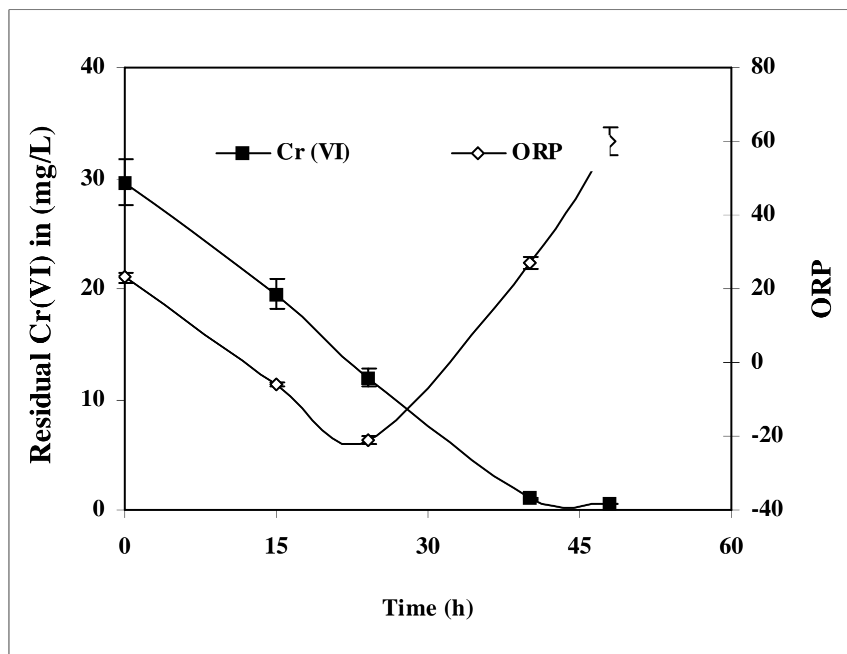
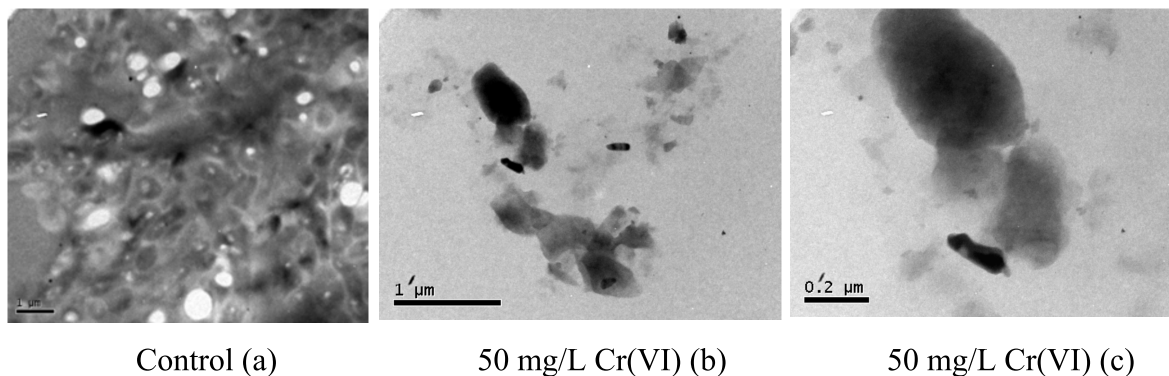


Fig. 2.6: Changes in ORP during reduction

TEM studies

During growth of the organism, medium which was yellow in colour due to Cr(VI) turned bluish green after conversion to Cr(III) and fraction of it was taken up by the biomass rendering bluish tinge to the biomass. Therefore, localization of chromium was studied by transmission electron microscopy (TEM). *S. griseus* cells grown with and without 50 mg/L of Cr(VI) for 48 h were used. TEM studies revealed the presence of dense spots inside the cells grown in presence of Cr(VI) indicating the localization of Cr(VI) inside the cell (Fig. 2.7 a-c).



Control (a) 50 mg/L Cr(VI) (b) 50 mg/L Cr(VI) (c)

Fig. 2.7: Transmission Electron Micrographs of *S. griseus* NCIM 2020 cells

CONCLUSIONS

Our studies revealed that majority of the actinomycetes tested were tolerant and capable of reducing Cr(VI) to Cr(III) during growth. Among the *S. griseus* strains showing chromate reduction, *S. griseus* NCIM 2020 was most efficient with complete conversion of 25 mg/L of Cr(VI) to Cr(III) within 24 h. At lower initial Cr(VI) concentration, reduction was nearly complete within 12 h while it was slower and required longer incubation time with higher initial Cr(VI) concentration. The rate of reduction by *S. griseus* for initial 89 mg/L chromate concentration was 2.85 mg Cr(VI) reduced/L/h in the initial period of 12 h which is one of the highest rate reported so far among gram positive bacteria. The organism was able to utilize variety of carbon/energy sources/electron donors for reduction. Reduction was unaffected by anions while cations inhibited the reduction to varying degrees with copper being least inhibitory. Copper enhanced chromate reduction by resting cells. After growth of the organism in medium containing Cr(VI), the colour of the medium which was yellow turned bluish green due to conversion of Cr(VI) to Cr(III) and a fraction of it was taken up by the biomass giving it bluish tinge. TEM studies of *S. griseus* cells grown in presence of 50 mg/L of Cr(VI) showed the presence of chromium in the cell.

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CHAPTER III

IMMOBILIZATION OF *S. griseus* FOR CHROMATE REDUCTION

ABSTRACT

Reduction of toxic Cr(VI) which is a mutagen and carcinogen to the less toxic Cr(III) by microorganisms is potentially a useful bioremediation process. Hexavalent chromium was efficiently reduced by *S. griseus* NCIM 2020 and this activity was associated with the cell. Cr(VI) reduction in batch mode was studied by free cells as well as by cells immobilized in different matrices. PVA-alginate immobilized *S. griseus* cells showed highest Cr(VI) removal efficiency with reduction rates similar to free cells and completely reduced 25 mg/L of Cr(VI) in 24 h. PVA-alginate immobilized cells could be reused four times with complete reduction of 25 mg/L of Cr(VI) in 24 h in each cycle. Chromate was completely reduced by PVA-alginate immobilized cells within 9 h from simulated effluent containing 25 mg/L of Cr(VI) supplemented with following metals: Cu(II), Mg(II), Mn(II) and Zn(II).

Since PVA-alginate immobilized *S. griseus* cells proved to be most effective, the same was used for Cr(VI) reduction in a bioreactor. Effect of parameters such as initial Cr(VI) concentration, flow rate and biomass concentration on chromate reduction were studied. The Cr(VI) reduction efficiency decreased with increasing Cr(VI) concentration from 2 to 12 mg/L and increased with increase in biomass concentration. However, increasing the flow rate from 2 to 8 ml/h did not significantly affect Cr(VI) reduction. The reduction was faster in simulated effluent than in synthetic medium and complete removal of 8 mg/L of Cr(VI) from simulated effluent and synthetic medium occurred in 2 and 12 h respectively. These results indicate that immobilized *S. griseus* cells can be used for bioremediation of chromate containing effluents and wastewaters.

INTRODUCTION

Chromate-containing wastes are generated from several industrial processes such as leather tanning, electroplating, paint, pigment dye manufacturing etc. and discharged in to rivers/ streams leading to wide spread pollution in the environment. Permissible level for discharge of toxic chromate, a potential carcinogen and mutagen, into water bodies is < 1 mg/L. Conventional methods of its remediation are either inefficient or suffer from high capital and operating costs and generate secondary wastes. The use of biological methods to remediate metal contaminated wastewaters is an emerging field and methods such as biotransformation and biosorption are attractive because they are less energy intensive and produce no secondary by-products. Biotransformation of Cr(VI) to the non-toxic trivalent form i.e. Cr(III) therefore offers a viable alternative. Microbial systems reducing highly toxic Cr(VI) to less toxic Cr(III) are reported in literature (Chen and Hao 1998). The potential of several bacterial strains to detoxify chromate has been described with a view of developing processes for microbiological detoxification of polluted waters.

Use of expensive electron donors is not feasible for practical applications in large-scale bioremediation projects. Hence use of whole cells having endogenous electron donors is advantageous. Methods using free cells for remediation suffer due to Cr(VI) toxicity and cell damage. Whole cell immobilization has advantages over free cells in being more stable. Immobilized whole cell systems are attracting worldwide attention due to their ease of regeneration, possibility of reuse, easier solid-liquid separation, minimal clogging in continuous systems. Cell immobilization can be accomplished using a variety of natural (viz. agar, carrageenan) and synthetic (viz. polyacrylamide, polyethylene glycol) matrices as supports. Natural matrices have the disadvantage of being prone to abrasion and are biodegradable. The choice of the immobilization support is a key factor in environmental application of immobilized biocatalyst.

Batch studies

There are reports on use of immobilized bacteria for Cr(VI) reduction in batch systems. Tucker et al. (1998) reported Cr(VI) reduction by *Desulfovibrio desulfuricans* immobilized in polyacrylamide gels. Cr(VI) reduction by immobilized cells of

Microbacterium liquefaciens MP30 on polyvinyl alcohol (Pattanapipitpaisal et al. 2001), *Bacillus* sp. ES 29 on celite, amberlite and Ca-alginate (Camargo et al. 2004a), *Serratia marcescens* as a stable biofilm on activated carbon (De Bruijn and Mondaca 2000), *Pseudomonas* immobilized in agar–agar films on the cellulose acetate membrane (Konovalova et al. 2003) are also reported.

Bioreactor studies

Most of the earlier studies on biological reduction of Cr(VI) were carried out in batch systems. The development of bioreactors consisting of a bed of chromate reducing bacteria immobilized to an inert support is considered as an economical, effective, safe and sustainable procedure for chromate detoxification in a continuous mode (Ganguli and Tripathi 2002). Bioreactors for continuous operation with immobilized microorganisms are being increasingly used for wastewater treatment achieving high performance and stability due to high cell densities, absence of cell washout, and extended biochemical or biotransformation reaction time. Various bioreactors such as continuous stirred tank (Bhide et al. 1996; Rajwade and Paknikar 1997), continuous-flow (Pattanapipitpaisal et al. 2001; Humphries et al 2005), membrane (Konovalova 2003), fixed film (Chirwa and Wang 1997a, b) and fed-batch (Fujii et al. 1990) have been used for reduction of Cr(VI). The potential of *Microbacterium liquefaciens* entrapped in PVA-alginate beads and the palladised biomass of *Desulfovibrio vulgaris and Desulfovibrio desulfuricans* have also been investigated for continuous removal of Cr(VI) from aqueous solutions (Pattanapipitpaisal et al. 2001; Humphries et al. 2004; Mabbett et al. 2004).

Actinomycetes have been extensively studied for antibiotic and enzyme production but their studies on application for metal transformation and bioremediation are meager. Though actinomycetes are also being investigated for chromate reduction in recent years, there are no reports on chromate reduction by immobilized actinomycetes (Laxman and More 2002; Desjardin et al. 2003; Camargo et al, 2004b; Polti et al. 2007).

As there is no information on Cr(VI) reduction by immobilized actinomycetes, the present study was undertaken with a view to examine the feasibility of using immobilized *S. griseus* cells for remediation of Cr(VI) containing effluents. The present chapter deals with the screening of various matrices for immobilization of *S. griseus* cells, their use for

Cr(VI) reduction including the factors influencing reduction in batch as well as in continuous bioreactor systems.

MATERIALS AND METHODS

Chemicals

Polyvinyl alcohol (PVA) Mol.wt 9000-10,000, 80 mol% of hydrolysation was obtained from **Aldrich**. Sodium alginate (medium viscosity) and agarose (Type VII, low gelling temperature) were obtained from **Sigma Chemicals Co, USA**. Analytical grade potassium dichromate from **Sara Merck, India** was used as source of hexavalent chromium. All other chemicals were of analytical grade.

Immobilization of S. griseus and screening of matrices

S. griseus was grown in MGYP medium for 48 h, harvested by centrifugation (10,000 x g, 30 min, 4ºC) under aseptic conditions and 2 g wet cells (100 mg dry weight) was suspended in 1 ml sterile water and used immediately for immobilization studies. PVA-alginate, PVA-borate, PVA-nitrate, agar, agarose and polyacrylamide were used as matrices for immobilization of whole cells of *S griseus* and evaluated for chromate reduction. Entrapment of cells in each matrix is carried as described below.

PVA-alginate

Polyvinyl alcohol (1 g) and sodium alginate (0.16 g) were mixed in 19 ml of sterile double distilled water (DDW). The solution was heated at 60°C to completely dissolve PVA and alginate. The PVA-alginate solution was cooled to 35°C and then mixed thoroughly with the cell suspension. The final concentration in the mixture was 5% (w/v) PVA and 0.8 % (w/v) sodium alginate. PVA-alginate cell beads were prepared by extruding the mixture as drops using a 10 ml disposable plastic syringe into 200 ml of 2% (w/v) $CaCl_2.2H_2O$ and kept at 4°C for 15 h for hardening (Pattanapipitpaisal et al. 2001).

PVA-borate

Polyvinyl alcohol (1 g) and sodium alginate (4 mg) were mixed in 19 ml of sterile double distilled water. The solution was heated at 60°C to completely dissolve PVA and alginate, cooled to 35°C and then mixed thoroughly with the cell suspension. The final concentration in the mixture was 5% (w/v) PVA and 0.02% (w/v) sodium alginate (0.02% alginate was added to prevent bead agglomeration). PVA-borate cell beads were

prepared by extruding the above cell suspension mixture as drops from a disposable syringe into 200 ml of saturated boric acid containing and 2% (w/v) $CaCl_2.2H_2O$ and immersing the beads for 24 h (Wu and Wisecarver 1992).

PVA-nitrate

Polyvinyl alcohol (1 g) and sodium alginate (4 mg) were mixed in 19 ml of sterile DDW. The solution was heated at 60°C to completely dissolve PVA and alginate, cooled to 35°C and then mixed thoroughly with the cell suspension. The final concentration in the mixture was 5% (w/v) PVA and 0.02 % (w/v) sodium alginate (0.02% alginate was added to prevent bead agglomeration). The above cell suspension was extruded as drops, into a 200 ml of solution comprising 50% (w/v) sodium nitrate and 2% (w/v) $CaCl_2.2H_2O$ and immersed for 2 h to form PVA-nitrate cells beads (Chang and Tseng 1998).

After the cell mixtures (PVA-alginate, PVA-borate, PVA-nitrate) were dropped into the solution, spherical gel-beads (3 mm in diameter) were formed without agglomeration and exhibited rubber like properties. This is because of the natural acidic polysaccharide being rapidly cross-linked with calcium.

Agar

The procedure was carried out according to the method described by Nilson et al. (1983). A 3% agar solution was homogenized by boiling, sterilized at 121°C for 15 min and cooled to 40°C. S. griseus cells (2 g) were mixed with 20 ml agar solution on a vortex mixer. The beads were prepared by extruding the mixture drop by drop into paraffin oil.

Polyacrylamide

Entrapment of *S. griseus* cells in polyacrylamide was carried out according to Pundle et al. (1988) *S. griseus* cells (2 g) were mixed with 20 ml solution containing acrylamide 15% (w/v), 0.8% (w/v) N,N' methylene bisacrylamide, 1% (v/v) TEMED and 0.5% (w/v) sodium alginate. This mixture was extruded as drops into a solution containing 2% (w/v) $CaCl_2.2H_2O$ and 0.5% (w/v) ammonium persulphate (APS), incubated for 1 h, washed with sterile distilled water to leach out alginate.

Agarose

Entrapment of *S. griseus* cells in agarose was carried out according to Shankar et al. (1985). The cells were mixed thoroughly with 20 ml of 2% (w/v) aqueous solution of agarose and 2% (w/v) sodium alginate. The resulting suspension (maintained at 40°C) was dropped into 2% $CaCl_2$ solution under mild stirring, incubated for 1 h. The immobilized beads were washed with sterile distilled water.

Cr(VI) reduction by free and immobilized S. griseus

Cr(VI) reduction studies were carried out in batch as well as in a bioreactor in continuous mode. All beads were washed 3 times with 200 ml sterile distilled water and used immediately for the Cr(VI) reduction studies.

Batch studies

Beads containing 2 g wet cells were added to 50 ml Broth-II medium or simulated effluent adjusted to pH 7 containing 25 mg/L of Cr(VI) in a 250 ml flask under aseptic conditions. The flasks were incubated at 28°C with shaking (180 rpm). Samples were taken at regular intervals and residual chromate concentration in the supernatants was measured and expressed as percentage of initial Cr(VI) concentration. Controls without added immobilized beads were included for each experiment and incubated under identical conditions.

Bioreactor design and operating conditions

The reactor was designed to operate in an upward flow mode under aerated conditions. The assembly consisted of a glass reactor with a flat base with 60 ml working volume. Inlet and outlet ports for the chromate containing influent and effluent were provided towards the bottom and top of the reactor respectively. The lines feeding the influent and the effluent were sterilized using 70% ethanol just before use. The reactor was operated at room temperature (28°C) and contents were mixed using a magnetic stirrer. Influent adjusted to pH 7 was fed continuously at the required flow rate from the bottom of the reactor using a programmable peristaltic pump. Another peristaltic pump was used to remove the treated effluent at the same flow rate as that of the influent.

Cr(VI) in the influent and effluent samples was periodically analyzed spectrophotometrically as described in Chapter 2. The residual Cr(VI) in the effluent was expressed as percentage of initial Cr(VI) concentration.

Data presented are mean of two independent experiments with error bars indicating standard error of mean (SEM). Wherever error bars are not seen, they are within the dimensions of the symbols.

RESULTS AND DISCUSSION

Batch Studies

Screening of immobilization matrices for Cr(VI) reduction

Performance of various matrices with respect to bead integrity and Cr (VI) reduction are presented in Fig. 3.1 and Table 3.1. Free as well as cells immobilized in PVA alginate and agarose completely reduced 25 mg/L of Cr(VI) within 24 h (Fig. 3.1). But reduction did not go to completion even after 96 h by PVA-nitrate and PVA-borate immobilized cells. Both these beads were unstable and disintegrated after 24 h (Table 3.1). No metal reduction was observed in absence of cells (beads without cells). Though beads without cells for each matrix were included as controls, only PVA-alginate beads (without cells) as representative are shown in Fig. 3.1. Among the matrices tested for whole cell immobilization of *S. griseus*, PVA-alginate proved to be most effective followed by agarose, agar and polyacrylamide with Cr(VI) reduction percentages of 100, 96.8, 90.4 and 80.3% respectively (24 h). Therefore further studies were carried out with PVA-alginate immobilized cells which showed reduction rates similar to that of free cells.

Humphries et al. (2005) found agar and agarose as the best matrices for whole cell immobilization of *Desulfovibrio vulgaris* NCIMB 8303 and *Microbacterium* sp. NCIMB 13776. PVA-alginate immobilized *S. griseus* cells that completely reduced 25 mg/L of Cr(VI) within 24 h seem to be more efficient compared to PVA-alginate immobilized cells of *Microbacterium liquefaciens* and agar immobilized cells of *Microbacterium* sp. *Microbacterium liquefaciens* required four days to reduce 100 μM (5.2 mg/L) sodium chromate to 2 μM and *Microbacterium* sp. NCIMB 13776 required 65 h to reduce 500 μM (26 mg/L) Cr(VI) to 261 μM (13.57 mg/L) Cr(VI). Unlike *S. griseus* which reduces Cr(VI) aerobically, these organisms are reported to reduce Cr(VI) only under anaerobic conditions using acetate or citrate as electron donors (Pattanapipitpaisal et al. 2001; Humphries et al. 2005).

Though PVA-nitrate and PVA-borate beads were unstable and disintegrated after 24 h, Cr(VI) reduction during this period was 28.6 and 44.6% respectively. Further incubation up to 48 h increased the reduction only marginally and no further reduction occurred even after prolonged incubation of 96 h which could be due to disintegration of

beads leading to exposure of the cells to Cr(VI). Similar observations were also made by Pattanapipitpaisal et al. (2001) and Humphries et al. (2005).

