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University of Calcutta, Kolkata 700 019, India

REVIEW

Exploring the potentials of chromate reducing microorganisms for bioremediation of chromium pollutants

ARUNDHATI PAL[a] AND A. K. PAUL[b]*

[a]*Department of Botany, Serampore College, 9, William Carey Road, Serampore, Hooghly, West Bengal 712 201 and*

[b] *Microbiology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata, West Bengal 700 019*

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Exploitation of chromium compounds for widespread industrial applications has led to discharge of Cr-laden effluents into the environment. The soluble hexavalent chromium [Cr(VI)] is a priority toxic, mutagenic and carcinogenic form, whereas its reduced trivalent form [Cr(III)] is insoluble and less toxic to biota. Conventional detoxification processes involving reduction of Cr(VI) to Cr(III) demand high energy and technology. Cr-resistant microorganisms are ubiquitous in terrestrial and aquatic environments which overcome the metal toxicity by transforming Cr(VI) to the less toxic Cr(III) via possible reduction systems. Bioreduction of chromate occurs directly as a result of bacterial metabolism or indirectly by the action of metabolic by-products of sulphate-reducing or iron-reducing bacteria. Chromate reduction may occur under aerobic or anaerobic or both conditions. While aerobic reduction is associated with soluble proteins and utilizes NADH/endogenous electron reserves as the electron donor, in anaerobic reduction Cr(VI) serves as the terminal electron acceptor through respiratory chains involving the transfer of reducing equivalents to Cr(VI) through cytochromes. Recent studies on chromium bioremediation involve the use of purified chromate reductase from bacteria which occurs either in the membrane or cytosolic fractions of the cells. The use of purified enzymes has the advantage of avoiding culture sensitivity to ambient toxicants. Moreover, cells and enzymes immobilized in different inert polymer matrices have been used for Cr(VI) reduction. Cr(VI) reducing micro-organisms have also been studied in batch, continuous and stirred batch reactor for large scale industrial purpose. In addition, novel engineered microbes and/or proteins with improved Cr(VI) reduction capability have been developed and introduced besides indigenous Cr(VI)-resistant microflora for better utilization and operation in closed bioreactor systems. With the advancement in biotechnology, it is speculated that application of effective Cr(VI)-reducing microbial cells and/purified enzymes under immobilization might be promising to alleviate bioremediation of chromium pollutants.

Key words: Chromium toxicity, bioreduction, Cr(VI) resistant microorganisms, chromate reductase, immobilization systems

INTRODUCTION

Heavy metal contamination has been identified as a serious global pollution. Industrial activities and sewage sludge applications largely contribute to a wide spread of these non-biodegradable elements in the terrestrial as well as aquatic ecosystems. Therefore, over the years, environmental pollution due to discarding of solid and/or liquid wastes containing heavy metals from industrial and manufacturing activities has received a lot of attention and legislation for the protection of the environment has become more rigid. Chromium is widely used in many industries and is considered one of the chief pollutants in the United States by the Environmental Protection Agency (EPA) (Cervantes *et al.*, 2001). In soils the most stable and common forms of chromium are trivalent Cr(III) and hexavalent Cr (VI) which display quite different chemical properties and affect living organisms in different ways. While hexavalent chromium is water-soluble and

E-mail: amalk_paul@yahoo.co.in; akpbot@caluniv.ac.in

highly toxic, the trivalent form is an essential micronutrient and relatively water insoluble and less toxic than Cr(VI) (Francisco *et al.*, 2002). Exploitation of Cr-compounds in electroplating, steel and automobile manufacturing, production of ferrochrome alloys, paint pigments and dyes, wood preservation and tanning industries all over the world in excess amount has led to the discharge of this hazardous contaminant in the environment and causes serious health hazards.

The remediation of Cr(VI)-contaminated soils and aquatic bodies, today, is essentially based on physical and chemical approaches, which include excavation or pumping of contaminated material, followed by the addition of reducing chemicals that lead to the precipitation and/or sedimentation of reduced chromium [Cr(III)], less toxic than Cr(VI) and greatly insoluble. However, the traditional approaches are disadvantageous mainly because they demand high energy and technology, require costly chemicals and additives and may cause health hazard during excavation of soil. The ability of several microbial groups (bacteria, fungi, microalgae) to reduce Cr(VI) to Cr(III) has been considered interesting for clean up of soil/water polluted with chromate. In fact, there is no doubt that the development of an effective biological system to alleviate the environmental problems associated with hexavalent chromium is highly desirable. Potentially bioremediation is cost-effective and environment friendly in comparison with physico-chemical treatments. The Cr(VI) bioremediation of soils can be performed *in situ* or *ex situ* using a bioreactor for treatment of soils or soil wash effluents (Viti and Giovannetti, 2007). The bioremediation approach offers some advantages compared with traditional techniques (Higgins *et al.*, 1997): i) it can be performed *in situ* without excavation of contaminated soils, ii) it can be applied to sites with high water table, iii) it can allow a continuous Cr(VI) stable reduction process, and iv) it does not destroy the site that is to be detoxified.

The present review is an attempt to provide an overview of i) the natural resources of chromium, their uses leading to environmental contamination and toxicity; ii) chromium resistant microorganisms with special emphasis on hexavalent chromium reduction during growth, by whole cells and their enzymes in free as well as in immobilized forms, and iii) exploring the potentials of Cr(VI)-reducing microorganisms for possible applications.

Chromium in the environment

Chromium (Cr), one of the most important transition metal, was discovered in Siberian red lead ore (Crocoite) in 1798 by French chemist Vanquelin. Chromite occurs exclusively in rocks formed by the intrusion and solidification of molten lava or magma which is very rich in iron containing minerals such as pyroxenes and olivines. Within these rocks, often referred to as ultramafic igneous rocks, chromium occurs as a chromium spinel, a highly complex mineral made up of magnesium as MgO and aluminium as Al_2O_3. Chromium is the most abundant of the Group V1A family of elements and at an average concentration of nearly 400 ppm in the earth's crust it is the 13th most common element. However, as with all minerals or elements, economic deposits occur only where it has been concentrated in nature. The chromium spinel is a heavy mineral and it concentrates through gravity separation from most of the other molten material in the magma during crystallisation from the cooling magma. Commercial chromite deposits are found mainly in two forms: stratiform seams in basin-like intrusions, often multiple seams through repeated igneous injections and the more irregular podiform or lenticular deposits. The best known example of a stratiform deposit is the Bushveld Igneous Complex of South Africa. This complex contains most of the world's chromite reserves. The Great Dyke of Zimbabwe, traversing nearly the length of the country, is very similar and has been linked to the Bushveld in geological history. These two features are well-known also for their important and very large commercial deposits of the platinum-group metals. Other stratiform deposits occur in Madagascar and in the Orissa district of India.

