MECHANISMS OF CHROMIUM(VI) RESISTANCE BY *ACINETOBACTER HAEMOLYTICUS*

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To those who love and care for me…

THANKS

I love you all too!

May you be blessed with good health and happiness!

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PREFACE

This thesis is the result of my work carried out in the Department of Chemistry and Department of Biological Sciences, Universiti Teknologi Malaysia between July 2006 to Jun 2008 under the supervision of Prof. Dr. Wan Azlina Ahmad and Dr. Shafinaz Shahir. Part of my work described in this thesis has been reported in the following publications:

1. Quek Hsiao Pei, Shafinaz Shahir, Liu Tao and Wan Azlina Ahmad, (2009). "Determination of Chromium(VI) Reduction by *Acinetobacter haemolyticus* using X-ray Absorption Fine Structure Spectroscopy", *Journal of Fundamental Sciences* (Accepted).

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ABSTRACT

Chromium (Cr), especially Cr(VI) is of particular environmental concern owing to its high solubility, bioavailability and toxicity. The reduction of Cr(VI) to innocuous Cr(III) is an important step in the remediation of Cr(VI)-contaminated environments. The understanding of how microorganisms resist metals can provide insight into strategies for their detoxification or removal from the environment. The present investigation was undertaken to study the Cr(VI) resistance mechanisms by *Acinetobacter haemolyticus*, a strain isolated from Cr(VI)-containing textile wastewater. In preliminary studies, the strain was shown to be able to tolerate Cr(VI) concentrations of 30 and 90 mg L^{-1} in Luria-Bertani (LB) agar and broth respectively. The Cr(VI) reduction capacity of *A. haemolyticus* was found to be greater when grown in higher percentage of LB broth than minimal salts broth. The Cr(VI) reduction also increased with lower initial concentration of Cr(VI) added after 5 hours. The x-ray absorption fine structure (XAFS) analysis displayed the ability of the strain to reduce Cr(VI) to Cr(III) which was octahedrally coordinated to oxygen. The Cr(III) was most likely to form complexes with carboxyl (COO^-) groups from the biomass based on Fourier-transform infrared (FTIR) analysis. The FTIR analysis also showed interactions of chromium with amino and hydroxyl groups. Field-emission scanning electron microscope (FESEM) showed that cells grown in the presence of Cr(VI) had a wrinkled appearance with a significant increase in size. No precipitates were found on the cell surface. However, precipitates were observed in the cytoplasmic region of the cells via transmission electron microscope (TEM) analysis, suggesting the transport of Cr(VI) into the cytoplasm and intracellular Cr(VI) reduction. Intracellular reduction of Cr(VI) was supported by a reductase test using soluble crude cell - free extracts. The specific reductase activity obtained was 0.52 µg Cr(VI) reduced per mg of protein an hour at pH 7.2 and 37 ˚C. In plasmid screenings, the strain was found to harbor a plasmid of about 12 kb. The findings showed that Cr(VI) resistance mechanisms of *A. haemolyticus* include the reduction of Cr(VI) to Cr(III), and intra- and extracellular sequestration of chromium.

ABSTRAK

Kromium (Cr), khasnya Cr(VI) merupakan ancaman utama kepada alam sekitar kerana mempunyai sifat keterlarutan, ketersediaan hayati dan ketoksikan yang tinggi. Penurunan Cr(VI) kepada Cr(III) adalah langkah penting dalam remediasi alam sekitar yang tercemar dengan Cr(VI). Kefahaman tentang mekanisme rintangan terhadap logam oleh mikroorganisma dapat memberi maklumat tentang cara detoksifikasi dan penyingkiran logam daripada alam sekitar. Kajian ini bertujuan untuk mengkaji mekanisme rintangan *Acinetobacter haemolyticus* terhadap Cr(VI), iaitu bakteria yang dipencilkan daripada air sisa tekstil yang mengandungi Cr(VI). Dalam kajian awal, *A. haemolyticus* didapati mempunyai kedayatahanan terhadap kepekatan Cr(VI) sebanyak 90 dan 30 mg L^{-1} dalam kaldu dan agar Luria-Bertani (LB). Kapasiti penurunan Cr(VI) oleh *A. haemolyticus* didapati lebih tinggi apabila dikulturkan di dalam medium yang mempunyai peratusan kaldu LB yang lebih tinggi berbanding kaldu garam minimal. Penurunan Cr(VI) juga meningkat apabila kepekatan asal Cr(VI) yang lebih rendah ditambah selepas 5 jam eraman. Analisis menggunakan spektroskopi serapan x-ray struktur halus (XAFS) menunjukkan keupayaan bakteria untuk menurunkan Cr(VI) kepada Cr(III) yang berkoordinat dengan oksigen secara oktahedral. Besar kemungkinan Cr(III) membentuk kompleks dengan kumpulan karboksil (COO^-) berdasarkan analisis spektroskopi inframerah (FTIR). FTIR juga menunjukkan interaksi antara kromium dengan kumpulan amino dan hidroksil. Melalui mikroskop imbasan elektron emisi medan (FESEM), bakteria yang dikulturkan dalam kehadiran Cr(VI) menunjukkan perubahan morfologi dari segi pertambahan saiz dan permukaan yang berkedut. Tiada mendakan kelihatan pada permukaan bakteria melalui FESEM tetapi mendakan kelihatan di kawasan sitoplasma dalam sel bakteria melalui mikroskop transmisi elektron (TEM). Ini mencadangkan terdapat pergerakan Cr(VI) ke dalam sitoplasma sel dan penurunan Cr(VI) secara intrasel. Keputusan daripada ujian punurunan Cr(VI) menggunakan ekstrak bebas sel membuktikan bahawa penurunan Cr(VI) berlaku secara intrasel. Aktiviti enzim penurunan tentu yang diperolehi adalah 0.52 µg Cr(VI) diturunkan per mg protin dalam masa 1 jam pada pH 7.2 dan 37 ˚C. Melalui penyaringan plasmid, *A. haemolyticus* didapati mempunyai satu plasmid bersaiz 12 kb. Hasil keseluruhan kajian ini menunjukkan mekanisme rintangan Cr(VI) oleh *A. haemolyticus* termasuk penurunan Cr(VI) kepada Cr(III), dan sekuestrasi kromium secara intra- dan ekstrasel.

