MICROBIAL ABILITIES TO DETOXIFY CHROMATE BY REDUCTION

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I hereby declare that the dissertation represents my own work. It has not been submitted before any diploma/degree or examination at any other Technikon/University.

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I hereby approve the final submission of the following dissertation.

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this 20 th day of March, 2001, at Technikon Natal.

DEDICATION

The source of light in all luminous objects, beyond the darkness of matter and is unmanifested. The knowledge, the object of knowledge, and the goal of knowledge...it is with such spiritual realization to which I salute, the object of my existence, GOD!

ABSTRACT

Hexavalent chromium [Cr(VI)] or chromate, is a toxic, water-soluble contaminant present in many soils and industrial effluents. As a result of contaminated discharges from industrial applications, and inappropriate waste-disposal practices, significant amounts of chromate have found their way into the environment. This poses a health risk to man as well as animals and plants due to the carcinogenicity, mutagenicity, and teratogenicity of chromate. In man, acute, high level exposures to Cr(VI) can result in ulceration of the skin, eyes, and mucous membranes. Exposure of plants to Cr(VI) can result in reduced biomass production, and in extreme cases, death. Upon reduction of Cr(VI) to trivalent chromium [Cr(III)], the toxic effects are significantly decreased because of a decrease in the solubility and bioavailability of Cr(III). Traditionally, Cr(VI) has been recovered from aqueous systems using processes exploiting the differential solubility properties described above. The use of chromate reducing bacteria represents a potential mechanism for the development of an efficacious, cost effective alternative to traditional chemical/physical processes for Cr(VI) recovery from the environment. Therefore, the aim of this research was to isolate and identify chromate reducing bacteria from soil, and characterise the chromate reductase enzyme in order to determine the potential of bacteria to detoxify chromate by reduction. Bacteria from soils and wastewater were examined for chromate reducing potential and identified on the basis of biochemical tests and API 20E. Organisms were isolated by the spread plate technique. Species of *Pseudomonas maltophilia, Bacillus subtilis, Acinetobacter calcoaceticus,* and *Cellumonas cellasea* were capable of catalyzing the reduction of Cr(VI) to Cr(III) in batch experiments. Reduction capability as high as 99% by the isolates was detected from an initial Cr(VI) concentration of 150 $mg.L^{-1}$ in batch cultures. Chromate reduction was determined by means of the diphenylcarbazide method and total chromium was measured by atomic absorption spectroscopy. *Pseudomonas maltophilia* was observed to be the most suited organism for the efficient detoxification of Cr(VI) due to its wide temperature and pH requirements, low substrate utilization, and tolerance to heavy metal ions of Cu^{2+}, Cd^{2+}, Zn^{2+}, and Ni^{2+} which commonly appear in industrial effluents along with Cr(VI). Reduction rate in a batch reactor for this organism was calculated to be 1.75 $mg.g^{-1}.h^{-1}$. Comparison of the rates of chromate reduction by Cr(VI) grown cells and cells grown without chromate indicated that the chromate reductase activity is constitutive. Reductase activity was detected by means of the lysozyme-EDTA method in aerobically grown cells, with highest specific activity in the cytoplasmic fraction of the cell. The Cr(VI)-reductase was found to be NAD(P)H-dependent and yielded an activity of 3.24 $mU.mg^{-1}$ of protein in the cytoplasmic fraction. Once optimization of the parameters in the batch reactor was achieved, cells of *Ps. maltophilia* was immobilized into polyacrylamide gel and packed in a column. Mass balance studies indicated that *ca* 147 $mg.L^{-1}$ chromate passing through the column undergoes reduction with an initial Cr(VI) concentration of 150 $mg.L^{-1}$ resulting in a Cr(VI) reduction efficiency of 98%. An amount of 0.11 $mg.L^{-1}$ remained in the cells, 0.11 $mg.L^{-1}$ in the cell wash water, and 1.65 $mg.L^{-1}$ was unaccounted for in the mass balance. Chromate reduction rate in the continuous-upflow reactor system was calculated to be 5.34 $mg.g^{-1}.h^{-1}$, which was 3-fold higher than that calculated for the batch reactor. Chromium-contaminated industrial effluent obtained from Sheffield, Natal, and Saayman Danks Electroplaters was pumped into the continuous-upflow reactor containing immobilized cells of *Ps. maltophilia* to determine the industrial applicability of the reactor to treat chromate-containing effluents. Complete Cr(VI) reduction was achieved in 18 h for the metal-plating wastewaters obtained from Sheffield, Natal, and Saayman Danks Electroplaters at concentrations ranging from 15.66 to 35.69 $mg.L^{-1}$. Results of the investigation confirmed the advantages of using immobilized cell systems through improved volumetric productivity by increased bacterial density in the bioreactor. The high rates of Cr(VI) reduction achieved during the exponential growth phase indicated that maximum removal rates are attained by the use of a continuous flow bioreactor running at a flow rate calculated to maintain bacteria in exponential growth phase. This research indicates that reduction of hexavalent chromium by *Ps. maltophilia* offers the potential to be developed into a viable technology for the remediation of Cr(VI) in the environment, at least in waste streams containing Cr(VI) levels up to 150 $mg.L^{-1}$.

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TABLE OF CONTENTS

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

APPENDICES

LIST OF TABLES

LIST OF FIGURES

TABLE OF ABBREVIATIONS

API	Analytical Profile Index
ASP	activated sludge process
ATP	Adenosine triphosphate
CM	cytoplasmic membrane
COD	Chemical oxygen demand
CSTR	Continous stirred tank reactor
E_h	electron potential
EPA	Environmental Protection Agency
EPR	electron paramagnetic resonance
EPS	Extracellular polymeric substances
GPC	gel permeation chromatography
GSSG-R	glutathione reductase
HRT	hydraulic retention time
IM	intermediate membrane
INEEL	Idaho National Engineering and Environmental Laboratory
MLVSS (VSS)	mixed liquor volatile suspended soilids (volatile suspended solids)
OM	outer membrane
ppb	parts per billion
SEM	Scanning electron micrograph
SRB	sulphate reducing bacteria
SRT	Sludge retention time
STW	Sewer Treatment Works
TDCr	total dissolved chromium

CHAPTER ONE

GENERAL INTRODUCTION AND SCOPE OF RESEARCH

1.1 WHAT IS CHROMIUM ?

Chromium (Cr) is a lustrous, silver grey metal, one of the less common elements in the earth's crust, which occurs only in compounds. The chief commercial source of chromium is the mineral chromite ($FeCr_2O_4$). Chromium is mined as a primary product and is not recovered as a by-product of any other mining operation. Chromium is released to land, surface water and ground water from industrial processes such as electroplating, dyeing, the automobile industry, leather tanning, stainless steel, anticorrosive agents, and pigment production industries. Chromium usually carries a +6 or +3 valence, however other forms do exist. Divalent chromium, Cr(II), is fairly unstable and is rapidly oxidized to Cr(III). Elemental chromium, Cr(0), is oxidized to Cr(III) unless it is made passive by superficial oxidation (Chrotoswski *et al*., 1991) and Cr(V) was established to be an intermediate during Cr(VI) reduction (Shi and Dalal, 1990). Hexavalent chromium [Cr(VI)] as chromate and dichromate ions (CrO_4^{2-} and $Cr_2O_7^{2-}$) are the major chromium species encountered. Cr(VI) is highly water-soluble and highly toxic due to its carcinogenocity, mutagenicity (Venitt and Levy, 1974), and teratogenicity (Bauthio, 1992) compared to trivalent chromium [Cr(III)], thus necessitating the treatment of Cr(VI)containing wastewater.

1.1.1 Aqueous Geochemistry of Chromium

In aqueous systems, chromium exists primarily in two oxidation states; Cr(VI) and Cr(III). Change in the oxidation state of chromium has a profound effect on toxicity and bioavailability (Mertz, 1979). The behaviour of chromium in the activated sludge process (ASP) was evaluated in a laboratory-scale study. Both pH and the oxidation state of chromium were confirmed as critical parameters in the ASP for evaluating the behaviour of chromium (Moore *et al.,* 1961). More than 60% of Cr(III) was removed when introduced into the effluent while less than 30% Cr(VI) was removed when added over a pH range from 5 to 9. The percentage removal of chromium was calculated based on TDCr (total dissolved chromium) in the effluent through a mass balance around the reactor. Dissolved constituents such as TDCr were defined as those that passed through a 0.45-μm membrane filter (Imai and Gloyna, 1990).

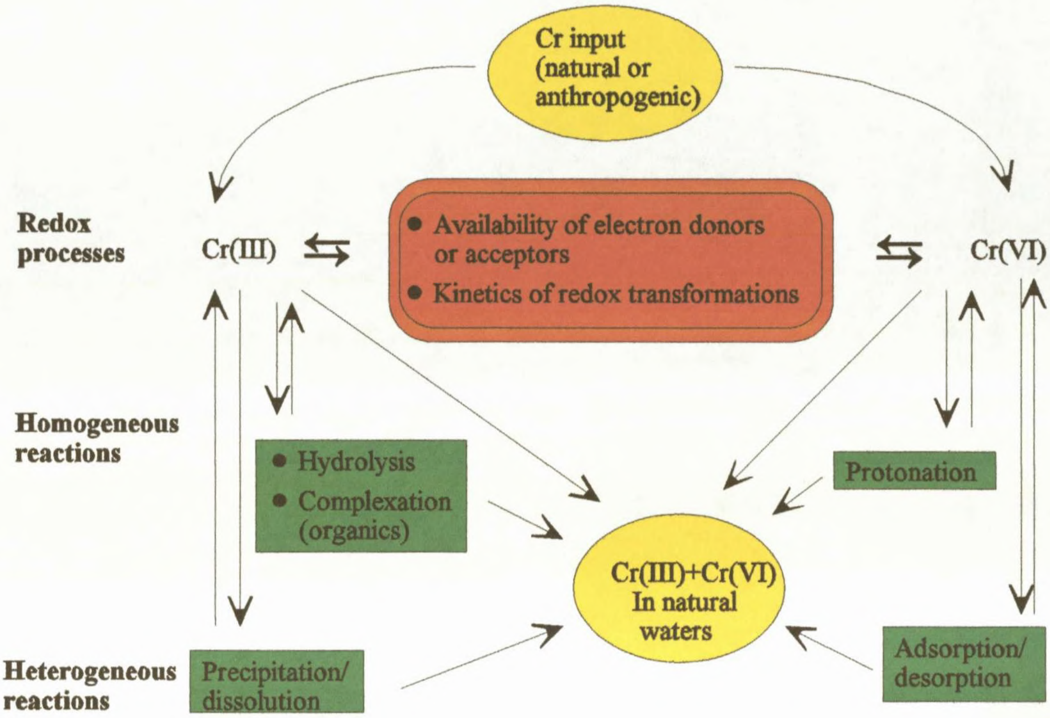


FIGURE 1.1 **Aqueous geochemistry of chromium (Richard and Bourg, 1991).**

The solubility of chromium is strongly dependent upon its oxidation state (Richard and Bourg, 1991). In addition to redox conditions, the effect of water chemistry (pH, competing ions, complexing agents) and natural solids (adsorbents) could also be quite significant (Mayer, 1981). As a result, hydrogeochemical cycles of chromium should be mainly controlled by the local environmental conditions, thus the establishment of equilibrium between Cr(III) and Cr(VI) was not rapid (FIG 1.1). Amacher *et al.*, (1988) reported that reversible reactions reached equilibrium within 24 h but the overall chromium retention reaction did not reach complete equilibrium even after two weeks. Chromium cycling in an aquatic environment (FIG 1.2) occurred in response to nutrient biogeochemistry (Richard and Bourg, 1991). When Cr was scavenged by particles, diagenetic processes could lend to remobilization of Cr either as chromate or as organic Cr(III) complexes (Eary and Rai, 1987).

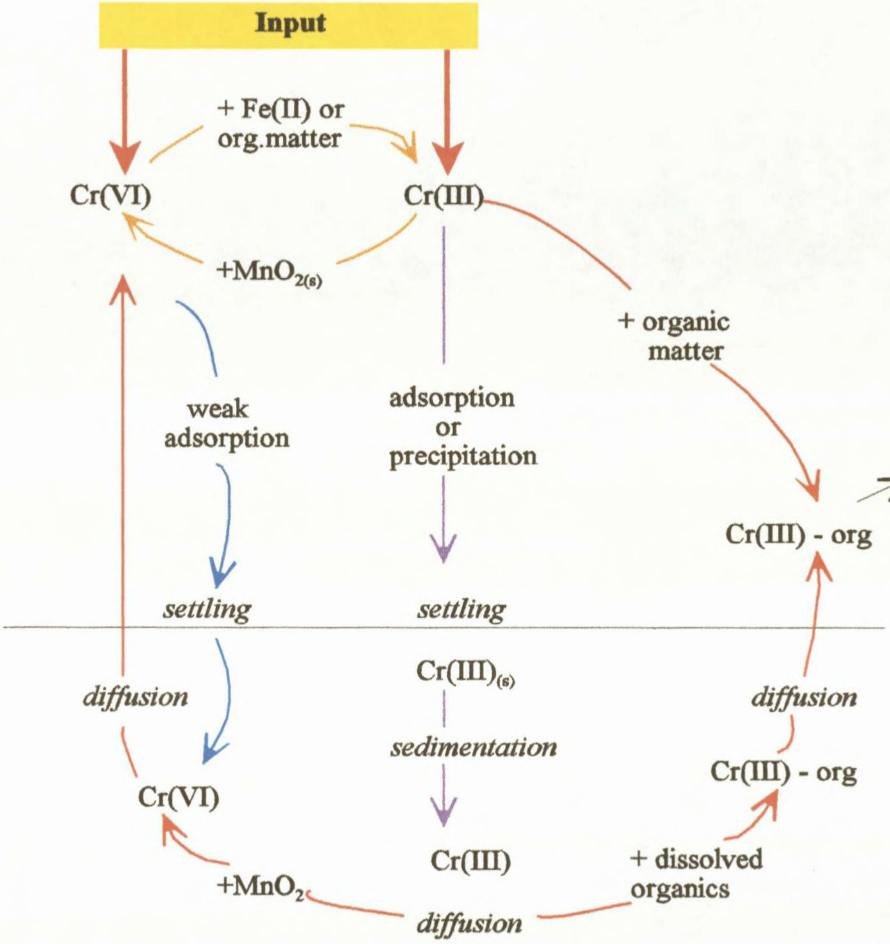


FIGURE 1.2 Chromium cycling in the aquatic environment (Richard and Bourg, 1991)

1.2 SOURCES OF Cr (VI) WASTEWATER CONTAMINATION

The largest contributor of heavy metals to municipal sewerage systems are the industries associated with metal finishing (TABLE 1.1). The major contaminants arising in the effluent are cyanides, heavy metals (nickel, copper, zinc, cadmium, tin, silver, and chromium), associated salts, oils and greases, cleaning aids and solvents. These arise from the discharge of acids, bright dips, process baths, rinses containing drag-out from previous process cycles and from metals leached from the work by the process chemicals (Grebenyuk *et al.*, 1989). An overview of a metal finishing process is illustrated in FIG 1.3. There are also ad-hoc accidental spills, leaks, and drips of process solutions to drain. Additional sources include sludges from the bottoms of baths, backwash from plating tank filter systems, and the dust and vapours released from the baths and process lines (Jacobs, 1992).

TABLE 1.1 **Primary contributors of Cr(VI) to municipal sewerage systems (Apel and Turick, 1992).**

Source	Amount released (mg.L^{-1})
Anticorrosive Agents	43
Tanning	44
Manufacture of dyes	37
Manufacture of inks	33
Metal rust proofing	41
Bright metal plating	49

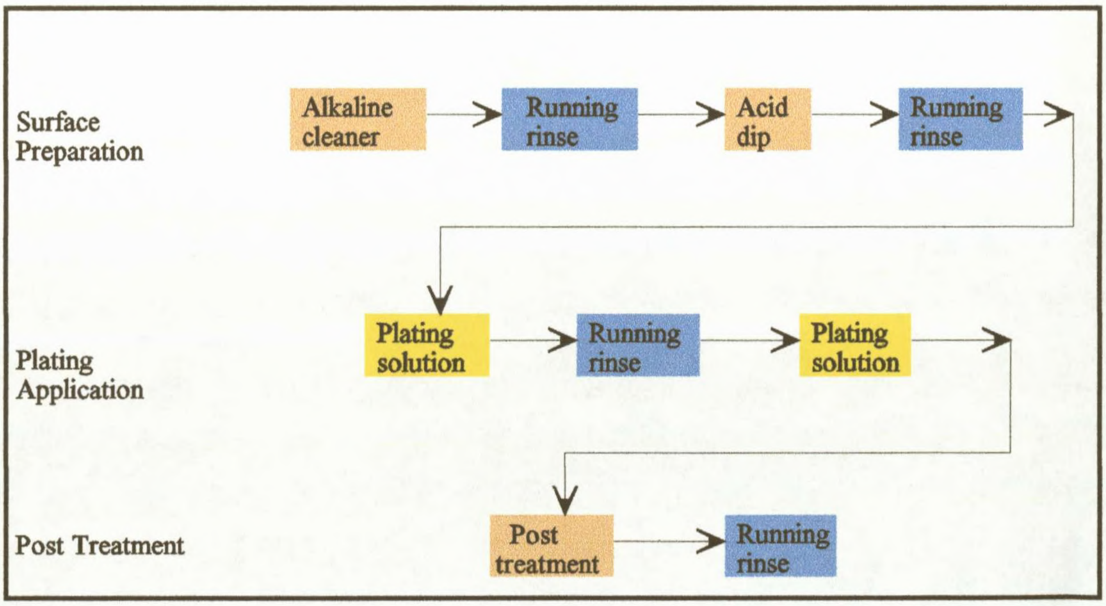


FIGURE 1.3 **Overview of the Electroplating/Metal Finishing Process (Cowan, 1998).**

The variations in process requirements for different plating operations, and consequently waste loads being generated at any particular time are illustrated in FIG 1.3. The general concentration range of chromium in chromium-plating solution is illustrated in TABLE 1.2. Most metal finishers have a range of pollutants, e.g., cyanide, chromium, other metals or greases and oils. Therefore, treatment to meet municipal discharge specifications combines several treatment technologies to satisfactorily treat their effluent constituents. In many facilities it is not considered viable to recover metals from waste waters or discharged sludges due to the extremely high purity required in the plating baths and the costs associated with such recovery as opposed to direct disposal and replacement (Palmer and Wittbrodt, 1991).

TABLE 1.2 General range of chromium in chromium-plating solutions (Cowan, 1998).

Type of solution	Chromic acid in $g.L^{-1}$	as Cr in $g.L^{-1}$
Bright chromium-plating	192 to 520	100 to 270
Hard chromium-plating	270 to 308	140 to 160

TABLE 1.3 Composition of raw waste stream from common metal plating (Cowan, 1998).

Constituent		Range ($mg.L^{-1}$)
Chromium :	Total	0.088 - 526
	Hexavalent	0.005 - 335
Copper		0.032 - 272.5
Nickel		0.019 - 2954
Zinc		0.112 - 252
Cyanide :	Total	0.005 - 150
	Amenable to chlorination	0.003 - 130
Fluorides		0.022 - 142
Cadmium		0.007 - 21.6
Lead		0.663 - 25.4
Iron		0.41 - 1482
Tin		0.060 - 103
Phosporous		0.020 - 144
Total Suspended Solids		0.100 - 9970

The contribution by each pollution source to the pollutant concentration of the final effluent can vary substantially. TABLE 1.3 illustrates the variation in effluent discharge quality that can result from a metal finishing operation,depicting the difficulties a control program has in ensuring a responsible compliance by the industry (Palmer and Wittbrodt, 1991, Nevski *et al.*, 1993).

1.3 TOXIC EFFECTS OF CHROMIUM

Chromium is an essential nutrient for plant and animal metabolism (glucose metabolism, amino- and nucleic acid synthesis). However, when accumulated at high levels, it can generate diseases (nausea, skin ulceration's, lung cancer) and, as concentrations reach 0.1 $mg.g^{-1}$ body weight, it can ultimately become lethal (Mertz, 1974 and Ajmal *et al.*, 1984). Cr(VI) can cause liver damage, internal hemorrhaging, and respiratory disorders in mammals. Chromate is also teratogenic, causing birth defects, to mammals including humans (Pettrilli and Miller, 1977; Keith and Tilliard, 1979). Inhalation of Cr(VI) has been known to cause cancer in both human and animals. Lung cancer due to exposure to Cr(VI) has been officially accepted as a cause in the United States and Germany (Hueper, 1986 and Mazierski, 1994).

Chromium can be found in concentrations ranging from 0.1 $mg.m^{-3}$ in air to $4g.kg^{-1}$ in soils (Merian, 1984). The major environmental exposure of humans to chromium occurs as a consequence of its presence in food. Chromium is also found in urban air and natural water supplies. In municipal drinking water, a concentration of 35 ppb (part per billion) has been reported (Casarett and Doull, 1980). The daily intake has been estimated at 60 mg, 10 mg of which is from drinking water.

1.3.1 Mechanism of Action of Chromium in the Cell

In contrast to Cr(III), Cr(VI) utilizes a general anion transport pathway to enter tissue cells. Once inside the cell, Cr(VI) is rapidly reduced through relatively unstable Cr(V) and Cr(IV) intermediates to kinetically stable Cr(III) species. Intracellular chromate reduction is required for chromium compounds to exert their genotoxic effects. Each of the major pathways of chromate reduction results in a different spectrum of cellular and/or genotoxic damage. Genotoxic effects of chromium include chromosome aberrations and sister chromatid exchanges (Snow, 1992; O'Flaherty, 1998).

1.4 ENVIRONMENTAL SIGNIFICANCE OF CHROMIUM

Potential approaches to Cr remediation have been intensively investigated. The element in toxicologically important concentrations may be found in effluents from cooling towers or the cooling water from electricity-generating stations and in contaminated soils or ground waters. Three approaches have been pursued: biosorption, enzymatic reduction, and abiotic reduction coupled to microbial sulphate reduction. The hexavalent form of Cr is characteristic of chromates, dichromates, and chromic trioxide, and the trivalent form characterizes Cr oxides and hydroxides. Cr(VI) is highly soluble, and hence less mobile if present in soil. Because of the differences in solubility of Cr(VI) and Cr(III), a reduction will result in precipitation of the element and thus diminished mobility and transport. Therefore, microbial Cr(VI) reduction may offer a solution in chromium contaminated wastes' (Jacobs, 1992).

The most extensive studies on the potential use of Cr(VI)-reducing microorganisms for the removal of Cr(VI) from waste streams have been conducted with *Enterobacter cloacae* strain HO1 (Wang *et al.*, 1989 and Ohake *et al.*, 1990b). Early studies with this organism revealed that during Cr(VI) reduction the yellow medium turns white and turbid, presumably as the result of the formation of insoluble Cr(III) hydroxide (Wang *et al.*, 1989). However, Cr(III) does not readily precipitate and cannot be effectively removed with centrifugation (Komori *et al.*, 1990a). Cr(III) was removed more effectively when *E. cloacae* was placed on one side of a semipermeable membrane and the Cr(VI)-containing medium was on the other (Komori *et al.*, 1990b). Cr(VI) diffused into culture and *E. cloacae* reduced the Cr(VI) to Cr(III),which was retained on the culture side, especially when an anion-exchange membrane that does not permit passage of Cr(III) was used (Komori *et al.*, 1990c).

Despite successful removal of Cr(VI) from culture medium with *E. cloacae*, removal from industrial effluents has been problematic as heavy metals and sulphate in the effluents can inhibit Cr(VI) reduction (Hardoyo and Ohtake, 1990; Ohtake *et al.*, 1990). Whether microbial Cr(VI) reduction would have advantages over simple chemical Cr(VI) reduction in the removal of Cr(VI) did not appear to have been evaluated.

1.5 WASTE HANDLING OF CHROMATE CONTAMINATED EFFLUENTS AND SLUDGES

The cost of handling and disposing of spent process baths and sludges containing heavy metals has increased significantly because of the increased regulatory requirements placed on the handling and disposal of hazardous wastes. It is a very rare facility that can satisfactorily, and legally, meet their permitted effluent limits without some type of pretreatment prior to discharge (Crnojevich *et al.*, 1990).

1.5.1 Chemical Reduction of Chromate

Liquors containing hexavalent chromium are discharged to the dedicated holding tank where the liquor pH is reduced to 2.5 - 3 by addition of hydrochloric acid prior to dosing with metabisulphite. The liquor pH is then adjusted with caustic soda to encourage precipitation of trivalent chromium sludge. The segregated chromium sludge can be dissolved using sulphuric acid to obtain a reusable liquor or can be transferred to the bulk liquor holding tanks for co-treatment, dewatering, stabilization and disposal.

1.5.2 Basis for Discharge Standards

Discharge standards for industrial sewer discharges were intended to prevent the introduction into a Sewer Treatment Works (STW) of pollutants that would interfere with its operation . These pollutants accumulate into waste sewage sludges at unacceptably high levels, or pass through the treatment works to exceed river discharge standards, and threaten aquatic life. The objective in setting specific discharge standards was to reduce the contaminants in metal finishing discharges. It should be reduced to environmentally acceptable levels while remaining technically feasible and affordable for the industry in relation to alternative metal control options, in line with the accepted international norms, with local considerations (Pollution Research Group, 1987).

For chromium, the specific standard on the basis of daily maximum and monthly average is 2.87 mg.L^{-1} and 0.08 mg.L^{-1} respectively as defined by the U.S. Environmental Protection Agency (EPA, 1987). However, in South Africa most municipalities prescribe a general discharge standard for chromium at 20 mg.L^{-1} (DWAF, 1995). The implementation of waste minimization or pretreatment programs within the industry was found to be expensive and unfavoured whilst discharge to sewer remains the easier and cheaper option. However, most of the industries do not comply with specifications and are thus fined accordingly. One of the main reasons for not complying was the high cost of chemical treatment options associated with treating heavy metals (Pollution Research Group, 1987).

1.6 CONVENTIONAL TREATMENT OF CHROMATE WASTE

Current technologies are quite satisfactory in terms of purging tannery waste waters from chromium and other heavy metals, but they systematically produce solid residues (sludges) containing toxic compounds whose final disposal is controlled land filling with related high costs (Tiravanti *et al.,* 1997). Conventional methods for the treatment of chromate include chemical reduction by using a reducing agent such as sodium sulphite, and adsorption ion exchange and chelating resins. However these methods consume high amounts of energy and large quantities of chemical reagents, and therefore more practical, economic methods need to be explored (Fujii *et al.*, 1994). The basic chromium reduction system (FIG 1.4) operates as a batch treatment system in which the wastewater is collected in a tank and then treated as a single batch. Although this conventional system is still used in some industries, others however utilize various systems eg. the baffled reaction chamber (FIG 1.5). Hexavalent chromium is very soluble at all pH values whereas trivalent chromium as a hydroxide reaches its least solubility at a pH of 7.5 (TABLE 1.4), (Nelson *et al.,* 1992).

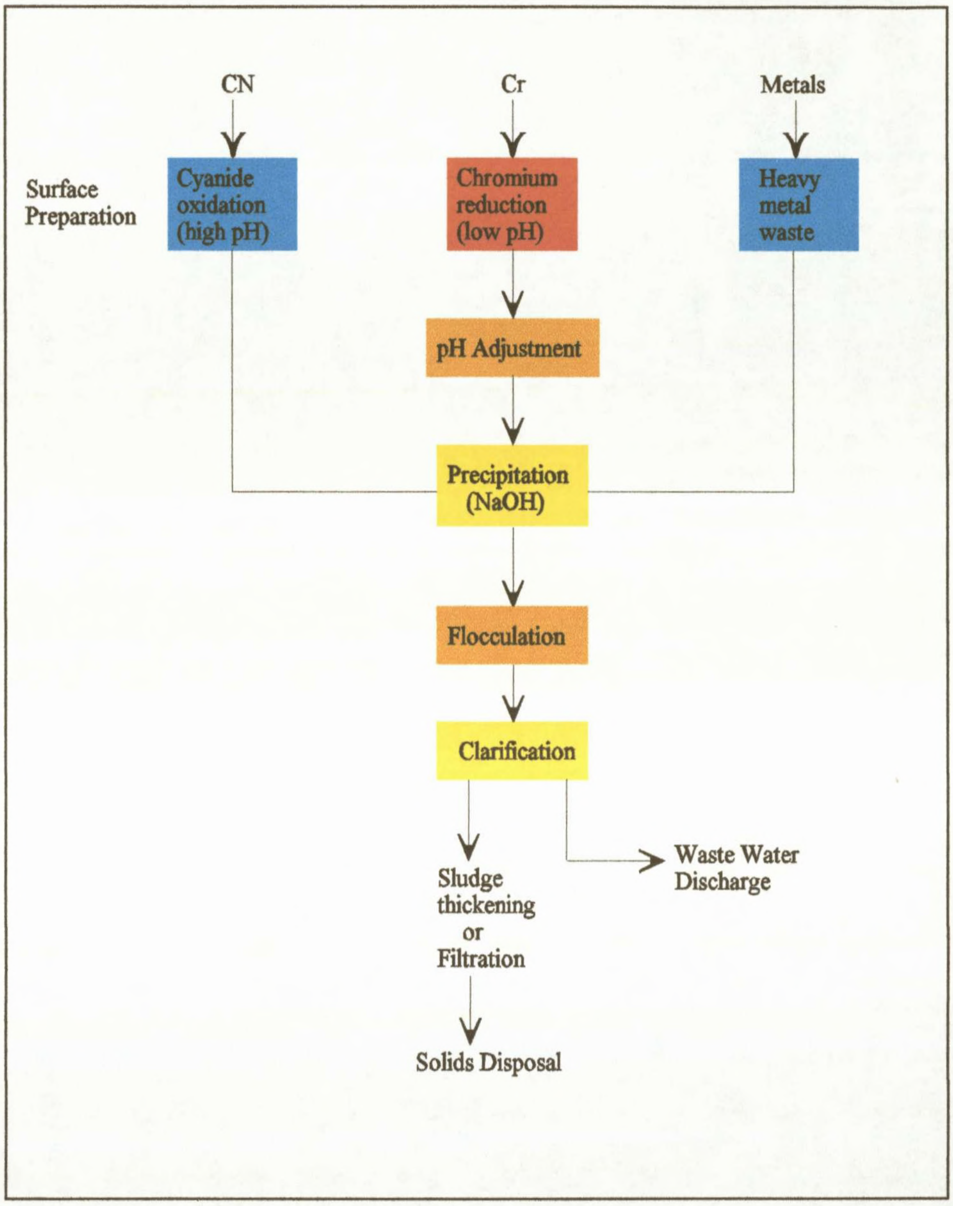
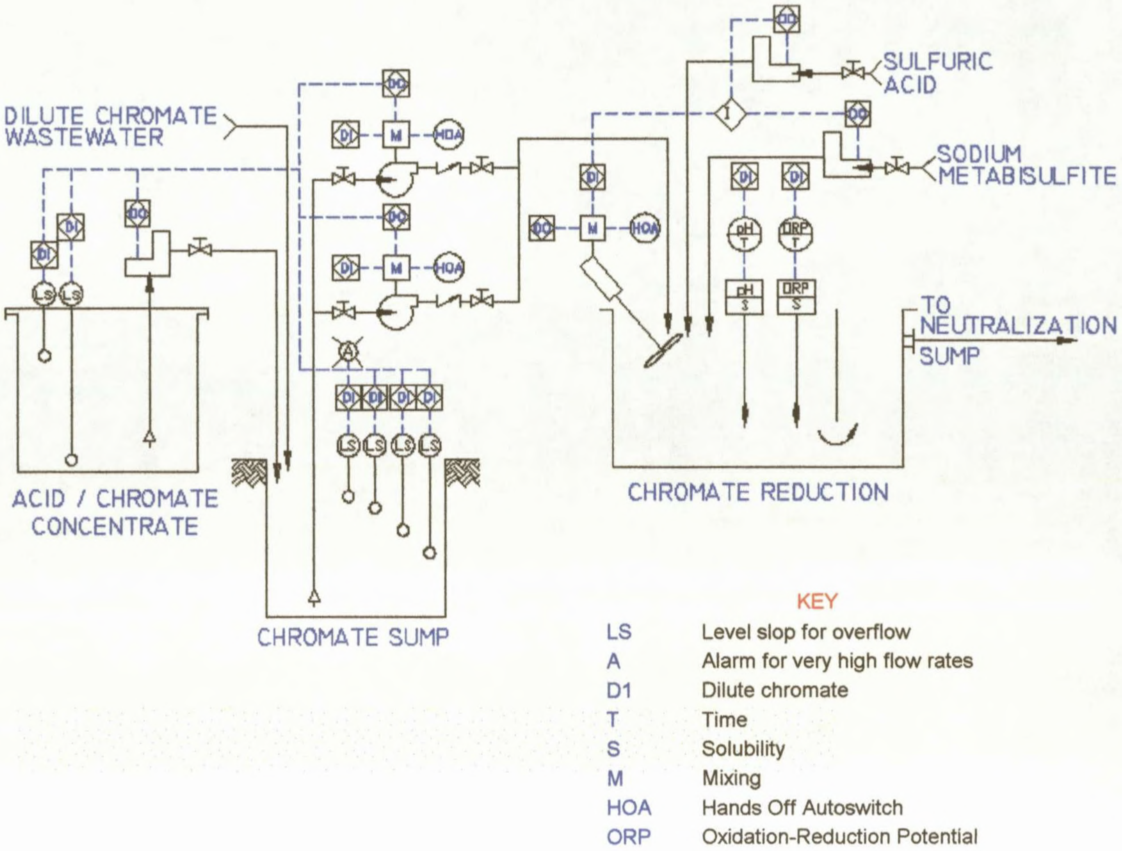


FIGURE 1.4 **Conventional ChromateTreatment Scheme (Cowan, 1998).**



KEY

LS	Level slop for overflow
A	Alarm for very high flow rates
D1	Dilute chromate
T	Time
S	Solubility
M	Mixing
HOA	Hands Off Autoswitch
ORP	Oxidation-Reduction Potential

FIGURE 1.5 **Baffled reactor system for the chemical treatment of chromium (Hoffland Environmental, 1999).**

TABLE 1.4 **Solubilty of Cr(III) at varying pH values (Nelson *et al.*, 1992).**

pH	Solubility (mg.L^{-1})
7.0	0.1
7.5	0.08
8.0	0.12
8.5	0.50
9.0	1.10
9.5	3.00
10.0	3.00

To chemically precipitate chromium from solution, the Cr(VI) must first be reduced to Cr(III) which would precipitate as a hydroxide compound at pH 9.5. In a baffled reaction chamber Cr(VI) reduction to Cr(III) occurs utilizing quadravalent sulphur as the reducing agent (FIG 1.5). The reaction zone maintained a pH of less than 3 with at least five minutes reaction time and a mixing rate of 2.5 - 3.0 tank turnovers per minute. The quadravalent sulphur was supplied by a solution of sodium metabisulphite or sulfur dioxide gas. When the pH was increased to 8.0 - 8.5, with either sodium hydroxide or lime (calcium hydroxide), chromium hydroxide precipitated from solution (Palmer and Wittbrodt, 1991).

