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Mechanisms of Bacterial Detoxification of Cr (VI) from Industrial Wastewater in the Presence of Industrial Effluent as Potential Energy Source

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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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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

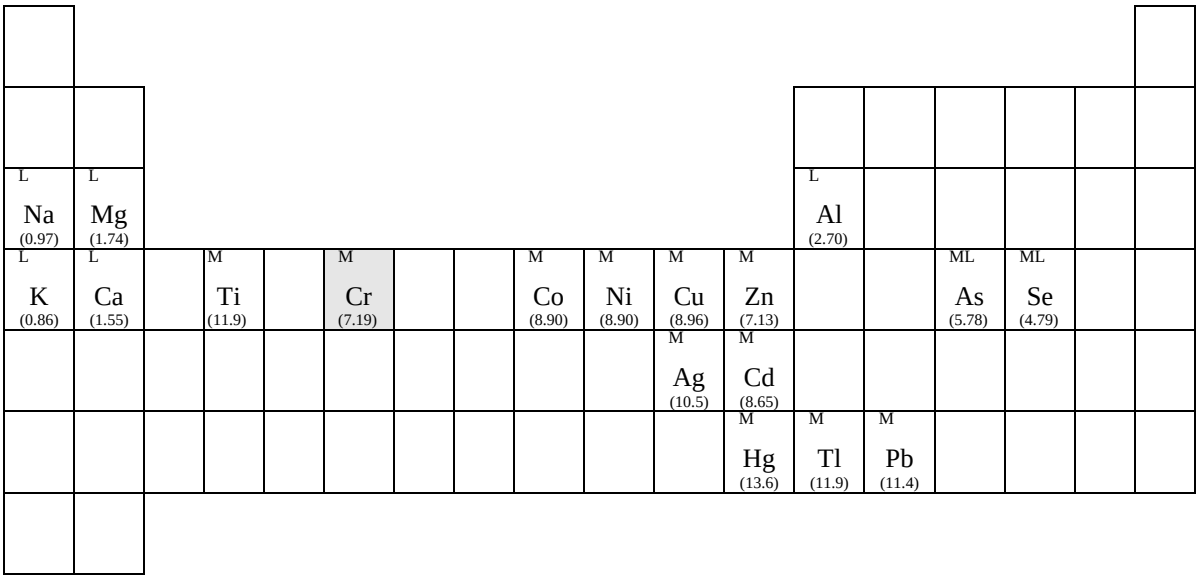
GENERAL INTRODUCTION

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.

CHAPTER II

CHROMIUM TOLERANCE AND REDUCTION CAPACITY OF *ACINETOBACTER HAEMOLYTICUS*

2.1 Introduction

This chapter reports the preliminary studies on the Cr(VI) tolerance and resistance properties of *Acinetobacter haemolyticus*, a locally-isolated strain from Cr(VI)-containing wastewater of a textile-related manufacturing premise.

Firstly, the effect of Cr(VI) on the growth patterns of *A. haemolyticus* in Luria-Bertani (LB) broth was studied. The Cr(VI) tolerance level of the strain was then investigated in LB broth and LB agar amended with varying concentrations of Cr(VI). The ability of *A. haemolyticus* to reduce Cr(VI) to its less toxic form, Cr(III), as a resistance mechanism during growth was investigated. This was carried out in LB broth followed by a study on the effects of the types of media, initial Cr(VI) concentration and the time of Cr(VI) inoculation into the media. The media used were composed of varying percentages of LB and minimal salts (MS) broth. To determine the Cr(VI) concentrations in the media, colorimetric method using 1,5-diphenylcarbazide (DPC) was employed. Total chromium was determined by atomic absorption spectrophotometric (AAS) technique.

The characterization of the bacteria is important to provide a background study for further works in elucidating the mechanisms of Cr(VI) resistance.

2.1.1 Heavy metal toxicity, tolerance and resistance

Toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular/molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects. In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Hall, 2002).

Tolerance is the acquired inability to make an adaptive immune response to antigens (Madigan and Martinko, 2006). On the other hand, resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions. Wherever there is a change in susceptibility that renders an agent ineffective against a certain organism, this organism is referred to as resistant. Susceptible organisms can become insensitive by mutation or by incorporation of the genetic information which encodes the resistance (Kümmerer, 2004).

Metal resistance in particular is defined as the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned. A particular organism may directly and/or indirectly rely on several survival strategies. For example, synthesis of metallothioneins or c-glutamyl peptides is a mechanism of Cu^{2+} resistance in *Saccharomyces cerevisiae*, but Cu-binding or precipitation around the cell wall and intracellular transport are also components of the total cellular response (Zafar *et al.*, 2007).

2.1.2 Cr(VI)-reducing bacteria

Microbial tolerance to Cr(VI) and reduction of Cr(VI) have been reported to be independent phenomena (Thacker *et al*., 2007; Megharaj *et al*., 2003; Ohtake *et al*., 1987). However, for reduction of Cr(VI) the cells should be able to tolerate Cr(VI) otherwise cell growth is inhibited. Cr(VI) in bacterial cells is reduced by

either physiological reducing agents such as glutathione or reductase enzymes (Puzon *et al*., 2002).

Chromium(VI)-reducing bacteria have also been isolated and characterized from chromium-contaminated soil, wastewater and industrial effluents (Pal and Paul, 2004). A group of 48 Cr(VI)-resistant isolates, with different colony types, was isolated from Cr-contaminated activated sludge (Francisco *et al.*, 2002). According to Abou-Shanab *et al*. (2007), Cr(VI) resistance and/or Cr(VI) reduction abilities were found in most of the isolated bacteria in their study. Approximately 54% of the isolated bacteria belonged to the genera *Acinetobacter* and *Ochrobactrum* whereas the remaining populations included strains identified as species of the β-*Proteobacteria* and high G+C Gram-positive bacteria. Indigenous bacteria in Cr-contaminated sediment from a stream in Dongducheon city, Korea also showed Cr(VI) reduction under aerobic condition (Lee *et al*., 2008). Pal and Paul (2004) reported the isolation of Cr(VI)-reducing bacteria from serpentine soil which was less studied.

Many other strains have been reported to reduce Cr(VI) including *Nesterenkonia* sp. strain MF2 (Amoozegar *et al*., 2007), *Achromobacter* sp. strain Ch1 (Zhu *et al*., 2006), *Sphaerotilus natans* (Caravelli *et al*., 2008), *Escherichia* (Shen and Wang, 1993), and *Arthrobacter* (Megharaj *et al*., 2003).

2.1.3 Mechanisms of Cr(VI) reduction by bacteria

Bacteria can reduce Cr(VI) under aerobic or anaerobic conditions through electron-transport systems containing cytochromes. The process involved in Cr^{6+} reduction can be under aerobic or anaerobic conditions. A diagram illustrating the mechanism of enzymatic Cr^{6+} reduction under aerobic conditions is shown in Figure 2.1 (upper) (Cheung and Gu, 2007).

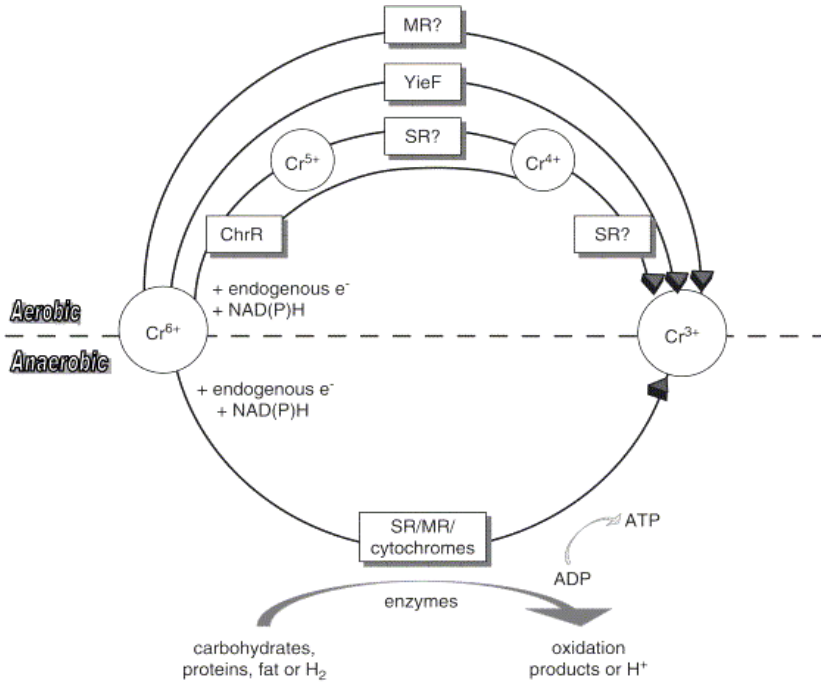


Figure 2.1: Plausible mechanisms of enzymatic Cr^{6+} reduction under aerobic (upper) and anaerobic (lower) conditions.

Under aerobic conditions, the reduction of Cr^{6+} to the stable end product, Cr^{3+} occurs as two- to three-steps with Cr^{5+} and/or Cr^{4+} as short-lived intermediates. The reduction of Cr^{5+} to Cr^{4+} and Cr^{4+} to Cr^{3+} is not known to be either spontaneous or enzyme-mediated. NADH, NADPH and electron from the endogenous reserve are implicated as electron donor in the Cr^{6+} reduction process. The ChrR transiently reduces Cr^{6+} with a one-electron shuttle to form Cr^{5+}, followed by a two-electron transfer to generate Cr^{3+}. Although a proportion of the Cr^{5+} intermediate is spontaneously reoxidized to generate ROS. Enzyme YieF is unique in that it catalyzes the direct reduction of Cr^{6+} to Cr^{3+} through a four-electron transfer, in which three-electrons are consumed in reducing Cr^{6+} and the other is transferred to oxygen (Cheung and Gu, 2007).

Under anaerobic conditions, natural metabolites of anaerobes such as H_2S produced by sulfate-reducing bacteria (SRB) are effective chemical Cr^{6+} reductants under anoxic environment. In the absence of oxygen, Cr^{6+} can serve as a terminal electron acceptor in the respiratory chain for a large array of electron donors,

including carbohydrates, proteins, fats, hydrogen, NAD(P)H and endogenous electron reserves. The typical anaerobic Cr^{6+} reduction is shown in Figure 2.1 (lower). SR and MR represent soluble and membrane-associated reductase, respectively (Cheung and Gu, 2007).

To date, study on mechanisms of Cr(VI) reduction has been carried out on *Vibrio fischeri* (Fulladosa *et al*., 2006), *Bacillus sphaericus* AND303 (Pal and Paul, 2004), *Pseudomonad* (CRB5) (McLean and Beveridge, 2001), *Escherichia coli* 33456 (Shen and Wang, 1994), and *Enterobacter cloacae* (Wang *et al*., 1990). However, the formation of chromium intermediates has been examined only in a few bacterial strains such as *Shewanella oneidensis* (Daulton *et al*., 2007; Neal *et al*., 2002), and *Pseudomonas ambigua* G-1 (Suzuki *et al*., 1992). Most importantly, little is known about the nature, localization and lifetimes and/or reactivities of these species in microorganisms (Codd *et al*., 2006). Up to date, the mechanisms of Cr(VI) resistance of *Acinetobacter haemolyticus* has not been carried out via instrumental analysis.

2.1.4 Chromium determination

Chromium is introduced into the environment by effluents in several industries where the highest concentrations of chromium, up to g L^{-1}, are found in samples of tanning, electroplating, automation and cement industries. The concentration of chromium can be ranging from ng L^{-1} in some consumer waters to over mg L^{-1} or up to g L^{-1} in natural waters or in river/reservoir waters with factories nearby (Gómez and Callao, 2006). It is important to control this element since it is both toxic and carcinogenic as mentioned in Chapter I (section 1.1.2.4).

As its toxicity depends on its state of oxidation, it is especially interesting to determine its most abundant species, Cr(III) and Cr(VI) (Gómez and Callao, 2006). Therefore, a great variety of methods have been proposed for the determination of chromium, being those based on atomic absorption spectrometry (AAS) as the most commonly used. Other methods for elemental chromium determination make use of inductively coupled plasma atomic emission spectrometry (ICP–AES), inductively

coupled plasma mass spectrometry (ICP–MS) and X-ray fluorescence (XRF). Only total chromium can be determined by these methods; therefore, a separation step prior to detection is required to obtain speciation information (Priego-Capote and Castro, 2006). The limit of detections (LOD) can range from ng L^{-1} to µg L^{-1}, depending on the preconcentration technique used. If preconcentration technique is not used, the limits of detections (LOD) are mg L^{-1} for flame atomic absorption spectrometry (FAAS) and µg L^{-1} for the other techniques (Gómez and Callao, 2006).

There are other analytical methods, which allow determination of only one of the two species. For instance, the environmental protection agency (EPA) recognises four methods for sample preparation of hexavalent chromium: 7195 (coprecipitation), 7196 (colorimetry with 1,5-diphenylcarbazide (DPC)), 7197 (chelation/extraction), and 7198 (differential pulse polarography). The colorimetric method based on the coloured complex formed between DPC and Cr(VI) is one of the most sensitive and selective for Cr(VI) determination. Nevertheless, the determination of both individual ionic forms is sometimes required. A simple alternative is the use of flow-injection (FI) methods, which enable to distinguish between both chromium species. One case in point is the inclusion of cationic and/or anionic resins in the FI manifold; another is based on the sequential injection of the sample and derivatization by DPC without and with previous addition of Ce(IV), which oxidizes Cr(III) to Cr(VI) (Priego-Capote and Castro, 2006).

Separation techniques, such as liquid chromatography (LC), ion chromatography (IC) and capillary electrophoresis (CE), are especially attractive for speciation studies, since they can differentiate various chemical forms of the same element. A previous step such as complexation is usually required in LC and CE. The necessity for complexation in CE is due to the different charge of the two chromium species, making it difficult for simultaneous determination in a single run (Priego-Capote and Castro, 2006).

Techniques such as IC can provide large linearity range for concentrated samples, whereas, for samples with low levels of chromium, techniques such as GF-AAS and ICP-AES may be suitable. GF-AAS can achieve very low LODs, but the relative standard deviations are higher than for ICP-AES (Gómez and Callao, 2006).

2.2 Materials and methods

2.2.1 Materials

All glassware used for experimental purposes were acid-washed with nitric acid (10%, v/v) and subsequently rinsed with double-distilled deionized (DDI) water to avoid metal contamination. Sterilization of glassware was carried out as when necessary by autoclaving at 121˚C, 115 kPa for 15 minutes. All chemicals used were of Analar grade.

2.2.2 *Acinetobacter haemolyticus*

A locally-isolated *Acinetobacter haemolyticus* (GenBank accession number EF369508) was used throughout this study. *Acinetobacter* is a Gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli genus. The colonies formed were circular, convex, smooth and translucent with entire margins (Nemec *et al.*, 2003).

2.2.3 Culture media

2.2.3.1 Luria-Bertani broth

A. haemolyticus was grown in LB broth which contained (g L^{-1}): tryptone (10.0) (Oxoid, UK), granulated yeast extract (5.0) (Merck, Germany), and sodium chloride (10.0) (Riedel-de-Haën, Germany). The medium was adjusted to pH 7.0 prior to autoclaving at 121˚C, 115 kPa for 15 minutes.

2.2.3.2 Luria-Bertani agar

Luria-Bertani agar was prepared by incorporating 13 g L^{-1} of agar (Merck, USA) to the LB medium. The medium was sterilized by autoclaving at 121˚C, 115 kPa for 15 minutes. The molten LB, which was held at about 45-55˚C was poured into sterile disposable Petri dishes (Sterilin, USA) and allowed to solidify. The plates were left to dry overnight at 30˚C in the incubator (INB 200-500, Memmert). For long-term storage, the LB plates were prevented from drying out by sealing with parafilm tape.

2.2.3.3 Luria-Bertani with Cr(VI) agar

Luria-Bertani agar was prepared by incorporating 13 g L^{-1} of agar (Merck, USA) to the LB medium. The medium was sterilized by autoclaving at 121˚C, 115 kPa for 15 minutes. The molten LB was added with Cr(VI) stock solution to the required concentration when the temperature was about 45-55˚C. The molten agar was then poured into sterile disposable Petri dishes (Sterilin, USA) and allowed to solidify. The plates were left to dry overnight at 30˚C in the incubator (INB 200-500, Memmert) prior to use.

2.2.3.4 Minimal salts broth

Minimal salts broth was prepared by dissolving 0.03 g NH_4C1, 0.03 g K_2HPO_4, 0.05 g KH_2PO_4, 0.01 g NaC1 and 0.01 g $MgSO_4.7H_20$ in 1 L of DDI water. The medium was autoclaved at 121˚C, 115 kPa for 15 minutes and stored at room temperature until use (Wang and Xiao, 1995).

2.2.3.5 Glycerol stock of cultures, 12.5% (v/v)

A loopful of pure culture was taken from LB agar plate and inoculated into LB broth (25 mL) in a 250 mL-Erlenmeyer flask. The culture was incubated for 12 hours with agitation at 200 rpm and 30˚C.

For long term storage of the bacteria, 2 mL of the culture in LB broth was transferred into 5 mL bijöu bottles and added with 2 mL of glycerol, 25% (v/v) (J.T. Baker, USA). The stock cultures were stored at -20˚C prior to use.

2.2.4 Chromium(VI) and Cr(III) stock solutions

Stock solution of Cr(VI) (1000 mg L^{-1}) was prepared by dissolving 2.829 g of $K_2Cr_2O_7$ (BDH-GPR™, UK) in 1 L of DDI water whereas, stock solution of Cr(III) (1000 mg L^{-1}) was prepared by dissolving 5.124 g of $CrCl_3.6H_2O$ (Fluka, Britain) in 1 L of DDI water. The solutions were then sterilized by using 0.2 μm pore size sterile cellulose-acetate filters (Whatman, England).

2.2.5 Characterization of *Acinetobacter haemolyticus*

2.2.5.1 Preparation of active culture

A glycerol stock culture as prepared in 2.2.3.4 was inoculated into LB broth (25 mL) in a 250 mL-Erlenmeyer flask. The culture was incubated for 12 hours with agitation at 200 rpm and 30˚C.

2.2.5.2 Effect of Cr(VI) on the growth of *Acinetobacter haemolyticus*

Growth profiles for *A. haemolyticus* in LB broth in the absence and presence of Cr(VI) at different concentrations were obtained. Active culture of *A. haemolyticus* (10%, v/v) as described in 2.2.6.1 was inoculated into 100 mL of LB broth and incubated for 60 hours, 30˚C at 200 rpm. The bacterial growth was determined periodically in terms of optical density at 600 nm (OD_{600}), with a UV spectrometer (Genesys 20, Thermo Spectronic). Controls without bacteria consisting of LB broth, with the presence and absence of Cr(VI) were also set up. Bacterial growth was expressed as the difference between the observed OD_{600} values and OD_{600} values for the control sets.

2.2.6 Screening for tolerance towards Cr(VI)

Two methods were employed to determine the tolerance of *A. haemolyticus* towards Cr(VI); the repli-plate and spread plate technique.

2.2.6.1 Repli-plate technique

LB broth was dispensed aseptically into the wells of the repli-plate dish (Sterilin, USA). The wells were added with increasing concentrations of Cr(VI) ranging from 5 to 150 mg L^{-1} except for the first row on the left of the plate which acted as controls. The wells were inoculated with mid-exponential phased cultures (5%, v/v). The repli-plate was incubated at 30˚C for 7 days. At the end of the incubation period, OD_{600} in each well was recorded. Growth in each well was expressed as % fraction of growth in the control wells.

2.2.6.2 Spread plate technique

The spread plate technique was used to determine the number of viable cells on LB agar plates supplemented with Cr(VI). Sterile LB agar plates was

supplemented with different concentrations of Cr(VI) ranging from 30 to 60 mg L^{-1}. Sterile LB agar plates without Cr(VI) acted as controls. Mid-exponential phased cultures (0.1 mL) were spread over the surface of the LB agar plates using a sterile glass spreader at dilutions of 10^{-5}, 10^{-6}, and 10^{-7}. The numbers of colonies were recorded after 24 hours of incubation at 30˚C.

2.2.7 Chromium(VI) reduction in LB broth

Active culture of *A. haemolyticus* (10%, v/v) (2.2.6.1) was inoculated into Erlenmeyer flasks containing 100 mL of LB broth supplemented with 30 and 60 mg L^{-1} of Cr(VI) and incubated at 30˚C and 200 rpm. Controls consisted of LB broth added with 30 and 60 mg L^{-1} of Cr(VI) without bacteria. At different time intervals within 48 hours, 5 mL of each sample were collected by centrifugation at 7000 rpm for 10 minutes. The supernatant was analyzed for residual Cr(VI) using the 1, 5-diphenylcarbazide (DPC) method as described in 2.2.10. Total chromium was also measured using an atomic absorption spectrometer (AAnalyst 400, Perkin Elmer) as mentioned in 2.2.11.

2.2.8 Chromium(VI) reduction in MS broth

2.2.8.1 Adaptation in MS broth

A. haemolyticus (10%, v/v) was inoculated into 25 mL of LB broth and incubated overnight at 30˚C and 200 rpm. The overnight culture (10% v/v) was inoculated into 25 mL of media with 30% MS, 70% LB broth and incubated at 30˚C with continuous agitation at 200 rpm for period between 24 and 72 hours. OD_{600} was recorded for determination of bacterial growth. The adaptation process was repeated in media with increasing percentage of MS broth compared to LB broth.

2.2.8.2 Chromium(VI) reduction in different media compositions

Active culture of *A. haemolyticus* (10%, v/v) (2.2.6.1) was inoculated into a series of Erlenmeyer flasks containing 25 mL of different media compositions; (A) 100% LB, (B) 70% LB, 30% MS, (C) 50% LB, 50% MS, (D) 30% LB, 70% MS, (E) 100% MS. Cr(VI) (30 and 60 mg L^{-1}) was added to the cultures at 0 hour and after 5 hours of growth. Controls consisted of the media added with 30 and 60 mg L^{-1} of Cr(VI) without bacteria. After 48 hours of incubation at 30˚C with continuous agitation at 200 rpm, 5 mL of each sample were collected by centrifugation at 7000 rpm for 10 minutes. The supernatant was analyzed for residual Cr(VI) using the 1, 5-diphenylcarbazide (DPC) method as described in 2.2.10. The percentage of Cr(VI) reduced was calculated as shown in equation 2.1.

$$\% \text{ Cr(VI) reduced} = \frac{[\text{Cr(VI)}]_c - [\text{Cr(VI)}]_x}{[\text{Cr(VI)}]_c} \times 100\% \qquad \text{(Equation 2.1)}$$

where:

$[Cr(VI)]_c$ - Residual Cr(VI) concentration in control (without cells)

[Cr(VI)] - Residual Cr(VI) concentration in each samples

The total cell count of each culture was also obtained using the spread plate technique. The cultures (0.1 mL) were spread over the surface of the LB agar plates without Cr(VI) using sterile glass spreader at dilutions of 10^{-5}, 10^{-6}, and 10^{-7}. The numbers of colonies were recorded after 24 hours of incubation at 30˚C. The percentage of growth was calculated as shown in equation 2.2.

$$\% \text{ cell growth} = \frac{CFU_x}{CFU} \times 100\% \qquad \text{(Equation 2.2)}$$

where:

CFU_x - CFU of cultures grown in different media compositions with Cr(VI)

CFU - CFU of cultures grown in 100% LB broth without Cr(VI)

2.2.9 Chromium(VI) analysis by DPC method

Cr(VI) reduction was estimated as the decrease in chromium concentration in supernatant with time using Cr(VI) specific colorimetric reagent 1,5-diphenylcarbazide (DPC) 0.25% (w/v) prepared in acetone (J.T. Baker, U.S.A.) to minimize deterioration. The reaction mixture was set up in 10 mL volumetric flask as follows: 200 µL or 400 µL sample or standard $K_2Cr_2O_7$ (10 mg L^{-1}) volume was made to 1 mL using DDI water followed by addition of 330 µL of 6M H_2SO_4 (QRëc) and 400 µL of DPC and final volume was made to 10 mL using DDI water. Measurements were made immediately at 540 nm using a UV spectrometer (Thacker *et al.,* 2006). The standard curve was plotted using 0.4-2.0 mg L^{-1} Cr(VI) prepared from Cr(VI) stock solution (1000 mg L^{-1}). All reaction mixtures were prepared in duplicates.

2.2.10 Total chromium analysis

In a 25 mL volumetric flask, 1 mL of sample was mixed with 24 mL of DDI water. The absorptions of chromium peak in the samples were recorded at a wavelength of 357.87 nm. The atomic absorption spectrometer used was calibrated using 0.4-2.0 mg L^{-1} Cr(VI) prepared from Cr(VI) stock solution (1000 mg L^{-1}).

2.3 Results and discussions

2.3.1 Effect of Cr(VI) on the growth of *Acinetobacter haemolyticus*

The growth of *A. haemolyticus* in LB broth with and without the presence of Cr(VI) was evaluated before the assays on Cr(VI) tolerance and reduction. The growth profile obtained is as shown in Figure 2.2.

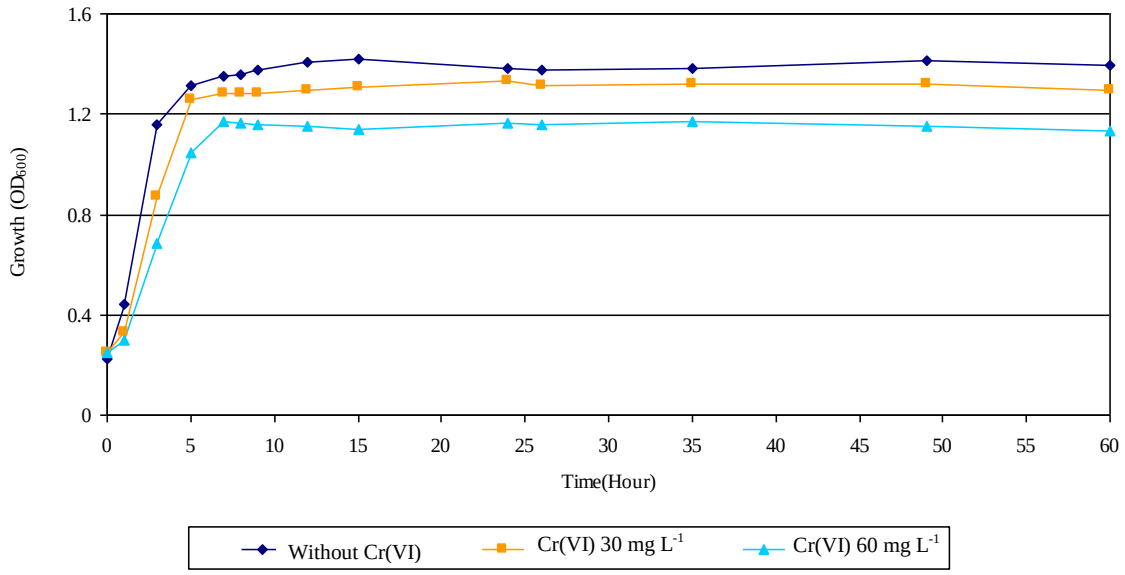


Figure 2.2: Growth of *Acinetobacter haemolyticus* in LB broth with and without Cr(VI).

The results show that Cr(VI) at concentrations of 30 and 60 mg L^{-1} does not particularly affect bacterial growth. It can be seen that the exponential growth period lasted for about 6 hours without any lag phase. The growth rate calculated based on the exponential phase of the graphs showed that the growth rate of *A. haemolyticus* in LB medium without Cr(VI) is 0.33 $hour^{-1}$. Although the growth patterns of *A. haemolyticus* in the presence of Cr(VI) seemed uninhibited, the growth rate in the presence of 30 and 60 mg L^{-1} of Cr(VI) were lower at 0.27 and 0.18 $hour^{-1}$, respectively. Nevertheless, *A. haemolyticus* exhibited good growth in the presence of

30 and 60 mg L^{-1} of Cr(VI). The OD_{600} are 1.24 and 1.16 respectively which are only slightly lower than OD_{600} of 1.41 for cells grown without Cr(VI). In most of the research works, for instance those conducted by Thacker and Madamwar (2005), nutrient-rich media with yeast extract and tryptone were used (Basu *et al*., 1997; Shakoori *et al*., 2000). In such conditions, the real toxicity of Cr(VI) may have been masked or decreased due to complexation of Cr(VI) with organic components. Therefore, the microbial metabolism was probably less affected (Caravelli *et al*., 2008).

2.3.2 Chromium(VI) tolerance of *Acinetobacter haemolyticus*

A. haemolyticus was tested for tolerance towards different concentrations of Cr(VI) using repli-plate dish and spread plate techniques.

The result from the repli-plate dish technique is as displayed in Figure 2.3.

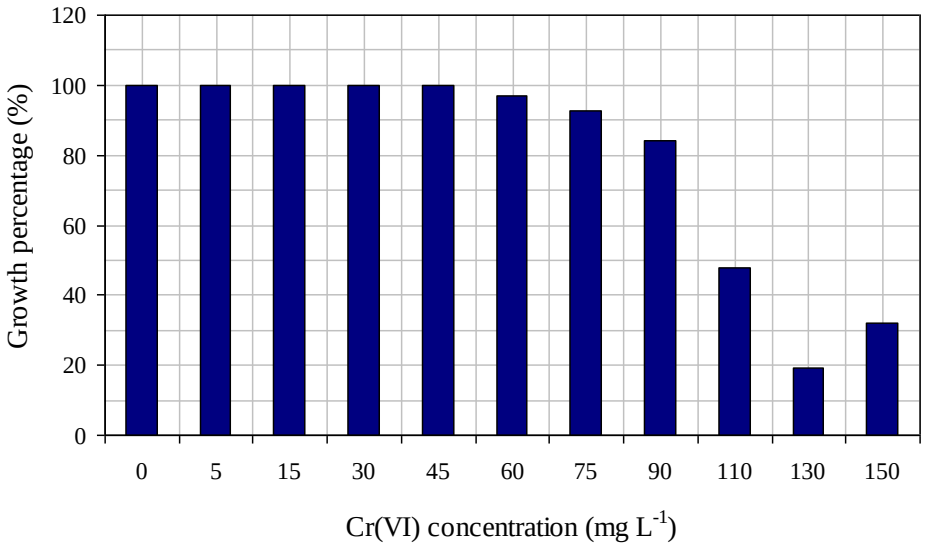


Figure 2.3: Effect of Cr(VI) in LB broth on growth of *Acinetobacter haemolyticus.*

A. haemolyticus was able to grow in LB broth containing chromium with no significant inhibition of growth up to 60 mg L^{-1}. Generally, with increasing Cr(VI)

concentration, the inhibition of growth increased but good growth was still seen in the medium containing up to 90 mg L^{-1} of Cr(VI). The percentage of bacterial growth dropped drastically to 48% in the presence of Cr(VI) at 110 mg L^{-1}. Similar trend of growth was observed by Sultan and Hasnain (2007). The reduced growth at high Cr(VI) concentrations was mentioned to be most likely related to the alteration of genetic material and altered metabolic and physiological reactions of bacteria (Losi *et al*., 1994).