Reduction of Cr(VI) by PVA-alginate immobilized *S. griseus* cells was slightly faster while polyacrylamide immobilized cells was slower than free cells (Fig.3.1). Tucker et al. (1998) reported reduction of 0.5 mM Cr(VI) (26 mg/L) to be slower with polyacrylamide-immobilized cells of *Desulfovibrio desulfuricans* than with freely suspended cells.

Table 3.1: Screening of matrices for Cr(VI) reduction

Immobilization matrix	Initial Cr (mg/L) 0 h	Residual (mg/L) 24 h	Cr(VI) reduction (%)	Bead Integrity
PVA-alginate	25.0	0	100	Retained
Agarose	24.9	0.8	96.8	Retained (cells leached after 4th recycle)
Agar	25.9	2.5	90.4	Cells leached out after 24h
Polyacrylamide-alginate	24.8	4.9	80.3	Retained
PVA-Borate	25.8	14.3	44.6	Beads dissolved after 24h
PVA-Nitrate	25.5	18.2	28.6	Beads dissolved after 24h

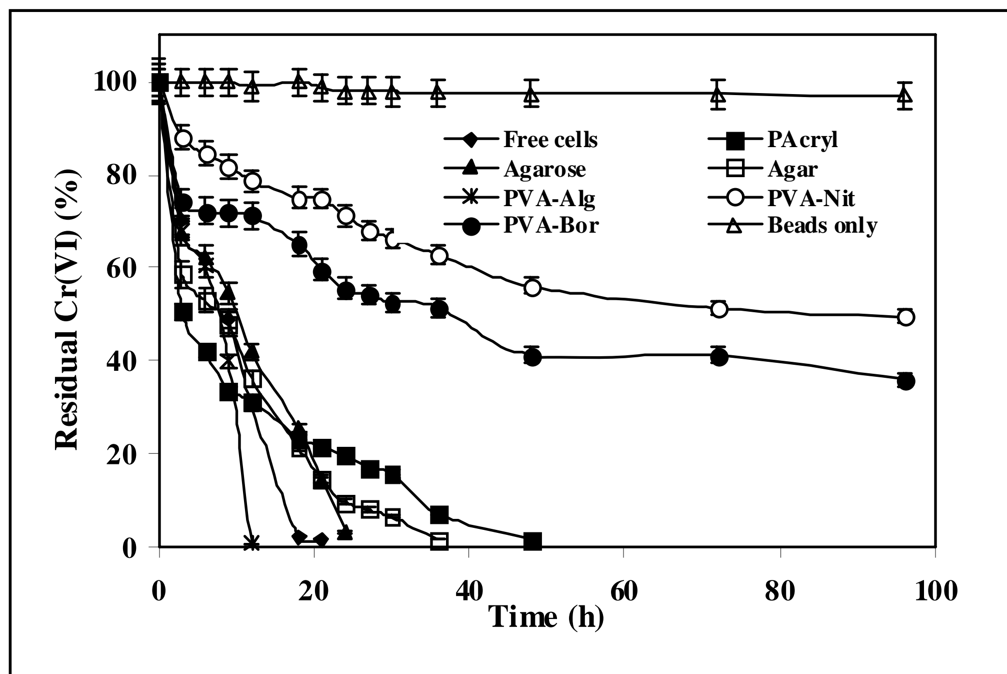

Fig 3.1: Chromate reduction by free and immobilized cells of *S. griseus*

Effect of cell concentration

Effect of cell concentration on reduction of 25 mg/L of Cr(VI) was studied using free and PVA-alginate immobilized cells at concentrations ranging from 1 to 4 g wet weight (dry weights ranging from 0.05 to 0.2 g). Chromate reduction increased with increase in cell concentration and 2 g wet cells (≈100 mg dry wt) completely reduced 50 ml of 25 mg/L of Cr(VI) in 24 h and higher cell densities had no additional benefit (Fig.3.2). Reduction by free cells was slightly higher compared to immobilized cells especially at lower cell densities (1 g wet cells). Though rate of reduction (mg Cr(VI) reduced/L/h) increased with increase in biomass concentration, specific reduction rate (mg Cr(VI) reduced/g biomass/h) decreased with increase in biomass concentration.

The effect of cell density on Cr(VI) reduction by free cells of *Bacillus coagulans* was studied by Philip et al. (1998). Rate of Cr(VI) reduction (mg Cr reduced/L/h) increased while specific reduction which is a measure of Cr(VI) reduction per unit weight of cells per unit time decreased with increase in biomass concentration. At an initial Cr(VI) concentration of 104 mg/L, as the biomass concentration increased from 0.32 to 1.96 g/L, specific reduction rate decreased from 153.53 to 50.51 mg Cr(VI) reduced/g

biomass. Similar trends were also observed with *Escherichia coli* and *P. fluorescence* LB-300 (DeLeo and Ehrlich 1994).

Humphries et al (2005) used 1.25 mg dry cell weight/ml for their studies on Cr(VI) reduction using immobilized *D .vulgaris* and *Microbacterium* sp. In batch mode agar and agarose immobilized *D. vulgaris* and *Microbacterium* sp. showed reduction occurring at an initial rate of 130 and 15 nmol/h/mg dry cell weight, respectively (both matrices).

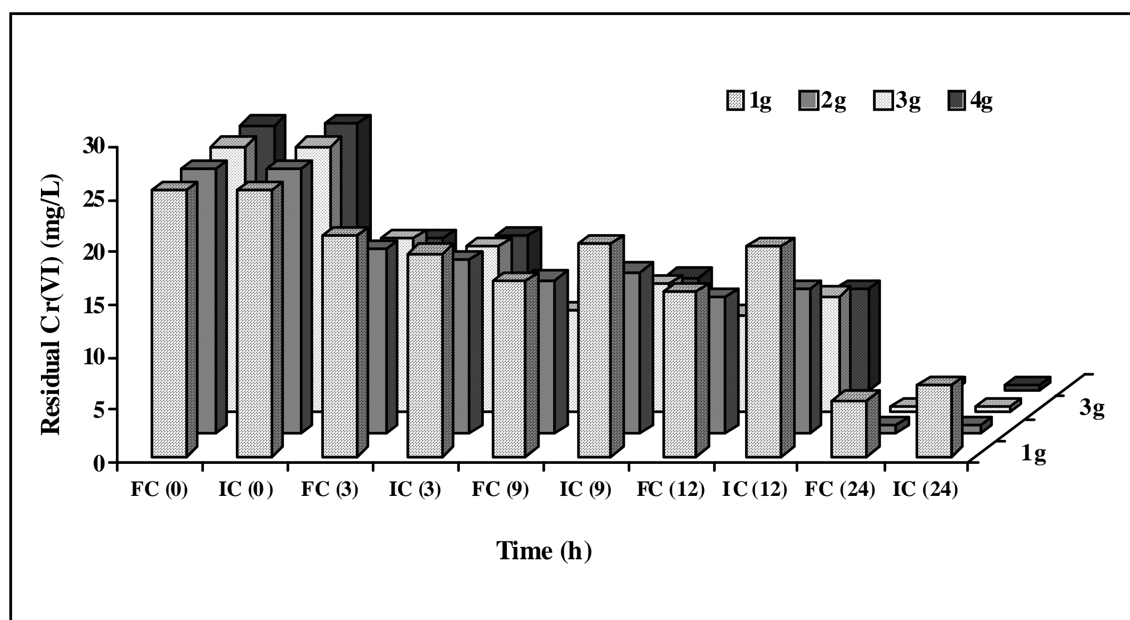

Fig 3.2: Effect of cell concentration on chromate reduction

FC: Free Cells, IC: Immobilized Cells. Figures in brackets represent time in hours.

Effect of Cr(VI) concentration

Cr(VI) reduction by free and PVA-alginate immobilized cells was studied in Broth-II medium containing 28 to 117 mg/L of Cr(VI). Both free and immobilized cells showed similar rates of Cr(VI) reduction. Reduction was faster and complete with lower initial Cr(VI) concentration while higher concentration required longer incubation time (Fig.3.3). The Cr(VI) reduction rates were comparable to those seen with free cells suggesting that there was no diffusional constraint for access of chromate or glucose, the electron donor for *S. griseus.*

Reduction of 28 mg/L of Cr(VI) by free as well as immobilized *S. griseus* cells was linear with incubation time and was complete in 24 h (Fig.3.3). But at Cr(VI) concentrations between 57 to 117 mg/L, initial rates of reduction were linear only up to 12-15 h and reduction continued up to 72-96 h at slower rates. In case of *Pseudomonas aeruginosa* A2Chr in batch culture, maximum Cr(VI) reduction occurred at the lowest Cr(VI) concentration i.e. 10 mg/L (Ganguli and Tripathi 2002). They found that 7.6 and 2.1 mg/L of Cr(VI) was reduced in 2 h from minimal medium containing 50 and 100 mg/L of Cr(VI) respectively. However in case of 100 mg/L of Cr(VI), reduction continued up to 4 h and reached a maximum of 3.9 mg/L of Cr(VI) and did not increase further.

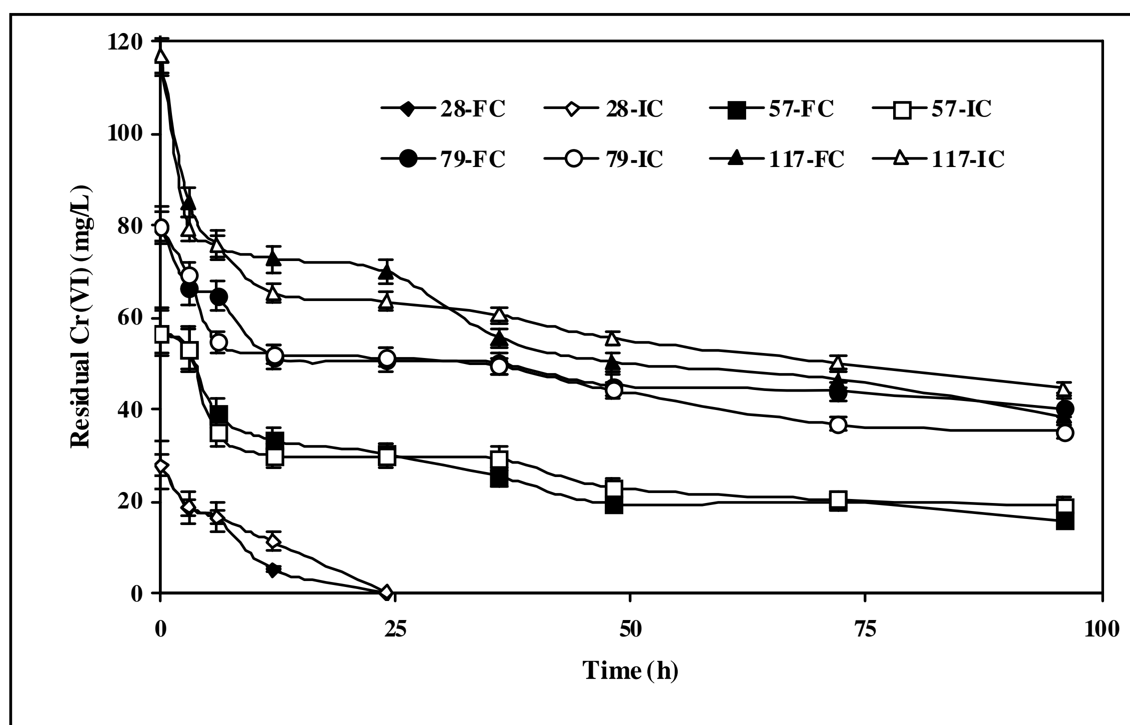

Fig 3.3: Effect of chromium concentration on chromate reduction

FC: Free Cells, IC: Immobilized Cells.

Reuse studies

For establishing the long-term stability and their reuse, repeated batch experiments were carried out using cells immobilized in agarose and PVA-alginate. After each incubation (24 h), beads were removed, washed with sterile water and transferred to

a fresh Broth-II medium containing 25 mg/L of Cr(VI) and incubated at 28ºC with shaking. This process was repeated for each reuse cycle. PVA-alginate immobilized cells could be used up to five reduction cycles and completely reduced 25 mg/L of Cr(VI) within 24 h each time. Reduction during the sixth use (fifth reuse) was only 50% which could be due to loss in cell viability (Fig. 3.4). Agarose immobilized cells completely reduced 25 mg/L of Cr(VI) till third reuse after which the cells leached out due to loss in bead integrity while free cells could not be used more than once due to cell lysis.

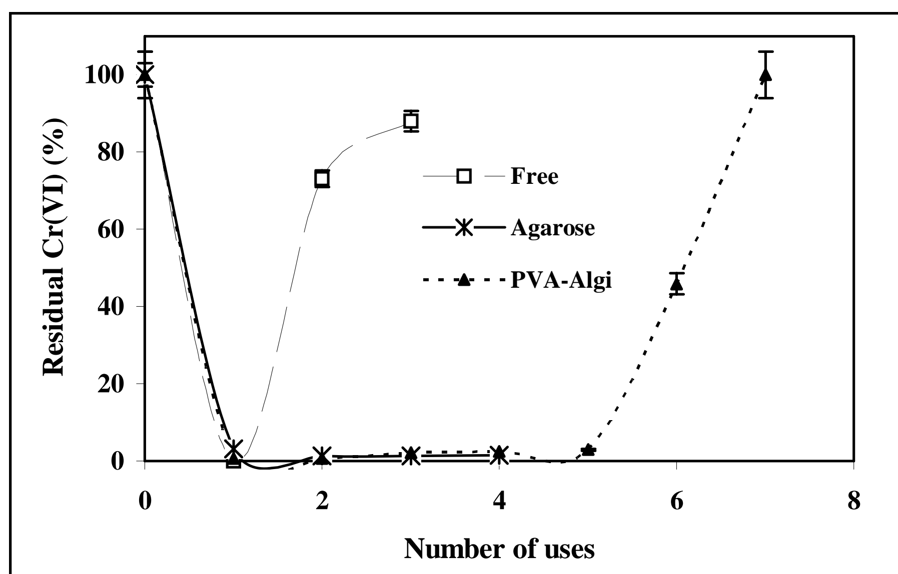

Fig 3.4: Reusability of immobilized cells for chromate reduction

Cr(VI) reduction by free and PVA-alginate immobilized cells in simulated effluent

In order to check the Cr(VI) removal from industrial effluents, reduction efficiency of free and PVA-alginate immobilized cells in a simulated effluent was evaluated. The simulated effluent was prepared as reported by Ohtake et al. (1990) with slight modifications and consisted of Broth-II medium containing following metals at the final concentrations as mentioned: Cr(VI) 25 mg/L, Cu(II), 28.6 mg/L, Mn(II) 21.6 mg/L, Zn(II) 0.1 mg/L, Mg(II) 3.3 mg/L. Free and PVA-alginate immobilized cells were added to the above medium and incubated at 28°C with shaking at 200 rpm on the rotary incubator shaker for 24 h. An un-inoculated control and cell free beads were maintained as controls.

Complete reduction of 25 mg/L Cr(VI) by immobilized cells occurred in 9 h (Fig. 3.5). Free cells required longer time (18 h) which may be due to free cells being more prone to toxicity of chromate as well as other metals present in the effluent while the immobilized cells may be protected from the toxic effects. Studies on chromate reduction reported by Konovalova et al. (2003) also support our observation that immobilized cells are protected from the excessive toxic action at high chromate concentration that improves cell activity compared with free cells. These results demonstrate the potential of the organism for chromate reduction from simulated effluents.

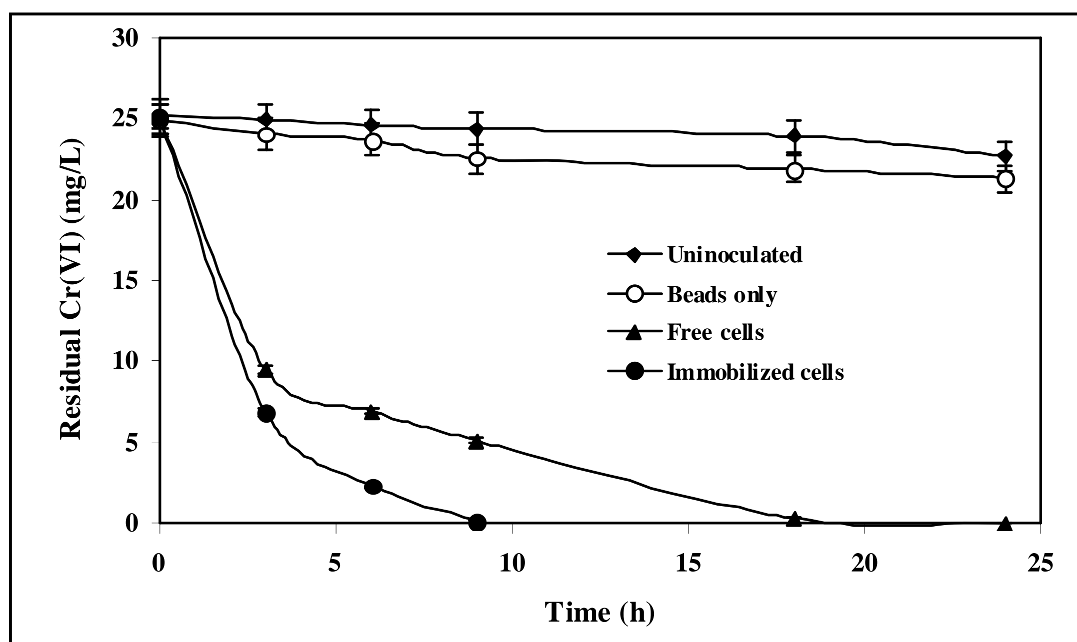

Fig 3.5: Chromate reduction in simulated effluent

It was noticed that reduction was faster in simulated effluent compared to minimal medium containing 25 mg/L of Cr(VI) both with free cells as well as PVA-alginate cells (Fig.3.6). The reason for this enhanced reduction could be due to stimulating effect of some of the ions especially Cu(II) present in the effluent. Earlier studies with free cells showed that Cu(II) enhanced reduction during growth as well as by resting cells. However, Ganguli and Tripathi (2002) found that Cr(VI) reduction from electroplating effluent was approximately 25% lower than that in minimal medium.

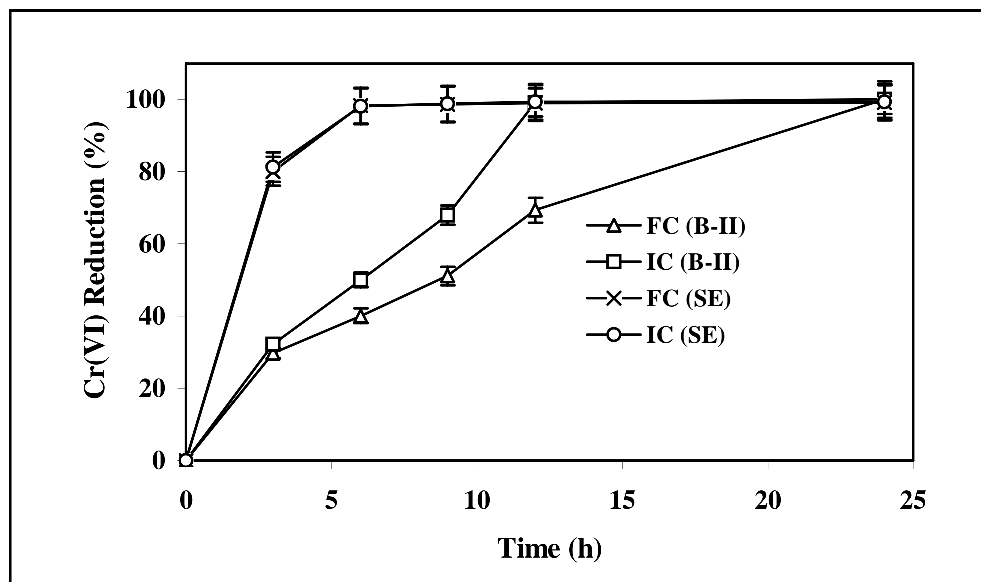

Fig 3.6: Chromate reduction in Broth (B-II) and simulated effluent (SE)

FC: free cells, IC: immobilized cells

These results showed that *S. griseus* entrapped in PVA-alginate beads could successfully remove Cr(VI) by reduction with rates similar to free cells.