1.6.1 Disadvantages of Chemical Reduction Systems

The disadvantages inherent in the reduction treatment are :

1. SO_2 is released in to the ambient air.
2. The reduction is approximately 88% complete.
3. The amount of residual Cr (V1) depends upon the time allowed for the reaction, the pH of the reaction mixture, and the concentration and type of reducing agent employed.
4. The reducing agent is nonspecific and therefore will reduce non-targeted compounds as well as targeted compounds thereby converting the non-targeted metals to more toxic or more mobile forms.
5. *In-situ* chemical treatment agents must be selected for compatibility with the environment.
6. *In-situ* chemical treatment systems have the potential for introducing oxidizing, reducing or neutralizing chemicals into the ground water system, but chemical addition to the *in-situ* environment may create a pollution problem in itself (Conner, 1990; Jacobs, 1992; Loomba and Pandey, 1992).

1.7 BIOLOGICAL TREATMENT TECHNOLOGIES

Metals and their salts have the capacity to inhibit biological activity. Although metals such as cobalt, copper, iron, chromium, manganese, molybdenum, and zinc are essential micro nutrients, they can become toxic to microbes at higher than trace concentrations, with the sensitivity being species dependent. Some microorganisms have developed mechanisms to protect themselves from the toxic effects of metals, including adsorption, oxidation, reduction, and methylation (Ohtake and Silver, 1994). Although these pathways are designed for protection from metals, they can be manipulated and thus are useful in the development of treatment methods for metal contamination.

Biological processes can alter the chemical state, form, or distribution of metals in several ways. Most of the potential techniques for biological treatment of metals contamination are being developed at bench and pilot scale.

Exploiting biological activity for the purpose of remediating metals contamination can take any one of several forms. Treatment can be conducted *in situ* or in more controlled scenarios such as aboveground reactor configurations (Farmer *et al.*,1995).

The more common biological mechanisms that can be exploited for remedial purposes include adsorption, oxidation reactions, reduction reactions, and methylation/demethylation reactions. Biological oxidation/reduction treatment avoids addition of environmentally unfriendly chemicals. Chemical oxidation/reduction processes on the other hand often use aggressive chemicals. An otherwise chemically feasible oxidation/reduction process may be unacceptable due to limitations on the types of chemicals that can be injected. Aboveground chemical treatment allows more flexibility in selection of chemicals, but still produces an aqueous waste stream that requires regeneration or cleanup and disposal. When available, biological mechanisms can achieve oxidation or reduction without the addition of acids, bases or oxidation/reduction agents (Macaskie and Dean, 1989; Wildeman *et al.*, 1995).

1.7.1 Microbial Potential for Chromate Reduction

It has long been known that microorganisms can bring about the reduction of a wide array of inorganic anions and cations (Romanenko and Korenkov, 1977; Ohtake *et al.*, 1987; Wang *et al.*, 1990). The reductions convert a higher oxidation state of the element to a lower one. Nitrate, sulphate, and carbonate are well-known examples of nonmetallic anions that are reduced microbiologically. Early studies showed that bacteria and fungi are capable of reducing ionic As, V, Te, S, and Mo as well as MnO_2 and chlorate (Bautista and Alexander, 1972), and enzyme preparations obtained from a bacterium which was observed to reduced As, Pb, Cu, Mo, U, Se, Bi, Te, Va, Mn, Fe, W, Ag, Au, Os, and Ru. Further research has focused on some of these as well as other elements, such as Hg and Cr (Woolfolk and Whiteley, 1962).

The microorganisms that carry out these reductions come from a variety of genera, and the transformations relate to the physiology of the organisms in different ways:

(a) Some of the reactions are catalyzed enzymatically, but some result from the excretion by the organism of a reducing compound that abiotically converts the ion or metallic compound from a higher to a lower oxidation state.

(b) Among the reactions catalyzed enzymatically, some serve a role in the physiology of the organism by acting as a terminal electron acceptor and permitting growth, much as O_2 functions for aerobes, simple organic molecules for fermentative microorganisms, sulphate for *Desulfovibrio*, nitrate for denitrifying bacteria, and CO_2 for methanogenic bacteria. Others, however, have no role in the physiology of the responsible species and represent merely fortuitous reductions coupled to enzymatic or microbial oxidation of some substrates (Bloomfield and Pruden, 1980).

For the reductions to occur, an energy source must be available. That energy source is usually organic, but it could be inorganic. Some of the reductions occur naturally in soils, sediments, and waters, but often the conversion is so slow or so much of the oxidized form of the element remains that remediation is necessary to allow for a more rapid conversion. From the toxicological and environmental viewpoints, the reductions are important for three reasons. The reductions may change the (a) toxicity, (b) water solubility, and/or (c) mobility of the element (Romanenko and Korenkov, 1977). An increase in water solubility and mobility can be exploited to bioremediate insoluble forms of an element in soil because the product of reduction would move out of the solids and into the water. Conversely, a decrease in solubility of an element as a result of reduction can be used to remove it from surface or groundwater (Aronstein *et al.*, 1995).

Species of *Alcaligenes, Bacillus, Corynebacterium, Enterobacter, Escherichia, Micrococcus, Pseudomonas, Vibrio*, and others have been reported to effect reduction (Kvasnikov *et al.*,1988; Cooke *et al.*,1995). Because of the toxicity of Cr(VI), the organisms of choice must have a high degree of tolerance, but such isolates have been obtained. Under suitable conditions, > 99% of Cr(VI) may be reduced (Cooke *et al.*,1995; Bhide *et al.*,1996). However, at high concentrations, the percentage of the toxic ion that is reduced diminishes appreciably (DeLeo and Ehrlich, 1994) probably because of the toxic effects of chromate.

Although the parameters for optimization of such organisms have been investigated, research is now focused on enzymes responsible for such reactions and optimization and feasibility of the various biological processes. *Shewanella putrefaciens* is a gram-negative facultative anaerobe that can reduce a number of metals at high rates. The reductase activity of this bacterium had been recently established (Myers and Myers, 2000) which is essential in understanding its potential effects on the fate of Cr(VI) in contaminated environments and its potential utility for the bioremediation of Cr(VI)-contaminated industrial effluents.

Since chemical methods of chromate reduction pose several disadvantages, the use of biological methods offers a remediation strategy that attempts to sustain a non-hazardous environment by not adding more toxic chemicals. Although the bulk of *in vitro* studies on microbial chromate reduction proved to be somewhat successful, more attention needs to be focused in the area of optimization and feasibility of a chromate bioremediation process. Thus far, the approaches to detoxify chromate that have been pursued were confined mainly to the use of synthetic chromate containing wastewater. Unfortunately, chromate-bearing waste waters from industrial processes generally contain a variance of other compounds. Therefore, organisms capable of reducing chromate in the presence of such compounds should be selected and conditions optimized to establish a process design that would be able to successfully detoxify chromate-contaminated effluents.

1.8 AIM AND OBJECTIVES OF THE STUDY

Accordingly, the primary aim of this investigation was to evaluate the potential of the biotransformation of Cr(VI) to Cr(III) by organisms indigenous to chromium-contaminated soils and industrial effluents. Development of a bioprocess to optimize the reduction of Cr(VI) and determination of the factors that may be important in the treatment of Cr(VI) seem warranted to attempt to elucidate successful Cr(VI) reduction of Cr(VI)-contaminated wastes.

To this end, the objectives of the research were as follows:

1. Screening of crude biomass from various sources for chromate reducing capability.
2. Isolation and identification of bacteria that are capable of chromate reduction.
3. Determination of location of enzyme for reduction.
4. Effect of various parameters (pH, temperature, competing ions, co-factors, concentration) on chromate reduction.
5. Optimization of a batch-scale reduction system for Cr(VI) reduction.
6. Determination of effectiveness of chromate reduction using polyacrylamide immobilized cells in a continuous up-flow reactor system.
7. Evaluation the capability of the isolated organisms to detoxify chromate-containing effluents.

CHAPTER TWO

LITERATURE REVIEW

2.1 PATH OF METALS IN THE ENVIRONMENT

Soil microflora may play a key role in modification of heavy metals added to soil as a result of man's industrial and agricultural activity. For such modification to occur, soils must contain populations of microbiota resistant to the toxic effects of a given metal and capable of altering the chemical form of the added metal. Through the years, toxicologists have developed a large body of data that considers the biological effect of heavy metals in the chemical forms (chlorides, sulphates, and nitrates) in which they enter the environment (Kvasnikov *et al.,* 1988).

Although these data are of great use in setting occupational health standards, and in determining effects that can be expected as a result of accidental exposures of the general populace to these compounds, they may not reflect the toxic potential of these metals for environmentally exposed populations over long periods of time. The obvious reason for this observation is that the toxicity of any element is, to a great extent, a function of its chemical form (Bremner, 1974). Determining potential effects on man of heavy metals in the environment requires knowledge of the chemical form to which man might be exposed. Thus, when considering metals through food chains as one form of exposure to man, the main factors determining chemical form (FIG. 2.1) should be regarded.

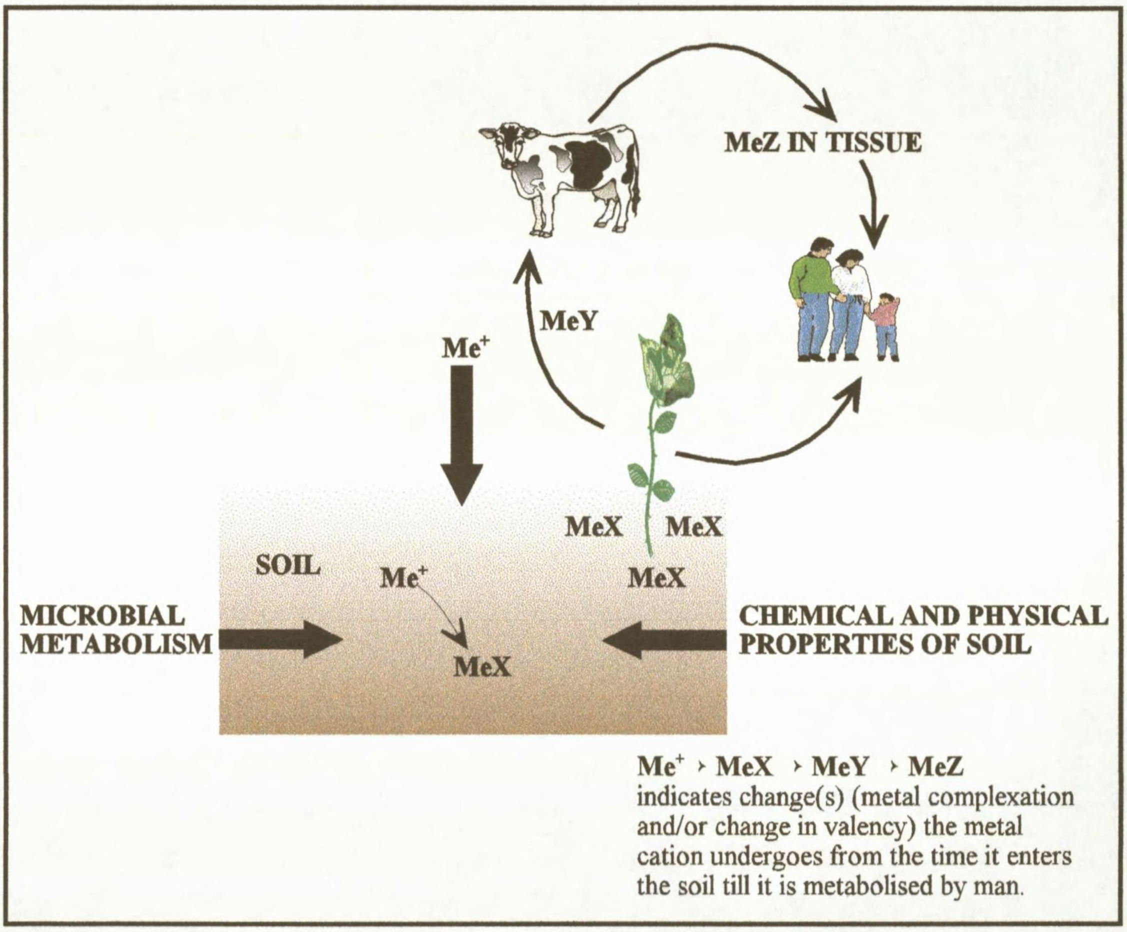
There are six basic areas requiring analysis:

1. The chemical form of the metal as it enters into soils.
2. Physical and chemical properties of the soil as they relate to the chemistry and solubility of the added metal.
3. Biochemical potential of soil as it relates to the "metabolism" of the added metal.

4. Transport and translocation of the most soluble or biologically available form of the metal into plant roots.

5. Plant metabolism as it relates to the translocated metal (if the plant component is consumed by man, the chemical form in the ingested tissue is of importance).

6. Transfer through gut and animal metabolism of the plant-incorporated metal (where, the form in the animal tissue determines the hazard of the metal to man) (White *et al.*, 1998a).



$Me^+ \rightarrow MeX \rightarrow MeY \rightarrow MeZ$
indicates change(s) (metal complexation and/or change in valency) the metal cation undergoes from the time it enters the soil till it is metabolised by man.

FIGURE 2.1 Postulated path of metals in the environment (White *et al.*, 1998).

Chromium modification, therefore, was analyzed as a function of growth of soil enrichment cultures on a range of carbon sources and in the presence and absence of the metal during a growth phase of the cultures. In order to determine whether the cultures transported, occluded, or associated with the metal, the distribution of chromium in the cellular and exocellular compartments of the cultures had to be evaluated. Gel permeation chromatography (GPC) of Cr-tagged materials verified changes in solubility of chromium in the exocellular media were accompanied by changes in the form of chromium compared to the medium alone (Barlett and James, 1988).

Thin-layer gel electrophoresis verified that alteration of Cr(VI) occurred on microbial growth and was visualized by staining with diphenylcarbazide. The exocellular soluble fraction of the fungal culture exposed to the chromium at the stationary phase contained three unidentified complexes, while only two were present in the continually exposed culture. All the complexes formed in the exocellular media were positively charged moving towards the negative pole. This suggested that soil microbiota, at least mixed culture, were capable of changing the valency of chromate in the media. It was also suggested that intermediates in the metabolism of the mixed cultures were able to reduce chromate, and that cultures stressed throughout growth by the presence of chromate maybe capable of different of further modification of the added metal (Barlett, 1991).

It has been reported that purified strains, like the mixed cultures, are capable of reducing chromate, both as a consequence of metabolism and, perhaps, as a result of more specific mechanisms of modification. Metabolites produced as a result of growth of chromium-resistant cultures appear to be capable of reducing chromate added to the milieu (Cotton and Wilkinson, 1988).

The results suggested that one may not have to postulate and then search for exotic classes of compounds to determine the potential for microbially mediated chromium modification and mobilization from soils. It may be some very common materials, such as amino acids, peptides, or saccharides, produced as a normal consequence of growth of chromium-resistant microbiota which may serve as vectors for chromium mobilization through soils to the roots of plants, to plant tissue, and then to man (Barlett and James, 1988).

2.2 BACTERIAL REDUCTION AS A MEANS OF BIOREMEDIATION

Waste treatment needs to be a low-cost process. Low cost and easily available materials such as calcinated coke and sawdust have been explored as Cr(VI) adsorbents. However, adsorption does not necessarily mean detoxification of Cr(VI). Since chemical reduction occurs only over a narrow range of pH, these methods require the use of chemical additives not only for Cr(VI) reduction, but also for pH adjustment. Furthermore, production of toxic gaseous byproducts such as SO_2 can also be a problem (Jacobs, 1992).

Bacterial reduction of Cr(VI) as a means of bioremediation has several potential advantages:

i. Reduction occurs under mild conditions;
ii. it requires neither chemical additives nor aeration;
iii. Anaerobic reduction minimizes excess sludge production in aqueous systems;
iv. no toxic byproduct is formed; and
v. the activity is reproducible and reusable (Bhide *et al.*, 1996).

There have been some attempts to use microorganisms as bioaccumulators of toxic heavy metals including Cr(VI). However, the process thus far appears successful only for dilute contaminated waters. Another attempt has been made to precipitate a wide variety of toxic metals by using microbes that release H_2S (Smillie *et al.*, 1981). This process may be a promising means of cleaning up dilute toxic wastes, but disadvantages of this process include slow rates of growth of obligate anaerobes and the production of unpleasant odours (Fude and Shigui, 1993).

Reduced chromium readily forms insoluble chromium hydroxides at a neutral pH. These chromium compounds are very stable, and they are unavailable to living organisms. The feasibility of the biological process to treat Cr(VI) on a laboratory scale has been successful and bioremediation is proving presently to be a very promising concept in terms of chromate detoxification as a full-scale process (Turick, 1997).

2.2.1 Chromate Reducing Microorganisms

Early investigations demonstrated that facultative anaerobes such as *Pseudomonas dechromaticans* (Romanenko and Korenkov, 1977), *Pseudomonas chromatophila* (Lebedeva and Lyalikova, 1979), and *Aeromonas dechromatica* (Kvasnikov *et al.*, 1985) reduced Cr(VI) from solution with the subsequent formation of a Cr(III) precipitate, presumably $Cr(OH)_3$. Studies have demonstrated that the capacity for Cr(VI) reduction is widespread and found in such organisms as *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa, Achromobacter eurydice, Micrococcus roseus,* and *Escherichia coil* (Gvozdyak *et al.*, 1987), as well as *Pseudomonas ambigua* (Horitsu *et al.*, 1987), *Pseudomonas fluorescens* (Bopp and Ehrlich, 1988), *D. desulfuricans* and *D. vulgaris* (Lovley and Phillips, 1988), *Enterobacter cloacae* (Wang *et al.*, 1989), *Streptomyces spp*. (Das and Chandra, 1990), and *Pseudomonas putida* (Ishibashi *et al.*, 1990).

Chromate reduction is not necessarily a Cr(VI)-resistance mechanism (Bopp and Ehrlich, 1988; Silver, 1992), and Cr(VI) reduction in *Ps. putida* and possibly other Cr(VI) reducers is a side activity for enzymes that have other, as yet unidentified, natural substrates (Ishibashi *et al.*, 1990; Cervantes, 1991). Although some microorganisms reduced Cr(VI) during anaerobic growth in media in which Cr(VI) was provided as the sole electron acceptor, in no instance had Cr(VI) reduction definitely been shown to yield energy to support anaerobic growth. For example, *Pseudomonas chromatophila* purportedly used Cr(VI) as an electron acceptor to support growth under anaerobic conditions with a variety of electron acceptors, including the nonfermentable substrate, acetate (Lebedeva and Lyalikova, 1979).

However, in the only data shown, most of the Cr(VI) reduction took place after growth stopped. No studies demonstrated that anaerobic growth depended upon the presence of Cr(VI) were presented. *Pseudomonas fluorescens* LB300, which reduced Cr(VI) while growing aerobically in a glucose medium, also grew in an anaerobic chamber containing oxygen-free N_2 on agar plates containing acetate as a potential electron donor and Cr(VI) as the potential electron acceptor (Bopp and Ehrlich, 1988). However, no Cr(VI) reduction occurred in anaerobic liquid cultures. Neither acetate oxidation nor Cr(VI) reduction under anaerobic conditions was documented. Further studies on whether electron transport to Cr(VI) can yield energy to support growth of this organism seem warranted given the difficulties in maintaining oxygen-free conditions in anaerobic chambers under a N_2 atmosphere.

Enterobacter cloacae strain HO1 reduced Cr(VI) while growing anaerobically in a medium that contained acetate and casamino acids as potential electron donors (Wang *et al.*, 1989). Oxygen rapidly inhibited Cr(VI) reduction, but reduction resumed when O_2 was removed . Amino acid mixtures have been reported as being the best electron donors for Cr(VI) reduction (Ohtake *et al.*, 1990a). The fate of the electron donors (i.e. whether they are oxidized to carbon dioxide or whether they are fermented) has not been reported. *Enterobacter cloacae* can grow anaerobically in the absence of added Cr(VI) and no evidence for Cr(VI)-dependent growth has been presented (Wang *et al.*, 1989). In fact, in one study, the number of viable cells decreased in the initial stages after Cr(VI) was added, and approximately 50% of the added Cr(VI) was reduced before viable cells numbers increased over what was presented prior to Cr(VI) addition (Ohtake *et al.*, 1990b).

2.2.2 Enzymes Involved in Cr(VI) Reduction

Cr(VI) reduction was found to be an enzymatically catalyzed reaction in the Cr(VI) reducers that have been studied in detail. For example, spent medium from cultures of *P. fluorescens* LB300 did not reduce Cr(VI), and washed cell suspensions reduced Cr(VI) only if glucose or another suitable electron donor was provided (Bopp and Ehrlich, 1988). Cyanide (10^{-3} M) inhibited Cr(VI) reduction in crude cell extracts, and the Cr(VI)-reducing capacity is lost when the membrane fraction was removed. Evidence for an enzymatic role in Cr(VI) reduction in *E. cloacae* was that: Cr(VI) was reduced faster with higher cell densities; no Cr(VI) reduction occured in cell-free controls; and inhibition of growth with antibiotics inhibited Cr(VI) reduction (Wang *et al.*, 1989). Cell-free filtrates of cultures did not reduce Cr(VI) (Wang *et al.*, 1990). Several metabolic poisons as well as molybdate, vanadate, and tellurate inhibited Cr(VI) reduction. Cr(VI) itself also inhibited Cr(VI) reduction. The rate of reduction declined as the concentration rose above 1 mM (Ohtake *et al.*, 1990b) . Temperature and pH optima for Cr (VI) reduction were characteristic of an enzymatically catalyzed reaction (Komori *et al.*,1990c).

The Cr(VI) reductase activity in *E. cloacae* was located in the membrane fraction (Wang *et al.*, 1990). When membrane vesicles were reduced with NADH and then exposed to Cr(VI) , the c - and b -type cytochromes were oxidized (Wang *et al.*, 1991). Further analyses have suggested that of the identifiable cytochromes in the membrane vesicles (c_{548}, c_{594}, c_{550}, b_{555}, b_{556}, and b_{558}), c_{548} might be specifically involved in Cr(VI) reduction, serving as a branch point between Cr(VI) and O_2 reduction.

In contrast to *Ps. dichromaticans* and *E. cloacae*, the Cr(VI)-reducing activity in *Ps. ambigua* and *Ps. putida* was found to be in the soluble fraction of the cell (Horitsu *et al.*, 1987, Ishibashi *et al.*, 1990). A 65-kDa protein had been purified from *Ps. ambigua* that could reduce Cr(VI) with NADH or NADPH serving as the electron donor (Suzuki *et al.*, 1992). The enzyme initially reduced Cr(VI) to Cr(V), which was subsequently reduced to Cr(III). NADH also reduced Cr(VI) to Cr(V) in the absence of the enzyme, but at slower rates than with the enzymes.

Washed cell suspensions of *D. desulfuricans* and *D. vulgaris* rapidly reduced Cr(VI) to Cr(III) under anaerobic conditions with H_2 as the electron donor (Lovley and Phillips, 1988). H_2-dependent, Cr(VI) reductase activity in the soluble, cell-free fraction of *D. vulgaris* was lost when the soluble fraction was passed over a cation-exchange column that removed cytochrome *c3*, a periplasmic protein. The capacity for Cr(VI) reduction was restored when cytochrome *c3* was added again. In the presence of H_2 and an excess of hydrogenase, cytochrome *c3* reduced Cr(VI) at a rate 50-fold faster than the maximum rate for Cr(VI) reduction by the Cr(VI) reductase purified from *Ps. ambigua.*

2.2.3 Kinetics and Modelling of Chromate Reduction

To optimize design and operation of biological processes for Cr(VI) reduction, a thorough understanding of the kinetic characteristics of microbial transformation is needed. Shen and Wang (1994), developed a kinetic model, which incorporated a finite Cr(VI) reduction capacity term for describing toxic effects of Cr(VI). It was also demonstrated that the rate of Cr(VI) reduction by enzymes can be expressed as the Monod equation (1), when soluble reductase activity is the predominant mechanism of Cr(VI) reduction in bacterial cells:

$$\frac{-dC}{dt} = \frac{k_m C}{K_c + C} X \qquad \textbf{(1)}$$

where C (mg.L^{-1}) is the concentration of Cr(VI) at time t (h); X (cells.L^{-1}) is the density of active bacterial cells at time t; k_m (mg Cr(VI).cell^{-1}.h^{-1}) is the maximum specific rate of Cr(VI) reduction; and Kc (mg Cr(VI).L^{-1}) is the half-velocity constant. However, the active cell concentration, X, may be assumed to decrease in proportion to the amount of Cr(VI) reduced due to the toxicity of Cr(VI):

$$X = X_0 - \frac{C_0 - C}{R_c} \qquad \textbf{(2)}$$

where C_0 (mg.L^{-1}) is the initial concentration of Cr(VI); X_0 (cells.L^{-1}) is the initial cell density of Cr(VI)-reducing strains; and Rc (mg Cr(VI).cell^{-1}) is the maximum Cr(VI) reduction capacity of cells. Substituting equation (2) into equation (1) yielded the following equation which describes the rate of Cr(VI) reduction:

$$\frac{-dC}{dt} = \frac{kmC}{Kc + C} \left(X0 - \frac{C_0 - C}{R_c} \right) \qquad \textbf{(3)}$$

Equation (3) can be integrated to yield equation (4):

$$t = \frac{Kc}{km\left(\frac{C_0}{R_c} - X_0\right)} \ln\left[\frac{CX_0}{C_0\left(X_0 - \frac{C_0 - C}{R_c}\right)}\right] + \frac{R_c}{k_m} \ln\left[\frac{X_0}{X_0 - \frac{C_0 - C}{R_c}}\right] \qquad \textbf{(4)}$$

Equation (4) can be solved only when X_0 is greater than $(C_0 - C)/R_c$. The initial density of bacterial cells, X_0, determines the maximum amount of Cr(VI) that can be reduced ($R_c X_c$) in a batch culture. Equation (4) is an appropriate expression to cope with both toxicity and mutation effects of Cr(VI) on Cr(VI) reduction. The consistency between model simulations and experimental data as demonstrated in studies by Shen and Wang (1997) verified the kinetic model which incorporates a finite Cr(VI) reduction capacity regardless of subsequent cell growth during Cr(VI) reduction. However, a modification of the developed model may be necessary for application in the field where many environmental factors such as pH, temperature, dissolved oxygen, and co-contaminants may influence the rate of Cr(VI) reduction which was illustrated by Shen and Wang (1994a,b), Wang and Shen (1995), and Wang and Xiao (1995).

2.3 FEATURES OF BACTERIAL CHROMATE REDUCTION

Enterobacter cloacae strain HO1, which was resistant to Cr(VI) under both aerobic and anaerobic conditions, could only reduce Cr(VI) anaerobically. Transformation from Cr(VI) to Cr(III) was confirmed using electron paramagnetic resonance (EPR) spectroscopy. After anaerobic incubation, the culture originated a signal that could be attributed to the paramagnetic species, Cr(III). The intensity of the signal, calculated from the height of the maximum peak of absorption, increased as the incubation proceeded , and the EPR signal was observed only when cells were grown anaerobically with Cr(VI). No Cr(VI) reduction was observed in the absence of bacterial cells. Neither fresh broth nor cell-free filtrate of anaerobic cultures showed reduction of Cr(VI) (Ohtake and Hardoyo, 1992).

Inhibition of cell growth by the addition of penicillin, cycloserine or chloramphenicol resulted in loss of Cr(VI) reduction activity. The reduction presumably took place on the cell surface. The reduced chromium existed mainly in the external medium, forming insoluble chromium hydroxides. Transmission electron microscopic examination of the centrifugal pellets revealed that electron-scattering particles were deposited at the outside of bacterial cells, and energy-dispersive X-ray analyses showed that these precipitates contained exclusively chromium (Fujii *et al.*, 1990). *Enterobacter cloacae* strain HO1 reportedly utilized toxic Cr(VI) anaerobically as a terminal electron acceptor (Ohtake *et al.*, 1992). The chromate reductase activity was preferentially associated with the membrane fraction of *E. cloacae* cells prepared as right-side-out membrane vesicles. Ascorbate-phenazine methosulfate functioned as an effective electron donor with such membrane preparations.

2.3.1 Factors Affecting Chromate Reduction

The rate of chromate reduction was found to be dependent upon cell density (Ohtake and Hardoyo, 1992). The rate of Cr(VI) reduction was also dependent on the amount of added Cr(VI). Increasing the concentration of Cr(VI) decreased the reduction rate, and the time required for complete reduction increased. *E. cloacae* HO1 cells were able to reduce completely concentrations as high as 520 $mg.L^{-1}$ within 11 h. Chromate reduction occurred at temperatures of 10 - 50^0C with the optimum temperature around 37^0C and over pH range 6.5-8.5 with the optimum at pH 7.0.

Chromate reduction by *E. cloacae* strain HO1 was sensitive to oxygen tension. Aeration eliminated the reductase activity of the culture grown anaerobically. The redox potential which changed from -60 to -220 mV did not influence the rate of Cr(VI) reduction. However, strict anaerobic conditions were not required for supporting the Cr(VI) reductase activity. A variety of organic substances were examined for their effects on Cr(VI) reduction. Amino acids including alanine, glutamine, glycine, methionine and threonine were effectively utilized as electron donors for Cr(VI) reduction. Aspartate, isocitrate, malate, oxalate and tartrate also stimulated Cr(VI) reduction by growing cultures. Glucose, pyruvate and lactate depressed Cr(VI) reduction, though they supported bacterial growth under anaerobic conditions. Metabolic poisons including carbonylcyanide-m-chlorophenyl hydrazone, 2,4-dinitrophenol, sodium cyanide, and formaldehyde inhibited Cr(VI) reduction. These metabolic poisons were reported to affect protein synthesis thus the enzyme responsible for Cr(VI) reduction was directly affected by these poisons. No inhibition was observed with antimycin, sodium azide and 2-hepylhydroxyquinolone-N-oxide probably because these chemicals had no effect on protein synthesis (Fujii *et al.*, 1990).

2.3.2 Biological Chromate Removal Strategies

Fed-batch cultures, where Cr(VI) was continuously added in small doses to minimize the toxic effect, showed elevated performance of detoxifying Cr(VI) in aqueous solutions. At a level of 52 $mg.L^{-1}$ Cr(VI), the specific rate of Cr(VI) reduction was to the extent of 22 mMol CrO_4^{2-} dry $cells^{-1}$ h^{-1}. Performance of the fed-batch process was strongly influenced by the feeding rate of carbon and energy sources, as well as Cr(VI).

The fed batch process has several potential advantages:

i. It was possible to keep Cr(VI) concentration below the toxic level.

ii. The feed of Cr(VI) is controlled by monitoring the medium colour which changed from yellow to white on Cr(VI) reduction and precipitation as $Cr(OH)_3$.

iii. An adequate population of Cr(VI) reducing cells could be maintained under Cr(VI) stress (Wang and Shen, 1997).

From a viewpoint of pollution control, it is desirable to remove total chromium from wastes (though this may not be an economic benefit). Two types of bioreactors were tested: dialysis-bag and anion-exchange membrane reactors. In the dialysis-bag reactor, *E. cloacae* cells were put in a semipermeable membrane bag, and the bag was submerged in Cr(VI) containing solution. The system has the advantage that precipitated reduced chromium could be collected inside the bag. Cr(VI) was allowed to diffuse into the culture which reduced Cr(VI) to Cr(III) inside the bag. Since reduced chromium readily forms insoluble chromium hydroxides, the greater part of reduced chromium was left immobilized inside the bag. Experiments with a bench-scale reactor showed that the chromium removal process was limited by Cr(VI) diffusion through the membrane (Ohtake and Hardoyo, 1992).

2.3.3 Dialysis-bag and Anion exchange Reactor for Chromate Reduction

Performance of a dialysis-bag reactor which contained cells of *Enterobacter cloacae* was strongly dependent on the size of the surface area of membrane, as well as the cell density in the dialysis bag. The dialysis bag reactor showed about 90% removal of total chromium at an initial Cr(VI) concentration of 208 $mg.L^{-1}$ in a time period of four days. About 10% of reduced chromium diffused out from the dialysis bag, before precipitating (Komori *et al.*, 1990b).

An anion-exchange membrane reactor was also proved effective for removing total chromium from wastes. Anion-exchange membrane allowed the transfer of chromate (a divalent anion, CrO_4^{2-}), whereas reduced chromium (a trivalent cation, Cr(III)) was prevented from diffusing back across the membrane. The unit consisted of two chambers between which an anion-exchange membrane (Selemion AMV; Asahi Glass Co., Tokyo, Japan) was clamped. One chamber was filled with Cr(VI) contaminated solution, while the other chamber contained anaerobic culture of *E. cloacae* HO1. Potassium chloride was used as the counter anion for CrO_4^{2-} diffusing across the membrane, and no electric potential was applied across the chambers. Successful removal of total chromium was observed in this type of bioreactor. About 94 % of the initial Cr(VI) was removed from waste solution within 50 h. This performance could be improved by increasing the surface area of the anion-exchange membrane (Srivasta *et al.*, 1986; Sag and Kutsal, 1989).