Chromium occurs ubiquitously in nature; its concentration in soil depends upon the parent rock type. The average Cr content in soil ranged from 10-50 mg/kg, however, soils derived from serpentine rocks contain 1000 to 3000 mg Cr/kg soil (Adriano, 1986). The chromium content in seawater varies strongly and is usually between 0.2 and 0.6 ppb. Rivers contain approximately 1 ppb of chromium, although strongly increased concentrations are possible. Chromium does not occur freely in nature and chromium compounds can be found in waters only in trace amounts. The element and its compounds can be discharged in surface water through various industries. The rise of Cr content

Table 1. Chromium compounds and their common uses in industries and manufacturing activities

Compound	Formula	Uses
Ammonium dichromate	$(NH_4)_2 Cr_2O_7$	Precursor for manufacturing chromium dioxide-magnetic media for production of high fidelity audio, data and video tapes
Barium chromate	$BaCrO_4$	Pyrotechniques and high temperature batteries
Cadmium chromate	$CdCrO_4$	Catalyst, and pigment
Cadmium dichromate	$CdCrO_4$, H_2O	Metal finishing
Calcium chromate	$CaCrO_4$	Metal primers, corrosion inhibitors, and high temperature batteries
Chrome sulphate	$Cr(OH)SO_4$	Leather tanning
Chromic acid	CrO_3	Wood preservation, and chrome plating
Chromic fluroborate	$Cr(BF_4)_3$	Chrome plating catalysts
Chromic napthenate	Not Definite	Textile preservation
Chromic phosphate	$CrPO_4$	Pigments, phosphate coating, wash primers
Chromium acetate	$Cr(OCOCH_3)_3$, x H_2O	Printing and dyeing textiles
Chromium chloride	$CrCl_3$	Chromatizing organo-chromium compounds
Chromium nitrate	$Cr(NO_3)_3$, $9H_2O$	Dye and pigment production
Chromium oxide	Cr_2O_3	Production of ferroalloy for stainless steel and non-ferrous superalloy for jet engines
Chromium sulphate	$Cr_2(SO_4)_3$	Leather tanning
Copper chromite	$CuCr_2O_4$	Catalyst, especially for automobiles
Copper dichromate	$CuCr_2O_7$, $2H_2O$	Wood preservative and catalyst
Ferrochromite	$FeCr_2O_4$	Alloy production and steel manufacturing
Lead chromate	PbCrO4	Printing ink, rubber, plastic and stationary industry
Lead chromate oxide	CrH4O5. 2Pb	Pigment production
Magnesium chromate	$MgCrO_4$, $5H_2O$	Corrosion inhibitor in gas turbines
Magnesium chromite	$MgCr_2O_4$	Refractory
Mercuric chromate	$HgCrO_4$	Antifouling formulation
Potassium chromate	K_2CrO_4	Enamels, leather finishing and rust proofing of metals
Potassium dichromate	$K_2Cr_2O_7$	Catalyst, lithographic chemicals and photographic engraving
Pyridine dichromate	$(C_5H_5NH)_2Cr_2O_7$	Photosensitiser in photoengraving and ceramics
Sodium chromate	Na_2CrO_4	Diagnostic radiopharmaceutical for intravenous administration
Sodium chromite	$NaCrO_2$	Additive and functional ingredients
Sodium dichromate	$Na_2Cr_2O_7$, $2H_2O$	Preparation of colored glass and ceramic glazes
Strontium chromate	$SrCrO_4$	Corrosion inhibiting pigment and plating additive
Zinc chromate	$ZnCrO_4$	Aviation primer for coating aluminium, magnesium and non-ferrous surfaces

in the ecosystem, however, is mostly due to anthropogenic discharges from industrial and manufacturing activities. According to Gadd and White (1993), more than 1,70,000 tons of Cr wastes are discharged annually into the environment and Cr-concentration can approach up to 2,70,000 mg/l in effluents released from metallurgical plants. Soluble chromates are converted to insoluble chromium (III) salts and consequently, availability for plants decreases. This mechanism protects the food chain from high amounts of chromium. Chromate mobility in soils depends on both soil pH and soil sorption capacity and on temperature. The guideline for chromium in agricultural soils is approximately 100 ppm.

Uses of chromium and its compounds

The name of the element "chromium" is derived from the Greek word "chrôma", meaning colour, because many of its compounds are intensely coloured. Chromium oxide was used by the Chinese in the Qin dynasty over 2,000 years ago to coat weapons such as bronze crossbow bolts and steel swords found at the Terracotta Army. It later came to the attention of the west when it was discovered by Louis Nicolas Vauquelin in the mineral crocoite [lead (II) chromate] in 1797. Crocoite was used as a pigment and after the discovery that the mineral chromite also contains chromium; this latter one was used to produce pigments as well. Chromium was regarded with great interest because of its high corrosion resistance property and hardness. A major development was the discovery that steel could be made highly resistant to corrosion and discoloration by adding chromium to form stainless steel. Development of the process of chrome plating (electroplating with chromium) is currently the highest volume uses of the metal. Chromium and ferrochromium are produced from the single commercially viable ore, chromite, by silicothermic or alumino-thermic reaction or by roasting and leaching processes.

Chromium is applied worldwide in amounts of approximately 20,000 tons per year. It may be polished and it does not oxidize when it comes in contact with air. It is applied for metal surface refinery and in alloys. Stainless steel consists of 12-15% chromium. Hexavalent chromium in industrial wastewaters mainly originates from tanning and painting. Chromium compounds are applied as pigments and 90% of the leather is tanned by means of chromium compounds. Wastewater usually contains about 5 ppm of chromium. Chromium may be applied as a catalyser, in wood impregnation, in audio and video production and in lasers. Chromite is the starting product for inflammable material and chemical production. In nuclear fission the ^{51}Cr isotope is released and this can be applied for medical purposes.

The bright colors of chromium compounds along with its strength, hardness, resistance to corrosion and oxidizing capabilities have led to their wide application in industrial sectors. A list of some chromium containing compounds and their popular uses is illustrated in Table 1. Chromium compounds are chiefly used in leather tanning, electro plating, metal cleaning and processing, wood preservation and alloy preparation industries. More than thirty different chromium compounds along with high and low grade chromite ores find application in metallurgical, chemical and refractory brick industries for pigment and paint manufacturing, metal finishing, etc. (Losi *et al.*, 1994). Chromium oxide is a magnetic compound and is used to manufacture magnetic tape, high-performance audio tape and standard audio cassettes. Chromates can prevent corrosion of steel under wet conditions and therefore chromates are added to drilling mud.