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LIST OF ABBREVIATIONS

Å	-	Ångström (1×10^{-10} metre)
A. haemolyticus	-	*Acinetobacter haemolyticus*
bp	-	base pairs
ccc	-	covalently-closed circle
CFE	-	cell-free extracts
CFU	-	colony forming unit
CN	-	coordination number
Cr(0)	-	chromium(0)
DDI	-	distilled de-ionized
DNA	-	deoxyribonucleic acid
DPC	-	1,5-diphenylcarbazide
EDTA	-	ethylene-diaminetetra-acetic acid
EDX	-	energy dispersive x-ray
EM	-	electron microscopy
EXAFS	-	extended x-ray absorption fine structure
FEFF	-	effective scattering amplitude
FESEM	-	field-emission scanning electron microscope
FTIR	-	Fourier-transform infrared
GeV	-	gigaelectronvolts
kb	-	kilo base pairs
kV	-	kilovolts
LB	-	Luria-bertani
mA	-	miliampere
MS	-	minimal salts
NADH	-	nicotinamide adenine dinucleotide
ng L^{-1}	-	nanograms per Litre
OD_{600}	-	optical density at 600 nm

PBS	-	phosphate buffered saline
pm	-	picometer (1×10^{-12} metre)
RNA	-	ribonucleic acid
ROS	-	reactive oxygen species
SDS	-	sodium dodecyl sulphate
USEPA	-	United States Environmental Protection Agency
w/v	-	weight per volume
XAFS	-	x-ray absorption fine structure
XANES	-	x-ray absorption near-edge structure