Bacterial reduction of chromate was further examined in a Cr(VI) contaminated industrial effluent. The effluent, taken from a manufacturing process of copper-chromium catalysts, contained 364 $mg.L^{-1}$ Cr(VI) and several metal cations including Cu^{2+}, Mn^{2+}, Zn^{2+} and Mg^{2+}. Since the industrial effluent didn't contain any organic compounds which were available to bacterial cells, a small supplement of broth was needed to provide carbon and energy sources. It was also necessary to dilute the waste solution prior to exposure to the bacteria. The original pH of the waste solution was 6.45, and changing the pH value did not allow the reduction of Cr(VI). Since *E. cloacae* HO1 was able to reduce as high as 520 $mg.L^{-1}$ of Cr(VI) in broth, some components, probably metal cations, seemed to inhibit the ability to reduce Cr(VI) in the waste solution (Ohtake *et al.*, 1990c; Komori *et al.*, 1989).

Indeed, when the waste solution was dialyzed through an anion-exchange membrane, across which cations were not allowed to diffuse, a substantial reduction of Cr(VI) occurred. The rates of Cr(VI) reduction were dependent on the amount of carbon and energy sources available for bacteria. The rates of Cr(VI) reduction were also strongly dependent on the cell density (Komori *et al.*, 1989). No Cr(VI) reduction was observed in the absence of bacterial cells. Increasing the cell density greatly increased the rates of Cr(VI) reduction.

2.3.4 Genetics of Chromate Reduction in Gram-negative Bacteria

Both resistance and reduction occurred, but resistance to chromate governed by plasmids of Gram-negative bacteria appeared to have nothing to do with chromate reduction. Furthermore, it was not clear whether the chromate reduction ability found with several bacterial isolates conferred resistance to CrO_4^{2-} or not (Ohtake and Silver, 1994). Plasmid-mediated chromate resistance was due to reduced cellular accumulation of chromate (Nies *et al.*, 1990; Ohtake *et al.*, 1987). Whether the reduced cellular accumulation resulted from accelerated efflux is unknown.

The two sequenced chromate resistance determinants (*chr* operons) from plasmids of *Ps. aeruginosa* (Cervantes *et al.*, 1990) and *A. eutrophus* (Nies *et al.*, 1990) were quite similar. Both *chr* operons contained the long *chrA* gene. ChrA was identified as a membrane protein in *E.coli*, although the cloned *chr* operon did not confer chromate resistance in *E.coli*. The actual role of ChrA in chromate transport and the energy coupling remains unknown (Cervantes *et al.*, 1990 and Nies *et al.*, 1990).

Bacterial reduction of chromate had been observed with numerous Gram-positive and Gram-negative bacteria, under both aerobic and anaerobic conditions (Cervantes and Silver, 1992; Ohtake and Silver, 1994). However, it is only recently that the enzymes carrying out chromate reduction have been characterized in much detail (Myers *et al.*, 2000). Chromate reduction activity could be associated with either the cytoplasmic fraction (Ishibashi *et al.*, 1990; Suzuki *et al.*, 1992) or cell membranes (Wang *et al.*, 1990). Ishibashi *et al.* (1990) preliminarily characterized a soluble chromate reduction activity from a *Ps.putida* strain and found the reduction activity was NAD(P)H-dependent. A cell-free extract from *Streptomyces* also reduced Cr(VI) to Cr(III) in the presence of NAD(P)H.

The chromate reductase from *Enterobacter cloacae* strain HO1 has been studied extensively (Ohtake and Silver, 1994). However, the basic reduction mechanism of the reductase enzyme has only recently being investigated and understood (Myers *et al.*, 2000). The *Enterobacter* strain confered resistance to chromate under both aerobic and anaerobic conditions (Wang *et al.*, 1990; Cervantes and Silver, 1992; Ohtake and Silver, 1994). It was believed that *E.cloacae* strain HO1 utilized Cr(VI) as a terminal electron acceptor (Ohtake and Silver, 1994; Ohtake *et al.*, 1990a,b). The chromate reductase from *Pseudomonas ambigua* strain G1 was purified and partially characterized (Suzuki *et al.*, 1992). The purified *Pseudomonas* enzyme reduced Cr(VI) to Cr(III) in vitro using NAD(P)H as electron donor with Cr(V) as a possible intermediate (Suzuki *et al.*, 1992).

2.4 MECHANISMS OF CHROMATE REDUCTION

2.4.1 Characterization of Cr(VI) Reductase

The reduction of Cr(VI) has been reported for a number of bacterial genera, including *Pseudomonas* (Bopp and Ehrlich, 1988; Gopalan and Veeramani, 1994; Bhide *et al.*, 1996; Turick and Apel, 1997), *Bacillus* (Campos *et al.*, 1995), *Enterobacter* (Wang *et al.*, 1989; Rege *et al.*, 1997), *Thiobacillus* (Sisti *et al.*, 1996) and sulfate reducing bacteria (Fude *et al.*, 1994). In some cases, the reduction of Cr(VI) was indirectly mediated by the production of reducing agents as metabolites; for example, *Thiobacillus ferrooxidans* generates sulphite and thiosulphate which could reduce Cr(VI) at pH 1.7 (Sisti *et al.*, 1996), and sulphate-reducing bacteria reduce Cr(VI) indirectly via the production of sulphide (Fude *et al.*, 1994). In other cases, the bacteria utilized sugars (e.g. molasses (Bhide *et al.*, 1996), glucose (Campos *et al.*, 1995) or sucrose (Rege *et al.*, 1997)) as carbon and energy sources to mediate Cr(VI) reduction via a CM-associated electron transport chain.

While localization of the Cr(VI) reductase activity in some strains has not been determined, the activities in *Bacillus* strain QC1-2 (Campos *et al.*, 1995) and *Pseudomonas putida* (Ishibashi *et al.*, 1990) were associated with the cytosolic and soluble fractions, respectively. In contrast, the Cr(VI) reductase activities in *Enterobacter* and *Ps. dechromaticans* appeared to be membrane-associated (Bopp and Ehrlich, 1988; Wang *et al.*, 1990). However, the Cr(VI) reductase activity in *Shewanella putrefaciens* MR-1 was found to be localized to the cytoplasmic membrane (CM), in comparison with the periplasmic localization of its fumarate reductase (Myers and Myers, 1992) and the outer membrane (OM) localization of its ferric reductase (Myers and Myers, 1993), suggesting that the Cr(VI) reductase activity was distinct from that of fumarate and iron reductases.

Cr(VI) reductase activity was found primarily in the cytoplasmic membrane. Heat-denatured CM showed no Cr(VI) reductase activity, or when formate was omitted (TABLE 2.1). The addition of ferric chloride did not change the rate of Cr(VI) reduction catalyzed by the CM (TABLE 2.1). Myers *et al.*, (2000) also tested for the ability of alternative electron donors to support Cr(VI) reduction. Relative to formate, neither L-lactate nor NAD(P)H supported Cr(VI) reduction whereas NADH supported even higher rates than formate (TABLE 2.2).

TABLE 2.1 **Effect of various treatments on the *in vitro* Cr(VI) reductase activity of the cytoplasmic membrane (Myers *et al.*, 2000).**

Treatment	Relative Cr(VI) reductase
Formate	1.0
Boiled	0.02
No formate	0.02
+ Fe	1.0

Formate = CM + 10 mmol.L^{-1} formate; Boiled = CM boiled for 10 min before use + 10 mmol.L^{-1} formate; No formate = CM only; +Fe = CM + 10 mmol.L^{-1} formate + 13 μmol.L^{-1} $FeCl_3$.

TABLE 2.2 **Effect of alternate electron donors on the relative *in vitro* Cr(VI) reductase activity of the cytoplasmic membrane (Myers *et al.*, 2000).**

Electron donor	Relative Cr(VI) reductase
Formate	1.0
NADH	4.48
NADPH	0.01
Lactate	0.01

Final concentration of each electron donor was 2.0 mmol.L^{-1} and activities are expressed relative to formate, which was arbitrarily set to 1.0.

Based on localization and other properties, the Cr(VI) reductase was distinct from the fumarate and Fe(III) reductases in *Shewanella putrefaciens* MR-1, and was not irreversibly inhibited by exposure to O_2 (Myers and Myers, 1992). Inhibitor studies suggested the involvement of a multi-component electron transport chain that could include cytochromes, quinones, flavoproteins, and proteins with iron sulfur centers. Additional studies on the Cr(VI) reductase activity in MR-1 would be useful in assessing its potential for the bioremediation of Cr(VI)-contaminated sites or industrial effluents.

2.5 APPLICATIONS OF CHROMATE REDUCTION

Reduction of chromate under aerobic conditions is not unique. Bopp and Ehrlich (1988) showed that although chromate sensitive strains *Ps. fluorescens* LB303 and *Ps. putida* AC10 could not grow at elevated chromate concentrations at which chromate-resistant *Ps. fluorescens* LB300 grew, non-growing, resting cell suspensions of all three strains could reduce chromate with glucose or NADH. Evidently high chromate concentrations prevent multiplication of the chromate sensitive strains but do not prevent them from reducing chromate. Thus, chromate resistance in *Ps. fluorescens* LB300 is not related to its ability to reduce chromate (TABLE 2.3).

TABLE 2.3 Minimum inhibitory concentrations (MIC) of chromate.

Strain	Origin	MIC K_2CrO_4 ($mg.L^{-1}$)
Ps. fluorescens LB300	River [a] isolate (pLHB1$^+$)	> 1000
Ps. fluorescens LB303	Spontaneous segregant (pLHB1$^-$)	10
Ps. fluorescens LB304	Transformant of LB303 (pLBH1$^+$)	> 1000
Ps. putida AC10	Lab strain	10

[a] pLHB1 is a chromate resistance plasmid originally isolated from *Ps. fluorescens* LB300.

The reduction by these strains appeared to be enzymatic, since it was catalyzed by cell extracts. Enzymatic chromate reduction by organisms such as *Ps. fluorescens* LB300 appeared to be a form of respiration in intact cells and may have beneficial environmental effects. The product of chromate reduction was Cr(III), which is several orders of magnitudes less toxic than Cr(VI) (Venitt and Levy, 1974; Nishioka, 1975; Mearns *et al.*, 1976; Petrilli and DeFlora, 1977). Furthermore, Cr(III) formed insoluble hydroxides at neutral pH and precipitates, thus making it less available to biological systems. The fact that chromate reductase activity was demonstrated in the S_{32} fraction with addition of NADH but not in the S_{150} fraction seemed to indicate that some or all of the enzymes necessary for the transfer of electrons from NADH to chromate are membrane-bound. The inhibition of chromate reduction by cyanide and azide and the ability of NADH to act as electron donor suggested that an electron transport system was involved (Bopp and Ehrlich, 1988).

Since LB300 and some other chromate-reducing pseudomonads were able to grow anaerobically using chromate as terminal electron acceptor (Romanenko and Korenkov, 1977; Lebedeva and Lyalikova, 1979; Kvasnikov *et al.*, 1985). *Pseudomonas fluorescens* LB300 was also able to grow anaerobically using nitrate as terminal electron acceptor, but unlike chromate, nitrate was not reduced under aerobic conditions. The chromate reductase in the three strains was similar to the reductase reported by Horitsu *et al.*, (1987) from *Ps. ambiguas* strain G-1 in that it can use NADH as electron donor for the reduction of chromate, but it also differed in that intact cells of the *Pseudomonas* strains could use exogenous glucose as an electron donor whereas intact cells of *Ps. ambigua* G-1 could not. Chromate reductase activity was constitutive in all three strains. In the chromate-resistant *Ps. fluorescens* LB300, no difference in rate of chromate reduction by resting cells grown with and without added chromate was noted. In the chromate-sensitive strains, the rate of chromate reduction by resting cells was essentially the same as in *Ps. fluorescens* LB300, even though the cells were grown without chromate.

The usual method for selecting strains capable of oxidizing or reducing metals involves enriching environmental samples in the presence of the metals of interest. In the case of chromium, this method would tend to select against metal sensitive strains with a capacity to reduce Cr(VI). Ohtake *et al.*, (1987) showed that chromate-resistance of *Ps. fluorescens* LB300 appears to be related to lessened chromate uptake relative to the plasmidless, chromate-sensitive strain LB303. They also showed that chromate uptake depended on a sulphate active transport system. Although the interactions of some bacteria with chromate are being clarified, more research is required before all mechanisms of chromate resistance and chromate reduction are fully understood.

2.5.1 Chromate Reduction in Soils

Since Cr(VI) utilizing bacteria are common to the environment, the possibilities of treating Cr(VI) in contaminated soils, in place has been explored. By exploiting the indigenous Cr(VI) utilizers through the addition of bacterial nutrients to soils or groundwater, these bacteria could bioreduce the Cr(VI) to Cr(III) without the need to excavate any soil, which is in many cases the only option. Since the Cr(III) is much less soluble than Cr(VI) it could adsorb to the soil and become immobilized, drastically minimizing the problem of Cr(VI) contamination. Results to date demonstrate that Cr(VI) utilizers in soils could easily be exploited for safe and economically practical bioreduction of Cr(VI) in place (Apel and Turick, 1991).

However, chromium-contaminated soils present a unique set of problems because the chemistry of chromium is so complex (Barlett, 1991). Hexavalent chromium exists in neutral-to-alkaline waste-amended soils principally as the soluble chromate anion (CrO_4^{2-}), or as moderately-to sparingly soluble chromate salts (e.g., $CaCrO_4$, $BaCrO_4$ and $PbCrO_4$). Oxidation and reduction reactions can convert Cr(III) to Cr(VI) and vice versa. The potential for this conversion from nontoxic Cr(III) to toxic Cr(VI) has complicated the task of determining whether a chromate-bearing waste or waste-contaminated soil is hazardous. Barlett and James, (1979) showed that a variable function (typically $< 15\%$) of Cr(III) as $Cr(OH)_3$ oxidized to Cr(VI) in laboratory tests. When soluble Cr(III) was added, the extent of oxidation was proportional to the level of easily reductable Mn(III, IV) hydroxides and oxides in the soil. But the extent of oxidation also depended on the form of added Cr(III) (soluble vs. insoluble; organically complexed vs. inorganic forms). Reduction reactions of Cr(VI) may occur simultaneously with oxidation of Cr(III) in heterogeneous soil materials containing organic matter, Fe(II) and Mn(III, IV) hydroxides and oxides (Lovely and Phillips, 1988). The relationship between Cr(III) and Cr(VI) depended on a balance between oxidation and reduction, as depicted in FIG 2.2. The soil pH was a master variable (a rolling weight on the see-saw) that controlled the balance between the two opposing redox reactions. Reduction of Cr(VI) by organic matter and other electron donors [e.g., Fe(II) and sulfides] was favoured by pH values < 6, and both oxidation and reduction may be inhibited under more alkaline conditions (Barlett, 1991). The coupling of oxidation and reduction reactions for Cr in soils also may be viewed as a cycle (FIG 2.3) in which Cr(III) and Cr(VI) are interconverted by a manganese redox cycle and the oxidation of organic matter. Irreversible reduction of Cr(VI) to insoluble forms of Cr(III) is the chemistry underlying "remediation by reduction" strategies. The interest is to produce a "clean soil" in which reduction of Cr(VI) to Cr(III) has occurred. Such a valence-specific approach to remediation of chromate contaminated sites is a significant departure from approaches used for other heavy metals when valence is not a criterion in the analysis, regulation, and remediation of contaminated soils (Palmer and Wittbrodt, 1991). However, managing and monitoring soil pH during remediation will also be needed. The environmental contamination associated with historic chromate production, metal processing, and other uses must be addressed to protect human health from the adverse effects of Cr(VI). Advances in the quantification of soilborne Cr(VI), the anticipated establishment of Cr(VI) cleanup standards, and the development of remediation methods offer the promise that the goal can be achieved.

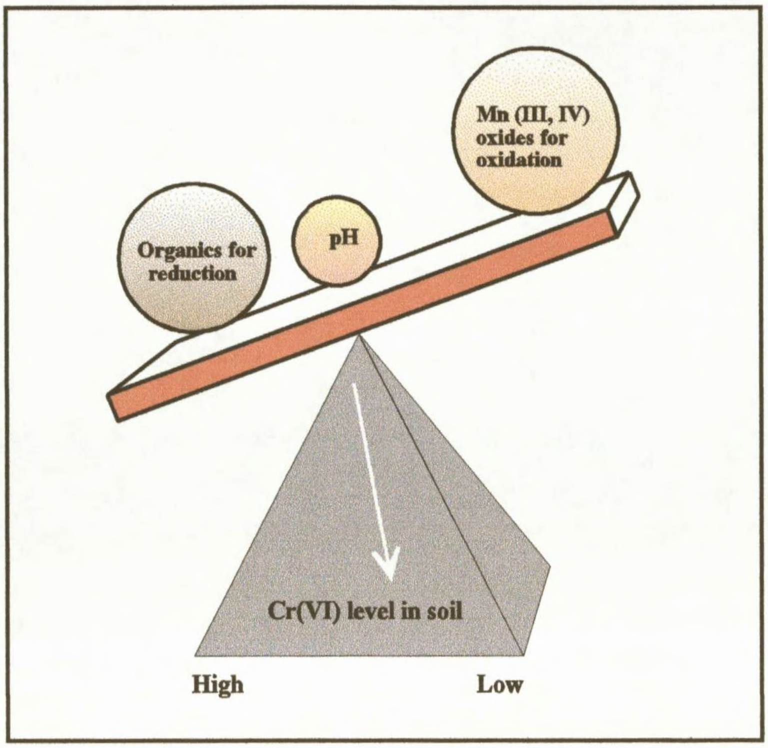


FIGURE 2.2 The role of pH on chromate reduction in soil (Barlett and James, 1988).

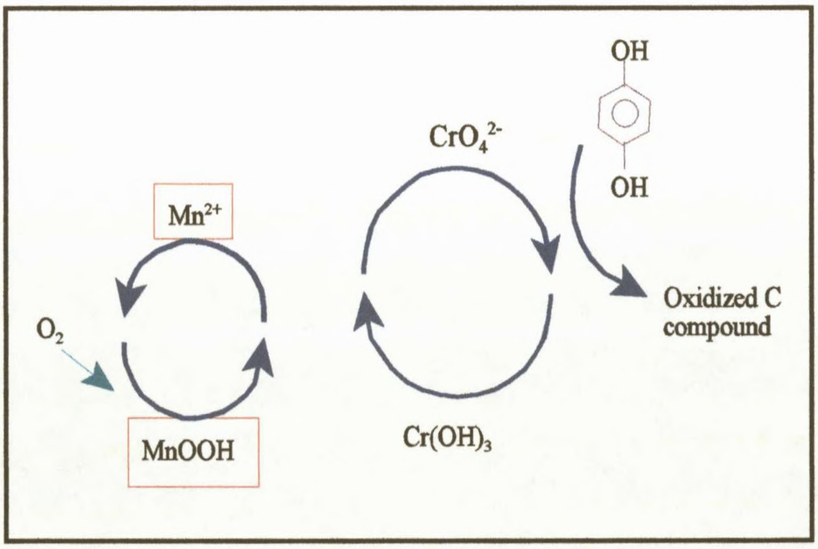


FIGURE 2.3 Interconversion of Cr(III) and Cr(VI) in soils governed by an Mn redox cycle and reactions (Barlett and James, 1988).

2.5.2 Chromate Reduction by a Consortium of Sulphate Reducing Bacteria

Sulphate reducing bacteria could withstand very high concentrations of toxic Cr(VI) and managed to immobilize substantial quantities of it. The addition of other toxic heavy metals (i.e., Zn) and even a potential radionuclide (i.e., U) did not alter these immobilization qualities, and these heavy metals themselves became incorporated into the Cr precipitate. Viable cells were responsible for the major immobilization power of the microbial consortium and bioreduction was necessary (Fude *et al.,* 1994).

Accordingly, at least two possibilities for the Cr immobilization are possible. First, the bacteria could directly reduce Cr(VI) to Cr(III) (Ishibashi *et al.,* 1990; Ohtake *et al.,* 1990c,d; Wang *et al.,* 1990). This type of reduction has been shown via a Cr(VI) → Cr(V) → Cr(III) pathway in *Pseudomonas ambigua* G-1 (Suzuki *et al.,* 1992). If such a possibility was correct, then both H_2S and Cr(III) would rapidly accumulate in the medium and the E_h would rapidly decrease. However, these H_2S and E_h phenomena were not evident. A second possibility was that the bacteria produce H_2S that acts as the reducing agent for Cr(VI), which also has been observed in marine environments (Smillie *et al.,* 1981). In such a case, H_2S would be reoxidized as it is produced by the bacteria and the E_h would not drop until all or most of Cr(VI) was reduced.

The reduction of Cr(VI) via an H_2S mechanism was corroborated when the cells were grown in the presence of molybdate, which acts as an inhibitor of sulphate reduction by microorganisms (Tugel *et al.,* 1986). So far, although enrichment was possible, pure cultures from the consortium using a variety of media and culturing conditions to obtain pure isolate was not achievable. Therefore, taxonomic identification of the gram-positive and gram-negative rods has not been possible. Presumably, there was a strong interdependence among the members of the SRB III consortium. The data suggested that the SRB III consortium may be useful for the bioremediation of fluid metal-refining wastes.

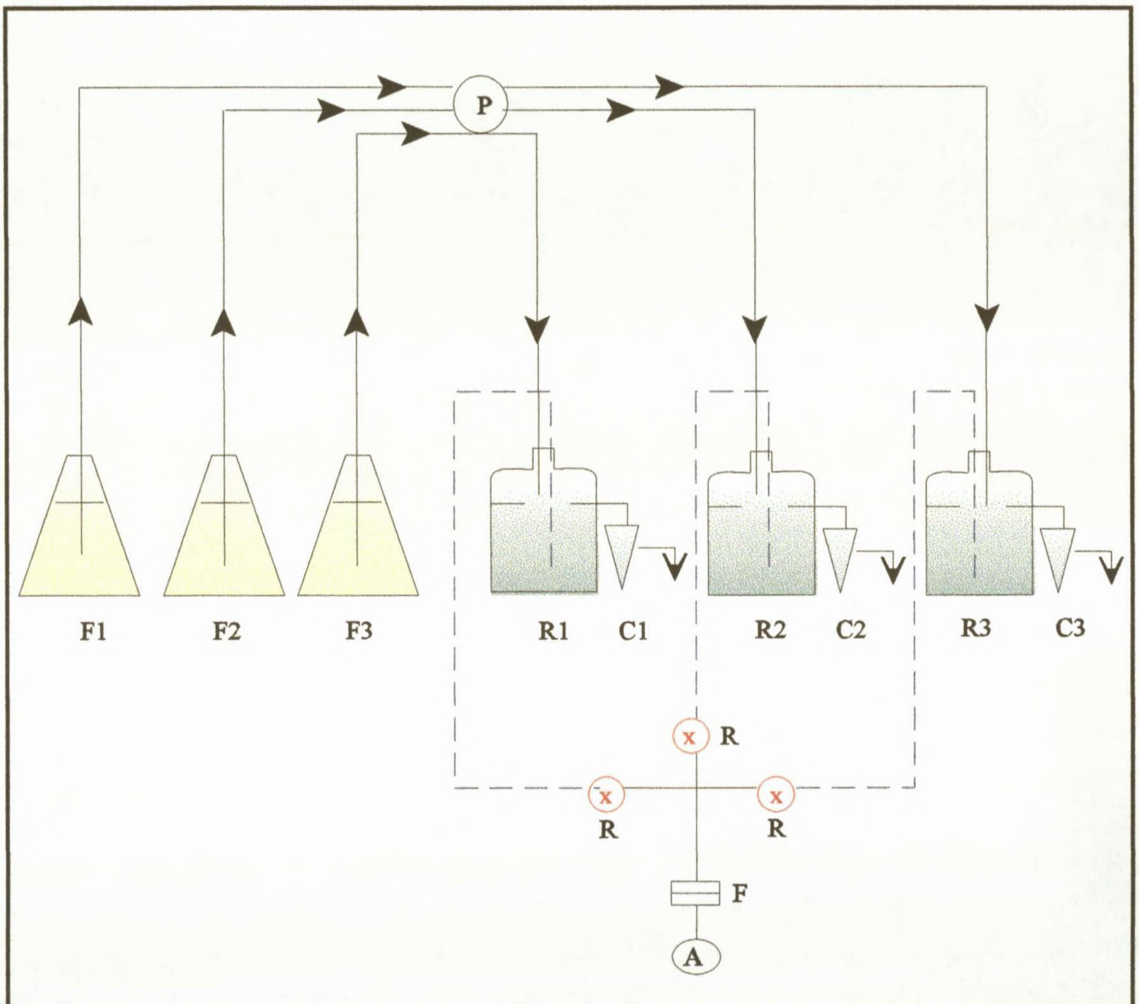
2.5.3 Continuous Microbial Chromate Reduction

Various strains of *Pseudomonas* sp. have been recognized as efficient chromate reducers (Bopp and Ehrlich, 1988). Under anaerobic conditions isolates like *Ps. dechromaticans* and *E. cloacae* HO1, resistant to Cr(VI) concentrations up to 1500 mg.L^{-1}, were reportedly effective in chromate reduction (Ilyaletdinov, 1988; Wang *et al.*, 1989). Pseudomonads like *Ps. fluorescens* LB 300 and *Ps. ambigua* G-1 have been used for the reduction of chromate aerobically (Bopp and Ehrlich, 1988; Horitsu *et al.*, 1987).

Coleman and Paran , (1991) studied the aerobic removal of Cr(VI) from synthetic wastewater containing 100 to 200 mg.L^{-1} Cr(VI) by a microbial consortium in laboratory continuous rotating biological contactor. The potential applicability of continuous microbial detoxification of Cr(VI) (10 to 40 mg.L^{-1}) with 99% efficiency has been reported on a pilot scale in a suspended growth, anaerobic system using *Ps.dechromaticans* for treating effluent from an automobile manufacturing unit admixed with municipal sewage (Ilyaletdinov, 1988).

The performance of biological systems for potential handling of industrial effluents can be augmented to counter the inhibitory effect of Cr(VI) by the addition of supplemental chromate-reducing cultures developed in retrofit enrichment reactors. The performance of the continuous reactor R2 using *Pseudomonas* sp. is shown in FIG 2.4 as a typical profile of MLVSS (Mixed Liquor Volatile Suspended Solids), pH, and chromate reduction efficiency. The MLVSS decreased by 50% during a HRT (Hydraulic Retention Time) of 72 h and chromate reduction efficiency gradually increased to the steady-state level of 88%. With a decrease in HRT to 60 h, VSS (Volatile Suspended Solids) level stabilized at 450 mg.L^{-1} and steady-state Cr(VI) reduction efficiency stabilized at 91% with essentially no change in the concentration of the active biomass (Gopalan and Veeramani, 1994).

The pH in the reactors was between 7.3 and 8.5 with maximum pH levels of 8.5, 7.8, and 7.5, corresponding to chromate reduction efficiencies of 86%, 88%, and 81% in reactors R1, R2, and R3, respectively, with a total HRT of 72 h. The characteristic green colour of the medium in the three reactors, vis-á-vis the yellow colour of the chromate in the feed, indicated reduction to the trivalent state.



Air (----); Liquid (—); P - peristaltic pump, A - compressed air; F - air filter; R - rotameter; Ri - reactors (i = 1, 2, 3); Ci - clarifiers (i = 1, 2, 3); Fi feed tanks (i= 1, 2, 3)

FIGURE 2.4 **Schematic representation of a continuous culture of *Pseudomonas* sp. for the reduction of chromate (Goopalan and Veeramani, 1994).**

A summary of the performance of the three reactors at steady - state conditions, at different levels of the operating parameters, led to the following major trends in behaviour during chromate reduction (Goopalan and Veeramani, 1994).

1. Suspended growth culture of *Pseudomonas* sp. in CSTR (Continuous Stirred Tank Reactor) were capable of efficient (80% to 100%) reduction of chromate [Cr(VI): 5 to 120 mg.L^{-1}] to trivalent chromium.
2. A high sludge growth retention time of 60 to 72 h was necessary, without recycling of biomass, to obtain a chromate reduction efficiency of 80% to 100% at influent Cr(VI) levels of 5 to 124 mg.L^{-1}.
3. A biomass concentration of 450 to 500 mg.L^{-1} of chromate-resistant *Pseudomonas* sp. was capable of sustaining the desired high chromate reduction efficiency at a sludge retention time (SRT) of 60 to 72 h, equivalent to the HRT.
4. Reactor pH in the range 7.5 to 8.5 was suitable for chromate reduction.
5. The total chromium concentration of the treated effluent indicated that maximum toxicity reduction of 80% to 100% was obtainable, and that detoxified chromium in the trivalent form was maintained in a soluble state.
6. The proportion of chromium associated with the sludge was rather low (below 3%).
7. Chemical oxygen demand (COD) removal efficiency (38% to 44%) was not significantly affected by the operating parameters.
8. The specific rate of chromate reduction was dependent upon the influent chromate levels.

The findings of the above study indicate the potential promise of using chromate-resistant isolates for detoxification of hexavalent chromium, and the need to improve chromate reduction efficiency for regulatory compliance by incorporating recycling of settled biomass into the system to increase sludge retention time. The resulting biological sludge containing chromium can be considered for potential chromium recovery by acid treatment or incineration methods.

2.5.4 Reduction and Precipitation of Chromate by Mixed Culture Sulphate-reducing Bacterial Biofilms

It has been reported that bacterial biofilms could influence metal availability when extracellular polymeric substances sorb and bind the metal species (Schorer and Eisele, 1997; White and Gadd, 1998). However, the mixed culture sulphate-reducing bacterial biofilm was found to have retained less than 10% of the total chromium. This also suggested that there was little or no cellular uptake of chromium by the sulphate-reducing bacteria. Extracellular polymeric substances (EPS) is negatively charged therefore it is unlikely that there would be significant sorption of negatively charged chromate ions. Cr(III) is the most stable form of chromium, being relatively kinetically inert in aqueous solution and is also unlikely to react with EPS or other medium constituents (Cotton and Wilkinson, 1988).

Mass balance studies of chromate reduction by *Pseudomonas fluorescens* and immobilized cells of *Desulfovibrio desulfuricans* have also shown that an insignificant amount of chromium was taken up by the bacterial cells (DeLeo and Ehrlich, 1994; Tucker *et al.*, 1998). The hexavalent chromium does not appear to have any significant effect on the utilization of the carbon source. Chromate anions have the same charge and a similar physical size to the sulphate anion and therefore have the potential to enter into sulphate transport pathways in biological and environmental systems. Chromate, like molybdate, is a known inhibitor of sulphate reduction and forms unstable analoges of active sulphate, depleting cells of ATP (Postgate, 1984).

Smith and Gadd, (2000) established that just over 10% of the sulphate reduction activity detected in the control biofilm was observed in the presence of hexavalent chromium. Consequently, very low levels of sulphide were produced, with control biofilms producing over 10 times more sulphide than in the presence of chromate. It is possible that the chromate anion could be used as an alternative electron acceptor to sulphate.

Tebo and Obraztsova, (1998) have demonstrated that *Desulfotomaculum reducens* was capable of growth with chromate in place of sulphate as electron donor. Hydrogen sulphide is a known reductant of hexavalent chromium.

Iron (II) and manganese (II) are also known to reduce chromium (Carter, 1995; James *et al.*, 1997; Sedlak and Chan, 1997). Smith and Gadd, (2000) demonstrated that Cr(VI) reduction by the mixed culture sulphate-reducing bacterial biofilms is a biologically mediated process. Mass balance calculations indicated that when sulphate-reducing bacteria was exposed to Cr(VI), the insoluble Cr(III) which was formed accumulates at the base of the biocell which was clearly visible by means of a scanning electron micrograph (FIG 2.5). Extracellular polymeric material released by bacteria was only observed in biofilms incubated without chromate (FIG 2.6). However, the precipitation of insoluble chromium may have concealed any extracellular polymeric materials in the bacterial biofilms incubated with chromate. Tucker *et al.*, (1998) suggested that the dissimilatory reduction of soluble Cr by *Desulfovibrio desulfuricans* is mediated by enzymatic reactions. Cytochrome c_3 was thought to be responsible for the reduction of chromate by *Desulfovibrio vulgaris* (Lovely and Phillips, 1994).

Lojou *et al.*, (1998) suggested that the metal reductase activity of *Desulfuromonas acetoxidans* and *D. vulgaris* was governed by the polyheme c-type cytochrome content. Soluble enzymes are thought to be responsible for the reduction of chromate by a *Bacillus* sp., *Pseudomonas fluorescens* LB 300 and *Escherichia coli* (Shen and Wang; 1993; Wang and Shen, 1997).

The reduction process using bacterial biofilms have the potential to be used in the biological remediation of chromium-contaminated wastewaters and soils. Sulphate-reducing bacteria play an important role in metal biogeochemistry. Their tolerance of environmental stress factors and their ability to carry out toxic metal reduction at near neutral pH may provide an advantage over traditional remediation methods where conditions are often required to be very acidic in order for metal reduction and removal to take place. It may be possible to use sulphate-reducing bacterial biofilms for the treatment of chromium-containing wastes in a bioreactor configuration, before discharge into the environment, thus minimizing the environmental hazard. The ultimate removal from solution by the sulphate-reducing bacterial biofilms may also provide a source for Cr(III) recovery (Smith and Gadd, 2000).

FIGURE 2.5 Scanning electron micrograph of sediment biomass with chromium precipitate at the base of the biocell. Bar represents 10 μm (Smith and Gadd, 2000).