Speciation and toxicity

Chromium naturally has four stable and eight instable isotopes. The ^{51}Cr, which is applied for diagnosis purposes, has an average degree of radioactivity. In dissolved form chromium is present as either anionic trivalent $Cr(OH)_3$ or as hexavalent CrO_4^{2-} forms. The amount of dissolved Cr^{3+} ions is relatively low, because these form stable complexes. In natural waters trivalent chromium is most abundant. Many chromium compounds are relatively water insoluble. Chromium (III) compounds are water insoluble because these are largely bound to floating particles in water. Chromium (III) oxide and chromium (III) hydroxide are the only water soluble compounds. Chromium (VI) oxide is an example of an excellently water soluble chromium compounds. Trivalent chromium has low affinity for oxygen, it complexes with ligands and forms insoluble oxides and hydroxides above pH 5.0. Hence, the bioavailability and toxicity of Cr(III) in natural environment is less as compared to its hexavalent form.

Cr(VI) on the other hand is a strong oxidizer, exists as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) and is highly soluble at neutral pH (Losi *et al.*, 1994). Cr(VI) compounds are mobile in soil or water systems and are easily absorbed in biological membranes leading to toxicity in all living forms. In presence of appropriate electron donor, Cr(VI) is readily reduced to Cr(III) state, this reduces the toxicity of hexavalent chromium.

Chromium is not an essential plant nutrient but it serves as an essential component of animal nutrition in trace amount, functioning mainly in glucose metabolism and has a possible role in fat utilization. Some microorganisms require chromate possibly as a cofactor for specific enzyme systems or other metabolic systems related to glucose utilization and enzyme stimulation (Hughes and Poole, 1989). It is universally observed that Cr(III) is the nutritionally useful one, while Cr(VI) renders toxic effects on the biota. Trivalent chromium is an essential trace element for humans. Together with insulin it removes glucose from blood and it also plays a vital role in fat metabolism. Chromium deficits may enhance diabetes symptoms. Chromium can also be found in RNA. The human body contains approximately 0.03 ppm of chromium and the daily intake strongly depends upon feed levels. Chromium deficits are very rare and chromium feed supplements are not often applied.

Hexavalent chromium is known for its negative health and environmental impact and its extreme toxicity. It causes allergic and asthmatic reactions, is carcinogenic and is 1000 times as toxic as trivalent chromium. Health effects related to hexavalent chromium exposure include diarrhoea, stomach and intestinal bleedings, cramps and liver and kidney damage. In human beings, absorption of Cr compounds through inhalation, skin and ingestion causes a series of adverse effects like ulceration, contact dermatitis, respiratory troubles, carcinomas, etc. (Cohen *et al.*, 1993). Chromium accumulation was also reported in agricultural crop plants growing in chromium-polluted soils (Khasim *et al.*, 1989).

The toxicity as well as mutagenic effect of chromate compounds on different groups of bacteria and fungi has been well documented. In general, Cr(VI) pollution adversely affect the generation time of bacteria, spore germination, mycelial proliferation, microbial respiration, photosynthetic and nitrogenase activity and viral infectivity. These effects have attributed to altered genetic material, metabolic activity and physiological reactions in the microbial cell. The effect of Cr(III) on bacterial cells was tested with the Pro-Tox (C) assay and its cellular uptake was measured with flame atomic absorption spectroscopy. The potential genotoxicity of Cr(III) was further examined by the study of its influence on a bacterial type II topoisomerase. Cr(III) was shown to cause DNA damage and inhibit topoisomerase DNA relaxation activity, probably by preventing the formation of the covalent link between enzyme and double helix. In addition, Cr(III) decreases the viability and/or proliferation rate of eukaryotic cells such as melanoma cells and ras-transformed human epithelial cells (MCF-10A neoT) (Jianlong *et al.*, 2004). Certain forms of hexavalent chromium are known respiratory carcinogens that induce a broad spectrum of DNA damage which may be promoted through Cr(VI)-induced inflammatory/immunological responses and alteration of survival signaling pathways. Cr(VI) enters the cell through non-specific anion channels and is metabolically reduced by agents including ascorbate, glutathione and cysteine to Cr(V), Cr(IV) and Cr(III). Cr(III) has a weak membrane permeability capacity and is thereby trapped within the cell where it can bind to DNA and producing genomic instability. Structural genetic lesions produced by the intracellular reduction of Cr(VI) include DNA adducts, DNA-strand breaks, DNA-protein crosslinks, oxidized bases, abasic sites and DNA inter- and intra-strand crosslinks. The damage induced by Cr(VI) can lead to dysfunctional DNA replication and transcription, aberrant cell cycle checkpoints, dysregulated DNA repair mechanisms, microsatellite instability, inflammatory responses and the disruption of key regulatory gene networks responsible for the balance of cell survival and cell death. Several lines of evidence have indicated that neoplastic progression is a result of consecutive genetic/epigenetic changes that provide cellular survival advantages and ultimately lead to the conversion of normal human cells to malignant cancer cells (Nickens *et al.*, 2010).

Chromium resistance in microorganisms

Resistance to chromium is found among vast number of terrestrial and aquatic microorganisms which have developed mechanisms to overcome the

metal toxicity by either transforming Cr(VI) to the less toxic Cr(III) via possible reduction systems or through intercellular or extracellular sequestration of metal ions, making them unavailable to the ecosystem. Chromium-resistant microflora have been isolated and described from water (de Vincente *et al.,* 1990), sediments, soil (Losi and Frankenberger, 1994), tannery (Khare and Tripathi, 2001) and industrial (Ganguli and Tripathi, 1999) effluents and heavy metal contaminated sludge samples (Francisco *et al.,* 2002). Similarly, chromium-resistant bacteria have also been reported to occur in the naturally occurring Cr-percolated ultramafic soil, which also show co-resistance to nickel and cobalt (Mengoni *et al.,* 2001; Pal and Paul, 2004).

Bacterial resistance to chromate has already been reported in *Pseudomonas ambigua*, *P. fluorescens, P. aeruginosa*, *Alcaligenes eutrophus*, *Streptococcus lactis*, *Enterobacter cloacae* and *Streptomyces* spp. The influence of Cr(VI) on the microbial community structure was analyzed in a river system subjected to long-term metal contamination, following sequencing of 16S rRNA genes cloned from DNA extracted from the river sediments. Shifts in the microbial community structure were analyzed by amplified ribosomal DNA restriction analysis fingerprinting. The isolates obtained were phylogenetically related to Actinobacteria, Firmicutes, Bacteroidetes and Proteobacteria, whereas *Acidobacteria* and *Deltaproteobacteria* were only revealed by clone analyses. Cr(VI)-resistant and Cr(VI)-reducing strains were isolated in all sites examined. However, each sample site had a microbial community with a different antibiotic resistance profile indicating that Cr influenced the microbial communities, altering their functional characteristics, community structure and the phylogenetic groups, but did not affect the structural diversity. Furthermore, the concentration of Cr(VI) in the sediments could not be correlated with generic diversity, neither with the ability of the microbial community to resist or to reduce higher Cr(VI) concentrations (Branco *et al.,* 2005). Exposure of Pseudomonas aeruginosa to high levels of Cr(VI) led to changes in the expression of proteins. Over expressed proteins included stress proteins, proteins involved in protein biosynthesis, proteins responsible for energy production, proteins involved in free radicals detoxification by the glutathione system, outer membrane proteins, MucD, while down-regulated proteins were identified as isocitrate dehydrogenase and 30S ribosomal protein S1. Under Cr(VI) exposure, upregulation of MucD (role in exopolysaccharide production) and outer membrane proteins concluded that the mechanisms of Cr(VI) resistance include production of exopolysaccharide and complexing of metal ions outside the cell (Kilic *et al.,* 2010).

