ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

BIOREMEDIATION OF HEXAVALENT CHROMIUM USING GRAM-NEGATIVE BACTERIA

BY

MARIAM MOHAMED ISMAEL

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF SHEFFIELD HALLAM UNIVERSITY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

JUNE 2014

ABSTRACT

Hexavalent chromium (Cr (VI)), the most toxic form of chromium, is widely used in industrial processes. As a result substantial amounts of Cr (VI) contaminated wastes are produced. The use of microbial cells as bioremediation of heavy metals is a potential alternative to conventional chemical methods.

In this work, laboratory- scale experiments were performed to investigate Cr (VI) removal using five environmental Gram-negative bacterial strains, three of which were nosocomial strains. The potential of live and autoclaved bacterial strains was investigated to mitigate Cr (VI) from its initial concentration of 2.54 mg/l. The autoclaved bacteria were used to determine whether Cr (VI) removal was dependent upon metabolism of the cells or a simple chemical reaction. The results showed notable reduction in Cr (VI) concentration (up to 87% and 23% using live and autoclaved bacteria, respectively).

Proteus mirabilis and *Methylococcus capsulatus* (Bath) bacterial strains were selected for further detailed analyses to investigate the enzyme system that is responsible for Cr (VI) reduction. To locate the cell compartment in which Cr (VI) removal took place in *P. mirabilis*, a standard bacterial cell fractionation method was used. The highest Cr (VI) removal activity resided in the cytoplasm, and there was also some activity in the cell membrane. No chromium VI removal was observed in the cell wall fraction.

The removal by *M. capsulatus* of Cr (VI) in high copper sulfate media was more rapid than in low copper sulfate media. Phenylacetylene, an inhibitor of soluble methane monooxygenase, completely inhibited Cr (VI) removal. The results reveals that pMMO, sMMO or other enzymes that induced by copper were involved in reducing or otherwise removing Cr (VI). The di-heme cytochrome *c* peroxidase is also a possible candidate enzyme of reducing chromium (VI), since it is known to be present in the periplasm and to play a role in reducing peroxides generated by oxidative metabolism.

Inductively coupled plasma mass spectrometry coupled with ion chromatography, for the determination of chromium species in *P. mirabilis* and *M. capsulatus*, showed that Cr (VI) was reduced and detoxified to less toxic and less soluble Cr (III). Furthermore, prominent changes in the polysaccharide, fatty acids, phosphate and proteins were

observed in FTIR spectra of *P. mirabilis* and *M. capsulatus* (Bath) with potassium dichromate. These changes were consistent with the adsorption of chromium.

BLAST searches using known chromate (VI) reducing enzymes from other bacteria showed a presence of four significant potential chromate reductase genes in the genome sequence of *P. mirabilis*.

During the growth of *M. capsulatus* (Bath), it was noticed that a contaminant bacterium appeared in some cell cultures. The contaminant bacterium was identified as *Bacillus licheniformis* (100%) using PCR and 16S rRNA sequencing. The mixed culture that contains *M. capsulatus* (Bath) plus *Bacillus licheniformis* was also tested for Cr (VI) mitigation.

The results of this work are a step forward in understanding the potential of environmental microorganisms for remediation of hexavalent chromium contamination. The future work may reveal more about the mechanism of Cr (VI) removal by the bacteria studied here as well as how they can be exploited for practical bioremediation.

ACKNOWLDGEMENT

All praise due to Allah, the most Gracious and Merciful, for giving me the strength, health and determination to complete my study.

I would like to express my deepest gratitude, honour and sincere appreciation to my director of study Professor Thomas Smith for his invaluable guidance, encouragement, constructive suggestions throughout the whole research endeavour.

My heartfelt appreciation goes to Dr Philip Gardiner for his help and motivation in his role as my second supervisor.

My special appreciation is also extended to the microbiology research group for their inspiration and constructive comments on my work during our meetings.

I would like to thank all staff, technicians and colleagues at BMRC for their advice, friendship and support.

Last but not least, I would like to thank my husband and my daughters for their love and endless encouragement. This work would not have been possible without their support.

DEDICATION

To those who are proud of my success;

My beloved husband and my dearest daughters

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

a.u.	Absolute unit
BSA	Bovine serum albumin
Cr (III)	Trivalent chromium
Cr (VI)	Hexavalent chromium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DPC	Diphenylcarbazide
EDTA	Ethylenediamine tetra acetic acid
FTIR	Fourier Transform Infrared
G	Acceleration due to gravity of the earth (m/s^2)
IC	Ion chromatography
ICP-MS	Inductively coupled plasma-mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
$K_2Cr_2O_7$	Potassium dichromate
Kb	Kilobase
KDa	Kilo Dalton
mg/l	Milligram per litre
$MgCl_2$	Magnesium chloride
mM	Millimole
µl	Micro litre
NaN_3	Sodium azide
NCIBM	National centre for biotechnology information

Nm	Nano meter
NMS	Nitrate mineral salts
OD	Optical density
PCR	Polymerase chain reaction
rpm	Revolution per minute
RuMP	Ribulose monophosphate
SDS	Sodium dodecyl suphate
Tris-HCl	Tris (hydroxyl methyl) methyl ammonium chloride
V	Volt
BLAST	Basic local alignment search tool

CHAPTER 1

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Chromium is a heavy metal like cadmium, lead, copper and nickel that can contaminate soils, ground water, sediments and surface waters. It is extremely toxic to biological and ecological systems. Heavy metals can be released by the discharge of effluent into the environment by a variety of industries such as electroplating, leather tanning, wood preservation, wood pulp processing, steel manufacturing, etc. (Faisal and Hasnanin, 2004). Such pollution is a danger to human health and the environment because of the low solubility of many heavy metals and their carcinogenic and mutagenic properties (Alloway, 1995; Diels *et al.*, 2002).

Physico-chemical methods have been devised for the treatment and removal of heavy metals from contaminated environments. These methods include electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption (Kadirvelu *et al.*, 2001; Kadirvelu *et al.*, 2002). Microbial reduction of heavy metals has been proposed as an alternative method of remediation. In this work, the potential of Gram-negative environmental bacteria was investigated as a means of hexavalent chromium remediation.

1.2 Chromium

Chromium (Cr) is a heavy metal that occurs naturally in the environment (primarily in rocks and minerals) in the form of metal-ores such as those containing iron. It is a transition element, located in group VI of the periodic table, and was discovered in 1797 by the French chemist, Louis-Nicolas Vaquelin (1763-1829) in a mineral known as Siberian red lead ore (crocoites). The word chromium was derived from the Greek word 'chroma' that means 'colour' because many chromium compounds have distinctive colours ranging covering the visible spectrum (Young, 2000).

Chromium is the 21st most abundant element in the earth's crust with an abundance of about 100-300 ppm (Barnhart, 1997). In its pure form, elemental chromium is a hard, steel grey, shiny metal that breaks easily. It has an atomic number of 24, melting point of 1900 ^{o}C, a boiling point of 2642 ^{o}C and a density of 7.1 g cm^{-3}. It is also

odourless, tasteless and malleable. Physical properties that greatly add to chromium's commercial importance are that it can be polished to a high shine, has high corrosion resistance and hardness. Chromium is a metal that does not react with water but does react with most metals. It combines slowly with oxygen at room temperature to form chromium oxide (Cr_2O_3). The chromium oxide form acts as a protective layer, preventing the metal from reacting further with oxygen (Young, 2000).

1.2.1 Chromium chemistry

Chromium is found in all compartments of the environment including air, water and soil. It has been reported that chromium contamination in soil and water is caused by leakage, unsuitable storage, or improper disposal practices of chromium wastes (Rock *et al.*, 2001). Chromium exists in nine valence states, ranging from -2 to +6 (Smith *et al.*, 2002). The most common oxidation states of chromium are +2, +3, and +6. The most stable forms of chromium are the trivalent Cr (III) and hexavalent Cr (VI) species, which display different chemical properties (Fendorf *et al.*, 1992; Fukai, 1967; Holleman *et al.*, 1985; Jan and Young, 1978; Kota and Stasicika, 2000; McGrath and Smith, 1990; Sarangi and Krishnan 2008; Shanker *et al.*, 2005). Naturally occurring chromium exists predominantly in its chromic (Cr^{3+}) form, while the chromate (CrO_4^{2-}) state is rarely found in nature.

Different types of chromium exhibit different properties, which are important for assessing the risk of potential harm to human health. The divalent (chromous) state is a strong reductant and is rapidly decomposed in air and water to form the relatively inert chromic (trivalent) compound, which is the most abundant form of chromium found naturally in the environment (Lai and Lo, 2008). Cr (III) is a hard acid, which exhibits a strong tendency to form hexacoordinate octahedral complexes with a variety of ligands such as water, ammonia, urea, ethylenediamine and other organic ligands containing oxygen, nitrogen or sulphur donor atoms (Kota and Stasicka, 2000). Cr (VI) is frequently encountered in the environment, but it is unstable in the presence of electron donors such as Fe (II) and organic matter with oxidizable groups. Cr (VI) usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions. Major forms of Cr

(VI) in natural waters are $HCrO_4^-$ and CrO_4^{2-} (Katz and Salem, 1994; Richard and Bourg, 1992).

Cr (VI) is a strong oxidizing agent particularly in acidic media and associates with oxygen, forming the chromate and dichromate anions (Lai and Lo, 2008). The relative proportion of these anions depends on pH and total Cr (VI) concentration. H_2CrO_4 is a strong acid and at pH > 1, its deprotonated forms prevails, while above pH7 only CrO_4^{2-} ions exist in solution throughout the concentration range. Between pH 1 and 6, $HCrO^{4-}$ is the predominant form up to 10^{-2} M Cr (VI), when it starts to condense yielding the orange-red $Cr_2O_7^{2-}$. Cr (VI) oxyanions can be reduced to Cr (III) by reducing agents such as organic matter or reduced inorganic species, which are widely found in soil, water and atmospheric systems (Kota and Stasicka, 2000). The predominant form of Cr (III) at pH less than 3 is Cr^{3+}. As pH increases, the hydrolysis of Cr (III) occurs and the most abundant species become $CrOH^{2+}$, $Cr(OH)_2^+$, $Cr(OH)_3^0$ and $Cr(OH)_4^-$ (Palmer *et al.*, 1991).