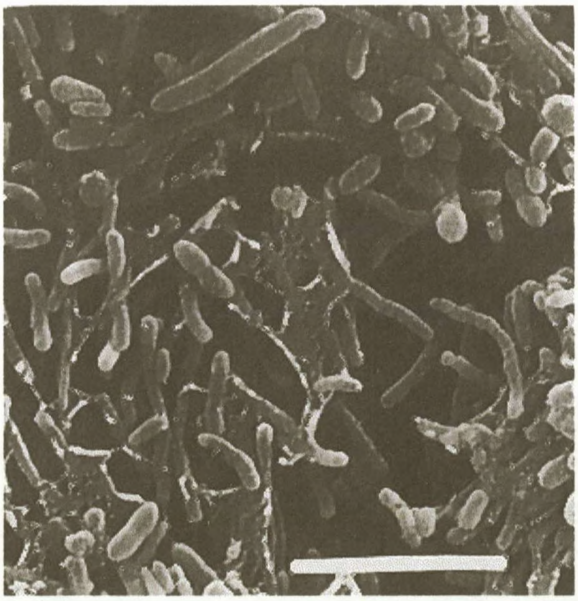


FIGURE 2.6 Scanning electron micrograph showing bacterial biofilm incubated without chromate with an accumulation of dehydrated extracellular polymeric material. Bar represents 5μm (Smith and Gadd, 2000).

2.5.5 Bioreduction of Chromate Contaminated Electroplating Wastewater and Groundwater

The main objective of the INEEL (Idaho National Engineering and Environmental Laboratory) Biotechnology Department was to develop a method in which bacteria convert Cr(VI) to the less toxic, less soluble, and less mobile trivalent chromium Cr(III). After bacterial conversion to Cr(III) the chromium could then either be removed from solution through filtration or sedimentation. Conversion of Cr(VI) to Cr(III) in groundwater would result in adsorption of Cr(III) to soil and organics, in most soils. With this technique, Cr(VI) could be treated in the following environments: groundwater, soil wash effluent, industrial waste streams, and unsaturated soil (Turick, 1997).

The approach involved using the microbial ecology and physiology of Cr(VI) utilizing, facultatively anaerobic bacteria. Cr(VI) utilizing bacteria were able to use Cr(VI) in an anaerobic environment to gain a growth advantage thereby out-growing bacteria that can't utilize Cr(VI). This strategy allowed the development of a bacterial population in a bioreactor or in the subsurface that was dominated by Cr(VI) reducing bacteria. One of the advantages was that Cr(VI) was converted (reduced) to a much less toxic and less soluble form of chromium, Cr(III), very efficiently (Turick and Apel, 1996).

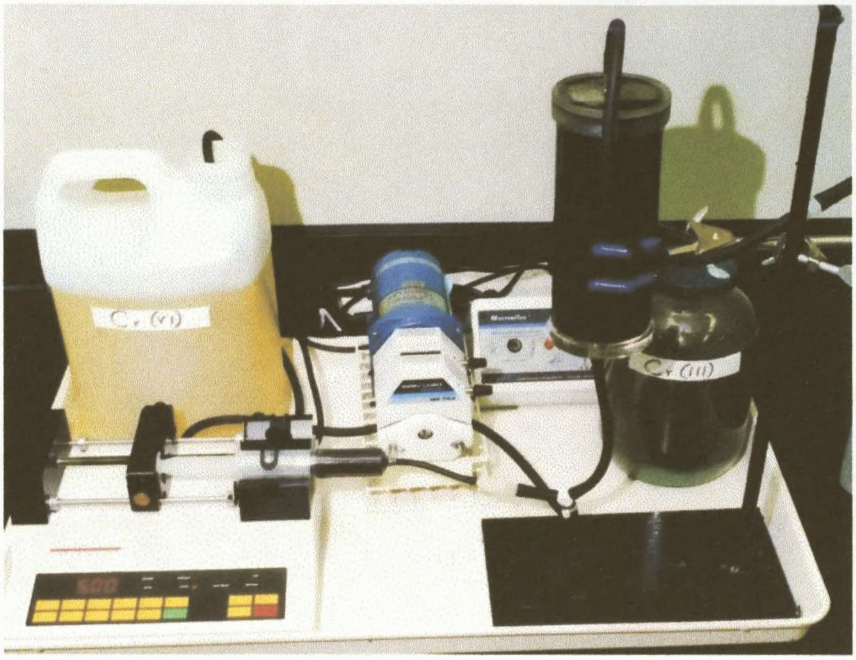


FIGURE 2.7 Bioreactor containing Cr(VI)-reducing bacteria for the reduction of Cr(VI) to Cr(III), Turick (1997).

FIGURE 2.8 Pilot-scale bioreactor containing Cr(VI) reducing organisms (Turick, 1997).

Chromate at a concentration of 250 ppm (characteristic yellow colour in container on left) was pumped into the cylindrical bioreactor containing Cr(VI) reducing bacteria in a laboratory scale study (FIG 2.7). The bacteria were immobilized into porous beads for increased volumetric productivity. The resulting Cr(III) exited the bioreactor into the container on the right. A characteristic greenish colour of Cr(III) formed a precipitate at the bottom. The carbon and energy source, molasses, was pumped into the reactor at a flowrate of 9 $mL.L^{-1}$ via syringe pump (Apel and Turick, 1992; Turick, 1997).

It has been found that Cr(VI) utilizing bacteria are common in Cr(VI) contaminated and non-contaminated soils. From this information a bioprocess has been designed that uses indigenous Cr(VI) utilizing bacteria. The conditions inside the reactor select for and encourage growth and population dominance of Cr(VI) utilizers. The conditions in the bioreactor select not just for Cr(VI) utilizing bacteria but the most efficient Cr(VI) utilizers (Turick and Apel, 1996).

This technology (FIG 2.8) has been deployed by a ferrochrome alloy company in Sweden with good results. Indigenous bacteria from a lagoon containing wastewater with 40-60 $mg.L^{-1}$ Cr(VI) was able to grow anaerobically in the 30 000 L bioreactor with only acetic acid as the carbon source. Cr(VI) reduction efficiency was over 98%. Because the lagoon water was pH 9, 99% of the resulting Cr(III) precipitates out of solution and is recovered in the bioreactor (Turick *et al.*, 1997).

It has been established that this bioprocess could be used for the treatment of soil wash effluent and industrial waste streams. It can also be used in conjunction with pump and treat technologies for groundwater remediation. This technology may be used alone and replace existing, less efficient technologies or in conjunction with conventional methods as a pretreatment step for treatment of surges of Cr(VI) or as post treatment polishing step (Apel and Turick, 1993; Schmieman *et al.*, 1997; Turick *et al.*, 1997).

Successful field trials of a Cr(VI) reducing bioreactor provided the following results:

- ☐ Electroplating wastewater and groundwater successfully treated with this bioreactor;
- ☐ an inexpensive feedstock was identified for bacterial reduction of Cr(VI);
- ☐ the bioreactor operated successfully in the range of 750 ppm to 120 ppb Cr(VI);
- ☐ the bioreactor operated successfully in the pH range of 4.3 - 9;
- ☐ nitrate did not inhibit bioreactor performance;
- ☐ need for chemical additives was reduced by as much as 90%; and
- ☐ bioreactor produced minimal biomass output.

(Turick and Apel, 1997).

2.6 CONCLUSIONS FROM REVIEW OF LITERATURE

The capacity of microorganisms to bring about chromate reduction has been investigated extensively. Unfortunately, chromate-bearing wastewaters from industrial processes generally contain heavy metal cations such as copper, cadmium, zinc and nickel, which are known to be toxic for most organisms and therefore possibly influence the performance of the chromate-reducing bacteria. The variability in requirements, e.g., presence of co-factors or specific carbon sources are also some of the selective conditions under which the Cr(VI)-reducers will successfully detoxify chromate. However, these requirements for the different bacteria in which previous studies were carried out make their results less applicable for industrial applications. Although biological Cr(VI) reduction is much preferred over chemical treatment due to its many advantages, the use of a bioprocess to treat Cr(VI)-contaminated soil wash effluents or ground water does present some problems.

These problems include:

- ☐ Owing to the expense of sterilization, a nonsterile soil effluent entering a bioreactor containing a pure culture would be prohibitive.

- ☐ In addition a nonsterile input of Cr(VI)-resistant nonreducing microorganisms into a bioreactor may eventually out compete a pure culture, rendering the bioprocess inefficient.

It is therefore necessary to develop a bioprocess with a strategy of selecting for and optimizing the growth of indigenous Cr(VI)-resistant microbes also capable of reducing Cr(VI). It is hypothesized that microbes indigenous to chromate-contaminated sites are capable of chromate reduction. It is also hypothesized that other heavy metal ions present in the waste effluents affect the rate of Cr(VI) reduction. It is also hypothesized that immobilization results in increased rate of Cr(VI) reduction.

CHAPTER THREE

ISOLATION AND IDENTIFICATION OF CHROMATE REDUCING BACTERIA FROM CHROMATE-CONTAMINATED SOILS AND WASTEWATER EFFLUENTS

3.1 INTRODUCTION

The ability of some microorganisms to reduce highly soluble and highly toxic Cr(VI) to less toxic, less soluble Cr(III) has led to the suggestion that Cr(VI)-reducing microorganisms might be useful agents for remediating Cr(VI)-contaminated waters and soils. A wide range of microorganisms are capable of enzymatic Cr(VI) reduction (Lovely, 1993), and it has been suggested that Cr(VI) is fortuitously reduced by enzymes that have other, as yet unidentified, natural substrates (Ishibashi *et al.*, 1990; Cervantes, 1991).

Reduction of Cr(VI) to Cr(III) represents a means by which toxicity is reduced and removal of chromium is facilitated. Reduction of Cr(VI) by microorganisms has been reported under a number of conditions and by a variety of bacterial species. The earliest reports were of an organism isolated from industrial sewage, *Pseudomonas dechromaticans*, which would use chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) as a terminal electron acceptor during anaerobic respiration (Romanenko and Korenkov, 1977).

Subsequently, a number of organisms have been identified that utilize Cr(VI), generally chromate or dichromate, as a terminal electron acceptor (Lebedeva and Lyalikova, 1979; Kvasnikov *et al.*, 1985; Gvozdyak *et al.*, 1986). Several additional chromate-reducing bacteria have been reported including other strains of *Pseudomonas* as well as *Achromobacter, Aeromonas, Bacillus, Enterobacter, Escherichia*, and *Micrococcus* species (Romanenko and Korenkov, 1977; Lebedeva and Lyalikova, 1979; Kvasnikov *et al.*, 1985; Gvozdyak *et al.*, 1986; Horitsu *et al.*, 1987; Bopp and Ehrlich, 1988; Wang *et al.*, 1989).

Cr(VI)-resistant bacteria have been isolated from Cr(VI)-contaminated sediments (Bopp *et al.*, 1983; Luli *et al.*, 1983; Kvasnikov *et al.*, 1985), and plasmid-coded resistance to Cr(VI) has been reported in *Ps.* (Bopp *et al.*, 1983), *Pseudomonas aeruginosa* (Summer and Jacoby, 1988), and *Alcaligenes eutrophus* (Nies and Silver, 1989). The mechanism of Cr(VI) resistance in the latter two strains involved reduced Cr(VI) uptake (Ohtake *et al.*, 1987; Nies and Silver, 1989). Chromate ion was observed to be accumulated intracellularly by the sulphate transport system (Ohtake *et al.*, 1987). The possibility of biological detoxification of chromium-containing sewage was first demonstrated by Romanenko and Korenkov (1977), who isolated and described a bacterial culture that reduces hexavalent chromium to the trivalent state.

In these studies both the absence and the existence of a relationship between the resistance of bacteria to hexavalent chromium and the ability to reduce it to the trivalent form was noted (Bopp and Ehrlich, 1980; Marques *et al.*, 1982; Luli *et al.*, 1983). The ability to reduce Cr(VI) is detected chiefly in species of the genus *Pseudomonas*, widespread in nature (Bopp and Ehrlich, 1980; Marques *et al.*, 1982; Bopp *et al.*, 1983; Shimada and Matsushima, 1983). It has been shown that adapted and non-adapted cells carry out this process (Bopp and Ehrlich, 1980).

Although several Cr(VI)-reducing bacteria were isolated from Cr(VI)-impacted environments (Romanenko and Korenkov, 1977; Lebedeva and Lyalikova, 1979; Kvasnikov *et al.*, 1985;), other investigators have demonstrated Cr(VI) reduction to occur with isolates from minimally Cr(VI)-impacted municipal wastes (Wang *et al.*, 1989) and pure cultures of laboratory strains that had not been previously exposed to Cr(VI) (Gvozdyak *et al.*, 1986). However, in this study, only chromate-contaminated sites were examined. It is hypothesized that bacteria isolated from Cr(VI)-contaminated soils and wastewater would be capable of Cr(VI) reduction.

Therefore, the aim was:

- to isolate organisms indigenous to Cr(VI)-contaminated sites and screen the isolates for reduction capability.

The objectives of this phase of study was:

- To screen samples for metal content;
- determine resistance capacity of isolate; and
- identify chromate reducing organisms that are capable of Cr(VI) reduction.

3.2 MATERIALS AND METHODS

3.2.1 Sample Collection and Preparation

Effluent samples taken in triplicate from each site were collected from sewage treatment plants (Northern Works, Darville Wastewater Works, and Hammarsdale Wastewater Works) as well as industrial effluents and soils obtained from electroplating industries (Saayman Danks, Sheffeild, and Natal Electroplaters). Soil samples were obtained from areas around the plating baths. All the samples were collected in sterile 1 L Schott bottles and stored at 4^{0}C before being used. The samples were digested by means of hot nitric acid (HNO_3) extraction (APPENDIX 1) and sludge digestion with aqua regia (APPENDIX 2) to determine the presence of heavy metal ions. Analysis for the presence of metal ions of copper, nickel, cadmium, and zinc was performed using Atomic Absorption Spectroscopy (APPENDIX 3). The samples from which isolates capable of Cr(VI) reduction were obtained was further analyzed for presence and quantity of heavy metal ions and those samples that demonstrated no Cr(VI) reduction were not further analyzed.

3.2.2 Screening of Crude Biomass for Cr(VI) Reduction

Crude biomass from the samples was screened for its ability to reduce chromate. Inoculum for the study was prepared by inoculating universal bottles containing 10 mL broth with 1 mL wastewater sample and soil dilutions (10^{-1} g.mL^{-1}) were also prepared. The culture media, Nutrient Broth (APPENDIX 4) and Trypicase Soy Broth (APPENDIX 5) in which the screening assay was conducted was formulated according to similar studies by Llovera *et al.*, (1993) and Wang and Shen, (1995) and adjusted to a pH of 7. Potassium chromate (K_2CrO_4)

which was used (APPENDIX 6) as hexavalent chromium was added to the media at final concentrations ranging from 25 mg.L^{-1} to 250 mg.L^{-1} (APPENDIX 7). Cultures were incubated at 30^{0}C on an orbital shaker at 100 rpm for agitation to ensure maximum diffusion of dissolved oxygen and maintenance of a homogenous suspension. Samples (10 mL) were analyzed periodically every three hours for Cr(VI) reduction by the diphenylcarbazide method (APPENDIX 8).

3.2.2.1 Analysis for Cr(VI) and Total Chromium

A colorimetric method that utilizes diphenylcarbazide (ASTM, 1991) was used to measure Cr(VI) in the supernatant samples which was centrifuged at 5000xg for 20 min in a Beckman J6-MC centrifuge. Concentration of Cr(VI) in the supernatant was measured at 540 nm using a UV spectrophotometer. The total chromium accumulated by cells, in cell wash water, and in the supernatant of culture fluids with cells removed by centrifugation was determined by digesting samples and reoxidizing and Cr(VI) with potassium permanganate (Ehrlich, 1983) followed by the colorimetric analysis for Cr(VI). The product of bacterial Cr(VI) reduction was inferred to be Cr(III) because it is the only stable, soluble chromium species formed by the reduction of Cr(VI) and because the starting Cr(VI) concentration in culture fluid samples could be restored by chemical oxidation with potassium permanganate to insure that all chromium was accounted for (Bopp and Ehrlich, 1988). Although Cr(II) exists, it is very unstable, readily autooxidizing to Cr(III). The concentration of trivalent chromium was calculated as the difference between the total and hexavalent chromium levels. Cultures that did not show the propensity to reduce Cr(VI) in any significant amount were subsequently discarded.

3.2.3 Isolation of Chromate-Reducing Bacteria

Bacterial isolates were obtained using the spread-plate method on Nutrient Agar after Cr(VI) reduction was evident in experiment 3.2.2. Plates were incubated at 30 ^{0}C until growth was obtained. Purity of the isolates was determined by performing Gram stains (APPENDIX 9) and by naked-eye examination of colony morphology. Susceptibility testing of pure isolates was also determined. Isolates were inoculated in Nutrient Broth with various concentrations of chromate ranging from 10 to 300 mg.L^{-1}, and incubated at 30^{0}C under aerobic conditions on a rotary shaker. Individual isolates were then analyzed to determine Cr(VI) reduction ability using the methods mentioned above. The growth was assayed by viable plate count method.

3.2.4 Identification of Isolates

All isolates showing the propensity to reduce Cr(VI) were Gram stained and identified using the API 20E system for Gram-negative rods and biochemical tests for Gram-positive rods. Isolates unable to reduce substantial quantities of Cr(VI) were discarded. Additional biochemical tests were performed to elucidate identification to species level (including cases of unacceptable/doubtful identification profiles).

3.2.5 Screening of Pure Isolates in a Batch System

Pure isolates were inoculated in 250 mL Erlenmeyer flasks containing 100 mL Nutrient Broth supplemented with Cr(VI) at a concentration of 150 mg.L^{-1}. The flasks were placed on an orbital shaker at 100 rpm at 30^{0}C. Reduction efficiency of the isolates was analyzed every three hours by aseptically removing 5 mL samples and determining the quantity of Cr(VI) reduced by the diphenylcarbazide method.

3.2.6 Chromate Reduction by Resting Cell Cultures

Resting cell cultures of the isolates were prepared by growing the cells in Nutrient Broth. Once sufficient growth had been obtained, cells were washed twice in phosphate buffer and resuspended in the same buffer. Chromate at a concentration of 150 mg.L^{-1} was then added to the buffer medium and the experimental conditions were same as that in experiment 3.2.5. The control that was setup contained only buffer and Cr(VI) but no cells.

3.3 RESULTS

3.3.1 Metal Ions Present in Samples

Samples were analyzed for the presence of heavy metal ions, but mainly to determine whether chromium was present in the electroplating and wastewater effluent and to what extent chromium-contamination was observed. The effluent obtained from the three electroplating industries, viz. Saayman Danks Electroplaters, Natal Electroplating and Sheffeild Electroplating as well as the effluent from Northern Wastewater Works indicated the presence of chromium, nickel, copper, zinc and cadmium (FIG 3.1)

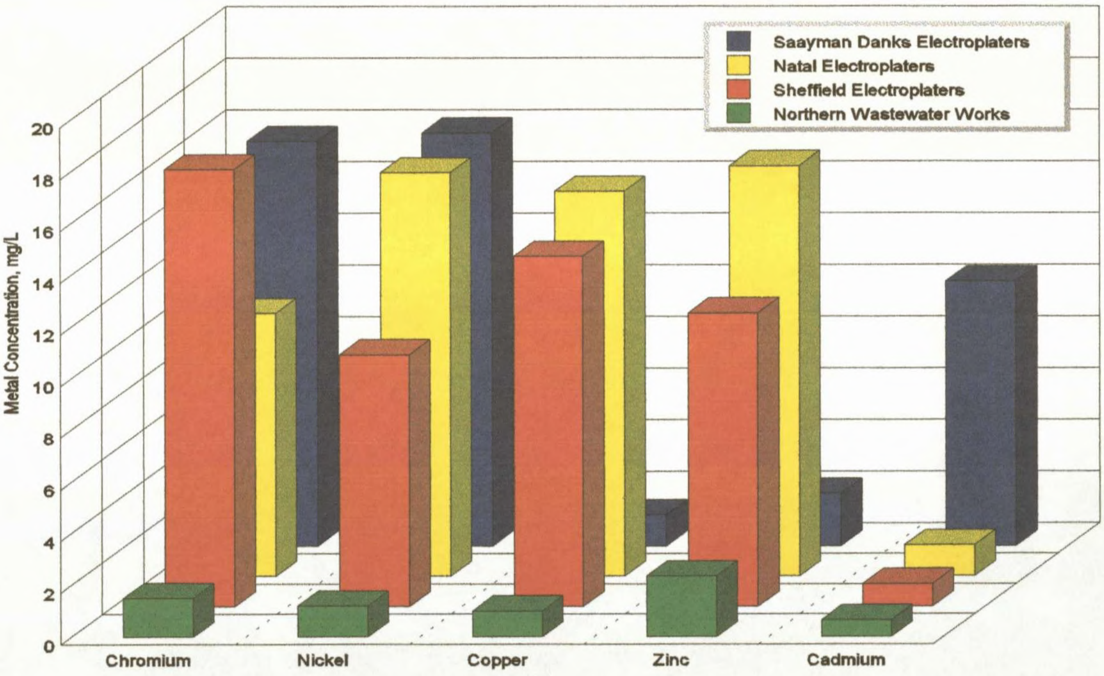


FIGURE 3.1 Heavy metal ions in electroplating and wastewater effluents obtained from Nothern Wastewater Works (🟩), Sheffield (🟥), Natal (🟨), and Saayman Danks (🟦) Electroplaters.

Since attempts were made to isolate organisms from soils, the soil samples were digested to examine if metals were present (FIG 3.2).

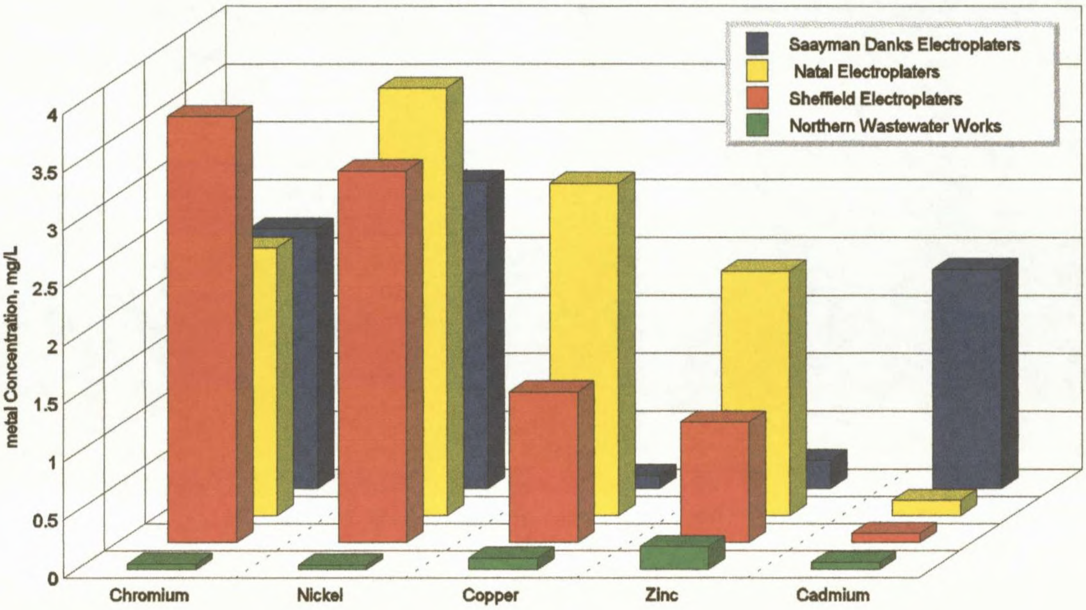


FIGURE 3.2 Heavy metal ions in soils and sludge obtained from Nothern Wastewater Works (🟩), Sheffield (🟥), Natal (🟨), and Saayman Danks (🟦) Electroplaters.

The amount of metals extracted from soils and sludge was less than that found in the effluent. As observed in FIG 3.1 the electroplating effluents contained chromium, nickel, copper, zinc and cadmium. Concentration of the metal ions varied from one effluent to the other since the electroplating industries work with specific metals depending on the type of polishing they do. Saayman Dank Electroplaters only work with chromium, nickel and cadmium, therefore only 1.21 mg.L^{-1}copper and 2.01 mg.L^{-1} zinc was observed in the effluent. Thus, high concentrations of chromium, nickel and cadmium detected in the effluents was expected. Sheffield and Natal Electroplaters showed high concentrations of chromium, nickel, copper and zinc since these are the metals that is used in the plating baths. Metal concentration observed in the wastewater effluent was quite low since Northern Wastewater Works receive mainly domestic sewage. Although these results indicate presence of chromium in the effluent in the range of 15 to 16 mg.L^{-1}, Cr(VI) concentrations as high as 35 to 49 mg.L^{-1} are generally observed. The presence of metal ions in the soil was largely attributed to the drag-out process . As indicated in FIG 3.2, the soils and sludge were chromium-contaminated.

3.3.2 Identification of Chromate-Reducing Organisms

A total of 12 bacterial isolates were obtained, however, only four of the 12 (from soils and wastewater samples) showed the ability to reduce chromate at a concentration as high as 150 mg.L^{-1}. The four isolates were subsequently identified as *Pseudomonas maltophilia, Acinetobacter calcoaceticus, Bacillus subtilis* and *Cellumonas cellasea.* The Gram-negative rods were identified using the API 20E system and the Gram-positive rods were identified on the basis of morphological and biochemical characteristics (TABLES 3.2 and 3.3). The sources of the organisms isolated is depicted in TABLE. 3.1.

TABLE 3.1 Sources of isolates and concentration chromium found in the soil and effluent samples.

Source	Organism	Sample type	[Cr(VI)], mg.L^{-1}
Saayman Danks Electroplaters	*A. calcoaceticus*	Effluent	15.66
Sheffield Electroplaters	*C. cellasea*	Soil	3.68
Natal Electroplaters	*Ps. maltophilia*	Soil	2.31
Northern Wastwater Works	*B. subtilis*	Effluent	1.50

TABLE 3.2 **Morphological and biochemical characteristics of *Pseudomonas maltophilia* and *Acinetobacter calcoaceticus*.**

Test or characteristic	*Pseudomonas maltophilia*	*Acinetobacter calcoaceticus*
Gram stain reaction	-	-
Cell type	Rod	Rod
Mobility	+	-
Nitrate reductase	-	-
Hydrogen sulfide	-	-
Indole	-	-
Voges-Proskauer	-	-
Citrate	-	-
O-Nitrophenyl-β-D-galactopyranoside	+	-
Urease	-	-
Lysine decarboxylase	-	-
Arginine dihydrolase	-	-
Ornithine decarboxylase	-	-
Oxidation-fermentation	+ [a]	-
Gelatin	+	-
Glucose	-	-
Mannose	-	-
Inositol	-	-
D-Sorbitol	-	-
L-Rhamnose	-	-
L-Arabinose	-	-
Oxidase	-	-
Growth on MacConkey	+	+
Pigment	+ [b]	-

[a] Reaction was that of oxidation.
[b] Yellow pigment on nutrient agar.
+ positive result.
- negative result.

The characteristics of *Ps. maltophilia* , *A. calcoaceticus, B. subtilis* and *C. cellasea* are shown in TABLES 3.2 and 3.3. On the basis of the morphological and biochemical properties using Bergey's Manual of Systematic Bacteriology, (1984), the isolates were identified at a confidence level of 98% and 99% respectively.

TABLE 3.3 **Morphological and biochemical characteristics of *Bacillus subtilis* and *Cellumonas cellasea.***

Test or characteristic	*Bacillus subtilis*	*Cellumonas cellasea*
Gram stain reaction	+	+
Cell type	Rod	Rod
Spore formation	+	-
Mobility	-	+
Nitrate reductase	-	-
Growth in air	+	+
Growth anaerobically	+	+
Catalase	-	+
Mannitol	+	+
Glucose	+	+
Voges-Proskauer	+	-
Oxidase	-	-
Mannitol	+	+
Pigment	-	+ [a]

[a] Yellow pigment on nutrient agar.

3.3.3 Chromate Reduction by Isolates

Isolates were also grown on nutrient agar supplemented with chromate at varying concentrations. Although *Ps. maltophilia* grew in the presence of 250 mg.L^{-1} Cr(VI) on agar medium (TABLE 3.4), the optimal concentration it was able to reduce Cr(VI) was at 150 mg.L^{-1} in a broth medium with > 99.9% reduction. The optimal concentration *A. calcoaceticus, B. subtilis* and *C. cellasea* were capable of Cr(VI) reduction was also at 150 mg.L^{-1} with reduction as high as 98, 95 and 97% respectively. Although *Ps. maltophilia* was capable of reducing 250 mg.L^{-1} Cr(VI), the rate of reduction, however was very slow. It is evident as seen in FIG. 3.3 that Cr(VI) reduction is directly proportional to time for all the isolates. Complete reduction was achievable by *Ps. maltophilia* in 60 hours. The other three isolates required 72 hours for complete reduction.

TABLE 3.4 **Chromate reduction after 72 h by the various isolates in broth culture supplemented with 150 mg.L^{-1} Cr(VI).**

Strain	Optimal chromate concentration [a] (mg.L^{-1})	% reduction [b] of 150mg.L^{-1} chromate
Ps. maltophilia	250	>99.9
A. calcoaceticus	150	98
B. subtilis	150	95
C. cellasea	150	97
Uninoculated control	-	0.05

[a] Isolates were inoculated on nutrient agar supplemented with chromate to determine Cr(VI) resistance.

[b] % reduction of the isolates was measured over 72 h in nutrient broth medium.

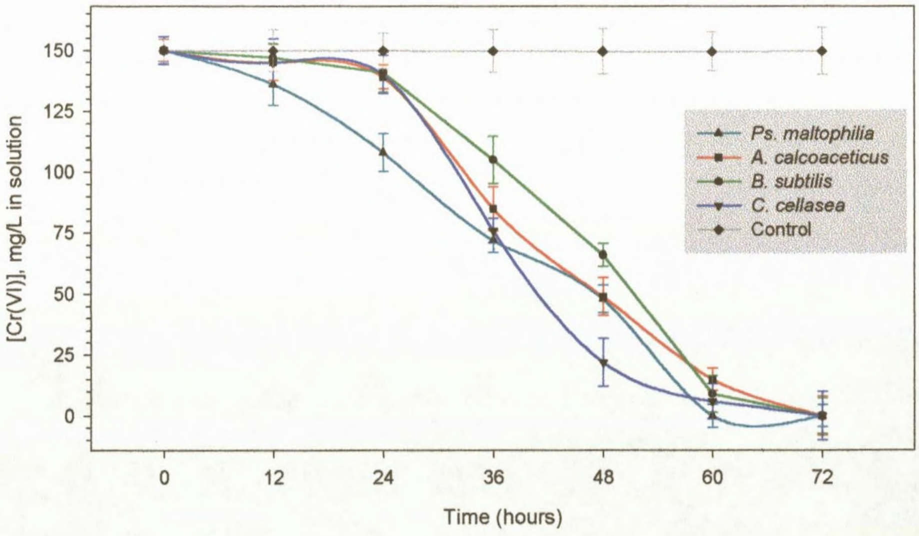


FIGURE 3.3 **Reduction of 150 mg.L^{-1} Cr(VI) in a batch bioreactor by isolates of *Ps. maltophilia* (▲), *A. calcoaceticus* (■), *B. subtilis* (●), *C. cellasea* (▼), and Control (◆).**

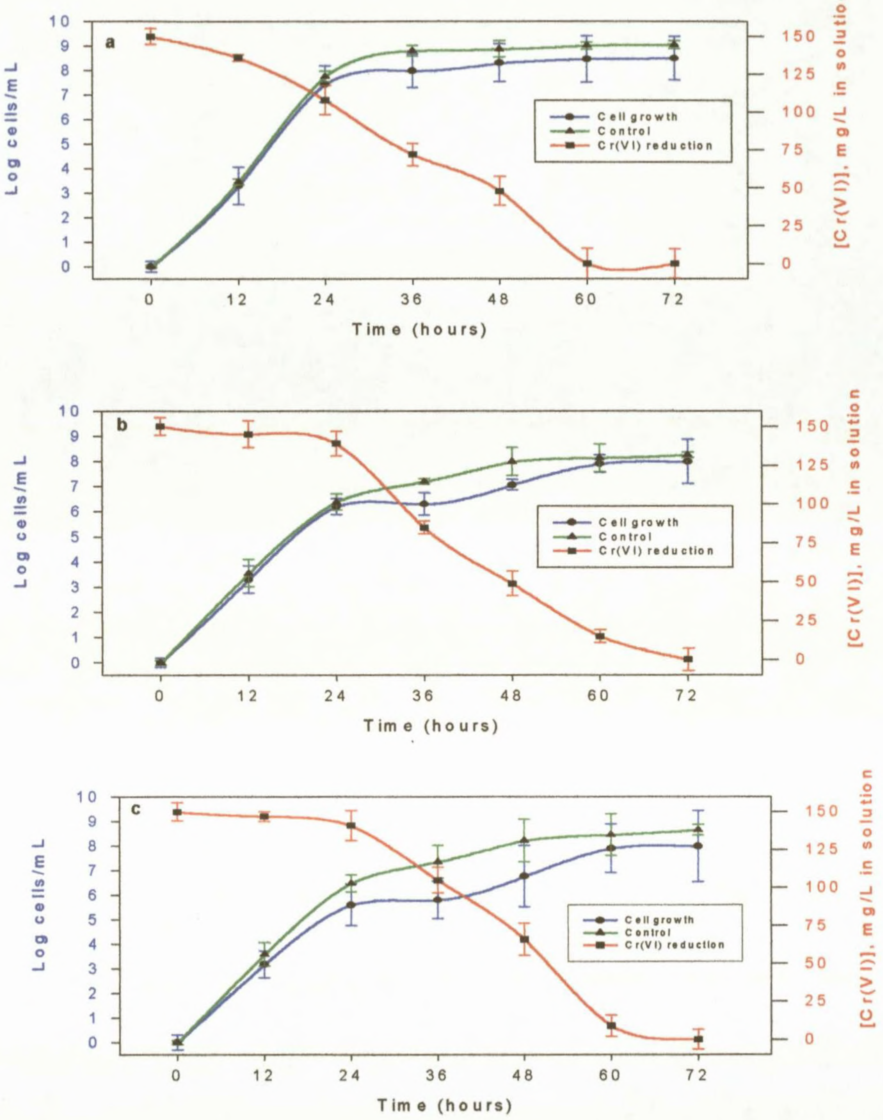


FIGURE 3.4 a-c. **Residual Cr(VI) in solution (■) and cell growth rate (●) of a, *Ps. maltophilia*, b, *A. calcoaceticus*, and c, *B. subtilis* in a batch reactor. Control (▲) contained no chromate.**

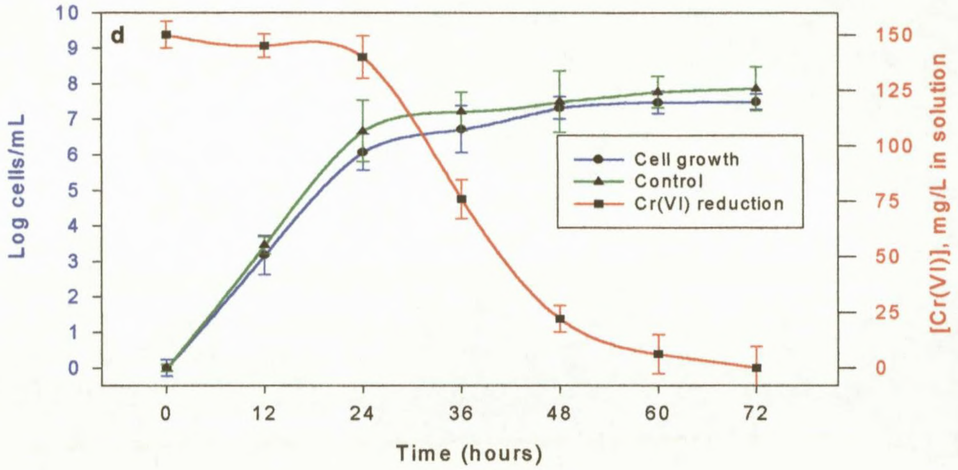


FIGURE 3.4 d. Residual Cr(VI) in solution (■) and cell growth rate (●) of *C. cellasea* in a batch reactor. Control (▲) contained no chromate.