However, the investigation on the tolerance level of *A. haemolyticus* towards Cr(VI) using the repli-plate method might be affected by limited diffusion of O_2 into the culture medium (Zakaria *et al.*, 2007). This would lead to limited cell metabolic activity as *A. haemolyticus* is an obligate aerobic organism. Furthermore, there are limitations in using optical density to measure cell growth which concurrently measures viable and dead cells; thus, leading to the use of spread plate technique to determine the growth inhibiting effect of Cr(VI) on *A. haemolyticus*.

As shown in Table 2.1, consistent results were obtained from *A. haemolyticus* inoculated on LB agar supplemented with varying concentrations of Cr(VI). *A. haemolyticus* exhibited good growth on LB agar with 30 mg L^{-1} of Cr(VI). The growth was only reduced by 16% compared to growth on LB agar without Cr(VI). However, growth was completely inhibited on LB agar with 40 mg L^{-1} of Cr(VI). This indicates that 40 mg L^{-1} of Cr(VI) is toxic to the bacteria.

Table 2.1: Total cell count of *Acinetobacter haemolyticus* on LB agar with Cr(VI).

Cr(VI) concentrations, mg L^{-1}	**Total cell count, CFU mL^{-1}**
0	5.5×10^7
30	4.6×10^7
40	0
50	0
60	0

In comparison, the inhibitory concentrations of Cr(VI) on the growth of *A. haemolyticus* on LB agar is significantly lower than in LB broth. *A. haemolyticus* exhibited good growth in LB broth and LB agar with 90 and 30 mg L^{-1} of Cr(VI), respectively. Generally, the toxicity in solid medium differed from those observed in liquid medium. This may be due to conditions of diffusion, complexation and availability of metals (Hassen *et al*., 1998).

In a study by Megharaj *et al*. (2003), it was found that *Bacillus* sp. and *Arthrobacter* sp. grew in tryptic soy broth (TSB) or minimal salts medium-glucose agar with 100 mg L^{-1} Cr(VI). Although both bacterial strains exhibited similar resistance on agar media supplemented with Cr(VI), *Bacillus* sp. did not grow (bacteriostatic) in liquid medium with 100 mg L^{-1} Cr(VI). The assessment of Cr(VI) toxicity on the bacteria was also affected by the use of nutrient-rich media. Further results showed that the Cr(VI) reduction ability of both bacteria in liquid medium was not related to resistance to Cr(VI) in agar medium. Therefore, it was suggested that the actual resistance of the bacteria to a pollutant should be determined preferably in liquid media after their isolation from agar media.

Although many studies exist concerning the metal tolerance of bacteria, it is difficult to make a meaningful comparison with literature because of the diversity of growth media and incubation conditions. Frequently, the media that best support the growth of the microorganisms studied are amended with various quantities of heavy metal salts and inoculated with the respective microorganism. The microbial growth is subsequently measured to determine the minimal inhibitory concentration. A number of problems are associated with this approach. Metal-binding capacity of the microorganisms, chelation to various components of the media, and formation of complexes can each cause a reduction in the activities of free metals. Therefore, the activity of a free metal ion, ordinarily considered to be the toxic metal species that ultimately determines the microbial response to the metal, rarely approaches the total metal concentration added to media. Of particular importance is the sorption or chelation of metals to unspecified organic compounds found in most biological media (Angle and Chaney, 1989).

Nonetheless, in this study, it is worth noting that *A. haemolyticus* consistently exhibited good growth in the presence of Cr(VI) up to 90 and 30 mg L^{-1} in LB broth and agar, respectively.

2.3.3 Chromium(VI) reduction capacity of *Acinetobacter haemolyticus*

The reduction capacity of the Cr(VI)-tolerant *A. haemolyticus* was evaluated in LB broth supplemented with Cr(VI) at 30 and 60 mg L^{-1}. After removal of cells, residual Cr(VI) concentrations and total Cr in the supernatants were analyzed at different time intervals within 48 hours. The graph of residual Cr(VI) in the supernatant versus time obtained are as shown in Figure 2.4.

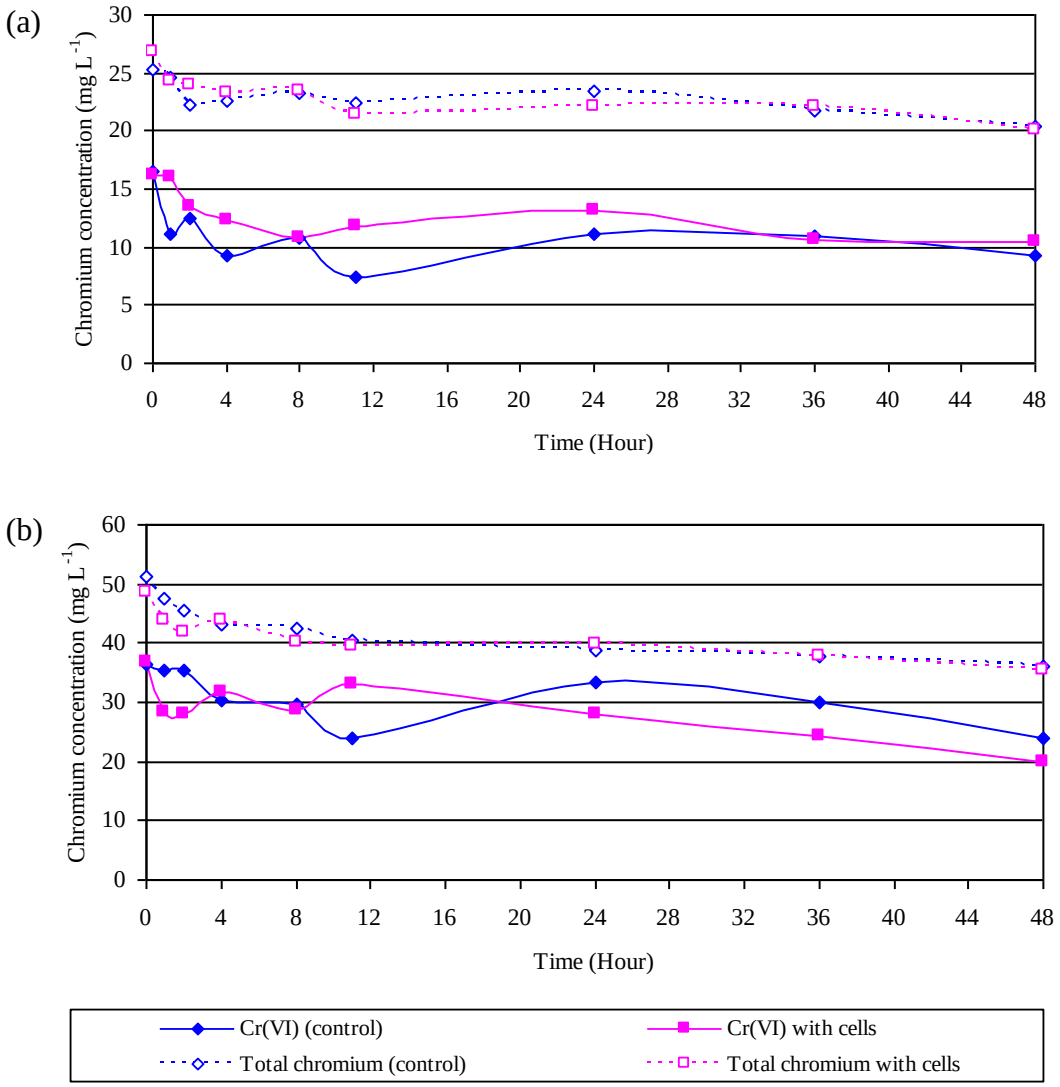


Figure 2.4: Changes in residual Cr(VI) in LB broth during growth of *Acinetobacter haemolyticus* with different initial Cr(VI) concentrations: (a) 30 mg L^{-1}; (b) 60 mg L^{-1}.

In the presence of cells, the residual concentration of Cr(VI) decreased with time for both initial Cr(VI) at 30 and 60 mg L^{-1}. The residual concentration of Cr(VI) in the controls without cells also showed significant Cr(VI) reduction. This suggests that the reduction may have been caused by components in the LB medium itself since no cells were inoculated into the medium. Medium components and carbon sources were reported to be able to interact with toxic metals to give misleading results (Clausen, 2000).

It was observed from both graphs (Figure 2.4) that the amount of total dissolved chromium also decreased simultaneously as Cr(VI) was reduced. Therefore, the dissolved Cr(VI) was presumably reduced to Cr(III) as a precipitate of $Cr(OH)_3$ form (Lee *et al.*, 2006). Thus, in this study, the concentration of Cr(VI) in the culture filtrate decreased with time with simultaneous increase in trivalent form. Similar observations were also found in the reduction Cr(VI) by indigenous bacteria from heavy metal-contaminated sediments grown in Postgate culture medium in both anaerobic and aerobic conditions (Lee *et al.*, 2006).

The colour of the medium was found to have changed from yellow to bluish brown with longer incubation time. Pal and Paul (2004) also reported significant discoloration of medium along with 62% reduction of Cr(VI) by isolates from serpentine soil grown in Vogel-Bonner (VB) broth. In a study using *Streptomyces griseus* grown in Broth-II and Mikami media (Laxman and More, 2002), the media colour change was reported to be due to the conversion of Cr(VI) to Cr(III) form.

The Cr(VI) reduction capacity of *A. haemolyticus* could not be ascertained in 100% LB medium due to the possible interactions between components of the medium with chromium. Subsequent attempts to adapt *A. haemolyticus* in 100% MS broth in order to evaluate the Cr(VI) reduction capacity was also unsuccessful. The adaptation was carried out by repeated sub-culturing into media with increasing ratio of MS to LB broth. The OD_{600} value recorded for growth of *A. haemolyticus* in 85% MS, 15% LB broth was 0.40. In 95% MS, 5% LB broth, the OD_{600} was only 0.17 which was about 12% of the growth in 100% LB broth (OD_{600} = 1.41).

Hence, growth media with varying percentages of LB to MS media were used to evaluate the Cr(VI) reduction capacity by *A. haemolyticus*. The effects of media on growth, initial Cr(VI) concentration and Cr(VI) inoculation time on Cr(VI) reduction by *A. haemolyticus* were also looked into.

Based on Figure 2.5 (line graph), the percentage of growth decreased in media compositions with increasing percentage of MS to LB broth. This is in agreement with the poor adaptation of *A. haemolyticus* in 100% MS broth mentioned earlier (section 2.3.3). *A. haemolyticus* grown in higher percentage of LB broth

showed better growth compared to MS broth. Samples that exhibited higher cell growth also showed greater percentage of Cr(VI) reduction (Figure 2.5 (bar graph)). Only slight Cr(VI) reduction was observed in the controls (without cells). This shows that *A. haemolyticus* is responsible for the reduction of Cr(VI). Cr(VI) reduction by bacteria grown in rich medium were also reportedly higher than in minimal medium in two separate studies (Pal and Paul, 2004; Sultan and Hasnain, 2007). Chromate reduction by washed cells of AND303 was studied in Vogel Bonner (VB) and MS broth supplemented with 20 mg L^{-1} of Cr(VI) where 62% reduction of Cr(VI) was recorded in VB broth. The reduction was comparatively much lower in MS broth (Pal and Paul, 2004). In the studies of Sultan and Hasnain (2007), Cr(VI) reduction by strain SDCr-5 was highest in Dey-Engley (DE) medium (containing g L^{-1}: tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0; $C_6H_8O_7$, 1.0; Na_2HPO_4, 6.9) (98%) followed by M9 supplemented with glucose (52%), nutrient broth (39%) and least in KSC (containing g L^{-1}: NH_4Cl, 0.03; K_2HPO_4, 0.03; KH_2PO_4, 0.05; NaCl, 0.01; sodium acetate, 2.0; $MgSO_4.7H_2O$, 0.01; $CaCO_3$, 0.005; $FeCl_3.7H_2O$, 0.005; casamino acids, 1.0) (16%).

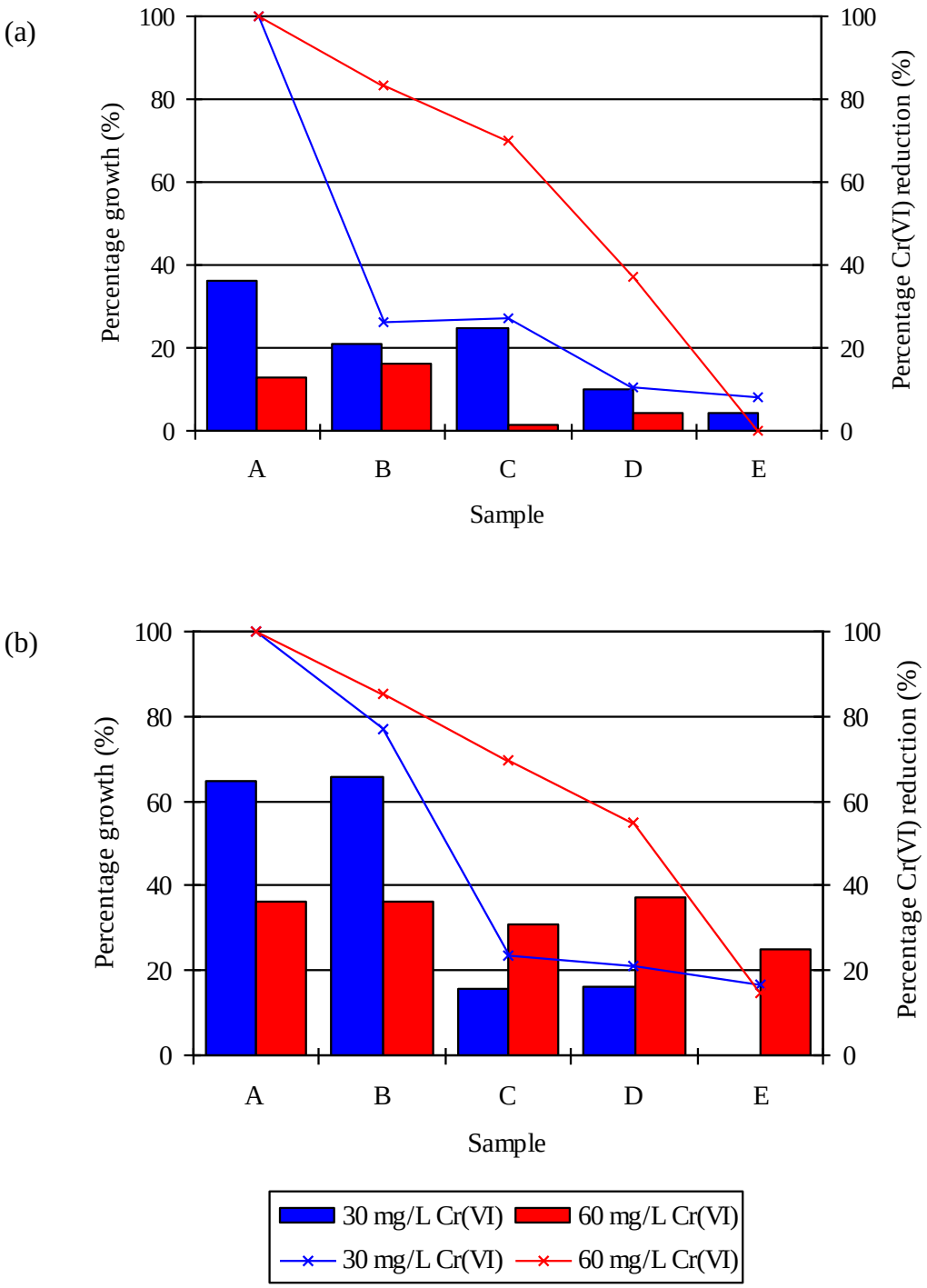


Figure 2.5: Comparison of percentage of growth (line graph) and Cr(VI) reduction (bar graph), in different media compositions with different initial Cr(VI) concentrations. Cr(VI) was inoculated (a) at 0 hour and (b) after 5 hours of growth. The media compositions used were: A. 100% LB; B. 70% LB, 30% MS; C. 50% LB, 50% MS; D. 30% LB, 70% MS; E. 100% MS.

The inoculation of Cr(VI) at 0 hour (Figure 2.5(a)) and after 5 hours (Figure 2.5(b)) of growth also affected the reduction of Cr(VI) by *A. haemolyticus*. Higher percentage of Cr(VI) reduction was observed when Cr(VI) was added after 5 hours of growth in all samples except C and E; both showed better Cr(VI) reduction with the addition of 30 mg L^{-1} Cr(VI) at 0 hour. The higher reduction percentage when Cr(VI) was added after 5 hours of growth might be attributed to the higher percentage of cells present. It has been reported by Wang and Xiao (1995) in a study using *Bacillus* sp. and *Pseudomonas fluorescens* LB300 that a high cell density is required for significant Cr(VI) reduction to occur. In contrast, it has also been reported that the conversion of Cr(VI) (20 and 50 mg L^{-1}) by *Streptomyces griseus* was marginally faster when added at the time of inoculation than after 24 hours of growth (Laxman and More, 2002).

In the study by Laxman and More (2002), the effect of initial Cr(VI) concentrations was more significant than the time of Cr(VI) inoculation on the rate of Cr(VI) reduction. The Cr(VI) conversion by *S. griseus* was faster where the initial Cr(VI) concentration was 20 mg L^{-1} compared to 50 mg L^{-1}. In another study by Lee *et al*. (2008) using indigenous bacteria, the removal efficiency of Cr(VI) also decreased with an increase of initial Cr(VI) concentration. Similar to both studies, Cr(VI) reduction by *A. haemolyticus* was also found to be generally greater with lower initial Cr(VI) concentration (Figure 2.5). Only samples C, D and E with 60 mg L^{-1} Cr(VI) concentration added after 5 hours of growth showed higher percentage of Cr(VI) reduction. Apart from that, greater Cr(VI) reduction with lower initial Cr(VI) concentration was consistently observed in samples where Cr(VI) was inoculated at 0 hour. Furthermore, the highest Cr(VI) reduction was observed in samples A and B which were inoculated with 30 mg L^{-1} of Cr(VI) after 5 hours of growth.

Generally, Cr(VI) reduction capacity of *A. haemolyticus* increased with higher percentage of cells which was largely affected by the compositions of growth media used and the initial Cr(VI) concentration inoculated into the media followed by the time of Cr(VI) inoculation.

Based on the preliminary studies carried out, it appeared that the Cr(VI) reduction property of *A. haemolyticus* is not directly related to the resistance level of

the cells. The growth of *A. haemolyticus* was uninhibited in the presence of 60 mg L^{-1} of Cr(VI) as illustrated previously in Figure 2.2. The increasing concentrations of Cr(VI) (30 and 60 mg L^{-1}) only lowered the growth rate. However, the reduction of Cr(VI) was found to be notably affected by the Cr(VI) concentrations inoculated into the growth media. Hence, the growth rate of *A. haemolyticus* with the presence of Cr(VI) could not be correlated with the amount of Cr(VI) reduced. Similarly, it was also shown for *Brucella* sp. (Thacker *et al*., 2007), *Bacillus* sp., *Arthrobacter* sp. (Megharaj *et al*., 2003) and *Pseudomonas* isolates (Ohtake *et al*., 1987) that resistance was unrelated to chromate reduction.

2.4 Conclusion

A. haemolyticus showed uninhibited growth patterns in LB broth supplemented with Cr(VI) at 30 and 60 mg L^{-1}. The strain was able to resist Cr(VI) by both tolerating and reducing Cr(VI). Good tolerance of *A. haemolyticus* towards Cr(VI) was observed in the presence of 30 and 90 mg L^{-1} of Cr(VI) in LB agar and broth respectively. The Cr(VI)-reducing capacity of *A. haemolyticus* was higher in rich medium (LB) and with lower initial concentrations of Cr(VI) added after 5 hours of growth.

CHAPTER III

POSSIBLE ROLE OF PLASMID TO MEDIATE CHROMIUM(VI) RESISTANCE IN *ACINETOBACTER HAEMOLYTICUS*

3.1 Introduction

This chapter discusses the possible role of plasmids in conferring Cr(VI) resistance in *A. haemolyticus*.

The presence of plasmids was screened in *A. haemolyticus* grown in the LB medium with and without Cr(VI) using three plasmid isolation and purification techniques. In order to estimate the size of the indigenous plasmid, restriction enzyme digestions were carried out. The possibility of the plasmid in mediating antibiotic resistance rather than for Cr(VI) resistance was also investigated. In order to ascertain the role of the plasmid harbored in the Cr(VI)-resistant strain, plasmid curing was attempted using two methods; sub-culturing and chemical methods.

3.1.1 Possible genetic mechanism of Cr(VI) resistance

In order to cope with chromate toxicity, microorganisms have evolved diverse resistance mechanisms (Figure 3.1). It has been demonstrated in a variety of bacterial species that chromate actively crosses biological membranes by means of the sulfate uptake pathway, which reflects the chemical analogy between these two oxyanions (Figure 3.1A) (Cervantes and Campos- García, 2007).

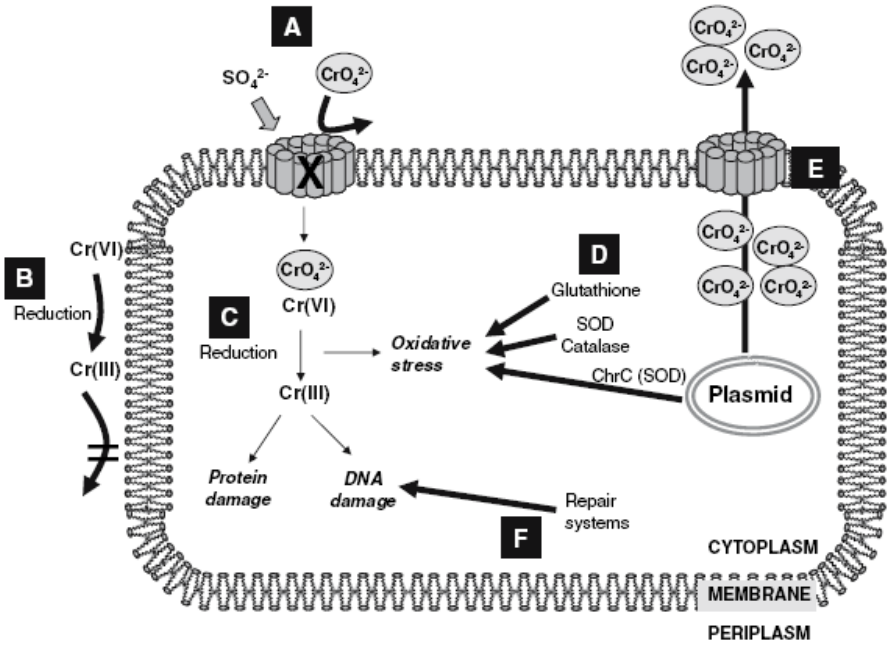


Figure 3.1: Mechanisms of chromate transport, toxicity and resistance in bacterial cells. Mechanisms of damage and resistance are indicated by thin and heavy arrows, respectively. (A) Chromosome-encoded sulfate uptake pathway which is also used by chromate to enter the cell; when it is mutated (X) the transport of chromate diminishes. (B) Extracellular reduction of Cr(VI) to Cr(III) which does not cross the membrane. (C) Intracellular Cr(VI) to Cr(III) reduction may generate oxidative stress, as well as protein and DNA damage. (D) Detoxifying enzymes are involved in protection against oxidative stress, minimizing the toxic effects of chromate. (E) Plasmid-encoded transporters may efflux chromate from the cytoplasm. (F) DNA repair systems participate in the protection from the damage generated by chromium derivatives (Ramírez-Díaz *et al*., 2008).

Inside the cell, Cr(VI) is readily reduced to Cr(III) by the action of various enzymatic or nonenzymatic activities; the Cr(III) generated may then exert diverse toxic effects in the cytoplasm (Figure 3.1C) (Cervantes *et al*., 2001). Chromate reduction is carried out by chromate reductases from diverse bacterial species generating Cr(III) that may be detoxified by other mechanisms. Though many bacterial species are reported to reduce Cr(VI) to Cr(III), the biochemical properties

of only a few Cr(VI) reductases have been elucidated. The diverse characteristics of these ancient enzymes and their wide distribution support the hypothesis that reduction of chromate is a secondary role for chromium reductases. Most characterized enzymes belong to the widespread NAD(P)H dependent flavoprotein family of reductases (Ramírez-Díaz *et al*., 2008).

Since the generation of reactive oxygen species (ROS) occurs during Cr(VI) reduction to Cr(III), the participation of bacterial proteins in the defense against oxidative stress induced by chromate represents an additional mechanism of chromate resistance (Figure 3.1D) (Ramírez-Díaz *et al*., 2008). Besides chromosomal genes, plasmids may also encode systems devoted to protect bacterial cells from the oxidative stress caused by chromate. Plasmid pMOL28 from *Cupriavidus metallidurans*, which encodes the ChrA chromate efflux pump, in addition encodes the ChrC and ChrE proteins that seem to be also involved in chromate resistance (Juhnke *et al*., 2002). These indirect systems of tolerance to chromium include mechanisms focused to maintain the integrity of the cells by protecting them from oxidative stress or to repair the damages caused by chromium derivatives (Ramírez-Díaz *et al*., 2008).

In addition, several examples of bacterial systems protecting from the oxidative stress caused by chromate have been described (Juhnke *et al*., 2002; Hu *et al*., 2005). After chromate exposure, these bacteria show a varied regulatory network that involves the expression of genes for several different metabolic processes as a chromium stress defensive strategy. These include genes for sulfur or iron homeostasis that probably lead to reduced uptake of Cr(VI) by the sulphate uptake pathway and with sulphur or iron homeostasis. Other mechanism of bacterial resistance to chromate involves the expression of components of the machinery for repair of DNA damage (Figure 3.1F) (Ramírez-Díaz *et al*., 2008).

The best characterized mechanisms of chromate resistance comprise efflux of chromate ions from the cell cytoplasm (Figure 3.1E) and the transformation of Cr(VI) to innocuous Cr(III) outside the cell (Figure 3.1B) (Ramírez-Díaz *et al*., 2008). Chromate efflux by the ChrA transporter has been established in *Pseudomonas aeruginosa* (Pimentel *et al*., 2002) and *Cupriavidus metallidurans*

(formerly *Alcaligenes eutrophus*) (Peitzsch *et al*., 1998) and consists of an energy dependent process driven by the membrane potential. The CHR protein family, which includes putative ChrA orthologs, currently contains about 135 sequences from all three domains of life.

Usually, the genes located in plasmids encode membrane transporters, which directly mediate efflux of chromate ions from the cell's cytoplasm. Microbial reduction of Cr(VI) to Cr(III) can be considered as an additional chromate resistance mechanism which is not usually a plasmid-associated trait (Cervantes *et al*., 2001). On the other hand, resistance systems encoded within bacterial chromosomes are generally related to strategies such as specific or unspecific Cr(VI) reduction, free-radical detoxifying activities, repairing of DNA damage, and processes associated with sulfur or iron homeostasis (Ramírez-Díaz *et al*., 2008).

3.1.2 Overview of metal and antibiotic tolerance amongst *Acinetobacter* strains

Species of *Acinetobacter* have been attracting increasing attention in both environmental and biotechnological applications. Some strains of this genus are known to be involved in biodegradation of a number of different pollutants such as biphenyl and chlorinated biphenyl, amino acids (analine), phenol, benzoate, crude oil, acetonitrile, and in the removal of phosphate or heavy metals (Abdel-el-Haleem, 2003).

Metal resistance is often associated with resistance to antibiotics. For example, 50% of the *Staphylococcus* spp. studied was found to display association between occurence of plasmids and resistance to antibiotics and heavy metals (Ugur and Ceylan, 2003). *Azotobacter chroococcum*'s resistance towards antibiotics and heavy metals were also suggested to be plasmid-mediated (Aleem *et al*., 2003). Akinbowale and co-authors (2007) showed that there was a tendency towards a higher frequency of streptomycin resistance among all the metal-resistant *Pseudomonas* isolates compared with the metal-sensitive isolates. It is suspected to be due to the streptomycin and metal resistance determinants being co-located in *Pseudomonas* spp.. Based on an investigation by Ready *et al*. (2007), the presence of

mercury (Hg) in amalgam resulted in an increase in the numbers of Hg-resistant bacteria and the Hg-resistant isolates also exhibited resistance to antibiotics.

Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance. Cross-resistance has the potential to occur when different antimicrobial agents attack the same target, initiate a common pathway to cell death, or share a common route of access to their respective targets. Co-resistance occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron (Chapman *et al*., 2003). The end result is the same: the development of resistance to one antibacterial agent is accompanied by the appearance of resistance to another agent. Importantly, a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Alonso *et al*., 2001).

3.1.3 Plasmid isolation

Plasmids are naturally occurring extra-chromosomal deoxyribonucleic acid (DNA) fragments that are stably inherited from one generation to another in the extra-chromosomal state. Plasmids are widely distributed in prokaryotes and range in size from approximately 1500 bp to over 300 kb. Most plasmids exist as closed-circular double stranded DNA molecules that often confer a particular phenotype onto the bacterial cell in which they are replicated. That is, the plasmid will often carry a gene that encodes resistance to either antibiotics or heavy metals, or that produces DNA restriction and modification enzymes, that the bacterium would not normally possess (Reece, 2004).

The structural differences between plasmid DNA and chromosomal DNA cause physicochemical differences that can be exploited to separate two types of DNA molecules. Methods for isolating plasmid DNA fall into three major categories. First are methods that rely on specific interaction between plasmid DNA and solid support. Examples are adsorption to nitrocellulose microfilter and hydroxyapatite

columns. Second are methods that cause selective precipitation of chromosomal DNA by various agents. These methods exploit the relative resistance of covalently closed circular DNA to extremes of pH, temperature, or other denaturing agents. Lastly, are methods based on differences in sedimentation behaviour between the two types of DNA. This is the approach of choice if highly purified plasmid DNA is required (Boyer, 2006).