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CHAPTER I

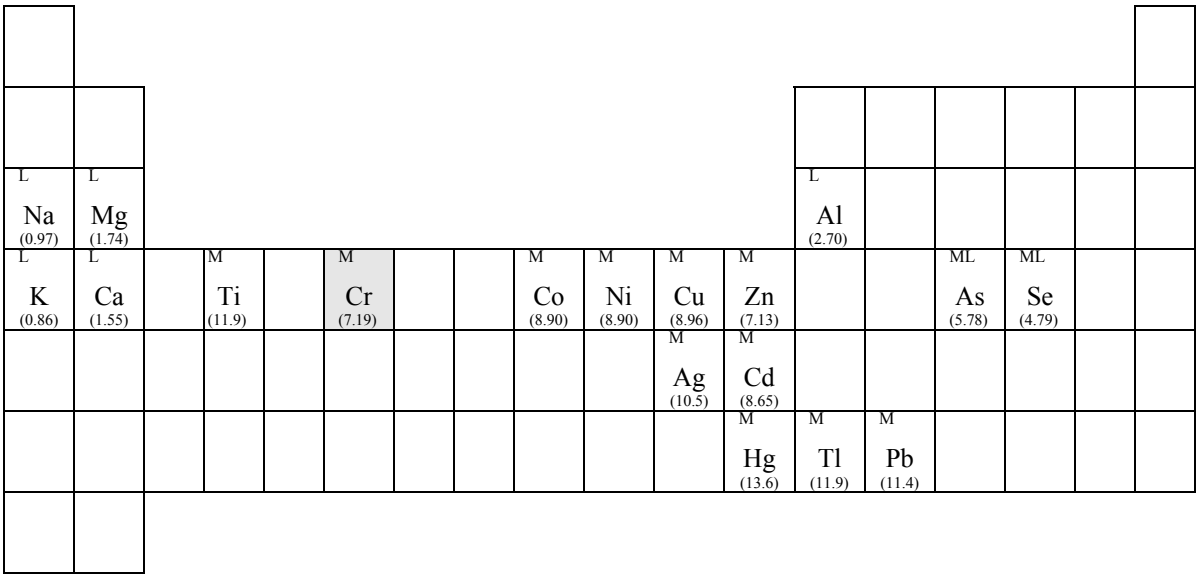
GENERAL INTRODUCTION

1.1 Introduction

A pollutant is defined as "a substance that occurs in the environment, at least in part as a result of human activities, and has a deleterious effect on the environment". The term pollutant is a broad term that refers to a wide range of compounds, from a superabundance of nutrients giving rise to enrichment of ecosystems to toxic compounds that may be carcinogenic, mutagenic, or teratogenic. Pollutants can be divided into two major groups, namely, those that affect the physical environment and those that are directly toxic to organisms, including human beings. The movement of pollutant and toxic compounds through the environment is called pollution and is very similar to the movement of energy and nutrients within the ecosystem or, on a larger scale, through the biosphere. This has affected the ecosystem and has caused health problems for the inhabitants residing near the factories. Efforts were made to treat these wastes so as to make them innocuous before discharge into public systems as people become more aware of the toxic effects of this waste and as federal and local laws imposed more stringent discharge norms. Initially the treatment procedures were based on physical and chemical methods, which proved to be inadequate and costly. Biochemical methods, which have inherent advantages, are still in their early stages of development (Doble and Kumar, 2005).

1.1.1 Heavy metal contamination problem

The term “heavy metal”, in spite of its widespread usage among professionals and laymen, does not have a rigorous scientific basis or a chemical definition. Although many of the elements listed under “heavy metals” have specific gravities greater than five, major exceptions to this rule remain. In hindsight, this group should preferably have been referred to as ‘toxic elements”, for they are all included in the United States Environmental Protection Agency’s (USEPA’s) list of priority pollutants. The periodic table containing the heavy metals that are of significant environmental concern is shown as in Figure 1.1. For comparison, commonly occurring light alkali and alkali-earth metals have also been included in the same figure.



Number in parenthesis represents the specific gravity of each element
Letters at the top left corner of each cell denote
L: Commonly occurring LIGHT metals
M: USEPA regulated HEAVY METALS
ML: USEPA regulated METALLOIDS

Figure 1.1 A modified periodic table showing commonly encountered regulated heavy metals, metalloids and unregulated light metals.

Strictly from a chemical point of view, heavy metals constitute transition and post-transition elements along with metalloids, namely, arsenic and selenium. They are indeed significantly heavier (i.e., higher specific gravities) than sodium, calcium and other light metals. These heavy metal elements often exist in different oxidation states in soil, water and air. The reactivities, ionic charges and solubilities of these metals in water vary widely. For their short- and long-term toxic effects, the maximum permissible concentrations of these heavy metals in drinking water as well as in municipal and industrial discharges are closely regulated through legislation. Nevertheless, barring the exceptions of cadmium, mercury and lead, heavy metals are also required micronutrients, i.e., essential ingredients for living cells. Toxicity effects of these elements are, thus, largely a function of concentration. These elements are beneficial and have more nutritional values lower than some critical dosages but become inhibitory to toxic with an increase in concentration. The threshold toxic concentrations differ for each heavy metal and are governed primarily by the chemistry of each heavy metal in question and associated physiological effects (Sengupta, 1994).

1.1.2 Chromium

Chromium (Cr) was first discovered in the Siberian red lead ore (crocoite) in 1798 by the French chemist Vauquelin. It is a transition element located in the group VI-B of the periodic table with a ground-state electronic configuration of Ar $3d^5\ 4s^1$ as shown in Figure 1.1 (Shanker *et al*., 2005).

Chromium exists in nine valence states ranging from -2 to +6, but mainly occurs as Cr(VI) in the divalent oxyanion chromate form and Cr(III) as trivalent cation which are of major environmental significance because of their stability in the natural environment (Thacker *et al*., 2006; Srivastava and Thakur, 2006). Gains of electron (reduction) by electron-poor, hexavalent chromium (Cr(VI)) convert this toxic, soluble anion (negatively charged species e.g. CrO_4^{2-}) to electron-rich, trivalent chromium (Cr(III)) cationic (positively charged, e.g. Cr^{3+}) form (Srivastava and Thakur, 2006).