It was observed that increased reduction occurs mainly during the stationary growth phase (FIG 3.4). Stationary growth was observed to be between 36h and 72h for *Ps. maltophilia* and *C. cellasea* (FIGS 3.4a,d). Growth for *A. calcoaceticus* and *B. subtilis* was observed to have two stationary phases between 24h and 36h, thereafter between 60h and 72h (FIGS 3.4b,c). However, the presence of two stationary phases was not evident in the controls for these organisms. *Ps. maltophilia* and *C. cellasea* were capable of reducing 75 mg.L^{-1} Cr(VI) in 36h during the log phase of growth and the same amount was reduced in 24 h during the stationary phase from an initial Cr(VI) of 150 mg.L^{-1}. Thus, the rate of reduction was greater during stationary growth phase. During the lag phase (0 to 12h), an insignificant amount of Cr(VI) was reduced (8 mg.L^{-1}) by *Ps. maltophilia*.

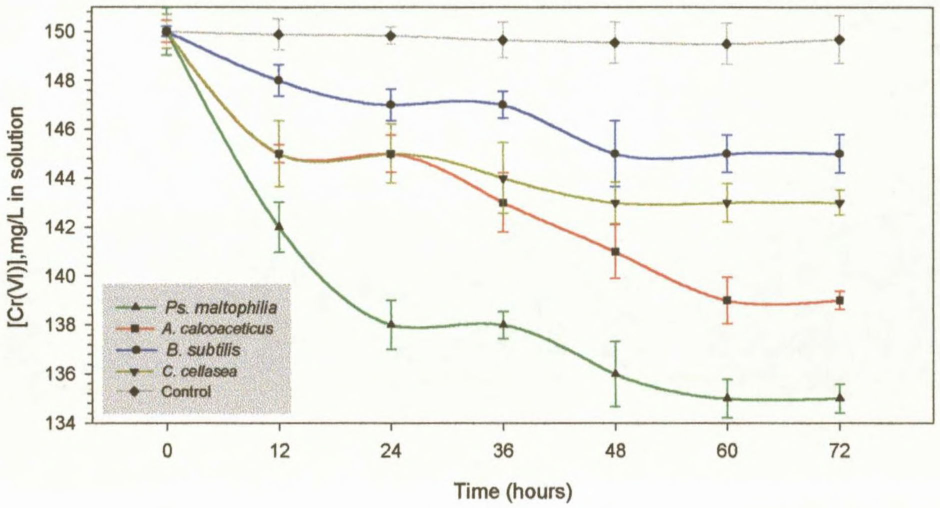


FIGURE 3.5 **Cr(VI) reduction by resting cell cultures of *Ps. mltophilia* (▲), *A. calcoaceticus* (■), *B. subtilis* (●), *C. cellasea* (▼), and Control (◆) at a cell concentration of 1 x 10^9 cells.mL^{-1}**

Cell growth as a function of reduction rates was also determined and it was evident from the results (FIGS. 3.4a-e) that a high cell concentration resulted in a high Cr(VI) reduction rate. Cell concentration increased with time undergoing a lag, log and plateau phase. Cell concentration reached a maximum for each of the isolates during the log phase of growth, thereafter remained fairly constant. Resting cell cultures were also prepared to determine whether Cr(VI) reduction was energy dependent. Cells were precultured in nutrient broth and then harvested by centrifugation, washed in phosphate buffer and resuspended in the same buffer. Results demonstrate no appreciable reduction in Cr(VI) (FIG. 3.5). Cell concentration of each of the cultures were approximately 3 x 10^8 cells.mL^{-1}. Cells at this concentration reduced Cr(VI) in nutrient broth but in a non-growth promoting medium Cr(VI) reduction was not evident.

3.4 DISCUSSION

Heavy metal ions in soils, electroplating and wastewater effluents were analyzed to determine metal content in the samples (FIGS. 3.1 and 3.2). Metal ions such as nickel, copper, zinc, cadmium and chromium where detected in the samples at varying concentrations. In the electroplating effluents, the concentration of a specific metal was greater than another depending on the type of metal polishing that the electroplating industry specializes in. The organisms obtained in this study was isolated from such samples therefore the quantity of metals in the samples had to be established. Chromium, at a concentration of approximately 15.66 mg. L^{-1} (FIG. 3.1) was detected in effluent obtained from Saayman Danks and soil from Sheffield Electroplating. Two Gram-negative rods and two Gram- positive rods were isolated from both soil and effluent samples (TABLE 3.1). *Ps. maltophilia* was isolated from a soil sample obtained from Natal electroplating and contained 2.3 mg.L^{-1} Cr(VI). *C. cellasea*, isolated from soil contained 3.68 mg.L^{-1} Cr(VI). *B. subtilis*, from Northern Works effluent containing 1.5 mg.L^{-1} Cr(VI) and *A. calcoaceticus* from Saayman Danks effluent containing 16 mg.L^{-1} Cr(VI). These organisms were identified on the basis of morphological and biochemical characteristics (TABLES 3.2 and 3.3). *Pseudomonas spp.* have been reported to reduce Cr(VI) (Romanenko and Korenka, 1977; Lebedeva and Lyalikova, 1979; Kvasnikov *et al.*, 1985; Deleo and Ehrlich; 1994). Subsequent studies have also demonstrated the capacity for Cr(VI) reduction in *Bacillus subtilis* (Gvozdyak *et al.*, 1986; Garbisu *et al.*, 1997). However, *A. calcoaceticus* and *C. cellasea* appears not to be common Cr(VI) reducers since these organisms has not been previously indentified as Cr(VI) reducers. TABLE 3.4 indicates the optimal concentration at which each of the organisms could grow in the presence of Cr(VI). *Ps. maltophilia* has the highest resistance of 250 mg. L^{-1} compared to the rest of the organisms that could grow in the presence of Cr(VI) at 150 mg. L^{-1} on nutrient agar.

The isolates were able to reduce high concentrations of Cr(VI) in a batch culture (FIG. 3.3) and Cr(VI) reduction increased with time. Over the period of observation (72 h), Cr(VI) was reduced >99.9% in a flask with an initial concentration of 150 mg.L^{-1}. Complete Cr(VI) reduction in *Ps. maltophilia* took 60 h compared to the other three cultures which required 72 h. Reduction at the beginning of incubation proceeded at a very slow rate and after approximately 48 hours, reduction progresses at a very fast rate. This change could be attributed to the organisms adapting to the presence of Cr(VI) and after this adaptation period, Cr(VI) reduction proceeded to an optimal.

Bacterial resistance to toxic chromate has been found in *Pseudomonas ambigua* (Horitsu *et al.*, 1983), *Ps. fluorescens* (Bopp *et al.*, 1983), *Ps. aeruginosa* (Brock and Gustafson, 1977; Summers and Jacoby, 1978), and *Streptococcus lactis* (Efstathiou and McKay, 1977). Except in the *Ps. ambigua* strain, the chromate resistance in these bacteria was plasmid determined. The colour of the medium changed from yellow to a dark greenish brown, and turbidity increased without delay as chromate decreased.

Pure cultures isolated from the contaminated environments reduced chromium with a reduction efficiency of approximately 99%. The medium chosen for these studies was a nutrient medium in order to facilitate rapid isolation of Cr(VI) reducers from soils and effluents. The rapid isolation of Cr(VI) reducers (less than 48 h) indicates the potential for incorporating this selection strategy into a bioprocess receiving nonsterile wastes from Cr(VI)-contaminated effluents. Another advantage of selecting for indigenous bacteria from contaminated environments may be minimization of inhibitory effects from other compounds that may be present along with Cr(VI), since viable indigenous organisms will have developed some degree of resistance to these compounds.

Total chromium concentrations in the cell-free supernatant were consistent with the initial concentrations of Cr(VI), demonstrating that Cr(VI) was reduced and Cr(III) was not or minimally adsorbed or accumulated by bacterial biomass. There was no evidence of Cr(III) precipitating out of solution or Cr(III)-associated bacterial growth inhibition as reported previously (Bopp and Ehrlich, 1988; Wang *et al.*, 1991). This may be due to the organic content of the medium allowing for Cr(III) complexation, thereby keeping Cr(III) in solution and minimizing any potential inhibitory characteristics.

Cell growth as a function of time in the presence of Cr(VI) was also determined in order to establish a relationship between rate of Cr(VI) reduction and cell concentration (FIGS. 3.4a-d). It was quite evident from the results that there is a direct relationship, as cell concentration increases, chromate concentration decreases. The control contained no chromate, thus cell growth reached an average concentration of 1 x 10^9 cells.mL^{-1} for each of the isolates. Comparative to the isolates in the presence of Cr(VI), it could be assumed that chromate inhibits cell growth to an extent. Minimal reduction occurred during the lag-phase of growth and the highest rates of reduction occurring during log-phase growth. Similar observations have been reported previously (Romanenko and Korenkov, 1977; Bopp and Ehrlich, 1988; Wang *et al.*, 1989; Apel and Turick, 1991) and are indicative of direct bacterial reduction of Cr(VI) associated with growth. In some cases Cr(VI)-reduction rates remained constant throughout the stationary growth phase. Similar results have also been reported previously (Lebedeva and lyalikova, 1979; Kvasnikov *et al.*, 1985) and were also associated with direct bacterial reduction of Cr(VI). The mechanism(s) of bacterial Cr(VI) utilization was not elucidated during this study, however, other investigators have reported that Cr(VI) can be utilized by numerous bacteria as a terminal electron acceptor (Romanenko and Korenkov, 1977; Lebedeva and Lyalikova, 1979; Gvozdyak *et al.*, 1986; Kvasnikov *et al.*, 1985, 1988; Wang *et al.*, 1989, 1991; Ohtake *et al.*, 1990; Lovely and Phillips, 1994).

The rates of chromate reduction were proportional to cell density. Increasing cell density greatly enhanced rates of chromate reduction, and at a density of 3 x 10^8 cells.mL^{-1}, 150 mg.L^{-1} Cr(VI) was completely reduced within 60 hours by *Ps. maltophilia* (FIG. 3.4a). *A. calcoaceticus, B. subtilis* and *C. cellasea* completely reduced 150 mg.L^{-1} Cr(VI) at an average cell density of 6 x 10^7 cells.mL^{-1} in 72 hours. The time required for complete reduction increased in proportion to the amount of added chromate. Examination of chromate reduction by resting cells was investigated to establish whether chromate reduction required an energy source. No concentration decrease of Cr(VI) occurred in the absence of metabolically active bacterial cells. Cell concentration in the control which contained only Nutrient Broth and cells with no Cr(VI) reached a maximum of 1 x 10^9 cells.mL^{-1} (FIG 3.3) but even at this concentration only as little as 2 mg.L^{-1} Cr(VI) was reduced in resting cell cultures (FIG 3.4a-d) as compared to growing cells. However, cells in nutrient broth at a concentration less than 1 x 10^9 cells.mL^{-1} were capable of >99.9% reduction. This implies that Cr(VI) reduction is energy-dependent.

3.5 CONCLUSIONS

It was evident from the results that organisms indigenous to chromate-contaminated sites are capable of efficient Cr(VI) reduction. Batch reactors were used to investigate reduction capabilities of the isolated organisms. Chromate reduction capability was established and the various parameters optimized . From the results obtained, indigenous microorganisms were shown to be capable of reducing Cr(VI) at a concentration as high as 150 $mg.L^{-1}$, although the concentration of Cr(VI) from where the organisms were isolated from was not greater than 16 $mg.L^{-1}$. It was also established that Cr(VI) resistance does not necessarily mean reduction. Although an organism can grow in the presence of a specific Cr(VI) concentration, it may not however, be able to reduce Cr(VI) at that particular concentration. Cr(VI) reduction appears to be energy-dependent since minimal reduction occurred when resting cells where suspended in a media that does not promote growth. It was also established that much of the Cr(VI) reduction took place during the stationary growth phase indication that the Cr(VI) metabolism is part of secondary metabolism. Bacterial cells alone were responsible for chromate reduction and the organic compounds present in the medium did not contribute to chromate reduction. In order to establish a reactor with optimal Cr(VI) reduction efficiencies, it is necessary to optimize parameters of pH, temperature, Cr(VI) concentration, substrate concentration, and presence of co-factors, thus the next chapter involves the establishment of optimal parameter requirements for an increased Cr(VI) reduction rate.

CHAPTER FOUR

CHROMATE REDUCTASE CHARACTERIZATION AND FACTORS AFFECTING ITS PRODUCTION

4.1 INTRODUCTION

Chromate-bearing wastewaters from industrial processes generally contain heavy metal cations such as Cu^{2+}, Cd^{2+}, Zn^{2+} and Ni^{2+} (Patterson, 1985), which are known to be toxic for most organisms and therefore possibly influence the performance of the chromate-reducing bacteria. Nonetheless, there is hardly any data published with respect to the performance of chromate reduction by the organisms in relation to metal ions. There is only one report published as to the effects of heavy metals on the anaerobic reduction of chromate by gram-negative *Enterobacter cloacae* (Hardoyo *et al.*, 1991).

The activities of non-growing cells are of fundamental importance in the area of biotechnology, since industrial processes might envisage the use of pre-grown, immobilized organisms as a filter through which metal-containing effluents could be passed. Resting cells of chromate-sensitive bacteria have been reported to be able to reduce chromate to the trivalent from (Bopp and Ehrlich, 1988). Resting cell biomass directs the energy obtained from the endogenous constituents toward a range of activities (Kjelleberg *et al.*, 1987). With non-proliferative cells, the envelope is the crucial structure that mediates the interaction with their environment (Brown and Williams, 1985). The influence of culture conditions on the envelope and other properties of bacteria has long been recognized. Thus, any study on the influence of several significant factors of the culture medium on the chromate reduction by bacterial cells is desirable.

Plasmids conferring resistance to heavy metals are commonly found in a variety of bacterial species of different origin (Summers, 1985; Foster, 1987) and in some cases the mechanisms of resistance have been elucidated (Silver, 1985; Silver and Walderberg, 1992). Several examples of plasmids governing resistance to CrO_4^{2-} have been reported (Bopp *et al.*, 1983) but the biochemical mechanism of resistance remains unknown. *Ps.* strain LB300, carrying the CrO_4^{2-} -resistance plasmid pLHB1 (Bopp *et al.*, 1983), showed a decrease in CrO_4^{2-} uptake as compared to the plasmidless strain LB303 (Ohtake *et al.*, 1987). In this paper it was reported that the characterization of CrO_4^{2-} -resistance plasmids isolated from *Ps. aeruginosa* clinical strains showed that one of these plasmids caused a reduction in the accumulation of CrO_4^{2-} in *Ps. aeruginosa* strain PU21.

Horitsu *et al.*, (1987) reported that *Pseudomonas ambigua* G-1 showed NADH-dependent Cr(VI)-reducing activity in the cell extract. Ishibashi *et al.*, (1990) found NAD(P)H-dependent Cr(VI)-reducing activity in the cell-free supernatant fluids from *Pseudomonas putida*. Das and Chandra (1990) have reported that Cr(VI) was reduced to Cr(III) in the presence of NAD(P)H in the cell extract of *Streptomyces* species. A membrane associated chromate reduction system was also reported. Ohtake *et al.*, (1990a,b,c) and Wang *et al.*, (1991) have shown that the reduction of Cr(VI) under anaerobic conditions by *Enterobacter cloacae* was caused by the respiratory chain system of the cell membrane.

Bopp and Ehrlich, (1988) isolated a strain of *Pseudomonas fluorescens* (LB300) that harbours a chromate resistance plasmid and reduction appears to be enzymatic, since it is catalyzed by cell extracts. Fractions of the crude cell extract were prepared by sonication followed by high speed centrifugation to obtain the fractions S_{32} and S_{150}. The fact that chromate reductase activity was demonstrated in the S_{32} fraction with addition of NADH but not in the S_{150} fraction seems to indicate that some or all of the enzymes necessary for the transfer of electrons from NADH to chromate are membrane bound. The inhibition of chromate reduction by cyanide and azide and the ability of NADH to act as electron donor suggests that an electron transport system was involved.

Electron spin resonance (ESR) measurements provided evidence for the formation of Cr(VI) intermediates in the enzymatic reduction of Cr(VI) by glutathione reductase (GSSG-R) in the presence of NAD(P)H, indicated an initial single-electron transfer step in the reduction mechanism (Shi and Dalal, 1990). Glutathione reductase is ubiquitously found in cellular systems and has important biological functions. Earlier studies on the mechanism of Cr(VI) reduction include those involving rat liver DT-diaphorases (DeFlora *et al.*, 1985), aldehyde oxidase

(Banks and Cooke, 1986), lactose (Goodgame and Joy, 1986), ascorbic acid, glucose, microsomes (Mikalsen *et al.*, 1989), and glutathione (Shi *et al.*, 1991). Glutathione reductase (GSSG-R) has been shown to be selectively inhibited by Cr(VI) in erythrocytes and also in liver in a NAD(P)H dependent manner (DeFlora *et al.*, 1984). Sugiyama *et al.*, (1989) reported that treatment of hamster V-79 cells with Cr(VI) results in a decrease of GSSG-R activity. They suggested that the inhibition of GSSG-R maybe involved in the toxic effect of Cr(VI). While the inhibition of GSSG-R by Cr(VI) was hypothesized to be linked to the reduction of chromium from Cr(VI) to Cr(III), the mechanism remains unclear (Sugiyama *et al.*, 1989).

Shewanella putrefaciens MR-1 can reduce a diverse array of compounds under anaerobic conditions, including manganese and iron oxides, fumarate, nitrate, and many other compounds (Lloyd and Macaskie, 1996; Myers and Myers, 1997). These reductive processes are apparently linked to a complex electron transport system. Formate-dependent reductase activity was detected in anaerobically grown cells of *S. putrefaciens* MR-1, with highest specific activity in the cytoplasmic membrane (Myers *et al.*, 2000).

Batch systems are usually used to study the effects of varying factors such as pH, temperature, Cr(VI) concentration, and biomass concentration on the removal of pollutants (Mertz, 1974). The engineering applications of batch-systems are limited to laboratory experimentation since effluent pollutants are discharged in mass volume. Batch systems were used to investigate the potential for the isolated organisms to reduce Cr(VI). The amount of Cr(VI) that a biological system can reduce is dependent on the number of active cells in the reactor. First, the growth of bacteria in the batch cultures is controlled by a maximum population density (Langand, 1983). Second, presence of Cr(VI) inhibits metabolic activity in cells (Yamamoto *et al.*, 1993).

The objective of this phase of study was :

- To locate the enzyme responsible for chromate reduction.
- Optimization of parameters for effective Cr(VI) reduction by the cells.

It must be emphasized however, that optimization of parameters was determined using whole cells and not enzyme fractions. Enzyme fractions that were prepared was not used since no significant Cr(VI) reduction was observed. This could be attributed to the fact that conditions required for enzymes are more demanding than that of whole cells and would add significantly to the cost of preparing and maintaining an industrial Cr(VI) reduction process.

4.2 MATERIALS AND METHODS

4.2.1 Isolation of Subcellular Fractions

Cells of the isolates were grown in one litre nutrient broth at 30 ^{0}C and were harvested in the late logarithmic phase, washed twice with phosphate buffer and suspended in 100 mL of the same buffer. For determination of protein concentrations (APPENDIX 11), cells at a concentration of 15 mg.mL^{-1} were first digested in 1 N NaOH for 5 min at 100 ^{0}C, and total protein of the cell pellet was determined by the Lowry method (Lowry, 1951).

Cytoplasmic membrane (CM), intermediate membrane (IM), outer membrane (OM) and soluble fractions (periplasmic plus cytoplasmic) were purified using an EDTA-lysozyme protocol (APPENDIX 12) described and utilized previously (Myers and Myers, 1992ab, 1993a, 1997abc). The IM is a hybrid of CM and OM and has been previously characterized from *Shewanella putrefaciens* MR-1 (Myers and Myers, 1992b); such intermediate hybrid membrane fractions have been observed in other bacteria (Osborne *et al.*, 1972; Kent and Wisnieski, 1983; Barbas *et al.*, 1986; Myers and Collins, 1987, 1988). For initial experiments, various amounts (0.09-0.52 mg) of subcellular fractions were used to confirm that the Cr(VI) reduction rates were a function of the amount of enzyme added (i.e. substrate was not limiting). For most experiments, 0.13-0.15 mg CM was used. Subcellular fractions for each of the isolates were incubated in glass tubes in a total volume of 2.5 mL containing 28 mmol.L^{-1} potassium phosphate (pH 7.5). Electron donors (NADP and NAD(P)H) were used as electron donors and added to the reaction mixture at a concentration of 1 mmol.L^{-1}.

4.2.2 Constitutive or Inducible Nature of Enzyme

For determining the constitutive or inducible nature of the reducing enzyme, each of the isolates were grown separately in two batches in the absence of Cr(VI). After a day, one batch of each isolate was induced with 150 mg. L^{-1} Cr(VI) while the other batch was grown without Cr(VI). Thereafter, Cr(VI) at 150 mg.L^{-1} was introduced into the second batch that was grown without Cr(VI). Reduction was assayed in both the batches. Chromate reduction in cultures grown without Cr(VI) would indicate that the Cr(VI) reductase is constitutive. However, if Cr(VI) reduction is evident only in cultures grown in the presence of Cr(VI), it would be indicative of an inducible Cr(VI) reductase.

4.2.3 Laboratory-scale Batch Setup

Isolates for the inoculation of experimental runs were cultivated in Nutrient Broth by batch in 1000 mL Erlenmeyer flasks with 800 mL in working volume on a shaker at 100 rpm under aerobic conditions at an initial Cr(VI) concentration of 150 mg.L^{-1}. The rate of chromate reduction by the isolates in the batch-wise operation was determined by measuring the difference between total chromium (Cr(VI) and Cr(III)) and Cr(VI). The time course for chromate concentration in the mixture of organisms and chromate at a given initial concentration was observed. The effect of the various parameters such as temperature, pH, Cr(VI) concentration, substrate concentration, and presence of heavy metal ions on Cr(VI) reduction was also determined.

4.2.4 Analysis of the Various Parameters to Optimize Chromate Reduction

The effect of the abovementioned parameters on chromate reduction were determined to optimize conditions for efficient chromate reduction. The pH range of the medium was from 2 to 12 and was changed appropriately to acidic or basic by adding either sodium hydroxide or hydrochloric acid. Temperature ranged from 20 to 40 ^{0}C and once this was optimized, concentration of nutrient broth ranged from 0.025% to 0.3%. Heavy metal ions viz., nickel ($NiCl_2.6H_2O$), copper ($(CuNO_3)_2.3H_2O$), zinc ($ZnSO_4.7H_2O$) and cadmium ($Cd(NO_3)_2.4H_2O$) at concentrations between 10 and 40 mg.L^{-1} were added to determine the effects on chromate reduction. Nickel, copper, zinc and cadmium were mixed to yield concentrations ranging from 5 to 20 mg.L^{-1} to determine the effect of a mixed metal solution on chromate reduction.

4.3 RESULTS

4.3.1 Location of Cr(VI) Reductase

Cells of each of the isolates were fractionated into membrane fractions (CM, IM and OM) and a soluble fraction (periplasmic plus cytoplasmic) by an EDTA-lysozyme protocol (Myers and Myers, 1992ab, 1993a, 1997abc). To assay for Cr(VI) activity *in vitro*, NADH and NAD(P)H was used as electron donors. The final concentration of each electron donor was 1.0 mmol.L^{-1}. NAD(P)H-dependent Cr(VI) reductase activity was readily detected in the CM (FIG 4.1) of cells from *Ps. maltophilia*.. Relative to the CM, the other subcellular fractions of *Ps. maltophilia* had much lower specific Cr(VI) reductase activities (TABLE 4.1). The other three isolates showed no appreciable Cr(VI) reduction in subcellular fractions, thus further experiments concerning enzyme fractions were carried out with *Ps. maltophilia*. Breakage of the cells of *Ps. maltophilia* led to an immense increase of Cr(VI) reductase activity in comparison with unbroken cells (TABLE 4.2). The spheroplast fraction contained 97% reductase activity, whereas the periplasmic fraction only contained 3% reductase activity. The soluble fraction (contains cytoplasmic, periplasmic and membrane fractions) of crude extracts accounted for 100% reductase activity since no activity was detected in the membrane fraction alone.

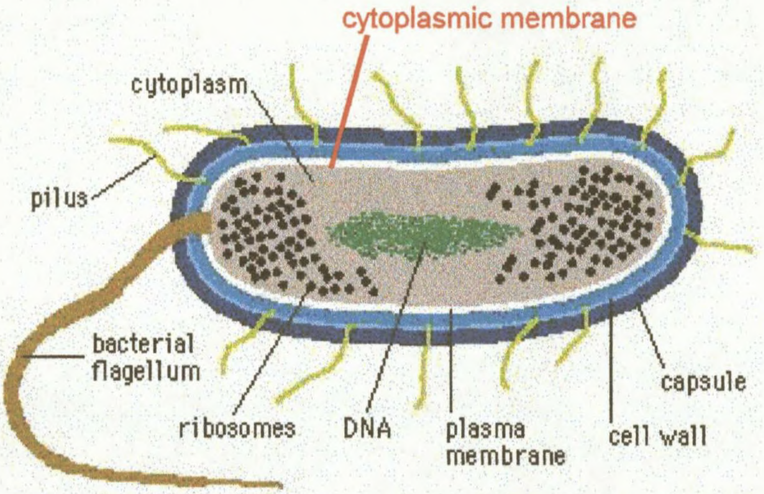


FIGURE 4.1 **Diagrammatic representation of a typical bacterial cell showing enzyme location in *Ps. maltophilia.***

Table 4.1 **Chromate reductase activity in various fractions prepared from isolates.**

Organism	**Electron donor**	**Specific activity (mU/mg of protein)[a]**		
		Cytoplasmic membrane	**Intermediate membrane**	**Outer membrane**
Ps. maltophilia	NAD(P)H	3.24	1.31	0.00
A. calcoaceticus	None[b]	0.54	0.22	0.00
B. subtilis	None[b]	0.00	0.00	0.00
C. cellasea	None[b]	0.00	0.00	0.18

[a] One micro unit is defined as 1 nmol of CrO_4^{2-} reduced per min.

[b] Both NADP and NAD(P)H were used, but the isolate required neither of the electron donors.

Table 4.2 **Localization of Cr(VI) reductase in subcellular fractions of *Ps. maltophilia*.**

Fraction	Protein (mg)	Cr(VI) reductase activity		
		Sp act (mU/mg of protein)	Total activity (mU)	%
*Whole cells vs. crude extract				
Whole cells	115 ± 5	4.5 ± 0.2	518 ± 15	100
Crude extract	120 ± 8	7.2 ± 0.5	864 ± 30	167
Periplasm vs. spheroplasts				
Periplasm	1.2 ± 0.2	1.3 ± 1	1.6 ± 2	3
Spheroplasts	9.1 ± 1.0	6.2 ± 1	56.4 ± 6	97
Soluble fraction vs. membrane fraction				
Soluble fraction	10.2 ± 1	6.2 ± 0.5	63.2 ± 4	100
Membrane fraction	48.1 ± 0.5	0.0 ± 1	0 ± 0.2	0

* Whole cell activity is regarded as 100%.

TABLE 4.3 **Constitutive versus induced cells assayed over a 60-h time period at $Cr(VI)_{initial} = 150$ mg.L^{-1}.**

Organism	Amt Cr(VI) reduced by constitutive enzymes (mg.L^{-1})	Amt Cr(VI) reduced by induced enzymes (mg.L^{-1})
Ps. maltophilia	150	150
A.calcoaceticus	135	137
B. subtilis	141	144
C.cellasea	144	144
Control	0.11	0.11

The Cr(VI) reductase was found to be constitutive in all four isolates (TABLE 4.3). In *Ps. maltophilia* and C. *cellasea*, 150 mg.L^{-1} and 144 mg.L^{-1} Cr(VI), respectively was reduced in 60h with induced and constitutive cells. However, there was an insignificant increase of 2 mg.L^{-1} in *A. calcoaceticus* and 3 mg.L^{-1} in *B.subtilis.*

4.3.2 Performance of Batch-scale Reactor for Biological Cr(VI) Reduction

The capability of the isolates to reduce chromate in a batch system was very successful with complete reduction of 150 mg.L^{-1} Cr(VI) within 60 hours by *Ps. maltophilia* and 72 hours for *A. calcoaceticus, B subtilis* and *C. cellasea*. A simple batch system was set up and chromate reduction was accompanied by a distinct colour change of the medium as compared to the control (FIG 4.2). As can be seen from the diagram, the colour of the control remained yellow, while the experimental changed from the original yellow to a dark greenish. Such a batch-scale reactor was setup to optimize parameters for chromate reduction so that conditions would be established to setup and operate other possible reactors that are capable of a higher Cr(VI) reduction rate as will be discussed further.

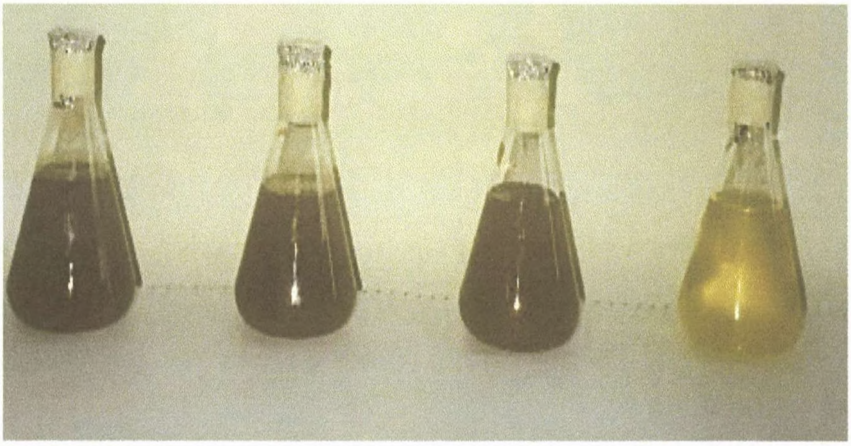


FIGURE 4.2 Batch-system for Cr(VI) reduction by isolates of (from left), *Ps. maltophilia, A. calcoaceticus, B. subtilis* and control showing no change in the colour of the medium.

4.3.3 Optimization of Parameters for Cr(VI) Reduction

As can be seen in FIG 4.3, the optimal temperature for Cr(VI) reduction is in the range of 20 to 30 ^{0}C. At 20^{0}C, reduction was observed to be much slower and the amount of Cr(VI) reduced in 72 h was 118.77 mg.L^{-1}, 83.70 mg.L^{-1}, 99.35 mg.L^{-1}, and 104.44 mg.L^{-1} for *Ps. maltophilia*, *A. calcoaceticus*, *B. subtilis*, and *C. cellasea*, respectively. Chromate reduction rate proved to be slowest at temperatures greater than 30^{0}C. The maximum concentration range at which reduction is optimal was also determined. At a Cr(VI) concentration range of 50 to 150 mg.L^{-1} complete reduction was observed (FIG 4.4). *Ps. maltophilia* was the only microorganism of the four not capable of reducing Cr(VI) at a concentration of 200 mg.L^{-1} and greater. *Ps. maltophilia* reduced Cr(VI) up to a concentration of 200 mg.L^{-1}, however, reduction rates were very slow at concentrations above 150 mg.L^{-1}. Results also indicate that the rate of reduction is directly dependent upon the amount of added carbon and energy sources (FIGS 4.5 a-d). As the concentration of nutrients increased, reduction rates increased and reduction was very low at low nutrient concentrations. Minimal reduction was observed at a concentration of 0.025 % for all of the isolates. The effect of metal ions such as Cu^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+} were examined to determine whether these metal ions inhibited chromate reduction. *Ps. maltophilia* was the only organism that showed a great degree of tolerance to these metal ions. The metal ions showed a high percentage of inhibition to *B. subtilis*. Cadmium was observed to be very inhibitory to both *B. subtilis* and *C. cellasea* with percent inhibition as high as 65 and 55% respectively. Metal ions in a mixture (FIG 4.7) appeared to be more inhibitory to the organisms than individual metal ions. Chromate reduction by the organisms was optimal at a pH range of 8 to 10 (FIG 4.8).