1.2.2 Industrial uses of chromium

Chromium compounds are used in several industrial processes and products including; dyes and pigments, additives in cooling water, chrome plating, leather tanning and wood preservation and cement manufacturing (Zayed and Terry, 2003). One of the most important applications of chromium is in the production of steel; high-carbon and other grades of ferro-chromium alloys are added to steel to improve mechanical properties, increase hardening and enhance corrosion resistance. It also added to cobalt and nickel-base alloys for the same purpose (Barlett, 1991; Katz and Salem, 1994). Chromium is also used in coatings, as it is applied on the surface of other metals for decorative purposes, to enhance resistance and to lower the coefficient of friction.

Cr (II) chloride is used as a reducing agent in organic chemistry and in metal plating In particular it is used to reduce alpha-haloketones to their parent ketones, epoxides to alkenes and aromatic aldehydes to their corresponding alcohols (Patnaik, 2003).

Cr (III) chloride is used for chromium plating, in leather tanning, as a waterproofing agent and as a catalyst for polymerization of alkenes. Cr (III) sulfate is used as the electrolyte to produce pure chromium metal. Other important applications of chromium III sulfate are as a mordant for treatment of fabrics before dyeing, to dissolve gelatin, to impart green colour to paints, varnishes, inks and ceramic glazes and as a catalyst. Cr (III) oxide is used as pigment for glass and fabrics. It is also used in metallurgy, as a component of refractory bricks, abrasives and ceramics and to prepare other chromium salts. Cr (III) fluoride is used in printing and dyeing woollens, mothproofing woollen materials, metal polishing and colouring marbles (Palmer and Wittbrodt, 1991; Sawyer *et al.*, 1994).

Cr (VI) oxide is used in purification of oil and for cleaning laboratory glassware and for chromium plating, copper stripping, as an oxidizing agent for conversion of secondary alcohols into ketones, and as a corrosion inhibitor, (Ayres, 1992; Nriagu and Pacyna, 1988; Patnaik, 2003).

Due to the wide use of chromium in various industrial activities, chromium is released into the environment in larger amounts, which account for 60–70% of the total emissions of atmospheric chromium (Alimonti *et al.*, 2000; Barceloux, 1999; Seigneur and Constantinous, 1995). These industries have become especially large contributors of chromium pollution, which can ultimately have significant adverse biological and ecological effects (Figure 1.1). In recent years, contamination of the environment by chromium has become a major concern (Hagendorfer and Goessler, 2008; Palmer, 2000; Riley *et al.*, 1992; Shanker, 2005). The difference in the toxicities of its different forms and its high concentration in the environment, speciation analysis of chromium is necessary for health and environmental considerations.

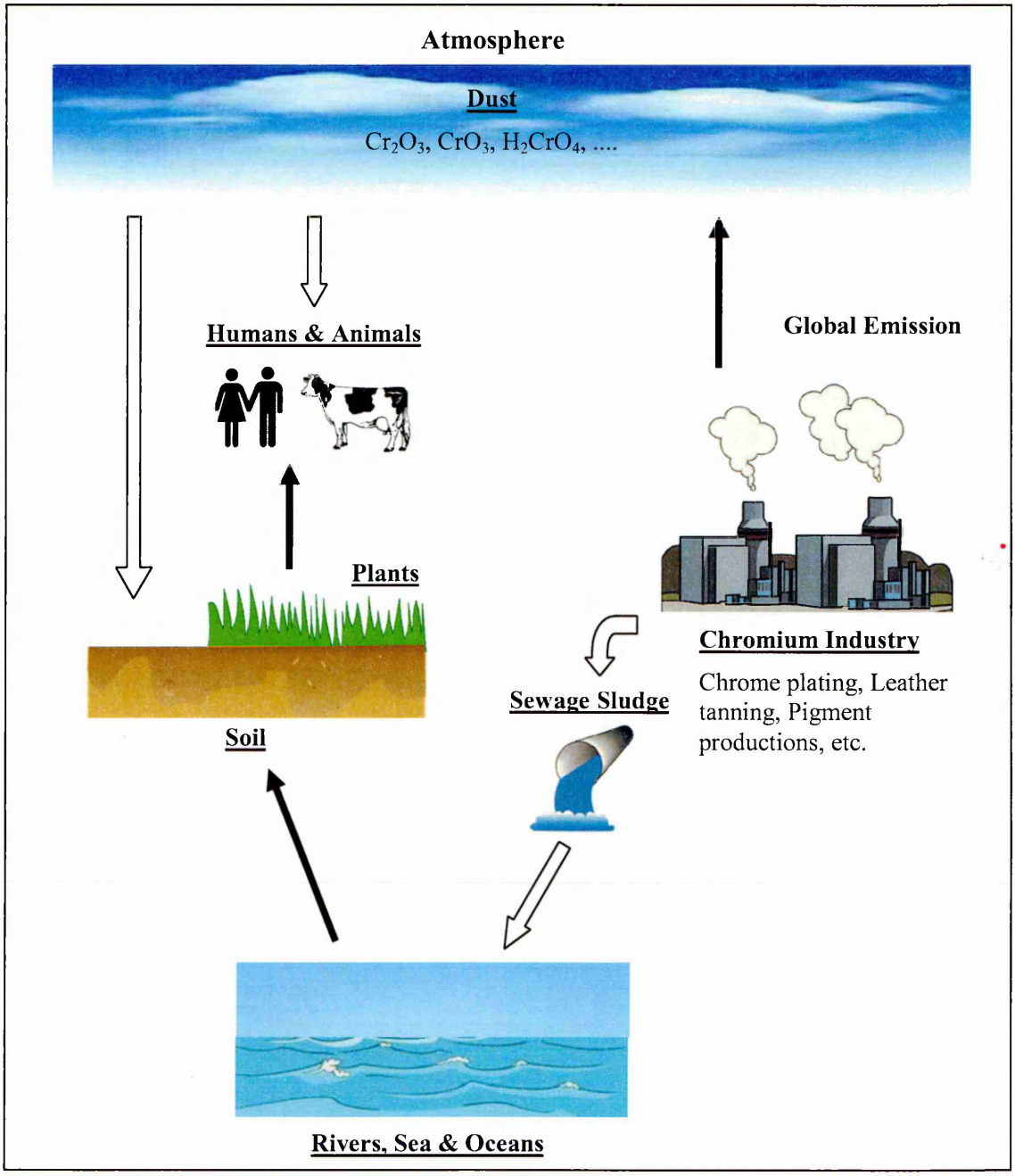


Figure 1.1: Chromium circulation in the polluted environment

1.2.3 Health issues

Chromium is unique among toxic elements in that environmental legislation regulates different species of chromium, specifically Cr (III) and Cr (VI), based on their differing toxicities. All other toxic elements such as lead, cadmium and arsenic are regulated based on their total concentrations, irrespective of their oxidation state (Cheung and Gu, 2002 & 2003; Kimbrough *et al.*, 1999; McLean et al., 2000). People can be exposed to chromium through breathing, eating or drinking and through skin contact with chromium or its compounds.

Cr (VI) is a known mutagen and carcinogen and it is more soluble and more mobile than Cr (III). Cr (VI) is considered the most toxic form of chromium to human, animals, and plants (Moore *et al.*, 1984). In addition to an increased risk of respiratory cancer, Cr (VI) is also known to be a skin irritant leading to ulcerations causing allergic contact dermatitis (Ajmal *et al.*, 1984; Nethercott, *et al.*, 1994; Seishima *et al.*, 2003; Wong and Trevors 1988). Other respiratory problems caused by Cr (VI) exposure include nasal irritations and bleeds (Kou, 1997), and lung carcinoma (Ajmal *et al.*, 1984; Wong and Trevors 1988). The reason for such toxicity arises from the possibility of free diffusion of Cr (VI) across the cell membrane and its strong oxidative potential (Turpeinen *et al.*, 2004).

Unlike Cr (VI), trivalent chromium is an essential nutrient for humans, even though in trace quantities. According to Anderson *et al.* (1992), most foods provide less than 2 μg of chromium per serving. The study reported the recommended daily chromium intakes, provided in the Dietary Reference Intakes (DRIs) developed by the Institute of Medicine of the National Academy of Sciences, which ranges between 50 to 200 μg (Institute of Medicine Food and Nutrition Board, 2001; National Research Council, Food and Nutrition Board, 1989).

Table 1.1 summarizes the daily adequate intakes (AIs) of chromium from food as found in several studies (Anderson *et al.*, 1992; National Research Council of Food and Nutrition Board, 1989).

Table 1.1: Adequate Intakes (AIs) for chromium

Age	Infants and Children (µg/day)	Male (µg/day)	Female (µg/day)	Pregnancy (µg/day)	Lactation (µg/day)
0 to 6 months	0.2				
7 to 12 months	5.5				
1 to 3 years	11				
4 to 8 years	15				
9 to 13 years		25	21		
14 to 18 years		35	24	29	44
19 to 50 years		35	25	30	45
Elder than 50 years		30	20		

Cr (III) has a vital role in the metabolism of glucose, fat and protein, by making the action of the hormone insulin more effective (Marques *et al.*, 2000). The low solubility retains Cr (III) in the solid phase as colloids or precipitates (Lin, 2002). Cr (III) salts such as chromium polynicotinate and chromium picolinate are used as micronutrients and dietary supplements (Bagchi *et al.*, 2001). Besides this, Cr (III) has been suggested as an element which can stabilize the tertiary structure of proteins and conformation of cellular RNA and DNA (Zetic *et al.*, 2001).

1.2.4 Traditional methods for chromium remediation

Several treatment technologies have been developed for removal of heavy metals, including hexavalent chromium, from the environment (e.g. from soil, industrial processes effluents and wastewater streams). Some of these methods include physical and chemical techniques, such as electrochemical precipitation, biosorption, membrane filtration, ion exchange, reverse osmosis and air and steam stripping (Ahluwalia and Goyal, 2007; Bhide *et al.*, 1996; Beleza *et al.*, 2001; Camargo *et al.*, 2003; Kadirvelu *et al.*, 2001 and 2002; Krishna and Philip, 2005; Mahajan, 1985; Nyer, 1992; Patterson, 1985; Sikaily *et al.*, 2007).