1.1.2.1 Hexavalent chromium

The ground state electron configuration of the chromium atom is:

$1s^2 \quad 2s^2 2p^6 \quad 3s^2 3p^6 \quad 3d^5 \quad 4s^1$

Divalent chromium compounds are basic, trivalent chromium compounds are amphoteric, and hexavalent chromium compounds are acidic. The acid anhydride (CrO_3), the acid chloride (CrO_2Cl_2), and a wide variety of metal chromates ($MCrO_4$) and metal dichromates (MCr_2O_7) are typical hexavalent chromium compounds. The acid functions have been evaluated:

$$H_2CrO_4 \rightarrow H^+ + HCrO_4^-, \qquad K_{a1} = [H^+][\, HCrO_4^-] \,/\, [\, H_2CrO_4]$$
$$HCrO_4^- \rightarrow H^+ + CrO_4^{2-}, \qquad K_{a2} = [H^+][\, CrO_4^{2-}] \,/\, [CrO_4^-]$$

and has the chromate-dichromate equilibrium:

$$2HCrO_4^- \leftrightarrow Cr_2O_7^{2-} + H_2O$$
$$[Cr_2O_7^{2-}] \,/\, [HCrO_4^-]^2 = K_{eq1}$$

or

$$2CrO_4^{2-} + 2H^+ \leftrightarrow Cr_2O_7^{2-} + H_2O$$
$$[Cr_2O_7^{2-}] \,/\, [CrO_4^{2-}]^2[H^+]^2 = K_{eq2}$$

Frequently cited values for K_{a1} range from 0.2 to 4, and those for K_{a2} range from 1×10^{-6} to 4×10^{-7}. Values for K_{eq1} and K_{eq2} range from 33 to 158, and from 1.2×10^{14} to 4.2×10^{14}, respectively. $HCrO_4^-$ ion is the dominant form of hexavalent chromium, 90% or more, in 1.00×10^{-3} to 1.00×10^{-5} M potassium dichromate solutions at pH 3 (Katz and Salem, 1994). Depending on pH, Cr^{6+} forms hydrochromate ($HCrO^{4-}$), chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) and is highly soluble in water. At pH values below 6.2, the hydrochromate anion is predominant while at pH above 7.8, the chromate ion dominates (Rodríguez *et al*., 2007).

The chromate ion is tetrahedral, and the structure of the dichromate ion corresponds to two tetrahedra linked through a corner oxygen. The Cr-O-Cr bond angles in the polymeric species are about 120˚, and the Cr-O bond lengths in the chromate and in the dichromate ions are 166 and 163 pm, respectively.

Aqueous solutions of hexavalent chromium compounds absorb in the ultraviolet and violet regions of the spectrum. Those of the chromates are distinctively yellow, dichromates are orange and the higher polymers are red. Aqueous solutions of potassium chromate absorb strongly at wavelengths of 370-373 nm and demonstrate a molar absorptivity of 4.5×10^3 L/(mol.cm). Aqueous solutions of potassium dichromate show absorption maxima near 350 and near 450 nm. The molar absorptivities are 2.5×10^3 and 3.7×10^2 L/(mol.cm), respectively (Katz and Salem, 1994).

1.1.2.2 Trivalent chromium

The major chemical properties of trivalent chromium compounds in aqueous solutions are characterized by the stability of the violet hexaaquochromium(III) ion, $[Cr(H_2O)_6]^{3+}$, and the tendency of the hexaaquochromium(III) ion to precipitate as polymers formed through oxo- and/or hydroxo bridging.

The electron configuration of trivalent chromium is:

$1s^2 \quad 2s^2 2p^6 \quad 3s^2 3p^6 3d^3$

In aqueous solution, the hexaaquochromium(III) ion, $[Cr(H_2O)_6]^{3+}$ demonstrates the octahedral geometry of d^2sp^3 hybridization and the kinetic inertness toward ligand exchange of the t_{2g}^3 state. As the pH of the aqueous system is raised, the hexaaquochromium(III) ion, an acid with a pK_a of approximately 4, is neutralized to species such as $[Cr(H_2O)_5(OH)]^{2+}$ and $[Cr(H_2O)_4(OH)_2]^{+}$. These species polymerize through oxo- and hydroxo bridging. Further deprotonation and

polymerization produce the hydrated chromium(III) oxide. When freshly precipitated, the hydrated chromium(III) readily dissolves in both acids and bases:

$$Cr_2O_3 \,.\, nH_2O + 2OH^- \rightarrow 2CrO_2^- + (n + 1)H_2O$$
$$Cr_2O_3 \,.\, nH_2O + 6H^- \rightarrow 2[Cr(H_2O)_6]^{3+} + nH_2O$$

The hexaaquochromium(III) ion is violet, and its absorption spectrum shows maxima at 404 and 570 nm. The molar absorptivities at both wavelengths are low. The absorption maxima change as the coordinated water is replaced by other ligands (Katz and Salem, 1994).

1.1.2.3 Essentiality of chromium

Chromium is a naturally occurring element found in many foods and drinking water, thus it makes its way into the body mainly from dietary intake. In addition, intake of chromium results from airborne dusts and mists, and cigarette smoke as well as from industrial and occupational exposures (Katz and Salem, 1994).

Chromium is an essential micronutrient required for the growth of many microorganisms for the maintenance of normal glucose, cholesterol and fatty acid metabolism (Thacker *et al*., 2006; Srivastava and Thakur, 2006).