Reports on chromate resistance in fungi are fewer and Cr(VI) is reported to be toxic on all groups of fungi (Babich *et al.*, 1992). The same holds good for fungal isolates from serpentine soils of Andaman. However, two isolates, *Mortierella* F604 and *Penicillium* F104 were the exceptions (Pal *et al.*, 2003) showing moderate resistance to chromium. Metal-resistant fungi belonging to *Aspergillus*, *Penicillium*, *Alternaria*, *Geotrichum*, *Fusarium*, *Rhizopus*, *Monilia* and *Trichoderma* were isolated from wastewater-treated soil and they demonstrated moderate tolerance to Cr (Zafar *et al.*, 2007). Marine seaweed (*Eucheuma* sp.) associated strains of *Aspergillus flavus* and *Aspergillus niger* were tested for their Cr(VI) tolerance and both the isolates showed luxuriant growth at different concentrations of Cr(VI), i.e., 25, 50 and 100 ppm (Vala *et al.*, 2004). Wild type yeasts were screened for their tolerance to chromium and it was observed that the yeast cultures proved to be generally more sensitive to Cr(VI) (concentration range: 0.1–0.5 mM) than to Cr(III) (0.25-5 mM) (Ksheminska *et al.,* 2005). Most of these fungal isolates were further used in removal of Cr(VI) from aqueous effluents for bioremediation.

Chromium-resistance mechanisms

Traditionally, several physico-chemical processes are available which reduce hexavalent chromate concentrations to levels that comply with statutory standards. Most commonly used processes include reduction-precipitation, ion exchange and reverse osmosis. However, the costs to set up the required equipment and to operate these processes are prohibitively high for large-scale industrial treatment plants and involve huge energy (Beleza *et al.,* 2001). Biological cell membrane is nearly impermeable to Cr(III) and thus its toxicity is nearly one thousandth times less than that of Cr(VI). Because the insolubility of Cr(III) facilitates its precipitation and removal, the biotransformation of Cr(VI) to Cr(III) has been considered as an alternative process for treating chromate contaminated wastes

(Cervantes *et al.*, 2001; Cheng and Gu, 2007).

Since the discovery of the first microbe capable of reducing Cr(VI) in the 1970s, the search for Cr(VI)-reducing microorganisms (both aerobic and anaerobic) has been enthusiastically pursued, with numerous strains being isolated from anthropogenically polluted as well as natural sediments (Cervantes *et al.*, 2001, Francisco *et al.*, 2002; Pal and Paul, 2004; 2005). Based on recent isolation and purification of chromate reductase enzymes from several bacteria, the biological processes for treating chromium contaminated sites are becoming very promising. Viable intact cells immobilized in inert support matrices have been utilized for metal detoxification particularly when using pathogenic microorganisms. The first step in reduction was binding of chromium on to the surface of the cells, which was confirmed by the Energy Dispersive X-ray spectrum and the extracellular substances on the surface possibly could have helped in the sequestration of chromium. The oxidation state of the chromium absorbed to the biomass dictates to what extent the reduction has taken place. The X-ray Photoelectron Spectrometer (XPS) confirm the presence of both hexavalent and trivalent oxidation states of chromium, which suggest the mechanism of adsorption in conjunction with the reduction at work on the surface of the cells. The trivalent form of chromium was known to readily precipitate as chromium hydroxide at pH 7.0.

Chromate reduction by growing and whole cells

Chromium-resistant microorganisms have developed strategies to detoxify their environment containing elevated levels of toxic chromate (Pattanapipitpaisal *et al.*, 2002) or dichromate (Camargo *et al.*, 2003). These generally involve 'bioreduction' of Cr(VI) to Cr(III). Bioreduction of chromate occurs directly as a result of bacterial metabolism or indirectly by the action of certain metabolites such as hydrogen peroxide (Bopp and Ehrlich, 1988). Chromium-resistant and reducing microbial strains are ubiquitous in nature. Comprehensive reviews on microbial reduction of chromate have been published (Ohtake and Silver, 1994; Wang and Shen, 1995). Table 2 summarizes a list of different microorganisms since 2002 which are directly or enzymatically involved in the reduction of Cr(VI).

Earlier studies evaluated Cr(VI) reduction in microbial cultures by observing the change in colour of the medium from yellow to white (Horitsu *et al.*, 1987; Shen and Wang, 1993). Later, Cr(V) was determined as an intermediate in *Pseudomonas ambigua*, which indicated that reduction phenomenon is a two-step reaction (Suzuki *et al.*, 1992). Transmission Electron Microscopic studies on cross-sections of Cr(VI)-reducing bacterial cells revealed electron dense particulates precipitating on the outer cell surface without any intracellular deposition. Energy Dispersive X-ray Absorption (EDXA) spectrum analysis over the dense particulates indicated that it was most likely amorphous Cr(III) hydroxide (Mclean and Beveridge, 2001). Energy Electron Loss Spectroscopic (EELS) studies on the electron dense particles deposited on the cell surface of chromate-reducing strain *Shewanella oneidensis* gave confirmatory evidence on the formation of trivalent chromium after reduction of Cr(VI) (Daulton *et al.*, 2002). In a recent study, Ravindranath *et al.*, (2011) used novel nanoparticle sensors and spectroscopic tools constituting surface-enhanced Raman spectroscopy (SERS) and Fluorescence Lifetime imaging (FLIM) to study intracellular chemical activities within single Cr(VI) reducing bacterium and this challenge with *Shewanella oneidensis* MR-1 has attracted wide interest from the research community because of its potential in reducing multiple chemical and metallic electron acceptors. While several biomolecular approaches to decode microbial reduction mechanisms exist, there is a considerable gap in the availability of sensor platforms to advance research from population-based studies to the single cell level. The uptake of chromate-decorated nanoparticles by cells was imaged by using TEM and Fluorescence Lifetime imaging confirmed the internalization of gold nanoprobes. Raman chemical imaging platform was utilized to monitor chromate reduction and localization within single cells. Distinctive differences in Raman signatures of Cr(VI) and Cr(III) enabled their spatial identification within single cells. A comprehensive evaluation of toxicity and cellular interference experiments conducted revealed the inert nature of these probes and that they are non-toxic. The experimental observations suggested the existence of internal reductive machinery and that reduction occurs at specific sites within cells instead of at disperse reductive sites throughout the cell as previously reported. While chromate-decorated gold