Some of these techniques present the disadvantages of producing chemical residues that are harmful to the environment. For instance, conventional chemical treatment involves the reduction of Cr (VI) to Cr (III) by a reducing agent at low pH and neutralization of the solution in order to precipitate Cr (III) as hydroxide (Wang and Shen, 1997). This method generates large amounts of secondary waste products. The electrochemical precipitation process, which is commonly used for treating wastewaters, creates sludge that requires subsequent disposal. Ion exchange is an alternative technology, although it is a non selective procedure and highly sensitive to the pH of the solution, and requires expensive equipment.

Biosorption of chromium from aqueous solutions is a relatively new process that has been investigated for removal of contaminants of aqueous effluents. In this process adsorbent materials derived from low-cost agricultural wastes can be used for the removal and recovery of chromium ions from wastewater streams (Basso *et al.*, 2002; Park *et al.*, 2006). The process of biosorption offers an economically feasible technology for efficient removal and recovery of metal(s) from aqueous solution. It has many attractive features including removal of metals over quite broad range of pH and temperature, its rapid kinetics of adsorption and desorption and low capital and operation cost. Biosorbent can easily be produced using inexpensive growth media or obtained as a by-product from industry (Ahluwalia and Goyal 2007). Biosorption allows significant cost savings in comparison with existing technologies, can be more effective in many cases than its closest rival, ion exchange can be easily converted to the biosorption

process (Volesky and Naja 2007). Non-living biomass appears to present specific advantages in the form of a biosorption process (Ozturk *et al.*, 2004). It contributed to removal of toxic Cr (VI) under all conditions tested including when the cells are no longer alive.

The membrane filtration technique has received significant attention for wastewater treatment (Pugazenthi *et al.*, 2005). It involves the application of hydraulic pressure to bring about the desired separation through a semipermeable membrane. This technique has several drawbacks apart from being expensive. It gives only incomplete removal of the metal that requires a high energy input and expensive reagents. It also generates toxic sludge and other waste products that require disposal. Chromium can also be removed using methods such as chemical precipitation, and membrane separation, although such approaches are not effective at metal concentrations below 100 mg l^{-1} (Kapoor and Viraraghavan, 1995).

The above mentioned approaches can be effective in reducing levels of a range of contaminants, but have several drawbacks, principally their technological complexity and lack of public acceptance. They also tend not to be cost-effective (Kadirvelu *et al.*, 2001; Vidali 2001; Volesky and May-Philips, 1995) and can result in generalised pollution (Kratochivil *et al.*, 1998). The relatively high costs of the chemical reagents and associated problems with sludge disposal make these methods somewhat limited in application. As a result of these and other shortcomings of physical and chemical treatment processes, low cost and effective alternatives are desirable for the remediation of environmentally toxic metals such as hexavalent chromium. In recent years, biological approaches have been considered as alternative means of remediating heavy metal contamination. Interest has been focused on the use of microorganisms to remove metal ions (Davis *et al.*, 2003; Mark, 2006; Munoz and Guieysse, 2006; Pena-Castro *et al.*, 2004). Therefore chromium reducing microorganisms may represent a cost efficient and highly effective technology for the removal or detoxification of the toxic forms of chromium. A number of aerobic and anaerobic microorganisms are capable of reducing Cr (VI). Thus, this work focuses on the use of environmental bacteria for bioremediation of Cr (VI).

1.3 Environmental bacteria

1.3.1 Bacterial cell structure

Bacteria can be classified into two distinct groups; Gram-positive and Gram-negative bacteria, based on the structural differences of their bacteria cell walls as shown in Figure 1.2. Gram-positive bacteria are the ones that retain the crystal violet dye when washed in a decolorizing solution, while, Gram-negative bacteria do not retain crystal violet dye in the Gram staining protocol.

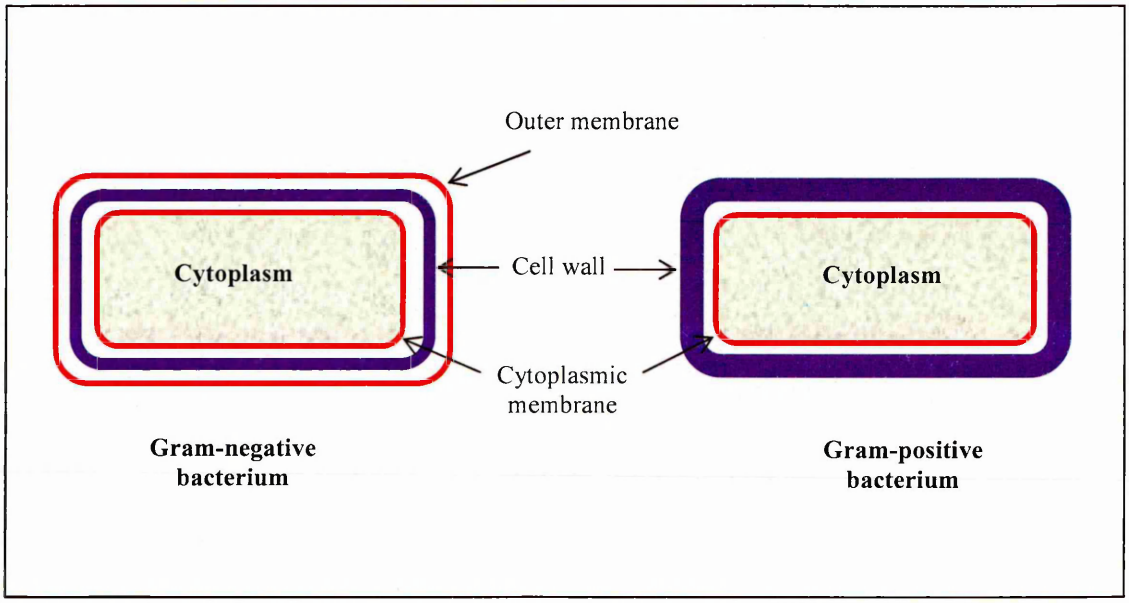


Figure 1.2: Cell structure of Gram-positive & Gram-negative bacteria

The outer membrane of the Gram-negative bacterial cell wall is made up of lipopolysaccharide and protein. It covers a thinner layer of peptidoglycan as compared to Gram-positive bacteria. The outer membrane of Gram-negative bacteria is spanned by porins (pore-like structures that permit passage of specific types of molecules. Below the outer membrane, exists the periplasmic space (the space between the inner and outer membranes, which contains the peptidoglycan and a large number of proteins). Some Gram negative bacteria also have flagella.

The Gram-negative bacteria include the *Cyanobacteria*, *Spirochaetes*, green sulfur and green non-sulfur bacteria and *Proteobacteria*. Important examples of the *Proteobacteria* include *Pseudomonas*, *Moraxella*, *Helicobacter*, *Stenotrophomonas*, *Legionella*, acetic acid bacteria; the *Enterobacteriaceae* includes *Escherichia coli*, *Salmonella*, *Shigella* and many others.

1.3.2 Methanotrophic bacteria

Methanotrophs or methanotrophic bacteria are a subset of a physiological group of bacteria known as methylotrophs, i.e. microorganisms able to grow using organic compounds that lack carbon-carbon bonds. Methanotrophs were first detected by Sohngen in 1906 and are distinguished from other microorganisms by their ability to utilize methane (CH_4) as their sole carbon and energy source (Anthony, 1982, 1986 & 1991; Dijkhuizen *et al.*, 1992; Hanson, 1992; Hanson *et al.*, 1991; Lidstrom, 1991; Whittenbury and Dalton, 1981; Whittenbury and Krieg, 1984). The best characterised methanotrophs are aerobic bacteria that play a major role in the global cycling of carbon, nitrogen and oxygen as well as in the degradation of hazardous organic material. Most methanotrophs discovered to date show optimal growth at moderate pH (5-6) and temperature ranges (20-35 °C), but psychrophilic (growth < 15 °C), thermophilic (growth > 40 °C), alkaliphilic (growth at pH > 9.0), and acidophilic (growth at pH < 5) methanotrophs have been isolated (Semrau *et al.*, 2010).

Due to the ability of methanotrophs to synthesise complex biomolecules from methane and also because they are able to oxidise a wide range of hydrocarbons and other chemicals, methanotrophs have attracted the considerable attention on the part of biotechnologists, Thus, they have found applications ranging from production of single cell protein (SCP) from natural gas to bioremediation of trichloroethylene-contaminated ground water. They are also potentially interesting for industrial applications such as production of bulk chemicals. Methanotrophs are widespread in nature, and have been isolated from a variety of environments, typically at the boundary between aerobic and anaerobic conditions where both oxygen and methane are found (Csaki, 2001; Hanson and Hanson, 1996; Lontoh and Semrau, 1998; Oremland and Culbertson, 1992).

Methane is the most stable carbon compound in anaerobic environments and is an important intermediate in the anaerobic mineralization of organic matter (Lontoh, 2000). Methane escapes from anaerobic environments to the atmosphere when it is not oxidized by methanotrophs. Methane is oxidized either photo-chemically in the atmosphere or biologically in terrestrial and aquatic systems. The release of methane to the atmosphere results in an increased rate of global warming and causes other changes in the chemical composition of the atmosphere (Ehalt and Schmidt, 1978; Lelieveld *et al.*, 1993). Figure 1.3, adapted from Ward *et al.* (2004), depicts the ocean, grasslands and desert as major methane sinks, while wetlands, agricultural and grazing lands form the major sources of methane.