The majority of plasmid preparation protocols rely on the 'alkaline lysis' method, which uses a narrow pH range (12.0–12.5) to selectively denature linear, but not covalently closed circular DNA. Birnboim and Doly (1979) used a selective alkaline denaturation of high molecular weight chromosomal DNA, leaving the plasmid DNA unharmed. The alkaline lysate is rapidly neutralized to form an insoluble aggregate consisting of genomic DNA, protein–detergent complexes and high molecular weight RNA. The plasmid DNA remained in the supernatant where it was later precipitated and collected by centrifugation.

Kado and Liu (1981) described a procedure for the detection and isolation of both small and large plasmids (2.6 to 350 X 10^6 Da) from species of *Agrobacterium, Rhizobium, Escherichia, Salmonella, Erwinia, Pseudomonas* and *Xanthomonas* using alkaline (pH 12.6) SDS and an elevated temperature to denature chromosomal DNA. Proteins and cellular debris are removed using a phenol-chloroform extraction. The clarified extract was suitable for agarose gel electrophoresis.

Alternative methods, such as the boiling method (Li *et al*., 2002) and microwave preparation (Marra *et al*., 1999) have been used to isolate plasmid DNA; however, the alkaline lysis method represents the most robust and stable platform for further development of high throughput purification methods (Dederich *et al*., .2002).

3.1.4 Plasmid curing

Most metal and antibiotic resistant bacteria isolated from environmental samples carry the resistance genes on plasmids. This is distinctively different from chromosomal encoded resistance to the same metal and/or antibiotic. If the selective

pressure of the metal or antibiotic is removed, it is possible that the plasmid will be spontaneously lost as it is no longer required (Trevors, 1985). Thus, elimination of plasmid DNA (curing) is an important step in identifying the phenotypic traits encoded by a given plasmid. For example, Ghosh *et al*. (2000) cured plasmids from acidophilic heterotroph, *Acidocella* sp. strain GS19h. The cured derivatives became sensitive to Cd^{2+} and Zn^{2+}, suggesting plasmid-mediated inheritance of metal resistance in this bacterium. By and large, plasmid curing studies have been focused on *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus* and, to a lesser extent, strains of degradative *Pseudomonads* (El-Mansi *et al*., 2000).

A multitude of different chemicals such as acriflavine (Mesas *et al*., 2004), mitomycin C (Kostal *et al*., 1998), ethidium bromide (Thakur *et al*., 2001), acridine orange (Hens *et al*., 2005), and sodium dodecyl sulphate (SDS) (El-Mansi *et al*., 2000; Bruins *et al*., 2003) have been used as curing agents. The mechanisms of these agents involved inhibiting DNA replication, intercalating with DNA, and causing changes to bacterial surface (El-Mansi *et al*., 2000). Spontaneous loss of plasmids through repeated sub-culturing have also been reported by Niazi *et al*. (2001) and Kostal *et al*. (1998) using *Bacillus* sp. and *Pseudomonas* C12B, respectively.

In the present study, plasmid curing method using SDS was attempted. One important feature that is shared between genomic (chromosome) and plasmid DNA is their direct attachment to cell membrane. SDS (an anionic detergent that is widely used for the disruption of the cell membrane) may at the right concentration, be capable of dislodging the indigenous plasmid free of its site of attachment which in turn leads to imperfect replication and unsuccessful segregation of the plasmid. Cured strains (plasmid-less cells) via SDS treatment can still survive in a rich medium. It is also possible for SDS once it reaches the cytoplasm, to interfere with cellular metabolism through its ability to dissociate proteins into their respective subunits thus rendering some enzymes partially or fully inactive. Consequently, a plasmid can be lost if any of the inactivated enzymes is functionally associated with replication and/or segregation of the plasmid (El-Mansi *et al*., 2000).

3.2 Materials and methods

3.2.1 Materials

3.2.1.1 Ampicillin stock solution

Stock solution of ampicillin (25000 mg L^{-1}) was prepared by dissolving 25 mg of ampicillin in 1 mL of DDI water. The solution was then sterilized by using 0.2 μm pore size sterile cellulose-acetate filters (Whatman, England) and stored in aliquots at -20˚C in sterile microcentrifuge tubes.

3.2.1.2 Luria-Bertani with ampicillin agar

LB agar was prepared by incorporating 13 g L^{-1} of agar (Merck, USA) to the LB medium (as described in 2.2.3.1). The medium was sterilized by autoclaving at 121˚C, 115 kPa for 15 minutes. The molten LB was added with ampicillin stock solution to the required concentration when the temperature was about 45-55˚C. The molten agar was then poured into sterile disposable Petri dishes (Sterilin, USA) and allowed to solidify. The plates were left to dry overnight at 30˚C in the incubator (INB 200-500, Memmert) prior to use.

3.2.2 Strains and growth conditions

3.2.2.1 Adaptation of *Acinetobacter haemolyticus* to Cr(VI)

A. haemolyticus (10%, v/v) was inoculated into 25 mL of LB broth and incubated overnight at 30˚C and 200 rpm. The overnight culture (10% v/v) was inoculated into 25 mL of LB broth containing 30 mg L^{-1} of Cr(VI). The remaining overnight culture was stored as glycerol stock solutions as described in 2.2.3.4. The

adaptation process was repeated by incubating overnight at 30˚C and 200 rpm using LB broth containing increasing concentrations of Cr(VI) with a maximum of 100 mg L^{-1}.

The adaptation of *A. haemolyticus* in varying Cr(VI) concentrations and storage as glycerol stock solutions with Cr(VI) was to maintain the culture of *A. haemolyticus* in the presence of Cr(VI) to discourage possible plasmid loss.

3.2.2.2 *Acinetobacter haemolyticus*

Wild-type and adapted strains (as described in 3.2.2.1) of *A. haemolyticus* were used in plasmid screenings. Active culture of the strains (10%, v/v) prepared as described in 2.2.6.1 were inoculated into LB broth supplemented with Cr(VI) (0, 30, and 60 mg L^{-1}). *A. haemolyticus* cultures were grown overnight (12 hours) at 30ºC with agitation at 200 rpm.

3.2.2.3 *Escherichia coli* JM109

E. coli JM109 with pUC19 plasmid was used as positive control in plasmid screenings. The glycerol stock cultures for long term storage and active cultures of *E. coli* JM109 were prepared as described in 2.2.3.4 and 2.2.6.1, except that the LB medium was supplemented with ampicillin (1 μg mL^{-1}).

Active culture of *E. coli* JM109 (10%, v/v) was inoculated into LB broth containing ampicillin (Sigma, USA) at a concentration of 1 μg mL^{-1}. The culture was grown overnight (12 hours) at 37˚C with agitation at 200 rpm.

3.2.3 Isolation and purification of plasmids

Three different alkaline lysis methods were employed for plasmid isolation; alkaline lysis with SDS (Sambrook and Russell, 2001), method of Kado and Liu (1981) and using QIAprep (Qiagen, Germany).

3.2.3.1 Isolation of plasmid DNA by alkaline lysis with SDS

To pellet the cells, 1.5 mL of each overnight culture was centrifuged at 14000 rpm for 1 minute at 4˚C in a microcentrifuge (Hettich Zentrifugen, Mikro 22R). The unused portions of the original cultures were stored at 4˚C. The medium were removed by aspiration, leaving the bacterial cells as dry as possible. The pellets were resuspended in 100 μL of ice-cold Alkaline Lysis Solution I (refer to Appendix A) by vigorous vortexing. Then, 200 μL of freshly prepared Alkaline Lysis Solution II (refer to Appendix A) was added to each bacterial suspension. The tubes were closed tightly and the contents were mixed by rapidly inverting the tube 5 times. The tubes were stored on ice. After that, 150 μL of ice-cold Alkaline Lysis Solution III (refer to Appendix A) was added. The tubes were closed and inverted several times to disperse Alkaline Lysis Solution III through the viscous bacterial lysates. The tubes were stored on ice for 5 minutes. The bacterial lysates were centrifuged at 14000 rpm for 5 minutes at 4˚C. The supernatant was transferred to fresh microcentrifuge tubes. Equal volumes of phenol:chloroform solution (refer to Appendix A) was added. The organic and aqueous phases were mixed by vortexing and then centrifuged at 14000 rpm for 2 minutes at 4˚C. The aqueous upper layer of each sample was transferred to a fresh microcentrifuge tube. Nucleic acids from the supernatants were precipitated by adding 2 volumes of ethanol at room temperature. The solutions were mixed by vortexing and allowed to stand for 2 minutes at room temperature. The precipitated nucleic acids were collected by centrifugation at 14000 rpm for 5 minutes at 4˚C. The supernatants were removed by gentle aspirations. In order to drain all of the fluid away, the tubes were left standing in inverted position. Drops of fluids adhering to the walls of the tubes were removed by pipetting. The pellets were added with 1 mL of 70% ethanol and inverted several times. The DNA of each sample was recovered by centrifugation at 14000 rpm for 2 minutes at 4˚C. All supernatants were removed by gentle aspiration. Beads of ethanol that formed on the sides of the tubes were removed. The open tubes were stored at room temperature until the ethanol had evaporated and no fluid was visible in the tubes (10 minutes). The nucleic acids were dissolved in 50 μL of TE (pH 8.0) buffer (refer to Appendix A) containing 20 μg/mL of DNase-free RNase A. The solutions were vortexed gently for few seconds and stored at -20˚C (Sambrook and Russell, 2001).

3.2.3.2 Method of Kado and Liu (1981)

For plasmid detection, 1 mL of the *A. haemolyticus* and *E. coli* overnight cultures (as described in 3.2.2.2 and 3.2.2.3, respectively) were pelleted by centrifuging at 14000 rpm for 7 minutes at 4˚C. The cell pellets were thoroughly suspended in 40 μL of E buffer (refer to Appendix A). The cells were lysed by adding 80 μL of lysing solution (refer to Appendix A), which was mixed by brief agitation. The solution was heated at 65˚C for 1 hour in a digital water bath (Daniel, ABAT 852), and 2 volumes of phenol-chloroform solution (1:1, v/v) (refer to Appendix A) were added. The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation at 14000 rpm for 5 minutes at 4˚C. Avoiding the precipitate at the interface, the upper aqueous phase was transferred to a fresh microcentrifuge tube by using a pipette. Samples were withdrawn to be used for electrophoresis directly (as described in 3.2.5) or stored at -20˚C.

To isolate plasmid suitable for restriction analysis, another method for rapid isolation of plasmid by Kado and Liu (1981) was used. The procedures employed were similar to the method as described above except that the pelleted cells were lysed using lysing solution directly without resuspension in E buffer. In addition, after aqueous DNA solution was extracted from the lysed cells, phenol and chloroform were removed by ether extraction.

Overnight cultures (1 mL) of *A. haemolyticus* (3.2.2.2) and *E. coli* (3.2.2.3) were pelleted by centrifugation at 14000 rpm for 7 minutes at 4˚C. The pelleted cells were lysed with 80 μL of the lysing solution (refer to Appendix A) and incubated at 65˚C for 1 hour in a thermomixer (Eppendorf, Thermomixer Comfort). Extraction with phenol-chloroform was carried out as described above. The aqueous DNA solutions were then freed of phenol by extracting twice with diethyl ether and transferring to fresh tubes, at which time 100 μL of 3 M sodium acetate were added. The plasmid DNA was precipitated by adding 800 μL of cold (-20°C) 95% ethanol (Fluka, Britain) and incubating the mixture in a dry-ice ethanol bath for 5 minutes. The precipitated plasmid DNA was pelleted by centrifugation for 15 minutes at 2˚C and suspended in 50 μL of autoclaved water (Kado and Liu, 1981). Samples were used for electrophoresis directly or stored at -20˚C.

3.2.3.3 Method of QIAprep Spin Miniprep™ using microcentrifuge

The method of Qiaprep was a modified method based on Sambrook *et al*. (1989), Birnboim and Doly (1979), Birnboim (1983), and Vogelstein and Gillespie (1979).

In the Qiaprep method, the overnight cultures (10 mL) of *A. haemolyticus* and *E. coli* (as described in 3.2.2.2 and 3.2.2.3, respectively) were centrifuged at 4000 rpm, 4˚C for 10 minutes in a centrifuge (Hettich Zentrifugen, Universal 32R). The pellets were resuspended in 500 μL Resuspension Buffer and transferred to a microcentrifuge tube. In order to lyse the cells, 500 μL of Lysing Buffer were added and mixed thoroughly by inverting the tube 15 times. Neutralization was then carried out by adding 700 μL of Neutralization Buffer and mixing immediately and thoroughly by inverting the tube 20 times. The Lyseblue reagent provided along with the kit was used to ensure complete lysis and neutralization. The mixtures were then centrifuged at 13,000 rpm for 10 minutes at 4˚C. The supernatants were applied to the QIAprep spin columns by pipetting and then centrifuged at 13,000 rpm for 1 minute at 4˚C. The flow-through from each spin column was discarded. The QIAprep spin columns were washed by adding 0.5 mL of Buffer PB and centrifuging for 1 minute. This step was required when purifying low-copy plasmids, where large culture volumes are used. Then, the QIAprep spin columns were washed by adding 0.75 mL Buffer PE and centrifuged at 13,000 rpm for 1 minute at 4˚C. The flow-throughs were discarded, and centrifuged for an additional 1 minute to remove residual wash buffer. Then, the QIAprep columns were placed in clean 1.5 ml microcentrifuge tubes. To elute DNA, 50 μL of Elution Buffer (10 mM Tris·Cl, pH 8.5) was added to the center of each QIAprep spin column. Elution Buffer was heated to 70˚C before eluting the plasmid DNA. The column was left to stand for 1 minute, and centrifuged at 13,000 rpm for 1 minute at 4˚C. Samples were used for electrophoresis directly or stored at -20˚C. The diagrammatic representation of the QIAprep spin procedure described is as shown in Figure 3.2.

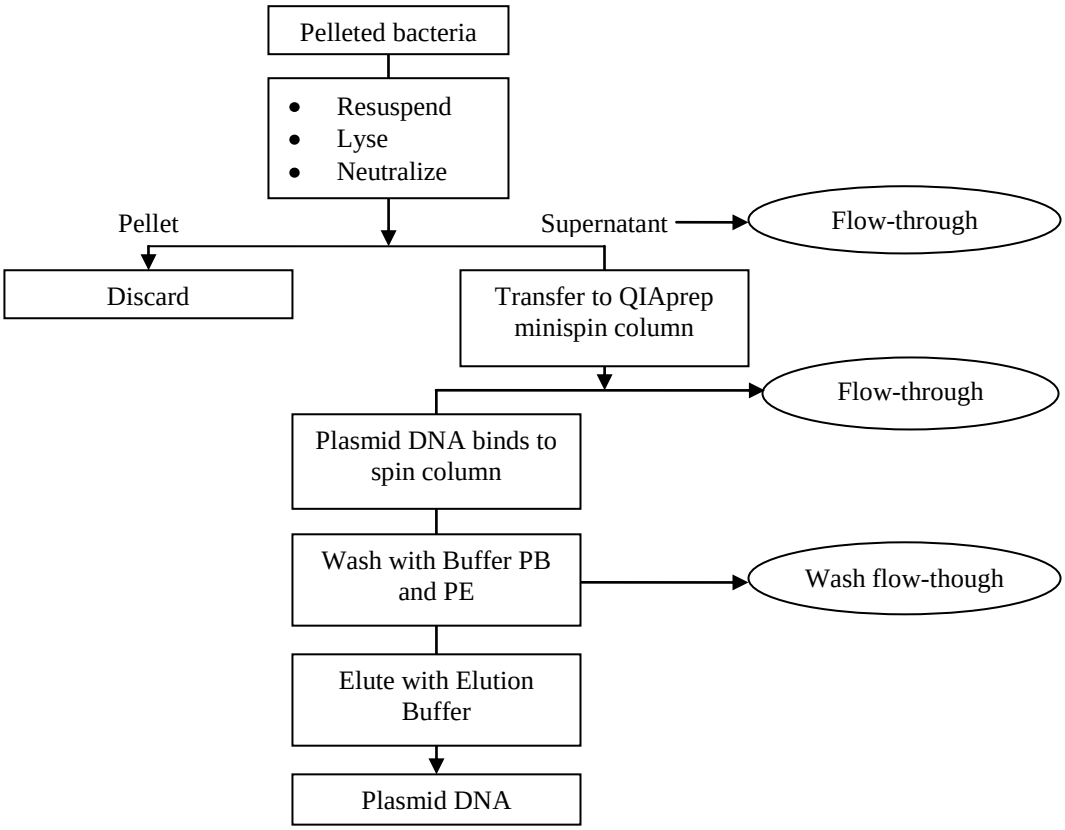


Figure 3.2: Diagrammatic representation of QIAprep spin procedure in microcentrifuges.

Due to the low yields of plasmid DNA, samples of cleared lysates after neutralization, flow-throughs before the washing steps and the wash flow-throughs (Figure 3.2) were kept to detect the loss of DNA samples during the plasmid isolation steps. The cleared lysates and the entire flow-throughs were precipitated by adding 0.7% (v/v) isopropanol and centrifuged at 13000 rpm for 30 minutes at 4˚C. The wash flow-through samples were centrifuged at 13000 rpm for 5 minutes at 4˚C after mixing with 0.7% (v/v) isopropanol and 0.1% (v/v) of 3 M sodium acetate (pH 5) (Unilab Reagent, Australia). All the supernatants were discarded and the pellets were resuspended in 20 µL of Elution Buffer and subjected to agarose gel electrophoresis as described in 3.2.5.

3.2.4 Restriction enzyme digestions

Restriction endonucleases *Pst*I and *Eco*RI obtained from Fermentas were used according to the recommendations by the manufacturer.

The components for restriction enzymes digestions were added in the following order; 16 µL of nuclease-free water, 2 µL of 10X recommended Tango Buffer, 1 µL of DNA sample, and 1 µL of restriction enzyme. The mixture was mixed gently by spinning down briefly followed by incubation at 37˚C for 1 hour. After incubation, the samples were used for electrophoresis as described in 3.2.5.

3.2.5 Gel electrophoresis

3.2.5.1 Preparation of DNA samples

The DNA samples (10 µL) were mixed with loading dye (Fermentas, Canada) in separate tubes to a ratio of 1:6 and carefully loaded into the preformed wells. MassRuler^{TM} DNA ladders (Fermentas, Canada) (5 µL) ranging from 80 to 10000 bp were loaded as molecular weight DNA markers.

3.2.5.2 Agarose gel electrophoresis

Agarose gel electrophoresis were performed using horizontal agarose (0.8%, w/v) (Vivantis, United Kingdom) slab gels in E buffer (refer to Appendix A) into which ethidium bromide (10 mg mL^{-1}) (Sigma, USA) had been added to yield a 0.1 µL mL^{-1} final concentration. The electrophoresis was run at a constant voltage of 100 V for large gels and 80 V for smaller gels at room temperature for 1 to 1.5 hours using the Mini-Sub Cell GT (Biorad). The DNA bands were visualized with ultraviolet light and photographed (Syngene Bio-imaging, Geneflash). The plasmid size estimates of the isolated plasmids were obtained by comparing their relative mobilities on agarose gel with standard molecular weight DNA markers (3.2.5.1).

3.2.6 Antibiotic and Cr(VI) effect on the presence of plasmids

3.2.6.1 Tolerance of *Acinetobacter haemolyticus* towards antibiotics

Active culture of *A. haemolyticus* (10%, v/v) as described in 2.2.6.1 was inoculated into 25 mL of LB broth and incubated for 8 hours, 30˚C at 200 rpm.

Serial dilutions of 10^{-5}, 10^{-6} and 10^{-7} of the culture at end log phase were spread on LB agar plates containing ampicillin (25000 mg L^{-1}) at final concentrations of 50, 100, 200 and 300 mg L^{-1} (as prepared in 3.2.1.2). The culture of *A. haemolyticus* inoculated onto sterile LB plates without ampicillin acted as the control. The plates were then incubated for 24 hours at 30˚C in an incubator and then the numbers of viable colonies were counted.

3.2.6.2 Preparation of cells for plasmid screening

Active culture of *A. haemolyticus* (10%, v/v) as described in 2.2.6.1 was inoculated into LB broth with ampicillin (300 mg L^{-1}), Cr(VI) (60 mg L^{-1}) and a mixture of ampicillin and Cr(VI) (300 mg L^{-1} and 60 mg L^{-1}).The active culture of *A. haemolyticus* inoculated into LB broth without Cr(VI) and ampicillin acted as the control. The cultures were grown overnight (12 hours) at 30˚C with agitation at 200 rpm. The overnight cultures were subjected to plasmid extraction using the procedure of Qiaprep method as described in 3.2.3.3.

3.2.7 Plasmid curing

Two plasmid curing methods were used; sub-culturing and chemical methods.

3.2.7.1 Sub-culturing

Active culture of *A. haemolyticus* (1%, v/v) prepared as described in 2.2.6.1 was inoculated into LB broth supplemented with 30 mg L^{-1} of Cr(VI). The culture was grown overnight (12 hours) at 30˚C with agitation at 200 rpm. An inoculum of the overnight culture (1%, v/v) was placed in fresh LB broth (without Cr(VI)) and the culture was incubated as described above. This inoculation and overnight growth cycle was repeated in fresh LB broth without Cr(VI) (Bopp *et al*., 1983).

Every growth cycle was followed by inoculation of the sub-culture on LB agar plates containing 30 mg L^{-1} of Cr(VI) to screen for Cr(VI) sensitivity. Inoculation of the sub-culture on LB agar plates without Cr(VI) acted as control. Plasmid screenings were occasionally carried out on the sub-cultures using the Qiaprep method as described in 3.2.3.3.

3.2.7.2 Chemical method using SDS (0.05%, w/v)

The method of Hardy and Kimber (1993) was employed with modification of the concentration of SDS used for plasmid curing.

Overnight inoculum of *A. haemolyticus* (10%, v/v) (as described in 2.2.6.1) was pipetted into 2 mL of curing broth (0.05% w/v SDS in LB broth) and incubated overnight at 30˚C, 200 rpm. The growth of bacteria in the curing broth was repeated several times to encourage plasmid loss and plasmid screenings were occasionally carried out using the method as described in 3.2.3.3.

Every growth cycle was also followed by inoculation of the sub-culture on LB agar plates containing 30 mg L^{-1} of Cr(VI) to screen for Cr(VI) sensitivity. Inoculation of the sub-culture on LB agar plates without Cr(VI) acted as control (Bruins *et al*., 2003).

3.3 Results and discussions

3.3.1 Plasmid screenings

To ascertain whether or not the Cr(VI) resistance in *A. haemolyticus* was plasmid-mediated, the strain was screened for the presence of plasmid. Plasmid screenings were carried out on the wild-type and Cr(VI)-adapted strains of *A. haemolyticus*. *E. coli* JM109 which carried the pUC19 plasmid was used as a positive control. The plasmid in the strain was maintained by cultivation in LB broth containing ampicillin. A variety of plasmid isolation procedures which included the alkaline lysis methods by Sambrook and Russell (2001), Kado and Liu (1981) and Qiagen kit (2005) were used.

Figures 3.3 and 3.4 show the agarose gel electrophoretic profile of plasmids isolated from *A. haemolyticus* and *E. coli* JM109 using Qiagen kit and plasmid detection method by Kado and Liu (1981), respectively.

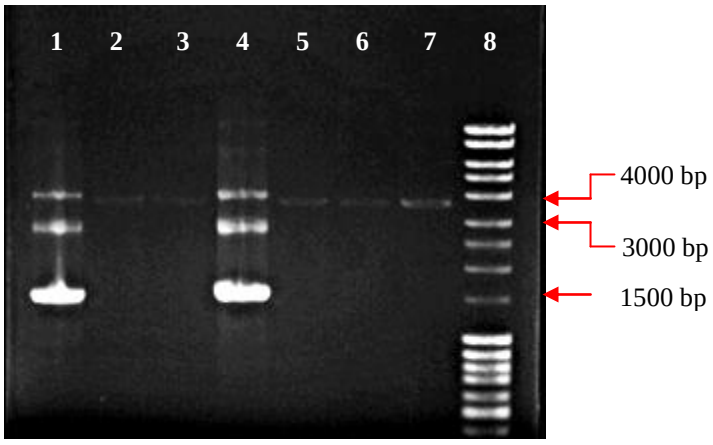


Figure 3.3: Agarose gel electrophoretic profile of plasmids isolated using Qiagen kit. Lane 1: pUC19; Lane 2: *A. haemolyticus* + 60 mg L^{-1} Cr(VI); Lane 3: *A. haemolyticus* + 30 mg L^{-1} Cr(VI); Lane 4: pUC19; Lane 5: *A. haemolyticus* + 60 mg L^{-1} Cr(VI); Lane 6: *A. haemolyticus* + 30 mg L^{-1} Cr(VI); Lane 7: *A. haemolyticus*; Lane 8: DNA Ladder Mix Range (10000 bp to 80 bp).

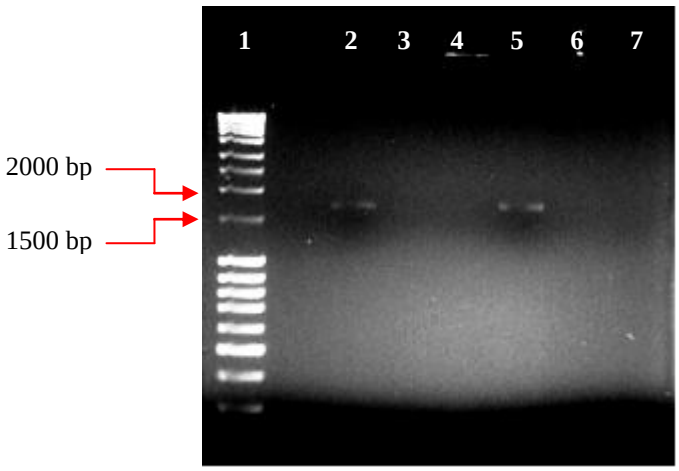


Figure 3.4: Agarose gel electrophoretic profile of plasmids from modified procedure of plasmid detection by Kado and Liu. Lane 1: DNA Ladder Mix Range (10000 bp to 80 bp); Lane 2: pUC19; 3. *A. haemolyticus*; Lane 4: *A. haemolyticus* + 30 mg L^{-1} Cr(VI); Lane 5: pUC19; Lane 6: *A. haemolyticus*; Lane 7: *A. haemolyticus* + 30 mg L^{-1} Cr(VI).

Amongst the three plasmid isolation procedures used, the Qiagen kit (Figure 3.3) showed the most distinctive plasmid band from the samples of *A. haemolyticus* and *E. coli* JM109 plasmid DNA. The alkaline lysis method with SDS by Sambrook and Russell (2001) failed to show any DNA bands from both strains whilst the method of Kado and Liu (1981) yielded only a faint band of the pUC19 plasmid (Figure 3.4). No plasmid from *A. haemolyticus* was isolated using the methods of Sambrook and Russell (2001) and Kado and Liu (1981). However, plasmids could be detected using the Qiagen kit.

Although all the plasmid isolation methods used were based on alkaline lysis, the results varied with each different method. This may be due to variation in the degree of lysis from strain to strain, dependent upon factors such as cell wall composition, pigmentation, and production of extracellular polysaccharides. Thus, it becomes difficult to judge the exact time of lysis sufficient enough to release the plasmid DNA, leaving the chromosomal DNA attached to the mesosomes with the cell wall and thus being precipitated out during the extraction procedure (Sambrook *et al*., 1989). Some studies have also reported problems in isolating plasmid DNA

from *Acinetobacter* sp., often because of unappreciated difficulties in lysing the cell wall of the cells (Towner, 2006).

The method of Qiagen provided the best results for plasmid visualization which was also simple and more rapid. The consistent appearance of the pUC19 plasmid (Figure 3.3, Lane 1 and 4) may be due to the different prominent forms of plasmids in the DNA sample (Schmidt *et al*., 1999). Agarose gel electrophoretic profiles of plasmid DNA isolated from *A. haemolyticus* using the Qiagen kit along with the marker DNA is shown in Figure 3.3.

Plasmid was found in *A. haemolyticus* grown without Cr(VI) (Figure 3.3, Lane 7), with 60 mg L^{-1} Cr(VI) (Figure 3.3, Lane 2, 5) and 30 mg L^{-1} Cr(VI) (Figure 3.3, Lane 3, 6). However, the plasmid bands were faint. The weak bands might represent the low yield of DNA from the plasmid isolation procedures. Therefore, the electrophoresis of the cleared lysates after neutralization, flow-throughs before and after the washing steps were carried out to detect the loss of plasmids during isolation and purification. However, the agarose gel electrophoresis profile showed no signs of DNA which indicated that there was no loss of plasmid.

Since there was no loss of plasmid during isolation procedures, additional steps were applied to ensure that the lysis and neutralization steps were complete. The Lyseblue reagent provided along with the kit was added into Lysing Buffer prior to the lysing step. After addition of the mixture into the resuspended cells, the number of inversions was increased (15-20 times) to obtain a homogeneous blue mixture which indicated complete lysis of the cells. Complete lysis will ensure that all the plasmids are released for further work. After addition of the Neutralization Buffer, the mixture was inverted for about 15-20 times until it became colourless indicating complete neutralization of the mixture.

Addition of Lyseblue reagent gave more intense plasmid bands as shown in Figure 3.5. This plasmid isolation technique was then used for the following studies. Interestingly, the plasmid of *A. haemolyticus* (Figure 3.5, Lane 2-4) was stably maintained even in the absence of Cr(VI) in the growth medium.

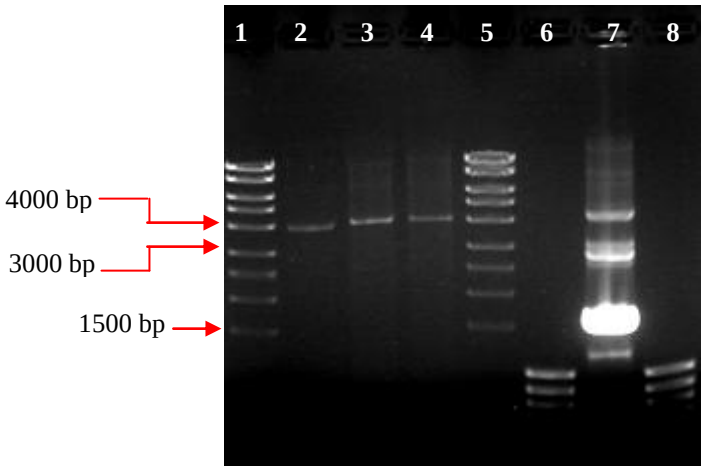


Figure 3.5: Agarose gel electrophoretic profiles of plasmids after addition of Lyseblue reagent. Lane 1 and 5: DNA Ladder High Range (10000 bp to 1500 bp): Lane 2: *A. haemolyticus*; Lane 3: *A. haemolyticus* + 30 mg L^{-1} Cr(VI); Lane 4: *A. haemolyticus* + 60 mg L^{-1} Cr(VI); Lane 6 and 8: DNA Ladder Low Range (1031 bp to 80 bp); Lane 7: pUC19.