Bioreactor Studies

Since PVA-alginate immobilized *S. griseus* cells could reduce Cr(VI) from aqueous solutions, studies were carried out for its continuous removal from Broth-II medium supplemented with chromate in a bioreactor. Effect of Cr(VI) and biomass concentrations, flow rate, influent composition were investigated. Finally Cr(VI) removal from simulated effluents was also studied. Control studies using cell-free beads, or beads challenged in the absence of electron donor (in absence of Broth-II medium or glucose), showed no significant Cr(VI) reduction. No bead breakdown or agglomeration was observed throughout the bioreactor operation. The set up of the bioreactor used for these studies is shown in Fig. 3.7.

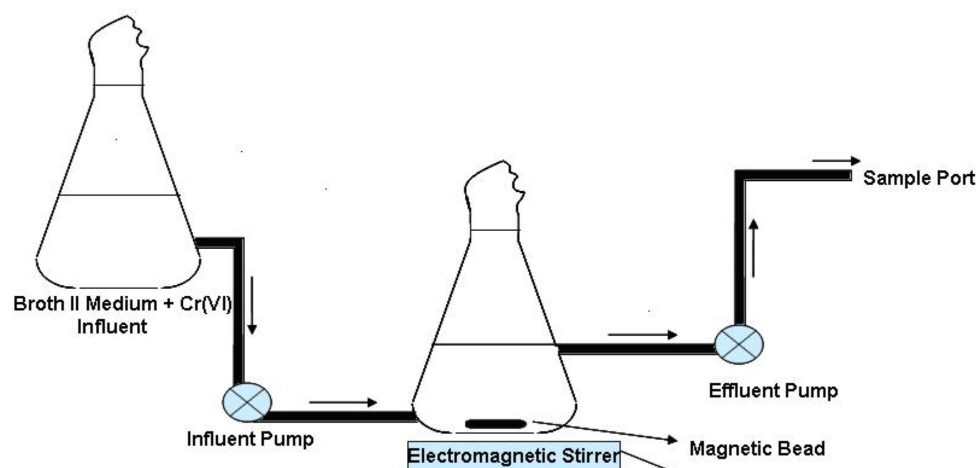

Fig. 3.7: Bioreactor system used for Cr(VI) reduction studies

Effect of Cr(VI) concentration

Effect of Cr(VI) concentration on reduction was studied with concentrations ranging from 2 to 12 mg/L with a flow rate of 4 ml/h. As shown in Fig. 3.8, reduction was faster and complete with lower Cr(VI) concentration (5.5 h for 2 mg/L, 7 h for 4 mg/L) while longer incubation time was required for higher Cr(VI) concentration (12 h for 8 mg/L, 14 h for 10 mg/L, 16 h for 12 mg/L) indicating that the reduction efficiency decreased with increase in Cr(VI) concentration in influent. Using this bioreactor near complete removal of Cr(VI) with a range of influent Cr(VI) concentrations (2-12 mg/L) and hydraulic retention times (5.5 h - 12 h) could be achieved.

Camargo et al. (2004a) studied Cr(VI) reduction by celite and Ca-alginate immobilized *Bacillus* sp. ES 29 with initial Cr(VI) concentrations ranging from 2 to 8 mg/L of Cr(VI) with a flow rate of 3 ml/h. Residual Cr(VI) concentrations in the effluents after 15 h from celite, Ca-alginate and amberlite columns were 0, 5 and 57.5% respectively. They found that rate of Cr(VI) reduction was inversely related to Cr(VI) concentration.

Fixed film bioreactor packed with glass beads containing intact cells of *Bacillus* sp. operated under steady state conditions with near complete removal of Cr(VI) with a range of influent Cr(VI) concentrations (10-200 mg/L) and hydraulic retention times (HRT) (6-24 h) was reported by Chirwa and Wang (1997a). They found that steady state Cr(VI) reduction efficiency decreased with increase in influent Cr(VI) concentration.

Cr(VI) reduction by *Bacillus coagulans* immobilized in polyacrylamide was studied in once-fed batch reactor by Philip et al. (1999). Approximately 50% reduction of 52 mg/L of Cr(VI) was observed after 24 h with very high biomass loading of 10 g dry weight of cells.

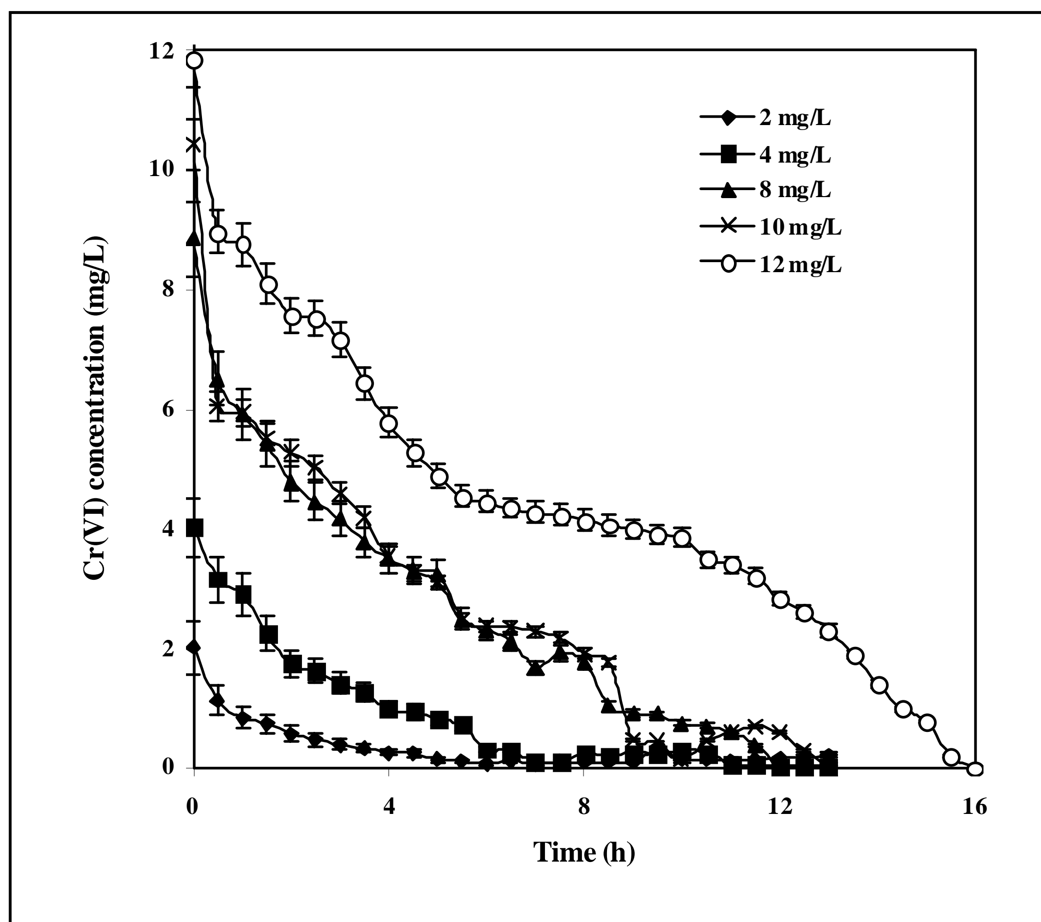

Fig.3.8: Effect of Cr(VI) concentration on reduction

In a continuous-flow system in 25 ml flow-through column, Pattanapipitpaisal et al. (2001) reported 90-95% chromate removal by *M. liquefaciens* MP30 immobilized in PVA-alginate beads from 50 μM Cr(VI) (2.6 mg/L) fed at a flow rate of 0.95 ml/h which maintained up to 20 days. Continuous chromate reduction by agar/agarose immobilized *Microbacterium* sp. NCIMB 13776 from 100 μM sodium chromate was carried out in 14 ml flow through packed bed bioreactor at a flow rate of 2.4 ml/h (Humphries et al 2005). Cordoba et al. (2008) reported Cr(VI) reduction by *Arthrobacter* sp. Cr47. They monitored time course of the Cr(VI) reduction in batch (12 ml void volume) and in

recirculating packed bed biofilm reactors (100 ml void volume) inoculated with *Arthrobacter* sp. Cr47. Under batch mode, complete reduction of 30 mg/L of Cr(VI) (100% removal) was attained in about 26 h, while in the recirculating reactor complete Cr(VI) removal from the 500 ml recirculation batch was attained in about 45 h, whereas in the reactor fed with the industrial model solutions it was achieved in 70 h..

Gram negative bacteria have also been investigated for chromate reduction in bioreactors. Continuous fixed film bioreactor of *P. fluorescens* LB300 packed with glass beads under steady-state conditions showed nearly complete removal from 30-100 mg/L of Cr(VI) influent at 3.6-14.2 min HRT (flow rates of 1.1-4.42 ml/h). Cr(VI) over loadings observed at 200 mg/L of Cr(VI) could be reversed by lowering the concentration to 10 mg/L of Cr(VI) (Chirwa and Wang 1997b). *P. aeruginosa* A2Chr in rotating biological contactor reduced 10, 25, 50 and 100 mg/L of Cr(VI) by 95, 35.2, 14.6 and 7% respectively in 8 h. However, the durability of the biofilms was limited to 10 h (Ganguli and Tripathi 2002). Shen and Wang (1995) reported 100% Cr(VI) reduction efficiency for 1.5 mg/L influent Cr(VI) concentration by free cells of *E. coli* ATCC 334456 in a two-stage continuous flow bioreactor. However, the efficiency decreased to 62.6% at 11.7 mg/L of Cr(VI) with retention times varying from 5 to 10.5 h.

Studies on continuous chromate reduction by agar/agarose immobilized *D. vulgaris* NCIMB 8303 under anaerobic conditions in 14 ml flow through packed bed bioreactor was reported by Humphries et al (2005). Sodium chromate solution (100 μM) was fed continuously at a flow rate of 2.4 ml/h. Agar immobilized cells removed 95% Cr(VI) which decreased to 60% by 24 h and complete loss of reduction was observed after 159 h. In case of agarose immobilized cells, they observed lower reduction ability after 30 h which they speculated to be due to cell washout, loss of matrix stability and bead integrity.

Effect of flow rate

Effect of flow rate on Cr(VI) reduction by PVA-alginate immobilized *S. griseus* in the reactor was studied by feeding the reactor with Broth-II medium containing 2 mg/L of Cr(VI) at flow rates of 4, 6 and 8 ml/h. Increasing the flow rate from 2 to 8 ml/h

did not significantly affect the reduction by the immobilized cells indicating that Cr(VI) can be reduced at the same rate even at a maximum flow rate of 8 ml/h (Fig. 3.9).

Similar observations of Cr(VI) reduction efficiency being unaffected by flow rates (highest flow rate tested was 5.3 ml/h) by *Bacillus* sp. in a fixed film bioreactor under steady state were made by Chirwa and Wang (1997a). Flow rate of 3 ml/h was found to be best with highest Cr(VI) reduction by immobilized intact cells of *Bacillus* sp. ES29 and increasing the flow rates up to 6 ml/h did not affect the rate of Cr(VI) reduction, but above 6 ml/h the reduction capacity decreased (Camargo et al. 2004a). DeLeo and Ehrlich (1994) observed increase in reduction of 10.75 mg/L of Cr(VI) influent from 28 to 57% with decrease in flow rate from 3.85 to 1.17 ml/h by *P. fluorescens* LB300 in a glass chemostat. The time required for complete removal of 10 mg/L of Cr(VI) was 11.7 to 38.5 h depending on cell density.

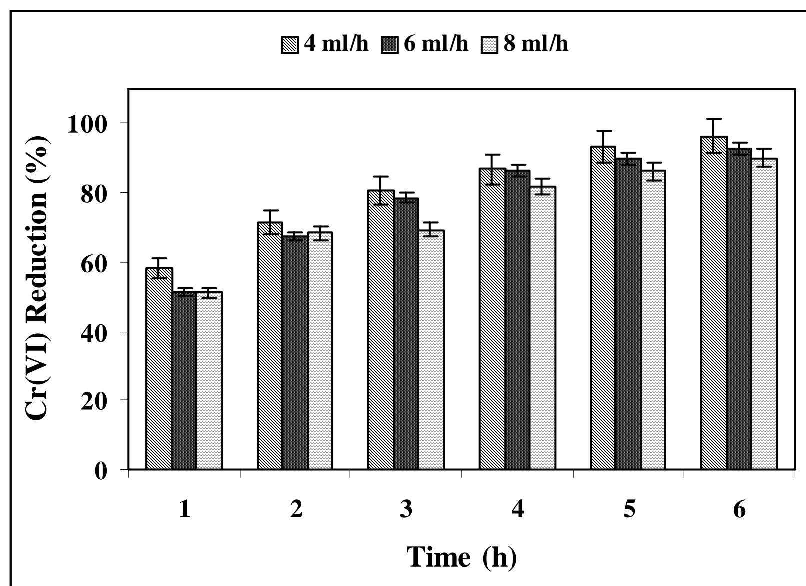

Fig. 3.9: Effect of flow rate

Effect of biomass concentration

Chromate reduction was studied with biomass concentrations of 2 and 4 g wet weight of cells (100-200 mg dry weight) with Cr(VI) concentration of 12 mg/L at a flow rate of 4 ml/h. Chromate reduction was higher with higher biomass concentration and

reduction using 0.1 and 0.2 g dry weight of cells was 77 and 98% in 12 h (Fig. 3.10). A similar trend of enhanced reduction with increase in cell density was observed with *P. fluorescens* LB300 (DeLeo and Ehrlich 1994) and *B. coagulans* (Philip et al. 1998).

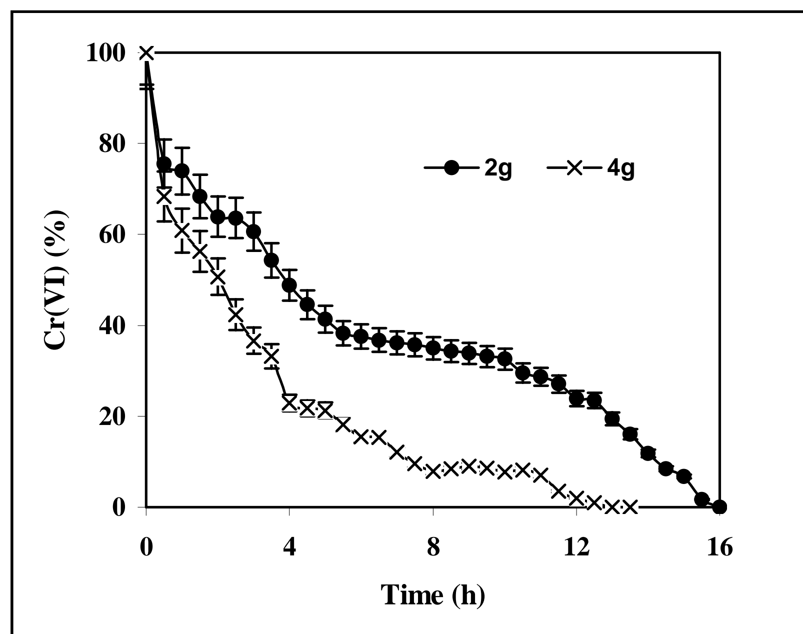

Fig. 3.10: Effect of biomass concentration

Effect of influent medium

To investigate the effect of influent composition on chromate reduction, Broth-II medium or Tris HCl buffer (pH 7) supplemented with 1% glucose, containing 4 mg/L of Cr(VI) was fed to the reactor continuously at a flow rate of 4 ml/h. Reduction of Cr(VI) in Broth-II minimal medium was faster and reached near completion (97%) in 7 h while reduction in Tris HCl buffer (pH 7) supplemented with 1% glucose was around 85% in 14 h indicating that besides glucose, other components of the medium also play an important role in faster and complete Cr(VI) reduction (Fig. 3.11).

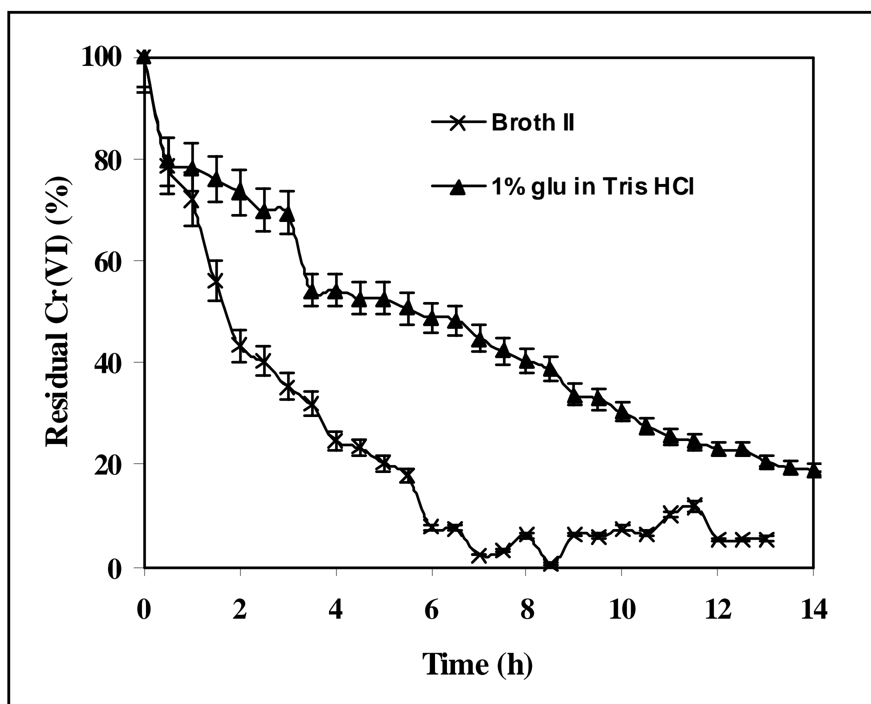

Fig. 3.11: Effect of influent medium

Treatment of simulated effluent

Chromate reduction from simulated effluent (SE) containing 8 mg/L of Cr(VI), 28.6 mg/L of Cu(II), 3.3 mg/L of Mg(II), 21.6 mg/L of Mn(II), 0.1 mg/L of Zn(II) was investigated in the reactor with an influent flow rate of 4 ml/h. Complete reduction of 8 mg/L chromate occurred in 2 h confirming our earlier results obtained in batch system viz. other metals in the SE had no inhibitory effect on reduction (Fig. 3.12). Broth-II medium containing Cr(VI) was included as control. As observed in batch system, Cr(VI) reduction in the bioreactor was faster (2 h) in a simulated effluent compared to that in Broth-II medium which took 12 h for complete reduction. This could be attributed to the stimulatory effect of the ions mainly Cu(II) in the simulated effluent. Batch studies as described earlier with free resting cells of *S. griseus* showed enhancement in Cr(VI) reduction by Cu(II). Addition of 0.4-0.6 mM Cu(II) resulted in 2.5 to 4 fold increase in

reduction compared to control without Cu(II). The stimulatory mechanism of Cr(VI) reduction activity by Cu(II) and other metals is not clear. However, Cu(II) is a prosthetic group for many reductase enzymes. Camargo et al. (2003) reported that the main function of Cu(II) is related to electron transport protection or acting as electron redox center and, in some cases, as a shuttle for electrons between protein subunits.

Reports on chromium removal from effluents are very few. Ganguli and Tripathi (2002) reported chromate reduction by *P. aeruginosa* A2Chr from chrome-plating effluent containing 10 to 100 mg/L of Cr(VI) in a dialysis bioreactor and rotating biological contactor with maximum reduction of 76 and 93% respectively from 10 mg/L of Cr(VI) in 8 h. *P. mendocina* in a 20 L continuously stirred tank reactor could treat 50 mg/L of Cr(VI) from chrome-plating effluent of automobile industry and 25-100 mg/L of Cr(VI) from cooling-tower effluent with Cr(VI) removal efficiency of 99.7% and 99.9% at a hydraulic retention time of 24 h and 4.5-8 h respectively (Rajwade et al. 1997; Bhide et al. 1996).