Table 2. Chromate reducing microorganisms and their redox conditions

Microorganism	Redox condition	Substrate / Electron donor	Reference
Bacteria			
Acinetobacter spp.	Anaerobic	-	Francisco *et al.*, 2002
Bacillus pumilis *Pseudomonas synrantha*	-do-	-	Pattanapipitpaisal *et al.*, 2002
Desulfovibrio vulgaris ATCC 29579	-do-	Citrate, EDTA	Mabett *et al.*, 2002
Dienococcus radiodurans R1	-do-	Lactate	Fredrickson *et al.*, 2000
Microbacterium liquefaciens MP 30	-do-	Acetate	Pattanapipitpaisal *et al.*, 2001
Serratia marcescens	-do-	-	Mondaca *et al.*, 2002
Shewanella oneidensis MR 1	-do-	Lactate	Vaimajala *et al.*, 2002
Thermoanaerobacter ethanolicus	-do-	Acetate, glucose	Roh *et al.*, 2002
Bacillus sphaericus AND 303	-do-	Glucose	Pal and Paul, 2004
Corynebacterium hoagii	-do-	-	Viti *et al.*, 2003
Pseudomonas aeruginosa A2Chr	-do-	NADH, NADPH	Ganguli and Tripathi, 2001
P. putida MK 1	-do-	NADH, NADPH	Park *et al.*, 2000
Streptomyces thermocarboxydus	-do-	Glucose	Desjardin *et al.*, 2002
Streptomyces griseus (NCIM 2020)	-do-	Carbon sources	Poopal and Laxman, 2009
Streptomyces sp. *Amycolatopsis* sp.	-do-	-	Polti *et al.*, 2007
Nesterenkonia sp. MF2	-do-	NaCl	Amoozegar *et al.*, 2007
Lysinibacillus fusiformis ZC1	-do-	Sodium acetate, NADH	He *et al.*, 2011
Aerococcus spp. S 31 *Micrococcus* spp. S 39	Aerobic and anaerobic	-	Srinath *et al.*, 2001
P. putida CRB 5	-do-	-	Mclean and Beveridge, 2001
Rhodobacter sphaeroides	-do-	NADH	Nepple *et al.*, 2000
Fungi			
Aspergillus flavus BX 1	Aerobic	Glucose	Wang *et al.*, 1998
Aspergillus sp. N2 *Penicillium sp.* N3	-do-	Acidic condition	Fukuda *et al.*, 2008
Microalgae			
Chlorella sp. R4	Aerobic	Light, acetate, glycerophosphate	Yewalkar *et al.*, 2007

nanosensors provided an improved means for the tracking of specific chromate interactions within the cell and on the cell surface, the single cell imaging tools could also be used to monitor the interaction of other toxic metal species.

Although Cr(VI) reduction was observed during growth of the microorganisms, cell multiplication was not necessarily required for the process. Resting cells of *P. fluorescens* and *E. coli* reduced chromate at the same rate as in the growth medium. In general, high cell densities are required for significant Cr(VI) reduction but the specific rate of Cr(VI) reduction by *E. coli* was higher at relatively lower cell densities. The rate of Cr(VI) reduction increased with increasing initial Cr(VI) concentration in *E. coli*

(Shen and Wang, 1994), although, the reverse was true for *Enterobacter cloacae* (Komori *et al.*, 1989). The optimal pH and temperature for Cr(VI) reduction generally coincide with the optimal growth conditions of the organism. Moreover, the chromate reducing bacteria utilize a variety of organic compounds as electron donors for bioreduction of Cr(VI). These mainly include natural aliphatic compounds like low molecular weight carbohydrates, amino acids and fatty acids (Wang and Shen, 1995). Hydrogen also served as the electron donor in *Desulfovibrio vulgaris* (Lovley and Philips, 1994). The rate of chromate reduction was not influenced by sulphate or nitrate, although, mercury and silver cations non-competitively inhibited chromate reductase activity. Metabolic poisons including carbonylcyanide-m-chlorophenylhydrazone, 2,4-dinitrophenol, sodium cyanide and formaldehyde were inhibitory to Cr(VI) reduction, but not with antimycin, sodium azide and 2-eptylhydroxyquinolone-N-oxide (Ohtake and Silver, 1994). Phenolic compounds also inhibit Cr(VI) reduction in several organisms (Shen and Wang, 1994; Wang and Xiao, 1995).

Bacteria may reduce chromate under aerobic or anaerobic or both conditions (Fig. 1). Aerobic reduction of chromate is normally associated with soluble proteins and utilizes NADH as the electron donor. In the absence of electron donors, organism may utilize endogenous electron reserves for chromate reduction. Under anaerobic phase, Cr(VI) serves as the terminal electron acceptor through respiratory chains involving the transfer of reducing equivalents to Cr(VI) through cytochrome c in *Enterobacter cloacae* (Ohtake *et al.*, 1990) and cytochrome b and d in *E. coli* (Shen and Wang, 1994). In *Desulfovibrio vulgaris,* cytochrome C_3 in the soluble protein fraction was responsible for Cr(VI) reduction (Lovley and Philips, 1994). Some microorganisms were capable of reducing chromate in aerobic as well as anaerobic conditions. However, in such strains reduction of Cr(VI) was better under anaerobic condition than in aerobic phase. Facultative anaerobes from tannery effluents showed >90% Cr(VI) reduction under anaerobic state, while only 10-50% reduction was achieved under aerobic condition (Srinath *et al.*, 2001). Chromate reduction was repressed by dissolved oxygen in *E. coli* ATCC 33456, where an apparent uncompetitive inhibition of oxygen was noted (Shen and Wang, 1994).