The deficiency of chromium has been implicated in impaired insulin action, which can cause glucose intolerance, elevated glucose blood levels, diabetes, elevated cholesterol levels, obesity and heart diseases, as well as other conditions not yet documented. Chromium is considered the cofactor for all the actions of the hormone insulin, primarily the regulation of carbohydrate, protein and fat metabolism. Signs of chromium deficiency are widespread; they tend to be associated with aging, and are consistent with the progressive decline in body and organ content of chromium from birth onward. Chromium deficiency impairs glucose utilization and disturbs protein and lipid metabolism (Katz and Salem, 1994).

1.1.2.4 Chromium toxicity

Some of the adverse effects of chromium compounds on human health were identified a century ago. Hexavalent chromium compounds are in general more toxic than trivalent chromium compounds. This observation is frequently interpreted as reflecting the stronger oxidizing power and the higher membrane transport of the former (Katz and Salem, 1994). Trivalent chromium on the other hand is largely insoluble and less toxic (Gonzalez *et al*., 2003). The solubility of trivalent chromium compounds is limited by the formation of several oxides and hydroxide species (Katz and Salem, 1994). However, at high concentration it is also toxic, carcinogenic and teratogenic (Thacker *et al*., 2006).

The ingestion of hexavalent chromium causes death. Occupational exposures to some chromium compounds have been shown to cause bronchial asthma, lung and nasal cancers, nasal and skin ulcers, and allergic reactions in the skin (Katz and Salem, 1994). The chromate anion is highly soluble and therefore can overcome the cellular permeability barrier (Thacker *et al*., 2006). The heavy metals oxyanions interfere with the metabolism of the structurally related non-metals in the living cells (Srivastava and Thakur, 2006).

Due to improper disposal, leakage and poor storage, chromate has become one of the most frequently detected contaminant at the waste sites (Thacker *et al*., 2006). Not only that chromate is dangerously toxic, it is also difficult to contain and spreads rapidly through aquatic systems and subterranean waterways (Gonzalez *et al*., 2003). Thus, chromium has been designated as the priority pollutant by USEPA (Thacker *et al*., 2006).

1.1.3 Chromium in the industries

Chromium occurs mainly as a result of human activities through production of waste water in metal smelting, electroplating, tanning, metallurgy and dyestuff industries. After processing, chromium occurs in several chemical species such as metallic chromium (Cr(0)), trivalent chromium (Cr(III)), and hexavalent chromium (Cr(VI)) (Gómez and Callao, 2006).

Metallic chromium is mainly found in alloys, such as stainless steel, but also in chrome-plated objects. It is the supreme additive, endowing alloys or materials with new properties, such as a resistance to corrosion, wear, temperature and decay, as well as strength, hardness, permanence, hygiene and colour (Gómez and Callao, 2006).

Chromium(III) exists in natural waters in hydrolyzed $Cr(H_2O)_4.OH_2^+$ form and complexes, and even adsorbed on colloidal matter. It is an essential micronutrient in the body and combines with various enzymes to transform sugar, protein and fat. Chromium(III) is also used in a number of commercial products, including dyes, paint pigments and salts for leather tanning (Gómez and Callao, 2006). The tendency for Cr(III) to form complexes with basic oxygen and/or nitrogen atoms in protein made possible the tanning of leather in hours rather than days, as was required with the vegetable tannins. The inertness of the trivalent oxide made chromium compounds useful as corrosion inhibitors and as agents for anodizing and plating metals (Katz and Salem, 1994).

Chromium(VI) is found as CrO_4^{2-} , $HCrO_4^-$ or $Cr_2O_7^{2-}$, depending on the pH of the medium. It occurs in a range of compounds used in industrial processes, such as chrome-plating (Gómez and Callao, 2006). The oxidizing properties of hexavalent chromium compounds have found applications in the synthesis of organic dyestuffs. The colours of trivalent and hexavalent chromium compounds coupled with appropriate solubility characteristics, made them attractive as pigments (Katz and Salem, 1994).

Chromium(VI) and Cr(III) enter the environment as a result of effluent discharged from industries and cooling-water towers. Chromium can also enter drinking water supply systems via corrosion inhibitors used in water pipes and containers or via contamination of underground water leaching from sanitary landfill. Chromium is an analyte of interest to the above industries and in the environment because, like other metals, it is not biodegradable. Once it enters the environment, its toxicity is determined to a large extent by its chemical form (e.g., Cr(VI) is much more toxic than Cr(III)). Changes in the oxidation state of an element can have a profound effect on bioavailability and toxicity (Gómez and Callao, 2006).

1.1.4 Treatment of metal-contaminated waste

Numerous industries (e.g. electroplating, metal-finishing operations, electronic-circuit production, steel and nonferrous processes, and fine-chemical and pharmaceutical production) discharge a variety of toxic heavy metals into the environment. Industry is compelled to treat waste liquids that contain appreciable quantities of heavy metals. For more than 35 years, legislation has required industry to remove metal pollutants from liquid discharges (Eccles, 1999).