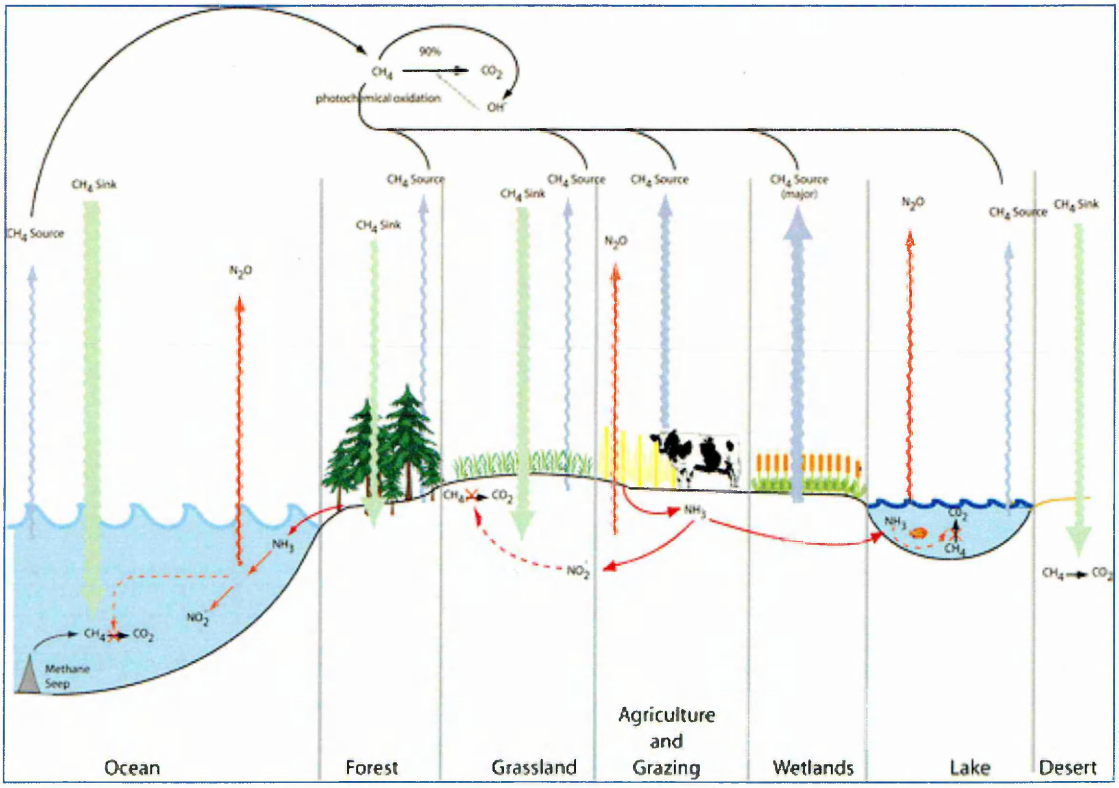


Figure 1.3: Global methane cycle (adapted from Ward et al., 2004)

1.3.2.1 Classification of methanotrophs

The first methanotrophic bacterium that was isolated in 1906 (Sohngen, 1906) was named *Bacillus methanicus*. Then, Whittenbury *et al.* (1970) isolated and characterized more than 100 new methane-utilizing bacteria and thus established the basis for the current classification of methanotrophs. Whittenbury separated the known methane-oxidizing bacteria into five groups; *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylocystis*, and *Methylosinus* based on morphological differences, types of resting stages, the fine structures of intracytoplasmic membranes, and some physiological characteristics (Whittenbury and Dalton, 1981; Whittenbury *et al.*, 1970).

The majority of well characterized methanotrophs are Gram-negative bacteria that are classified as type I or type II methanotrophs according to whether they belong to the γ or α- subdivisions of the proteobacteria respectively although additional groups of methanotrophs are now known (Smith and Murrell, 2009).

1.3.2.2 Methanotrophs and the bioremediation process

Because of the wide substrate range of methane monooxygenase enzymes (MMOs), methanotrophs have attracted the interest of researchers involved in the development of biological methods for degradation of toxic chemicals and organic synthesis (Higgins *et al.*, 1982; Large and Bamforth, 1988). The MMO enzymes, found in methanotrophic bacteria, catalyze the conversion of methane to methanol using dioxygen as the oxidant at ambient temperatures and pressures. These systems have also attracted considerable attention in studying the mechanism of biological methane oxidation (Anthony, 1982; Bedard and Knowles, 1989; Crabtree, 1995; Smith and Murrell, 2009).

Methane and methanol have been important starting materials for the chemical industry for many years and methanotrophs have also been used for the production of single-cell protein as food for farmed fish and livestock. In recent years the biotechnological potential of methanotrophs has been increased by the isolation and characterization of a large number of new methane-utilizing microbes, and studies of

their physiology and genetics (Dalton and Stirling, 1982; Davies and Whittenbury, 1970; Haber *et al.*, 1984; Higgins *et al.*, 1980; Large and Bamforth, 1988; Quayle, 1980; Whittenbury *et al.*, 1970). Recently, methanol has largely replaced methane as a potential feedstock for industrial fermentations involving methanotrophic bacteria. Methanol is abundant, stably priced, relatively safe to use, and easily stored and transported.

The first step in the pathway for methane oxidation is oxidizing methane to methanol by MMO enzymes (Anthony, 1982 & 1986; Dalton, 1991; Dalton and Leak, 1985; Lipscomb, 1994). MMOs are monooxygenases that use two electrons to split the covalent bonds of O_2 (Figure 1.4). One of the oxygen atoms is reduced to form H_2O and the other is incorporated into methane to form CH_3OH.

There are two major pathways for carbon assimilation in methanotrophs; the type I methanotrophs assimilate carbon into cell biomass predominantly by the ribulose monophosphate (RuMP) pathway, and the type II methanotrophs use the serine pathway. Approximately 50% of the carbon is assimilated into cell biomass as formaldehyde and the remaining 50% is further oxidized to CO_2 by formaldehyde dehydrogenase (FADH) and formate dehydrogen (FDH) (Vorholt, 2002).

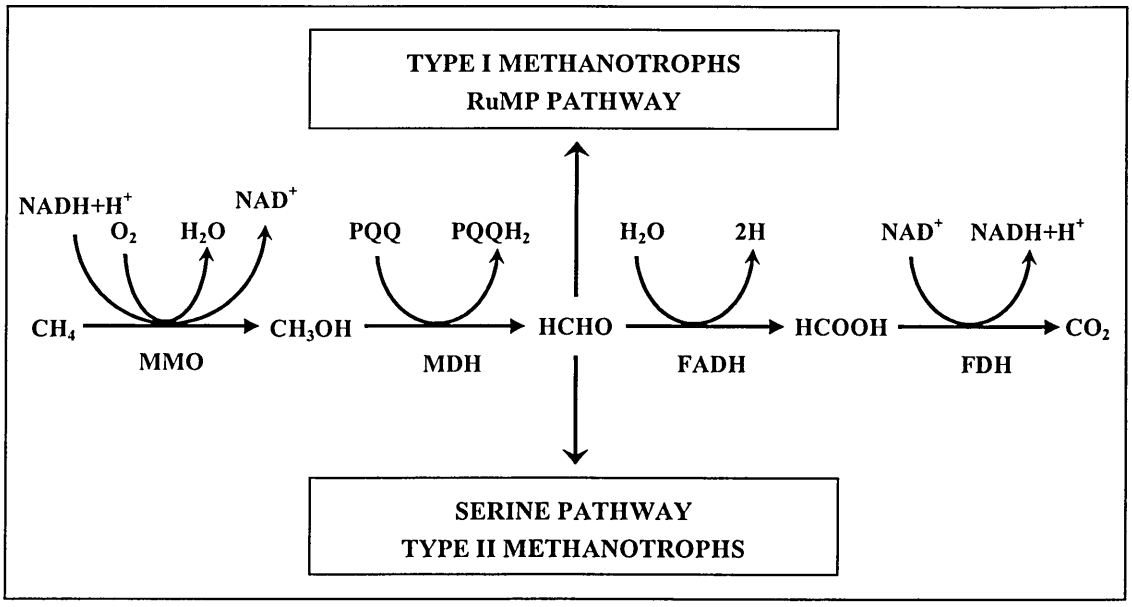


Figure 1.4: Pathways for methane oxidation and assimilation of formaldehyde

1.3.2.3 Enzymology of methanotrophs

Methane-oxidising bacteria possess two distinct types of MMO enzymes: soluble, cytoplasmic enzyme complex (sMMO) and a membrane-bound, particulate enzyme system (pMMO). The single factor that governs expression of the two types of MMO in these organisms is the concentration of available copper (Lee and Lipscomb, 1999; Lloyd *et al.*, 1997). At high copper-to-biomass ratio pMMO is produced, whereas the soluble form of the enzyme (sMMO) is expressed when the copper-to-biomass ratio is low (Jahng and Wood, 1996; Murrell 2000; Prior and Dalton, 1985a; Stanley *et al.*, 1983; Smith and Dalton, 1989; Smith and Murrell, 2008). Several research studies reported that the pMMO enzyme is found in most known methanotrophs and is located in the cytoplasmic membrane.

Methanotrophs that possess only pMMO, such as *Methylomonoas methanica* and *Methylomicrobium album.* Type II methanotrophs *Methylyocella sylvestris* has been shown to possess sMMO but not pMMO. *Methylococcus capsulatus* (Bath) type I and *Methylosinus trichosporium* OB3b (type II) can produce either form of MMO (Smith and Murrell 2009).

1.3.2.3.1 Particulate methane monooxygenase (pMMO)

The pMMO, shown in Figure 1.5, is a multiple copper containing enzyme, although the number and roles of copper ions in the active form of the enzyme continues to be unclear, as the possible involvement of iron in the metal centres of the enzyme (Lieberman and Rosenzweig, 2005; Martinho *et al.*, 2007). pMMO has proven more diffcult to study than sMMO because is loses activity when solubilised (Myronova *et al.*, 2006; Nguyen *et al.*, 1994; Nguyen *et al.*, 1996; Semrau *et al.*, 1995; Lieberman and Rosenzweig, 2005). The first reliable purification procedure for isolation of the pMMO complex from *M. capsulatus* was achieved by Smith and Dalton (1989) using dodecyl β-D maltoside as the solubilising agent.

The active pMMO complex consists of two components, the hydroxylase (pMMOH) which comprise three subunits (α, β, γ) of approximate masses 47, 24, and 22

kDa and a putative reductase (pMMOR) comprising of 63 and 8 kDa proteins (Basu *et al.*, 2003; Zahn and DiSpirito, 1996).