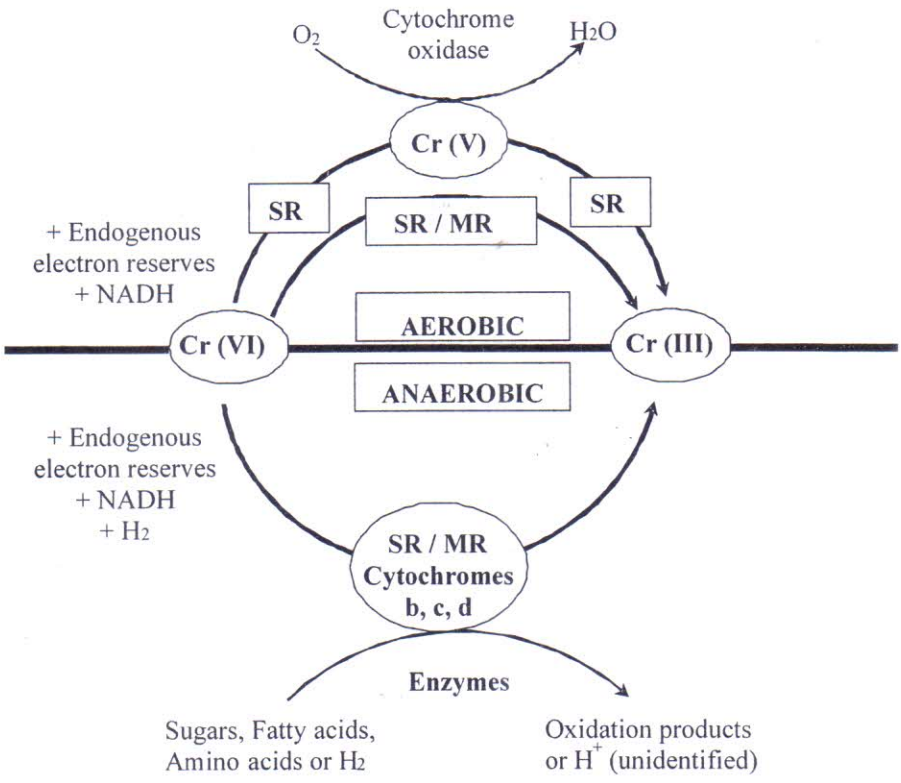

Fig. 1. Schematic diagram showing bioreduction of Cr(VI) to Cr(III) under aerobic and anaerobic conditions with the involvement of soluble reductase (SR) and / or membrane reductase (MR) from different microorganisms.

Enzymatic reduction of Cr(VI)

A comparative account of hexavalent chromium reduction by crude and/or partially purified bacterial enzymes is presented in Table 3. Recent studies on chromate reduction involve the use of purified chromate reductase from bacteria. Chromate reductase was found to occur in the membrane fraction of *Pseudomonas fluorescens*, *Enterobacter cloacae* (Ohtake *et al.*, 1990) and in the cytosolic fraction of *P. ambigua* (Suzuki *et al.*, 1992), *P. putida* (Park *et al.*, 2000) and *Bacillus* sp. (Camargo *et al.*, 2003; Pal and Paul, 2005). The enzymes require NADH, NAD(P)H or H_2 as electron donors and possibly involve cytochromes during chromate reduction. Myers *et al.*, (2000) suggested that cytochrome C548 was specifically involved in reduction of Cr(VI) by membrane vesicles of *Shewanella putrefaciens* MR-1. In presence of H_2 and excess hydrogenase, cytochrome C_3 in the soluble cell-free extract of *Desulfovibrio vulgaris* reduced Cr(VI) 50 times faster than that of *Pseudomonas ambigua* using NADH or NAD(P)H as electron donors (Lovley and Philips, 1994). The Cr(VI) reductase was characterized from soluble cell-free extract of *Pseudomonas putida* MK-1 and purified to homogeneity using ammonium sulphate, anion exchange chromatography, chromatofocussing and gel filtration. Enzyme activity was NADH or NAD(P)H dependent and X-ray Absorption Near Edge Spectroscopy confirmed the conversion of Cr(VI) to Cr(III) during enzyme reaction. 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).

The chromate reductase ChrR-coding gene, chrR, 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). This enzyme ChrR was described as a dimeric flavoprotein catalyzing the reduction of Cr(VI) optimally at 70°C (Ackerley *et al.*, 2004). An open reading frame, yieF, on the E. coli chromosome with no assigned function was found to have a high homology to chrR. This gene was cloned and the encoded protein, YieF, showed maximum reduction of Cr(VI) at 35°C (Park *et al.*, 2002). Recently, a membrane associated chromate reductase was identified from the proteome of B. megaterium TKW3 detected on a two dimensional electrophoresis gel (Cheung *et al.*, 2006). 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

Table 3 : Hexavalent chromium reduction by crude and/or partially purified bacterial enzymes

Bacteria	Remark	References
Ochrobactrum sp.	Cr(VI) reductase activity localized in the cell free extract	Thaker and Madamwar, 2004
Bacillus sphaericus AND303	Cr(VI) reductase activity is constitutive and localized in cell free extract, NADH used as electron donor	Pal and Paul, 2005
Pseudomonas ambigua G1	Reduced Cr(VI) using NADH and NAD(P)H as electron donor	Suzuki *et. al.*, 1992
Pseudomonas putida	Reduced Cr(VI) using NADH and NAD(P)H as electron donor	Park *et. al.*, 2000
Bacillus sp. ES29.	Reduced Cr(VI) using NADH as electron donor, reduction facilitated in presence of Cu(II)	Camargo *et. al.*, 2003
Staphylococcus sp.	Reduced 100 µM Cr(VI) in 150min, reduction depend on Cr(VI) concentration	Mistry *et. al.*, 2010
Escherichia coli ATCC 33456	Cytosolic reductase purified as 84 and 42 kDa protein, NADPH used as electron donor	Bae *et al.*, 2005
Streptomyces griseus (NCIM 2020)	Constitutive Cr(VI) reductase, NAD(P)H enhanced reductase activity	Poopal and Laxman, 2009
Pseudomonas sp. G1DM21	Cell free extract reduced 90% of 100 µM Cr(VI) in 120 min, NADH used as electron donor. Relative molecular mass native Cr(VI) reductase is 61.7 kDa	Desai *et al.*, 2008

of the Cr(V) intermediate is spontaneously re-oxidized 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 because 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 (Park *et al.*, 2002).

Lysinibacillus fusiformis ZC1 isolated from wastewater of a metal electroplating factory displayed high chromate resistance (MIC 60 mM) and resistances to multiple metals (Cu, Ni, Co, Hg, Cd and Ag) and a metalloid (As). This bacterium exhibited an extremely rapid Cr(VI) reduction capability and completely reduced 1mM K_2CrO_4 in 12 h. By whole genome sequence analysis, strain ZC1 was found to contain large numbers of metal(loid) resistance genes. Specifically, a chrA gene encoding a putative chromate transporter was identified conferring constitutive chromate resistance in both phenotypic and gene expressions. Expression of adjacent putative chromate reduction related genes, nitR and yieF, was found to be constitutive and these multiple NADH-dependent chromate reductase genes present in the bacterium might be responsible for the rapid detoxification of Cr(VI) and survival strategy in the harsh wastewater environment (He *et al.*, 2011).