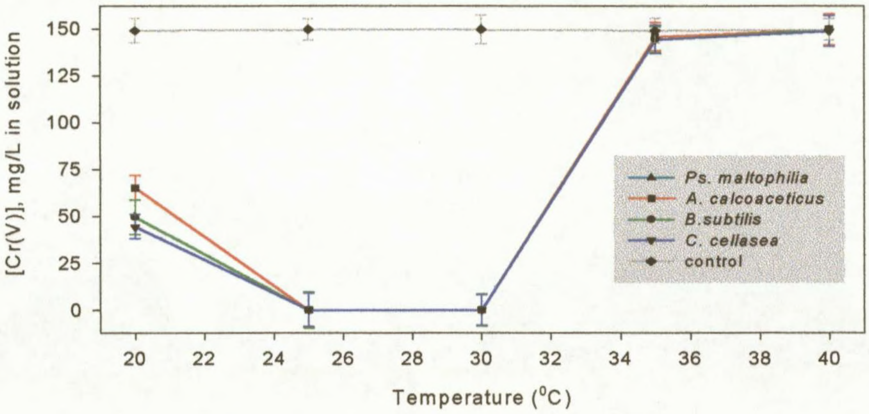


FIGURE 4.3 Effect of temperature on chromate reduction in *Ps. maltophilia, A. calcoaceticus, B.subtilis,* and *C. cellasea* at an initial [Cr(VI)] of 150 mg.L^{-1}.

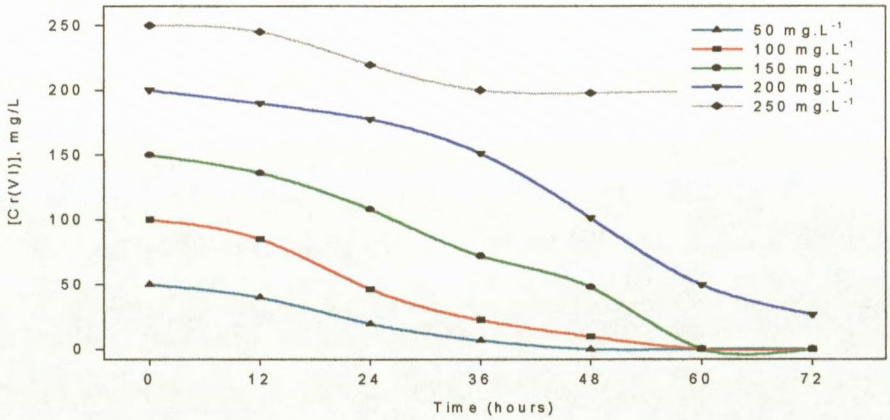


FIGURE 4.4 Effect of increasing CrO_4^{2-} concentration on chromate reduction in *Ps. maltophilia.*

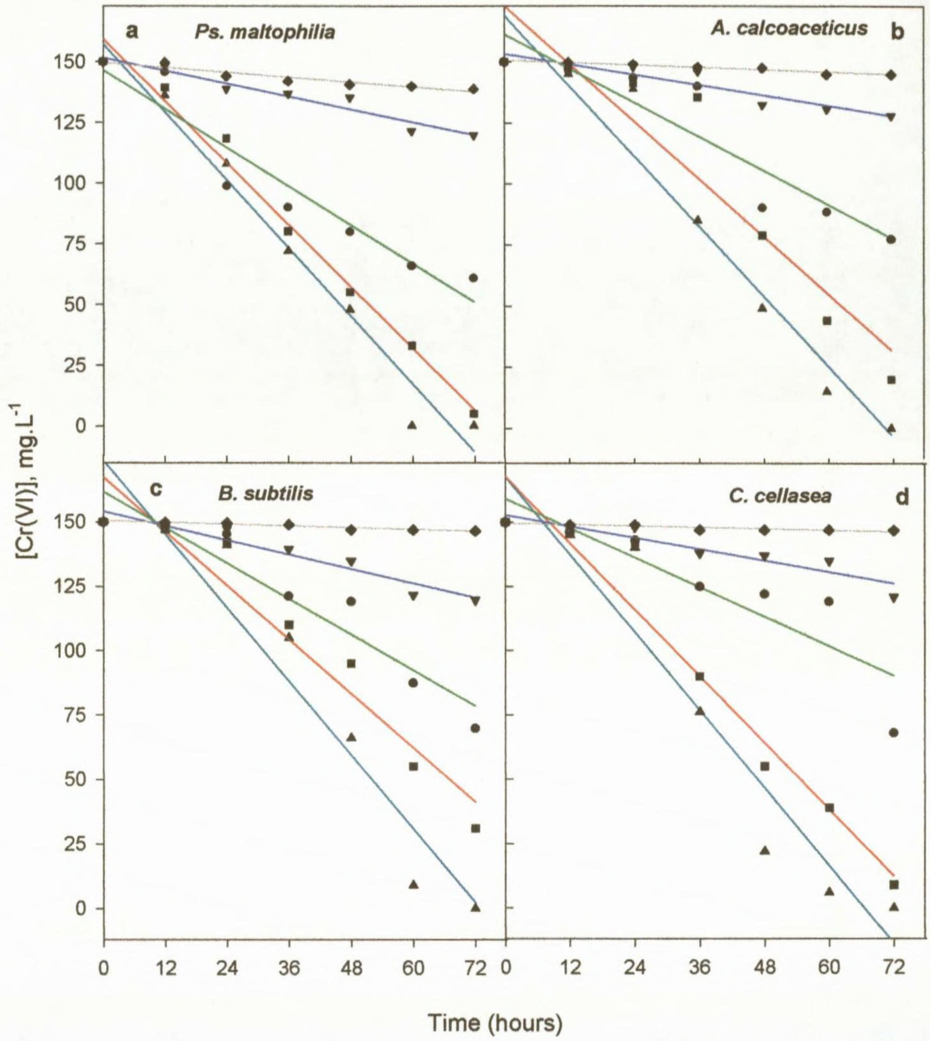


FIGURE 4.5 a-d **Dependence of chromate reduction by (a), *Ps. maltophilia*, (b), *A. calcoaceticus*, (c), *B. subtilis*, and (d), *C. cellasea* on added carbon and energy sources. Nutrient broth medium was added to Cr(VI) solution at concentrations of 0.3% (▲), 0.15 % (■), 0.1 % (●), 0.05 % (▼), and 0.025 % (◆).**

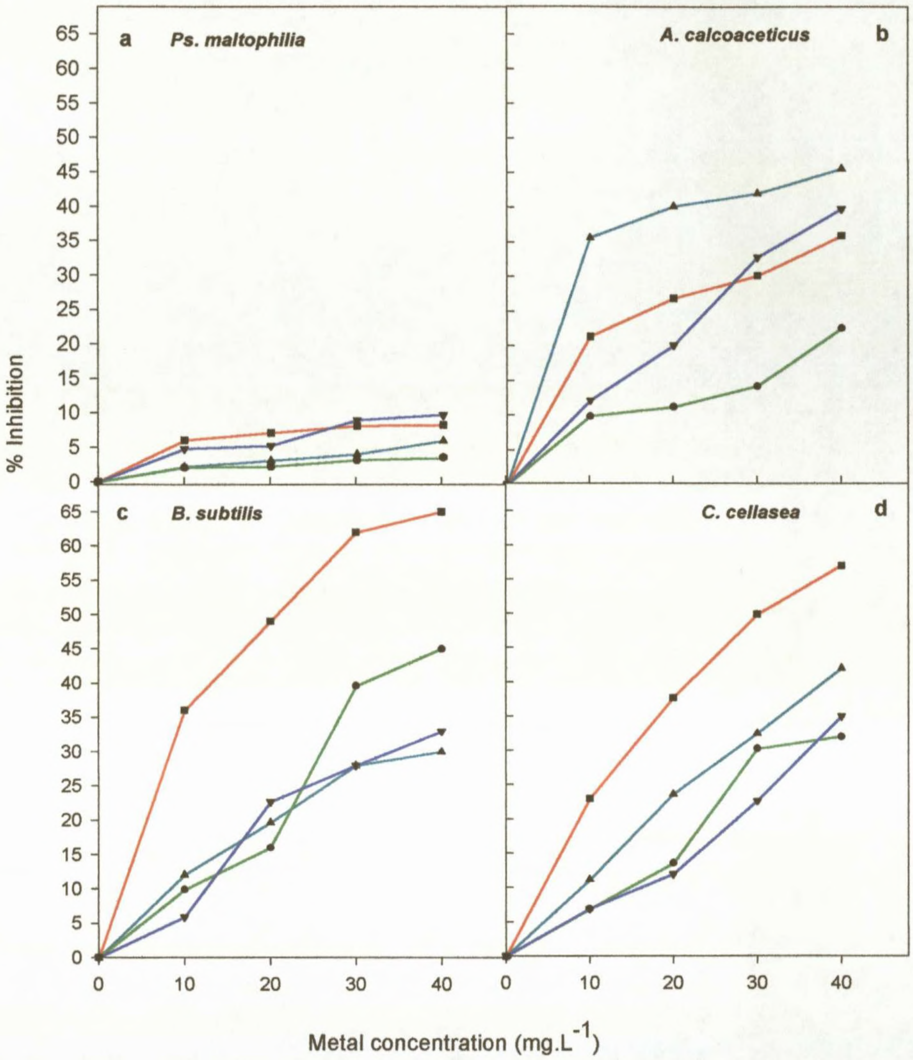


FIGURE 4.6 a-d **Effects of heavy metals on chromate reduction by Cu^{2+} (▲), Cd^{2+}(■), Ni^{2+} (●), and Zn^{2+}(▼) on isolates of (a), *Ps. maltophilia*, (b), *A. calcoaceticus*, (c), *B. subtilis*, and (d), *C. cellasea.***

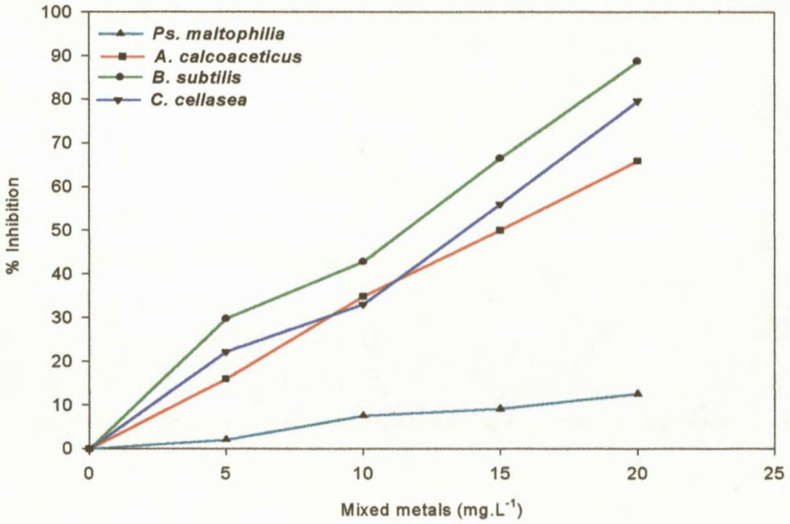


FIGURE 4.7 Effect of mixed metal ions of Cu^{2+}, Cd^{2+}, Ni^{2+}, and Zn^{2+} at various concentrations on chromate reduction on cultures of *Ps. mltophilia* (▲), *A. calcoaceticus* (■), *B. subtilis* (●), *C. cellasea* (▼),

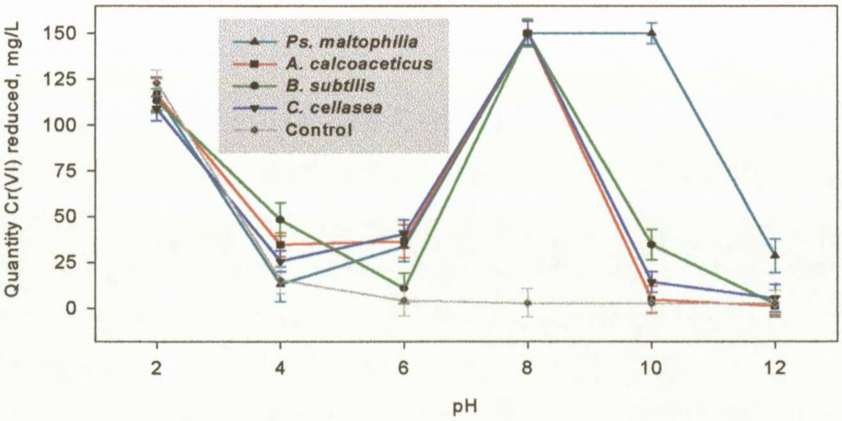


FIGURE 4.8 Effect of pH on chromate reduction by isolates of *Ps. mltophilia* (▲), *A. calcoaceticus* (■), *B. subtilis* (●), *C. cellasea* (▼), and Control (◆), over 72h.

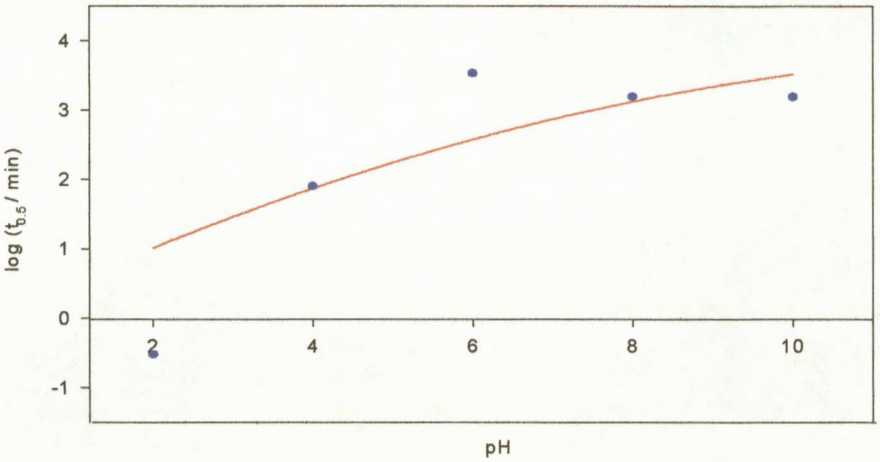


FIGURE 4.9 a **Time required to reach 50% reduction of the initial [Cr(VI)] ($t_{0.5}$), $[Cr(VI)]_{initial}$ = 150 mg.L^{-1} by *Ps. maltophilia.***

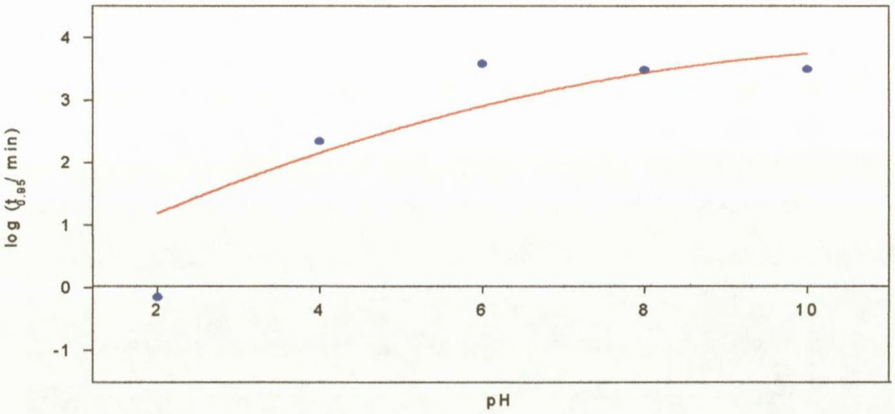


FIGURE 4.9 b **Time required to reach 95 % reduction of the initial [Cr(VI)] ($t_{0.95}$), $[Cr(VI)]_{initial}$ = 150 mg.L^{-1} by *Ps. maltophilia.***

TABLE 4.4 **A comparison between initial and final pH values for Cr(VI) reduction by *Ps. maltophilia* in an unbuffered nutrient broth medium at an initial Cr(VI) concentration of 150 mg.L^{-1}.**

Intial pH	Final pH
2.0 ± 0.1	2.1 ± 0.1
4.0 ± 0.1	4.1 ± 0.2
6.0 ± 0.1	5.4 ± 0.3
8.0 ± 0.1	6.8 ± 0.3
10.0 ± 0.1	7.1 ± 0.3

It is evident from TABLE 4.4 that final pH in an unbuffered medium resulted in a drop in pH as initial pH value increases from 6.0 to 10.0. At a pH of 2.0 and 4.0, however, there is a small increase in pH of approximately 0.1. Solubility of Cr(III) is maximum between pH 6.0 and 8.0 (refer to results in CHAPTER 1, TABLE 1.3), and the effect of pH on solubility will be discussed later in this chapter.

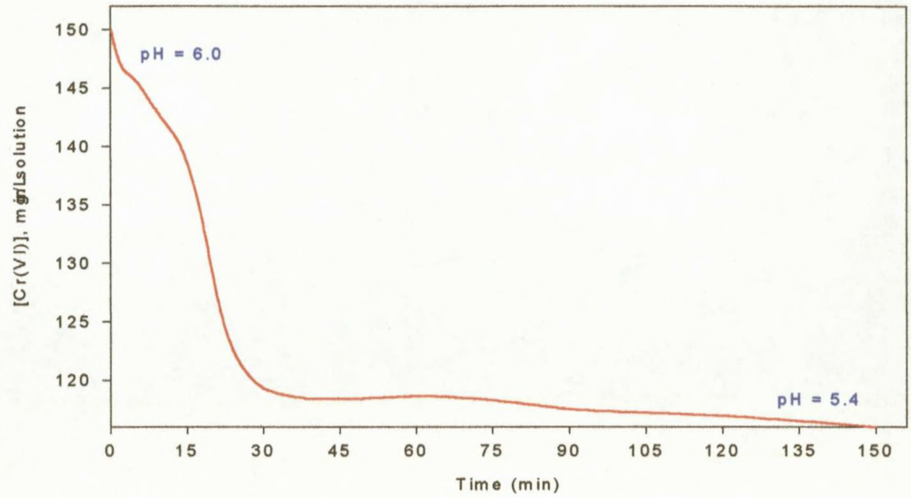


FIGURE 4.10 a Change in medium pH during Cr(VI) reduction by *Ps. maltophilia*, $pH_{initial} = 6.0 \pm 0.1$.

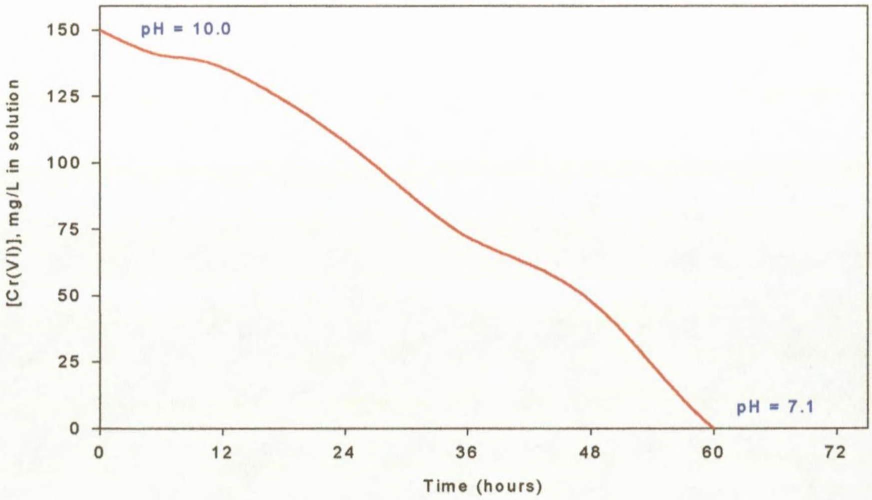


FIGURE 4.10 b Change in medium pH during Cr(VI) reduction by *Ps. maltophilia*, $pH_{initial} = 10.0 \pm 0.1$.

To facilitate the comparison of results under different reaction conditions, results were given in terms of the percentage reduction of the $[Cr(VI)]_{intial}$ vs. time required. Initial pH of the reaction mixture had a strong and definite effect on reduction times for $2.0 \leq pH_{intial} \leq 10.0$, the time required to achieve 50% reduction of the $[Cr(VI)]_{initial}$ ($t_{0.5}$) increased logarithmically with increasing $pH_{initial}$ (FIG 4.9a). Under the same reaction conditions the time required to reach 95% reduction of $[Cr(VI)]_{initial}$ ($t_{0.95}$) showed a similar trend, increasing logarithmically with increasing pH, at $2.0 \leq pH_{initial}$ (FIG 4.9b). A discontinuity was, however, observed at $pH_{initial} > 8$ where the time required to reach 95% reduction, was no longer directly logarithmically related to the $pH_{initial}$. At $pH_{initial} > 10$ very long retention times, were needed to reduce Cr(VI) successfully. For instance at $pH_{initial} = 11.0$, it took 50 h to reach 50% reduction and 102 h to reach 95% reduction of $[Cr(VI)]_{initial}$. At $pH_{initial} = 12.0$ only 4% reduction of $[Cr(VI)]_{initial}$ had occurred after 7 d. No further investigations into the reduction of Cr(VI) were therefore conducted at pH > 10. Looking at the results shown in FIGS 410a and 4.10b, three phenomena warrant explanation: a) what causes the reaction rate to increase at lower pH values; b) why a logarithmic increase in the time required to reach 50% reduction of $[Cr(VI)]_{initial}$ was observed for increasing pH ($2.0 \leq pH \leq 10.0$); and c) why this logarithmic increase only persisted between pH 2 and 6 to reach 95% reduction of the $[Cr(VI)]_{initial}$. The explanations for the occurrence of such phenomena will be further discussed.

4.4 DISCUSSION

Reductase activity was detected in the cytoplasmic membrane (FIG 4.1) in cells of *Ps. maltophilia* suggesting that the Cr(VI) reductase is intracellular and therefore no activity was detected in the outer membrane. Subcellular fractions of *A. calcoaceticus, B. subtilis*, and *C. cellasea* showed no appreciable Cr(VI)-reductase activity (TABLE 4.1). The reason for minimal activity in these subcellular fractions could be that the enzymes isolated were sensitive to specific conditions. The enzymes for each of the organisms may have different parameter requirements thus could not function at a temperature of 28 ^{0}C at a pH of 8.0 which were the optimum conditions required for whole cells. Electron donors, NADH or NAD(P)H were not required for the organisms except for the Cr(VI)-reductase isolated from *Ps. matolphilia.* The Cr(VI)-reductase activity of *Ps. maltophilia* could be measured with NAD(P)H as a physiological electron donor but not with NADH. The same was also reported for *Pseudomonas ambigua* G-1 (Suzuki *et al.*, 1992). While the localization of the Cr(VI) reductase activity in some strains has not been determined, the activities in *Bacillus* strain QCI-2 (Campos *et al.*, 1995) and *Pseudomonas putida* (Ishibashi *et al.*, 1990) are associated with the cytosolic and soluble fractions, respectively. In contrast, the Cr(VI) reductase activities in *Enterobacter* and *Ps. dechromaticans* appear to be membrane associated (Bopp and Ehrlich, 1988; Wang *et al.*, 1990). The NAD(P)H-dependent Cr(VI)-reductase yielded an activity of 3.24 mU.mg^{-1} of protein (TABLE 4.1). Further experiments were carried out with this organism and its subcellular fractions to determine percentage activity that each subcellular fraction constitutes (TABLE 4.2). Crude cell extract demonstrated a 1.5 fold incease of Cr(VI)-reductase activity in comparison to whole cells. The spheroplast fraction contained 97% reductase activity, whereas the periplasmic fraction contained 3% activity. The soluble fraction contained 100% activity while the membrane fraction contained no activity. Thus from these results it is distinct that reductase activity is contained in the cytoplasmic fraction of the cell isolated from *Ps. maltophilia*. Reductase activity was observed in the crude extract and soluble fraction since both these fractions contained cytoplasmic, periplasmic and membrane fractions.

Experiments were also performed to determine whether the enzymes were constitutive or inducible. Cr(VI)-reductase activity was found to be constitutive in all four isolates investigated in this study since no difference in rate of chromate reduction with and without added chromate was noted (TABLE 4.3). Das and Chandra, (1989) also found the Cr(VI)-reductase of *Streptomyces* to be of a constitutive nature.

Suzuki *et al.*, (1992) proposed the catalytic mechanism of purified Cr(VI) reductase. The proposed mechanisms demonstrated that the enzyme catalyzes the reduction of Cr(VI) to Cr(III) by two (or more) steps. First, Cr(VI) accepts one electron from one NADH or NAD(P)H molecule (depending on which electron donor is required) and generates Cr(V) as an intermediate. Second, the Cr(V) intermediate accepts two electrons from two molecules of the same coenzyme. The first step proceeding more rapid than the second one. Reports showed that Cr(VI) is reduced nonenzymatically by some bioreductants, such as NAD(P)H, ascorbic acid, glutathione, and reducing sugars (Goodgame and Joy, 1986; Suzuki, 1986; Branca *et al.*, 1990; Shi and Dalal, 1990; Suzuki and Fukuda, 1990; Aiyar *et al.*, 1991).

Electron spin resonance analysis revealed that the reduction of Cr(VI) caused by these reagents proceeded up to Cr(V). It was also suggested that Cr(V) is actually a toxic and mutagenic agent (Kawanishi *et al.*, 1986; Aiyar *et al.*, 1991; Kortenkamp *et al.*, 1990). Some reports showed that DT-diaphorase (Flora *et al.*, 1985), aldehyde oxidase (Banks and Cooke, 1986), glutathione reductase (Shi and Dalal, 1990), and cytochrome P-450 (Garcia and Jennette, 1981; Suzuki, 1986; Mikalsen *et al.*, 1991) have Cr(VI)-reducing activity. However, it is still unclear whether these enzymes contribute to the detoxification or rather to the toxification. It is necessary to elucidate the behavior of the intermediates formed during reduction in order to achieve a good understanding of the overall mechanism of Cr(VI) toxicity and its detoxification.

Batch-scale reactors are reportedly successful in determining parameters for optimal Cr(VI) reduction (Komori *et al.*, 1990a). Thus batch reactors were used in an attempt to optimize parameters of temperature, pH, nutrient concentration, presence of heavy metal ions and increasing Cr(VI) concentration on chromate reduction. Batch reactors for *Ps. maltophilia, A. calcoaceticus,* and *B. subtilis* can be seen in FIG 4.2. There was a distinct colour change from yellow to a dark greenish in the experimental which was not observed in the control. Turick, (1997) also reported of such a colour change. This colour change could be attributed to an increase in turbidity due to increased biomass concentration and formation of $Cr(OH)_3$. Other investigations, however, reported a colour change from yellow to white formed as a result of $Cr(OH)_3$ precipitation (Garbisu *et al.*,1997). In the aforementioned investigation as well as investigations conducted by Turick, (1997), an unbuffered nutrient-rich medium was used viz., nutrient broth and molasses, respectively compared to the buffered chemically defined medium used by Garbisu *et al.*, (1997). Therefore the contradictions in the colour change of the medium could be due to the fact that in a buffered medium Cr(III) formed as a result of Cr(VI) reduction, precipitates out as a hydroxide and in an unbuffered medium, no precipitate was observed since hydroxides are not necessarily formed. The absence of a precipitate, however may also be due to the organic content of the medium allowing for Cr(III) complexation, thereby keeping Cr(III) in solution and even minimizing any potential inhibitory characteristics.

The temperature range at which complete Cr(VI) reduction was achieved was 25-30^{0}C with an optimum of 28^{0}C (FIG 4.3). All isolates were capable of reduction at 20^{0}C, however at this temperature, the quantity Cr(VI) reduced was between 85 mg.L^{-1} and 105 mg.L^{-1}. Chromate reduction was very sensitive at temperatures above 30^{0}C with no reduction occurring above 30^{0}C. The reason for these organisms choosing the stated temperature range could be that the isolates were obtained from soils and wastewaters to which they are indigenous. The temperature in such environments usually range from 20-30^{0}C, thus it could explain why such a temperature range would be much suited for successful and optimal Cr(VI) reduction. This temperature range is in correlation with much of the results obtained from previous studies. *Enterobacter cloacae* that was isolated from activated sludge required a temperature of 30^{0}C for Cr(VI) reduction in a fed-batch system (Fujii *et al.*, 1994; Rege *et al.*, 1997). DeLeo and Ehrlich, (1994) isolated *Pseudomonas fluorescens* from a river sediment and conducted Cr(VI) experiments in a batch system at a temperature of 25^{0}C. Resting cell cultures of *Agrobacterium radiobacter* isolated from soil also required a temperature of 30^{0}C (Llovera *et al.*, 1993). All these organisms were indigenous to either wastewater or soil, consequently the required temperature would be that closest to the temperature of the natural environment.

The time course data on Cr(VI) concentrations of the batch culture is shown in FIG 4.4. The optimal concentration at which Cr(VI) could be reduced was 150 mg.L^{-1} by *A. calcoaceticus. B. subtilis,* and *C. cellasea.* The effect of increasing initial chromate concentration had a direct effect on the rate of chromate reduction. Only *Ps. maltophilia* was capable of Cr(VI) reduction at a concentration 200 mg.L^{-1}. The rate of reduction with increasing Cr(VI) concentration was inversely proportional to time. A longer time period was required to reduce 200 mg.L^{-1} Cr(VI) as compared to the time required to reduce 50 mg.L^{-1} Cr(VI) and complete reduction was still not achieved at 200 mg.L^{-1}.

In this experiment, the initial concentration of Cr(VI) was varied from 50 to 250 mg.L^{-1} (FIG 4.4). One distinct phenomenon noted here is that addition of Cr(VI) led to a transient decrease in cell viability which was measured by the plate count method. The rate and extent of decrease in viable cell concentration were dependent on the concentration of added Cr(VI). The average rate of Cr(VI) reduction increased with increasing initial Cr(VI) concentration until an optimum of 150 mg.L^{-1} was reached over an incubation period of 72 h. The average rate began to decrease at Cr(VI) concentrations higher than 150 mg.L^{-1}. The results indicate that chromate (CrO_4^{2-}) with its toxicity and high oxidative potential may inhibit biological activity. Evidently high chromate concentrations prevent multiplication of the organisms but do not prevent them from reducing chromate. Thus it could be assumed that chromate resistance in these organisms is not related to its ability to reduce chromate. Earlier work (Bopp *et al.*, 1983) showed that the determinant for chromate resistance resides on a plasmid which is contrary to the results obtained in this study. The reason for this contradiction is that, if chromate reductase is a plasmid mediated enzyme then more would be transcribed if the concentration of chromium is increased. However, the increased chromate concentration in the culture media hindered the rate of reduction when chromate concentration was higher than 150 mg.L^{-1}.

No chromate reduction was observed without adding carbon and energy sources. To test the effect of added carbon and energy sources on chromate reduction, varying concentrations of nutrient media with 150 mg.L^{-1} Cr(VI) was inoculated with the cultures and the reduction rate was noted (FIG 4.5a-d). Clearly, the rates of chromate reduction were dependent on the amount of carbon and energy added. Rates of chromate reduction increased with increasing concentration of nutrients.It was thought that the bacterial ability to reduce chromate might be inhibited by some components of the waste solution. The waste solution contained several metal ions and since heavy metals were known to be toxic to microorganisms, inhibition of chromate reduction by these heavy metal ions was also tested. In the experiment, bacterial cells were inoculated into nutrient broth medium at an initial density of 3 x 10^8 cells.mL^{-1} with an initial Cr(VI) concentration of 150 mg.L^{-1}. Enzymes points out the possibility of developing cell-free systems for treating Cr(VI)-containing wastes. The percent inhibition was calculated from the initial rate of chromate reduction relative to that of the control culture without added metal cations. As expected, the higher the concentration of metal used the higher the percent inhibition of chromate reduction. Degrees of inhibition caused by metal cations were $Zn^{2+}>Cd^{2+}>Cu^{2+}>Ni^{2+}$ for *Ps. maltophilia*, $Cu^{2+}>Zn^{2+}>Cd^{2+}>Ni^{2+}$ for *A. calcoaceticus*, $Cd^{2+}>Ni^{2+}>Zn^{2+}>Cu^{2+}$ for *B. subtilis*, and $Cd^{2+}>Cu^{2+}>Zn^{2+}>Ni^{2+}$ for *C. cellasea* at maximum metal concentrations of 40 mg.L^{-1} (FIG 4.6a-d). Similar results indicating the strong inhibition indicating the strong inhibition caused by heavy metal cations on the reduction of chromate were reported by Hardoyo *et al.*, (1991) for anaerobically grown *Enterobacter cloacae*. Garbisu *et al.*, (1997) also reported the inhibitory effects of metal ions on *Bacillus subtilis* and found the degrees of inhibition to be $Cd^{2+} >Ni^{2+} >Zn^{2+} >Cu^{2+}$. The results by Garbisu *et al.*, (1997) also indicated that although *B. subtilis* cells could reduce Cr(VI) in the presence of different heavy metal cations, the performance of a potential biological treatment of chromate-bearing wastewaters would be significantly improved by removing toxic heavy metal cations from industrial effluent. However, conventional methods for removing metals from effluents (ion exchange, adsorption on coal, activated carbon, etc.) are expensive due to their requirements for high energy or large quantities of chemical adsorbents. In this context, the removal of heavy metals by microbial cells, mainly through their sorption to cell surfaces, has been recognized as a potential alternative to the existing technologies for the recovery of metals from industrial wastewaters (Sampedro *et al.*, 1995). Additionally, if the concentrations of metals in the effluent are low, appropriate dilution could help to overcome their inhibition effect on the biological reduction of chromate. However, much research is needed to unravel the specific inhibition mechanisms for each heavy metal.