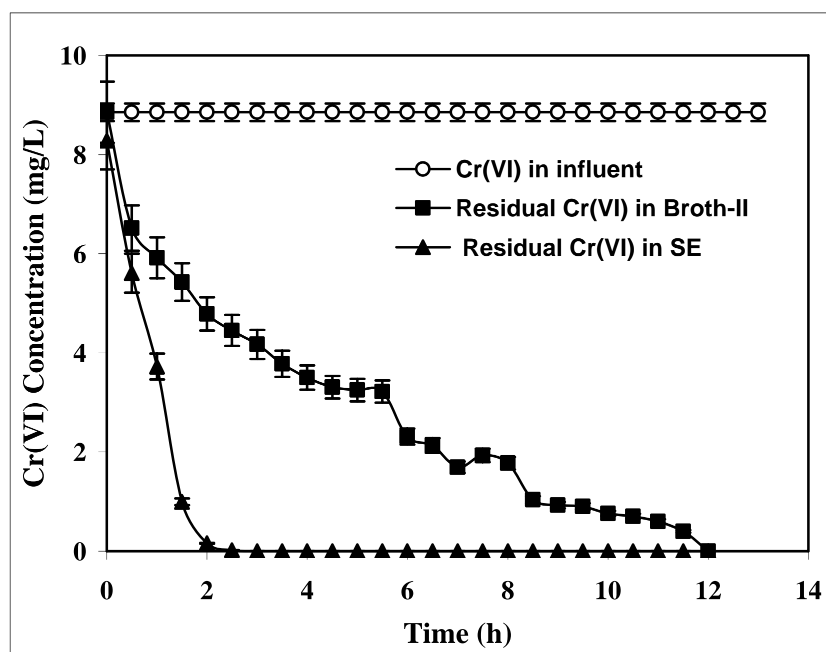

Fig. 3.12: Chromate reduction from simulated effluent and Broth-II medium

CONCLUSIONS

The present investigation has revealed that among the various matrices used for immobilization, PVA-alginate was found to be the best method for entrapment for *S. griseus* cells. PVA-alginate immobilized cells could successfully reduce Cr(VI). Chromate removal observed in batch experiments indicated that no hindrance to accessibility of Cr(VI) or electron donors to cells present in the immobilized beads. It was found that reduction was slower at higher Cr(VI) concentrations while 2 g wet biomass was optimal for completely reducing 50 ml of 25 mg/L of Cr(VI) in 24 h. The ability of PVA-alginate immobilized cells for repeated use up to five times without compromising on its performance, makes it a promising system for development of a continuous bioprocess for treatment of Cr(VI) contaminated effluents.

The potential of immobilized *S griseus* cells for Cr(VI) removal in a continuous system was examined. The results demonstrated for the first time the applicability of immobilized *S griseus* cells for reduction of chromate from minimal medium (Broth-II) and from simulated effluents in a continuous manner under aerobic conditions. Studies of various parameters influencing reduction showed that flow rates from 2 to 8 ml/h had no significant effect on reduction while increasing the chromate concentrations from 2 to 12 mg/L slightly decreased the reduction from 100 to 70% in 5.5 h and required longer incubation for complete reduction. Cr(VI) reduction in a simulated effluent was faster than synthetic medium indicating the possible stimulatory effect of metal ions. The present study indicates the potential of immobilized cells of *S. griseus* for bioremediation of chromate-contaminated effluents.

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CHAPTER IV

ISOLATION, PARTIAL PURIFICATION AND CHARACTERIZATION OF THE CHROMATE REDUCTASE

ABSTRACT

Chromate reduction *in S. griseus* was associated with the cells and no extra-cellular activity was detected. Sonication was found to the best method of cell breakage to release the intracellular enzyme. The enzyme was constitutive and did not require presence of chromate during growth for expression of activity. Chromate reduction was observed with cell free extract (CFE) without added NADH and addition of NADPH/NADH resulted in 2-3-fold increase in activity. Ammonium sulphate precipitated fraction of cell free extract (40-60%) which contained major portion of chromate reductase activity was used for property studies. Reductase activity was optimally active at 28°C and pH 7. Effect of metal ions on chromate reductase showed mercury to be highly inhibitory (relative activity of 21%) while copper had slight stimulatory effect. No inhibition was observed in presence of sodium azide, Ag(I), Mn(II) etc. Fraction eluted with 0.1 M NaCl after DEAE cellulose column chromatography of the dialyzed 40-60% ammonium sulphate precipitate sample showed 5 protein bands on SDS PAGE. Molecular weight of the partially purified enzyme was found to be approximately 46-48 kDa.

EPR studies revealed the presence of Cr(V) and Cr(III) species during reduction of Cr(VI) by the reductase. The presence of Cr(V) as a transient intermediate in the conversion of Cr(VI) to Cr(III) indicated one-electron reduction as the first step during the reduction. Ultracentrifugation of cell free extract showed the localization of chromate reductase activity to be in the soluble fraction and no activity was associated with membrane fraction.

INTRODUCTION

Cr(VI) reductase is a central enzyme in the Cr(VI) reduction system in many soils and enteric bacteria, which enables them to reduce Cr(VI) to Cr(III), which readily forms an insoluble less toxic chromium hydroxide at neutral pH, and thus, is relevant to an understanding of the detoxification and ultimate remediation of Cr(VI) pollution. These proteins have recently raised enormous interest because of their central role in mediating chromium toxicity and their potential use in bioremediation.

Hexavalent chromium i.e. Cr(VI), a widespread industrial waste is a strong oxidant and is very toxic, carcinogenic and mutagenic. It is highly soluble and therefore easily spreads in the environment whereas Cr(III) is less toxic and less soluble (Philip et al. 1998). Thus, one way of detoxifying Cr(VI) is reduction of Cr(VI) to Cr(III), which is possible by chemical or microbial means. Conventional methods such as chemical reduction/ ion exchange require high energy inputs, leads to formation of large quantity of chemical sludge, is less specific and is unsuitable for effluents containing low concentrations of the metal while the microbial method does not require high energy input and involves reduction or bio-accumulation/absorption or an efflux pump (Philip et al. 1998; Alvarez et al. 1999; Cervantes et al. 2001).

Enzymatic reduction of chromate

Chromate reduction is carried out by diverse bacterial species (Cervantes et al. 2001; Cervantes and Campos-Garcia 2007). Aerobic bacteria such as *Escherichia coli* ATCC 33456, *Bacillus subtilis*, *Pseudomonas ambigua* G-1 and *Pseudomonas putida* can reduce chromate by NAD(P)H-dependant chromate reductases (Garbisu et al. 1998; Ishibashi et al. 1990; Park et al. 2000; Shen and Wang 1993; Suzuki et al. 1992; Campos et al. 1995). The reductases can be soluble in the cytoplasm (Campos et al. 1995; Bae et al. 2005), or periplasmic (Camargo et al. 2004; Rajwade et al. 1999) or membrane-bound (Wang et al. 1989; Das and Chandra 1990). In aerobic systems, most of the chromate reductases reported are soluble in the cytosol and reduce Cr(VI) to Cr(III) inside or outside the plasma membrane (Camargo et al. 2003). Under aerobic conditions, the enzymatic reduction of Cr(VI) generally involves a soluble cytosolic chromate reductase and both NAD(P)H and endogenous cell reserves may serve as the electron donors for

Cr(VI) reduction (Suzuki et al. 1992, Shen and Wang 1993). Anaerobic Cr(VI) reduction in *Desulfovibrio vulgaris* is mediated by membrane bound cytochrome, c_3 (Lovely and Philips, 1994) or cytoplasmic membrane fraction (Myers et al. 2000). The enzymatic reduction involves a membrane-bound chromate reductase during anaerobic respiration where chromate acts as the terminal electron acceptor (Bopp and Ehrlich 1988; Wang et al. 1989). Das and Chandra (1990) reported a membrane associated chromate reductase from *Streptomyces* sp. that reduced Cr(VI) to Cr(III) in the presence of NAD(P)H. *Arthrobacter crystallopoietes* produced a periplasmic chromate reductase that was stimulated by NADH (Camargo et al. 2004).

Cr(VI) reduction by bacteria may be inhibited by Cr(VI), oxygen (Shen and Wang 1993), heavy metals like Ag(I), Ba(II), Cd(II), Fe(II), Hg(II), and Zn(II) (Bae et al. 2005; Elangovan et al. 2006; Desai et al. 2008), and phenolic compounds. However, Cu(II) and Ni(II) are reported to enhance the reduction (Desai et al. 2008; Elangovan et al. 2006; Camargo et al. 2003). The optimum pH and temperature observed for Cr(VI) reduction generally coincide with the optimal growth conditions of cells. However, there are few exceptions, for example chromate reductase from *P. putida* has an optimum temperature of 80°C (Park et al. 2000).

The first enzyme described with the ability to transform Cr(VI) to Cr(III) was a Cr(VI) reductase from chromate-resistant *Enterobacter cloacae* HO1 (Ohtake et al. 1990). This is a membrane-associated enzyme and utilizes toxic chromate as an electron acceptor (Wang et al. 1990). Though microbes of genera *Pseudomonas, Arthrobacter, Escherichia* and *Bacillus* have been reported to reduce Cr(VI) through soluble chromate reductase, only a few of them from *Pseudomonas* and *Bacillus* have been characterized (Park et al. 2000; Camargo et al. 2003; Megharaj et al. 2003; Bae et al. 2005; Elangovan et al. 2006). Ishibashi et al. (1990) has reported the partial purification of a soluble NAD(P)H dependant, Cr(VI) reductase from *P. putida* PRS 2000. Park et al. (2000) first reported the purification of bacterial chromate reductase from *P. putida* MK1 to homogeneity. ChrR from *P. putida* KT2440 is currently the best studied Cr(VI) reductase. ChrR is a soluble flavoprotein and functions as a 50-kDa dimer and shows a NADH-dependent reductase activity (Ackerley et al. 2004; Gonzalez et al. 2005). This multifunctional protein, besides its role as Cr(VI) reductase, also reduced quinones,

potassium ferricyanide, and 2,6-dichloroindophenol, but did not appear to have activity with U(VI), V(V), Mn(IV), and Mo(VI) . Studies with enzyme mutants showed that ChrR protects against chromate toxicity; this is possibly because it preempts chromate reduction by the cellular one-electron reducers, thereby minimizing generation of reactive oxygen species (ROS) (Ackerley et al. 2004). During Cr(VI) reduction, ChrR shows a quinone reductase activity that generates a flavin semiquinone. By this reaction, the enzyme transfers 25% of the NADH electrons to superoxide anion and probably produces the Cr(V) species transiently (Fig. 4.1). Indeed, ChrR in one pathway reduces Cr(VI) to Cr(III), generating intermediary Cr(V) and superoxide anion, and by an additional mechanism reduces quinones, which provide shielding against ROS (Fig. 4.1).

NAD(P)H dependent Cr(VI) reductase from *Pseudomonas ambigua* G-1 that reduced Cr(VI) to Cr(III) via formation of Cr(V) as an intermediate has been purified (Suzuki et al. 1992). The 65 kDa protein, (38 fold purification) reduced Cr(VI) with NADH and NADPH serving as the electron donors. A periplasmic chromate reductase with a molecular weight of 183 kDa from *P. mendocina* was purified (Rajwade et al. 1999). The chromate reductase from *Bacillus* sp. is not yet fully characterized and properties of a crude chromate reductase enzyme from *Bacillus* sp. ES29 has been reported (Camargo et al. 2003). Several bacterial Cr(VI) reductases, some conferring resistance to chromate, have been subsequently characterized. Properties of some of the bacterial reductases are presented in Tables 4.1a and 4.1b. These enzymes commonly show a NADH:flavin oxidoreductase activity and can use Cr(VI) as electron acceptor (Gonzalez et al. 2005).

The first available report on chromate reductase from actinomycetes is by Das and Chandra (1990) who partially purified a membrane associated chromate reductase from *Streptomyces* sp. The other report on chromate reduction by actinomycete is by Desjardin et al. (2003) who showed that cell free supernatants of *Streptomyces thermocarboxydus* NH50 reduced Cr(VI) to Cr(III). They found it contained small molecular weight reducing agents in contrast to enzymes which brought the chromate reduction. They had used these cell free supernatants for bioremediation of Cr(VI) contaminated soils.

It seems that various reductases in the cell can function in chromate reduction. Ishibashi et al. (1990) suggested that the ability to reduce chromate may be a secondary

function for Cr(VI) reductases, which have a different primary role other than Cr(VI) reduction. The nitroreductases NfsA/NfsB from *Vibrio harveyi* possess a nitrofurazone nitroreductase as primary activity and a Cr(VI) reductase activity as a secondary function (Kwak et al. 2003). These secondary functions may be related to the bacterial enzymatic adaptation to the relatively recent increase of Cr(VI) content in the environment (Ramirez-Dıaz et al. 2008). It seems that various reductases in the cell can function in chromate reduction. *P. ambigua* chromate reductase (Suzuki et al. 1992) has high homology with *Escherichia coli* NfsA (59%) and *Vibrio harveyi* NfsA (58%) nitroreductase (Kwak et al. 2003).

In conclusion, Cr(VI) reduction seems to be an efficient system of resistance to chromate in bacteria; however, the use of alternative substrates in addition to Cr(VI) by chromate reductases suggests that this reduction activity has been an adaptive mechanism promoted by recent chromate exposure.

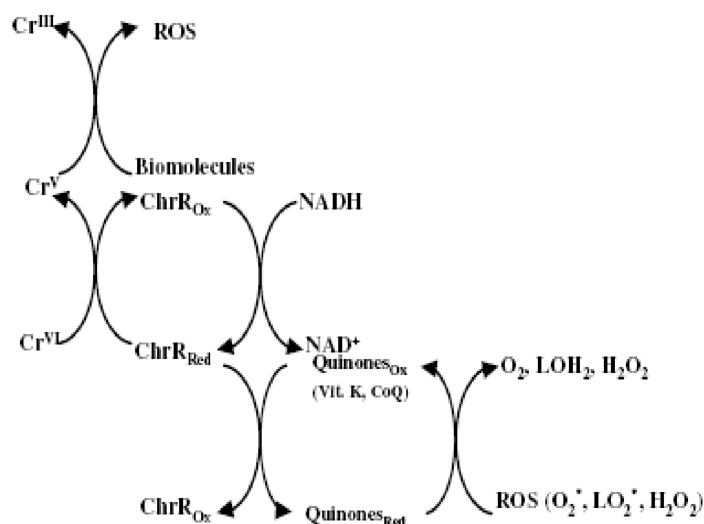


Fig. 4.1 Model for chromate reduction and protection mechanism by *Pseudomonas putida* ChrR chromate reductase (Ramirez-Dıaz et al. 2008)**.**

The present chapter describes isolation, partial purification and characterization of the chromate reductase from *S. griseus* NCIM 2020.

Table 4.1a: Properties of Bacterial Cr(VI) reductases (Anaerobic and/or Aerobic)

Bacteria	Reduction Conditions	Enzyme location	Electron donor	Molecular Mass (kDa)	Opt pH	Opt Temp (°C)	Reference
D. vulgaris	Ana	S and M	H_2	NA	NA	NA	Lovely and Phillips 1994
E. cloacae	Ana	M	NADH	NA	7.0	30	Wang et al. 1989
Shewanella putrefaciens MR-1	Ana	M	NADH & formate	NA	7.5	23	Myers et al. 2000
Bacillus QC1-2	Aer and Ana	S	NADH	44 (GF) 24 (SDS PAGE)	7.0	37	Campos-García et al. 1997
E. coli ATCC 33456	Aer and Ana	S	NA	NA	6.5	37	Shen and Wang 1993
E. coli ATCC 33456	Aer and Ana	S	NAD(P)H	84 (GF) 42 (SDS PAGE) Dimeric	6.5	37	Bae et al. 2005
Pseudomonad CRB5	Aer and Ana	S	Not required	NA	NA	NA	Mclean and Beveridge 2001
P. fluorescens LB 300	Aer and Ana	M	NADH	NA	6.5	NA	Bopp and Ehrlich 1988
Rhodobacter sphaeroides	Aer and Ana	S	NADH	42 (GF)	7.0	30	Nepple et al. 2000
Thermus scotoductus SA-01	Aer and Ana	M	NAD(P)H NADH preferred	48 (SDS PAGE) covalently bound FAD coenzyme	6.5	65	Opperman and Heerden 2008
Thermus scotoductus SA-01	Aer and Ana	S	NAD(P)H	36 (SDS PAGE) containing non covalently bound FMN factor	6.3	65	Opperman et al. 2008

Table 4.1b: Properties of Bacterial Cr(VI) reductases (Aerobic)

Bacteria	Reduction Conditions	Enzyme location	Electron donor	Molecular Mass (kDa)	Opt pH	Opt Temp (°C)	Reference
Arthrobacter sp.	Aer	S	NADH	NA	NA	NA	Meghraj et al. 2003
Arthrobacter crytallopoietes ES 32	Aer	P	NADH	NA	7	30	Camargo et al. 2004
Bacillus sp.	Aer	S (Activity enhanced by M fraction)	Not required	NA	7	30	Wang and Xiao 1995
Bacillus coagulans	Aer	S	NADPH	NA	7.0	NA	Philip et al. 1998
Bacillus subtilis	Aer	S	NAD(P)H	NA	NA	NA	Garbisu et al. 1998
Bacillus ES29	Aer	S	NADH	NA	7.0	30	Camargo et al. 2003
Bacillus sp.	Aer	S	NADH	NA	NA	NA	Meghraj et al. 2003
Bacillus sphaericus AND 303	Aer	S	NADH, glucose, yeast extract	NA	6.0	30	Pal et al. 2005
Bacillus megaterium TKW3	Aer	M	NADH	NA	NA	NA	Cheung and Gu 2006
Bacillus sp. RE	Aer	S	NAD(P)H	NA	6	30	Elangovan et al. 2006
Pseudomonas ambigua G-1	Aer	S	NADH	NA	NA	NA	Horitsu et al. 1987
P. putida	Aer	S	NAD(P)H	NA	6.5-7.5	50	Ishibashi et al. 1990
P. ambigua	Aer	S	NAD(P)H	65	8.6	50	Suzuki et al. 1992

Pseudomonas maltophila	Aer	M	NAD(P)H	NA	7.5	-	Blake et al. 1993
Pseudomonas aeruginosa HP014	Aer	S	NAD(P)H	NA	7.0	37	Oh and Choi 1997
Pseudomonas mendocina	Aer	P	NAD(P)H	183 (GF) 65 and 29 (SDS-PAGE)	8	70	Rajwade et al. (1999)
P. putida MK1	Aer	S	NAD(P)H	20 (SDS-PAGE)	5	80	Park et al. 2000
P. aeruginosa	Aer	P	NAD(P)H	30	-	-	Ganguli and Tripathi 2001
P. putida	Aer	S (some activity in periplasm	NADH	20 (SDS-PAGE)	8.5	75	Renuga et al. 2008
Streptomyces sp.	Aer	S	NAD(P)H	NA	NA	NA	Das and Chandra 1990

Abbreviations : Ana- anaerobic; Aer- aerobic; M-membrane; S- soluble; P- periplasmic; NA- not available; GF- Gel Filtration

MATERIALS AND METHODS

Chemicals

Pure and analytical grade chemicals were used in all the experiments. Peptone, yeast extract, malt extract, glucose were supplied by **M/s Hi Media chemicals, India**. Di-phenylcarbazide, NADH and NADPH were procured from **Sigma Chemicals Co, USA**. Potassium dichromate was obtained from **Sara Merck, India**.

Investigation of extracellular/ intracellular nature of Cr(VI) reduction

Cells grown for 48 h in Broth-II medium containing 25 mg/L of Cr(VI) were collected by centrifugation at 8,000 rpm, 15 min. The culture supernatant (≈ 50 ml) was filter sterilized on sterile 0.22 µm Millipore filters. Cells (2 g wet weight) and the filter sterilized culture supernatant were separately incubated with fresh Broth-II medium containing 25 mg/L of Cr(VI) under sterile conditions at 28°C, 180 rpm up to 96 h and residual Cr(VI) in the medium was determined.