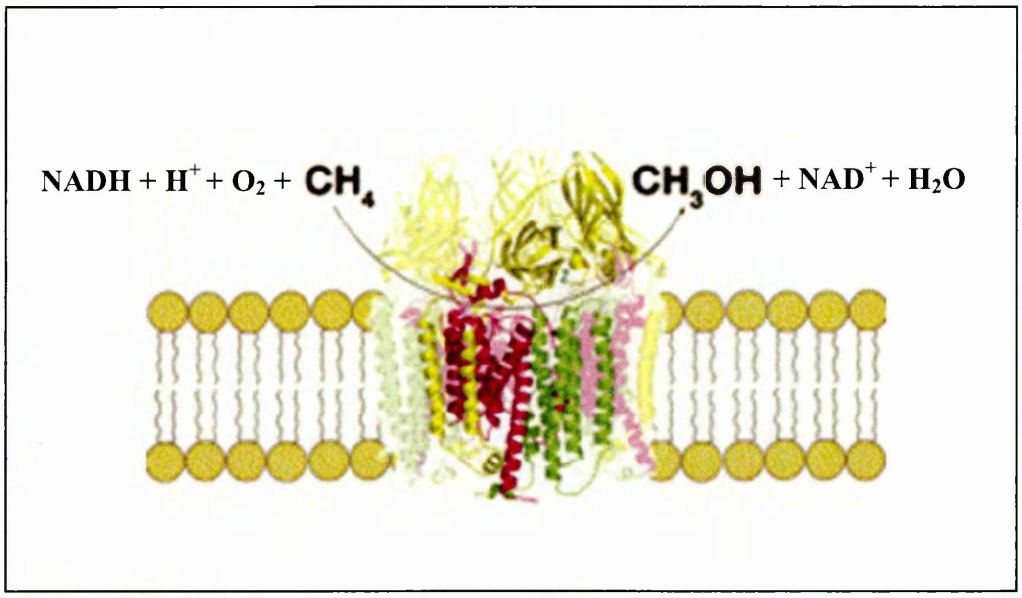


Figure 1.5: pMMO crystal structure showing the proposed association of the enzyme with the lipid bilayer of the membrane (adapted from Lieberman and Rosenzweig, 2005)

1.3.2.3.2 Soluble methane monooxygenase (sMMO)

Unlike pMMO, the soluble methane monooxygenase (sMMO) is cytoplasmic in location, expressed when copper is limiting for growth and only found in some methanotrophs (Murrell *et al.*, 2000a). The sMMO is a non-haem iron-containing enzyme complex consisting of three components. It has been investigated by several research groups (Fox *et al.*, 1990; Liu *et al.*, 1995; Lee *et al.*, 1993). The crystal structure of the sMMO system is illustrated in Figure 1.6.

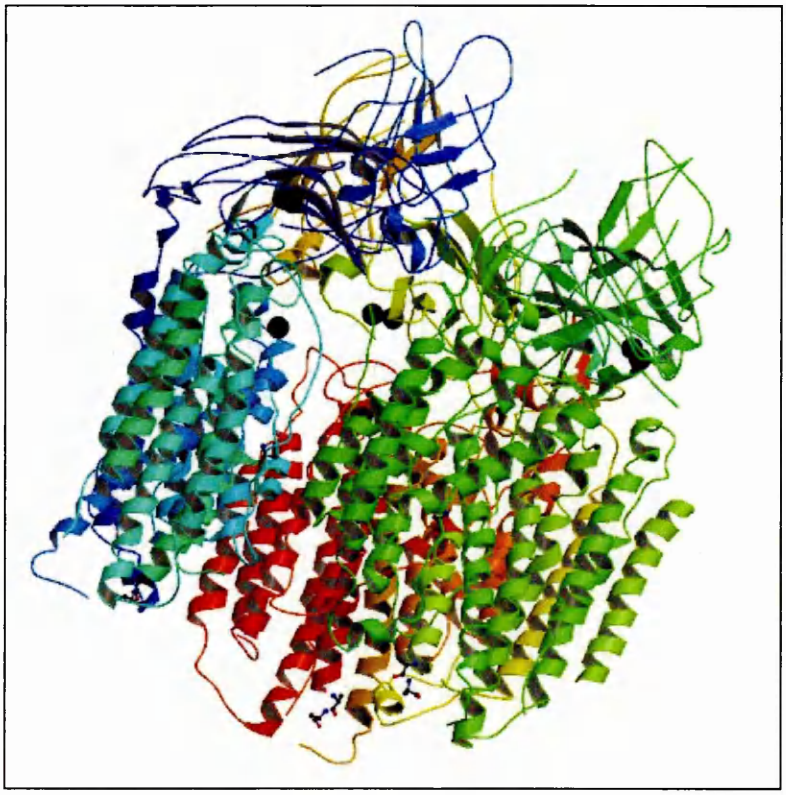


Figure 1.6: sMMO crystal structure (adapted from Lieberman and Rosenzweig, 2005)

The sMMO is a well characterized enzyme, which has broad substrate specificity, oxidizing a wide range of aliphatic and aromatic compounds (Dalton 1992; Semrau *et al.*, 1995; Zahn and DiSpirito 1996; Burrows *et al.,* 1984; Stanley *et al.*, 1983). It has three components: a hydroxylase, a reductase, and a regulatory protein required for activity of sMMO (Colby *et al.*, 1977; Dalton, 1978, 1979; Fox *et al.*, 1989; Hiep 1998; Lipscomb, 1994; Murrell and McDonald, 2001; Pilkington and Dalton, 1990; Wallar and Lipscomb, 1996, 2001). As illustrated in Figure 1.7, the hydroxylase component is composed of three subunits with molecular masses of approximately 60 kDa (α-subunit), 50 kDa (β-subunit) and 22 kDa (γ-subunit). The reductase component is NADH-dependent and composed of one polypeptide with a molecular mass of 38-40 kDa containing both FAD and Fe-S cofactors (Fox *et al.*, 1989). Component B is a 15-17 kDa regulatory protein (Green and Dalton, 1985; Wallar and Lipscomb, 2001).

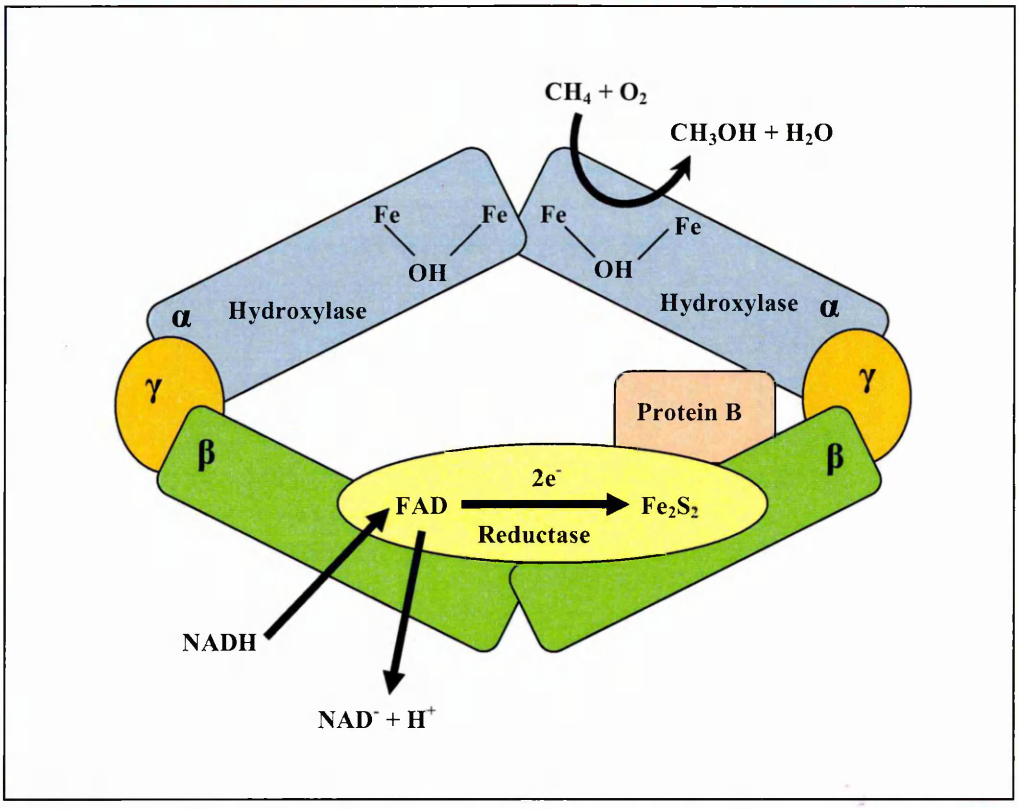


Figure 1.7: Model of sMMO complex from Methylococcus capsulatus (Bath) (adapted from Bowman et al., 1991).

1.4 Bioremediation of chromium

Bioremediation is the use of biological organisms such as bacteria, fungi, green plants or their enzymes to reduce, remove, or immobilize contaminants (McCullough *et al.*, 1999; Volesky, 1986). The popularity of bioremediation approaches is attributed to their low cost, high efficiency, minimisation of chemical and biological sludge and possibility of metal recovery. Biological treatment could significantly reduce the costs for chemicals and energy compared with conventional physico-chemical treatments described earlier.

Biological reduction of the more toxic Cr (VI) to the less toxic Cr (III) can be achieved by the use of microorganisms as biotechnological tool for remediation of

chromate-polluted wastewater. Bioremediation of toxic chromium compounds is of a special interest since this metal has found a variety of industrial applications (Barnhardt, 1997) and as a consequence it causes severe environmental problems due to its extreme toxicity to living organisms (Cieslak-Golonka, 1995; Cohen *et al.*, 1993). A large number of bacterial strains have been evaluated for reducing Cr (VI) to Cr (III) (Cher and Hao, 1996; Fude *et al.*, 1994; Lovely and Philips, 1994; White *et al.*, 1997).

In general bioremediation of heavy metals operates by conversion of the metal ions into insoluble forms by specific enzyme-mediated reactions that remove them from the aqueous phase (Park *et al.*, 2000). A number of bacteria possess chromate reductase activity that can convert Cr (VI) to Cr (III), which is much less toxic and less soluble, and thus reduction by these enzymes provides a means of chromate bioremediation (Gadd and White, 1993).

1.4.1 Types of microorganisms capable of hexavalent chromium bioremediation

Microbial reduction of Cr (VI) is considered to be an effective alternative method to the use of chemicals (Cervantes *et al.*, 2001). Since the discovery of the first microbe capable of reducing Cr (VI) in the 1970s (Romanenko and Korenkov, 1977), a number of bacterial strains (both aerobic and anaerobic) have been isolated and investigated for Cr (VI) reduction such as *B. cereus*, *B. subtilis*, *Pseudomonas. aeruginosa*, *Pseudomonas ambigua*, *Pseudomonas fluorescens*, *E. coli*, *Achromobacter eurydice*, *Micrococcus roseus*, *Desulfovibrio desulfuricans* and *D. vulgaris* (Lovley, 1993 & 1994). *E. coli* (ATCC 33456) and *Shewanella alga* (BrY-MT) have also been shown to reduce Cr (VI) (Blake II *et al.*, 1993; Camargo *et al.*, 2003; Cheung *et al.*, 2006; Guha *et al.*, 2001; Shakoori *et al.*, 1999 & 2000; Shen and Wang, 1994; Wang and Xiao, 1995).