3.3.2 Estimation of plasmid size

To estimate the size of the plasmid isolated from *A. haemolyticus*, restriction enzyme digestions were carried out. Molecular weight estimations based on the restriction fragments size have been used in determining the size of large plasmids. For example, *Hin*dIII and *Eco*RI were used to determine the size of an 85-kb plasmid in *Pseudomonas* sp. strain IST 103 (Thakur *et al*., 2001). According to Barton *et al*. (1995), supercoiled plasmids migrated at rates that were not a simple function of their molecular weights, making size determinations problematic. Sizes of large plasmids were reliably estimated after S1 nuclease converted the supercoiled plasmids into full-length linear molecules.

In this study, the restriction enzymes used were *Pst*I and *Eco*RI. The restriction enzymes chosen were based on a study by Suzuki *et al*. (1992). An NAD(P)H-dependent Cr(VI) reductase (molecular weight = 65000 bp) was purified from a Cr(VI)-resistant bacterium, *Pseudomonas ambigua* G-1. The sequence of the *chr*R gene encoding the well-characterized enzyme has been deposited in GenBank

(accession number D83142) (Appendix B). Based on the NEBcutter V2.0 by New England Biolabs Inc. (Figure 3.6), *Pst*I could be used as a double cutter restriction enzyme for the *chr*R gene. Hence, *Pst*I was used to examine if the gene in *A. haemolyticus* was homologous to that in *P. ambigua*. *Eco*RI, that does not cut the *chr*R gene, was used as control.

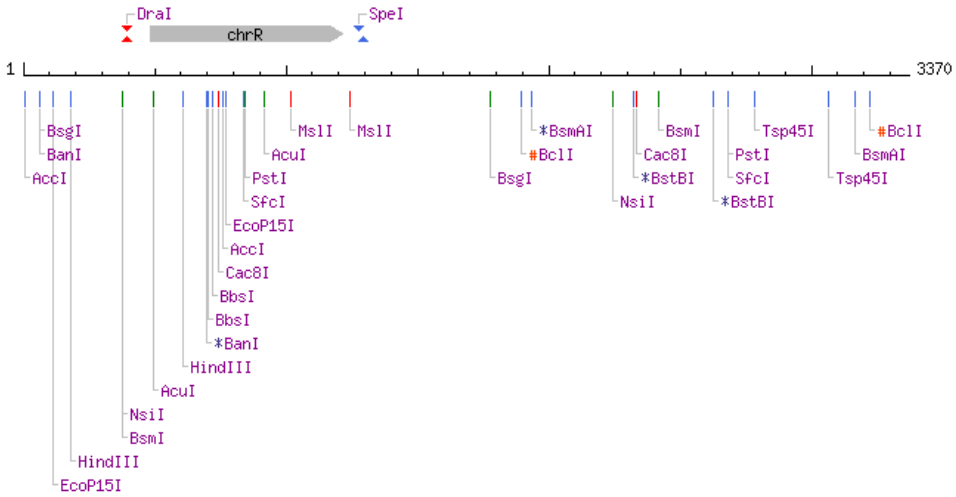


Figure 3.6: Restriction map of the *chr*R gene (D83142) encoding for chromate reductase from *Pseudomonas ambigua* (Suzuki *et al*., 1992).

The result of the restriction enzyme analysis is as displayed in Figure 3.7. The molecular weights of the unknown digested plasmids were estimated by comparing the relative mobilities on agarose gel with standard DNA size markers as illustrated in Figure 3.8. Based on the equation from the graph which is y = -0.2492x + 4.476, the correlation coefficient obtained was 98.88.

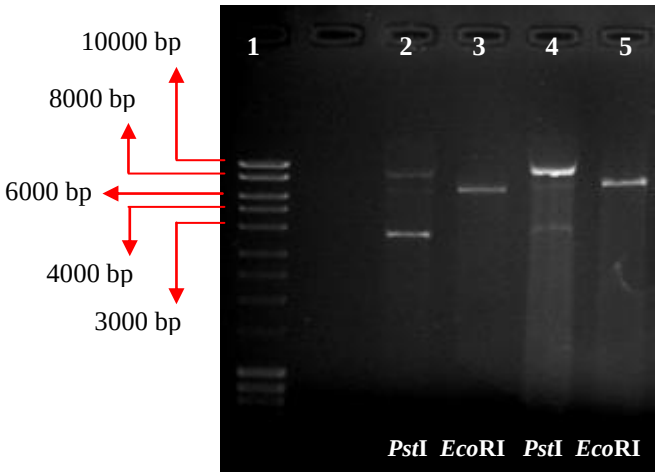


Figure 3.7: Agarose gel electrophoretic profiles of digested plasmids. Lane 1: DNA Ladder Mix Range (10000 bp to 80 bp); Lane 2-3: *A. haemolyticus*; Lane 4-5: *A. haemolyticus* + 30 mg L^{-1} Cr(VI).

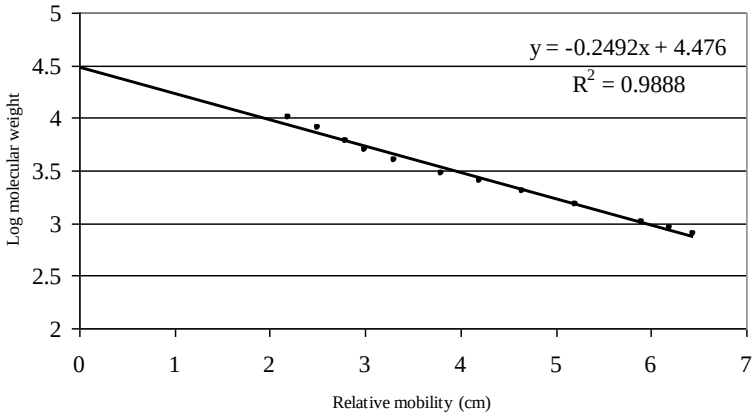


Figure 3.8: Molecular size versus relative mobility plot of the DNA Ladder Mix Range (10000 bp to 80 bp).

The digestion of plasmid using *Eco*RI (Figure 3.7, Lane 3 and 5) yielded only one plasmid band, whereas, the digestion of plasmid DNA of *A. haemolyticus* grown in the absence of Cr(VI) with *Pst*I (Figure 3.7, Lane 2) resulted in the formation of two fragments of plasmids at 7550 and 4133 bp. The plasmids of *A. haemolyticus* grown in LB broth with 30 mg L^{-1} of Cr(VI) (Figure 3.7, Lane 4) also showed two fragments after *Pst*I digestion with molecular weights of 7550 and 4504 bp. Based on the restriction fragments size after digestions with *Pst*I, the molecular weight was

calculated to be 12 kb (11683 and 12054 bp for *A. haemolyticus* grown in the absence and presence of Cr(VI) at 30 mg L^{-1} respectively).

A recent study by Pardesi *et al*. (2007) reported that majority (98.3%) of the 118 *Acinetobacter* sp. isolates (48 isolates were grouped as *A. haemolyticus*) from human skin showed a maximum of 3 plasmids. The molecular weights of the plasmids found in the isolates were found to be between 1.5 and 40 kb. Many other studies have reported *Acinetobacter* isolates that carry multiple indigenous plasmids of variable molecular size (Towner, 2006). According to Towner (2006), most indigenous plasmids from *Acinetobacter* sp. seem to be relatively small (<23 kb). Several examples of plasmids in *Acinetobacter* sp. responsible for other resistance mechanisms (other than antimicrobial agents) are as shown in Table 3.1.

Table 3.1: Examples of plasmid(s) isolated from *Acinetobacter* sp..

Isolate(s)	**Origin of isolate**	**Plasmid(s)**	**MW[a] (kb)**	**Role of plasmid(s)**	**Reference**
A. calcoaceticus RA57	Not stated	pSR1 pSR2 pSR3 pSR4	5.1 5.4 10.5 20	Crude oil degradation[b]	Rusansky *et al*. (1987).
A. calcoaceticus BD413	Not stated	pWM10 pWMll pWM12	7.4 2.4 2.2	Cryptic	Minas and Gutnick (1993).
A. haemolyticus (A19), *A. baumannii* (A18, A16, A13), *A. genospecies 3* (A15)	Rhizosphere of wheat	pUPI126	40	Indole-3-acetic acid production, selenium, tellurium, lead resistance	Huddedar *et al*. (2002).
A. baumannii BL88	Environmental isolates	pUPI199	54	Silver resistance	Deshpande and Chopade (1994).

[a]MW = molecular weight

[b]mediated by pSR4 plasmid

In the present study, the single plasmid isolated from *A. haemolyticus* (12 kb) was within the range of the molecular weight reported for *A. calcoaceticus* RA57 (Table 3.1). *A. calcoaceticus* RA57 harbored four plasmids with molecular weights ranging from 5.1 to 20 kb. The pSR4 plasmid was found to mediate for crude oil degradation.

Based on the restriction enzyme analysis, *Pst*I was able to digest the plasmid isolated from *A. haemolyticus*. Hence, it may be possible that a gene homologous to *chr*R gene (from *P. ambigua*) (Suzuki *et al*., 1992) to be present in the plasmid. However, further studies need to be carried out to determine if the *chr*R gene was present in the plasmid of *A. haemolyticus*.

Therefore, in this study, the role of the plasmid in *A. haemolyticus* was still unknown. The plasmid was found to be present regardless whether Cr(VI) was present or absent in the growth medium. This suggests that the plasmid might also code for some other novel property/ies or may be cryptic in nature (Pardesi *et al*., 2007).

3.3.3 Resistance towards ampicillin and Cr(VI)

Plasmids in *Acinetobacter* sp. have been widely reported to mediate other properties; for example, the ability to grow and disperse crude oil (Rusansky *et al*., 1987) and more prominently, resistance towards antibiotics (Weyrich *et al*., 2006). *Acinetobacter* sp. has been reported to be multidrug-resistant as it is resistant to most types of antibiotics for example, polymixins (Falagas and Bliziotis, 2007), aminoglycosides (Magnet *et al*., 2001), and β-lactamases (Costa *et al*., 2000). Weyrich *et al*. (2006) reported that *A. baumanii* was resistant to carbapenems which was mediated by a plasmid-encoded metallo-ß-lactamase. The correlation between antibiotic and metal resistance is also well established in clinical and environmental isolates of *Acinetobacter* (Kunte *et al*., 1993; Shakibaie *et al*., 1999; Huddedar *et al*., 2002).

In this study, the existence of the plasmid was independent of the presence of Cr(VI) in the growth medium. Thus, the possible involvement of the plasmid in resistance towards antibiotics rather than for Cr(VI) was examined. *A. haemolyticus* was first screened for tolerance towards ampicillin followed by isolation of plasmid from *A. haemolyticus* grown in the presence of ampicillin using Qiagen kit (3.2.3.3).

The surface spread method was used to determine the tolerance of *A. haemolyticus* towards ampicillin. Table 3.2 shows the total cell count of *A. haemolyticus* grown in the LB agar containing the antibiotic. The strain was able to grow well in the presence of ampicillin at a concentration of 300 mg L^{-1}. Therefore, for plasmid screenings, *A. haemolyticus* was grown in LB broth with 300 mg L^{-1} of ampicillin.

Table 3.2: Total cell count of *Acinetobacter haemolyticus* on LB agar with ampicillin.

Ampicillin concentrations, mg L^{-1}	**Total cell count, CFU mL^{-1}**
50	5.0×10^8
100	5.4×10^8
200	4.3×10^8
300	4.2×10^8

Figure 3.9 shows the agarose gel electrophoresis profile of plasmid for *A. haemolyticus* grown in LB broth in the presence of Cr(VI) and/or ampicillin. The control was *A. haemolyticus* grown in the absence of both inhibiting substances.

The supplementation of ampicillin in the growth medium did not significantly affect the appearance of plasmid in *A. haemolyticus* to signify its role in resistance to the β-lactam antibiotic. The plasmid bands for *A. haemolyticus* grown in the presence of ampicillin and/or Cr(VI) (Figure 3.9, Lane 3, 4 and 5) were similar to that obtained without ampicillin (Figure 3.9, Lane 2). No association between the plasmid profile and antibiotic resistance of *A. haemolyticus* was observed.

All *Acinetobacter* sp. isolates with a 40 kb plasmid have been reported to display intermediate to low level resistance to some antibiotics (cefotaxime, ceftazidime, ticarcillinclavulinic acid, ciprofloxacin) tested (Thakur *et al.*, 2007). Eight isolates having low molecular weight plasmids in addition to 40 kb plasmid were highly susceptible to most of the 30 antibiotics tested. Curing experiments proved that other antibiotic resistance seen in the *Acinetobacter* sp. isolates was not plasmid-encoded. In another study, the gene encoding an aminoglycoside 6'-N-acetyltransferase which modifies amikacin was suggested to be located in the chromosomes of *A. haemolyticus* (Lambert *et al*., 1993). The existence of chromosomal-mediated antibiotic resistance led to the probability that the resistance towards ampicillin in *A. haemolyticus* was not plasmid-mediated. Therefore, the role of plasmid in antibiotic resistance could not be determined, leading to the ambiguity in the role of the plasmid harbored by *A. haemolyticus* in Cr(VI) resistance.

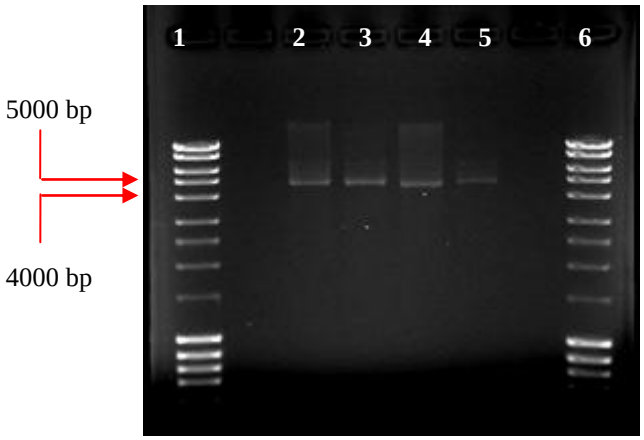


Figure 3.9: Agarose gel electrophoretic profiles of plasmids. Lane 1 and 6: DNA Ladder Mix Range; Lane 2: *A. haemolyticus*; Lane 3: *A. haemolyticus* + 60 mg L^{-1} Cr(VI); Lane 4: *A. haemolyticus* + 300 mg L^{-1} ampicillin; Lane 5: *A. haemolyticus* + 60 mg L^{-1} Cr(VI) + 300 mg L^{-1} ampicillin.

3.3.4 Attempts to cure plasmid in *Acinetobacter haemolyticus*

A direct experiment was required to conclude whether or not Cr(VI) resistance was a plasmid-mediated character. Therefore, plasmid curing was

attempted to note the loss, if any, of Cr(VI) resistance in *A. haemolyticus*. The methods employed were the sub-culturing and chemical method (refer to 3.2.7.1 and 3.2.7.2, respectively).

In the sub-culturing method, the potential spontaneous loss of plasmid was tested by screening for plasmids in sub-cultures of *A. haemolyticus* for approximately 72 generations in LB broth without the supplementation of Cr(VI). The sub-cultures were also inoculated onto LB agar plates containing 30 mg L^{-1} of Cr(VI) to investigate the effect of sub-culturings on growth. However, no spontaneous loss of plasmid was observed as sub-cultures of *A. haemolyticus* were still able to grow on LB agar containing 30 mg L^{-1} of Cr(VI).

In the second method, *A. haemolyticus* was repeatedly sub-cultured in LB broth in the presence of 0.05 % (w/v) SDS followed by inoculation on LB agar plates without Cr(VI) and with 30 mg L^{-1} of Cr(VI). The sub-cultures were also subjected to plasmid screenings using Qiagen kit method. The agarose gel electrophoretic profile is as shown in Figure 3.10.

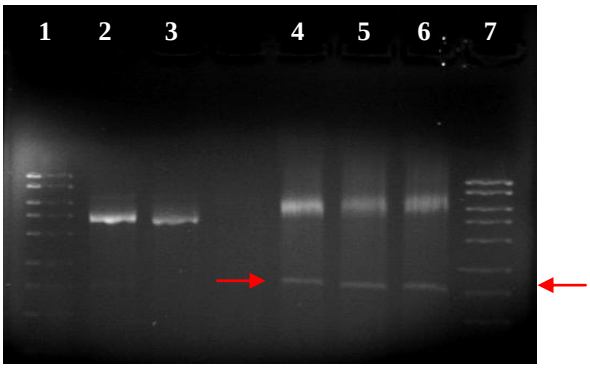


Figure 3.10: Agarose gel electrophoretic profiles of plasmid isolated from *Acinetobacter haemolyticus* subjected to plasmid curing using SDS. Lane 1: DNA Ladder Mix Range (10000 bp to 80 bp); Lane 2: *A. haemolyticus*; Lane 3: *A. haemolyticus* + 30 mg L^{-1} Cr(VI); Lane 4-7: 13^{th}, 15^{th} and 17^{th} sub-cultures of *A. haemolyticus* in LB broth containing 0.05% (w/v) SDS.

Plasmid curing in LB broth containing 0.5% of SDS was used in a study by Bruins *et al*. (2003) where plasmid loss was confirmed after a second serial attempt to cure plasmid from *Pseudomonas pickettii* that confers cadmium resistance. However, in this study, the appearance of plasmid bands for the 13th, 15th and 17th sub-cultures (Figure 3.10, 4-6) was still observed although the intensity was lower with higher number of sub-culturings. The result obtained also showed the appearance of another plasmid of lower molecular weight which was not previously observed (Figure 3.10, arrow). Similarly, Thakur *et al*. (2001) reported the appearance of another plasmid in *E. coli* JM109 after transformation of a 4-kb plasmid from *Pseudomonas* sp. IST103 (a stable bacterial consortium was capable of utilizing pentachlorophenol as sole carbon and energy source) into the strain. The authors postulated that the plasmid may have evolved in the transformed strain due to adaptability in pentachlorophenol. In the present study, it is also possible that the presence of SDS may have caused the plasmid in *A. haemolyticus* to evolve.

Figure 3.11 displays the percentage of viable cells grown on LB agar plates containing Cr(VI) relative to cells grown without Cr(VI). The cells were inoculated onto LB agar plates from every SDS-treated sub-culture in LB broth containing 0.05% (w/v) SDS.

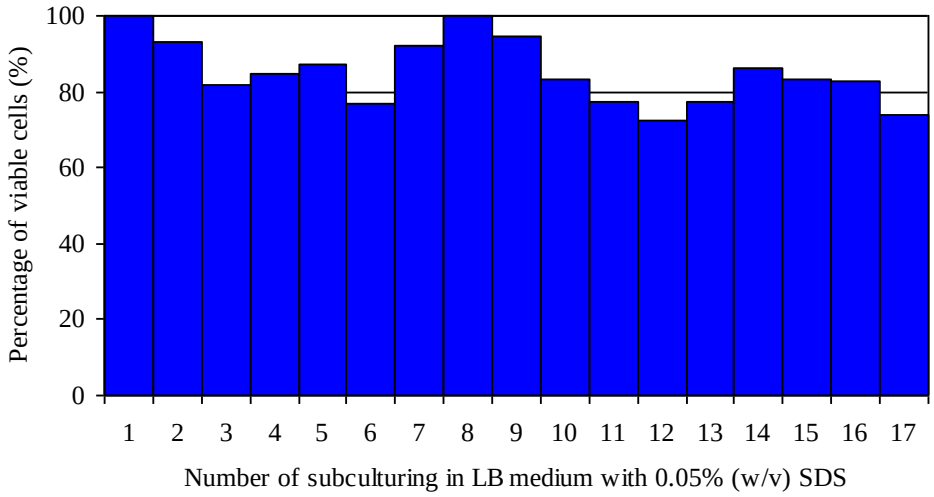


Figure 3.11: Percentage growth of *Acinetobacter haemolyticus* sub-cultures on LB agar plates supplemented with 30 mg L^{-1} Cr(VI) relative to growth of *Acinetobacter haemolyticus* without Cr(VI).

Based on the graph (Figure 3.11), the growth of *A. haemolyticus* ranged between 74% and 100%. Therefore, *A. haemolyticus* was still able to grow well on LB agar with Cr(VI) at 30 mg L^{-1} without showing any significant decline in growth. Good growth percentage was observed. However, the size of colonies on LB agar plates containing Cr(VI) (Figure 3.12 (b)) was significantly smaller compared to the colonies on LB agar plates without Cr(VI) (Figure 3.12 (a)).

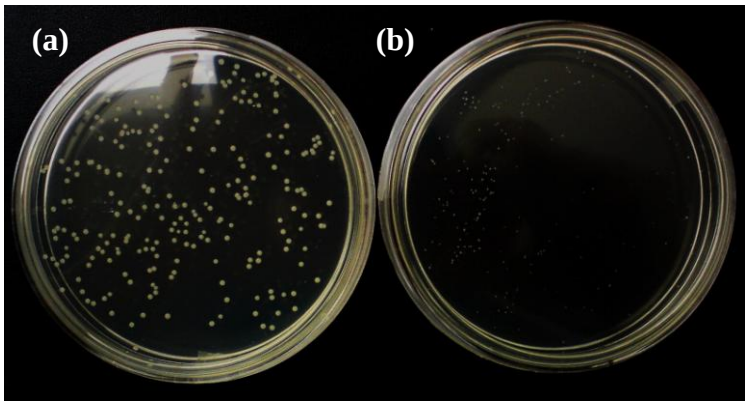


Figure 3.12: Growth of SDS-treated cells on LB agar plates (a) without and (b) with 30 mg L^{-1} Cr(VI) after 24 hours.

From the results on plasmid curing it is most unlikely that Cr(VI) resistance is plasmid encoded in *A. haemolyticus*. Thus, the resistance to Cr(VI) is possibly conferred chromosomally. Unfortunately, due to time constraints, the subsequent work for locating the genetic determinant of Cr(VI) resistance in the chromosomes would be suggested for future research.

Some literature suggest that Cr(VI) resistance may be mediated by genes on bacterial chromosomes. Park *et al*. (2000) purified 600-fold a soluble Cr^{6+} reductases, ChrR, from *Pseudomonas putida* MK1. The ChrR-coding gene, *chrR* was identified from the genomic sequence of *Pseudomonas putida* MK1. In another example, *chr*R was found located on the chromosome of *Ralstonia metallidurans* strain CH34 (Juhnke *et al*., 2004). Therefore, there is a possibility that the genes responsible for Cr(VI) reduction may be in the chromosomes of *A. haemolyticus*.

Nevertheless, as previously suggested (Ramírez-Díaz *et al*., 2008), some of the genes involved in Cr(VI) resistance are localized on plasmid. Chromate resistance determinants have been described on plasmids in several bacteria, especially in *Pseudomonas* (Wasi *et al*., 2008). A study by Bopp *et al*. (1983) reported that the degree of chromate resistance of chromate-resistant (Cr^r) *E. coli* LB317 was 40-fold greater than that of the corresponding plasmidless strain (AC80), whereas the *Pseudomonas fluorescens* plasmid-bearing strain (LB300) was 200-fold more resistant to chromate than was its plasmidless counterpart (LB303). However, unlike Cr(VI) resistance determinants, Cr(VI) reduction determinants were not essentially found on plasmids. *P. fluorescens* SM1 was found to carry the dichromate resistance as well as reduction determinant on the same plasmid (with a molecular weight of approximately 43.6 kb) (Wasi *et al*., 2008). Furthermore, the plasmid in *A. haemolyticus* could be digested using *Pst*I (as discussed in 3.3.2), similar to the *chr*R gene in *P. ambigua* (Suzuki *et al*., 1992). Therefore, it cannot be *a priori* excluded that the ability of *A. haemolyticus* to grow in the presence of high Cr(VI) concentrations could be related to the presence of plasmids.

3.4 Conclusion

This study showed that *A. haemolyticus* harbors a plasmid with a molecular weight of approximately 12 kb which could be digested with *Pst*I. However, the role of the plasmid in Cr(VI)-resistance could not be ascertained as attempts to define the role of the plasmid in ampicillin resistance and to cure the plasmid were unsuccessful. Further work needs to be done to study the potential of chromosomal-mediated Cr(VI) resistance in the strain thus leading to the ability of the strain to survive Cr(VI)-contaminated environments.

CHAPTER IV

INSTRUMENTAL ANALYSIS ON CHROMIUM REDUCTION BY *ACINETOBACTER HAEMOLYTICUS*

4.1 Introduction

This chapter elucidates the Cr(VI) reduction mechanisms by *A. haemolyticus* in terms of the possible surface interactions involved, localization of chromium on the cell surface and intracellularly, and the local coordination environment of the reduced Cr(VI).

The methods employed included field-emission scanning electron microscope coupled with energy dispersive X-ray (FESEM-EDX) analysis was carried out to obtain information on the changes in the cell morphology in the presence of Cr(VI). Fourier-transform infrared (FTIR) spectroscopy was done to determine the functional groups on the cell surface that were involved in the interactions with Cr(VI). In order to determine the intracellular localization of Cr(VI), transmission electron microscope (TEM) technique was used. X-ray absorption of fine structure (XAFS) spectroscopy was used to investigate the final oxidation state of chromium and the local coordination of chromium in *A. haemolyticus*. The X-ray absorption of near-edge structure (XANES) spectra provided information on the oxidation state of chromium in *A. haemolyticus* during the reduction of Cr(VI), whereas, the extended X-ray absorption of fine structure (EXAFS) spectra provides information on the coordination environment such as the nearest neighbouring atoms. Using crude cell-free extracts (CFE) of *A. haemolyticus* obtained via ultrasonication, the localization (intra- or extracellularly) of Cr(VI) reduction was investigated.

4.1.1 Analysis on Cr(VI) reduction mechanisms

Bacterial mechanisms of resistance to chromium exposure involve direct strategies aimed at transforming Cr(VI) to innocuous Cr(III) outside the cell or at extruding toxic chromate ions from the cytoplasm (Cervantes and Campos-García, 2007). Cr(VI) reduction is a complex process with multiple possible pathways and unstable redox intermediates with intermediate valence between the valences of the stable end members (Daulton *et al*., 2007).

To understand microbial reduction processes, techniques capable of providing submicron-scale information are necessary (Srivastava and Thakur, 2007). Electron microscopy (EM) analytical techniques, such as energy-dispersive X-ray analysis (EDX), have been systematically employed in biological sciences to identify chemical elements in cells (Andrade *et al*., 2004). According to Srivastava and Thakur (2007), conventional TEM studies of microbial reduction of metals have provided important information necessary for understanding microbial reduction.

Identification of the functional groups capable of interacting with metal ions has also been studied using FTIR spectroscopy (Quintelas *et al*., 2008; Lameiras *et al*., 2008; Pandi *et al*., 2007). Interactions between *Rhodococcus opacus* (Bueno *et al*., 2008) and *Termitomyces clypetus* (Das and Guha, 2007) with metal ions and Cr(VI) respectively had been evaluated by both FTIR and SEM-EDX analyses. A schematic diagram by Das and Guha (2007) based on FTIR study, desorption experiment, and the fluorescence detection of non-metal ions using complexes between phosphate and tris(3-aminopropyl)amine is as shown in Figure 4.1. The interaction of the Cr(VI)-biomass (*Termitomyces clypeatus*) displayed the different functional groups of the biomass that are involved in chemical reactions, e.g. ionic interaction, hydrogen bonding or ion–dipole interaction and complexation with chromate ions.

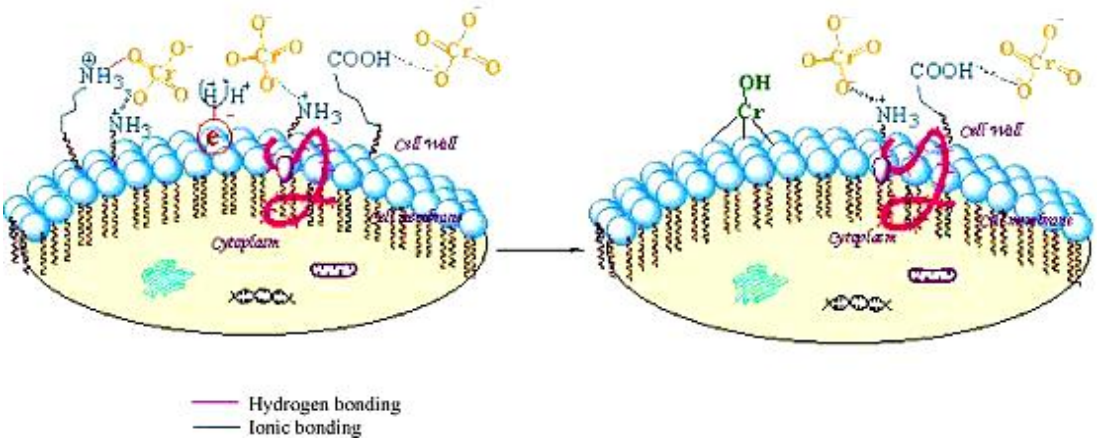


Figure 4.1: A schematic diagram of Cr(VI)-biomass interaction; Cr^{6+} initially binds with the functional groups of the biomass and then reduced to Cr^{3+}.

Recently, a synchrotron-based XAFS technique has provided means for studying biological systems. XAFS offer direct information of metal speciation immobilized in the biomass (Parsons *et al*., 2002). XAFS is a direct and non-destructive technique to determine the oxidation state of chromium in solids with a detection limit for chromium oxidation states in solids in the parts per million (ppm) range.

The enzymatic mechanisms of bacterial reduction of Cr(VI) have also been established. Recently, three bacterial enzymes with Cr(VI)-reducing property have been characterized by Barak *et al*., (2006); ChrR of *Pseudomonas putida* and *Escherichia coli* (ChrR of *E. coli* was formerly referred to as YieF) (Park *et al.*, 2000; Ackerley *et al*., 2004a) and NfsA of *E. coli* (Ackerley *et al*., 2004b). ChrR seems to provide an antioxidant defense mechanism to *P. putida* by protecting cells against H_2O_2 stress (Gonzalez *et al*., 2005). The *E. coli* YieF protein shares sequence homology with the *P. putida* ChrR enzyme (Ackerley *et al*., 2004a). However, YieF has a different reaction mechanism in which the enzyme simultaneously transfers three electrons to chromate to produce Cr(III) and one electron to molecular oxygen generating ROS. Another example is the NfsA of *E. coli* which is a flavoprotein that is able to reduce chromate to Cr(III) (Ackerley *et al*., 2004b).