Methods of cell breakage for enzyme extraction

Cells grown in MGYP medium for 48 h were harvested by filtration followed by washing with 25 mM Tris HCl buffer, pH 7 (three times). Wet cells (2 g) were suspended in 4 ml of the same buffer and used for each method of cell breakage. Three different methods of cell breakage were employed viz. grinding with neutral alumina (Das and Chandra 1990), crushing the frozen cells with liquid nitrogen and sonication (Camargo et al. 2003). Cells were frozen with liquid nitrogen and crushed on ice for 20 min, dispensed in buffer and processed further. Alternatively, cells were sonicated in an ice bath with an ultrasonic probe. Power was applied for 4 min with 10 s pulses at 60 W each. The broken cell suspension was centrifuged at 4°C, 12,000 x g for 10 min. The cell free extract (CFE) was treated as the source of enzyme and used for Cr(VI) reduction studies.

Conversion of Cr(VI) to Cr(III) by cell free extract (CFE)

Changes in Cr(VI) and Cr(III) during reduction were monitored by diphenylcarbazide (DPC) method described earlier and absorbance at 595 nm respectively (Das and Chandra 1990). One millilitre of reaction mixture containing 25

mM Tris HCl buffer (pH 7), 64 mg/L of Cr(VI) and CFE (obtained after sonication) was incubated at 28°C for 2 h (longer incubation period was required to detect noticeable changes in absorbance at 595 nm). The CFE was heated at 100°C for 5 min and used as a control.

Effect of chromium as inducer and growth medium

To ascertain the constitutive/inducible nature of chromate reductase, cells grown for 48 h under identical conditions in Broth-II medium with and without 25 mg/L of Cr(VI) were sonicated and Cr(VI) reduction by CFE was examined. Effect of growth medium was studied by estimating the reductase activity in CFE obtained from cells grown for 48 h in Broth-II and MGYP media.

Fractional ammonium sulphate precipitation

The CFE from cells grown on MGYP was used for fractional precipitation studies. The enzyme was fractionated with ammonium sulfate at following saturations (%): 0-20, 20-40, 40-60, 60-80. The supernatant obtained from each step (12,000 g, 15 min) was used for the next fractionation step. The pellet from each fraction was separately re-suspended in 25 mM Tris HCl, pH 7.

Effect of pH and temperature on reductase activity

Crude CFE and partially purified enzyme (40-60% ammonium sulphate precipitate) were used for determination of optimum pH and temperature. For determination of optimum temperature, activity was estimated at pH 7 and temperatures ranging from 20 to 60°C. Optimum pH was determined by estimating the activity at 28°C and pH values ranging from 5 to 9. Citrate (pH 5 and 6); Tris HCl (pH 7, 8 and 9) buffers were used. Relative activity was expressed as percentage of the highest activity taken as 100%. Temperature stability was determined by incubating CFE at 28°C up to 2 h. Samples were removed at intervals of 30 min and the residual activity was measured and expressed as percentage of initial activity taken as 100%.

Effect of Metal ions and inhibitors

Effect of metal ions and inhibitors on reductase activity was studied by incubating partially purified enzyme (40-60% ammonium sulphate precipitate) with metal ions, EDTA, sodium azide at 1mM final concentration and 0.01% formaldehyde at 28°C for 30 min. Relative activity was expressed as percentage of control (no additive) taken as 100%.

Localization of chromate reductase activity by ultracentrifugation

Intracellular or membrane associated nature of Cr(VI) reductase was investigated as follows: *S. griseus* cells grown in MGYP for 48 h were sonicated as described earlier and the suspension was centrifuged at 12,000 x g, 20 min, 4°C. Cell debris settled at the bottom as pellet. The supernatant (S_{12}) was further centrifuged at 150,000 x g, 1 h at 4°C in Beckman Coulter Optima XL-100k ultracentrifuge to separate membrane fraction (in the form of a pellet) from the soluble fraction (S_{150}). Reductase activity in each of the fractions ($S_{12,}$ S_{150} and the membrane fraction) was determined as described below.

Identification of intermediate formed during reduction by EPR

Reduced form of chromium was characterized by Electron Paramagnetic Resonance (EPR) Spectrometry. One ml of reaction mixture containing 1mM Cr(VI) as $K_2Cr_2O_{7,}$ 1 mM NADH in 25 mM Tris HCl (pH 7) and enzyme (40-60% ammonium sulphate precipitate) was incubated at 28°C for 2 h. EPR spectra (X band) were obtained with Bruker EMX EPR Spectrometer with a Teflon capillary tube (20 cm long and 0.5 mm internal diameter) as a cell. The magnetic field (g value) was calibrated using a ER035M NMR Gaussmeter. Microwave frequency was calibrated with a frequency control fitted in a Bruker ER041XG-D Microwave bridge. Experimental parameters were as follows: Center Field = 3520 G, Sweep width = 160 G, Freq. = 9.76 GHz, Power = 4 mW, Gain = 2 x 105, modulation amplitude = 1.6, number of scans =5, analysis temperature = 25°C.

Analytical and SDS PAGE

Anodic and SDS PAGE (pH 8.8) were performed in Tarson make mini vertical dual slab gel unit (10 x 10 cm) according to Laemmli (1970) using 10% gel with 100 V and constant current of 3 mA per well for 1-2 h. Sample for SDS PAGE was boiled with 2% SDS for 5 min. 10-20 μg protein was loaded in each well and protein bands were visualized by silver staining (Heukeshoven and Dernick 1985).

Adsorption of Cr(VI) reductase to DEAE-cellulose: effect of pH on binding

Prior to DEAE-cellulose column chromatography, preliminary experiments were carried out to find optimum pH for binding the enzyme to DEAE-cellulose. DEAE-cellulose was equilibrated with 25 mM buffers with pH ranging from 6 to 9. 40-60% ammonium sulphate fraction was used for binding at each pH and incubated at 4°C for 1 h. After the incubation, the supernatant was separated by centrifugation at 12,000 x g, 10 min. and activity in the supernatant was checked and treated as unbound enzyme.

DEAE-cellulose column chromatography

10 g of DEAE-cellulose equilibrated with 25 mM Tris HCl buffer pH 7 was packed in a glass column (1.5 × 20 cm). 10 mg of enzyme protein (dialyzed 40-60% ammonium sulphate fraction) was loaded. The column was thoroughly washed with three column volumes of same buffer to remove unbound enzyme. The bound enzyme was eluted with 25 mM Tris HCl buffer pH 7 containing NaCl gradient (0.1M to 0.5 M) at a flow rate of 10 ml/h. One ml fractions were collected and reductase activity and protein in the fractions were determined.

Cr(VI) reductase assay

Reductase assay was carried out by incubating 1 ml of assay mixture containing 0.4 mM Cr(VI), 1.6 mM NADH and appropriately diluted enzyme in 25 mM Tris HCl buffer, pH 7 at 28°C, 30 min. The residual Cr(VI) in the assay mixture was estimated by diphenyl carbazide method described earlier. One unit of activity is defined as the amount of enzyme that reduced 1 nanomole of Cr(VI) per min under the assay conditions.

Protein estimation

Protein in the sample was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Investigation of extracellular/ intracellular nature of Cr(VI) reduction

Cr(VI) reduction was seen during growth as well as by resting cells of the organism indicating that the reductase activity is associated with cells. However, it was of interest to know if the organism secreted any extracellular reductase activity in to the medium. No chromate reduction was observed when the culture supernatant was incubated with Cr(VI) even after prolonged time (96 h) while complete reduction within 24 to 48 h was noticed when the cells were incubated with Cr(VI) indicating the intracellular nature of the reductase activity. Morales et al. (2007) reported chromate reduction in *Streptomyces* sp. CG252 to be cell associated and no activity was found in culture supernatant.

Methods of cell breakage

Since the reductase activity was intracellular, it was necessary to break the cells to release the enzyme. Residual Cr(VI) concentration was measured after incubating 64 mg/L of Cr(VI) with 0.5 ml of the cell free extract (CFE) obtained from each method of cell breakage for 2 h. Among the three methods tested for cell breakage, sonication was found to be the best method which showed highest decrease in residual Cr(VI) concentration (35.31%) followed by liquid nitrogen (12.03%) while grinding with neutral alumina was poor (4.56%). Hence for all further experiments sonication was used for enzyme extraction.

Conversion of Cr(VI) to Cr(III) by cell free extract (CFE)

Observed decrease in Cr(VI) concentration was in fact due to formation of Cr(III) was confirmed by monitoring the increase in absorbance at 595 nm which was due to formation of Cr(III). In presence of CFE, there was decrease in initial Cr(VI) concentration from 63.606 to 22.462 mg/L (as estimated by DPC) with simultaneous increase in absorbance at 595 nm from 0.048 to 0.308 indicating the formation of Cr(III). No reduction was observed by the CFE heated at 100°C for 5 min. The above results clearly indicated that CFE obtained after sonication was able to reduce Cr(VI) and the reduction by *S. griseus* appears to be enzymatic since it was catalyzed by the

proteinaceous and heat labile CFE. In case of *B. subtilis,* chromate reductase present in the soluble fraction when heated at 100°C for 10 min lost ability to reduce Cr(VI) indicating that the enzyme was heat labile (Garbisu et al. 1998). Similar observations of loss in activity and heat labile nature of enzyme were made by others (Ishibashi et al. 1990; Horitsu et al. 1987). Only the soluble fraction from *Streptomyces* sp. CG252 was capable of Cr(VI) reduction and very little/ no activity was present either in the culture supernatant or cell debris. The activity present in the soluble fraction was abolished upon heating for 20 min at 100°C (Morales et al. 2007).

Loss in activity on dialysis and restoration by addition of NADH

CFE obtained after sonication was dialyzed overnight against 25 mM Tris HCl pH 7 buffer at 4°C. One millilitre of reaction mixture containing 25 mM Tris HCl buffer pH 7 and 30 mg/L of Cr(VI) was incubated with dialyzed or un-dialyzed CFE and residual Cr(VI) was estimated by DPC. As shown in Table 4.2, the reductase activity was lost on dialysis and dialysed CFE showed only 2.77% reduction compared to undialysed CFE which showed around 26.32% reduction. However, the activity was completely restored by the addition of 1.6 mM NADH (final concentration) to dialysed CFE suggesting the loss of cofactor(s) required for reduction on dialysis. Similar observation was made by Horitsu et al. (1987) in case of *P. ambigua* G-1.

Table 4.2: Loss in activity on dialysis and restoration on addition of NADH

Component	Residual Cr(VI) (mg/L)	Cr(VI) Reduction (%)
Undialysed CFE	22.385	26.32
Dialysed CFE	29.540	2.77
Dialysed CFE + NADH	22.260	26.73

Effect of addition of NAD(P)H on Cr(VI) reductase activity

Since NADH restored reductase activity of dialysed CFE, effect of addition of NADPH on chromate reduction by undialyzed CFE was also investigated. NADH was included for comparison. CFE alone and NAD(P)H were included as controls. No decrease in Cr(VI) was observed in NAD(P)H alone. CFE without NADH or NADPH showed around 21% decrease in residual Cr(VI) concentration and reductase activity was around 8.53 U/ml (Table 4.3). Addition of either NADH or NADPH significantly

increased Cr(VI) reduction with residual Cr(VI) concentrations around 27.5 to 32%. Reductase activities of around 26 to 28 U/ml were obtained which were nearly 3 times higher than in controls without added NAD(P)H (Table 4.3). There was no preference for electron donor and both NADH and NADPH acted equally well.

Table 4.3: Effect of NAD(P)H addition on chromate reduction

	0 min	30 min		
	Initial Cr (mg/L)	**Residual Cr (mg/L)**	**Residual Cr (%)**	**Reductase Activity (U/ml)**
NAD(P)H	25.01	23.50	94.03	-
CFE	21.18	16.57	78.92	8.53
CFE+ NADH	21.23	5.77	27.49	27.94
CFE+NADPH	21.08	6.71	31.96	26.30

Reductase activity was determined as described earlier. NAD(P)H blank was devoid of CFE

Our work is in agreement with other studies where NAD(P)H dependant enzymatic reduction under aerobic conditions is reported (Elangovan et al 2006; Pal and Paul 2004; McLean and Beveridge 2001; Park et al. 2000; Campos et al. 1995; Suzuki et al. 1992). NADH and NADPH also served as electron donors for reduction by *Streptomyces* sp. (Das and Chandra 1990). Addition of 1 mM NADH enhanced the Cr(VI) reductase activity in the cell-free extracts of all the three isolates of *Bacillus* sp. G1DM20, G1DM22 and G1DM64 (Desai et al. 2008). In contrast, chromate reductase from *P. ambigua* G-1 required NADH but not NADPH as electron donor for Cr(VI) reduction (Horitsu et al. 1987). Chromate reductase from *P. aeruginosa* HP014 required either NADH or NADPH for activity. However, NADPH gave only 50% activity compared to NADH (Oh and Choi 1997).

Unlike *P. putida* which had absolute requirement of NADH for activity, CFE from *S. griseus* was able to reduce chromate even in absence of exogenous addition of NAD(P)H since the CFE itself appears to have the cofactors necessary for reduction. Similar observations were also made by Horitsu et al. (1987) with chromate-resistant *P. ambigua* G-1 strain which used internally stored reserves as electron donors. Addition of NAD(P)H significantly enhanced chromate reduction by 2-3 folds in *S. griseus*. Camargo et al. (2003) reported similar results with *Bacillus* sp. E29 where addition of NADH increased the reduction by 4.6 times.

Partial inhibition of Cr(VI) reduction by the resting cells of this organism by azide (Laxman and More 2002) and the ability of NADH/NADPH to act as electron donors for reduction by the CFE suggests that an electron transport system is involved.

Effect of chromium as inducer and growth medium

The reductase activities in CFEs from cells grown in absence and presence of Cr(VI) were 21.89 ± 2.32 and 25.47 ± 0.96 U/ml respectively. These results indicate chromate reductase to be constitutive in nature with slight induction in presence of Cr(VI). Therefore, it is apparent that addition of Cr(VI) during growth was not necessary for the expression of reductase activity. Constitutive nature of the reductase was also reported in *Streptomyces* sp. (Das and Chandra 1990). Reductase activities from *Bacillus* sp. G1DM20, G1DM22 and G1DM64 (Desai et al. 2008) *B. subtilis* (Garbisu et al. 1998) were mainly associated with the soluble fraction of the cells and was expressed constitutively. The constitutive nature of reductase from pseudomonad CRB5 was also reported where presence of Cr(VI) was not required for reductase production (McLean and Beveridge 2001). Mabrouk (2008) found no difference in reductase activity between *Streptomyces* sp. CG252 grown in the presence or absence of Cr(VI) indicating that the activity was not being induced by the presence of Cr(VI).

The activities in CFEs from cells grown in MGYP and Broth-II medium were 23.58 ± 1.07 and 22.32 ± 1.64 U/ml respectively. Growth medium and addition of Cr(VI) to growth medium had no significant effect on the reduction confirming that the activity was constitutive.

Localization of chromate reductase activity by ultracentrifugation

Fractionation of crude sonicated cell free extract by ultracentrifugation showed that the Cr(VI) reductase activity was located in the soluble fraction (Table 4.4). Around 90% activity was found in this fraction while no significant reduction was observed with the membrane fraction. Around 63.03 and 36.76% of total protein was found in soluble and membrane fractions respectively.

Das and Chandra (1990) reported Cr(VI) reduction in *Streptomyces* sp. by the particulate cell fraction obtained by centrifugation at 105,000 x g for 1h that reduced

82.7% Cr(VI), in presence of NADH. The aerobic mechanism of Cr(VI) reduction is generally associated with the soluble fraction that utilizes NADH as an electron donor (Suzuki et al. 1992; Shen and Wang 1993; Oh and Choi 1997). Microbes of genera *Pseudomonas*, *Arthrobacter*, *Escherichia* and *Bacillus* have been reported to reduce Cr(VI) through soluble chromate reductases (Park et al. 2000; Camargo et al. 2004; Campos-Garcia et al. 1997; Bae et al. 2005).

Table 4.4: Localization of chromate reductase activity by ultracentrifugation

Fraction	Activity (IU/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Total Activity (Units)	Total protein (mg)	Activity Recovery (%)	Protein Recovery (%)
Cell Free Extract (S_{12})	31.07	16.39	1.90	372.84	196.70	100.00	100.00
Soluble Fraction (S_{150})	27.31	10.33	2.64	327.72	123.98	87.90	63.03
Membrane Fraction	0	72.31	-	0	72.31	0	36.76

Fractional ammonium sulphate precipitation

Fractional ammonium sulphate precipitation of cell free extract showed that the major portion of reductase activity was found in the fraction at 40-60% ammonium sulfate saturation followed by the fraction at 20-40% saturation. Though major portion of protein was recovered, activity was very low at 0-20% saturation (Table 4.5). Highest specific activity of 5.06 U/mg of chromate reductase was found in fraction at 40-60% saturation followed by the fraction at 20-40% saturation with a specific activity of 4.55 U/mg. The specific activity of the fraction at 0-20% saturation was nearly similar to that of CFE.

Table 4.5: Fractional ammonium sulphate precipitation

Saturation (%)	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg Protein)
CFE	22.70	10.97	2.07
0-20	8.76	33.00	2.02
20-40	22.73	4.99	4.55
40-60	39.64	7.83	5.06
60-90	1.72	2.90	0.59

Effect of pH and temperature on reductase activity

Though both CFE as well as 40-60% ammonium sulphate fraction showed optimum activity at pH 7, CFE showed a broader peak compared to partially purified enzyme (Fig. 4.2). Most of the bacterial reductases are optimally active near neutral pH (Table 4.1a and b). The only exception of an acidic reductase optimally active at pH 5 being reported from *P. putida* MK1 (Park et al. 2000) while reductases optimally active in slightly alkaline range are reported from *P. ambigua* (Suzuki et al. 1992), *P. mendocina* (Rajwade et al. 1999) and *P. putida* (Renuga et al. 2008).

CFE as well as partially purified enzyme (40-60% saturation ammonium sulphate fraction) showed maximum activity at 28°C. Both the enzyme preparations showed 25% activity at 20°C. However at 40°C, CFE and partially purified reductase showed 75-80% and around 40% activities respectively. Activities decreased with further increase in temperature (Fig. 4.2). Optimum temperatures of most of the chromate reductases reported so far fall in the range of 30 to 40°C with exceptions like reductases from *P. putida* MK1 (Park et al. 2000) and *P. putida* (Renuga et al. 2008) which had very high temperature optima of 80 and 75°C respectively. In view of their application for metal remediation of contaminated site/effluents, it is preferable that the Cr(VI) reductases are optimally active at ambient temperature.

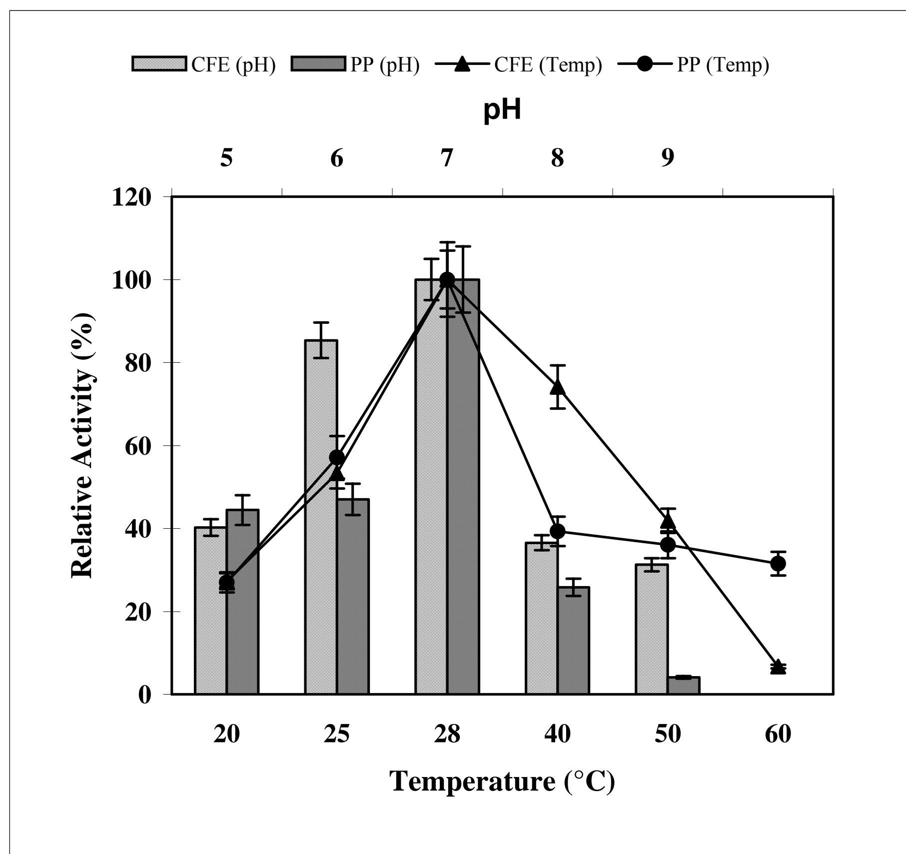


Fig. 4.2: Effect of pH and Temperature
(CFE- cell free extract; PP- partially purified enzyme)

Temperature stability of crude chromate reductase (CFE)

Crude reductase (CFE) was stable at 28°C up to 60 min with nearly 90% activity being retained after which there was gradual loss in activity and around 30% of its original activity was retained after 2 h (Fig 4.3). The purified reductase from *P. putida* was stable between 30 to 50°C up to 30 min but appreciable inactivation occurred at higher temperatures (Park et al. 2000). Chromate reductase from *Bacillus* sp. was most stable below 30°C after 60 min incubation which is its optimum temperature for activity and retained 80% of the residual activity but further increase of temperature resulted in rapid loss (Elangovan et al. 2006).