Bacterial strains such as *Acintobacter haemolyticus* (Ahmad *et al.*, 2013), *Desulfomicrobium norvegicum* (Michel *et al.*, 2003), *Bacillus* (Camargo *et al.*, 2003), *Shewanella* (Guha *et al.*, 2001; Middleton *et al.*, 2003; Viamajala *et al.*, 2003) *Desulfovibrio* (Chardin *et al.*, 2002), *Escherichia coli* (Puzon *et al.*, 2002), *Pseudomonas*

(McLean and Beveridge, 2001), and *Alcaligenes* (Peitzsch *et al.*, 1998) can reduce Cr (VI) to Cr (III).

Sulfate-reducing bacteria (SRB) have been extensively studied for reduction of metals, including Cr (VI) (Chardin *et al.*, 2002; Lovley, 1995; Michel *et al.*, 2001). *Enterobacter cloacae* HO1 is a Cr (VI) reducing facultative anaerobe. In this species, oxygen inhibits Cr (VI) reduction as does the presence of other toxic metals (Kato and Ohtake, 1991; Ohtake *et al.*, 1990; Wang *et al.*, 1989). Other reported Cr (VI) reducing anaerobes include *Microbacterium sp.* MP30 (Pattanapipitpaisal *et al.*, 2001), *Geobacter metallireducens* (Lovley *et al.*, 1993), *Shewanella putrefaciens* MR-1 (Myers *et al.*, 2000), *Pantoea agglomerans* SP1 (Francis *et al.*, 2000), *Agrobacterium radiobacter* EPS-916 (Llovera *et al.*, 1993) and a consortium that can both reduce Cr (VI) and degrade benzoate (Shen *et al.*, 1996). Several attempts have been reported of Cr (VI) reductase purification from pseudomonads (Ackerley *et al.*, 2004; Cheung *et al.*, 2006; Kwak *et al.*, 2003; Park *et al.*, 2002).

A number of studies have shown biosorption of chromium species by yeast (Rapoport and Muter, 1995; Raspor *et al.*, 2000), especially *Saccharomyces cerevisiae* which is a waste product of industrial fermentations (Engl and Kunz, 1995; Krauter *et al.*, 1996; Volesky and May-Phillips, 1995). *S. cerevisiae* can accumulate chromium and cadmium (Brady and Duncan, 1994) and other metals (Raspor *et al.*, 2000). Among other yeast, Cr (VI) resistant *Candida* sp. and *Rhodosporidium* strains have been isolated from industrial wastes (Pepi and Baldi, 1992), *Schizosaccharomyces pombe* wild type and mutant strains have been studied for uptake and tolerence of Cr (VI) (CzakoVer *et al.*, 1999), and *Candida intermedia* has been used to study the Cr (III) accumulation (Batic and Raspor, 2000).

Faisal and Hasnain (2004) reported that chromium resistant bacteria isolated from effluent of tanneries could resist up to 250 μgml^{-1} of Cr (VI) in the medium. They also reported that the amount of chromium accumulated by the cells increased with increase in concentration of chromium from 100 to 1000 μgml^{-1}. Megharaj *et al.* (2003) also reported another bacterial strain, which was isolated from polluted soil, could resist up to 100 μgml^{-1} of Cr (VI). Besides chromium, bacterial strains such as those reported by Filali *et*

al. (2000) are resistant to a broad range of heavy metals (Mn, Ni, Zn, Pb, Cu and Co) and antibiotics (ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline). The simultaneous presence of resistances to metals and antibiotics is consistent with the co-localisation of genes specifying resistance to metals and antibiotics on the same plasmids and also suggests that such strains could be used for the bioremediation of multiply contaminated industrial effluents.

The first enzyme described with the ability to transform Cr (VI) to Cr (III) was a Cr (VI) reductase from chromate-resistance *Enterobacter cloacae* HO1 (Ohtake *et al.*, 1990). This is a membrane-associated enzyme that transfers electrons to Cr (VI) via NADH-dependent cytochromes (Wang *et al.*, 1990). Ishibashi *et al.* (1990) partially purified a soluble Cr (VI) reductase from *Ps. putida* PRS2000. Suzuki *et al.* (1992) reported a 38-fold purification of a soluble Cr (VI) reductase from *Ps. ambigua*. In *Ps. aeruginosa*, Cr (VI) resistance is attributed to the decreased uptake and enhanced efflux of Cr (VI) by the cell membrane (Bopp *et al.*, 1983; Ohtake *et al.*, 1987; Alvarez *et al.*, 1999; Aguilera *et al.*, 2004). A similar mechanism of resistance has been reported for *Alcaligenes eutrophus* CH34 (Valls *et al.*, 2000; Vaneechoutte *et al.*, 2004). Conversely, other investigations with *Ps. fluorescens* showed that Cr (VI) resistance does not depend upon the capacity of the organism to reduce this chemical (Bopp and Ehrlich, 1988). Thus, Cr (VI) reduction activity was found to be same in sensitive (wild-type) and resistant (mutant) strains when assayed at sub-lethal Cr (VI) levels. Other pseudomonads capable of reducing Cr (VI) include a close relative of *Pseudomonas synxantha* and an unidentified species (Gopalan and Veeramani, 1994; McLean *et al.*, 2000).

In a recent study reported by Alhasin *et al.* (2010), a methane-oxidizing bacterium has been investigated in Cr (VI) reduction. The study demonstrated the ability of *Methylococcus capsulatus* (Bath) strain to remediate Cr (VI) over a wide range of concentration (1.4 – 1000 mg l^{-1}).

1.4.2 Mechanisms of bacterial chromium reduction

Chromate crosses the biological membranes of the bacterial cell, as illustrated in Figure 1.8, by the means of sulfate uptake pathway, which reflects the chemical analogy between these two oxyanions. Inside the cell, Cr (VI) is reduced to Cr (III) by action of various enzymatic or nonenzymatic activities; the Cr (III) generated may then exert diverse toxic effects in the cytoplasm (Ramirez-Diaz *et al.*, 2008).

At the extracellular level, Cr (VI) rapidly enters to the cytoplasm where it may exert its toxic effects (Katz and Salem 1993; Wong and Trevors 1988). In the cytoplasm, Cr toxicity is mainly related to the process of Cr (VI) reduction to lower oxidation states (i.e. Cr (III) or Cr (V)) during which free radicals may be formed (Kadiiska *et al.*, 1994; Shi and Dalal 1990). It has been reported in several research studies (Cervantes and Campos-Garcia 2007; Nies *et al.*, 1998) that enzymes catalysing chromate reduction in bacterial isolates may be encoded either by plasmids or by chromosomal genes.

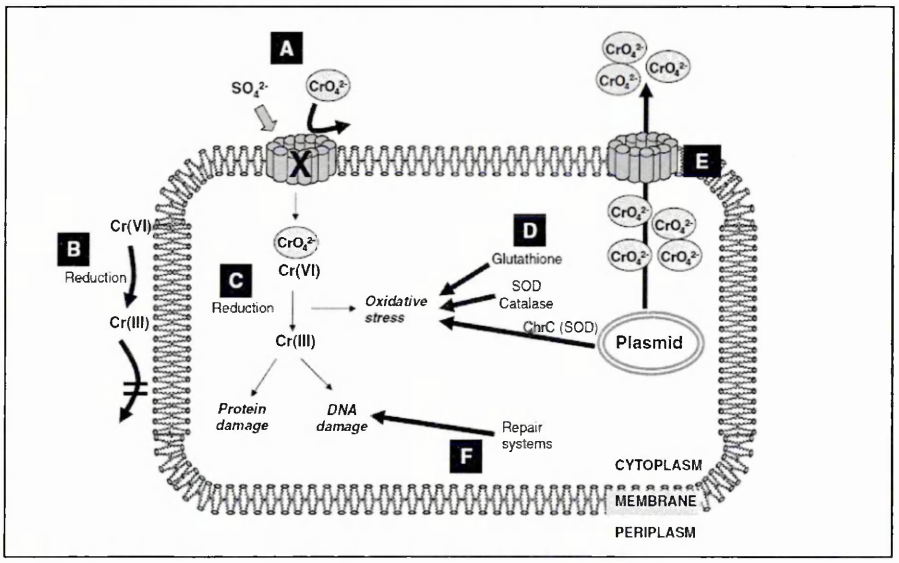


Figure 1.8: Mechanisms of chromate transport, toxicity and resistance in bacterial cells (Adapted from Ramirez-Diaz et al., 2008)

In Figure 1.8, 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 this pathway 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 Cr derivatives.

Several bacterial Cr (VI) reductase enzymes show an NADH: flavin oxidoreductase activity and can use Cr (VI) as electron acceptor (Gonzalez *et al.*, 2005). The ability to reduce chromate may be a secondary function for the "Cr (VI) reductase" enzymes, which may have a different natural role within the cell besides Cr (VI) reduction. For instance the nitroreductases NfsA/NfsB from *Vibrio harveyi* is a nitrofurazone nitroreductase that also exhibits Cr (VI) reductase activity as a secondary function (Kwak *et al.*, 2003). Similarly, ferric reductase (FerB) from *Paracoccus dentrificans* can use both Fe (III)-nitrilotriacetate and Cr (VI) as substrates (Mazoch *et al.*, 2004).

Park *et al.* (2000) studied ChrR from *Pseudomonas putida* as a Cr (VI) reductase. This enzyme, which is a 50 kDa dimer, is a soluble flavin mononucleotide-binding protein with NADH-dependent reductase activity. This multifunctional protein, besides its role as a Cr (VI) reductase, also reduces ferricyanide (Ackerley *et al.*, 2004). Studies with enzyme mutants showed that ChrR protects against chromate toxicity; this is possibly because it prevents the production of reactive oxygen species (ROS) by reactions involving Cr (VI) (Ackerley *et al.*, 2004).