In order to explain the increase in reaction rate with decreasing pH, one needs to take into account the speciation of Cr(VI) under these conditions. The *E*h-pH diagram shown in FIG 4.11 (Rai *et al.*, 1989) provides a generalized depiction of the major aqueous chromium species and redox stabilities under conditions of chemical equilibrium. The dominant Cr(VI) species are $HCrO_4^-$ at pH 1-6 and CrO_4^{2-} at pH > 6. Chromium (VI) also forms other species, such as $HCr_2O_7^-$ and $Cr_2O_7^{2-}$. However, their formation requires Cr(VI) concentration > 10^{-2} mol.dm^{-3} (Baes and Mesmer, 1976). The increase in reaction rates with decreasing pH can therefore probably be attributed to CrO_4^{2-} (see redox stabilities FIG 4.11).

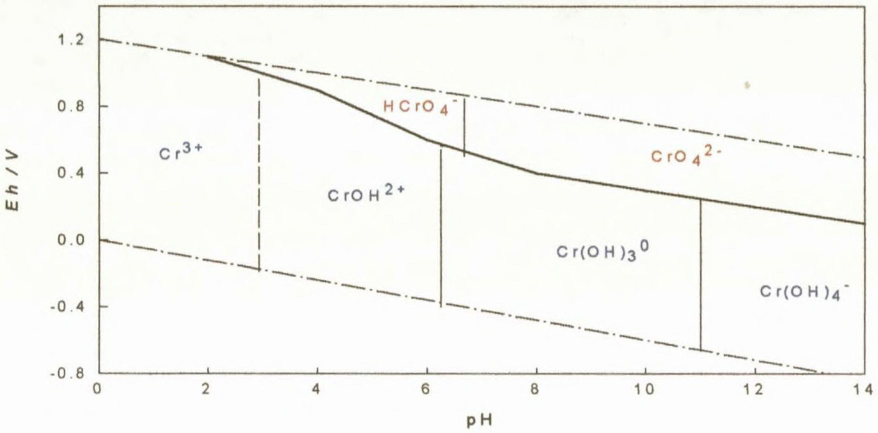


FIGURE 4.11 ***E*h-pH diagram for aqueous chromium species (Rai *et al.*, 1989).**

A logarithmic increase in time required to reach a certain percentage reduction of the $[Cr(VI)]_{initial}$, for increasing pH, can be explained if the Cr(VI)-cell concentration reaction system's rate law has a first-order dependence on the $[H^+]$. For example a simple rate law might be:

$$\frac{-d[Cr(VI)]}{dt} = k[H^+][Cr(VI)][cells]$$

This can be simplified to:

$$\frac{-d[Cr(VI)]}{dt} = k'[Cr(VI)]$$

$$\text{with } k' = k\,[H^+][\text{cell}]$$

The half life, $t_{0.5}$, for a first-order reaction is given by :

$$t_{0.5} = \frac{\ln 2}{k'}$$

Which means that :

$$t_{0.5} \propto \frac{1}{[H^+]} \quad , \quad \text{since } k' \propto [H^+]$$

Therefore :

$$\log(t_{0.5}) \propto \log\left(\frac{1}{[H^+]}\right) = \text{pH}$$

(Beukes *et al.*, 1999)

In the same way log $(t_{0.95}) \propto$ pH . The logarithmic increase in time required to reach a 50% reduction of the $[Cr(VI)]_{initial}$ for increasing pH, can therefore be explained by the fact that the Cr (VI)-cell concentration reaction system's rate law has a first order dependence on the $[H^+]$. As long as the pH remains constant, this direct relationship will remain. Nelson *et al.*, (1992) stated that Cr(VI) is very soluble at all pH values, whereas Cr(III) reaches its least solubility at a pH of 7.5 (refer to TABLE 1.3 in CHAPTER 1). During this investigation, unbuffered nutrient broth was used. Any drift in pH, due to the formation of reaction products, would obviously influence the reduction rate. To investigate this possibility, some initial and final pH values, at a fixed $[Cr(VI)]_{initial}$ and cell density were measured (Table 4.3). From these results it is clear that there is a slightly upward drift in pH at $2.0 \leq \text{pH} \leq 6.0$, which is due to the consumption of H^+ ions. At pH > 6 a downwards drift in pH was observed. The amount of pH drift also becomes significantly larger as the initial pH increase from 6 to 10. A feasible explanation for this depression in pH at higher initial pH values is the release of H^+ ions due to hydrolysis of the Cr(III) ions produced by the reaction, which dominates the consumption of H^+ ions. Above pH =6, hydrolysis of the Cr(III) ions become significant (Richard and Bourg, 1991). The effect of pH drift on the reaction is also reflected by the shapes of the kinetic traces at different pH values. At pH =6.0, where the pH drift was relatively small, typical first-order-like time traces were observed (see Fig. 4.10 a). The time traces observed at pH =10.0 exhibited a shape generally associated with autocatalytical processes (see Fig. 4.10 b). At first the reaction rate is slow, it then accelerates either by the formation of a species which accelerates the reaction rate or by the consumption of species which retards the reaction and eventually the reaction rate slows down again due to the depletion of one or more of the reagents. Indeed, the unbuffered reduction of aqueous Cr(VI) by the cells, at $pH_{initial} > 6$, can be seen as an autocatalytic process. The kinetic trace in Fig. 4.10 b can be explained as follows: initially the reaction rate is slow, then as reduction starts taking place the pH starts drifting down which accelerates the reduction rate and eventually the reduction rate slows down due to the depletion of Cr(VI). In the previous paragraph it was shown that $\log (t_{0.95}) \propto pH$, if the pH remains constant. The fact that a significant downwards drift in pH was observed at $pH_{initial} > 6$, means that the direct relationship between $\log (t_{0.95})$ and pH is no longer valid, explaining the discontinuity at pH > 6 in Fig. 4.8 b.

4.5 CONCLUSIONS

Cr(VI) reductase activity was found to be associated with the CM of aerobically grown cells of *Ps. maltophilia* with NAD(P)H serving as an electron donor. The Cr(VI) reductase was observed to be constitutive in all four isolates. Additional studies on the Cr(VI) reductase activity in *Ps. maltophilia* will be useful in assessing its potential for the bioremediation of Cr(VI)-contaminated sites or industrial effluents.

In summary, laboratory-scale batch reactors for the organisms provided the following conclusions:

2. The optimum temperature for successful Cr(VI) reduction is 20-30^{0}C for the organisms.
3. Batch cultures of *Ps. maltophilia, A. calcoaceticus. B. subtilis*, and *C. cellasea* were capable of 100% reduction of chromate with a $[Cr(VI)]_{initial} = 150$ mg.L^{-1} and $[cell]_{initial} = 3 \times 10^8$ cells.mL^{-1}.
4. The specific rate of chromate reduction is dependent upon initial Cr(VI) concentration and pH.
5. Unbuffered nutrient broth at a percentage concentration of 0.3% was observed to be optimal for Cr(VI) reduction.

6. Degrees of inhibition caused by metal cations were $Zn^{2+}>Cd^{2+}>Cu^{2+}>Ni^{2+}$ for *Ps. maltophilia*, $Cu^{2+}>Zn^{2+}>Cd^{2+}>Ni^{2+}$ for *A. calcoaceticus*, $Cd^{2+}>Ni^{2+}>Zn^{2+}>Cu^{2+}$ for *B. subtilis*, and $Cd^{2+}>Cu^{2+}>Zn^{2+}>Ni^{2+}$ for *C. cellasea* at maximum metal concentrations of 40 $mg.L^{-1}$.
7. Batch reactor pH in the range 7.0 to 10.0 is suitable for chromate reduction.
8. The pH of the reaction mixture that is not in the range stated above has a negative effect on the reduction rate of Cr(VI) by the organisms.
9. During the unbuffered reduction of Cr(VI), pH drift takes place, which influences the reduction process.
10. Maximum Cr(VI) reduction of 100% is obtainable in 60 h, and the detoxified chromate in the trivalent form is maintained in a soluble state.

The fact that the process described here is enzymatic suggests that there is substantial opportunity for enhancing the rate and extent of this process. Techniques designed to specifically isolate organisms with high rates of Cr(VI) reduction could yield better Cr(VI) reducers. Furthermore, once the Cr(VI)-reducing enzymes are identified, there is potential to use molecular techniques to magnify the activity in whole cells or to produce large quantities of Cr(VI)-reducing enzymes for use in cell-free systems. It was also evident from the results that a high bacterial density eventuates in an increased Cr(VI) reduction rate. Thus, immobilization of cells onto appropriate immobilizing agents is considered to yield increased volumetric productivity.

CHAPTER FIVE

CONTINUOUS-UPFLOW REACTOR SYSTEM FOR CHROMATE REDUCTION

5.1 INTRODUCTION

A bioprocess operating in conjunction with soil washing and pump-and-treat technologies could be developed to reduce Cr(VI). Turick and Apel, (1997) demonstrated a packed bed, anaerobic bioprocess, incorporating a mixed culture of Cr(VI) reducers from soil. Bio-Sep beads (Bio-Sep™, DuPont, Newark, DE) were used as the solid support for the mixed soil cultures with a liquid volume of 750 mL (FIG 5.1). This bioprocess has been shown to operate with a continuous stream of Cr(VI) ranging from 140-750 $mg.L^{-1}$ at a dilution rate of 0.5 d^{-1}. Cr(VI) reduction occurred at a rate of 4 $mg.L^{-1}.h^{-1}$. Although these Cr(VI) reduction rates were considered low, the results demonstrated the possibility of developing a bioprocess using a mixed culture of soil isolates to reduce Cr(VI) continuously.

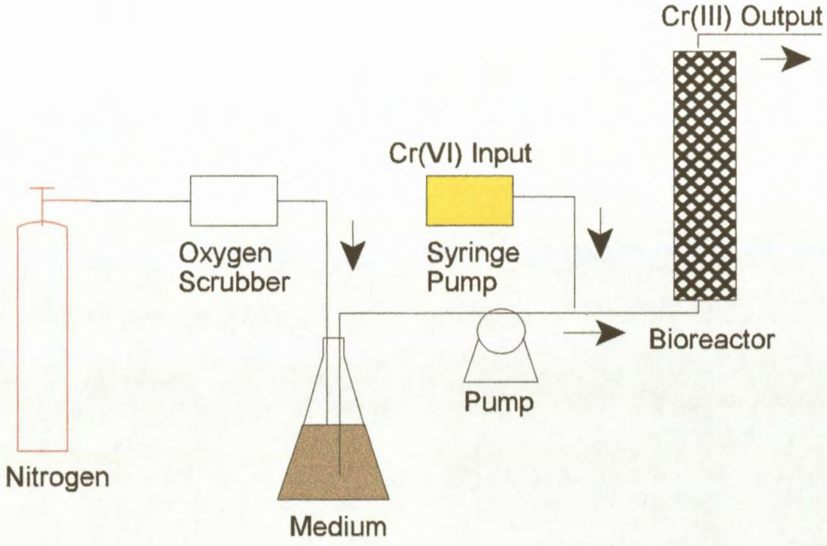


FIGURE 5.1 Schematic representation of a Cr(VI)-reducing bioprocess using a packed-bed bioreactor (Turick *et al.*, 1997).

Turick *et al.*, (1997) investigated an increase in bacterial density in a bioreactor and subsequent volumetric productivity of the bioprocess. The increased porosity of the beads allowed for potentially higher densities of bacteria owing to entrapment and immobilization. Approximately 95% of the Cr(VI) entering the bioreactor was reduced to Cr(III) that resulted in a reduction efficiency of 260 mg.L^{-1}.h^{-1} at a dilution rate of 2 h^{-1}.

In this chapter, polyacrylamide immobilized bacterial cells were used to reduce Cr(VI) from solution in a laboratory-scale batch reactor. Suspended cells will be impractical in full-scale reactors because firstly, they must be removed from the effluent solution by filtration, settling, or some other process and secondly, reuse of the cells will be difficult since precipitated metals will be interspersed with the cells. Thus, a more practical method for Cr(VI) reduction in laboratory reactors was investigated. Intact cells of *Ps. maltophilia* was immobilized in polyacrylamide gel and used to reduce chromate from solution.

The aim of this phase of study involved:

- The development of a continuous-upflow bioreactor packed with immobilized bacterial cells for the treatment of Cr(VI)-containing industrial effluents.

The main objectives were:

- Cell immobilization into polyacrylamide gel.
- Stabilization of reactor containing using synthetic effluent containing Cr(VI) up to 150 mg.L^{-1}.
- Treatment of chromate-contaminated industrial effluents.

5.2 MATERIALS AND METHODS

5.2.1 Immobilization of Cells in Polyacrylamide Gel

Cells of *Ps maltophilia* were harvested by centrifugation at 5800 x *g* for 30 min, washed with sterile phosphate buffer at a pH of 7.0 and centrifuged again. The biomass (1g wet mass) was resuspended in 20 mL sterile, deionized water supplemented with 2.5 g acrylamide monomer and 0.25 g *N,N'*-methylenebisacrylamide as a cross-linking agent. A small aliquot of this solution was collected for protein determination. The polymerizing reaction was initiated by adding 2.5 mL of a 2.5% (w/v) solution of potassium persulphate and accelerated by adding 2.5 mL of a 5% (w/v) of 3-dimethylaminopropionitrile. The suspension was stirred gently until the gel began to set and was then refrigerated at 4^{0}C for 1h. After the gel had set, it was aseptically cut into approximately 4 mm cubes and washed with sterile phosphate buffer at a pH 0f 7.0 before being placed into the column reactor (Pons and Fusté, 1993).

5.2.2 Bacterial Growth Analysis

In order to establish a growth curve of the immobilized cells in the reactor, the same amount of polyacrylamide immobilized cells that was placed into the reactor was put into 250 mL conical flasks. The volume was the same as that in the continuous reactor as well as the contact time of 6.64 h. Thereafter, the polyacrylamide immobilized cells were homogenized and plate counts on nutrient agar by means of the poured plate method were performed to determine cell concentration and protein concentration was also performed to determine total cell concentration.

5.2.3 Bioreactor Description

The bioreactor consisted of a 50 mL glass cylinder with a diameter of 2.5 cm and height of 15 cm sealed with stoppers at either end. Liquid volume was 15 mL with the polyacrylamide immobilized cells making up the remaining volume. The operational temperature in the reactor was maintained between 25-30^{0}C (approximate room temperature) and pH at 8.0. Nutrient broth with 150 mg.L^{-1} Cr(VI) entered the bioreactor via a peristaltic pump. The nutrient broth containing Cr(VI) was circulated through the reactor with a peristaltic pump positioned upstream of the reactor. The pump was cycled on and off with an automatic timer at hourly intervals to achieve an average flow rate of approximately 2-5 mL.h^{-1}. The effluent was then analyzed for Cr(VI) reduction. The reactor was set up similar to that in FIG 5.1, except that it was an aerobic process (thus a nitrogen and an oxygen scrubber was not needed) and the medium and Cr(VI) was not mixed separately prior to entering the bioreactor.

5.2.4 Chromium and pH analysis

Influent samples were taken periodically and analyzed for pH and Cr(VI). Hexavalent chromium, total chromium, and pH of the effluent were monitored in the reaction effluent. In batch studies conducted it had been established that an unbuffered medium result in a decrease in pH which is not desired. Thus a modified nutrient broth containing a buffer was established (APPENDIX 13). Effluent samples for analysis were collected in sterile polyethylene vials.

5.2.5 Chromate Reduction of Industrial Effluent

In the experimental work carried out thus far, synthetic effluents were used to establish the performance of a reactor. However, in order to determine the industrial applicability of the reactor, chromate-containing effluents from electroplating industries were used to determine the effectiveness of such a bioprocess . Therefore, industrial effluent was used, but nutrients still had to be added for effective Cr(VI) reduction. Modified nutrient broth at a concentration of 0.3% was added to the industrial effluent.

5.3 RESULTS

5.3.1 Performance of a Continuous -upflow Reactor System

Reduction of Cr(VI) occurred at a much faster rate with immobilized cells in a continuous reactor (FIG 5.4) compared to freely suspended cells in a batch reactor (refer to FIG 3.4 in CHAPTER 3). Cr(VI) reduction efficiencies greater than 99% were measured. In the continuous reactor 99% reduction efficiency within a time of 24 h was observed, whereas the same reduction efficiency was observed in batch reactors within 60 h. There was a noted colour change from yellow to light brown as can be seen in FIG 5.2. The colour of the effluent was much lighter than that seen in the batch reactor (FIG 5.3).

5.3.2 Mass balance for Cr(VI) Reduction by *Ps. maltophilia* in a Continuous Reactor

Continuous flow cultures showed an increase in Cr(VI) reduction with an increased mean cell residence time. The data indicated that Cr(VI) reduction onto the polyacrylamide cubes during experimental operation was not a significant factor. Approximately 99% of the Cr(VI) entering the bioreactor was reduced to Cr(III), resulting in a reduction efficiency of 5.34 $mg.g^{-1}.h^{-1}$ (TABLE 5.1). Initial cell concentration immobilized onto polyacrylamide was 1.000 $mg.L^{-1}$ and gradually increased to 1.150$mg.L^{-1}$. The residence time of the influent in contact with the cells was calculated to be 6.64 h. Approximately 148 $mg.L^{-1}$ Cr(VI) was reduced in 18 h and an average of 1.65 $mg.L^{-1}$ was unaccounted for with the rest being either in the bacterial cells or in the wash water (TABLE 5.2).

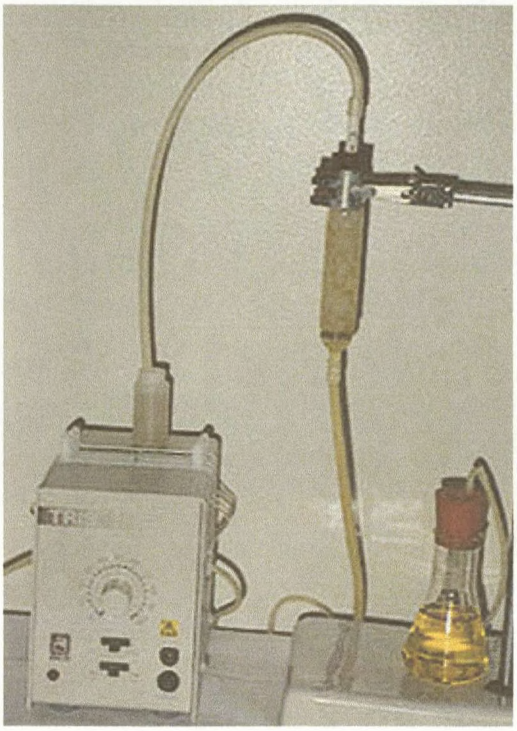


FIGURE 5.2 **Continuous-upflow reactor system containing immobilized cells for Cr(VI) reduction.**

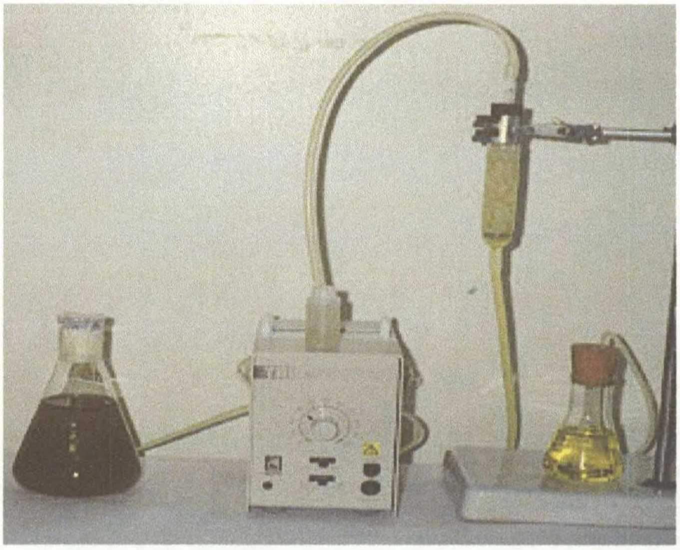


FIGURE 5.3 **Differences in colour of effluent in batch (left) and continuous (right) resulting from Cr(VI) reduction.**

TABLE 5.1 Reduced Cr(VI) and cell concentration as a function of time.

Contact time (h)	Reduced Cr(VI)[*] ($mg.L^{-1}$)	Cell conc ($g.L^{-1}$)	Cr(VI) reduction rate ($mg.g^{-1}.h^{-1}$)
6	28.98	1.000	4.83
16	78.01	1.075	4.53
24	147.35	1.150	5.34

[*] Initial Cr(VI) concentration = 150 $mg.L^{-1}$.

TABLE 5.2 Mass balance of chromium in a continuous-upflow reactor.

Sample no.	1	2	3
Initial Cr(VI) in supernatant[a] ($mg.L^{-1}$)	150	150	150
Final Cr(VI) in supernatant[a] ($mg.L^{-1}$)	2.65	2.21	1.78
Total Cr in supernatant[a] ($mg.L^{-1}$)	148.26	147.35	148.42
Cr(III) in supernatant[a,b] ($mg.L^{-1}$)	147.61	146.14	147.64
Total Cr in cells[a] ($mg.L^{-1}$)	0.09	0.11	0.13
Total Cr in washwater[a] ($mg.L^{-1}$)	1.02	1.19	1.13
Cr(VI) not accounted for ($mg.L^{-1}$)	1.28	2.56	1.10

[a] Average of two values.

[b] Difference between total Cr in supernatant and final Cr(VI) in supernatant.

5.3.3 The Effect of Flowrate on Cr(VI) Reduction

Cr(VI) reduction was determined with varying flowrates to establish an optimal rate at which Cr(VI) would successfully be reduced (FIG 5.4). It is evident from the graph that a high flowrate results in an increased time required to reduce Cr(VI). Chromate reduction is inversely proportional to flowrate, since the rate of Cr(VI) reduction increases with a decrease in flowrate. At a flowrate of 2.26 mL.h^{-1}, 148 mg.L^{-1} Cr(VI) was completely reduced in 24 h. However, at flowrates of 4.85 and 3.26 mL.h^{-1}, only 77 mg.L^{-1} and 50 mg.L^{-1} Cr(VI) was reduced, respectively in 24 h. At these flowrates (4.85 and 3.26 mL.h^{-1}), Cr(VI) would eventually be reduced at an initial concentration of 150 mg.L^{-1}, however the time required for complete reduction would be much longer. Therefore, a flowrate of 2.26 mL.h^{-1} was used throughout the experiment.

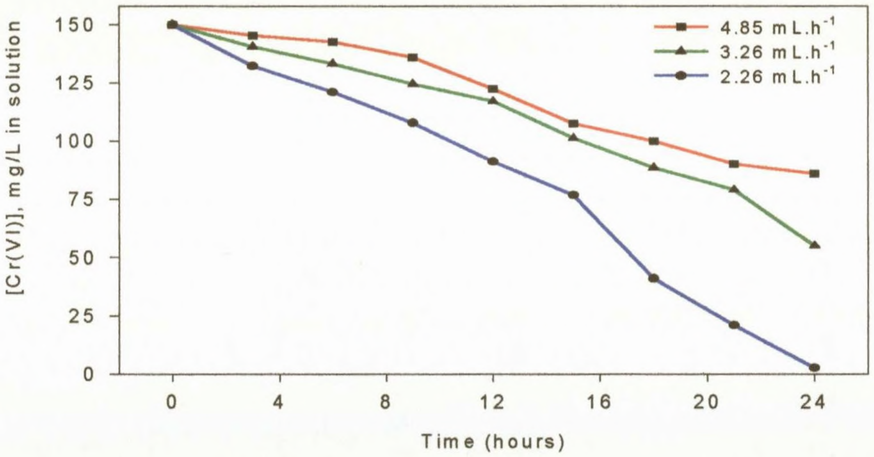


FIGURE 5.4 **Chromate reduction at different flowrates, 4.85 mL.h^{-1} (■), 3.26 mL.h^{-1} (▲), and 2.26 mL.h^{-1} (●) of influent entering the bioreactor by immobilized cells of *Ps. maltophilia* in a continuous-upflow reactor.**

5.3.4 Determination of Biomass Concentration in the Continuous Reactor

It was well established in the previous chapter that increased biomass concentration greatly influences the rate of Cr(VI) reduction in a batch system. Therefore, biomass concentration was determined to establish whether this phenomenon was also true for continuous systems using immobilized cells. From the graph (FIG 5.5), it is apparent that there is a very slight increase in cell concentration over a time of 36 h. Protein content of the cells was also calculated to determine the total cell concentration to establish the rate of Cr(VI) reduction. The graph (FIG 5.5) depicts an increase in viable cell concentration, from an initial concentration of 2.76 x 10^5 to 3.7 x 10^8 cells.mL^{-1}. There is a slight increase in cell concentration between 0 and 24 h, thereafter a plateau phase is observed. FIG 5.6 depicts results of Cr(VI) reducing bioreactor studies in terms of Cr(VI) reduction rate with Cr(VI)$_{initial}$ at 150 mg.L^{-1} and a flowrate of 2.26 mL.h^{-1}.

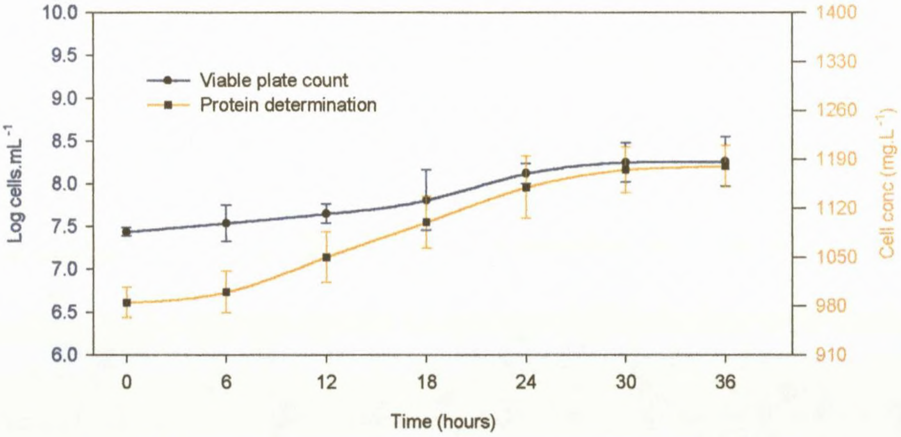


FIGURE 5.5 **Biomass concentration by viable plate count method (●) and protein determination (■) in a continuous reactor.**

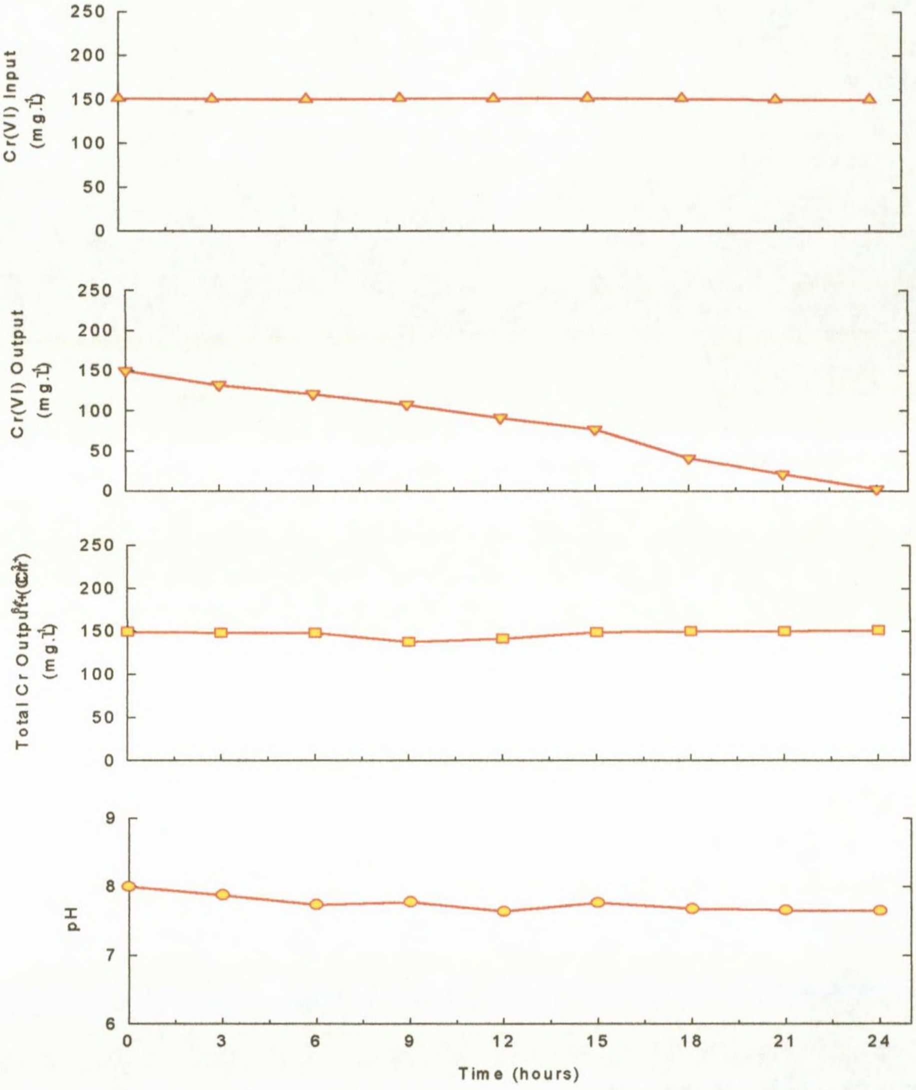


FIGURE 5.6a-d **Reduction of 150 mg.L^{-1}Cr(VI) in a continuous reactor with a) Cr(VI) input (▲), b) Cr(VI) output (▼), c) Total Cr output (■), and (d) pH (●).**

5.3.5 Treatment of Chromium-contaminated Industrial Effluents

Pseudomonas maltophilia was capable of successful Cr(VI) reduction of the various chromium-contaminated effluents. This organism was capable of complete Cr(VI) reduction within 18 h at concentrations ranging from 15.66 to 35.69 mg.L^{-1} (FIG 5.7). The effluent samples was composed of heavy metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+} (TABLE 5.3). Complete reduction in a batch system was accomplished in 21 h and 24 h for 15.66 and 27.85 mg.L^{-1}, respectively. At a $Cr(VI)_{initial}$ of 35.69 mg.L^{-1}, a time period greater than 24 h was required for complete reduction. There was a gradual decrease in Cr(VI) between 0 and 9 h at a $Cr(VI)_{intial}$ of 35.69 mg.L^{-1}, thereafter there was a sharp decline in reduction rate, until 99.5% reduction was achieved in 15 h (FIG 5.8a-c). However, at $Cr(VI)_{initial}$ of 15.66 and 27.85 mg.L^{-1}, respectively, Cr(VI) reduction progressed gradually until complete reduction was achieved. The pH of the system was maintained at 7.5 and no drastic changes from this value had been observed.

TABLE 5.3 Composition of metal plating wastewater

Sample	Cr^{6+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	Cd^{2+}
Sheffield Electroplaters	35.69	15.94	14.33	11.20	1.02
Natal Electroplaters	27.85	16.22	17.41	17.11	2.08
Saayman Danks	15.66	11.66	1.95	15.89	10.0

All values are represented in mg.L^{-1}.