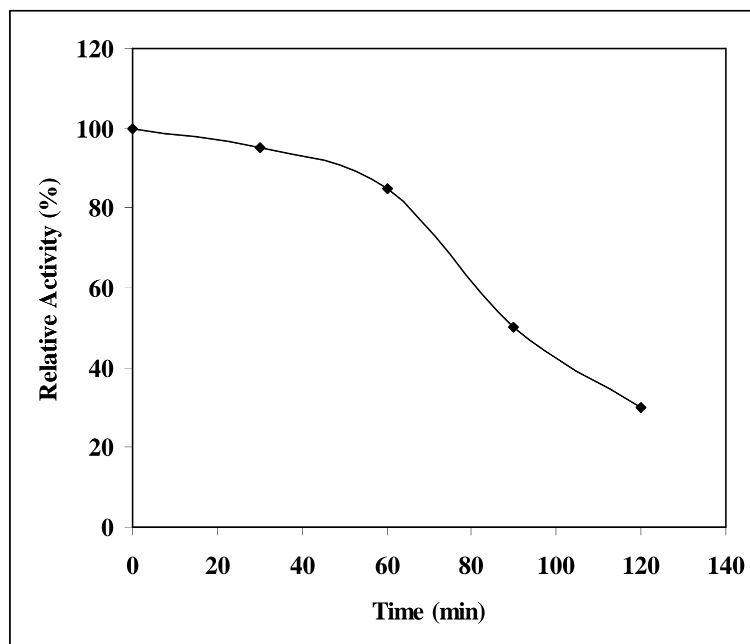


Fig 4.3: Temperature stability of CFE at 28°C

Effect of metal ions and inhibitors on chromate reductase activity

Effect of metal ions and inhibitors on partially purified reductase from *S. griseus* (40-60% ammonium sulphate fraction) is presented in Table 4.6. Among the metals tested, Hg(II) and Cd(II) were found to be most inhibitory with nearly 80% and 60% inhibition respectively. The inhibition by Hg(II) suggests possible involvement of thiol group in the catalysis. Similar inhibition by Hg(II) has also been reported for other chromate reductases. Hg(II) is reported to inhibit chromate reductases from *P. putida* (Ishibashi et al. (1990), from *E. coli* ATCC 33456 (Bae et al. 2005) and *Bacillus* sp. RE (Elangovan et al. 2006). Cd(II) produced significant inhibition of reductase activity from *P. putida* (Park et al. 2000); *E. coli* ATCC 33456 (Bae et al. 2005); Cd(II) (100 µM) strongly inhibited cell free Cr(VI) reductase of *Bacillus sphaericus* AND 303 (Pal et al. 2005).

Co(II), Mn(II), Zn(II) and Ni(II) showed around 20% inhibition of partially purified reductase from *S. griseus* while Ag(I) and Mn(II) were least inhibitory with only 10% inhibition. In case of chromate reductase from *E. coli* ATCC 33456, there was more

than 50% inhibition by Ag(I), Cd(II), Zn(II) (Bae et al. 2005). Ag(I), Fe(II), Ba(II) and Zn(II) inhibited chromate reductase from *Bacillus sp* from 40–75% (Elangovan et al. 2006). Metal ions like, Ni(II), Co(II), Cd(II) and Pb(II) inhibited chromate reduction by *Bacillus sphaericus* AND 303 (Pal et al. 2005).

Table 4.6: Effect of metal/inhibitor on chromate reductase activity

Metal/Inhibitor (1mM)	Relative activity (%)
None	100.00
Hg	20.61
Cd	40.04
Zn	73.91
Ni	77.86
Mg	77.86
Co	79.26
Ag	86.59
Mn	91.88
Cu	118.00
Ca	102.4
EDTA	86.78
Formaldehyde*	39.22
NaN_3	99.87

* 0.01%

No inhibition of reductase from *S. griseus* was observed with Ca(II) and Cu(II). Chromate reductase from *Bacillus* sp. RE was enhanced by Cu(II) and Ni(II) by 51 and 14%, respectively (Elangovan et al. 2006). Mg(II) and Ca(II) had no effect on chromate reductase from *Bacillus* sp. RE (Elangovan et al. 2006). In case of chromate reductase from *Thermus scotoductus* SA-01 activity was dependent on the presence of the divalent metal Ca(II) or Mg(II) which increased the activity > 4-fold (Opperman et al. 2008). Enhancement of chromate reductase activity by Cu(II) was reported from *Bacillus* sp. (Camargo et al. 2003). On the other hand, Cu(II) has been known to inhibit the chromate reductase from *P. putida* (Park et al. 2000). In case of chromate reductase from *Thermus scotoductus* SA-01 activity was inhibited Zn(II), Mn(II) and EDTA (Opperman et al. 2008).

Sodium azide showed no inhibition while formaldehyde showed 60% inhibition on chromate reductase from *S. griseus*. There was only marginal inhibition by EDTA. Sodium azide did not inhibit chromium reduction in *Streptomyces* (Das and Chandra,

1990) and in *Pseudomonas mendocina* (Rajwade et al. 1999). Cyanide and azide prevent *de novo* protein synthesis but do not inhibit the activity of any reductase enzymes that are already present (Garbisu et al. 1998). No inhibitory effects on aerobic Cr(VI) reduction were demonstrated by addition of cyanide, azide, and rotenone into both intact cell cultures and supernatant fluids of *E. coli* ATCC 33456 (Shen and Wang 1993). Respiratory inhibitor sodium azide completely inhibited Cr(VI) reduction by growing cells of *B. megaterium* TKW3 however, no inhibitory effect of sodium azide on Cr(VI) reduction was found by soluble and membrane fractions S_{27k} (Cheung et al. 2006).

Effect of copper on chromate reductase activity

Effect of copper on chromate reductase was studied by incubating ammonium sulphate precipitated enzyme (40-60% saturation) with copper concentrations ranging from 0 to 1mM at 28°C for 30 min. Relative activity was expressed as percentage of activity of control without Cu(II) taken as 100%. It was seen that Cu(II) marginally enhanced reductase activity in the concentration range tested (Table 4.7). Batch studies showed enhancement of Cr(VI) reduction by free cells of *S. griseus* by Cu(II) where addition of 0.4 mM Cu(II) resulted in complete reduction of 25 mg/L of Cr(VI) in 6 h, while only 40% reduction was observed in its absence. The addition of Cu(II) did not affect Cr(VI) reduction under aerobic conditions by soluble chromate reductase of *P. putida* (Park et al. 2000) or pseudomonad CRB5 (McLean and Beveridge 2001). Camargo et al. (2003) reported for the first time that 1mM Cu(II) substantially stimulated the activity of the CFE of *Bacillus* sp. ES 29. Most studies have reported an inhibitory influence of Cu(II) on Cr(VI) reduction. Ohtake et al. (1990) reported 32% inhibition of Cr(VI) reduction by a membrane-associated chromate reductase under anaerobic conditions by 0.5 mM of Cu(II). The stimulatory mechanism of Cr(VI) reduction activity by Cu(II) and other metals is not clear. However, Cu(II) is a prosthetic group for many reductase enzymes. The main function of Cu(II) has been reported to be related to electron transport protection or acting as electron redox center and, in some cases, as a shuttle for electrons between protein subunits (Camargo et al. 2003). The highest concentration of Cu(II) used in the present study was 1mM and it is possible that higher

concentrations beyond 1mM would have further enhanced the reductase activity of *S. griseus*.

Table 4.7: Effect of copper on chromate reductase activity

Copper (mM)	**Relative Activity (%)**
0.0	100
0.4	94
0.6	100
0.8	113
1.0	118

Identification of intermediate formed during reduction by EPR

An EPR analysis was performed to identify any intermediates formed during reduction of Cr(VI) to Cr(III). Incubation of chromate ($K_2Cr_2O_7$) solution with partially purified reductase (40-60% ammonium sulphate precipitate) in presence of NADH gave 2 signals (Fig 4.4). A major signal due to Cr(III) (g value = 1.980) and a minor signal due to Cr(V) (g value =1.977) were observed. During incubation in absence of enzyme and NADH the signal due to Cr(III) and Cr(V) did not appear. These findings show that the chromate reductase from *S. griseus* reduced Cr(VI) to Cr(III) with at least two reaction steps via the formation of Cr(V) as an intermediate, which being highly unstable is converted to stable Cr(III).

Das and Chandra (1990) reported the formation of Cr(V) as an intermediate during Cr(VI) reduction in *Streptomyces* sp. transiently formed Cr(V) was detected as an intermediate in *P. ambigua,* indicating that the reduction of Cr(VI) to Cr(III) is at least a two-step reaction. Indirect evidence suggested that the end product of the reaction was Cr(III) (Suzuki et al. 1992). Direct evidence to show that Cr (VI) was completely and quantitatively transformed to Cr(III) has been provided in case of *E. coli* (Shen and Wang 1993) and *Agrobacterium radiobacter* (Llovera et al.1993). In case of anaerobically grown *Shewanella putrefaciens* MR-1, Cr(VI) reductase associated with the cytoplasmic membrane was found to reduce Cr(VI) to Cr(V) suggesting a one-electron reduction as the first step (Myers et al. 2000). Their EPR data indicated presence of Cr(V) and Cr(VI) reduction must therefore proceed through an initial single electron transfer leading to the formation of Cr(V).

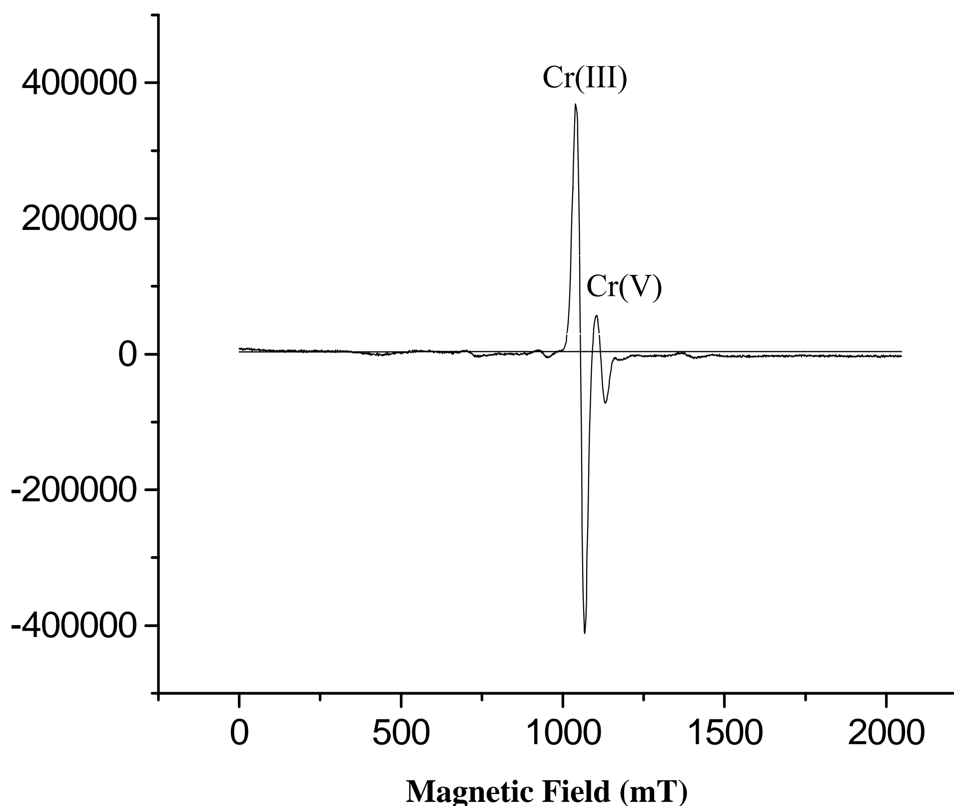


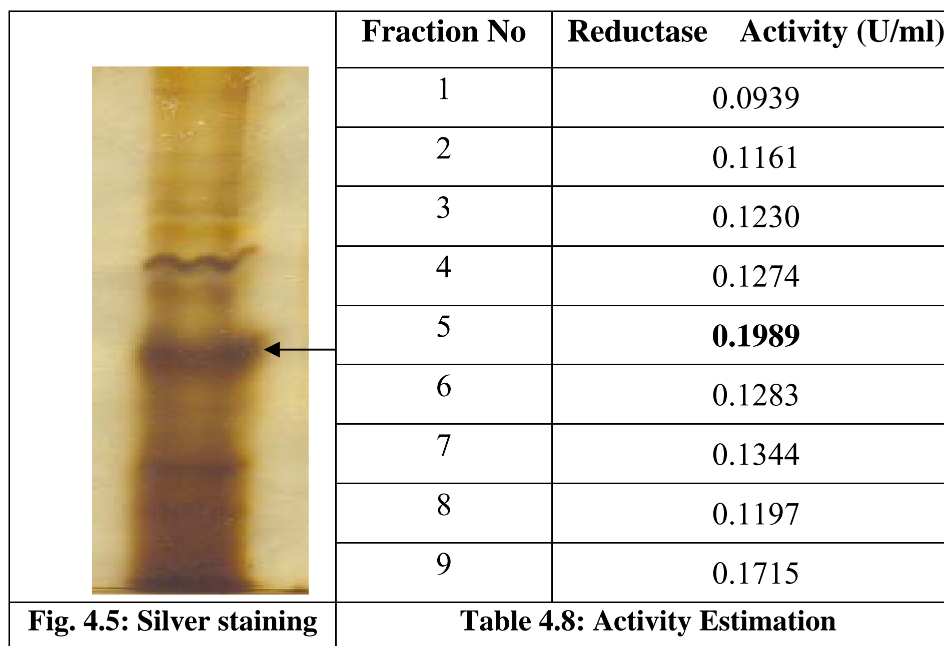
Fig 4.4: EPR spectra of Cr(VI) reductase from *S. griseus*

Park et al. (2000) have shown quantitative conversion of Cr(VI) to Cr(III) during the enzyme reaction using XANES. At 2.7 min of the reaction, Cr(VI) constituted more than 90% of the total chromium in the sample, but by 40 min, it was depleted to less than 10%, with a proportionate increase in Cr(III). The sensitivity of their assay was not high enough to permit determination of unstable and transient Cr(V) species.

Analytical PAGE- anodic system (pH 8.8)

Dialyzed 40-60% ammonium sulphate precipitated enzyme was loaded on analytical PAGE, 8.8 system. To locate reductase activity on the gel, sample was loaded in duplicate and one strip was used for protein staining and the other strip was used for activity estimation. For activity estimation, the gel was cut into 9 equal pieces, each piece was homogenized, extracted with 1 ml of 25 mM Tris HCl buffer, pH 7 and clear

supernatant obtained after centrifugation was used for chromate reductase assay. Activity in each fraction was estimated and fraction 5 showed maximum reductase activity (Table 4.8). Protein was stained with silver staining and a prominent protein band was observed which corresponded to the fraction 5 where maximum activity was detected (Fig. 4.5).



Fraction No	Reductase Activity (U/ml)
1	0.0939
2	0.1161
3	0.1230
4	0.1274
5	**0.1989**
6	0.1283
7	0.1344
8	0.1197
9	0.1715

Fig. 4.5: Silver staining **Table 4.8: Activity Estimation**

Anodic PAGE of chromate reductase (40-60% ammonium sulphate precipitate)

Adsorption of Cr(VI) reductase to DEAE-cellulose: effect of pH on binding

Preliminary studies of batch adsorption of dialysed 40-60% ammonium sulphate fraction on DEAE - cellulose showed that highest residual Cr(VI) at pH 7 indicating that maximum binding of reductase occurred at this pH (Fig. 4.6). Lowest residual Cr(VI) was observed at pH 9 followed by pH 6 indicating that least binding of enzyme at these pH values.

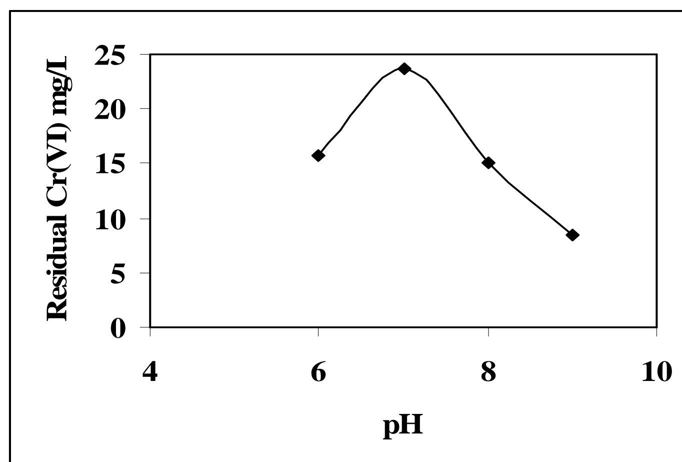


Fig 4.6: Effect of pH on binding of Cr(VI) reductase to DEAE-cellulose

***DEAE*-cellulose column chromatography**

Dialyzed 40-60% ammonium sulphate precipitate fraction was applied to DEAE-cellulose column and the bound enzyme was eluted with NaCl gradient (0.1 to 0.5M) in 25mM Tris HCl pH 7. The enzyme was eluted at 0.1M NaCl concentration with maximum activity being recovered at this concentration (Fig 4.7).

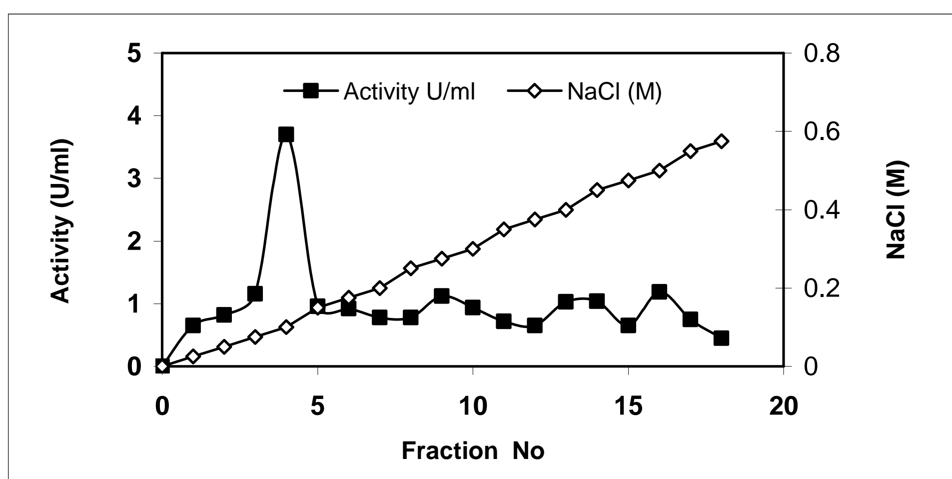


Fig 4.7: Elution of DEAE-cellulose bound chromate reductase

Fractions having activity were concentrated by speed vac and used for molecular weight determination by SDS-PAGE.