1.5 Aims and objectives of the study

The main aim of this study is to investigate the capability of five Gram-negative bacterial strains for remediation of hexavalent chromium. Three of the bacterial strains

employed in this research were nosocomial isolated bacteria; *Ps. aeruginosa* 090124-B isolated from left leg ulcer, *E. cloacae* 090226-B isolated from a sputum sample. The third bacterial strain is *P. mirabilis* 090321-A, which was isolated from a wound swab and to the author's knowledge is the first strain of *P. mirabilis* to be tested for chromate VI bioremediation activity. Bacteria isolated from hospital infections have been found in other previous studies to contain genes that confer antibiotic resistance to inorganic ions derived from mercury (Porter *et al.*, 1982; Nasaru *et al.*, 2004), cadmium (Nucifora *et al.*, 1989), silver (Gupta *et al.*, 2001), and arsenic (Silver *et al.*, 1981). Such bacteria possess heavy metal resistance genes that may be present on chromosomes, plasmids, or transposons (Silver and Phung, 2005). Here, similar strains were investigated for their ability to reduce chromium (VI) and to bio-absorb chromium species.

E. coli DH5α bacterial strain, which was obtained from the microbiology laboratory of the Biomedical Research Centre (BMRC) at Sheffield Hallam University, was also used as a benchmark for Cr (VI) reduction activity. In this study, further detailed analyses were as carried out using *M. capsulatus* (Bath), which was obtained from NCIMB, Scotland, UK.

The present work addresses bioremediation and has the following objectives:

- To implement techniques of analysis by monitoring bioremediation of chromium (VI) as described in the literature using the proposed Gram-negative bacterial strains.
- To select appropriate media for cultivation of bacterial strains to avoid competing chemical reactions of the chromium VI directly with components of the medium.
- To identify the enzymes that are responsible for Cr (VI) reduction or removal in *P. mirabilis* and *M. capsulatus* (Bath) bacterial strains.
- To identify the chromium species resulting from the bioremediation process of Cr (VI) using *P. mirabilis* and *M. capsulatus* (Bath) bacterial strains.

1.6 Outline of the thesis

The organization of the thesis reflects the sequence of steps involved in the bioremediation of hexavalent chromium using Gram-negative bacteria. A brief outline of the contents of the thesis is as follows:

- Chapter 1 presents background, literature review of chromium and environmental bacteria.
- Chapter 2 describes the materials and methods and media selection for bacterial strains cultivation.
- Chapter 3 investigates Cr (VI) interaction with Gram-negative bacteria.
- Chapter 4 focuses on further analyses during the bioremediation process of Cr (VI) remediation using *P. mirabilis*.
- Chapter 5 also concentrates on detailed analyses of Cr (VI) remediation using *M. capsulatus*.
- Chapter 6 presents overall conclusions and discussion of the results, together with possible future research directions.

2.1 Materials

2.1.1 Chemicals

1, 5-Diphenylcarbazide (DPC) reagent, sodium azide, and components of NMS and M9 media (for composition of media shown below) were supplied by Sigma-Aldrich, UK. Methane (99.95%) was supplied by Scientific and Technical Gases Ltd., UK. Acetone and sulfuric acid were supplied by Science, UK. Nutrient agar and nutrient broth no.2 were supplied by Sigma-Aldrich, UK. Potassium dichromate, chromium (III) nitrate nonahydrate, and 99% phenyl acetylene were supplied by Sigma, chemical, UK. Tris-HCl was supplied by Fisher Scientific and lysozyme was obtained from Sigma–Aldrich, UK.

2.1.2 Media

Three types of media were initially proposed to be used for bacterial cultivation. Their compositions are described in the following sub-sections. All solutions and growth media were prepared with de-ionized water and sterilised by autoclaving at 15 psi for 15 minutes at 121 °C.

2.1.2.1 Nutrient broth No. 2 Medium

Nutrient Broth No.2, is a general purpose liquid medium for the cultivation of microorganisms. The composition of this medium (supplied by Sigma-Aldrich UK) is shown in Table 2.1. A suspension 28 g of this medium in 1000 ml of distilled water (pH 7.5) was sterilised by autoclaving at 121 °C for 15 minutes.

Table 2.1: Composition of Nutrient broth No. 2 Medium

Ingredient	Quantity
Meat peptone	4.3 g/l
Casein peptone	4.3 g/l
Sodium chloride	6.4 g/l

2.1.2.2 M9 Medium

M9 medium is a chemically defined growth medium used for bacterial cultures (Atlas, 1993). It was prepared in the laboratory as described in Table 2.2. All constituents were added, except $MgSO_4.7H_2O$, glucose, Thiamine.HCl and $CaCl_2$ solutions, to deionized water and pH was adjusted to 7.0. After autoclaving, the mixture was allowed to cool down to room temperature. Then, $MgSO_4.7H_2O$, glucose, Thiamine.HCl and $CaCl_2$ solutions were aseptically added to the mixture, which was immediately distributed into autoclaved 250 ml flasks.

Table 2.2: Composition of M9 Medium

Ingredient	Quantity
Na_2HPO_4	6.0 g/l
KH_2HPO_4	3.0 g/l
NH_4Cl	1.0 g/l
NaCl	0.5 g/l
Glucose solution (20 g/l)	10.0 ml
$MgSO_4.7H_2O$ solution (246.5 g/l)	1.0 ml
Thiamine.HCl solution (10 mg/l)	1.0 ml
$CaCl_2$ solution 14.7 g/l	1.0 ml

2.1.2.3 NMS Medium

The nitrate mineral salts (NMS) medium was used for growing methanotrophic bacteria (Dalton & Whittenbury, 1976; Whittenbury *et al.*, 1970). All reagents were autoclaved and separately, sterilised phosphate buffer (pH 6.8) was added as detailed in Table 2.3 after the medium had cooled to room temperature.

Table 2.3: Compositions of NMS medium

Ingredient	Volume
10 x NMS Salts: KNO_3 (10 g) + $MgSO_4.7H_2O$ (10 g) + $CaCl_2.2H_2O$ (2 g) + H_2O to make 1 litre and stored at 4 °C.	100 ml
Na molybdate solution: $NaMoO_4.2H_2O$ (0.5 g) + H_2O to make 1 litre and stored at 4 °C.	1 ml
NMS Trace Elements: $CuSO_4.5H_2O$ (100 mg) + $FeSO_4.7H_2O$ (500 mg) + $ZnSO_4.7H_2O$ (400 mg) + H_3BO_4 (15 mg) + $CoCl_3.6H_2O$ (50 mg) + Na_2EDTA (250 mg) + $MnCl_2.6H_2O$ (20 mg) + $NiCl_2.6H_2O$ (10 mg) + H_2O to make 1 litre and stored at 4 °C.	1 ml
Fe EDTA Solution: Fe EDTA (3.8 g) + H_2O to make 100 ml and stored in the dark at 4 °C.	0.1 ml
Water	to 1 litre
NMS Phosphate Buffer Solution: Na_2HPO_4 (49.7 g) + KH_2PO_4 (39 g) + H_2O to 1 litre.	10 ml

2.1.3 Bacterial strains

Five Gram-negative bacterial strains were used in this study. Three of them were nosocomial isolated bacteria, which were obtained from a previous study of hospital microorganisms in this research group. *Pseudomonas aeruginosa* 090124-B was isolated

from left leg ulcer, *Enterobacter cloacae* 090226-B was isolated from a sputum sample and *Proteus mirabilis* 090321-A was isolated from wound swab. The other two strains; *Escherichia coli* DH5α was obtained from the microbiology laboratory of the Biomedical Research Centre (BMRC) at Sheffield Hallam University and *Methylococcus capsulatus* (Bath) was obtained from NCIMB, Scotland, UK.

2.1.3.1 Cultivation and maintenance of bacterial strains

E. coli DH5α, *E. cloacae*, *P. mirabilis* and *Ps. aeruginosa* were routinely cultivated in 250 ml conical flask containing 50 ml of M9 medium and incubated in an orbital shaker (180 rpm) at 37 °C.

M. capsulatus (Bath) were grown and propagated aerobically in 250 ml conical quickfit flasks containing 50 ml of a NMS medium in the presence of methane as the source of carbon and energy. Flasks were sealed with suba-seals (Fisher Scientific, UK) to facilitate the addition of methane using a sterile plastic syringe (Fisher Scientific, UK) fitted with hypodermic needle (Fisher Scientific, UK). 50 ml of air was then aseptically replaced with 60 ml of methane. The flasks were then incubated at 45 °C and 180 rpm in the orbital incubator shaker.

The bacterial growth, during the cultivation process, was monitored by measuring the optical density (at 600 nm) using 6715 UV/Vis spectrophotometer (JENWAY). All strains were stored at -80 °C for long-term storage in the presence of 50 % (v/v) sterile glycerol, after flash freezing in liquid nitrogen.

The bacterial strains were also cultivated on solid media. *E. coli*, *E. cloacae*, *P. mirabilis* and *Ps. aeruginosa* were grown on nutrient agar plates (28 g/l) and incubated at 37 °C. While, *M. capsulatus* (Bath) was grown on NMS agar plates (15 g of bacteriological agar was added to 1 litre of NMS medium prior to autoclaving) in the presence of methane (1:4 v/v in air) as the source of carbon and energy were incubated in aerobically stainless steel jar at 45 °C.

2.1.3.2 Bacterial strain purity check

The purity of *M. capsulatus* (Bath) was routinely checked by plating cultures onto nutrient agar plates, which were incubated at 37 °C for 24 hours. *M. capsulatus* does not grow on nutrient agar and so if any microorganisms grew on these plates this indicated contamination.

2.1.4 Equipment

- 3505 pH meter JENWAY designed and manufactured in UK, by Bibby Scientific Ltd.
- Spectrophotometer 6715 UV/Vis. spectrophotometer JENWAY single holder, supplied by Bibby Scientific Ltd., UK.
- Inductively coupled plasma-optical emission spectrometry (ICP-OES) ACTIVA HORIB JOBIN YVON Gen Co., made in France.
- A Hewlett Packard 4500 quadrupole inductively coupled plasma-mass spectrometer (ICP-MS), model number HP 4500 G 1820A, manufactured in Japan.
- Autoclave was purchased from Classic Prestige Medical, UK.
- Orbital incubator shaker was obtained from Gallenkamp, UK, and orbital incubator shaker S1 50, STUART Scientific, made in UK. Incubator Heraeus Kendro laboratory products, made in Germany.
- Modulyod 230 Freeze Dryer purchased from Thermo Electron Corporation, UK.
- Sorvall RT 6000D Centrifuge supplied by Thermo Electron Corporation, UK.
- RC6 centrifuge Thermo scientific Sorvall RC6 plus centrifuge Thermo Electron Corporation, UK.
- Optima ultracentrifuge Beckman Coulter made in USA.
- French press cell Disruptor Thermo Electron Corporation, UK.
- FTIR, Perkin Elmer Spectrum, UK.