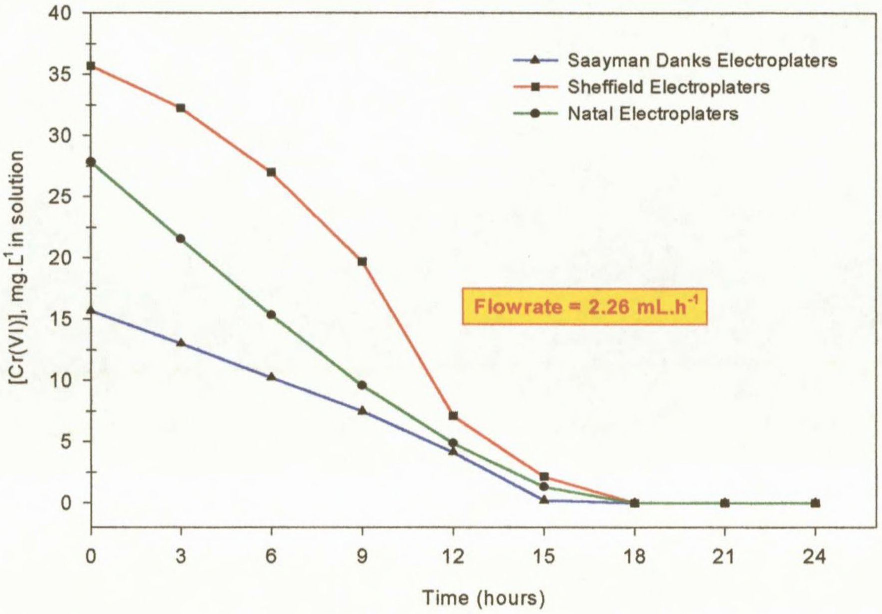


FIGURE 5.7 **Treatment of industrial wastewater in a continuous reactor at varying $[Cr(VI)]_{initial}$ of 15.66 mg.L^{-1} from Saayman Danks Electroplaters (▲), 35.69 mg.L-1 from Sheffield Electroplaters (■), and 27.85 mg.L-1 from Natal Electroplaters (●).**

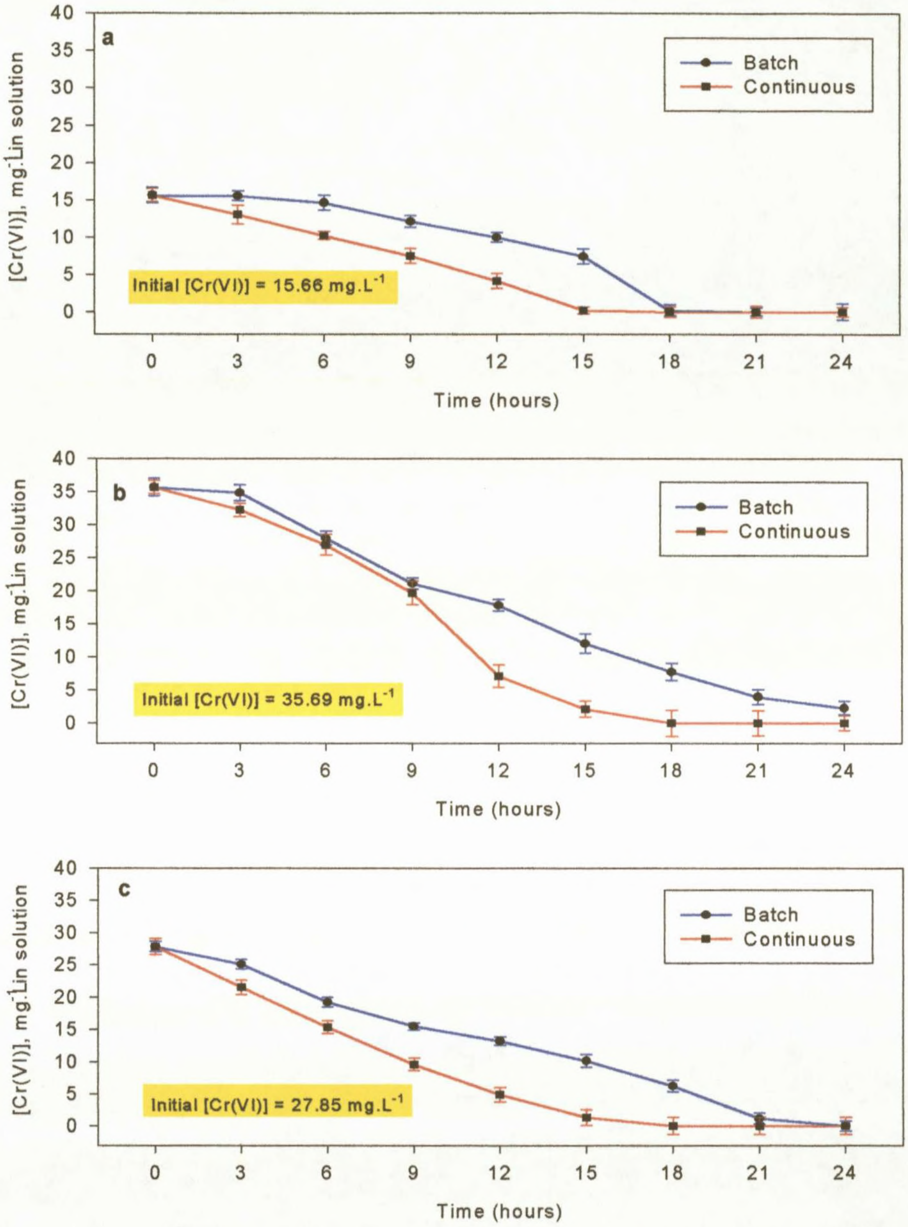


FIGURE 5.8 a-c **Cr(VI) reduction in batch (●) and continuous systems (■)at Cr(VI)$_{initial}$, a) 15.66 mg.L^{-1}, b) 35.69 mg.L^{-1}, and c) 27.85 mg.L^{-1} of industrial effluents.**

5.4 DISCUSSION

It is apparent from the results as can be seen in FIGS 5.2 and 5.3 that Cr(VI) reduction eventuates a distinct colour change of the medium. However, the colour change of the medium in the continuous reactor comparative to that in the batch reactor seems to differ. The colour in the batch reactor appears to be much darker than the continuous reactor. This is probably because of an increase and eventual settling of the biomass during reduction in the batch reactor. In the continuous reactor there is minimal biomass that leaves the reactor and a lighter effluent colour results. Although most of the parameters in the continuous reactor were the same as the batch, it did differ with regard to one important parameter, which was oxygen. In the batch reactor, aeration was provided by means of agitation, and in the continuous reactor, no aeration was provided. Thus the amount of oxygen that entered the system would have been far less than that in the batch reactor. However, this factor proved not to be an essential one when it came to Cr(VI) reduction in the continuous reactor. Cr(VI) reduction resulted in a 147 mg.L^{-1} CrVI) reduction in 24 h yielding a reduction rate of 5.34 mg.g^{-1}.h^{-1} (TABLE 5.1). In a batch reactor, 42 mg.L^{-1} Cr(VI)was reduced in 24 h with cell concentration of 1g.L^{-1}, yielding a reduction rate of 1.75 mg.g^{-1}.h^{-1}. The average cell density in the late log phase in a continuous reactor was calculated to be approximately 3 x 10^{6} cells.mL^{-1} resulting in a cell concentration of 1075 mg.L^{-1} calculated by means of protein determination (FIG 5.5).

FIG 5.4 depicts the flowrates of the effluent in the reactor, and it is quite apparent from the graph that the lower the flowrate, the greater would be the rate of reduction. The greater the contact time, the greater is the amount of Cr(VI) reduced (TABLE 5.1). The reason for this would be the fact that the Cr(VI) is exposed to the immobilized cells for a much longer time as opposed to a short time period. It is can be seen from the results in TABLE 5.1 that a time of 6 h resulted in only 28.98 mg.L^{-1} CrVI) being reduced, whereas at a cell concentration (1.150 g.L^{-1}) a contact time of 24 h resulted in 147.35 mg.L^{-1} Cr(VI) being reduced. Thus, it could be noted that although a higher cell density would results in an increased Cr(VI) reducing efficiency, increased contact time between the cells and Cr(VI) is just as important for an improved Cr(VI) reduction efficiency.

A mass balance of chromium in a continuous upflow reactor at a flow rate of 2.26 mL.h^{-1} revealed that an amount of 0.11 mg.L^{-1} chromium was taken up by the cells (TABLE 5.2). It also showed that the chromium in solution was quantitatively reoxidizable to Cr(VI). Mass balance studies also showed that the majority of the chromate was converted to insoluble form and the bacterial biomass did not retain a significant amount of chromium. This was also evident for *Pseudomonas fluorescens* and *Desulfovibrio desulfuricans* (DeLeo and Ehrlich, 1994; Tucker *et al.,* 1998). Tucker *et al.,* (1998) found that 86% of an initial 500 μmol.L^{-1} Cr(VI) was removed over 24 h by immobilized cells of *Desulfovibrio desulfuricans*, while over a 5 day period, 83% removal of the initial 960 μmol.L^{-1} Cr(VI) was obtained using a mixed consortium of sulphate-reducing bacteria (Fude *et al.,* 1994).

The performance of the continuous reactor using *Ps. maltophilia* as a typical profile of chromate reduction efficiency for a period of 24 h at various chromate flowrates is shown in FIG 5.4. At a flowrate of 2.26 mL.h^{-1}, 99% Cr(VI) reduction was achieved (FIG 5.4). It is also evident from the graph that the rate of Cr(VI) reduction decreases with increased flowrate. The reason could be that with a low flowrate, Cr(VI) is exposed for a longer time period to the bacterial cells for reduction to take place. It was reported by Wang, (1996) that oxygen transfer is often the rate limiting step in a suspended cell culture, and it is more so in an immobilized cell culture. Since the reductase enzyme of this organism is located in the soluble fraction of the cell, i.e. the cytoplasm and not extracellular or membrane associated could also pose the possibility as to why a low flowrate would be advantageous. The Cr(VI) reductase enzyme being located in the cytoplasm of the cell, would result in Cr(VI) not being directly in contact with the enzyme in the initial stages as opposed to if the enzyme were to be extracellular. This was reported to be a problem of mass transfer resistance imposed by the fact that the Cr(VI) has to diffuse to the enzymes reaction site (Wang, 1996). In the case of an extracellular reductase, as Cr(VI) passes through the column, the metal ion would be in direct contact with the enzyme and it is assumed that reduction rate would be slightly faster. However, in the case of an intracellular enzyme, the mechanism of Cr(VI) reduction is still not well understood.

In the batch system, increased bacterial density was observed to influence the rate of Cr(VI) reduction (refer to results in CHAPTER 3, FIG 3.5). Cr(VI) reduction was observed to occur between the late log phase of growth and the early stationary phase in the batch reactor. However, in the continuous system a modest increase in bacterial growth was detected (FIG 5.5). The reason is that cells for immobilization were obtained in the late log phase of growth, therefore it was expected that any increase in cell concentration would be minimal. Turick *et al.*, (1997) stated that improved volumetric productivity was probably achieved by increasing bacterial density in the bioreactor through immobilization. In the stated investigation it was observed that improvement in the rate of Cr(VI) reduction could be presumed to be owing to increased bacterial density. However, the results in the current study showed that although there was an increase in cell concentration within the system, improved Cr(VI) reduction rate was not totally attributed to cell concentration. Tucker *et al.*, (1998) reported that Cr(VI) reduction by polyacrylamide immobilized cells of *Desulfovibrio desulfuricans* occurred at a much slower rate than by suspended cells which was contrary to the results obtained in the current study. This contradiction in results could be attributed to the presence of other metal ions such as Mo(VI), Se(VI), and U(VI) with lactate as an electron donor in the experimental design by Tucker *et al.*, (1998). It was also stated that slower rates suggest that transport limitations may be controlling the rate of reduction in immobilized cell systems.

An immobilized cell bioreactor is said to be well suited for those cells whose growth phases and product formation are uncoupled. Cell biomass and primary metabolites are growth associated metabolites, but secondary metabolites such as enzyme production are produced during the stationary phase. The uncoupling of the phases means that productive cells cannot compete with the nonproductive cells in a continuously operated suspension fermentor. The reason is reported to be due to the fact that productive cells spend the nutritional and energy resources producing compounds in quantities far above the amount necessary for their survival, instead of reproducing themselves to propagate further. On the contrary, cell growth in an immobilized cell reactor must be severely limited if gel swelling or breakage is to be avoided. However, once the cells are immobilized, the cell viability must be concomitantly sustained over a long period of time.

Approximately 98.5% of the Cr(VI) entering the reactor was reduced to Cr(III) (FIG 5.6a and 5.6b), resulting in a reduction efficiency of 5.34 $mg.g.h^{-1}$. Throughout the experiment, the total Cr concentrations in the effluent increased with time and were similar to Cr(VI) influent concentrations (FIG5.6c), indicating that sorption in the reactor was minimal. The pH of the reactor remained fairly constant (FIG 5.6d), varying little from the input values of 8.0. When batch studies were performed, pH values showed a decrease from pH 8.0 to pH 6.8. This problem of an undesirable decrease in pH was overcome by the use of a modified nutrient broth containing a buffer, thus pH of the system did not change significantly so as to affect reduction rates.

Chromium-contaminated industrial effluent obtained from Sheffield Electroplaters, Natal Electroplaters and Saayman Danks Electroplaters was pumped into the reactor. Industrial effluent was used to determine whether the reactor could be used in an industrial application to treat the wastewater. However, the reactor still required an energy source for Cr(VI) reduction, therefore Cr(VI)-contaminated wastewater had to be mixed with modified nutrient broth. The composition of the metal plating wastewater included metals such as Cu^{2+}, Zn^{2+}, Ni^{2+}, and Cd^{2+} (TABLE 5.3). The composition of the wastewater had to be determined to establish whether metal ions affect Cr(VI) reduction. Although it had been established in batch systems that these metal ions had no drastic effect on Cr(VI) reduction (refer to FIGURE 4.6 in CHAPTER 4) particularly in *Ps. maltophilia*, the concentration of metals in the wastewater varies from time to time. However, industrial wastewater not only contains contaminant metal ions, but organic compounds and various other ions such sulphates, nitrates, and phosphates as well. The presence and quantity of these compounds were not tested for since it was assumed that these ions would not affect Cr(VI) reduction to any significant degree. Fujii *et al.*, (1994) also determined the compositions of metal plating wastewater. The following ions were found to be present in the wastewater, $Cr0_3$, SO_4^{2-}, Cr^{3+}, Cu^{2+}, Zn^{2+}, and Ni^{2+}. The results in the investigation demonstrated no inhibitive effect on chromate reduction by the constituents present in the wastewater.

Chromium contaminated industrial effluent was successfully treated in the continuous reactor (FIGURE 5.7). Complete Cr(VI) reduction was achieved in 18 h for all of the metal-plating wastewaters tested. At Cr(VI) concentrations of 27.85 and 15.66 $mg.L^{-1}$, there seemed to be a gradual increase in Cr(VI) reduction with time. However, at a concentration of 35.69 $mg.L^{-1}$, a gradual increase in Cr(VI) was demonstrated between 0 and 9 h, thereafter a sharp decline between 9 and 12 h was observed (FIG 5.7). It could be assumed that metal ions present in the waste solution are simultaneously being biosorbed by *Ps. maltophila* while reducing Cr(VI). It could be possible that at lower Cr(VI) concentrations, although biosorption might be taking place as Cr(VI) passes through the column, reduction is not affected at these comparatively low Cr(VI) concentrations (27.85 and 15.66 mg.L^{-1}). This would explain why there was a sharp decline when Cr(VI) concentration was 35.69 mg.L^{-1}. If such a phenomenon is true, that metal ions present in the metal plating wastewater might undergo biosorption then it also assumed that as Cr(VI)$_{intial}$ increases, Cr(VI) reduction rate is affected. The reason could be that metal ions are in competition to simultaneously be biosorbed or reduced (as in the case of Cr(VI)). Biosorption of the metal ions could well be the reason as to why Cr(VI) reduction was uninhibited by the presence of metal ions.

Differences in Cr(VI) reduction in batch (with freely suspended cells) and continuous systems (immobilized cells) were determined to establish which of these reactors would be a better option for treatment of Cr(VI) contaminated wastes. Results indicate that both batch and continuous reactors were capable of effective Cr(VI) reduction (FIG 5.8). However, the rate of Cr(VI) reduction in each of the systems differed from one another. In the continuous reactor, 100% reduction was achieved in 18 h. In the batch reactor, 100% reduction was achieved in a time period of 60 h. However, in both the reactors, it was evident that Cr(VI) reduction was directly proportional to time. In the batch reactor, a gradual Cr(VI) reduction rate is observed at all Cr(VI)$_{initial}$ concentrations. A sharp decline between 9 and 12 h was not evident in the batch reactor at Cr(VI)$_{initial}$ of 35.69 mg.L^{-1}. This could be explained by the fact that diffusion of substances to the reaction site and oxygen transfer is more rate limiting in an immobilized system compared to suspended cell culture (Wang, 1996). In the batch reactor only 93.47% Cr(VI) reduction was achievable in 24 h (FIG 5.8 b). It is evident from the results that although both the reactors were capable of effective Cr(VI) reduction, the time required by a batch reactor was longer than a continuous reactor.

5.5 CONCLUSIONS

Pseudomonas maltophilia exhibited a capability for chromate reduction in the continuous-upflow reactor. The tolerance of this organism to environmental stress factors facilitates the development of a novel bioreactor to treat chromium-contaminated wastewaters. This investigation indicates that reduction of hexavalent chromium by *Ps.*

maltophilia offers the potential to be developed into a viable technology for the remediation of hexavalent chromate in the environment, at least in waste streams containing Cr(VI) levels up to 150 mg.L^{-1}. The high rates of Cr(VI) reduction achieved during exponential growth phase indicates that maximum removal rates can be attained by using a continuous flow bioreactor running at a flowrate calculated to maintain the bacteria in exponential growth phase.

In summary, laboratory-scale continuous reactors for *Ps. maltophilia* provided the following conclusions:

1. Mass balance studies indicated that much of the chromate passing through the column undergoes reduction and an insignificant amount is unaccounted for with the rest being either in the bacterial cells or in the cell wash water.
2. Reduction efficiency was calculated to be 5.34 mg.g^{-1}.h^{-1} in a continuous reactor and 1.75 mg.g.h^{-1} in a batch reactor, thus Cr(VI) reduction was almost double in a continuous reactor compared to the batch experiments. Batch reactors were also capable of Cr(VI) reduction , however, comparative to continuous reactors, batch systems proved to be less efficient. Batch reactors are still very important for optimizing experimental parameters.
3. This investigation also confirmed the advantages of using immobilized cell systems through improved volumetric productivity by increased bacterial density in the bioreactor. Complete reduction of Cr(VI)-contaminated industrial effluents was accomplished by *Ps. maltophilia* in 18 h at initial concentrations ranging for 15.66 mg.L^{-1} to 35.69 mg.L^{-1}.
4. It was also established that although metal ions present in the metal plating waste solution did not inhibit Cr(VI) reduction at the specific concentrations, higher concentrations could affect reduction efficiency. Therefore, it may be necessary to dilute waste solutions prior to treatment. The continuous-upflow reactor proved to be a practical means of treating Cr(VI) wastes but further research would aid in understanding bacterial-Cr(VI) interactions within a reactor system.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

The results of this study indicate that bacteria are capable of reducing Cr(VI). Four organisms, viz., *Ps. maltophilia*, *A. calcoaceticus*, *B. subtilis*, and *C. cellasea* were isolated from chromium contaminated sites. All these organisms were capable of Cr(VI) reduction to various degrees. The organisms were indigenous to chromium-contaminated sites. However, organisms capable of Cr(VI) reduction were reportedly isolated from non-contaminated environments as well (Turick *et al.*, 1996). Indigenous Cr(VI) reducing microorganisms from contaminated sites should provide the means for developing a bioprocess to reduce Cr(VI) in nonsterile effluents such as those from soil washes.

Parameters for Cr(VI) reduction was determined for all four of the organisms to establish which of these organisms was by far the most suited for successful Cr(VI) reduction. *A. calcoaceticus, B. subtilis,* and *C. cellasea* comparative to *Ps. maltophilia* were restricted in efficient chromate reduction in terms of pH, temperature, increased Cr(VI) concentration, nutrient concentration, and tolerance to metal ions. Tolerance to metal ions was a very important factor to consider in the choice of organism, since metal plating effluents contains various metal ions. The results of this study unquestionably indicate that *Ps. maltophilia* was by far the most suitable organism to successfully carry out the chromate reduction process. Preference for this organism was based on results obtained in batch studies according to its wide pH requirement (pH 7.5 - 10.0), temperature range (20-30^{0}C), nutrient concentration (0.15-0.3%), and tolerance to heavy metal ions of Cu^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+}. It was observed that in a batch reactor, Cr(VI) reduction in an unbuffered nutrient medium caused a drop in pH which affected chromate reduction. A pH drop from 8.0 to 6.8 resulted in a decrease in Cr(VI) reduction rate since the optimal pH range was observed to be pH 8.0. In order to overcome this problem, a modified phosphate buffered nutrient medium was used. *Ps. maltophilia* was capable of growing in the presence of 250 mg.L^{-1} Cr(VI) on agar medium, but reduction rate at this concentration was very slow at this concentration in broth culture with only 50 mg.L^{-1} being reduced in 36 h and thereafter no further reduction was observed. Another trait of this organism was its assumed biosorptive capabilities which might explain its high tolerance to the metal ions present in the industrial effluents. However, further research needs to be conducted to confirm this assumption. Optimal Cr(VI) concentration that *Ps. maltophilia* was capable of reducing was established to be 150 mg.L^{-1} in 60 h and reduction rate was calculated to be 1.75 mg.g^{-1}.h^{-1}. The other three organisms required 72h to reduce 150 mg.L^{-1} Cr(VI) to a final concentration of approximately 3.75 mg.L^{-1}.

Enzyme location was determined for the organisms and the Cr(VI) reductase was found to located in the cytoplasmic fraction of the cell for *Ps.maltophilia* and *A. calcoaceticus*. No enzyme activity was detected in cell fractions of *B. subtilis* and *C. cellasea* which was assumed to be due to such requirements as an alternate electron donor, since only NADH and NAD(P)H were used. *Ps. maltophilia* required the electron donor NAD(P)H for Cr(VI) reduction and *A.calcoaceticus* did not require neither NADH nor NAD(P)H as an electron donor. The specific activity for *A. calcoaceticus* was comparatively insignificant yielding an activity of just 0.54 mU.mg^{-1} of protein. The NAD(P)H-dependent reductase enzyme from *P. maltophilia* yielded a specific activity of 3.24 mU.mg.$^{-1}$ of protein. The enzymes were found to be constitutive in all the organisms since reduction occurred in both induced and uninduced cells. Currently whole cell systems are being used for the successful treatment of Cr(VI) wastes. Extensive investigations are however, being conducted on utilizing cell-free systems by use of enzymes to determine the feasibility in an industrial application (Tucker *et al.*, 1998; Turick, 1998; Myers *et al.*, 2000).

Based upon the extensive batch studies on chromate reducing characteristics, a bioreactor system was established for the treatment of chromate wastewater. Optimal operating conditions and the performance of the bioreactor were clarified. Cells of *Ps. maltophilia* immobilized onto polyacrylamide gel was packed in a continuous upflow column. Results indicated a reduction rate of 5.34 mg.g^{-1}.h^{-1}, this is almost 3-fold the reduction rate calculated in the batch reactor which was 1.75 mg.g^{-1}.h^{-1}. Biomass density was established to be one of the key factors in increased Cr(VI) reducing efficiency. Thus, it was quite evident that a continuous reactor was a more practical means of treating chromium-contaminated wastes and the bioreactor produced minimal biomass output.

6.2 CONCLUSIONS

1. Organisms indigenous to Cr(VI) contaminated sites were capable of efficient Cr(VI) reduction in both batch and continuous reactors.
2. Four organisms identified viz., *Pseudomonas maltophilia, Bacillus-subtilis, Cellumonas cellasea,* and *Acinetobacter calcoaceticus* showed the potential for complete Cr(VI) reduction.
3. The reductase enzyme responsible for chromate reduction was located in the cytoplasmic fraction of the cell.
4. Apart from *Ps. maltophilia* being the organism best suited for Cr(VI) reduction of the four organisms investigated, this isolate was not as fastidious to the various parameter requirements as the others.
5. A continuous reactor containing immobilized cells of *Ps. maltophilia* indicated greater Cr(VI) reduction efficiencies as compared to batch reactors.
6. Immobilized cells and free cell suspensions were capable of reducing Cr(VI) up to an optimal concentration of 150 $\mathrm{mg.L^{-1}}$. Cell density was established to be one of the key parameters in increased Cr(VI) reduction rate in a continuous reactor.
7. *Pseudomonas maltophila* was capable of complete reduction of Cr(VI) from a metal plating effluent at an initial concentration of 35.69 $\mathrm{mg.L^{1}}$, and it was capable of Cr(VI) reduction at much higher concentrations in synthetic metal solutions. Minimal inhibition of Cr(VI) reduction activity by heavy metals present in the plating effluent was observed by this organism. The ability of *Ps. maltophilia* to reduce Cr(VI) by reduction has been achieved in laboratory scale for the effective treatment of chromate-contaminated effluents as an alternative bioremediation strategy as compared to chemical methods of treatment.

6.3 RECOMMENDATIONS

⇨ Although the results above have begun to clarify the interactions of some bacteria with chromate, more research is required before all mechanisms of chromate resistance and chromate reduction are fully understood.

⇨ Further characterization of the enzyme would enable the potential to use molecular techniques to magnify the activity in whole cells or to produce large quantities of Cr(VI)-reducing enzymes for use in cell free systems.

⇨ Biosorptive capabilities of *Ps. maltophilia* should be explored, so that a waste solution containing various metal ions could simultaneously undergo biosorption as well reduction (as in the case with chromate).

⇨ The ability of *Ps. maltophilia* to reduce chromate reportedly enables the cells to increase their tolerance towards Cr(VI) and thus should be determined.

⇨ Cheaper and more easily obtainable feed substrates such as molasses should be examined as alternative energy and carbon sources.

⇨ Determination of oxygen utilization rates and its contribution to the Cr(VI) reduction process should be investigated.

⇨ Reduction of Cr(VI) and production of Cr(III) with appropriate agents to remove or immobilize it and to prevent its reoxidation could be an effective *in situ* strategy for the long-term detoxification of chromate-polluted environments.

⇨ The operational advantages of a bioprocess compared to conventional Cr(VI) treatment technologies had been established in this investigation, however, economic advantages are just as important to consider.

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APPENDICES

APPENDIX 1

HOT NITRIC ACID EXTRACTION TO DETERMINE PRESENCE OF METALS IN SOILS

- Add 50 mL of a 7.0 N HNO3 to 1 g of soil sample.
- Digest gently at 95^{0}C for 2 hours, stirring occasionally.
- If excessive foaming occurs, then remove from heat periodically until foaming subsides.
- Maintain at least 25 mL in the beaker by adding 7.0 N HNO_3 as necessary.
- Cool and dilute with 10 mL 1.0 N HNO_3.
- Filter through a Whatman no. 42 filter paper into a volumetric flask.
- Rinse with 1.0 N HNO_3 and dilute to volume.
- Digested sample is then analyzed using atomic absorption spectroscopy.

APPENDIX 2

SLUDGE DIGESTION IN AQUA REGIA

- One litre of sludge is dried at 105^{0}C overnight.
- 0.5 g of the dried sludge is weighed in the beaker.
- Aqua regia at a ratio of 1:3 (HNO_3 : HCl) is added to obtain a volume of 12 mL.
- Heat the mixture slowly in a fume cupboard until all brown fumes disappear.
- Filter the digested sludge to remove solid particles and transfer into a 100 mL volumetric flask.
- Analyze sample using atomic absorption spectroscopy.

APPENDIX 3

SETTINGS FOR ATOMIC ADSORPTION SPECTROPHOTOMETRY

Setting	**Metal**				
	Cr	**Cd**	**Cu**	**Zn**	**Ni**
Wavelength	428.9 nm	228.8 nm	218.2 nm	213.9 nm	352.4 nm
Spectral band	0.5 nm	0.5 nm	0.2 nm	1.0 nm	0.5 nm
Lamp Current	7.0 mA	3.5 mA	3.5 mA	5.0 mA	3.5 mA
Flame Stoichiometry	reducing	oxidizing	oxidizing	oxidizing	oxidizing
Fuel	acetylene	acetylene	acetylene	acetylene	acetylene
Support	air	air	air	air	air

APPENDIX 4

PREPARATION OF NUTRIENT BROTH

Ingredients

Peptone	10.0 g
Beef extract	6.0 g
Distilled water	1000 mL

Preparation

All ingredients are heated and continuously stirred until clarified. Remove from heat and adjust pH to 7.0 and autoclave at 121^{0}C for 20 minutes.

APPENDIX 5

PREPARATION OF TRYPTICASE SOY BROTH

Ingredients

Trypticase	15.0 g
Phytane	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 mL

Preparation

All ingredients are heated and continuously stirred until clarified. Remove from heat and adjust pH to 7.0 and autoclave at 121^{0}C for 20 minutes.

APPENDIX 6

PREPARATION OF METAL STOCK SOLUTIONS

Analytical grade metal salt was added to a 1000 mL volumetric flask and made up to the mark with deionized water. The mass of metal salt added for each metal was as follows:

Metal species	Metal salt	Mass added ($g.L^{-1}$)
Cr^{6+}	K_2CrO_4	
Cd^{2+}	$Cd(NO_3)_2.4H_2O$	2.7316
Cu^{2+}	$Cu(NO_3)_2.3H_2O$	3.0829
Zn^{2+}	$ZnSO_4.7H_2O$	4.3971
Ni^{2+}	$NiCl_2.6H_2O$	4.0420

APPENDIX 7

PREPARATION OF METAL STANDARDS

Stock solutions of 1000 mg.L^{-1} of the required metal salts, K_2CrO_4, $Cd(NO_3)_2.4H_2O$, $Cu(NO_3)_2.3H_2O$, $ZnSO_4.7H_2O$, and $NiCl_2.6H_2O$ of analytical grade were prepared in one litre A-grade volumetric flasks concentrations of 30 to 250 mg.L^{-1} were prepared in 100 mL A-grade volumetric flasks using A-grade pipettes.

Conc required (mg.L^{-1})	Vol stock soln added (mL)	Deionized water
25	2.5	97.5
50	5.0	95.0
75	7.5	92.5
100	10.0	90.0
125	12.5	87.5
150	15.0	85.0
200	20.0	80.0
250	25.0	75.0

APPENDIX 8

DETERMINATION OF HEXAVALENT CHROMIUM (ADAPTED SABS 206 AND MERCK METHOD)

Principle

In slightly acid solution, hexavalent chromium reacts with diphenylcarbazide to produce a reddish-violet colour, the intensity of which is measured spectrophotometrically.

Sensitivity:

The limit is 0.01 mg.l^{-1} when a 5 cm light path is used for photometric measurement at 540nm.

Procedure (Conducted in triplicate)

(a) Preparation of calibration carve: Standard

Make a 1:10 dilution of the initial standard solutions of 10, 20, 30, 40 and 50 ug/lm resulting in 1, 2, 3, 4 and 5 ug.mL^{-1}.

- Add 5 mL of each of the standards into 25 mL conical flasks.
- Follow with addition of 1.666 mL of H_2SO_4.
- Add 1. 501mL of deconized water.
- Add 0.166 mL of diphenylcarbazide reagent.
- Allow to stand for 5 minutes.

(b) Preparation of test samples

- Dilute test sample (1:10) ie., 0.5ml of sample added to 4.5ml deconized water- however, if sample is too concentration then increased dilution factor eg 1:20.
- Balance of the additions are the same as for the standard preparations.
- If number of samples is large, add reagent prior to reading sets of samples, 50 samples do not stand for too long resulting in the colour fading.

APPENDIX 8 *continued*

ACKNOWLEDGMENTS

Reading spectrophotometrically at 540nm and use deionized water as a blank. If necessary, oxidize trivalent chromium with ammonium peroxodisulfate. This is usually done when chromium is present in the trivalent form only. Plot a curve of the absorbances against the masses (in μg) of chromium present in the respective standards. Subsequently, read concentrations of the test samples from the standard curve.

APPENDIX 9

PREPARATION OF GRAM STAIN

Preparation

Solution 1: Prepare the following separately, then combine

A		B	
Crystal violet	2 g	Ammonium oxalate	0.80 g
Ethanol, 95%	20 mL	Distilled water	80 mL

Solution 2:

Iodine	1 g
Potassium iodide	2 g
Distilled water	300 mL

Solution 3:

Safranin (2.5% in 95% ethanol)	10 mL
Distilled water	100 mL

Procedure

- Stain with crystal violet for one minute.
- Wash off the stain with tap water.
- Apply Gram's iodine for one minute.
- Wash off the Gram's iodine with tap water.
- Add 95% alcohol drop by drop until the alcohol runs clear.
- Wash off with 95% alcohol with tap water.
- Counterstain with safranin for 45 seconds.
- Wash off the safranin with tap water.
- Blot dry with bibulous paper and examine under oil immersion.

APPENDIX 10

PREPARATION OF SPORE STAIN

Preparation

Solution 1:

Malachite green	5.0 g
Distilled water	100 mL

Solution 2:

Safranin (2.5% in 95% ethanol)	10 mL
Distilled water	100 mL

Procedure

- Smear inoculum onto glass slide.
- Allow the smear to air dry, then heat fix.
- Flood smears with malachite green and place on a warm hot plate, allowing the preparation to steam for 2 to 3 minutes.
- Remove slides from hot plate, cool, and wash under running tap water.
- Counterstain with safranin for 30 seconds.
- Wash with tap water.
- Blot dry with bibulous paper and examine under oil immersion.

APPENDIX 11

PROTEIN DETERMINATION IN CELLS

Principle

In a two-step reaction, Folin's reagent reacts with the aromatic amino acids in protein, after treatment with alkaline copper, to give a blue colour.

Outline

Solubilize sample in 0.1 N NaOH, add alkaline copper solution and then diluted Folin's solution, and read in a spectrophotometer.

Materials

2% sodium carbonate in 0.1 N NaOH (A)

0.5% copper sulphate in 1% sodium citrate solution (B)

1 mL (B): 50 mL (A) - make up with constant mixing and use on same day (C)

1 mL Folin Ciocalteau, 1:3 mL water (D)

Bovine Serum Albumin (BAS) standard solution, 50 ug. mL^{-1} in 0.1 N NaOH

1.0 N NAOH

Protocol

- Dissolve cell pellet in 1.0 N NaOH and dilute to 0.1 N.
- Add 1.0 mL of reagent (C) to 200 uL of protein sample, mix, leave for 10 minutes.
- Add 100 uL of reagent (D), with constant mixing, leave for 40 minutes.
- Read on a spectrophotometer at 700 nm against a reagent blank and with a BSA standard, 50 ul. mL^{-1}.

APPENDIX 12

PREPARATION OF PERIPLASMIC, CYTOPLASMIC, AND MEMBRANE FRACTIONS BY LYSOZYME-EDTA METHOD

- Prepare the cells by washing and resuspending in Tris-HCl. The rest of the procedure should be done on ice.
- Resuspend the bacterial pellet in 80 μL of a freshly prepared solution of 1 mg.mL^{-1} lysozyme, 30 mM Tris at pH 8.0, sucrose (20% w/v), 1mM EDTA at pH8.0. Leave on ice for 10 minutes.
- Centrifuge in the cold for 1 minute at 5000 x g.
- Remove the supernatant **(periplasmic fraction).**
- Resuspend the pellet in 200 μL of 100 mM Tris at pH 8.0.
- Freeze (dry ice) and thaw (water-bath at 37^{0}C) three times to open the cells.
- Centrifuge in the cold for 5 minutes at 12000 x g.
- Remove the supernatant **(cytoplasmic fraction).**
- Resuspend the pellet in 200 μL Tris-HCl **(membrane fraction).**

APPENDIX 13

PREPARATION OF MODIFIED NUTRIENT BROTH

Ingredients

Bacto-peptone	5.0 g
Glucose	2.0 g
KH_2PO_4	0.49 g
K_2HPO_4	1.11 g
Distilled water	1000 mL

Preparation

All ingredients are heated and continuously stirred until clarified. Remove from heat and adjust pH to 7.0 and autoclave at 121^{0}C for 20 minutes.