4.1.2 Electron microscopy (EM)

EM involves the use of electrons to illuminate a specimen and create an enlarged image. Two techniques of EM are SEM and TEM, a comparison of which is given in Table 4.1 (Wischnitzer, 1981). A modern SEM provides an image resolution typically between 1 nm and 10 nm, not as good as in the TEM but much superior to the light microscope. In addition, SEM images have a relatively large depth of focus: specimen features that are displaced from the plane of focus appear almost sharply in-focus. The SEM uses a thick (bulk) specimen, into which the electrons may penetrate several micrometers (at an accelerating voltage of 30 kV), so the x-ray intensity is higher than that obtained from thin specimen used in TEM (Egerton, 2005).

Table 4.1: Comparison of scanning and transmission electron microscope.

Scanning Electron Microscope	Transmission Electron Microscope
An image can be obtained by any signal generated by the incident electrons.	An image can be obtained only from the focusable transmitted electron.
Only one minute spot of the specimen is irradiated at a time.	A relatively large area of the specimen is continuously irradiated.
Specimen observation at progressively higher magnification is usually possible without any change in focus or image brightness.	Adjustment of focus and image brightness are usually necessary as magnification is progressively increased (automated in several models).
The specimen need not be cut into thin sections to be examined.	Primarily, only thin sections can be examined.
Specimen preparation is simpler.	Specimen preparation is more complex.

4.1.2.1 Scanning electron microscope (SEM)

A SEM is based on secondary emission of electrons. Electrons can be "reflected" (back-scattered) from a bulk specimen but another possibility is for the incoming (primary) electrons to supply energy to the atomic electrons that are present in a solid, which can then be released as secondary electrons. These electrons are emitted with a range of energies making it more difficult to focus them into an image by electron lenses. However, the alternative mode of image formation uses a scanning principle where primary electrons are focussed into a small-diameter electron probe that is scanned across the specimen. When applied at right angles to the beam, the electrostatic or magnetic fields can be used to change its direction of travel (Egerton, 2005).

The emissions of electrons from a substance can be induced by the application of heat (thermionic emission), strong electrical fields (high-field emission or field emission), electromagnetic radiation (photoelectric emission) or atomic particles (secondary emission). Only thermionic and field emissions are used to generate the electron beam in electron microscopes. The main advantage of using a field emission source in electron microscopy is the large number of electrons that are emitted in a relatively small beam spot. A smaller beam spot diameter results in increased resolution of the image. This is because a field emission source produces an electron beam containing so many electrons that the beam spot diameter can be smaller and still have sufficient electrons to form an image (Lee, 1993).

4.1.2.2 Transmission electron microscope (TEM)

In TEM, electrons penetrate a thin specimen and are then imaged by appropriate lenses as found in the biological light microscope. TEM is capable of displaying magnified images of a thin specimen, typically with a magnification in the range of 10^3 to 10^6 (Egerton, 2005).

In TEM, essentially all of the incoming electrons are transmitted through the specimen, provided it is suitably thin (Egerton, 2005). Each electron passing through

the specimen has a number of possible fates. The most significant are: It is undeflected (i.e. transmitted without interacting with any atoms); It is deflected but loses no energy (i.e. elastically scattered); It loses a significant amount of energy and is probably deflected (i.e. inelastically scattered). As a result, secondary electrons or X-rays may be excited. If all these types of electrons are allowed to carry on down the microscope, they will all contribute to the image and all regions of the specimen will look the same. There would be no contrast between areas of different thickness or different compositions. In order to create contrast in the image, the scattered electrons must be separated from the unscattered electrons using the objective lens aperture (Chescoe and Goodhew, 1990).

4.1.3 Fourier-transform infrared (FTIR) spectroscopy

Infrared spectroscopy is very useful for obtaining qualitative information about molecules. However, for absorption in the infrared region to occur, there must be a change in the dipole moment (polarity) of the molecule. A diatomic molecule must have a permanent dipole (polar covalent bond in which a pair of electrons is shared unequally) in order to absorb but larger molecules do not (Christian, 2004).

Absorbing (vibrating) groups in the infrared region absorb within a certain wavelength region and the exact wavelength will be influenced by neighboring groups. Absorption in the 6 to 15 μm is very dependent on the molecular environment and this is called the fingerprint region (Christian, 2004).

Although the most important use of the infrared spectroscopy is in identification and structure analysis, it is useful for quantitative analysis of complex mixtures of similar compounds because some absorption peaks for each compound will occur at a definite and selective wavelength with intensities proportional to the concentration of absorbing species (Christian, 2004).

4.1.4 X-ray absorption fine structure (XAFS) spectroscopy

XAFS spectroscopy has the ability to determine atom types and numbers in the near vicinity of the atom whose absorption spectrum is being measured, as well as the distance of these atoms from the absorber. The main advantages of XAFS that make it one of the few structural methods applicable to natural and hydrated samples are as follows: the technique has elemental selectivity by tuning to the absorption edge of interest; no long-range (crystalline) order in the sample is required; and measurements are performed on the "as prepared" samples (i.e., no drying or ultrahigh vacuum environment is necessary). The local structural information obtained from those measurements and from spectra of appropriately chosen, well-characterized standards enable identification and quantification of the functional groups responsible for the bacterial surface complexation, thereby providing a rigorous test of the adsorption model (Boyanov *et al*., 2003).

XAFS consists of two different complimentary techniques, the X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS). The XAFS spectra are split into two distinct regions: a near-edge region that includes the fine structure associated with the edge itself, and an extended fine-structure region that consists of the weak oscillatory structure that may persist to as much as 1000 eV above the edge. These two regions are referred to as the XANES and EXAFS spectra, respectively (Huggin *et al*., 1999).

4.1.4.1 Extended x-ray absorption fine structure (EXAFS) spectroscopy

The EXAFS is the fine structure in the x-ray absorption coefficient starting somewhat past an absorption edge and extending typically 1000 eV further, as shown in Figure 4.2, for the *K*-edge of copper metal. Fine structure also exists nearer the absorption edge, but because the interaction of the ejected photoelectron with the potential of the surrounding atoms is still strong in this region, the simplifying single-scattering assumption leading to EXAFS cannot be made (This is discussed later in 4.1.4.2) (Koningsberger and Prins, 1988).

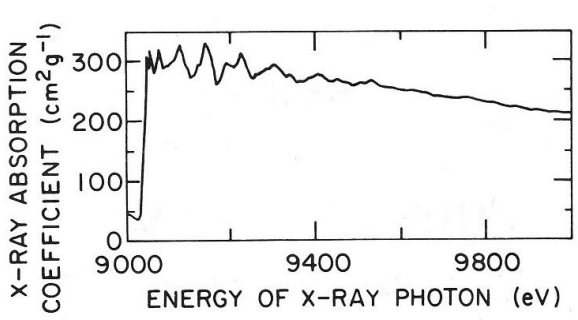


Figure 4.2: The x-ray absorption coefficient for the *K*-edge of copper metal.

The absorption edge corresponds to an x-ray photon having enough energy to just free a bound electron in the atom. When the electrons are in the most tightly bound $n = 1$ shell the edge is called the *K*-edge. For the next most tightly bound shell of atoms, the n = 2 shell, the corresponding edges are called the *L*-edges. At present these edges are the only ones used to observe EXAFS, though in principle, $n = 3$ or higher shells could be used. X-ray absorption in the photon range up to 40 keV, the range of most importance for EXAFS, is dominated by photoelectron absorption where the photon is completely absorbed, transferring its energy to excite a photoelectron and leaving behind a core hole in the atom (Koningsberger and Prins, 1988).

4.1.4.2 X-ray absorption near-edge structure (XANES) spectroscopy

The XANES is an absorption spectrum covers the range between the threshold and the point at which the EXAFS begins. This is admittedly a rather loosely defined spectral range, its limits being in principle different for each system. Figure 4.3 shows some experimental data in which the different character of the two spectral regimes can clearly be appreciated (Koningsberger and Prins, 1988).

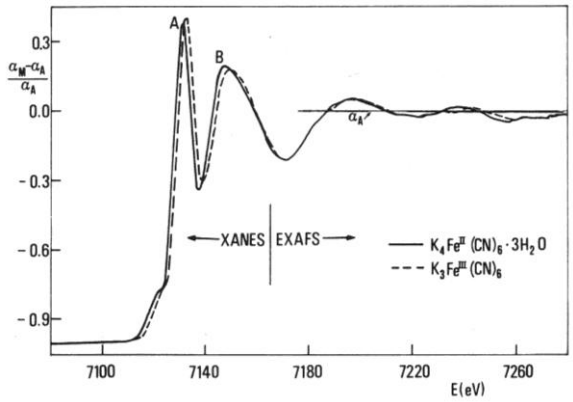


Figure 4.3: *K*-edge absorption spectra of iron in $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$.

In the XANES regime, multiple scattering of the excited electron confers sensitivity to the details of the spatial arrangement of atoms neighbouring the absorbing one. The details include their radial distance, orientations relative to one another, bond angles, and so on. Changes in the charge distribution around a given atom in different chemical environments can alter core-level binding energies and thus produce absorption edge shifts that show up in the XANES. A practical point worth noting is that XANES structures are usually much stronger than the EXAFS oscillations (Figure 4.3) (Koningsberger and Prins, 1988).

Generally, the XANES spectrum is used as a ‘‘fingerprint’’ to identify the form or forms of the element in the material under investigation, whereas the EXAFS region can be mathematically manipulated to yield a ‘‘radial structure function’’ (RSF) from which the local structure around the absorbing element may be inferred (Huggin *et al*., 1999).

4.2 Materials and methods

4.2.1 Field-emission scanning electron microscope coupled with energy dispersive x-ray (FESEM-EDX) spectroscopy

4.2.1.1 Preparation of bacterial cultures

Active culture of *A. haemolyticus* (10%, v/v) as described in 2.2.5.1 was inoculated into 25 mL of LB broth and incubated for 5 hours, 30˚C at 200 rpm. The mid-exponential phase cultures (0.1 mL) of *A. haemolyticus* were then spread over the surface of the LB agar plates with 30 mg L^{-1} Cr(VI) using a sterile glass spreader at dilutions of 10^{-6} and 10^{-7}. In this study, sterile LB agar plates supplemented with 30 mg L^{-1} Cr(VI) was used. LB agar plates without Cr(VI) inoculated with bacteria acted as controls. All the plates were incubated for 24 hours at 30ºC prior to FESEM sample preparation.

4.2.1.2 FESEM sample preparation and instrumentation

The cells prepared as described in 4.2.1.1 were removed from LB agar plate into a fixative which consisted of 2.5% of glutaraldehyde (Sigma,USA) in DDI water for 1-2 hours at room temperature. Fixed cells were washed twice with DDI water, and post-fixed with 2% osmium tetroxide (Fluka, Switzerland) for 1 hour. The pellets were subsequently dehydrated using a series of alcohol (10%, 30%, 50%, 70%, and 100% ethanol in DDI water) for 5 minutes each and left to dry overnight in a desiccator. The specimens were mounted onto the sample holder with carbon-conductive adhesive tapes and coated with platinum using a sputter coater (Auto fine coater JFC-1600, JEOL) prior to viewing using a field-emission scanning electron microscope (FESEM JSN-670 1F, JEOL) fitted with EDX analysis.

4.2.2 FTIR spectroscopy

4.2.2.1 Preparation of bacterial cultures

Active culture of *A. haemolyticus* (10%, v/v) were inoculated into a series of Erlenmeyer flasks containing 200 mL of LB broth and incubated at 30˚C and 200 rpm. After the cultures reached mid-exponential phase, stock solutions of filter-sterilized Cr(VI) was added to give final concentrations of 30, 60 and 100 mg L^{-1}. Control experiment consisted of *A. haemolyticus* (10%, v/v) grown in LB broth. After 24 hours of incubation, the cultures were pelleted by centrifugation at 7000 rpm for 10 minutes at 4˚C. The bacterial suspensions were washed three times with 0.85% sodium chloride solution followed by DDI water and dried in air at 50˚C for 8 hours (Kamnev *et al*., 1997).

4.2.2.2 Sample preparation and FTIR spectra acquisition

Finely ground air-dried bacterial cell samples were pressed by a manual hydraulic press into spectroscopic quality KBr (Riedel-de Haën, Germany) pellet with a sample/KBr ratio of about 1/100. The disks were then fixed in the FTIR spectrometer for analysis. The spectra were collected at a resolution of 4 cm^{-1} and the number of scans for each spectrum was a minimum of 5 (mid-infrared region, 400-4000 cm^{-1}) using an FTIR spectrometer (FTIR 1600, Perkin-Elmer). Background correction for atmospheric air was used for each spectrum.

4.2.3 TEM analysis

4.2.3.1 Preparation of bacterial cultures

Active culture of *A. haemolyticus* (10%, v/v) as described in 2.2.5.1 was inoculated into 25 mL of LB broth and incubated for 5 hours, 30˚C at 200 rpm. The mid-exponential phase cultures (0.1 mL) of *A. haemolyticus* were then spread over the surface of the LB agar plates with 30 mg L^{-1} Cr(VI) using a sterile glass spreader at dilutions of 10^{-6} and 10^{-7}. LB agar plates without Cr(VI) inoculated with bacteria acted as controls. All the plates were incubated for 24 hours at 30˚C prior to TEM sample preparation.

4.2.3.2 TEM sample preparation and instrumentation

The cells prepared as described in 4.2.3.1 were removed from agar plate into a fixative which consisted of 4% of glutaraldehyde (Sigma, USA) in PBS buffer, 0.1 M for 1 hour at room temperature. Fixed cells were washed three times with 0.1 M PBS buffer and post-fixed with 2% osmium tetroxide (aqueous solution) (Ted-Pella, California) for 1 hour. The cells were subsequently dehydrated using a series of alcohol (30%, 50%, 70%, and 90% ethanol in DDI water) for 5 minutes each followed by two 5-minute washes in 100% ethanol and two 5-minute washes in 100% acetone. The dehydrated cells were then embedded in epoxy resin. Increasing concentrations of epoxy resin (Ted-Pella, California) in acetone (50%, 100%) were infiltrated into the pellets for 15 minutes each. After that, cell pellets were infiltrated a second time with fresh epoxy resin (100%) and cured at 60˚C overnight. Specimens of 90 nm thickness were sectioned from the embedded blocks using a Leica UltraCut UCT ultramicrotome and mounted on 200-mesh copper TEM grids. The specimens were stained with uranyl acetate (Ted-Pella, California) and post-stained with lead citrate (Ted-Pella, California) for 5 minutes each. The samples were viewed using a transmission electron microscope (Tecnai G^2, Philips), operated at 80 kV.

4.2.4 XAFS spectroscopy

4.2.4.1 Sample preparation

Active culture of *A. haemolyticus* (10%, v/v) were inoculated into 4 Erlenmeyer flasks containing 400 mL of LB broth each and incubated at 30˚C and 200 rpm. After the cultures reached mid-exponential phase, stock solutions of filter-sterilized Cr(VI) were added to give final concentration of 60 mg L^{-1}. After 48 hours of incubation, the cultures were pelleted by centrifugation at 13000 rpm for 5 minutes, 4˚C. The cell suspensions were then divided into 2 sets of Eppendorf tubes. One set of the cell suspensions was rinsed twice with phosphate bufferred saline (10X, pH 7.4) (PBS) (Fluka, Switzerland) solution to remove any unbound chromium from the samples. The pellet was air-dried.

Both the samples in Eppendorf tubes were placed in flasks sealed with butyl rubber stoppers and purged with N_2 gas for about 8 hours, to dry the homogeneous wet cell suspensions and preserve the samples. For XAFS measurements, bacterial suspensions were loaded directly into a slotted sample holder, sealed with Kapton tape.

4.2.4.2 Data collection and analysis

Chromium K-edge XAFS spectra were collected at the XDD beamline, Singapore Synchrotron Light Source (SSLS), operated at electron energy, 0.7 GeV and beam current 200 mA. The bacterial biomasses from 4.2.4.1 were measured in fluorescence at room temperature using a Lytle-type fluorescence detector. The reference chromium models were measured using transmission mode. The compounds used were Cr foil, $Cr(NO_3)_3$, $Cr(NO_3)_3$(aq), CrOOH, Cr-acetate, and $K_2Cr_2O_7$. A channel-cut silicon (111) double-crystal monochromator with an entrance slit of 1 mm was used for all XAFS measurements. Energy calibration was carried out by Cr foil. For adsorption samples, multiple scans were collected and averaged over to improve signal to noise ratio.

Data analysis was carried out using WINXAS 2.3. Background subtractions were performed by applying linear and polynomial fits to pre- and post-edge regions, respectively. The spectra were then converted into k space (wave vector space $Å^{-1}$) from E space. The conversion to k space was based on the energy of the photoelectrons ejected from the samples. The EXAFS were extracted after background correction using a spline of seven knots taken between 2.0 $Å^{-1}$ and 12.2 $Å^{-1}$. The spectra were then k^3 weighted and Fourier transformed into R space. The same procedures were applied to reference samples. The phase shift and backscattering amplitude were theoretically calculated from CrOOH using FEFF6 and s2 was determined to be 0.82 by fitting CrOOH and was fixed during the fit to other spectra. The input files for the FEFF6 fitting were created using the Atoms software and crystallographic data in the literature. The interatomic distances (R), coordination numbers (CN), and Debye-Waller factors (σ^2) on the first coordination shell were obtained by fitting to the first peak in Fourier transform.

4.2.5 Chromium(VI) reductase assay using crude cell-free extracts (CFE)

4.2.5.1 Preparation of crude CFE

The crude CFE were prepared based on the methods by Thacker *et al*. (2006) and Thacker *et al*. (2007). Mid-exponential phase culture in 200 mL LB broth, was harvested by centrifugation at 6000 g for 20 minutes at 4ºC, washed twice with 20 mL of 10 mM Tris-HCl buffer, pH 7.2 and resuspended in 30 mL of the same buffer. Cells were disrupted by sonication (Vibra Cell, Sonic and Materials Inc.) for 20 minutes in cold condition. The resultant homogenate was centrifuged at 8000 g for 30 minutes at 4ºC; the supernatant (CFE) was used for chromate reductase assay (see 4.2.5.2). Protein estimation was done by Lowry *et al*. (1951) method (see 4.2.5.3). Supernatant obtained after harvesting of the cells was also filter sterilized and used for chromate reductase assay.

4.2.5.2 Chromium(VI) reductase assay

The methods by McLean and Beveridge (2001) and Pal and Paul (2004) were adopted in the Cr(VI) reductase assays.

Cr(VI) reduction assays using crude CFE or supernatant (10 mL) from 4.2.5.1 were conducted at 30°C with agitation at 120 rpm in Erlenmeyer flasks containing 5 mg L^{-1} of Cr(VI) under aerobic condition. One set of LB broth only and autoclaved crude CFE acted as controls.

Each treatment was done in duplicate and samples were collected at regular time interval for 8 hours. All the samples were immersed in hot water bath at about 80ºC for 1 hour immediately after collection to stop the reactions. Cr(VI) was determined by the colorimetric DPC method as described in 2.2.10.

4.2.5.3 Protein estimation

Standards (0.5 mL) containing a range of 10-100 µg mL^{-1} protein were prepared from a stock solution of a 1 mg mL^{-1} BSA (see Appendix A). Crude CFE samples were set up in two dilutions to a final volume of 0.5 mL. Sample buffer (0.5 mL) was also set up. DDI water (0.5 mL) was used as blank. Lowry assay mix (2.5 mL) (see Appendix A) was added to each tube, vortexed and left to incubate at room temperature for 10 minutes at room temperature in the dark. Folin-Ciocalteu reagent (0.25 mL) (see Appendix A) was then added to each tube and vortexed immediately. After incubation in the dark at room temperature for 30 minutes, each tube was measured at 750 nm. The data for unknown was corrected by subtracting value for buffer. Standard curve for the known amount of standard protein was plotted. The protein concentration of the crude CFE was determined based on the plot.

4.3 Results and discussions

4.3.1 FESEM-EDX analysis

FESEM was employed in this study to observe the effect of Cr(VI) on the morphology of *A. haemolyticus*. EDX was carried out to further confirm the presence of chromium. *A. haemolyticus* cells used were grown on LB agar containing 30 mg L^{-1} of Cr(VI), whereas cells grown in the absence of Cr(VI) acted as control.

FESEM micrographs and EDX spectra obtained from *A. haemolyticus* grown without Cr(VI) (control) and exposed to 30 mg L^{-1} Cr(VI) are presented in Figure 4.4. The FESEM micrographs show that *A. haemolyticus* grown without Cr(VI) appeared as coccobacilli (Figure 4.4A, C) with rough surface. The average diameter of the cells was 0.577 μm (n = 3). However, after 24 hours of growth on LB agar plates containing 30 mg L^{-1} of Cr(VI), the morphology of *A. haemolyticus* cells (Figure 4.4B, D) were irregular and enlarged with the appearance of wrinkles on the surface. The average diameter of the cells exposed to Cr(VI) was 0.704 μm (n = 3). Interestingly, numerous pilus-like structures were also observed when the cells were exposed to Cr(VI).

Srivastava and Thakur (2007) also reported on the morphology of *Acinetobacter* sp. grown with and without Cr(VI). The *Acinetobacter* sp. cells incubated without Cr(VI) were short rod-shaped with smooth surface. However, in this study, *A. haemolyticus* cells were found to be in coccobacilli shapes with rough surfaces. Despite the differences, after incubation in the presence of Cr(VI), both the *Acinetobacter* sp. and *A. haemolyticus* showed morphological changes. Ridges were observed in the cell walls of *Acinetobacter* sp. (Srivastava and Thakur, 2007), whereas, *A. haemolyticus* had wrinkled appearance. In another example by Lin *et al.* (2006), the surface structures of basalt-inhabiting *Arthrobacter* K-2 grown on agar plates with Cr(VI) were also found to have wrinkled surface.

The appearance of pilus-like structures between *A. haemolyticus* cells exposed to Cr(VI) during growth was similarly found in *Shewanella oneidensis* MR-

1 during transition of growth from 3˚C to 22˚C. The structures were suspected to be sex pili to enhance genetic or nutrients exchange. However, the reason for the appearance of the pilus-like structure in *S. oneidensis* was not explained (Abboud *et al*., 2005).

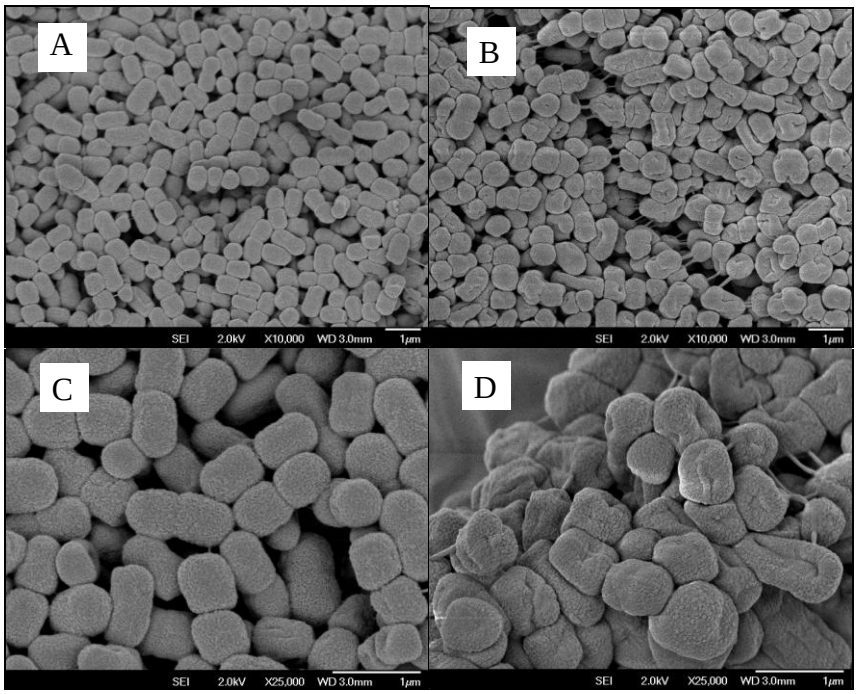


Figure 4.4: Scanning electron micrographs of *Acinetobacter haemolyticus* cells grown on LB agar (A, C) without Cr(VI) (control) and (B, D) with 30 mg L^{-1} Cr(VI); magnification, (A, B) 10 k and (C, D) 25 k.

The diameter of *A. haemolyticus* grown with Cr(VI) was notably larger than the cells grown without Cr(VI). The changes in size may have resulted in the OD_{600} measurements (as discussed in 2.3.1) to not accurately reflect cell growth (Ackerley *et al*., 2006). The cells used in this study was grown on LB agar plates with and without Cr(VI) via spread plate technique. Although the cell count in both conditions were found to be similar, the size of the colonies was notably smaller with the presence of Cr(VI). This may signify the retardation of cell separation which eventually caused cell enlargement. However, the possibilities of other reasons leading to cell enlargement are not ruled out.

Enlargement or elongation of cells were also observed for other bacteria exposed to Cr(VI). Non-adapted *E. coli* K-12 cells exposed to 250 M potassium chromate (K_2CrO_4) exhibited extreme filamentous morphology within 3 hours of Cr(VI) challenge (Ackerley *et a*l., 2006). An enlarged or elongated cell size has also been observed following exposure to other stress conditions, such as high salt (Vijaranakul *et al*., 1995), low-temperature growth at 3°C (Abboud *et al*., 2005), and UV radiation (Qiu *et al*., 2005), and may likely constitute a general cellular response to environmental stress. The increased cell size of *Staphylococcus aureus* under high NaCl was correlated with shorter interpeptide bridges, less cross-linked peptidoglycan, and retarded cell separation (Vijaranakul *et al*., 1995).

To determine the presence of chromium on the cell surface, elemental analysis via EDX spectroscopy was carried out. No chromium was detected on the surface of the cells grown on LB agar without Cr(VI) (Appendix C). Analysis on the cell surface of *A. haemolyticus* cells grown in the presence of Cr(VI) (Appendix C) revealed the presence of little chromium peaks. However, no precipitates were found on the cell surface of *A. haemolyticus* unlike as reported by Srivastava and Thakur (2007). This probably implies that chromium was most likely adsorbed onto the surface of *A. haemolyticus* instead of forming precipitates. The adsorbed chromium was assumed to be Cr(III) because chromate anions cannot bind to electronegative surface functional groups found on gram-negative envelopes (McLean and Beveridge, 2001). The main elements co-localized with chromium were the typical biological elements (Niftrik *et al*., 2008); carbon, oxygen, nitrogen, phosphorus and sulphur (Table 4.2).

Table 4.2: Contents of elements in *Acinetobacter haemolyticus* grown on LB agar without Cr(VI) and with 30 mg L^{-1} Cr(VI).

Element	Cr weight based on EDX analysis (%)	
	without Cr(VI)	**with Cr(VI)**
C K	37.49	27.96
N K	7.30	2.14
O K	13.32	12.65
P K	3.32	4.82
S K	0.69	1.12
Cr L	-	0.19
Total	100	100

4.3.2 TEM analysis

To locate the intracellular accumulation of chromium, TEM analysis was carried out. TEM was performed on *A. haemolyticus* cells grown on LB agar containing 30 mg L^{-1} of Cr(VI) and cells grown without the presence of Cr(VI) acted as control.

The thin sections of *A. haemolyticus* grown without Cr(VI) (Figure 4.5A, C) also appeared as coccobacilli with inner and outer sections of the bacteria clearly discernible similar to the results obtained by Zakaria *et al*. (2007).

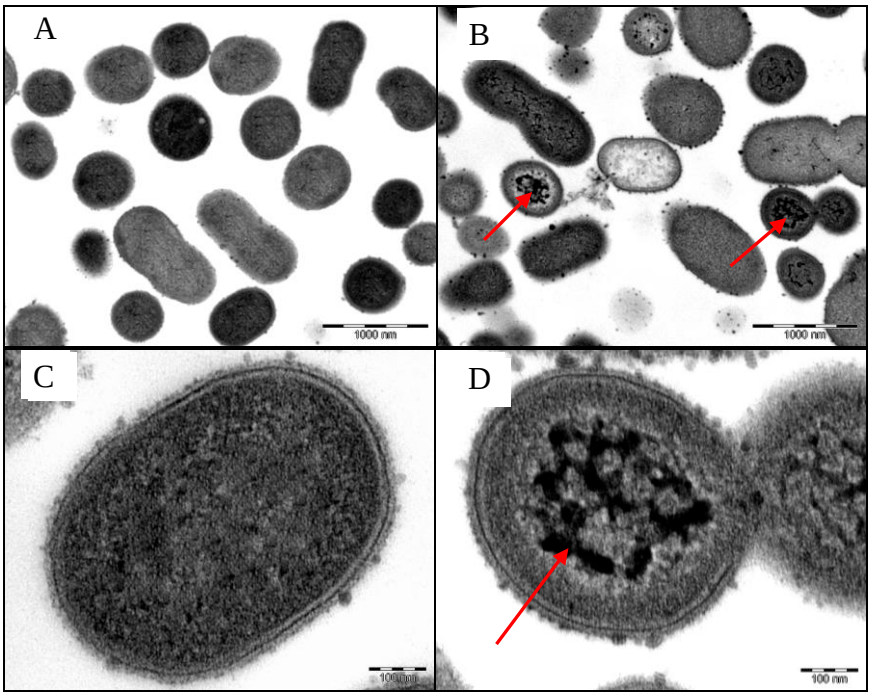


Figure 4.5: TEM images of thin sections of *Acinetobacter haemolyticus* cells grown (A, C) without Cr(VI) (control) and (B, D) with 30 mg L^{-1} of Cr(VI). Arrows in (B, D) indicates electron-opaque particles; magnification, (A, B) 21 k and (C, D) 110 k.

The TEM images of *A. haemolyticus* grown on LB agar with Cr(VI) (Figure 4.5B, D) revealed the presence of electron opaque particles that were not found in the

thin sections of *A. haemolyticus* grown on LB agar without Cr(VI). The electron-opaque particles were located in the cytoplasm and less on the cell envelopes. However, it was observed that the precipitates were not uniformly distributed in the cell. Similar observation was found in *Pseudomonas aeruginosa* samples where one cell may have bound large amounts of lanthanum (La) while an adjacent cell had little or none. There may be physiological reasons why individual cells within a culture take up metals differently (Mullen *et al*., 1989).