Molecular weight determination by SDS PAGE

40-60% ammonium sulphate precipitate and enzyme eluted with 0.1M NaCl from DEAE-cellulose column were loaded under non-denaturing conditions on SDS-PAGE (10%) along with molecular weight markers. 40-60% ammonium sulphate precipitate showed 9 bands while DEAE eluted enzyme showed 5 bands on silver staining (Fig.4.8 a and b). The molecular weight was found to be approximately 46-48 kDa. The molecular weights of Cr(VI) reductases from *Bacillus* QC1-2 (Campos-Garcia et al. 1997), *P. mendocina* (Rajwade et al. 1999), *P. putida* MK 1 (Park et al 2000), *E. coli* ATCC 33456 (Bae et al. 2005) and *P. putida* (Renuga et al. 2008) by SDS PAGE were found to be 24, 65 and 29, 20, 42 and 20 kDa respectively Table 4.1). The molecular weight of *S. griseus* which is 46-48 kDa which is similar to the one reported for *E. coli* ATCC 33456 by Bae et al. 2005.

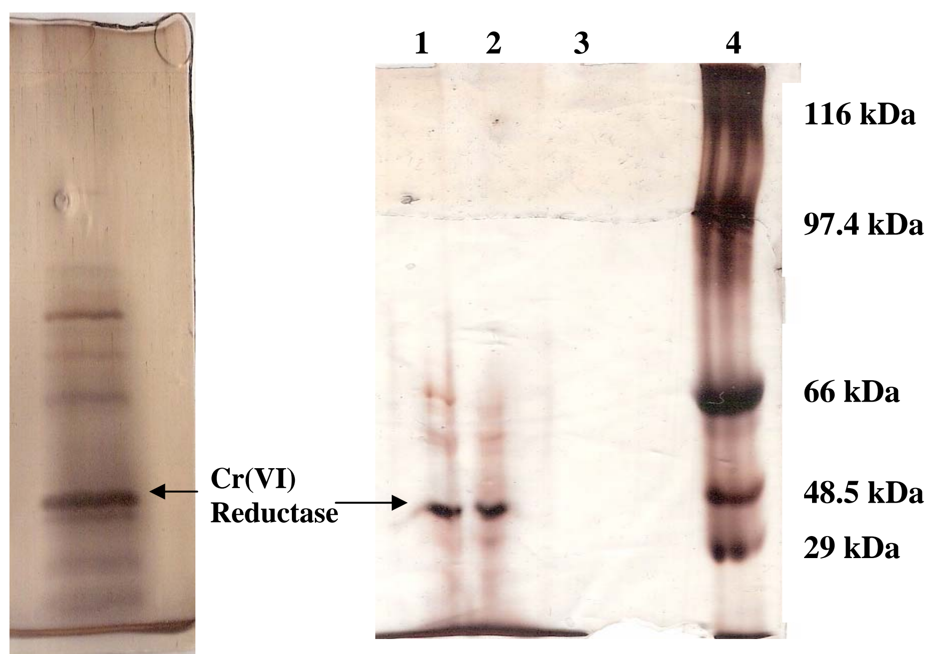


Fig 4.8a: 40-60% ammonium sulphate precipitated enzyme

Fig 4.8b:10% Non-denaturing SDS PAGE
Lane 1 and 2 DEAE-cellulose eluted enzyme
Lane 4 Molecular weight marker

CONCLUSIONS

Chromate reduction was associated with the *Streptomyces griseus* cells and sonication was found to be the best method of cell breakage to release the enzyme. The enzyme was constitutive and did not require presence of chromate during growth for expression of activity. Chromate reduction with cell free extract (CFE) was observed without added NADH. Reductase activity which was lost on dialysis was restored on addition of NADH. Addition of NADH/NADPH enhanced chromate reduction by 2-3 folds. The enzyme was optimally active at 28°C and pH 7 and had no preference for NADH or NADPH. Hg(II) and Cd(II) inhibited *S. griseus* reductase (CFE) by 80 and 60% while Ca(II) and Cu(II) had no inhibitory effect.

Reduction of Cr(VI) by the cell free chromate reductase from *S. griseus* in presence of NADH as a co-factor has been shown to yield Cr(V) as well as Cr(III). The presence of Cr(V) as a transient intermediate during the conversion of Cr(VI) to Cr(III) was confirmed by EPR spectroscopy indicating a one-electron reduction as the first step. Ultracentrifugation studies of cell free extract of *S. griseus* revealed that the chromate reductase activity was found to be in the soluble fraction and no activity was detected in the membrane fraction.

Fractional ammonium sulphate precipitation of the cell free enzyme showed that major portion of chromate reductase activity was found in 40-60% ammonium sulphate precipitate fraction. Partially purified reductase was obtained by passing dialyzed 40-60% ammonium sulphate precipitate fraction on DEAE and eluting with 0.1M NaCl in 25 mM Tris HCl pH 7. The molecular weight of the enzyme was around 46-48 kDa by SDS PAGE.

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CHAPTER V

INVESTIGATION OF THE GENETIC DETERMINANTS OF CHROMATE RESISTANCE/REDUCTION

ABSTRACT

A chromate resistant strain of *Streptomyces griseus* capable of reducing hexavalent chromium was found to harbor a giant linear plasmid. Its presence was confirmed by the analysis of the total cellular DNA by pulsed field gel electrophoresis. Transformation of *E. coli* JM109 with purified plasmid DNA resulted in acquisition of resistance to chromate in the transformants. Most importantly, the plasmid transfer was found to confer chromate reduction ability on to the *E. coli* transformants.

INTRODUCTION

Microorganisms have evolved diverse resistance mechanisms to cope with chromate toxicity. These systems include direct strategies that involve the efflux of toxic chromate ions from the cytoplasm or the transformation of Cr(VI) to innocuous Cr(III) outside the cell. Several probable Cr(VI) membrane transporters have been identified and they have been grouped into a large super family. However, only two bacterial transporters for efflux of chromate are well characterized.

Many bacterial species are reported to reduce Cr(VI) to Cr(III), but the biochemical properties of few Cr(VI) reductases have been elucidated. The diverse characteristics of these ancient enzymes and their wide distribution support the hypothesis that reduction of chromate is a secondary role for chromate reductases. Diverse bacterial species seem to display indirect systems of tolerance to chromate. After chromate exposure, these bacteria show a varied regulatory network that involves the expression of genes for several different metabolic processes as Cr stress defensive strategy. These include genes for sulfur or iron homeostasis and ROS (reactive oxygen species) detoxification. These indirect systems of tolerance to chromate include mechanisms focused to maintain the integrity of the cells by protecting them from oxidative stress or to repair the damages caused by Cr derivatives.

Chromate resistance/reduction mechanisms

Chromium transport and the CHR superfamily of transporters : exclusion by permeability barrier

The CHR (Chromate resistance) superfamily of transporters is a wide spread group of proteins probably involved in chromate or sulfate transport (Nies et al. 1998). The chromate transporters have probably evolved recently as a result of chromate exposure by bacteria. The databases of the CHR protein family currently contain 135 sequences of homologs, including proteins from eukaryotes (Cervantes and Campos-Garcı´a 2007). With the exception of *P. aeruginosa* and *Cupriavidus metallidurans* chromate resistance ChrA proteins, the function of other CHR homologs have not yet been analyzed in detail. CHR homologs exist in two sizes (Nies et al. 1998): (1) Small proteins or SCHR (about 200 aa) possess only one domain. Sequence analysis suggests

that these proteins may form a paralog group inside the CHR superfamily. (2) Large proteins or LCHR with about 400 aa (except eukaryotic proteins of 500–600 aa) with two homologous domains. The LCHR proteins are arranged in six subfamilies from bacteria (LCHR1 to LCHR6), and one subfamily from fungi. The LCHR1 subfamily contains all the Gram positive homologs. The ChrA proteins from *C. metallidurans* and *P. aeruginosa* with demonstrated function in chromate efflux, are located into the LCHR2 and LCHR5 subfamilies respectively. The LCHR4 subfamily includes a protein from *Desulfovibrio vulgaris* and the only protein from an Archaea (*Methanococcus jannaschii*).

Chromate reduction: Extracellular/Intracellular

Bacterial reduction of metallic ions has been shown to occur for U(VI), Se(VI), Cr(VI), Mo(VI), Se(IV), Hg(II), Ag(I) and others (Lovley 1993; Bradley and Obraztsova 1998). A wide range of bacteria have been identified that are capable of carrying out complete reduction of Cr(VI) to Cr(III) by oxidation-reduction reactions of biotic and abiotic nature. Microbial reduction of Cr(VI) to Cr(III) can be considered as an additional chromate resistance mechanism which is not usually a plasmid-associated trait (Cervantes et al. 2001). Cr(VI) reduction outside the cell generates Cr(III) which cannot cross cellular membranes. Three Cr(VI) reduction mechanisms have been described (Cervantes and Campos-Garcı´a 2007):

(i) In aerobic conditions, chromate reduction has been commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors.

(ii) Under anaerobiosis, some bacteria, like *Pseudomonas fluorescens* LB300 (Bopp and Ehrlich 1988), can use Cr(VI) as an electron acceptor in the electron transport chain.

(iii) Reduction of Cr(VI) may also be carried out by chemical reactions associated with compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione. For instance, riboflavin derivatives FAD and FMN are essential coenzymes for chromate-reducing flavoenzymes (Masayasu 1991). The most characterized enzymes belong to the widespread NAD(P)H-dependent flavoprotein family of reductases.

Protection against oxidative stress

Since the generation of reactive oxygen species (ROS) occurs during Cr(VI) reduction to Cr(III), the participation of bacterial proteins in the defense against oxidative stress induced by chromate represents an additional mechanism of chromate resistance. *E. coli* displays several chromate protective systems, including the activation of enzymes such as superoxide dismutase (SOD) and catalase. Additionally, chromate exposure in *E. coli* led to the depletion of the pools of glutathione and other thiols, suggesting that these compounds have an important detoxifying role against Cr(VI) (Ackerley et al. 2006). *Caulobacter crescentus* and *Shewanella oneidensis* MR-1 employ different processes to counteract oxidative stress upon exposure to chromate. Cr(VI)-exposed cultures showed the up-regulation of genes involved in the response to heavy-metal toxicity and cellular detoxification (Hu et al. 2005, Chourey et al. 2006).

Besides chromosomal genes, plasmids may also encode systems devoted to protect bacterial cells from the oxidative stress caused by chromate. Plasmid pMOL28 from C. *metallidurans*, which encodes the ChrA chromate efflux pump, in addition encodes the ChrC and ChrE proteins that seem to be also involved in chromate resistance (Juhnke et al. 2002).

In conclusion, enzymes that participate in detoxification of ROS generated after Cr(VI) exposure and reduction may be involved in the protection against the deleterious effects of chromate.

Transmembrane efflux of chromate

The efflux of chromate is a resistance mechanism conferred by the ChrA protein. ChrA is encoded by plasmids pUM505 of *P. aeruginosa* and pMOL28 from *C. metallidurans* (previously *Alcaligenes eutrophus* and *Ralstonia metallidurans*) (Cervantes et al. 1990; Nies et al. 1990). ChrA from *P. aeruginosa* functions as a chemiosmotic pump that effluxes chromate from the cytoplasm using the proton motive force (Alvarez et al. 1999; Pimentel et al. 2002).

Efflux of chromate is inhibited by sulfate, suggesting that this analog oxyanion may also bind to the ChrA protein (Pimentel et al. 2002). In fact, it has been proposed

that ChrA may function as a chromate/sulfate antiporter (Nies et al. 1998) nevertheless, sulfate transport by the ChrA proteins has not yet been determined.

In summary, the efflux of chromate seems to be an efficient and widespread mechanism of resistance, which prevents the accumulation of this toxic ion inside the cell.

DNA repair

Another defensive shield against Cr toxicity is the protection of bacterial cells from DNA damage caused by chromium compounds. Cr(VI) has long been known to induce the *E. coli* SOS repair system that protects DNA from oxidative damage (Llagostera et al. 1986). Components of the recombinational DNA repair system, like DNA helicases RecG and RuvB, were also shown to participate in the response to DNA damage caused by chromate in *P. aeruginosa* (Miranda et al. 2005). Similarly, in 24 h Cr(VI) exposed *S. oneidensis*, the SO0368, *uvr*D, and *hrp*A genes, which encode helicases, were induced (Chourey et al. 2006). *C. crescentus* also showed the up-regulation of genes related to repair of DNA damage (endonucleases, RecA protein) in response to Cr(VI) treatment (Hu et al. 2005).

Plasmid mediated resistance

Plasmid-associated bacterial resistance to Cr(VI) has been reported in *Streptococcus lactis* (Efstathiou and Mckay 1977), *Pseudomonas* sp. (Summers and Jacoby 1978), and *Alcaligenes eutrophus* (Cervantes and Silver 1992; Nies and Silver 1989; Peitzsch et al. 1998). Studies with *Pseudomonas fluorescens* LB300 showed that loss of plasmid resulted in loss of Cr(VI) resistance whereas transformation of plasmid less Cr sensitive strain with the purified plasmid DNA resulted in regaining of the Cr(VI) resistant ability of the strain (Bopp et al. 1983).

Earlier it was reported that resistance to chromate governed by bacterial plasmids appears to have nothing to do with chromate reduction (Cervantes and Ohtake 1988; Cervantes and Silver 1992; Ohtake et al. 1987). Furthermore, it was not clear whether the chromate reduction ability found with several bacterial isolates confers resistance to chromate (Ohtake and Silver 1994). Ohtake et al. (1987) reported that plasmid-determined chromate resistance results from reduced uptake of chromate by the resistant

cells, but they were unable to determine whether there was chromate efflux (as in other resistance mechanisms) or a direct block on uptake. A variety of chromate-resistant bacterial isolates have been reported, including strains from environmental and clinical settings; in these natural isolates, chromate tolerance was found to be usually associated with plasmids (Cervantes and Silver 1992). Bacterial resistance to chromate has been found in *Pseudomonas ambigua* (Horitsu et al. 1983), *P. fluorescens* (Bopp et al. 1983), *P aeruginosa* (Cervantes and Ohtake 1988; Summers and Jacoby 1978), *Alcaligenes eutrophus* (Nies and Silver 1989), *Streptococcus lactis* (Efstathiou and McKay 1977) and *Enterobacter cloacae* (Wang et al. 1990). Except for *P. ambigua* and *E. cloacae* strains, chromate resistance was found to be plasmid mediated. Two plasmid mediated chromate resistance systems were cloned and sequenced by Cervantes et al. (1990) and Nies et al. (1989, 1990). The involvement of sulfate transport in plasmid-conferred chromate resistance has been ruled out in both *P. fluorescens* (Ohtake et al. 1987) and *A. eutrophus* (Nies et al. 1989): resistant strains with plasmids transported sulfate with a similar kinetics to that shown by plasmid-less chromate-sensitive strains. Bopp and Ehrlich (1988) also showed that Cr(VI) reduction is independent of the chromate resistance mechanism conferred by plasmid pLHB1 of *P. fluorescens*. However, Dhakephalkar et al. (1996) reported that plasmid pARI180, from a *Pseudomonas mendocina* strain, determined both chromate resistance and reduction.

Plasmids

Most *Streptomyces* strains contain plasmids, nearly all of which are self-transmissible fertility factors. Both linear and circular plasmids occur. The linear plasmids are between 12 kb -1Mb in size and like the chromosome have Terminal Inverted Repeats (TIRs) and proteins at the 5' ends. Linear plasmids ranging in size from 12 kb -1Mb have been reported in more than 10 *Streptomyces* spp. and have been shown to carry genes encoding antibiotic biosynthesis, resistance to heavy metal and ability to break down xenobiotics. In the last decade, new plasmid borne resistance associated with metal mediated selection pressures from clinical, agricultural or industrial origins have been reported. These plasmids encode resistance genes that have potential use in biotechnology such as cloning, manufacture of biosensors and bioremediation processes.

In the present study, genetic basis of chromate resistance and reduction in *S. griseus,* the relationship between a giant linear plasmid from *S. griseus* and chromate resistance were investigated. Giant linear plasmid from *Streptomyces* was also transferred to *E. coli* (Inter-generic transfer).

MATERIALS AND METHODS

Materials

Lysozyme and Proteinase K were obtained from Sigma, Chemical Co, USA. Low range and mid range PFG markers were obtained from New England Biolabs. All other chemicals used in the present study were of analytical grade.

Bacterial strains and growth media

Streptomyces griseus NCIM 2020 was grown in Broth-II medium (pH 7.0) supplemented with 25 mg/L of Cr(VI) as $K_2Cr_2O_7$, unless otherwise specified. *E. coli* JM109 (plasmidless, chromate sensitive) strain used in this study was grown in Broth-II medium/ modified Luria Bertani Broth (g/L): Tryptone-10, Yeast Extract-5, pH 7.0.

Isolation of genomic DNA

Isolation of genomic DNA was carried out as described by Kieser et al. (2000). The quantification of DNA was done by measuring the absorbance of the sample at 260 nm on spectrophotometer and purity was checked on 0.8% agarose gel electrophoresis.

Isolation of plasmid DNA

Giant linear plasmid (GLP) from *S. griseus* was isolated by alkaline lysis method (low copy number) as described in QIAGEN protocol without column or by elution from pulsed field gels (Kieser et al. 2000).

Agarose gel electrophoresis (0.5%)

Run conditions - 60 V, 1 h. The agarose gel was stained with ethidium bromide (15 min) and observed under UV transilluminator.

Curing of S. griseus chromate resistant phenotype

Plasmid curing was attempted to check if loss of plasmid led to loss in Cr(VI) reduction and resistance ability. Plasmid curing was carried out using the following methods as described by Trevors (1986).

1. Intercalating dye (Ethidium Bromide)
2. Elevated growth temperature

3. Continuous subculture in nutrient rich medium

1. Intercalating dye (Ethidium Bromide)

Overnight grown culture (12 h) was inoculated in Broth-II medium (pH 7.0). Filter sterilized ethidium bromide was added at concentrations ranging from 1.0 to 100 μg/ml. The culture was incubated overnight at its optimum growth temperature (28°C) and the tube containing the highest concentration of curing agent that still allows bacterial growth was used as a source of inoculum for plating cells on Broth-II agar plates. After colony growth, individual colonies were screened for loss of the phenotype under investigation. The colonies from the master plate were replica plated on 25 mg/L of Cr(VI) containing Broth-II agar plates and tested for sensitivity to Cr(VI).

2. Elevated growth temperature

Elevated incubation temperature (5-7°C above the normal or optimal growth temperature) can be employed as a curing method. *S. griseus* was incubated at elevated temperature (37°C) until it reached late log phase, at which time it was diluted (1:20) and re-incubated at 37°C until late log phase is reached again. Serial dilutions were prepared and plated to obtain single colonies which were tested on 25 mg/L of Cr(VI) containing Broth-II agar plates.

3. Continuous subculture in nutrient rich medium

S. griseus was incubated at 28°C in MGYP medium (nutrient rich medium) until it reached late log phase, at which time it was diluted (1:20) and reincubated at 28°C. This cycle was repeated 5-10 times. Serial dilutions were prepared and plated to obtain single colonies which were tested on 25 mg/L of Cr(VI) containing Broth-II agar plates.

Pulse field gel electrophoresis (PFGE)

DNA plugs for PFGE analysis were prepared by a modification of the procedure used by Kieser et al. (1992). Mycelia were embedded in 1% inCert Agarose (FMC BioProducts, Rockland, Maine) and treated with lysozyme for 2 h at 37°C, followed by 48 h at 55°C in a 1 mg/ml concentration of proteinase K in NDS solution (0.5 mM

EDTA, 10 mM Tris HCl [pH 9.0], 1% Sarkosyl). Plugs were rinsed with T_{20}-E_{50} (20 mM Tris HCl, 50 mM EDTA; pH 8.0) three times for 1 h at 4°C.