A soluble Cr(VI) reductase was purified from the cytoplasm of Es*cherichia coli* ATCC 33456 (Bae *et al.*, 2005). The molecular mass was estimated to be 84 and 42 kDa by gel filtration and SDS-polyacrylamide gel electrophoresis, respectively, indicating a dimeric structure. The Cr(VI) reductase from *E. coli* ATCC 33456 does not have an immunologically protein related to the other Cr(VI) reducing strains. Thermus scotoductus SA-01, a South African gold mine isolate, has been shown to be able to reduce a variety of metals, including Cr(VI). Opperman *et al.*, (2008) characterized the purification of a novel chromate reductase from the isolate and found the enzyme to be a homodimeric protein (oxidoreductase), with a monomer molecular mass of approximately 36 kDa, containing a non-covalently bound flavin mononucleotide cofactor. The chromate reductase is optimally active at a pH of 6.3 and at 65°C and requires Ca or Mg for activity. Sequence analysis shows the chromate reductase to be related to the old yellow enzyme family, in particular the xenobiotic reductases involved in the oxidative stress response.

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.*, 1997)

Indirect chromate reduction

The reduction of Cr(VI) can also occur indirectly by bacterial activity. Indirect (or non-enzymatic) reduction of chromate by microbes generally involved a biotic-abiotic coupling system and was mediated by metabolic by-product produced in anaerobic environment by sulphate-reducing or iron-reducing bacteria. Fe(II) or S^{2-} produced by a variety of microorganisms through dissimilatory reduction pathways, catalyze the reduction of chromate. This technology was promising for both anaerobic bioreactor system as well as *in situ* applications (Nevin and Lovley, 2002). The sulphate reducing bacteria (SRB) use an organic compound (e.g., formate) or molecular H_2 as the electron donor and SO_4^{-2} as the electron acceptor in dissimilatory sulfate reduction. Certain high valence metal ions, such as Cr(VI), can be used as electron sinks in lieu of sulfate, resulting in metal reduction (Mabbett *et al.*, 2002), although the ability of SRB to grow at the expense of chromate has only been demonstrated in one case (Tebo and Obraztsova, 1998). The process of indirect reduction of chromate using iron reducing bacteria consists of two reactions. The Fe(II) produced by reducing bacteria is cycled back to Fe(III) by abiotic chromate reduction. At the ecological level, this process represents a significant role, because it permits the uninterrupted regeneration of the Fe(III), terminal electron acceptor in anaerobic conditions. In sulphate rich soil environments, when anaerobic conditions are present, such as in flooded compacted soils, the reduction of Cr(VI) by sulphide produced through sulphate reducing bacteria, which couple the oxidation of organic sources to the reduction of sulphate, is an

important mechanism to detoxify the environment from hexavalent chromium.

Dissimilatory Fe(III) reduction by *Shewanella alga* ATCC 51181, a facultative anaerobe, provided a primary pathway for Cr(VI) reduction by microbially induced ferrous ions (Wielinga *et al.*, 2001). H_2S, produced by sulphate reducing bacteria, in sulphate rich soil or marine environment have been implicated in Cr(VI) reduction. Chemoautotrophs belonging to the Thiobacilli group of bacteria, that drive energy from oxidation of inorganic sulphur compounds, produced sulphite and thiosulphate which catalyzes the reduction of Cr(VI). *Thiobacillus ferroxidans*, growing on elemental sulphur, has been used to reduce Cr(VI) under aerobic condition (Qui Intana *et al.*, 2001). In *Desulfomicrobium norvegicum*, a hydrogenase and a c-type cytochrome catalyzed Cr(VI) reduction. *Desulfotomaculum reducens* MI-1 was capable of utilizing Cr(VI) as sole electron acceptor, this capability was only reported in another SRB consortium (Cheung and Gu, 2007).

Cr (VI) reduction by immobilized cells and enzymes

For industrial purposes Cr(VI) reduction by freely suspended cells is disadvantageous because of difficult biomass/effluent separation. These problems can be overcome by the use of immobilized cell packed-bed reactors. Bacterial cells and enzymes immobilized in different polymer matrices like agar, agarose, polyacrylamide, calcium alginate, diatomite, polyvinyl alchohol, etc. have been used for Cr reduction and proved to be effective (Table 4). These immobilized cells being more stable, can be reused, easy to regenerate with easier solid-liquid separation (Humphries *et al.*, 2005; Elangovan *et al.*, 2010). Under immobilization, cells are protected from the excessive toxic action at high chromate concentration that improves cell activity compared with free cells. Cr(VI) reduction by immobilized cells have been used in different systems like packed bed biofilm reactor, membrane bioreactor or column bioreactors operating under batch, continuous or stirred mode (Pattanapipitpaisal *et al.*, 2001; Cordoba *et al.*, 2008).

The immobilization matrices that gave the highest Cr(VI) reducing efficiency in batch suspensions containing *Desulfovibrio vulgaris* and *Microbacterium* sp. were agar and agarose, with reduction occurring at an initial rate of 130 and 15 nmol/h/l per mg dry cell wt, respectively (both matrices). Immobilization of cells did not appear to affect Cr(VI) reducing ability (as compared to free cells), suggesting low mass transfer limitations. In a continuous-flow system, Cr(VI) reduction initially occurred significantly more efficiently in *D. vulgaris* agar-immobilized cell columns, but by 24 h similar results were obtained with each organism and by 159 h Cr(VI) reduction had ceased in all columns. Continuous-flow studies showed that agarose is a poor immobilization matrix for application to Cr(VI) reduction. Although Cr(VI) reduction by agar immobilized cells of *D. vulgaris* and *Microbacterium* sp. in continuous mode occurred at nearly 60% conversion, the longevity of the columns is relatively

Table 4: Hexavalent chromium reduction by immobilized bacterial cells and enzymes

Bacteria	Remark	References
Microbacterium liquefaciens MP30	PVA immobilized bacteria were able to reduce 100 µM Cr(VI) in 4 days, whereas whole cells reduced the same amount in 2 days	Pattanapipitpaisal *et. al.*, 2001.
Streptomyces griseus	PVA alginate immobilized cells were able to reduce 25 mg of Cr(VI) in 24 h The immobilized cells could be reused 5 times	Poopal and Laxman, 2008.
Arthrobacter rhombi	Enzyme immobilized in calcium alginate, Ca^{2+} enhanced the enzyme activity Sodium pyruvate , NADH and propionic acid served as electron donors	Elangovan *et. al.*, 2010.
Intrasporangium sp. strain Q5-1	Cells were immobilized in compound matrices containing 4% PVA, 3% sodium alginate, 1.5% active carbon and 3% diatomite Acetate was the most efficient carbon source for stimulating Cr(VI) reduction	Yang *et. al.*, 2009

short; which may be attributable to biochemical constraints since the agar beads retain excellent integrity and would be the immobilization matrix of choice for future studies on biosystem stability (Humphries *et al.*, 2005).