The effluent quality of any discharge from an industrial treatment process must meet the minimum requirements of the Environmental Quality Act 1974 (issued by the Department of Environment, Malaysia). The limits set down by the Environmental Quality (Sewage Industrial Effluent Regulations, 1979) are as presented in Table 1.1.

Table 1.1: Parameters limits of effluent.

	Parameter	Unit	Standard	
			A	B
(i)	Temperature	°C	40	40
(ii)	pH value	-	6.0-9.0	5.5-9.0
(iii)	BOD at 20°C	mg L^{-1}	20	50
(iv)	COD	mg L^{-1}	50	100
(v)	Suspended solids	mg L^{-1}	50	100
(vi)	Mercury	mg L^{-1}	0.005	0.05
(vii)	Cadmium	mg L^{-1}	0.01	0.02
(viii)	Chromium, Hexavalent	mg L^{-1}	0.05	0.05
(ix)	Arsenic	mg L^{-1}	0.05	0.10
(x)	Cyanide	mg L^{-1}	0.05	0.10
(xi)	Lead	mg L^{-1}	0.10	0.5
(xii)	Chromium trivalent	mg L^{-1}	0.20	1.0
(xiii)	Copper	mg L^{-1}	0.20	1.0
(xiv)	Manganese	mg L^{-1}	0.20	1.0
(xv)	Nickel	mg L^{-1}	0.20	1.0
(xvi)	Tin	mg L^{-1}	0.20	1.0
(xvii)	Zinc	mg L^{-1}	2.0	2.0
(xviii)	Boron	mg L^{-1}	1.0	4.0
(xix)	Iron (Fe)	mg L^{-1}	1.0	5.0
(xx)	Phenol	mg L^{-1}	0.001	1.0
(xxi)	Free Chlorine	mg L^{-1}	1.0	2.0
(xxii)	Sulphide	mg L^{-1}	0.50	0.50
(xxiii)	Oil and Grease	mg L^{-1}	Not detectable	10.0

Standard A for discharge upstream of drinking water take-off

Standard B for inland waters

1.1.4.1 Conventional treatment

The simplest and cheapest method of removing most heavy metals from solution is to increase the pH of the effluent, thus converting the soluble metal into an insoluble form (i.e. its hydroxide). Precipitation by adjusting the pH is, however, not selective. Any iron (ferric ion) present in the liquid effluent will be precipitated first, followed by other heavy metals (Cu, Pb, Zn, Cd). Consequently, precipitation by alkali addition (usually lime) produces large quantities of solid sludge for disposal. Nonetheless, precipitation processes can be highly efficient as they rely mainly on the insolubility of the precipitate, and secondarily on the effectiveness of solid–liquid separation. The former can be influenced by the presence of metal-

complexing agents such as cyanide or the ability of the metal to exist in an anionic form, such as Cr as chromate (CrO_4^{2-}). Solid-liquid separation can be improved either mechanically or chemically; in the latter case, polyelectrolytes or flocculants such as aluminium are generally used (Eccles, 1999).

The most commonly used conventional processes to remove Cr(VI) are: (a) reduction to Cr(III) followed by precipitation as chromium hydroxide, (b) removal by ion exchange and (c) removal by adsorption. These methods are costly due to operational, treatment and sludge disposal costs (Fiol *et al*., 2008).

According to Eccles (1999), the costs in using industrial waste-water treatment processes involve factors such as: (a) concentration of the metal in solution; (b) the operational mode of the equipment; (c) the need for secondary treatments, such as regeneration of the granulated activated carbon (GAC) or ion-exchange resins; (d) the selectivity of GAC or ion-exchange resins, coupled with their respective capacities for the target metal(s); and (e) disposal of secondary wastes such as sludge.

1.1.4.2 Biological treatment

Recently, research for new and innovative technologies has centered on the biological treatment methods (Morales-Barrera *et al*., 2008). Bioremediation is the use of microorganisms to break down toxic and hazardous compounds in the environment (Acquaah, 2004). It generally utilizes microbes (bacteria, fungi, yeast, and algae), although higher plants are used in some applications. The two main biological treatment processes under investigation are: the adsorption of Cr(VI) onto microbial cells (i.e. biosorption), and the reduction of Cr(VI) to Cr(III) by enzymatic reaction or indirectly by reducing compounds produced by micro-organisms (i.e. biotransformation) (Cheung and Gu, 2003; Desjardin *et al*., 2003). The biological reduction of hexavalent chromium has attracted increased interest, since this process may not only relieve the toxicity of chromium that affect living organisms, but may also aid in the precipitation of chromium at near-neutral pH (mainly as $Cr(OH)_3$) for further physical removal (Cheung and Gu, 2003).