There are examples of bacterial studies using TEM where electron-opaque particles were observed in the cell cytoplasm of cultures grown in media supplemented with Cr(VI). Strain MR-4 cells grown in Shewanella medium (SM) with 100 μM Cr(VI) and 30 mM Tris or Hepes illustrated the extensive deposition of $Cr(OH)_3$ precipitates both intracellularly and extracellularly in the case of Hepes but showed no evidence of precipitation in the presence of Tris. The deposition of $Cr(OH)_3$ around the periphery of the cell in the Hepes case is assumed to be due to the nearby reduction of Cr(VI) and subsequent precipitation of Cr(III). The intracellular localization of $Cr(OH)_3$ suggests that the fraction of the Cr(III) produced does not precipitate extracellularly. Cr(VI) first enters the cells through the cellular membrane and reduced to Cr(III) in the cytoplasm. Cr(III) is impermeable to biological membranes. Hence, Cr(III) generated inside the cell binds to protein and interacts with nucleic acids (Daulton *et al*., 2001).

TEM examination on the effect of Cr(VI) on *Acinetobacter* sp. (PCP3 strain) by Srivastava and Thakur (2007) revealed circular electron dense precipitates within the cell cytoplasm. The biosorbed Cr(VI) was assumed to be Cr(III), as Cr(VI) is reduced to Cr(III) in the living cells due to reducing environment and enzymes present inside the cell (Srivastava and Thakur, 2007). Cr(III) is then free to bind to ionic sites and once bound, will act as a template for further heterogeneous nucleation and crystal growth. The result of this study indicated the penetration of chromium into the cells and the chromium accumulation within the cells as precipitates. This could be due to precipitation of Cr(III) in the form of hydroxyl and carboxyl group (McLean and Beveridge, 2001). Unfortunately, it was hypothesized by Bencheikh-Latmani *et al*. (2007) that intracellular precipitation of Cr(III) (as $Cr(OH)_3$) is responsible for the deleterious effect of Cr(III) on growth of *Shewanella*

sp. strain MR-4. Apparently, Cr(VI) as the chromate ion is not, in itself, a toxic species because it has no effect on cell growth prior to the onset of reduction.

In this study, it is worth noting that the electron-opaque particles found in cells grown on LB agar with Cr(VI) appeared to be dense and located mostly in the cytoplasm unlike that reported by Zakaria *et al*. (2007) where only fine particles were observed in *A. haemolyticus* grown in nutrient broth with 50 mg L^{-1} of Cr(VI). This probably implies that the state of growth medium (solid or liquid) has an effect on the uptake of Cr(VI) into the cells where the uptake may be higher on solid medium. This is probably the reason that *A. haemolyticus* exhibited higher tolerance towards Cr(VI) in LB broth compared to LB agar at 90 and 30 mg L^{-1}, respectively as described in 2.3.2.

4.3.3 FTIR analysis

Based on the results from FESEM-EDX analysis, chromium was found to be sequestered on the cell surface via adsorption. Chromium found on the cell surface could be the result of surface complexation. Thus, FTIR analysis was carried out to investigate the surface functional groups involved in the adsorption of chromium.

The FTIR spectra of *A. haemolyticus* grown in LB broth without Cr(VI) and with Cr(VI) at 30, 60 and 100 mg L^{-1} were taken in the range of 400-4000 cm^{-1}. The spectrum of the biomass (Figure 4.6) display a number of absorption peaks, reflecting the complex nature of the biomass.

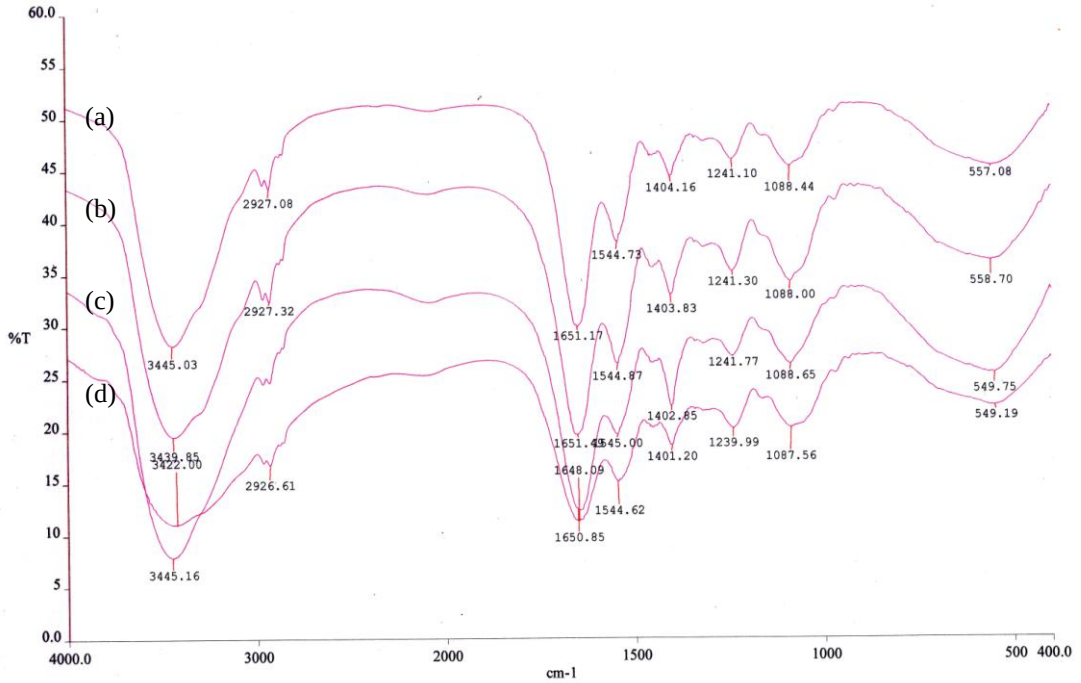


Figure 4.6: FTIR spectra of *Acinetobacter haemolyticus* cells grown (a) without Cr(VI) (control), and with (b) 30, (c) 60, (d) 100 mg L^{-1} of Cr(VI).

The infrared absorption frequencies of each peak and the corresponding functional groups of *A. haemolyticus* (without chromium) (Figure 4.6(a)) are as displayed in Table 4.3. FTIR spectrum of *A. haemolyticus* grown in LB broth without Cr(VI) exhibit the presence of amino, carboxyl, hydroxyl, and sulphonate groups. The broad absorption peak around 3450-3200 cm^{-1} is indicative of the existence of the -OH groups and the -NH groups (Park *et al*., 2005; Mungasavalli *et al*., 2007). In a report by Bai *et al*. (2002), the broad adsorption peak at 3400-3290 cm^{-1} represents -OH groups of the glucose and the -NH stretching of protein. The absorption peaks in the range 1540-1640 cm^{-1} can also be attributed to primary and secondary amide bands of the amide bond of protein peptide bonds which correspond to -NH bending. The appearance of strong band within 1100-1000 cm^{-1} is due to C-O bond, which is the characteristic peak for polysaccharides (Das and Guha, 2007). The small characteristic band region at 800-850 cm^{-1} suggests the presence of sulphonate (SO_2O^-) group on the cell surface (Das and Guha, 2007). Phosphate functional groups such as P=O, orthophosphate (PO_4^{3-}), and P-OH have characteristic absorption peaks at 1150, 1100-1030, and 1040-910 cm^{-1}, respectively. However, the

peaks occur around the same wavelength as C-N stretching which is between 1350-1000 cm^{-1} (Mungasavalli *et al*., 2007).

Table 4.3: Functional groups of *Acinetobacter haemolyticus* grown in LB broth without Cr(VI) and the corresponding infrared absorption wavelengths.

Observed wavelength (cm^{-1})	**Wavelength (cm^{-1})**	**Assignment**	**References**
3422	3500 – 3200 3500 - 3100	Bonded hydroxyl group Primary and secondary amines and amides stretching (N–H stretching)	Mungasavalli *et al*., 2007.
1651 1545	1670–1640 1546 1690–1640 1640–1550	C=O chelate stretching C-N stretching and N-H deformation Imines and oximes Primary and secondary amines and amides (N–H bending)	Park *et al*., 2005; Lameiras *et al*., 2008; Mungasavalli *et al*., 2007.
1451	1450	Asymmetric bending of the CH_3 of the acetyl moiety	Park *et al*., 2005.
1403 1241	1398 1238	COO- anions -SO_3 groups	Lameiras *et al*., 2008.
1089	1350–1000 1300–1000	Amines (C–N stretching) C-O stretching of COOH	Mungasavalli *et al*., 2007.

In the FTIR spectra of *A. haemolyticus* grown in LB broth with 30, 60 and 100 mg L^{-1} of Cr(VI) (Figure 4.6(b), (c), (d)), changes were observed in the region of 1655-750 cm^{-1} and 3450-2800 cm^{-1}. These changes indicated that there was a metal binding process taking place on the surface of the cells with certain functional groups (Bueno *et al.*, 2008). It is likely that the oxidation of the biomass during Cr(VI) biosorption had resulted in changes in the absorption frequencies of the various functional groups present in the biomass (Park *et al*., 2005). The functional groups involved in the interaction with chromium were ionizable functional groups that

included amino, carboxyl, and hydroxyl groups (Table 4.4). These are reported to be able to interact with protons or metal ions (Bueno *et al*., 2008). Based on Table 4.4, the contribution of amino groups towards the interaction was evident. This is because the amino and carboxyl groups, and nitrogen and oxygen of the peptide bonds are available for coordination bonding with metal ions such as Cr(VI) (Pandi *et al*., 2007). In the spectra of chromium-treated cyanobacteria, significant alteration was observed for the characteristic peaks of $COOH^-$ group; thereby implying complexation of chromium with protein molecules in the species studied (Pandi *et al*., 2007). Furthermore, since carbohydrates, hexosamines, and proteins are the major cell wall constituents, the contributory role of amino groups in chromium binding could be assumed (Mungasavalli *et al*., 2007).

Table 4.4: Comparison of functional groups of *Acinetobacter haemolyticus* grown in LB broth with Cr(VI) and the corresponding infrared absorption wavelengths.

Wavelength (cm^{-1})				**Assignment**	**References**
Control	**Cells with Cr(VI) ($mg\ L^{-1}$)**				
	30	**60**	**100**		
3422	3445	3440	3445	Bonded hydroxyl group Primary and secondary amines and amides stretching (N–H stretching)	Mungasavalli *et al*., 2007.
1648	1650	1651	1651	C=O chelate stretching	Park *et al*., 2005.
1240	1242	1241	1241	SO_3 groups	Lameiras *et al*., 2008.
1088	1089	1088	1088	C-O stretching of COOH	Mungasavalli *et al*., 2007.

Based on the result from both the FTIR and FESEM-EDX analyses, chromium was suggested to have complexed with ionizable functional groups (amino, carboxyl, and hydroxyl groups) on the cell surface of *A. haemolyticus*.

4.3.4 XAFS analysis

The preceding results obtained from TEM, FESEM-EDX, and FTIR spectroscopy analyses could not be used to determine the final oxidation state of chromium. Therefore, further work was carried out using XAFS spectroscopy which is based on the absorption of high-energy monochromatic X-rays by an element in the characteristic absorption edge region, and supplies two useful spectra; XANES and EXAFS (Gardea-Torresdey *et al*., 2002). In this study, XANES analysis will be discussed in detail and supported with EXAFS studies. XANES spectroscopy was employed to provide direct evidence of the reduced chromium deposited in *A. haemolyticus* since the analysis involved slight manipulation of bacterial samples and could be used to accurately determine the valences of the target elements.

For XAFS analysis, *A. haemolyticus* cells were grown in LB broth to mid-exponential phase before addition of 60 mg L^{-1} of Cr(VI). The culture was harvested after 48 hours and one set of the cell suspensions was washed with PBS buffer. Each sample from both the washed and unwashed samples was air-dried to obtain the concentrated samples.

Figure 4.7 shows the XANES spectra of the concentrated and non-concentrated unwashed suspensions. Since both the spectra appeared to be similar and the concentrated sample yielded better absorption with less noise than the non-concentrated sample, only the concentrated suspensions were used for XANES analysis.

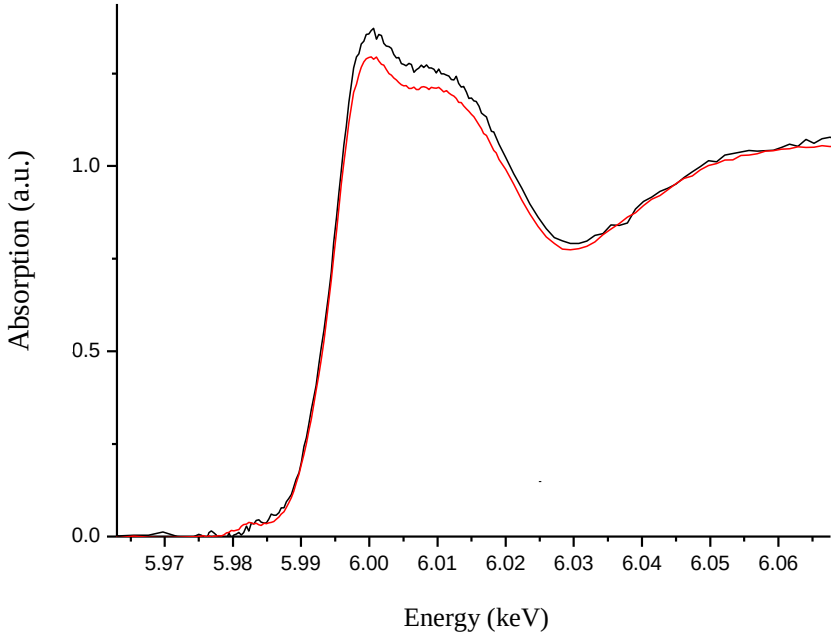


Figure 4.7: XANES spectra of chromium in unwashed concentrated (—) and non-concentrated (—) *Acinetobacter haemolyticus* cells.

The XANES spectra of chromium in concentrated *A. haemolyticus* cells, together with the XANES spectra of the reference compounds of Cr foil, Cr-acetate, $Cr(NO_3)_3$, $Cr(NO_3)_3$(aq), and $K_2Cr_2O_7$ is shown in Figure 4.8. The reason for originally choosing the five chromium compounds as references is as follows: Cr foil (Cr(0)), Cr-acetate, $Cr(NO_3)_3$, $Cr(NO_3)_3$(aq) (Cr(III)), and $K_2Cr_2O_7$ (Cr(VI)) to compare XANES spectra patterns according to the oxidation state of chromium, while Cr(III) references as Cr-acetate, $Cr(NO_3)_3$, and $Cr(NO_3)_3$(aq) were chosen to examine the effects of the element atom linked to the chromium atom in the XANES spectra. $K_2Cr_2O_7$ and Cr-acetate was used to determine the possibility of long range ordering of chromium in *A. haemolyticus*. In agreement to the spectra obtained by Park *et al*. (2008), the XANES data for Cr(VI) reference, $K_2Cr_2O_7$ show the well-defined Cr(VI) pre-edge peak characteristic at 5.984 keV (Figure 4.10). The pre-edge peak is attributed to the electron transition from chromium 1s orbital to 3d (Peterson *et al*., 1997). The lack of the pre-edge feature for any of the other reference compounds indicates that the chromium was in its reduced valence state, such as Cr(III). As seen in the XANES spectra, the absorption patterns of chromium reference compounds with different oxidation states are distinguishable from each

other. The XANES results of the Cr(III) reference compounds show a small pre-edge feature. The pre-edge feature is indicative of Cr(III) bound to oxygen ligands in an octahedral arrangement of atoms. The small pre-edge features are present for octahedral Cr(III) at 5990.5 and 5993.5 eV due to 1s to 3d (t_{2g}) and 1s to 3d (e_g) electronic transitions, respectively (Peterson *et al*., 1997).

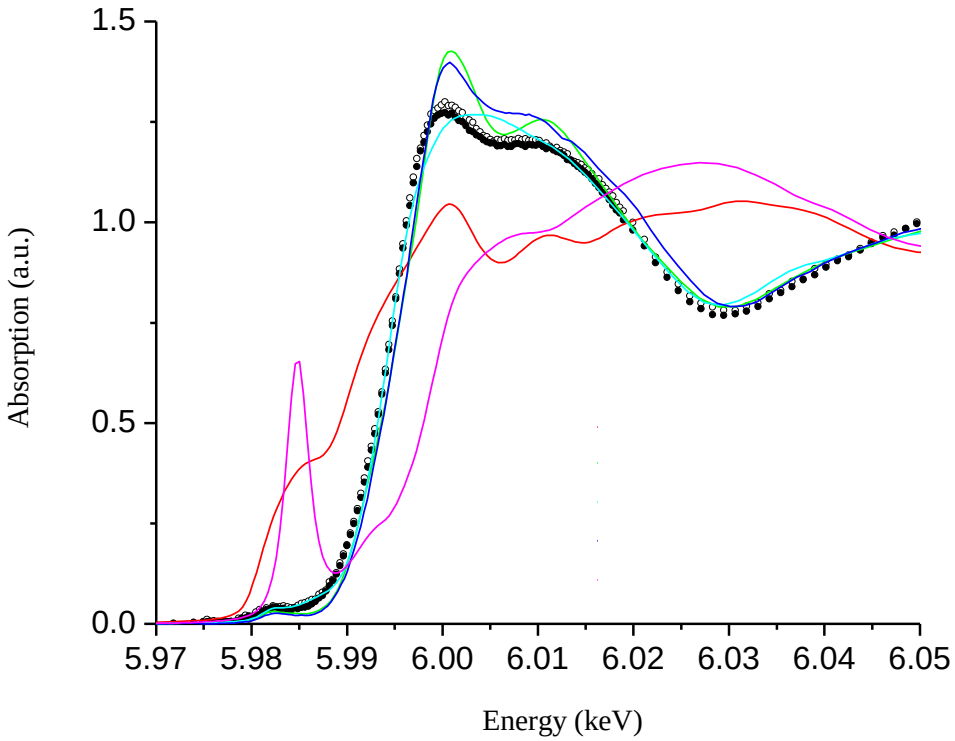


Figure 4.8: XANES spectra at chromium K-edge in unwashed (○) and washed (●) *Acinetobacter haemolyticus* cells, Cr foil (—), $Cr(NO_3)_3$ (—),$Cr(NO_3)_3$(aq) (—), Cr-acetate (—), and $K_2Cr_2O_7$ (—) standards.

The results presented in Figure 4.8 indicate that the XANES spectra of the bacterial biomass were identical to that of the Cr(III) reference compounds and the absence of a well-defined pre-edge peak at 5.984 keV and the chemical shift demonstrate that only octahedral-coordinated Cr(III) existed in the *A. haemolyticus* cells. Both bacterial biomass (washed and unwashed) showed no evidence of an absorption peak for Cr(VI) from the starting $K_2Cr_2O_7$ in the growth medium despite of incomplete reduction of Cr(VI) initially added into the medium. The chromium in

the bacterial biomass also did not correlate to the spectrum of Cr foil. Based on the results obtained, XANES was successfully and directly employed to determine the oxidation state of chromium in *A. haemolyticus* cells after growth in LB broth supplemented with 60 mg L^{-1} of Cr(VI). The reduction in the oxidation state of Cr(VI) reacted with biomass has been previously observed in the Cr K-edge XANES for a number of different biomaterials such as hops (Parsons *et al*., 2002), native microbial community (Oliver *et al*., 2003), saltbush (*Atriplex canescens*) (Sawalha *et al*., 2005), and brown seaweed (*Ecklonia*) (Park *et al*., 2008). Interestingly, the washed and unwashed *A. haemolyticus* biomasses appeared to be similar and thus, reflecting the strong adsorption/absorption of chromium onto the cell surface or intracellularly despite washes with PBS buffer which served to strip any adventitiously bound chromium ions.

To determine the geometry of the chromium complex, the chromium pre-edge features of the biomasses and reference compounds were enlarged as shown in Figure 4.9. The geometry of the complex formed between Cr(III) and bacterial cells can be determined if identical or very similar to that of one of the model compounds. The spectra of chromium in the *A. haemolyticus* biomasses closely matched that of Cr-acetate showing their almost similar molecular geometries. Cr-acetate has multi-nuclear geometry in aqueous solution; the nearest neighbours for Cr(III) are 5 oxygen atoms either from acetate COO^- or from water molecules (Gardea-Torresdey *et al*., 2002). Therefore, the structure may be in an octahedral or pentahedral arrangement. At this local structure, the pre-edge peaks of *A. haemolyticus* biomasses and Cr-acetate show a slightly enhanced intensity compared to octahedrally coordinated geometry as in $Cr(NO_3)_3$ and $Cr(NO_3)_3.4H_2O$. Thus, the pre-edge spectra of the reference compounds, $Cr(NO_3)_3$ and $Cr(NO_3)_3.4H_2O$ were different from those of the *A. haemolyticus* biomasses and Cr-acetate. This implies that the chromium was not bound to the nitrogen atom of amino and nitrile groups (Park *et al*., 2008). The chromium in $Cr(NO_3)_3.4H_2O$ in aqueous state complexes with H_2O and NO_3 as neighbours to form highly symmetrical complexes. The difference in the spectra indicates that the Cr(III) complexes formed in the biomasses are in a non-centro-symmetric geometry.

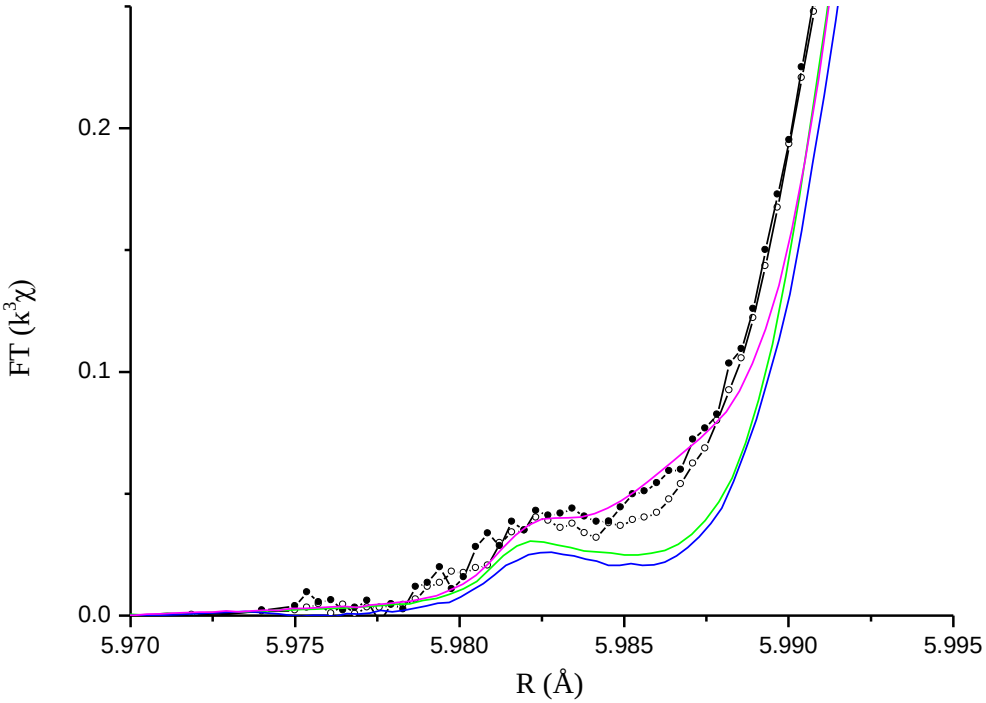


Figure 4.9: Pre-edge spectra of XANES at chromium K-edge in unwashed (○) and washed (●) *Acinetobacter haemolyticus* cells, $Cr(NO_3)_3$ (—),$Cr(NO_3)_3$(aq) (—), and Cr-acetate (—) standards.

Figure 4.10 shows the Fourier-transformed EXAFS (FT-EXAFS) spectra of the chromium reference compounds as well as the *A. haemolyticus* biomass to determine the neighbouring atoms of Cr(III) in *A. haemolyticus* biomass. The FT-EXAFS oscillations for the biomass was also almost similar to Cr-acetate which corroborates the result (Figure 4.8) mentioned earlier that Cr(III) was bound to oxygen ligands (Gardea-Torresdey *et al*., 2002).

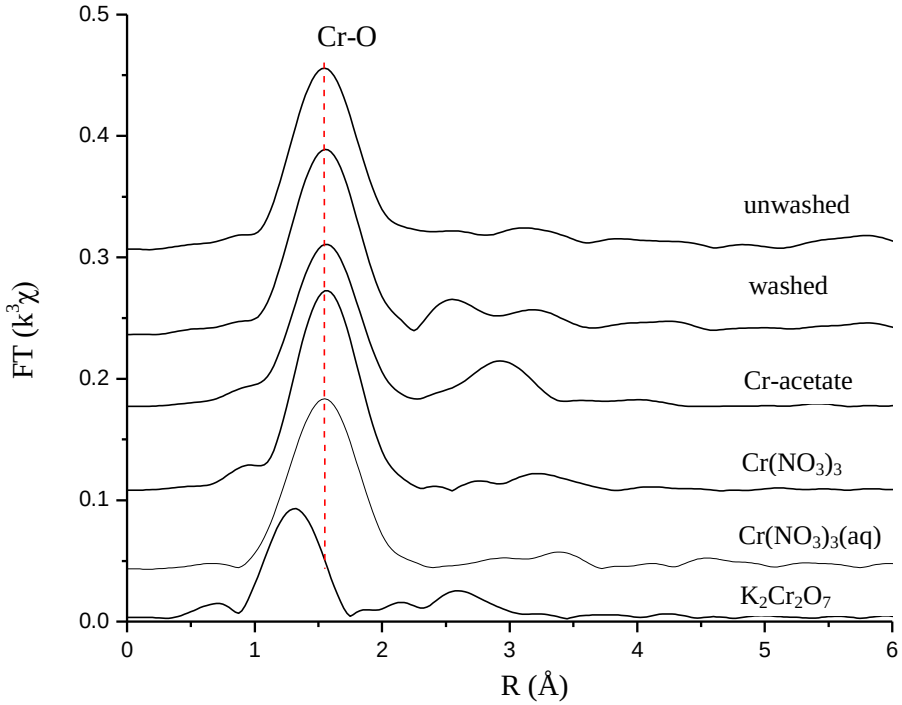


Figure 4.10: Fourier-transform spectra of chromium in unwashed and washed *Acinetobacter haemolyticus* cells, Cr-acetate, $Cr(NO_3)_3$, $Cr(NO_3)_3$(aq), and $K_2Cr_2O_7$ standards.

Figure 4.11 demonstrates that the first-shell peak of each sample is satisfactorily fitted. The bonding distances of the chromium oxygen atoms in the biomass was approximately the same lengths (Table 4.5) based on the FEFF fittings of the backtransformed EXAFS of the chromium in the biomass and Cr(III) reference compounds, respectively; 1.98 Å for both Cr(III) reference compounds ($Cr(NO_3)_3.4H_2O$ and Cr-acetate) whereas, 1.97-1.98 Å for the washed and unwashed biomass. The longer Cr-O interatomic distance in the biomass reveals that the chromium in these samples is in the form of Cr(III). The fact that the oxidation state associated with Cr(VI) is greater than that with Cr(III) results in the shorter first-shell interatomic distance because of the stronger attractive force between Cr(VI) and its neighbouring oxygen (Wei *et al*., 2007). The interatomic distance of Cr(III) and oxygen reported is between 1.96-1.99 Å (Park *et al*., 2008; Gardea-Torresdey *et al*., 2000; Parsons *et al*., 2002) whereas for Cr(VI) and oxygen is between 1.64-1.67 Å (Parsons *et al*., 2002; Wei *et al*., 2007; Sawalha *et al*., 2005).

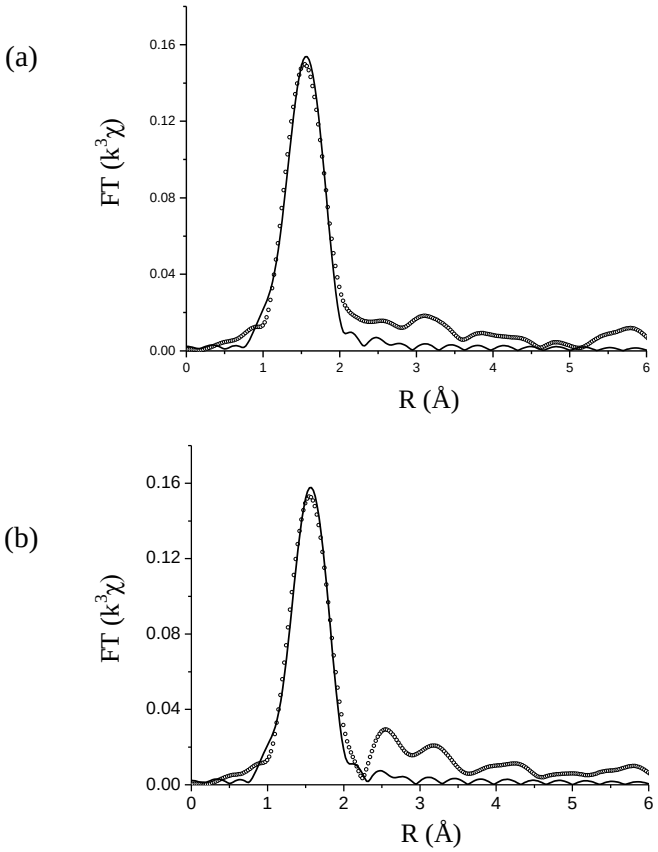


Figure 4.11: FEFF fittings of the Fourier transforms of EXAFS of the chromium in (a) unwashed, and (b) washed *Acinetobacter haemolyticus* cells (○) with Cr-O (—).

Table 4.5 also shows the coordination numbers (CN) of the Cr(III) bound to the bacterial biomass which is almost similar to that of the reference compounds. In a study by Parsons *et al*. (2002) using hops biomasses, the CN may be determined by the availability or the orientation of the free oxygen ligands on the biomass. The orientation and availability of free oxygen atoms on the biomass may also slightly change the geometry of the complex formed. The CN values also support an octahedral coordination of Cr-O in adsorbed samples. However, these Cr-O bonds are dispersive and very different from the perfect octahedral geometry. Their spatial configurations are not as perfect as the octahedral geometry in $Cr(NO_3)_3$. It is therefore deduced that Cr-O in the biomass with Cr may be partially from water molecules and partially from ligands of biomass.

Table 4.5: FEFF fittings of chromium in *Acinetobacter haemolyticus* grown in LB broth with 60 mg L^{-1} of Cr(VI).

Sample	**Bond**	**CN**[a]	**R(Å)**	**σ^2(Å^2)**
Washed biomass	Cr-O	5.6	1.97	0.0018
Unwashed biomass	Cr-O	6.0	1.98	0.0027
$Cr(NO_3)_3$ (aq) (Reference compound)	Cr-O	5.7	1.98	0.0024
Cr-acetate (Reference compound)	Cr-O	5.4	1.98	0.0030

[a] CN represents the coordination number (number of neighbouring atoms), R is the interatomic distances given in angstroms, and σ^2 is the Debye-Waller factor given in angstroms squared.