Potentials and applications of Cr(VI)-reducing microorganisms

The use of micro-organisms for large scale industrial purpose has also been studied and proved to be effective. A pilot scale trickling filter were constructed for bioremediation of Cr(VI) using indigenous bacteria from industrial sludge. Amongst the three operating modes present, batch, continuous and stirred batch reactor, with recirculation, the latter achieved the highest removal efficiency which accounts for 530 g Cr(VI)/m^2d, indicating it to be a feasible solution for environmental pollution (Dermou et al., 2005). Cr(VI) reduction was monitored in batch operated packed bed biofilm reactors (12 ml void volume) and in recirculating packed bed biofilm reactors (100 ml void volume) inoculated with *Arthrobacter* Cr47 (Cordoba *et al.*, 2008). Under batch mode, the reduction reaction by the biofilm fit well to an exponential-decay model with a first order kinetic parameter. In the re-circulating reactor, monitored after 4 weeks from inoculation and fed with laboratory solutions, the removal rate was 0.79 mg/l/h. In the reactor fed with the industrial model solutions, the maximum Cr(VI) removal rate attained was 0.49 mg/l/h. The *Arthrobacter* sp. packed bed biofilm reactors achieved Cr(VI) reduction rates comparable to other aerobic and anaerobic fixed film bioreactors.

Chromate reduction was studied in a membrane bioreactor under action of *Pseudomonas* bacteria immobilized in agar-agar films on the surface of synthetic membrane. Immobilized cells are protected from the excessive toxic action at high chromate concentration that improves cell activity compared with free cells. Almost complete chromate reduction was observed at stepwise introduction of chromate in feed solution allowing maintenance of optimal chromate concentration. Reduction is suppressed by high metabolite concentrations, which reached on the sixth step of chromate adding in studied system. Cells ability to reduce chromate is restored after changing of feed and receiving solutions allowing remediation of Cr(VI)-contaminated water in semi-batch operation of membrane bioreactor (Konovalova *et al.*, 2003). Continuous Cr(VI) reduction was carried out in 25 ml column packed with PVA immobilized *Microbacterium* sp. cells. The system was able to reduce 50 µM Cr(VI) in 20 days (Pattanapipitpaisal *et al.*, 2001). Bioremediation of Cr(VI) contaminated effluents using microbial cells thus is an effective and eco-friendly option in pollution management. The application of pure or mixed bacterial cultures for Cr(VI) biotransformation followed by chemical flocculation of $Cr(OH)_3$ as a combined treatment for industrial wastes was carried out by Garavaglia *et al.*, (2010). *Pseudomonas veronii* 2E, *Delftia acidovorans* AR, *Klebsiella oxytoca* P2 and *Klebsiella ornithinolytica* 1P, isolated from polluted environments showed a decrease from 38.83 to 74.32%, in 0.05 mM of initial Cr(VI). As revealed DGGE experiments, *P. veronii* 2E and *K. ornithinolytica* 1P could develop together in co-cultures and in these conditions a 72.88% of Cr(VI) present was removed. Although the pH of the cultures was alkaline, the precipitation of $Cr(OH)_3$ as sediment was not detected.

Chromate-reduction was performed under continuous-feed conditions in a fixed-film column bioreactor originally inoculated with a bacterial consortium containing *Desulfomicrobium norvegicum* and fed with H_2. With 500 mg/l of sulphate in the feed solution, total chromate-reduction was observed in the effluent whereas sulphate-reduction was strongly decreased, as also confirmed by measurements of isotopic ratios for sulphur. In the absence of sulphate, a chromate-reduction activity was lowered and reduction was H_2-dependent. Molecular biology techniques revealed the bacterial population in the effluent which contained *D. norvegicum* together with other microorganisms *Acinetobacter*, *Acetobacterium* and *Rhodocyclus*. A H_2- and CO_2-consuming bacterial population thus may be used in a globally autotrophic process to reduce chromate at low sulphate concentration, thus avoiding excess sulphide production (Battaglia-Brunet *et al.*, 2007).

Genetically engineered microorganisms may have higher activity in transforming metals. However, the release of such organisms into the environment is still of concern. Although this issue has been dealt with by many regulatory agencies and scientists, but guidelines with universal acceptance is presently unavailable. The use of purified enzymes

has the advantage of avoiding culture sensitivity to ambient toxicants, but such catalysts may be too costly for widespread environmental applications. Nevertheless, the purification and characterization of the functional enzymes may facilitate their genetic and/or protein engineering and enhance operational efficiency. Maximum enzyme expression of the desired gene in slow growing bacteria may minimize biomass formation and reduce clogging, which is desirable for the large-scale Cr(VI) cleanup. A recent attempt to isolate Cr(VI) reductase with the 2-DE proteomics techniques shed more light on a convenient alternative approach for extensive enzyme purification. Nevertheless, extreme ambient conditions (e.g. pH and temperature) in heterogeneous sites of the environment may inactivate or even denature the introduced enzymes, limiting the scope of their use and perhaps requiring prior modulation of the physical conditions. Technological breakthroughs, particularly in the enzyme immobilization, should help to overcome these barriers and make *in situ* bioremediation application a reality (Cheung and Gu, 2007).

CONCLUSION

Microbial reduction of hexavalent chromate to relatively insoluble and considerably less toxic trivalent chromium is a potential remediation strategy for chromium-contaminated soils in the twenty first century. Nevertheless, in spite of considerable advances in the processes made in recent years, some points still need to be examined in details before applying bioremediation technologies to large-scale soil and wastewater reclamation systems. The knowledge of the mechanisms involved in the process of microbial resistance system must be studied in depth including how some abiotic factors affect the rate of Cr(VI)-reduction. The capability of indigenous bacteria in reducing Cr(VI) to Cr(III) is to be quantified and the optimal conditions are to be defined in order to improve the ability of specific microbial strains to play their role under stressed conditions in polluted-environments. Moreover, indigenous microbes obtained from sites contaminated with chromium are endowed with intrinsic characteristics, which facilitate their exploitation in *in situ* bioremediation processes. This may solve the legal and ethical problems related with the introduction of engineered microorganisms into the environment.

Novel engineered microbes and/or proteins with improved CrVI) reduction capability can be developed and introduced besides indigenous Cr(VI) resistant microflora for better utilization and operation in *ex situ* closed bioreactor systems. With molecular engineering, it will be possible to enhance Cr(VI)-reduction activities of indigenous bacterial strains that express such activities at high levels under poor nutrient and stressful environmental conditions (Gonzalez *et al.*, 2003). Finally, the most suitable Cr(VI) bioremediation system can be developed successfully by assembling the advantages of all available technologies together with the characteristics of the contaminated site which should also be taken into consideration.

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