Bioremediation has already proven itself to be a cost-effective and beneficial addition to chemical and physical methods of managing wastes and environmental pollutants. New bioremediation approaches are emerging based on advances in molecular biology and process engineering. Recently developed rapid-screening assays can identify organisms capable of degrading specific wastes and new gene-probe methods can ascertain their abundance at specific sites. New tools and techniques for use of bioremediation in situ, in biofilters, and in bioreactors are contributing to the rapid growth of this field. (Bonaventura and Johnson, 1997).

Microorganisms have the ability to accommodate a variety of pollutants, both organic and inorganic, it is important to appreciate from the outset that microorganisms cannot destroy metals. However, they can influence metals' mobility in the environment by modifying their chemical and/or physical characteristics (Eccles, 1999). In addition, bioremediation may also play an increasing role in concentrating metals and radioactive materials to avoid toxicity or to recover metals for reuse. An added advantage of using microbes is that they can biodegrade organic chemicals; purposeful enhancement of this natural process can aid in pollutant degradation and waste-site cleanup operations (Bonaventura and Johnson, 1997).

1.1.5 Metals and microorganisms

Human activities, such as mining operations and the discharge of industrial wastes, have resulted in the accumulation of metals in the environment. It has been reported that microorganisms become adapted to these environments by the acquisition of specific resistance systems (Yilmaz, 2003). The interest in the interactions of heavy metals with microorganisms has increased.

1.1.5.1 Heavy metal stress on microbial community

Low concentrations of certain transition metals such as cobalt, copper, nickel and zinc are essential for many cellular processes of bacteria. However, higher concentrations of these metals often are cytotoxic. Other heavy metals, including

lead, cadmium, mercury, silver and chromium have no known beneficial effects to bacterial cells and are toxic even at low concentrations (Abou-Shanab *et al*., 2007).

The study of the interaction between heavy metals and microorganisms has focused in particular on the selection of metal-resistant microorganisms from polluted environments (Hassen, 1998; Pal and Paul, 2004; Abou-Shanab *et al*., 2007). The results by Akinbowale *et al*. (2007) indicate that aeromonads and pseudomonads resistant to antibiotics and heavy metals are easily recovered from farm-raised fish and sediments. The possibility of using these bacteria for detoxifying polluted environments (Srivastava *et al*., 2007; Congeevaram *et al*., 2007) was also looked into. Wastewater from aquaculture contributes to the antibiotic and metal resistance found in the environment (Akinbowale *et al*., 2007). The increase in tolerance towards toxic metals and antibiotic resistance among aquatic bacterial populations is also an indication of risk to the safety of the aquatic ecosystem, fish fauna, and ultimately human health (Pathak and Gopal, 2005).

Past studies have shown that chronic metal stress affects the structure of microbial communities, resulting in decreased biomass, activity and microbial diversity. Despite toxic stress, microorganisms that tolerate toxic stress conditions or more rapidly decompose pollutants are more likely to survive (Francisco *et al*., 2002). Consequently, metal-tolerant bacteria can be readily isolated from environments containing elevated levels of toxic metals. Some have adapted and some are endemic to their environment, while the environmental conditions may have selected for others (Clausen, 2000). In polluted soils, microbial survival depends on intrinsic biochemical and structural properties, physiological, and/or genetic adaptation including morphological, changes of cells, as well as environmental modifications of metal speciation. Microbes apply various types of resistance mechanisms in response to heavy metals. Bacterial communities in serpentine soil were reported to tolerate spiking of metals, such as nickel and zinc, more than those of unpolluted soils (Abou-Shanab *et al*., 2007).

For example, *Providencia sp.* was isolated from the contaminated sites of chemical industries. The bacterial isolate could grow and reduce chromate at a concentration ranging from 100–300 mg L^{-1} and at a concentration of 400 mg L^{-1}, pH

7 and temperature 37ºC. It also exhibited multiple heavy metal (Ni, Zn, Hg, Pb, Co) tolerance (Thacker *et al*., 2006).

1.1.5.2 Mechanisms of metal resistance by bacteria

There are four known mechanisms of bacterial heavy metal resistances. The first mechanism is by keeping the toxic ion out of cell by altering a membrane transport system involved in initial cellular accumulation. The second mechanism is the intracellular or extracellular sequestration by specific mineral-ion binding components (analogous to the metallothioneins of eukaryotes and the phytochelatins of plants, but generally at the level of the cell wall in bacteria). The third method is the most commonly found mechanism of plasmid-controlled bacterial metal ion resistance, involving highly specific cation or anion efflux systems encoded by resistance genes (analogous to multidrug resistance of animal tumor cells). The fourth known mechanism involves detoxification of the toxic cation or anion by enzymatically converting it from a more toxic to a less toxic form. This last surprising mechanism does indeed occur, as best known for detoxification of inorganic and organomercurials. It may also be used for oxidation of As(III) and the reduction of Cr(VI) to less toxic forms, but these known microbial processes here have not been associated with plasmids (Silver, 1992).