In addition, the fitting results (Table 4.5) show only Cr(III) was present and that Cr(III) was neighbouring with oxygen ligands. The similarity of the biomass spectra to Cr-acetate spectra indicates that the Cr(III) was bound to more oxygen from bacteria than H_2O as in the case of Cr-acetate (Liu, personal communication, 2008). Since the XAFS analysis suggests that the neighbouring atoms of Cr(III) is O and that Cr(III) coordinates less with H_2O, it is suggested that Cr(III) formed complexes with the carboxyl (COO^-) group from *A. haemolyticus*. FTIR studies (as discussed in 4.3.3) have shown that the functional groups that interact with chromium are carboxyl, amino, and hydroxyl groups which correlate with the elements found to co-localize with chromium on the cell surface via EDX elemental analysis (as discussed in 4.3.1). However, the XAFS spectrum taken from a sample reflects the average local environment of all probed atoms in it (Boyanov *et al*., 2003). Thus, to be able to separate, identify, and quantify the binding mechanism of chromium in the multiple binding-site case of the biomass requires a clear understanding of the isolated contributions in the spectra from all potential ligands (intra and extracellularly). The interactions of chromium with the possible ligands will not be discussed here as the main objective of this study is to obtain the oxidation state of chromium and the local coordination environment of the element in *A. haemolyticus* biomass.

4.3.5 Chromium(VI) reduction by crude CFE

The TEM analysis in section 4.3.2 showed that chromium precipitates were localized intracellularly (cytoplasmic area). However, it is unknown whether the reduction of Cr(VI) was intra- or extracellular. It is unclear whether cellular uptake of toxic Cr(VI) occurs with reduction localized to the cytoplasm or periplasm, and/or electrons are transferred outside of the cells to reduce chromium extracellularly. Thus, to determine the localization of Cr(VI) reduction by *A. haemolyticus*, the supernatant from the growth medium, and the crude CFE from late-exponential phase culture was used for Cr(VI) reductase assay.

Cr(VI) reductase assay was carried out on supernatants from *A. haemolyticus* grown in LB broth to determine if active enzyme was released from the cell into the culture medium. This could be through secretion or through cell lysis (McLean and Beveridge, 2001). The controls used consisted of LB broth only and autoclaved crude CFE. The controls and samples added with 5 mg L^{-1} of Cr(VI) were withdrawn at 0, 4^{th} and 8^{th} hour for residual Cr(VI) measurements.

Crude CFE was also used to measure the Cr(VI) reductase activity. The specific activity obtained by crude CFE of *A. haemolyticus* was 0.52 µg Cr(VI) reduced mg of $protein^{-1}$ h^{-1} at pH 7.2 and 37˚C. Table 4.6 shows the specific activity of cell fractions in other Cr(VI)-reducing microorganisms. The specific activity by cell fractions of other microorganisms were relatively higher than *A. haemolyticus* except *Brucella* sp.

Table 4.6: Specific activity of cell fractions in Cr(VI)-reducing microorganisms.

Microorganism	Fraction	Condition	Specific activity (µg Cr(VI) reduced mg of protein^{-1} h^{-1})	References
Brucella sp.	Cell-free extract	pH 7.2, 37˚C	0.052	Thacker *et al*., 2007.
Providencia sp.	Cell-free extract	pH 7.2, 37˚C	3.68	Thacker *et al*., 2006.
Pseudomonad (CRB5)	Crude soluble	Not stated	980	McLean and Beveridge, 2001.

Table 4.7 shows the percentage of Cr(VI) reduction with time for both the supernatants and crude CFE (relative to LB broth only and the autoclaved crude CFE, respectively).

Table 4.7: Percentage reduction of Cr(VI) in supernatant and crude cell-free extracts from *Acinetobacter haemolyticus*.

% Cr(VI) reduction / Hour	Supernatant	Crude cell-free extracts
0	0	11
4	0	45
8	0	64

Based on the results, Cr(VI) reduction activity was evident in the crude CFE of *A. haemolyticus*. Approximately 64% (relative to the Cr(VI) concentration in autoclaved crude CFE) of a 5 mg L^{-1} of Cr(VI) was reduced at the 8th hour of incubation under aerobic condition. Essentially, very low Cr(VI) reductase activity was obtained in heat-denatured crude CFE (via autoclaving). The supernatant after

harvesting the cells also showed no reduction of Cr(VI) (relative to LB broth). This implies that the Cr(VI) reductase activity is associated with the soluble fraction of the cells and not extracellular (Thacker *et al*., 2006). Similar results were obtained by McLean and Beveridge (2001) where Cr(VI) reduction by a Pseudomonad (CRB5) was largely contained in the soluble fractions. Since reduction is associated with the soluble fraction, the enzyme responsible could be either cytoplasmic or periplasmic in origin (McLean and Beveridge, 2001).

As mentioned earlier, Cr(VI) reduction under aerobic condition is commonly associated with soluble chromate reductases that use NADH or NADPH as cofactors (Cervantes and Campos-García, 2007). There are reports where Cr(VI) reduction activity is enhanced with the addition of external electron donors such as NADH and lactate. The addition of NADH enhanced the reduction of Cr(VI) by CFE of *Providencia* sp. (Thacker *et al*., 2006), *Pseudomonas ambigua* G-1 (Suzuki *et al*., 1992), and *E. coli* ATCC 33456 (Shen and Wang, 1993). In a study by Viamajala *et al*. (2007), *Cellulomonas* sp. ES6 was found to be able to reduce Cr(VI) without the addition of lactate, which suggests that *Cellulomonas* sp. ES6 can use endogenous electron donors for Cr(VI) reduction. Since the CFE of *A. haemolyticus* also showed substantial Cr(VI) reduction without the addition of external electron donors, it is suggested that *A. haemolyticus* can also use endogenous electron donors for Cr(VI) reduction.

4.4 Conclusion

XAFS studies confirmed that Cr(VI) is reduced to Cr(III) and subsequently the reduced chromium is bound to an oxygen-containing ligand in an octahedral geometry. Based on FTIR analysis, the ligand was deduced to be most likely a carboxyl group. Other groups on the cell surface of *A. haemolyticus* that were involved in the complexation with chromium were amino and hydroxyl. Results from the FESEM-EDX analysis confirmed the presence of chromium element on the cell surface of *A. haemolyticus* cells grown in LB broth with Cr(VI). The cells were also found to be enlarged with wrinkles on the cell surfaces. TEM analysis revealed the presence of electron-opaque particles in the cell cytoplasmic area, suggested to be

chromium precipitates. Based on the Cr(VI) reductase test using crude CFE of *A. haemolyticus*, Cr(VI) reductase activity was found to be associated with the intracellular fraction of the cells. The specific activity obtained by crude CFE of *A. haemolyticus* was 0.52 µg Cr(VI) reduced mg of protein^{-1} h^{-1} at pH 7.2 and 37˚C.

CHAPTER V

CONCLUSION

5.1 Conclusion

Chromium occurs mainly as a result of human activities through production of waste water in metal smelting, electroplating, tanning, metallurgy and dyestuff industries. Thus, effective remedial processes are necessary to overcome the mutagenic and carcinogenic properties of Cr(VI). Bioremediation has already proven itself to be a cost-effective and beneficial addition to chemical and physical methods of managing wastes and environmental pollutants. Implementation of these techniques requires knowledge of the mechanisms involved in Cr(VI) detoxification by the microorganism involved. The present study provides a greater understanding of Cr(VI) resistance and reduction by *A. haemolyticus*, a locally-isolated strain from Cr(VI)-containing wastewater of a textile-related manufacturing premise.

In preliminary studies (Chapter II), the growth of *A. haemolyticus* in the presence of Cr(VI) was observed. This was followed by evaluation on the ability of *A. haemolyticus* to tolerate and reduce Cr(VI). The results from the study showed that *A. haemolyticus* demonstrated good growth in the presence of 30 and 60 mg L^{-1} of Cr(VI) as compared to without Cr(VI). *A. haemolyticus* was found to be tolerant towards 30 and 90 mg L^{-1} of Cr(VI) in LB agar and broth, respectively. The Cr(VI) resistance of *A. haemolyticus* could also be partly due to the capacity of this strain to reduce Cr(VI) to the less toxic Cr(III). The reducing capacity was found to depend on the growth medium, initial concentration of Cr(VI) and time of Cr(VI) inoculation. *A.*

haemolyticus displayed greater Cr(VI) reduction in rich medium (LB broth) and with lower initial concentrations of Cr(VI) (30 mg L^{-1}) added after 5 hours of growth.

In Chapter III, *A. haemolyticus* was screened for the presence of plasmid and restriction enzyme digestions were carried out. *A. haemolyticus* was found to harbour a 12 kb-plasmid. The restriction enzyme, *Pst*I was found to be able to digest the plasmid from *A. haemolyticus.* Thus, it is possible for a gene homologous to *chr*R (encoding chromate reductase from *P. ambigua*) (Suzuki *et al*., 1992) to be present in the plasmid. However, the role of the plasmid in Cr(VI)-resistance could not be determined as attempts to define the role of the plasmid in ampicillin resistance and to cure the plasmid were unsuccessful. It is suggested that in order to characterize the Cr(VI) reductase in *A. haemolyticus*, cells can be grown with Cr(VI) at varying concentrations and without Cr(VI) to compare the inducibility of Cr(VI) reductase. This is followed by the extraction of the soluble fraction by subcellular fractionation and checking for nucleotide homology with the *Pseudomonas putida* chromate reductase gene (Park *et al*., 2000).

The mechanisms of Cr(VI) resistance by *A. haemolyticus* were investigated via instrumental analyses as discussed in Chapter IV. The studies included the use of FESEM-EDX, TEM, FTIR, XAFS spectroscopic techniques and Cr(VI) reductase assay. XAFS analysis confirmed the ability of *A. haemolyticus* to reduce Cr(VI) to Cr(III). The results which comprised of XANES and EXAFS analyses showed that only Cr(III) bound to oxygen from the biomass in an octahedral geometry was present. It was deduced that Cr(III) was most likely bonded to a carboxyl group from the biomass based on the FTIR analysis. The FTIR analysis also indicated interaction of chromium with other ionizable functional groups (amino and hydroxyl) on the cell surface of *A. haemolyticus*. The FESEM-EDX analysis showed that chromium element was present on the surface of *A. haemolyticus* cells grown in LB broth with Cr(VI). The cells grown in the presence of Cr(VI) appeared to be enlarged and wrinkled. No precipitates were found on the cell surface. However, electron-opaque particles, suggested to be chromium precipitates, were observed in the cell cytoplasmic area via TEM analysis. This suggests that the Cr(VI) was transported into the cytoplasm, and reduced intracellularly. The Cr(VI) reductase test using crude CFE of *A. haemolyticus* showed that the Cr(VI) reductase activity was associated

with the intracellular fraction of the cells. The specific activity obtained by crude CFE of *A. haemolyticus* was 0.52 µg Cr(VI) reduced mg of protein^{-1} h^{-1} at pH 7.2 and 37˚C. In order to obtain higher specific reductase activity, the optimization of conditions for the Cr(VI) reductase assay and purification of the crude CFE may be attempted in future research.

Despite Cr(VI) toxic stresses, *A. haemolyticus* was found to have evolved resistance mechanisms towards Cr(VI) which included extracellular and intracellular sequestration, binding to cell surface, and enzymatic reduction of Cr(VI) to Cr(III). *A. haemolyticus* can be used as a promising microorganism for Cr(VI) reduction from industrial wastewaters.

5.2 Suggestions for future work

Based on the work carried out in this thesis, *A. haemolyticus* was found to exhibit resistance towards Cr(VI) by various mechanisms. However, further studies can be carried out to gain more details on the resistance and reduction of Cr(VI) by *A. haemolyticus*.

Polymerase chain reaction (PCR) analysis can be carried out to determine if the *chr*R gene found in *Pseudomonas ambigua* (Suzuki *et al*., 1992) may be present in the plasmid from *A. haemolyticus*. The possibility of the Cr(VI) resistance and reduction determinants to be found in the chromosomes can also be investigated.

In the present study, the mechanisms of Cr(VI) resistance by *A. haemolyticus* was investigated by instrumental techniques such as XAFS, FTIR, FESEM-EDX, and TEM. These techniques employed only provided information based on bulk analyses. Specific analysis like electron energy loss spectroscopy (EELS) coupled to TEM could be used. The mechanisms of Cr(VI) reduction and precipitate formation by *Shewanella oneidensis* under anaerobic conditions was investigated using a newly developed TEM-EELS techniques (Daulton *et al*., 2007). TEM-EELS can determine the oxidation state of chromium over a wide valence range (Cr(0)-Cr(VI)) at high-spatial resolution (Daulton and Little, 2006). This is accomplished by comparing the

fine structure of Cr-$L_{2,3}$ adsorption edges of unknowns to the measured correlation between parameterized L-edge fine structure and valence for chromium standards with varied chemistry. Thus, for future work, TEM-EELS can be used to directly determine the localization of Cr(VI) reduction and determine the oxidation states of the reduced chromium in *A. haemolyticus*.

REFERENCES

Abboud, R., Popa, R., Souza-Egipsy, V., Giometti, C.S., Tollaksen, S., Mosher, J.J., Findlay, R.H., and Nealson, K.H. 2005. Low-Temperature Growth of *Shewanella oneidensis* MR-1. *Applied and Environmental Microbiology*, 71, 811-816. American Society for Microbiology.

Abdel-El-Haleem, D. (2003). Minireview: *Acinetobacter*: Environmental and Biotechnological Applications. *African Journal of Biotechnology*, 2, 71-74. Academic Journals.

Abou-Shanab, R.A.I., Berkum, P., and Angle, J.S. (2007). Heavy Metal Resistance and Genotypic Analysis of Metal Resistance Genes in Gram-positive and Gram-negative Bacteria Present in Ni-rich Serpentine Soil and in the Rhizosphere of *Alyssum murale*. *Chemosphere*, 68, 360-367. Elsevier.

Ackerley, D. F., Barak, Y., Lynch, S. V., Curtin, J., and Matin, A. (2006). Effect of Chromate Stress on *Escherichia coli* K-12. *Journal of Bacteriology*, 188, 3371-3381. American Society for Microbiology.

Ackerley, D. F., Gonzalez, C. F., Park, C. H., Blake, R., Keyhan, M., and Matin, A. (2004a). Chromate-Reducing Properties of Soluble Flavoproteins from *Pseudomonas putida* and *Escherichia coli*. *Applied Environmental Microbiology*, 70, 873-882. American Society for Microbiology.

Ackerley, D. F., Gonzalez, C. F., Park, C. H., Blake, R., Keyhan,, M., and Matin, A. (2004b). Mechanism of Chromate Reduction by the *Escherichia coli* Protein, NfsA, and The Role of Different Chromate Reductases in Minimizing

Oxidative Stress during Chromate Reduction. *Environmental Microbiology*, 6, 851–860. Blackwell Publishing.

Acquaah, G. (2004). *Understanding Biotechnology: An Integrated and Cyber-Based Approach.* (1st ed.). New Jersey: Pearson Education, Inc. 294-295.

Akinbowale, O.L., Peng, H., Grant, P., and Barton, M.D. (2007). Short Communication: Antibiotic and Heavy Metal Resistance in Motile Aeromonads and Pseudomonads from Rainbow Trout (*Oncorhynchus mykiss*) Farms in Australia. *International Journal of Antimicrobial Agents*, 30, 177–182. Elsevier.

Aleem, A., Isar, J., and Malik, A. (2003). Impact of Long-term Application of Industrial Wastewater on the Emergence of Resistance Traits in *Azotobacter chroococcum* Isolated from Rhizospheric Soil. *Bioresource Technology*, 86, 7–13. Elsevier.

Alonso, A., Sánchez, P., and Martínez, J.L. (2001). Environmental Selection of Antibiotic Resistance Genes. *Environmental Microbiology*, 3, 1-9. Blackwell Science.

Amoozegar, M.A., Ghasemi, A., Razavi, M.R., and Naddaf, S. (2007). Short Communication: Evaluation of Hexavalent Chromium Reduction by Chromate-Resistant Moderately Halophile, *Nesterenkonia* sp. Strain MF2. *Process Biochemistry*, 42, 1475-1479. Elsevier.

Andrade, L.R., Farina, M., and Filho, G.M.A. (2004). Effects of Copper on *Enteromorpha flexuosa* (Chlorophyta) in vitro. *Ecotoxicology and Environmental Safety*, 58, 117-125. Academic Press.

Angle, J.S., and Chaney, R.L. (1989). Cadmium Resistance Screening in Nitrilotriacetate-buffered Minimal Media. *Applied and Environmental Microbiology*, 55, 2101-2104. American Society for Microbiology.

Bai, R.S., and Abraham, T.E. (2002). Studies on Enhancement of Cr(VI) Biosorption by Chemically Modified Biomass of *Rhizopus nigricans*. *Water Research*, 36, 1224-1236. Pergamon.

Barak, Y., Ackerley, D.F., Dodge, C.J., Banwari, L., Alex, C., Francis, A.J., and Matin, A. (2006). Analysis of Novel Soluble Chromate and Uranyl Reductases and Generation of an Improved Enzyme by Directed Evolution. *Applied and Environmental Microbiology*, 72, 7074–7082. American Society for Microbiology.

Barton, B.M., Harding, G.P., and Zuccarelli, A.J. (1995). A General Method for Detecting and Sizing Large Plasmids. *Analytical Biochemistry*, 226, 235-240. Academic Press.

Basu, M., Bhattacharya, S., and Paul, A.K. (1997). Isolation and Characterization of Chromium-resistant Bacteria from Tannery Effluents. *Bulletin of Environmental Contamination and Toxicology*, 58, 535–542. Springer-Verlag New York.

Baumann, P. (1968). Isolation of *Acinetobacter* from Soil and Water. *Journal of Bacteriology*. 96, 39-42. American Society for Microbiology.

Bencheikh-Latmani, R., Obraztsova, A., Mackey, M., Ellisman, M., and Tebo, B. (2007). Toxicity of Cr(III) to *Shewanella* sp. Strain MR-4 during Cr(VI) Reduction. *Environmental Science and Technology*, 41, 214-220. American Chemical Society.

Birnboim, H.C., and Doly, J. (1979). A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA. *Nucleic Acid Research*, 7, 1513-1523. Information Retrieval Ltd.

Birnboim, H.C. (1983). A Rapid Alkaline Extraction Method for the Isolation of Plasmid DNA. *Methods in Enzymology*, 100, 243-255. Elsevier.

Bonaventura, C., and Johnson, F. M. (1997). Healthy Environments for Healthy People: Bioremediation Today and Tomorrow. *Environmental Health Perspectives*, 105, Supplement 1.

Bopp, L.H., Chakrababarty, A.M., and Ehrlich, H.L. (1983). Chromate Resistance Plasmid in *Pseudomonas fluorescens*. *Journal of Bacteriology*, 155, 1105-1109. American Society for Microbiology.

Boyanov, M.I., Kelly, S.D., Kemner, K.M., Bunker, B.A., Fein, J.B., and Fowles, D.A. (2003). Adsorption of Cadmium to *Bacillus subtilis* Bacterial Cell Walls: A pH-dependent X-ray Absorption Fine Structure Spectroscopy Study. *Geochimica et Cosmochimica Acta*, 67, 3299–3311. Elsevier.

Boyer, R. (2006). *Biochemistry Laboratory: Modern Theory and Techniques*. St. Francisco: Pearson Education.

Bruins, M.R., Kapil, S., and Oehme, F. W. (2003). Characterization of a Small Plasmid (pMBCP) from Bovine *Pseudomonas pickettii* that Confers Cadmium Resistance. *Ecotoxicology and Environmental Safety*, 54, 241–248. Elsevier.

Bueno, B.Y.M., Torem, M.L., Molina, F., and Mesquita, L.M.S. (2008). Biosorption of Lead(II), Chromium(III) and Copper(II) by *R. opacus*: Equilibrium and Kinetic Studies. *Minerals Engineering*, 21, 65-75. Elsevier.

Camargo, F.A.O., Okeke, B.C., Bento, F.M., Frankenberger, W.Y. (2005). Diversity of Chromium-resistant Bacteria Isolated from Soils Contaminated with Dichromate. *Applied Soil Ecology*, 29, 193–202. Elsevier.

Caravelli, A.H., Giannuzzi, L., and Zaritzky, N.E. (2008). Reduction of Hexavalent Chromium by *Sphaerotilus natans* a Filamentous Micro-organism Present in Activated Sludges. *Journal of Hazardous Materials*. 156, 214–222. Elsevier.

Cervantes, C., Campos-García, J., Devars, S., Gutiérrez-Corona, F., Loza-Tavera, H., Torres-Guzmán, J.C., and Moreno-Sánchez, R. (2001). Interactions of Chromium with Microorganisms and Plants. *FEMS Microbiology Reviews*, 25, 335-347. Elsevier.

Cervantes, C., and Campos-Garcia, J. (2007). *Reduction and Efflux of Chromate by Bacteria. Molecular Microbiology of Heavy Metals*. Berlin: Springer-Verlag.

Chapman, J.S. (2003). Disinfectant Resistance Mechanisms, Cross-resistance, and Co-resistance. *International Journal of Biodeterioration and Biodegradation*, 51, 271–276. Elsevier.

Chescoe, D., and Goodhew, P.J. (1990). *The Operation of Transmission and Scanning Electron Microscopes*. Oxford: Oxford University Press.

Cheung, K.H., and Gu, J-D. (2007). Mechanism of Hexavalent Chromium Detoxification by Microorganisms and Bioremediation Application Potential: A Review. *International Biodeterioration & Biodegradation*, 59, 8–15. Elsevier.

Cheung, K.H., and Gu, J-D. (2003). Reduction of Chromate (CrO_4^{2-}) by an Enrichment Consortium and an Isolate of Marine Sulfate-reducing Bacteria. *Chemosphere*, 52, 1523–1529. Pergamon.

Christian, G.D. (2004). *Analytical Chemistry*. (6^{th} ed.). USA: John Wiley and sons.

Clausen, C. A. (2000). Isolating Metal-tolerant Bacteria Capable of Removing Copper, Chromium, and Arsenic from Treated Wood. *Waste Management and Research*, 18, 264-268. ISWA.

Codd, R., Lay, P.A., Tsibakhashvili, N.Y., Kalabegishvili, T.L., Murusidze, I.G., Holman, H-Y.N. (2006). Chromium(V) Complexes Generated in *Arthrobacter oxydans* by Simulation Analysis of EPR Spectra. *Journal of Inorganic Biochemistry*, 100, 1827-1833. Elsevier.

Congeevaram, S., Dhanarani, S., Park, J., Dexilin, M., and Thamaraiselvi, K. (2007). Biosorption of Chromium and Nickel by Heavy Metal Resistant Fungal and Bacterial Isolates. *Journal of Hazardous Materials*, 146, 270–277. Elsevier.

Costa, S.F., Woodcock, J., Gill, M., Wise, R., Barone, A.A., Caiaffa, H., and Levin, A.S.S. (2000). Original Article: Outer-membrane Proteins Pattern and Detection of β-lactamases in Clinical Isolates of Imipenem-resistant *Acinetobacter baumannii* from Brazil. *International Journal of Antimicrobial Agents*, 13, 175–182. Elsevier.

Das, S.K., and Guha, A.K. (2007). Biosorption of Chromium by *Termitomyces clypeatus*. *Colloids and Surfaces B*: *Biointerfaces*, 60, 46–54. Elsevier.

Daulton, T.L., and Little, B.J. (2006). Determination of Chromium Valence Over the Range Cr(0)–Cr(VI) by Electron Energy Loss Spectroscopy. *Ultramicroscopy*, 106, 561–573. Elsevier.

Daulton, T.L., Little, B.J., Lowe, K., and Jones-Meehan, J. (2001). In-Situ Environmental Cell–Transmission Electron Microscopy Study of Microbial Reduction of Chromium(VI) Using Electron Energy Loss Spectroscopy. *Microscopy and Microanalysis*, 7, 470–485. Microscopy Society of America.

Daulton, T.L., Little, B.J., Jones-Meehan, J., Blom, D.A., and Allard, L.F. (2007). Microbial Reduction of Chromium from the Hexavalent to Divalent State. *Geochimica et Cosmochimica Acta*, 71, 556–565. Elsevier.

Dederich, D.A., Okwuonu, G., Garner, T., Denn, A., Sutton, A., Escotto, M., Martindale, A., Delgado, O., Muzny, D.M., Gibbs, R.A., and Metzker, M.L. (2002). Glass Bead Purification of Plasmid Template DNA for High Throughput Sequencing of Mammalian Genomes. *Nucleic Acids Research*, 30, e32. Oxford University Press.

Deshpande, L.M., and Chopade, B.A. (1994). Plasmid Mediated Silver Resistance in *Acinetobacter baumannii*. *Biometals*, 1, 49-56. Springer Netherlands.

Desjardin, V., Bayard, R., Lejeune, P., and Gourdon, R. (2003). Utilisation of Supernatants of Pure Cultures of *Streptomyces thermocarboxydus* NH50 to Reduce Chromium Toxicity and Mobility in Contaminated Soils, *Water, Air, and Soil Pollution: Focus,* 3, 153–160. Kluwer Academic Publishers.

Doble, M., and Kumar, A. (2005). *Biotreatment of Industrial Effluents*. United States of America: Elsevier Butterworth Heinemann.

Eccles, H. (1999). Biotopic: Treatment of Metal-contaminated Wastes: Why Select a Biological Process? *Tibtech December*, 17, 462-465. Elsevier.

Egerton, R.F. (2005). *Physical Principle of Electron Microscopy: An Introduction to TEM, SEM, and AEM*. United Sates of America: Springer Science + Business Media.

El-Mansi, M., Anderson, K.J., Inche, C.A., Knowles, L.K., and Platt, D.J. (2000). Isolation and Curing of the *Klebsiella pneumoniae* Large Indigenous Plasmid using Sodium Dodecyl Sulphate. *Research in Microbiology*, 151, 201–208. Elsevier.

Falagas, M.E., and Bliziotis, I.A. (2007). Pandrug-resistant Gram-negative Bacteria: The Dawn of the Post-antibiotic Era? *International Journal of Antimicrobial Agents*, 6, 630-636. Elsevier and International Society of Chemotheraphy.

Fiol, N., Escudero, C., and Villaescusa, I. (2008). Chromium Sorption and Cr(VI) Reduction to Cr(III) by Grape Stalks and Yohimbe Bark. *Bioresource Technology*, 99, 5030–5036. Elsevier.

Francisco, R., Alpoim, M.C., and Morais, P.V. (2002). Diversity of Chromium-resistant and -reducing Bacteria in a Chromium-contaminated Activated Sludge. *Journal of Applied Microbiology*, 92, 837–843. The Society for Applied Microbiology.

Fulladosa, E., Desjardin, V., Murat, J., Gourdon, R., and Villaescusa, I. (2006). Cr(VI) Reduction into Cr(III) as a Mechanism to Explain the Low Sensitivity of *Vibrio fischeri* Bioassay to Detect Chromium Pollution. *Chemosphere*, 65, 644-650. Elsevier.

Gardea-Torresdey, J.L., Dokken, K., Tiemann, K.J., Parsons, J.G., Ramos, J., Pingitore, N.E., and Gamez, G. (2002). Infrared and X-ray Absorption Spectroscopic Studies on the Mechanism of Chromium(III) Binding to Alfalfa Biomass. *Microchemical Journal,* 71, 157-166. Elsevier.

Ghosh, S., Mahapatra, N.R., Ramamurthy, T., and Banerjee, P.C. (2000). Plasmid Curing from an Acidophilic Bacterium of the Genus *Acidocella*. *FEMS Microbiology Letters*, 183, 271-274. Elsevier.

Gómez, V., and Callao, M.P. (2006). Chromium Determination and Speciation Since 2000. *Trends in Analytical Chemistry*, 25, 1006-1015. Elsevier.

Gonzalez, C.F., Ackerley, D.F., Lynch, S.V., and Matin, A. (2005). ChrR, a Soluble Quinone Reductase of *Pseudomonas putida* that Defends against H_2O_2. *The Journal of Biological Chemistry*, 280, 22590–22595. The American Society for Biochemistry and Molecular Biology.

Gonzalez, C.F., Ackerley, D.F., Park, C.H., Matin, A. (2003). A Soluble Flavoprotein Contributes to Chromate Reduction and Tolerance by *Pseudomonas putida*. *Acta biotechnology*, 2-3, 233-239. WILEY-VCH Verlag GmbH & Co.

Hall, J.L. (2002). Cellular Mechanisms for Heavy Metal Detoxification and Tolerance. *Journal of Experimental Botany*, 53, 1-11. Society for Experimental Biology.

Hardy, K. (1981). *Bacterial Plasmids*. (2nd ed.). England: Van Nostrand Reinhold (UK) Co. Ltd.

Hardy, K.G., and Kimber, G. (1993). *Plasmids: A Practical Approach*. Oxford: IRL Press.

Hassen, A., Saidi, N., Cherif, M., and Boudabous, A. (1998). Resistance of Environmental Bacteria to Heavy Metals. *Bioresource Technology,* 64, 7-15. Elsevier.

Hens, D.K., Niyogi, S.K., and Kumar, R. (2005). Epidemic Strain *Shigella dysenteriae* Type 1 Dt66 Encodes Several Drug Resistances by Chromosome. *Archives of Medical Research*, 36, 399-403. Elsevier.

Hu, P., Brodie, E.L., Suzuki, Y., McAdams, H.H., and Andersen, G.L. (2005). Whole-genome Transcriptional Analysis of Heavy Metal Stresses in *Caulobacter crescentus*. *Journal of Bacteriology*, 187, 8437-8449. American Society for Microbiology.

Huddedar SB, Shete AM, Tilekar JN, Gore SD, Dhavale DD, Chopade BA. (2002). Isolation, Characterization of Plasmid pUPI126 Mediated Indole Acetic Acid (IAA) Production in *Acinetobacter* from Rhizosphere of Wheat. *Applied Biochemistry and Biotechnology*, 102-103, 21-39. Humana Press.

Huggins, F.E., Najih, M., Huffman, G.P. (1999). Direct Speciation of Chromium in Coal Combustion by-products by X-ray Absorption Fine-structure Spectroscopy. *Fuel*, 78, 233–242. Elsevier.

Juhnke, S., Peitzsch, N., Hübener, N., Grose, C., and Nies, D.H. (2004). Erratum: New Genes Involved in Chromate Resistance in *Ralstonia metallidurans* Strain CH34. *Archive of Microbiology*, 181, 390. Springer-Verlag.