Plugs were subjected to PFGE along with standard PFGE markers by using a clamped homogeneous electric field system (CHEF DR-III; Bio-Rad, Melville, N.Y.) in 0.5 x TBE buffer (1 x TBE is 98 mM Tris HCl, 89 mM boric acid and 62 mM EDTA) at 14°C. The PFGE gel was stained with ethidium bromide and observed under UV transilluminator.

Transformation studies in E. coli

E. coli JM109 was transformed with purified plasmid DNA from *S. griseus* by electroporation. Electroporation was carried out with BioRad Genepulser (BioRad) as follows. Electrocompetent cells of *E. coli* JM109 (Invitrogen) (25 µl) prepared as described in the BTX protocol (1992) and the plasmid DNA (5 µl/ 1 µg DNA) were added to a cuvette with a 1 mm gap between the electrodes (BTX) previously kept on ice. Immediately after electroporation at 2.5 kV, 100 Ω and 25 µF, 970 µl of SOC medium (Sambrook et al. 1989) was added to the cuvette and the cell suspension was transferred to a sterilized polypropylene tube and incubated at 37°C for 60 min with shaking. *E. coli* cells were spread on Broth-II agar plates containing 25 mg/L of Cr(VI) and cultivated at 37°C to check *E. coli* transformants.

Chromate reduction by E. coli transformants

The chromate reduction ability of *S. griseus* (plasmid), *E. coli* JM109 and *E. coli* JM109 (plasmid) transformants was compared in Broth-II/ LB without NaCl (*E. coli*) medium containing 25 mg/L of Cr(VI). In case of *S. griseus,* inoculum was developed by inoculating spores from 7 to 10 days slant. The experimental flasks were inoculated with 24-48 h old inoculum (10% v/v). Flasks were incubated at 28°C with shaking on the rotary shaker at 200 rpm. 24-48 h old inoculum (10%) *E. coli* JM109 was added to Broth-II medium containing 25 mg/L of Cr(VI) and incubated at 28°C with shaking on the rotary shaker at 200 rpm. The Cr(VI) concentration was determined and reduction efficiency of the cultures was expressed as percent difference in the Cr(VI) concentration

at 0 h and after 24 h growth. Uninoculated Broth-II medium containing 25 mg/L of Cr(VI) served as control.

RESULTS AND DISCUSSION

Genetic determinants of chromate resistance and reduction in S. griseus

There are no reports on plasmid encoded Cr(VI) resistance in *Streptomyces*, while very few reports are available in literature on mercury resistance encoded by transferable giant linear plasmids in *Streptomyces* (Ravel et al. 1998). Initial attempts to locate circular plasmids (10-20 kb) in *S. griseus* strain using agarose gel electrophoresis were unsuccessful. A single band was detected in the gel close to the loading well but it did not migrate further even on prolonged gel run indicating the absence of circular plasmids (Fig 5.1). Plasmid curing with ethidium bromide showed that all the colonies on the master plate were able to grow on Cr(VI) containing plate indicating that there was no plasmid loss. *S. griseus* was able to grow at elevated temperature (37°C). The single colonies obtained were able to grow on Cr(VI) containing plates indicating that there was no plasmid loss. Incase of frequent subculture on nutrient rich medium it was observed that the single colonies obtained were able to grow on Cr(VI) containing plates indicating that there was no plasmid loss. Thus, curing studies showed that ethidium bromide, incubation in a nutrient rich medium, or incubation at an elevated temperature (37°C) were unable to eliminate the chromate resistance phenotype (Cr^R) in *S. griseus*.

Therefore, presence of linear plasmids was investigated since *Streptomyces* are known to harbor linear plasmids in addition to a wide range of circular plasmids. The presence of linear plasmids in *Streptomyces* sp. was first described by Hayakawa et al. (1979). Large linear plasmids ranging in sizes from 12 kb to 1 Mb have been reported in many *Streptomyces* sp. and have been shown to carry genes encoding antibiotic biosynthesis, resistance to heavy metal and ability to break down xenobiotics. The discovery of pulse-field gel electrophoresis (PFGE) has facilitated the molecular examination of giant linear plasmids.

A survey of the total DNA from Cr(VI) reducing *S. griseus* by pulsed field gel electrophoresis was conducted to investigate the presence of giant linear plasmids. PFGE gel was run with low range pulse field gel (PFG) marker (0.1-200 kb)-Ladders of λ DNA concatamers and λ DNA-Hind III fragments and mid range PFG marker (15-300 kb)-Ladders of λ DNA concatamers with XhoI digested lambda DNA and *S. griseus* total

DNA using pulse time of 30s for 24 h under conditions of 6 V/cm at 14°C. The lanes containing *S. griseus* total DNA showed the presence of a single plasmid band (Fig. 5.2). The molecular weight of the plasmid was approximately 200 kb.

Transformation studies in E. coli

Transformation of *E. coli* JM109 with purified plasmid DNA resulted in simultaneous acquisition of resistance to chromate and the appearance of plasmid in the transformants. Most importantly, the plasmid transfer was found to confer chromate reduction ability to the *E. coli* transformants (Table 5.1). During the transformation studies with *E. coli* JM109, colonies appeared on Broth-II medium containing 25 mg/L of chromate. Agarose gel electrophoresis of the plasmid DNA preparation of *E. coli* JM109 transformants revealed the presence of a single band of plasmid DNA corresponding to that in *S. griseus*. No band was detected in the plasmid DNA preparation of *E. coli* JM109 and it was unable to grow in presence of chromate. These results indicated that plasmid replicated in *E. coli* JM109 and possessed genetic information necessary for the expression of chromate resistance and reduction.

Table 5.1: Chromate reduction by *S. griseus* and *E. coli* JM109 strains

Strain	**Growth in presence of 25 mg/L of Cr(VI)**	**Percentage of reduction of 25 mg/L Cr(VI) (24 h)**
S. griseus	+	>99.9
***E. coli* JM109**	-	0
***E.coli* JM109 (transformant)**	+	50

Note: + indicates growth, - indicates no growth (monitored as increase in turbidity)

In conclusion, the giant linear plasmid of 200 kb from *S. griseus* carries the genes responsible for resistance to chromate and these genes are expressed in *E. coli.* Plasmid-associated bacterial resistance to Cr(VI) has been reported in *Streptococcus lactis* (Efstathiou and Mckay 1977), *P. aeruginosa* (Cervantes and Ohtake 1988; Summers and Jacoby 1978) *Pseudomonas. fluorescens* (Bopp et al. 1983) and *Alcaligenes eutrophus* (Cervantes and Silver 1992; Nies and Silver 1989; Peitzsch et al. 1998). Dhakephalkar et al. (1996) reported that plasmid pARI180, from a *Pseudomonas mendocina* strain, determined both chromate resistance and reduction.

During the present study curing could not be achieved due to large size of the plasmid whereas studies with *Pseudomonas fluorescens* LB300 showed that loss of plasmid resulted in loss of Cr(VI) resistance whereas transformation of plasmid less Cr sensitive strain with the purified plasmid DNA resulted in regaining of the Cr(VI) resistant ability of the strain (Bopp et al. 1983). However in *S. griseus*, chromate resistance (governed by plasmid) and reduction seem to be linked. Similar observations have been made in *Pseudomonas mendocina* MCM B-180 by Dhakephalkar et al. (1996) while other reports claim resistance and reduction to be separately controlled (Cervantes and Ohtake 1988; Cervantes and Silver 1992; Ohtake et al. 1987). Ohtake et al. (1987) reported that plasmid-determined chromate resistance results from reduced uptake of chromate by the resistant cells, but they were unable to determine whether there was chromate efflux or a direct block on uptake.

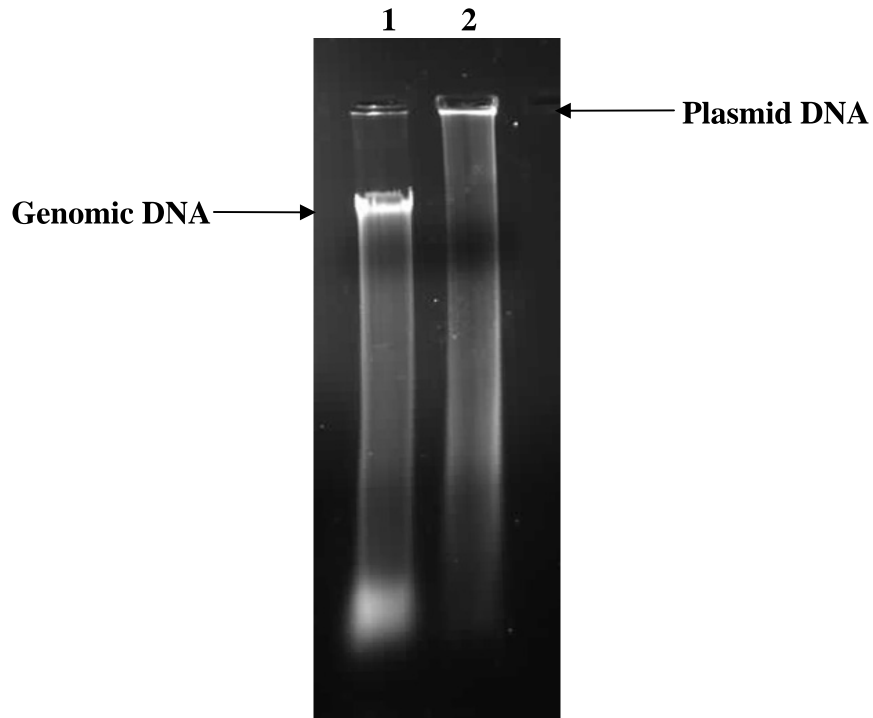

Fig 5.1: Agarose Gel Electrophoresis -

Lane 1-Genomic DNA Lane 2 – Plasmid DNA

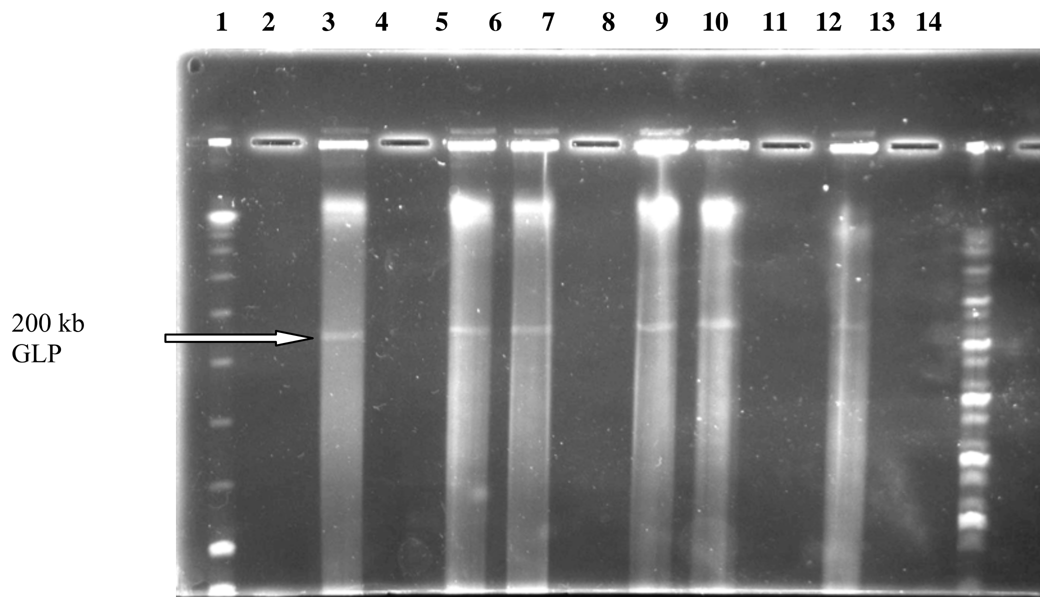

Fig 5.2: Pulse Field Gel Electrophoresis of Total Genomic DNA from *S. griseus.* Lane 1. Low range marker; Lane 13. Midrange marker; Lanes 3, 5, 6, 8, 9, 11 *S. griseus* total DNA

CONCLUSIONS

Initial attempts to locate circular plasmids (10-20 kb) in *S. griseus* strain using agarose gel electrophoresis were unsuccessful. Curing studies showed that ethidium bromide, incubation in a nutrient rich medium, or incubation at an elevated temperature (37°C) were unable to eliminate the chromate resistance phenotype (Cr^R) in *S. griseus*. A survey of the total cellular DNA from Cr(VI) reducing *S. griseus* by pulsed field gel electrophoresis indicated the presence of 200 kb giant linear plasmid. Transformation of *E. coli* JM109 with purified plasmid DNA resulted in simultaneous acquisition of resistance. Most importantly, the plasmid transfer was found to confer chromate reduction ability to the *E. coli* transformants. Chromate resistance and reduction was found to be plasmid mediated in *S. griseus*.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

Studies on chromate tolerance and reduction in actinomycetes

Actinomycetes mainly belonging to *Streptomyces* were screened for Cr(VI) tolerance and reduction. Eighteen out of the Nineteen actinomycetes tested were found to be tolerant to 25 mg/L Cr(VI). Thirteen among the tolerant strains showed Cr(VI) reduction, out of which eleven were *S. griseus* strains. Rate of chromate reduction varied with the organism and ranged between 0.3012 to 1.042 mg Cr(VI) reduced /L/h. Most efficient among them was a *S. griseus* strain (NCIM 2020) which completely reduced 25 mg/L Cr(VI) within 24 h which was taken up for detailed study. *S. griseus* reduced Cr(VI) in the form of chromate and dichromate equally well. The organism was tolerant up to 40 mg/L Cr(VI) and inhibition of 60% and above was observed beyond Cr(VI) concentration of 70 mg/L.

Reduction was nearly complete within 12 h at lower initial Cr(VI) concentration while it was slower and required longer incubation time with higher initial Cr(VI) concentration. Rate of reduction (mg Cr(VI) reduced/L/h) and specific reduction rate (mg Cr(VI) reduced per gram of biomass) increased with increase in initial Cr(VI) concentration but decreased with incubation time. The highest rate of reduction for *S. griseus* is 2.85 mg Cr(VI) reduced/L/h for the initial period of 12 h with 89 mg/L of Cr(VI) concentration. To our knowledge, this is one of the highest rate reported so far among gram positive bacteria. The specific reduction rate increased from 11.45 to 182.31 mg Cr reduced /g biomass with increase in initial Cr(VI) concentration from 15 to 89 mg/L of Cr(VI).

There was absolute requirement of electron donor for Cr(VI) reduction and variety of carbon sources were utilized by the organism for reduction during growth. Maximum Cr(VI) reduction was observed in presence of glucose followed by glycerol and acetate. Reduction was unaffected by anions while cations inhibited reduction to varying degrees. Copper enhanced chromate reduction by resting cells TEM studies indicated the presence of chromium in the biomass.

Cr(VI) reduction by immobilized *S.griseus* - batch studies

Among the matrices tested for whole cell immobilization of *S. griseus,* PVA-alginate was found to be the best for chromate reduction. Free and immobilized cells showed similar rates of Cr(VI) reduction. Increase in biomass concentration increased rate of chromate reduction and 2 g wet biomass corresponding to 100 mg dry weight was sufficient to completely reduce 50 ml of 25 mg/L of Cr(VI). Reduction was faster and complete with lower initial Cr(VI) concentrations while higher concentration required longer incubation time. PVA-alginate immobilized cells could be successfully reused 5 times to completely reduce 25 mg/L of Cr(VI) within 24 h in each cycle. Immobilized cells completely reduced Cr(VI) from simulated effluent containing Cu(II), Mg(II), Mn(II) and Zn(II) within 9 h while free cells required 18 h which could possibly be due to other metal ions present in the effluent being more toxic to free cells than immobilized cells. These findings demonstrated the utility of immobilized *S. griseus* for treating chromate containing aqueous solutions/ industrial wastes.

Cr(VI) reduction by immobilized *S. griseus* - bioreactor studies

At a fixed flow rate of 4 ml/h, reduction rate decreased with increasing Cr(VI) concentration from 2 to 12 mg/L. However, increasing the flow rate from 2 to 8 ml/h did not significantly affect the reduction of 2 mg/L Cr(VI) by the immobilized cells. Cr(VI) reduction increased with increase in biomass concentration but the increase was not linear. Reduction of 12 mg/L of Cr(VI) was only 77% in 12 h using 2 g wet cells while it reached 98% in 12 h with 4 g wet *S. griseus* cells. Cr(VI) reduction in Broth-II minimal medium by PVA-alginate immobilized *S. griseus* cells was faster and reached near completion (97%) in 7 h while in presence of 1% glucose in Tris HCl pH 7.0 buffer it was only 85% in 14 h suggesting that glucose alone is not sufficient. Cr(VI) from simulated effluent (SE) containing Cu(II), Mn(II) and Zn(II) was completely reduced in 2 h by immobilized cells which was faster compared to Broth-II medium which took 12 h for complete reduction. This could be attributed to the stimulatory effect of the Cu(II) ions in the SE. The present study indicates the potential of immobilized cells of *S. griseus* for bioremediation of chromate-contaminated effluents.

Isolation, partial purification and characterization of chromate reductase

The chromate reductase from *S. griseus* was constitutive and intracellular. Reductase activity of CFE which was lost on dialysis could be restored with NADH addition. There was no preference for electron donor and both NADH and NADPH acted equally well. Two-three fold increase by NAD(P)H addition. Reductase was optimally active at 28°C and pH 7 and around 90% of the original activity was retained after 60 min incubation at room temperature. Inhibition of reductase by the metals (1 mM) was in the following order: Hg>Cd>Zn>Ni≥Mg>Co>Ag>Mn>Cu. Among the metals tested, Hg(II) and Cd(II) were found to be most inhibitory with nearly 80% and 60% inhibition respectively. Co(II), Mn(II), Zn(II) and Ni(II) showed around 20% inhibition while Ag(I) and Mn(II) were least inhibitory with only 10% inhibition. Sodium azide showed no inhibition while formaldehyde showed 60% inhibition.

Ultracentrifugation of CFE showed reductase activity to be associated with soluble fraction and no activity found in the membrane fraction. EPR studies revealed that Cr(VI) was converted to Cr(III) via the formation of Cr(V) as an intermediate. Reductase was partially purified by fractional ammonium sulphate precipitation and DEAE column chromatography. The molecular weight of the partially purified enzyme according to non-denaturing SDS PAGE was approximately 46-48 kDa.

Investigation of the genetic determinants of chromate resistance/reduction

Initial attempts to locate circular plasmids (10-20 kb) in *S. griseus* strain using agarose gel electrophoresis were unsuccessful. Curing studies showed that ethidium bromide, incubation in a nutrient rich medium, or incubation at an elevated temperature (37°C) were unable to eliminate the chromate resistance phenotype (Cr^R) in *S. griseus*. A survey of the total cellular DNA from Cr(VI) reducing *S. griseus* by pulsed field gel electrophoresis indicated the presence of 200 kb giant linear plasmid. Transformation of *E. coli* JM109 with purified plasmid DNA resulted in simultaneous acquisition of resistance. Most importantly, the plasmid transfer was found to confer chromate reduction ability to the *E. coli* transformants. Chromate resistance and reduction was found to be plasmid mediated in *S. griseus*.

The observation that reductase activity was found to be predominant in actinomycetes mainly belonging to *Streptomyces* is an important finding in the context that these organisms are widely distributed in nature. *S. griseus* can form dormant spores, which are resistant to heat and desiccation, can be easily stored, transported and readily germinated into vegetative cells by the addition of simple nutrients. These attributes make this organism a promising candidate for use in knowledge-based toxic metal remediation systems. An additional benefit of this organism is that it can effectively reduce Cr(VI) when grown on a simple mineral medium.

Future scope

Further work is needed to purify the reductase from *S. griseus* to homogeneity, investigate if the ability to reduce chromate in this organism is a secondary function of an enzyme with a different primary role other than Cr(VI) reduction. The response of the organism to chromate stress by secretion of enzymes like superoxide dismutase, catalase etc. also needs to be investigated. Cloning and molecular regulation of the gene encoding the reductase, the effect of a knockout mutation of this gene on *S. griesus* physiology, and comparative molecular features of the various enzymes that exhibit chromate reductase activity are some of the long term goals.