The largest group of resistance systems functions by energy-dependent efflux of toxic ions. Fewer involve enzymatic transformations (oxidation, reduction, methylation, and demethylation) or metal-binding proteins (for example, metallothionein SmtA, chaperone CopZ and periplasmic silver binding protein SilE). Some of the efflux resistance systems are ATPases and others are chemiosmotic ion/proton exchangers. For example, Cd^{2+}-efflux pumps of bacteria are either inner membrane P-type ATPases or three polypeptide RND chemiosmotic complexes consisting of an inner membrane pump, a periplasmic-bridging protein and an outer membrane channel. In addition to the best studied three-polypeptide chemiosmotic system, Czc (Cd^{2+}, Zn^{2+}, and Co^{2+}), others are known that efflux Ag^{+}, Cu^{+}, Ni^{2+}, and Zn^{2+}. Resistance to inorganic mercury, Hg^{2+} (and to organomercurials, such as CH_3Hg^{+} and phenylmercury) involve a series of metalbinding and membrane

transport proteins as well as the enzymes mercuric reductase and organomercurial lyase, which overall convert more toxic to less toxic forms. Arsenic resistance and metabolizing systems occur in three patterns, the widely-found *ars* operon that is present in most bacterial genomes and many plasmids, the more recently recognized *arr* genes for the periplasmic arsenate reductase that functions in anaerobic respiration as a terminal electron acceptor, and the *aso* genes for the periplasmic arsenite oxidase that functions as an initial electron donor in aerobic resistance to arsenite (Silver and Phung, 2005).

For chromate, the mechanism of resistance involves cellular uptake; it is not known as yet whether there is a block directly on uptake or accelerated chromate efflux (Silver, 1992).

1.1.5.3 Plasmids conferring resistance to metals

Bacterial plasmids contain specific genetically determined resistances to a wide range of toxic heavy metal ions, including Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Bi^{3+}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, TeO_3^{2-}, Tl^{+}, Pb^{2+}, Zn^{2+}, and other metals of environmental concern (Silver, 1992).

Plasmids found in enterobacteria can confer resistance to the ions of arsenic, silver, copper, mercury and tellurium. Staphylococcal plasmids can confer resistance to arsenic, bismuth, cadmium, copper, lead, mercury and zinc compounds; *Pseudomonas* plasmids can confer resistance to chromium, mercury and tellurium ions. Resistance to ions such as Hg^{2+}, Ag^{+} or TeO_3^{2-} can be increased more than 100-fold by these plasmids (Hardy, 1981).

Plasmid genes conferring resistance to mercuric ions are especially common. About 25% of conjugative R plasmids found in enterobacteria and about 75% of R plasmids from *Pseudomonas aeruginosa* confer resistance to Hg^{2+}. Plasmids from *P. aeruginosa* strains isolated from patients are more likely to have genes coding for Hg^{2+} resistance than genes for antibiotic resistance (Hardy, 1981).

Resistance to mercuric ions is brought about by a plasmid-determined reductase which reduces Hg^{2+} to volatile Hg^{0}. This is insoluble in water and is rapidly released as a vapour when mercuric-resistant bacteria are grown in liquid medium containing mercuric ions. The vapour can be collected in a condenser to yield liquid metallic mercury. Plasmids conferring mercuric-resistance also specify a mechanism for the uptake of mercuric ions. The genes for the reductase and for transport are part of an operon which is inducible by Hg^{2+}. Transposon Tn *501* confers resistance to mercuric ions. Resistance to cadmium and arsenate are caused by plasmid determined efflux mechanisms (Hardy, 1981).

Plasmids are extra chromosomal DNA that are not associated with the nucleus of the cell. By altering the plasmids or adding to them, biodegradation may be accelerated or altered (Hardy, 1981). Additional bacterial systems that reduce more toxic Cr(VI) to less toxic reduced Cr(III) exist but the genetic determinants for these systems have not been identified as chromosomal or plasmid (Silver, 1992).

Several strains belonging to the genus of *Acinetobacte*r have been attracting growing interest from medical, environmental and a biotechnological point of view. *Acinetobacter* are known to be involved in biodegradation, leaching and removal of several organic and inorganic man-made hazardous wastes (Abdel-El-Haleem, 2003). According to Baumann (1968), the genus *Acinetobacter* is usually isolated from water and soil with the species *haemolyticus* reported to be isolated mostly from soil.

1.1.6 Objective and Scope of thesis

The aim of the work described in this thesis is to study the mechanisms related to Cr(VI) reduction by a locally isolated strain of *Acinetobacter haemolyticus.* Initial investigations on the tolerance towards Cr(VI) and Cr(VI) reduction capacity of *A. haemolyticus* were carried out. *A. haemolyticus* was then screened for the presence of plasmids using a few plasmid isolation techniques followed by attempts to determine the role of the plasmid. The Cr(VI) reduction mechanisms was studied via instrumental analysis.

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