Juhnke, S., Peitzsch, N., Hübener, N., Grose, C., and Nies, D.H. (2002). New Genes Involved in Chromate Resistance in *Ralstonia metallidurans* strain CH34. *Archive of Microbiology*, 179, 15-25. Springer-Verlag.

Kado, C.I, Liu, S.T. (1981). Rapid Procedure for Detection and Isolation of Large and Small Plasmids. *J. Bacteriol.*, 145, 1365-1373.

Kamnev, A.A., Ristić, M., Antonyuka, L.P., Chernyshev, A.V., and Ignatov, V.V. (1997). Fourier Transform Infrared Spectroscopic Study of Intact Cells of the Nitrogen-fixing Bacterium *Azospirillum brasdense*. *Journal of Molecular Structure*, 408/409, 201-205. Elsevier.

Katz, S.A., and Salem, H. (1994). *The Biological and Chemistry of Environmental Chromium*. USA: VCH Publishers, Inc.

Koningsberger, D.C., and Prins, R. (1988). *X-ray Absorption: Principles, Applications, Techniques of EXAFS, SEXAFS, and XANES*. United Sates of America: John Wiley and sons, Inc..

Kostal, J., Suchanek, M., Klierova, H., Demnerova, K., Kralova, B., and McBeth, D. (1998). *Pseudomonas* C12B, an SDS-degrading Strain, Harbours a Plasmid Coding for Degradation of Medium Chain Length *n*-alkanes. *International Biodeterioration and Biodegradation*, 31, 110-117. Elsevier.

Kümmerer, K. (2004). Reviews: Resistance in the Environment. *Journal Antimicrobial Chemotheraphy*, 54, 311 – 320. The British Society for Antimicrobial Chemotherapy.

Kunte, D.P., Dhakephalkar, P.K., Mukerjee, A., Patil, A., Kumbhar, A.S., Padhye S.B., and Chopade, B.A.. (1993). Metals in Medicine: Elimination of Plasmid Mediated Antibiotic and Metal Resistance in *Acinetobacter baumannii* C11 by Naturally Occurring Quinones and their Metal Complexes. *Journal of Inorganic Biochemistry*, 51, 387. Elsevier.

Lambert, T., Gerbaud, G., Galimand, M., and Courvalin, P. (1993). Characterization of *Acinetobacter haemolyticus* aac(6')-Ig Gene Encoding an Aminoglycoside 6'-N-Acetyltransferase Which Modifies Amikacin. *Antimicrobial Agents and Chemotheraphy*, 37, 2093-2100. American Society for Microbiology.

Lameiras, S. Quintelas, C., and Tavares, T. (2008). Biosorption of Cr (VI) using a Bacterial Biofilm Supported on Granular Activated Carbon and on Zeolite. *Bioresource Technology*, 99, 801-806. Elsevier.

Laxman, R.S., and More, S. (2002). Reduction of Hexavalent Chromium by *Streptomyces griseus*. *Minerals Engineering*, 15, 831–837. Elsevier.

Lee, R.E. (1993). *Scanning Electron Microscopy and X-Ray Microanalysis*. United Sates of America: Prentice-Hall.

Lee, S.E., Lee, J.U., Lee, J.S., and Chon, H.T. (2006). Effects of Indigenous Bacteria on Cr(VI) Reduction in Cr-Contaminated Sediment with Industrial Wastes. *Journal of Geochemical Exploration*, 88, 41–44. Elsevier.

Lee, S.E., Lee, J.U., Chon, H.T., and Lee, J.S. (2008). Reduction of Cr(VI) by Indigenous Bacteria in Cr-Contaminated Sediment Under Aerobic Condition. *Journal of Geochemical Exploration*. *Journal of Geochemical Exploration*, 96, 144-147. Elsevier.

Li, Y., Knobloch, O., and Hahn, H.P. (2002). An Extended Boiling Method for Small-scale Preparation of Plasmid DNA Tailored to Long-range Automated Ssequencing. *Journal of Biochemical and Biophysical Methods*, 51, 69-74. Elsevier.

Li, Y., Low, G.K.C., Scott, J.A., and Amal, R. (2007). Microbial Reduction of Hexavalent Chromium by Landfill Leachate. *Journal of Hazardous Materials*, 142, 153–159. Elsevier.

Lin, Z., Zhu, Y., Kalabegishvili, T.L., Tsibakhashvili, N.Y., and Holman, H.Y. (2006). Effect of Chromate Action on Morphology of Basalt-Inhabiting Bacteria. *Materials Science and Engineering*, 26, 610-612. Elsevier.

Liu, T. (2008). Singapore Synchrotron Light Source, National University of Singapore, Singapore. *Personal communication.*

Losi, M.E., Amrhein, C., Frankenberger, W.T. (1994). Environmental Biochemistry of Chromium. *Review Environmental Contamination Toxicology,* 36, 91–121.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein Measurements with Folin Phenol Reagents. *J. Biol. Chem.* 193, 265–275.

Madigan, M. T., and Martinko, J. M. (2006). *Brock Biology of Microorganisms.* New Jersey: Pearson Prentice Hall.

Magnet, S., Courvlin, P., and Lambert, T. (2001). Resistance-Nodulation-Cell Division-Type Efflux Pump Involved in Aminoglycoside Resistance in *Acinetobacter baumannii* Strain BM4454. *Antimicrobial Agents and Chemotherapy*, 45, 3375–3380. American Society for Microbiology.

Marra, M.A., Kucaba,T.A., Hillier,L.W., and Waterston,R.H. (1999) High-Throughput Plasmid DNA Purification for 3 cents per Sample. *Nucleic Acids Research*, 27, e37. Oxford University Press.

McLean, J., and Beveridge, T.J. (2001). Chromate Reduction by a Pseudomonad Isolated from a Site Contaminated with Chromated Copper Arsenate. *Applied and Environmental Microbiology*, 67, 1076-1084. American Society for Microbiology.

Megharaj, M., Avudainayagam, S., and Naidu, R. (2003). Toxicity of Hexavalent Chromium and Its Reduction by Bacteria Isolated from Soil Contaminated with Tannery Waste. *Current Microbiology*, 47, 51–54. Springer-Verlag New York.

Mesas, J.M., Rodríguez, M.C., and Alegre, M.T. (2004). Short Communication: Plasmid Curing of *Oenococcus oeni*. *Plasmid*, 51, 37–40. Elsevier.

Mickel, S., Arena, V.J., and Bauer, W. (1977). Physical Properties and Gel Electrophoresis Behaviour of R-12 Derived Plasmid DNAs. *Nucleic Acid Research*, 4, 1465-1482. Information Retrieval Ltd.

Minas, W., and Gutnick, D. (1993). Isolation, Characterization, and Sequence Analysis of Cryptic Plasmids from *Acinetobacter calcoaceticus* and Their Use in the Construction of *Escherichia coli* Shuttle Plasmids. *Applied and Environmental Microbiology,* 2807-2816. American Society for Microbiology.

Morales-Barrera, L., Guillén-Jiménez, F.M., Ortiz-Moreno, A., Villegas-Garrido, T.L., Sandoval-Cabrera, A., Hernández-Rodríguez, C.H., and Cristiani-Urbina, E. (2008). Isolation, Identification and Characterization of a *Hypocrea tawa* Strain with High Cr(VI) Reduction Potential. *Biochemical Engineering Journal*, 40, 284-292. Elsevier.

Mullen, M.D., Wold, D.C., Ferris, F.G., Beveridge, T.J., Flemming, C.A., and Bailey, G.W. (1989). Bacterial Sorption of Heavy Metals. *Applied and Environmental Microbiology*, 55, 3143-3149. American Society for Microbiology.

Mungasavalli, D.P., Viraraghavan, T., and Jin, Y.C. (2007). Biosorption of Chromium from Aqueous Solutions by Pretreated *Aspergillus niger*: Batch and Column Studies. *Colloids and Surfaces A: Physicochemical Engineering Aspects*, 301, 214–223. Elsevier.

Neal, A.L., Lowe, K., Daulton, T.L., Jones-Meehan, J., and Little, B.J. (2002). Oxidation State of Chromium Associated with Cell Surfaces of *Shewanella oneidensis* during Chromate Reduction. *Applied Surface Science*, 202, 150–159. Elsevier.

Nemec, A., Dijkshoorn, L., Cleenwerck, I., Baere, T. D., Janssens, D., Reijden, T. J. K., Ježek, P., and Vaneechoutte, M. (2003). *Acinetobacter parvus* sp. nov., a Small-Colony-Forming Species Isolated from Human Clinical Specimens. *International Journal of Systematic and Evolutionary Microbiology*, 53, 1563–1567. IUMS.

Niazi, J.H., Prasad, D.T., and Karegoudar, T.B. (2001). Initial Degradation of Dimethylphthalate by Esterases from *Bacillus* sp. *FEMS Microbiology Letters*, 196, 201-205. Elsevier.

Niftrik, L.V., Geerts, W.J.C., Donselaar, E.G.V., Humbel, B.M., Yakushevska, A., Verkleij, A.J., Jetten, M.S.M., and Strous, M. (2008). Combined Structural and Chemical Analysis of the Anammoxosome: A Membrane-Bounded Intracytoplasmic Compartment in Anammox Bacteria. *Journal of Structural Biology*, 161, 401-410. Elsevier.

Ohtake, H., Cervantes, C., and Silver, S. (1987). Decreased Chromate Uptake in *Pseudomonas fluorescens* Carrying a Chromate Resistant Plasmid. *Journal of Bacteriology*, 169, 3853-3856. American Society for Microbiology.

Oliver, D.S., Brockman, F.J., Bowman, R.S., and Kieft, T.L. (2003). Vadose Zone Processes and Chemical Transport: Microbial Reduction of Hexavalent Chromium under Vadose Zone Conditions. *Journal of Environmental Quality*, 32, 317–324.

Pal, A., and Paul, A. K. (2004). Aerobic Chromate Reduction by Chromium-resistant Bacteria Isolated from Serpentine Soil. *Microbiological Research*. 159, 347-354. Elsevier.

Pandi, M., Shashirekha, V., and Swamy, M. (2007). Bioabsorption of Chromium from Retan Chrome Liquor by Cyanobacteria. Article in press.

Pardesi, K.R., Yavankar, S.P., and Chopade, B.A. (2007). Plasmid Distribution and Antimicrobial Susceptibility Patterns of *Acinetobacter* genospecies from Healthy Skin of a Tribal Population in Western India. *Indian Journal of Medical Research*, 125, 79-88. University of Pune, Ganeshkhind.

Park, C.H., Keyhan, M., Wielinga, B., Fendorf, S., and Matin, A. (2000). Purification to Homogeneity and Characterization of a Novel *Pseudomonas putida* Chromate Reductase. *Applied and Environmental Microbiology*, 66, 1788-1795. American Society for Microbiology.

Park, D., Yun, Y.S., and Park, J.M. (2005). Studies on Hexavalent Chromium Biosorption by Chemically-Treated Biomass of *Ecklonia* sp.. *Chemosphere*, 60, 1356–1364. Elsevier.

Park, D., Yun, Y., and Park, J.M. (2008). XAS and XPS Studies on Chromium-binding Groups of Biomaterial during Cr(VI) Biosorption. *Journal of Colloid and Interface Science*, 317, 54-61. Elsevier.

Parsons, J.G., Hejazi, M., Tiemann, K.J., Henning, J., and Gardea-Torresdey, J.L. (2002). An XAS Study of the Binding of Copper(II), Zinc(II), Chromium(III) and Chromium(VI) to Hops Biomass. *Microchemical Journal*, 71, 211–219. Elsevier.

Pathak, S.P., and Gopal, K. (2005). Occurrence of Antibiotic and Metal Resistance in Bacteria from Organs of River Fish. *Environmental Research*. 98, 100-103. Elsevier.

Pattanapipitpaisal, P., Brown, N.L., and Macaskie, L.E. (2001). Short Contribution: Chromate Reduction and 16S rRNA Identification of Bacteria Isolated from a Cr(VI)-Contaminated Site. *Applied Microbiology Biotechnology*, 57, 257–261. Springer-Verlag.

Peitzsch, N., Eberz, G., and Nies, D.H. (1998). *Alcaligenes eutrophus* as a Bacterial Chromate Sensor. *Applied and Environmental Microbiology*, 64, 453–458. American Society for Microbiology.

Peterson, M., Brown, G.E., Parks, G.A., and Stein, C.L. (1997). Differential Redox and Sorption of Cr(III/VI) on Natural Silicate and Oxide Minerals: EXAFS and XANES Results. *Geochimica et Cosmochimica Acta*, 61, 3399-3412. Elsevier.

Pimentel, B.E., Moreno-Sánchez, R., and Cervantes, C. (2002). Efflux of Chromate by *Pseudomonas aeruginosa* Cells Expressing the ChrA Protein. *FEMS Microbiology Letters*, 212, 249-254. Elsevier.

Priego-Capote, F. and Luque de Castro, M.D.L. (2006). Speciation of Chromium by In-Capillary Derivatization and Electrophoretically Mediated Microanalysis. *Journal of Chromatography A*, 1113, 244-250. Elsevier.

Puzon, G.J., Petersen, J.N., Roberts, A.G., Kramer, D.M. and Xun, Luying. (2002). A Bacterial Flavin Reductase System Reduces Chromate to a Soluble Chromium(III)–NAD^+ Complex. *Biochemical and Biophysical Research Communications*, 294, 76–81. Academic Press.

Qiu, X., Sundin, G.W., Wu, L., Zhou, J., and Tiedje, J. M. (2005). Comparative Analysis of Differentially Expressed Genes in *Shewanella oneidensis* MR-1 Following Exposure to UVC, UVB, and UVA Radiation. *Journal of Bacteriology*, 187, 3556–3564. American Society for Microbiology.

Quintelas, C., Fernandes, B., Castro, J., Figueiredo, H., and Tavares, T. (2008). Biosorption of Cr(VI) by a *Bacillus coagulans* Biofilm Supported on Granular Activated Carbon (GAC). *Chemical Engineering Journal*, 136, 195-203. Elsevier.

Ramírez-Díaz, M.I., Díaz-Pérez, C., Vargas, E., Riveros-Rosas, H., Campos-García, J., Cervantes, C. (2008). Mechanisms of Bacterial Resistance to Chromium Compounds. *Biometals*, 21, 321-332. Springer.

Ready, D., Pratten, J., Mordan, N., Watts, E., Wilson, M. (2007). The Effect of Amalgam Exposure on Mercury- and Antibiotic-Resistant Bacteria. *International Journal of Antimicrobial Agents*, 30, 34–39. Elsevier.

Reece, R.J. (2004). *Analysis of Genes and Genomes*. United Kingdom: John Wiley and Sons, Ltd.

Rodríguez, M. C., Barsanti, L., Passarelli, V., Evangelista, V., Conforti, V., and Gualtieri, P. (2007). Effects of Chromium on Photosynthetic and Photoreceptive Apparatus of the Alga *Chlamydomonas reinhardtii*. *Environmental Research*, 105, 234–239. Elsevier.

Rusansky, S., Avigad, R., Michaeli, S., and Gutnick, D.L. (1987). Involvement of a Plasmid in Growth on and Dispersion of Crude Oil by *Acinetobacter calcoaceticus* RA57. *Applied and Environmental Microbiology*, 53, 1918-1923. American Society for Microbiology.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. (2nd ed.). New York: Cold Spring Harbor Laboratory Press.

Sambrook, J., and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*. (3rd ed.). New York: Cold Spring Harbor Laboratory Press.

Sawalha, M.F., Gardea-Torresdey, J.L., Parsons, J.G., Saupe, G., and Peralta-Videa, J.R. (2005). Special article: Determination of Adsorption and Speciation of Chromium Species by Saltbush (*Atriplex canescens*) Biomass Using a Combination of XAS and ICP–OES. *Microchemical Journal*, 81, 122–132. Elsevier.

Schmidt, T., Friehs, K., Schleef, M., Voss, C., and Flaschel, E. (1999). Quantitative Analysis of Plasmid Forms by Agarose and Capillary Gel Electrophoresis. *Analytical Biochemistry*, 274, 235–240. Academic Press.

Sengupta, A. K. (1994). *Principles of Heavy Metals Separation: An Introduction. Environmental Separation of Heavy Metals – Engineering Processes*. USA: Lewis Publishers.

Shakibaie, M.R., Dhakephalkar, P.K., Kapadnis, B.P., and Chopade, B.A. (1999). Removal of Silver from Photographic Wastewater Effluent using *Acinetobacter baumannii* BL54. *Canadian Journal of Microbiology*, 45, 995-1000. NRC Research Press.

Shakoori, A.R., Makhdoom, M., and Haq, R.U. (2000). Hexavalent Chromium Reduction by a Dichromate-Resistant Gram-positive Bacterium Isolated from Effluents of Tanneries. *Applied Microbiology Biotechnology*, 53, 348-351. Springer-Verlag.

Shanker, A.K., Cervantes, C., Loza-Taverac, H., and Avudainayagam, S. (2005). Review Article: Chromium Toxicity in Plants. *Environment International*, 31, 739– 753. Elsevier.

Shen, H. and Wang, Y.T. (1994) Modeling Hexavalent Chromium Reduction in *Escherichia coli* 33456. *Biotechnology and Bioengineering*, 43, 293-300. John Wiley & Sons, Inc.

Shen, H., and Wang, Y.T. (1993). Characterization of Enzymatic Reduction of Hexavalent Chromium by *Escherichia coli* ATCC 33456. *Applied and Environmental Microbiology*, 59, 3771-3777. American Society for Microbiology.

Silver, S. (1992). Plasmid-determined Metal Resistance Mechanisms: Range and Overview. *Plasmid*, 27, 1-3.

Silver, S., and Phung, L.T. (2005). A Bacterial View of the Periodic Table: Genes and Proteins for Toxic Inorganic Ions. *Journal of Industrial Microbiology Biotechnology*. Society for Industrial Microbiology.

Srivastava, S., and Thakur, I.S. (2006). Evaluation of Bioremediation and Detoxification Potentiality of *Aspergillus niger* for Removal of Hexavalent Chromium in Soil Microcosm. *Soil Biology and Biochemistry.* 7, 1904-1911. Elsevier.

Srivastava, S., and Thakur, I.S. (2007). Evaluation of Biosorption Potency of *Acinetobacter* sp. for Removal of Hexavalent Chromium from Tannery Effluent. *Biodegradation*, 18, 637-646. Springer.

Srivastava, S., Ahmad, A.H., Thakur, I.S. (2007). Short Communication: Removal of Chromium and Pentachlorophenol from Tannery Effluents. *Bioresource Technology*, 98, 1128–1132. Elsevier.

Sultan, S., and Hasnain, S. (2007). Reduction of Toxic Hexavalent Chromium by *Ochrobactrum intermedium* Strain SDCr-5 Stimulated by Heavy Metals. *Bioresource Technology*, 98, 340–344. Elsevier.

Suzuki, T., Miyata, N., Horitsu, H., Kawai, K., Takamizawa, K., Tai, Y., and Okazaki, M. (1992). NAD(P)H-Dependent Chromium(VI) Reductase of *Pseudomonas ambigua* G-1: a Cr(V) Intermediate Is Formed during the Reduction of Cr(VI) to Cr(III). *Journal of Bacteriology*, 174, 5340-5345. American Society for Microbiology.

Thacker, U, and Madamwar, D. (2005). Reduction of Toxic Chromium and Partial Localization of Chromium Reductase Activity in Bacterial Isolate DM1. *World Journal of Microbiology and Biotechnology*, 21, 891–899. Springer.

Thacker, U., Parikh, R., Shouche, Y., and Madamwar, D. (2006). Hexavalent Chromium Reduction by *Providencia* sp.. *Process Biochemistry*, 41, 1332–1337. Elsevier.

Thacker, U., Parikh, R., Shouche, Y., and Madamwar, D. (2007). Reduction of Chromate by Cell-free Extract of *Brucella* sp. Isolated from Cr(VI) Contaminated Sites. *Bioresource Technology*, 98, 1541–1547. Elsevier.

Thakur, I.S., Verma, P.K., and Upadhaya, K.C. (2001). Involvement of Plasmid in Degradation of Pentachlorophenol by *Pseudomonas* sp. from a Chemostat. *Biochemical and Biophysical Research Communications*, 286, 109-113. Academic Press.

Towner, K. (2006). The Genus *Acinetobacter*. *Prokaryotes*, 6, 746–758. Springer-Verlag.

Trevors, J.T. (1985). Bacterial Plasmid Isolation and Purification. *Journal of Microbiological Methods*, 3, 259-270. Elsevier.

Ugur, A., and Ceylan, Ö. (2003). Occurrence of Resistance to Antibiotics, Metals, and Plasmids in Clinical Strains of *Staphylococcus* spp. *Archives of Medical Research*, 34, 130–136. Elsevier.

Viamajala, S., Smith, W.A., Sani, R.K., Apel, W.A., Petersen, J.N., Neal, A.L., Roberto, F.F., Newby, D.T., and Peyton, B.M. (2007). Isolation and Characterization of Cr(VI)-Reducing *Cellulomonas* spp. from Subsurface Soils: Implications for Long-term Chromate Reduction. *Bioresource Technology*, 98, 612–622. Elsevier.

Vijaranakul, U., M. J. Nadakavukaren, B. L. M. de Jonge, B. J. Wilkinson, and R. K. Jayaswal. (1995). Increased Cell Size and Shortened Peptidoglycan Interpeptide Bridge of NaCl-stressed *Staphylococcus aureus* and Their Teversal by Glycine Betaine. *Journal of Bacteriology*, 177, 5116–5121. American Society for Microbiology.

Vogelstein, B., and Gillespie, D. (1979). Preparative and Analytical Purification of DNA from Agarose (NaI/acetone precipitation/DNA-glass complexes/ molecular hybridization). *Proceedings of the National Academy of Sciences*, 76, 615-619. National Academy of Sciences.

Wang, P.C., Mori, T., Toda., K., and Ohtake, H. (1990). Membrane-Associated Chromate Reductase Activity from *Enterobacter cloacae*. *Journal of Bacteriology*, 172, 1670-1672. American Society for Microbiology.

Wang, Y.T., and Xiao, C. (1995). Factors Affecting Hexavalent Chromium Reduction in Pure Cultures of Bacteria. *Water Research*, 29, 2467-2474. Pergamon.

Wasi, S., Jeelani, G., and Ahmad, M. (2008). Biochemical Characterization of a Multiple Heavy Metal, Pesticides and Phenol Resistant *Pseudomonas fluorescens* Strain. *Chemosphere*, 71, 1348-1355. Elsevier.

Wei, Y., Hsu, L., Wang, H.P., and Chen, K. (2007). XAS Study of Chromium Recoverable from Plating Sludge. *Journal of Electron Spectroscopy and Related Phenomena*, 156–158, 204–207. Elsevier.

Weyrich, P., Borgmann, S., Mayer, F., Heeg, P., Riessen, R., and Kotter, I. (2006). Fatal Multidrug-resistant *Acinetobacter baumannii* Sepsis in a Patient with Travel History and Recent Onset of Systemic Lupus Erythematosus: A Case Report. *International Journal of Hygiene and Environmental Health*. Article in Press. Elsevier.

Wischnitzer, S. (1981). *Introduction to Electron Microscopy*. (3rd ed.). New York: Pergamon.

Wu, W., Welsh, M.J., Kaufman, P.B., and Zhang, H.H. (1997). *Methods in Gene Biotechnology*. New York: CRC Press Lcc.

Yilmaz, E.I. (2003). Metal Tolerance and Biosorption Capacity of *Bacillus circulans* Strain EB1. *Research in Microbiology*, 154, 409–415. Elsevier.

Zafar, S., Aqil, F., and Ahmad I. (2007). Metal Tolerance and Biosorption Potential of Filamentous Fungi Isolated from Metal-Contaminated Agricultural Soil. *Bioresource Technology*, 98, 2557-2561. Elsevier.

Zakaria, Z. A., Zakaria, Z., Surif, S., and Ahmad, W. A. (2007). Hexavalent Chromium Reduction by *Acinetobacter haemolyticus* Isolated from Heavy Metal-Contaminated Wastewater. *Journal of Hazardous Materials*, 146, 30-38. Elsevier.

Zhu, W., Chai, L., Ma, Z., Wang, Y., Xiao, H., and Zhao, K. (2006). Anaerobic Reduction of Hexavalent Chromium by Bacterial Cells of *Achromobacter* sp. Strain Ch1. *Microbiological Research.* Article in Press.

APPENDIX A: Preparation of buffers and solutions

EDTA, 0.5 M (pH 8.0) stock solution

EDTA, 0.5 M (pH 8.0) stock solution was prepared by adding 186.1 g of disodium EDTA (Fluka, Britain) in 800 mL of DDI water and stirred vigorously on a magnetic stirrer. The pH was adjusted to pH 8.0 with NaOH pellets (Merck, Germany). The solution was then dispensed into aliquots and sterilized by autoclaving. The disodium salt of EDTA would not go into solution until the pH of the solution was adjusted to approximately 8.0 by the addition of NaOH (Sambrook and Russell, 2001).

Tris-Cl, 1 M (pH 8.0) stock solution

To prepare Tris-Cl, 1 M (pH 8.0) stock solution, 121.1 g of Tris base (Sigma, USA) was dissolved in 800 mL of DDI water. The pH was adjusted to 8.0 by adding concentrated HCl (fuming hydrochloric acid) (Merck, Germany). The solution was allowed to cool to room temperature before final adjustments to the pH was made. The volume of the solution was adjusted to 1 L with DDI water. The solution was dispensed into aliquots and sterilized by autoclaving (Sambrook and Russell, 2001).

TE buffer

TE buffer consisted of 100 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) which was sterilized by autoclaving and then stored at room temperature (Sambrook and Russell, 2001).

TE-saturated phenol/ chloroform

TE-saturated phenol/ chloroform solution was prepared by thawing crystals of phenol (Schärlau, Spain) in a 65˚C water bath with occasional shaking. Equal parts of TE buffer and thawed phenol was mixed and let stand until the phases were separated at room temperature. An equal part of the lower, phenol phase was mixed with an equal part of chloroform (Merck, Germany):isoamyl alcohol (Univar, Australia) (24:1) (Wu *et al*., 1997).

Alkaline Lysis Solution I

Alkaline Lysis Solution I consisted of 50 mM glucose (Riedel-de-Haën, Germany), 25 mM Tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0) (Sambrook and Russell, 2001).

Alkaline Lysis Solution II

Alkaline Lysis Solution II which consisted of freshly diluted NaOH from a 10 N stock to a final concentration of 0.2 N NaOH and 1% (w/v) SDS (Sigma, USA) was prepared fresh and used at room temperature (Sambrook and Russell, 2001).

Alkaline Lysis Solution III

Alkaline Lysis Solution III consisted of 3 M potassium acetate (98.14 g/mol) (Unilab, Australia) and 11.5% (v/v) glacial acetic acid (60.05 g/mol) (Merck, Germany). The solution was stored at 4˚C and transferred to an ice bucket just before use (Sambrook and Russell, 2001).

Phenol-chloroform solution (1:1, v/v)

To prepare phenol-chloroform solution (1:1, v/v), crystallized phenol was liquefied at 50˚C and mixed with chloroform (50:50, v/v) (Kado and Liu, 1981).

Lysing solution

The lysing solution contained SDS (3%, w/v) and 50 mM Tris (pH 12.6). The solution was adjusted to pH 12.6 by adding 1.6 mL of 2 N NaOH by using a glass electrode (Mettler Toledo 320 pH Meter). Then, the volume was adjusted to 100 mL with DDI water and the solution was filtered by using a 0.2 µm pore size membrane filter paper (Whatman, England) (Kado and Liu, 1981).

E buffer

All gel electrophoresis experiments were performed in the following standard E buffer: 0.04 M Tris-acetate, 0.02 M sodium acetate, and 2 mM EDTA, pH 8.1 made from stock solutions 1 M tris-base which was adjusted to pH 8.3 with glacial acetic acid, 1 M sodium acetate and 0.5 M disodium EDTA pH 8.13 (Mickel *et al*., 1977).

Lowry assay mix

Lowry copper reagent (100 mL) was prepared by dissolving 0.5 g $CuSO_4.5H_2O$ (GCE, France) and 1 g sodium citrate (Merck, Germany) in DDI water. Lowry alkaline reagent (1 L) was prepared by dissolving 20 g sodium carbonate (BDH, New Jersey) and 4 g sodium hydroxide in DDI water. To obtain Lowry assay mix, 1 mL Lowry copper reagent was mixed with 50 mL Lowry alkaline reagent. The Lowry assay mix was prepared fresh prior to protein estimation.

Folin-Ciocalteu reagent

Folin-Ciocalteu reagent (Merck, Germany) was prepared fresh by mixing 10 mL Folin reagent with 10 mL of DDI water.

Bovine serum albumin (BSA) stock solution

BSA stock solution (100 mg L^{-1}) was prepared by dissolving 0.05 g BSA (Sigma-Aldrich, USA) in 500 mL DDI water.

APPENDIX B: *Pseudomonas* sp. *chr*R gene for Cr(VI) reductase (Suzuki *et al*., 1992)

```
LOCUS       PSECHRR                 3370 bp    DNA     linear   BCT
06-FEB-1999
DEFINITION  Pseudomonas sp. chrR gene for Cr(VI) reductase, complete
            cds.
ACCESSION   D83142
VERSION     D83142.1  GI:1199489
KEYWORDS    chrR; Cr(VI) reductase.
SOURCE      Pseudomonas sp.
ORGANISM    Pseudomonas sp.
            Bacteria; Proteobacteria.
REFERENCE   1  (bases 1 to 3370)
AUTHORS     Suzuki,T.
TITLE       Cloning and expression of Cr(VI) reductase gene (chrR)
JOURNAL     Unpublished
REFERENCE   2 bases 1 to 3370)
AUTHORS     Suzuki,T.
TITLE       Direct Submission
JOURNAL     Submitted (18-JAN-1996) Tohru Suzuki, Gifu University,
            Department of Agriculture; Yanagido 1-1, Gifu, Gifu 501-
            11, Japan(E-mail:suzuki@cc.gifu-u.ac.jp, Tel:058-293-
            2909, Fax:058-293-2905)

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                     FKRLQSAGKLEGVDIVADSAENVLVGVADVSLFAQNFVVAAESMGYG
                     ICYIGGVRNKPEEISELFNLPEYVFPLFGLTIGVPARRNEVKPRLPV
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```

APPENDIX C: Elemental compositions of *Acinetobacter haemolyticus* cells grown on LB agar (A) without Cr(VI) (control) and (B) with 30 mg L^{-1} Cr(VI). Inner graph: FESEM of the samples analyzed with EDX probe.