2.2 Methods

2.2.1 Chromium (VI) standard curves using diphenylcarbazide (DPC) assay

2.2.1.1 Preparation of potassium dichromate stock solution

Chromium (VI) solution (1.27 mg/l) was prepared by dissolving 3.6 mg of $K_2Cr_2O_7$ and 10 ml of concentrated sulfuric acid into 900 ml distilled water to make up a solution of 1000 ml.

Six test tubes were obtained and labelled (1, 2, 3, 4, 5 and 6). The quantities of $K_2Cr_2O_7$ and H_2SO_4 (0.18 M) were added to each test tube as shown in Table 2.4 below:

Table 2.4: Amounts of $K_2Cr_2O_7$ and H_2SO_4 used for the standard curve

Tube no.	1	2	3	4	5	6
$K_2Cr_2O_7$ (ml)	0.00	0.4	1.0	2.0	4.0	10
H_2SO_4 (ml)	10	9.6	9.0	8.0	6.0	0.0

The content of each test tube was mixed by shaking. Then, 0.5 ml of diphenylcarbazide solution (0.5 g in 200 ml of acetone) was added to each test tube. The contents of each test tube were mixed and left to stand for 5 minutes for colour development. The absorbance of each sample was measured using the spectrophotometer at a wavelength of 540 nm (Herrmann, 1994). A standard curve of known Cr (VI) concentrations was prepared in the same medium used for the experiments. The standard curve showed a high degree of linearity. Figure 2.1 shows representative standard curve in water, which showed a high correlation coefficient $R^2 = 0.9978$. This curve was then used to estimate Cr (VI) concentration in the presence of media employed and lysozyme solution and tris-HCl solutions (full details are shown in Appendix A).

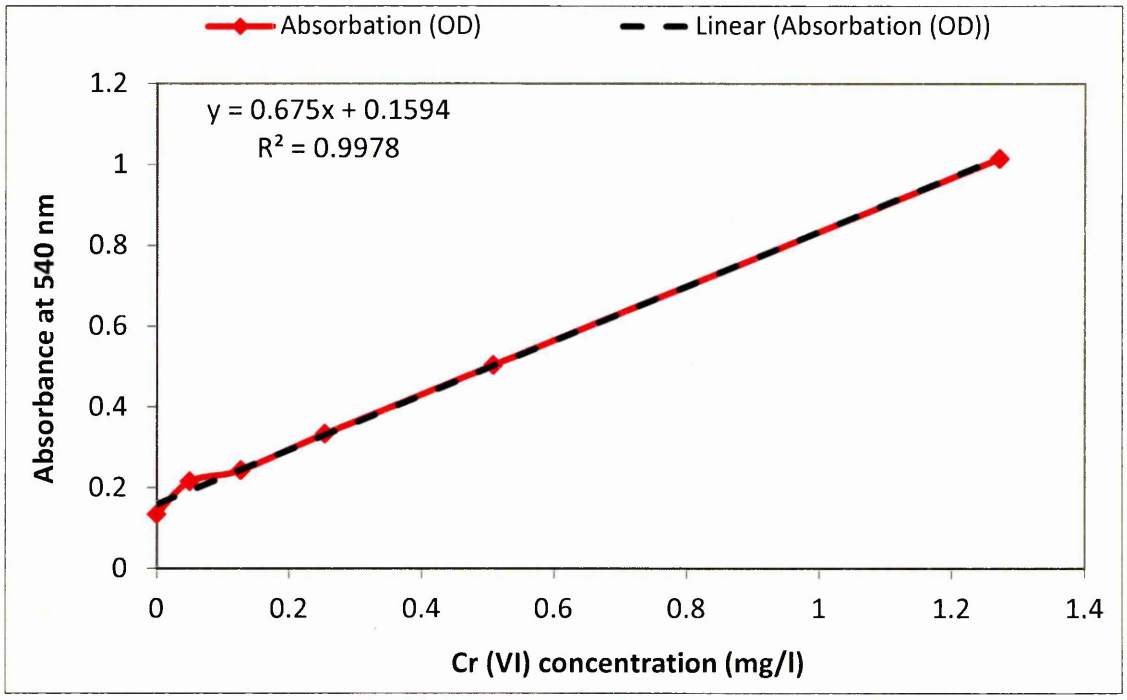


Figure 2.1: Standard curve for Cr (VI) concentration using the DPC assay

2.2.2 Standard curve for Cr (VI) concentration in M9, NMS and nutrient broth no.2 media, lysozyme and tris-HCl solutions

2.2.2.1 Materials

Cr (VI) solution (1.27 mg/l), 3 M sulfuric acid solution, and 1, 5-diphenylcarbazide solution (0.5 g in 200 ml acetone) were prepared as described above. Three types of media (M9, NMS and nutrient broth no.2), lysozyme (0.5 mg/l) and tris-HCl (50 mM) solutions (pH 7.5) were used to verify their reaction with Cr (VI) prior the bioremediation process.

2.2.2.2 Chromate assay

The same method described in section 2.2.1 was followed with slight modification, as follows: six test tubes were obtained and labelled (1, 2, 3, 4, 5 and 6). 5 ml of utilised media and 0.6 ml of 3 M H_2SO_4 were added to each tube. The amounts of $K_2Cr_2O_7$ and deionised water were added to each test tube according to Table 2.5:

Table 2.5: Amounts of $K_2Cr_2O_7$ and deionised H_2O

Tube no.	**1**	**2**	**3**	**4**	**5**	**6**
$K_2Cr_2O_7$ (ml)	0.00	0.4	1.0	2.0	4.0	0.0
H_2O (ml)	4.4	4.0	3.4	2.4	0.4	0.0

The content of each test tube was mixed by shaking. 0.5 ml of diphenylcarbazide solution (0.5 g in 200 ml acetone) was added to each test tube. The contents of the test tubes were mixed and left to stand for 5 minutes for colour development. The absorbance of each sample was measured at 540 nm. The standard curves for Cr (VI) concentration in M9, NMS and nutrient broth no. 2 media, lysozyme solution and tris-HCl are shown in Figure 2.2.

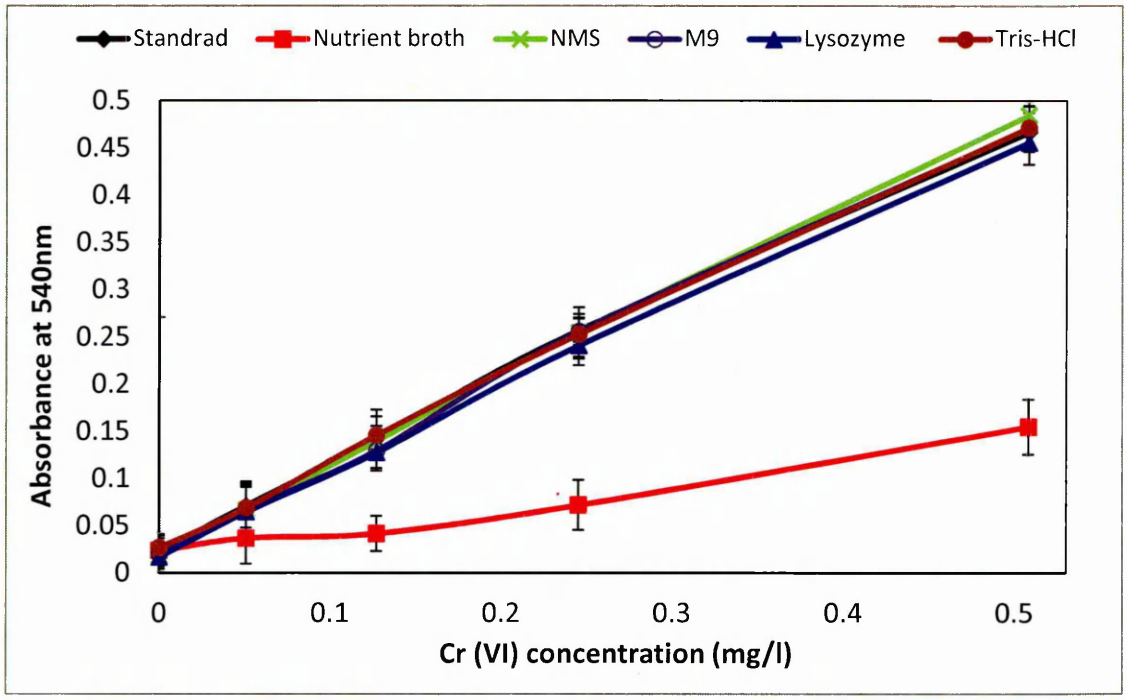


Figure 2.2: Standard curve for Cr (VI) concentration for several media

It was found that the absorbance in the diphenylcarbazide assay with increasing Cr (VI) concentration in nutrient broth no. 2 medium is far lower than that observed in other media and solutions. This was attributed to the reaction that took place between Cr (VI) and the nutrient broth no. 2 medium, which contains meat and casein peptone. Thus, nutrient broth no. 2 medium was excluded from use throughout the work.

2.2.3 Laboratory-scale bioremediation reactions

When liquid bacterial cultures had grown to a sufficient extent (OD_{600} = 0.3-1.0), 1 ml of filter sterilised $K_2Cr_2O_7$ was added to the bacterial culture to give a final Cr (VI) concentration of 2.54 mg/l. Then, 4 ml samples of the mixture were taken at different incubation times (0, 4, 12, 24, 36, 48, 60, 84, 96, 108 and 120 hours). At each incubation time, the mixture was centrifuged at room temperature at 5000 *g* for 5 minutes. Supernatant samples were then separated from the pellets.

Lysozyme solution (4 ml) was added to the pellets, to digest cell-wall peptidoglycan. The lysozyme solution consisted of 50 µl of 0.5 mg/ml fresh lysozyme solution in 1:1 v/v of tris-HCl (20 mM at pH 7.5) and NaCl (20 mM). The pellets were incubated at 37 °C and 180 rpm for 1 hour in orbital incubator shaker. A schematic diagram of the bioremediation experiment is shown in Figure 2.3.

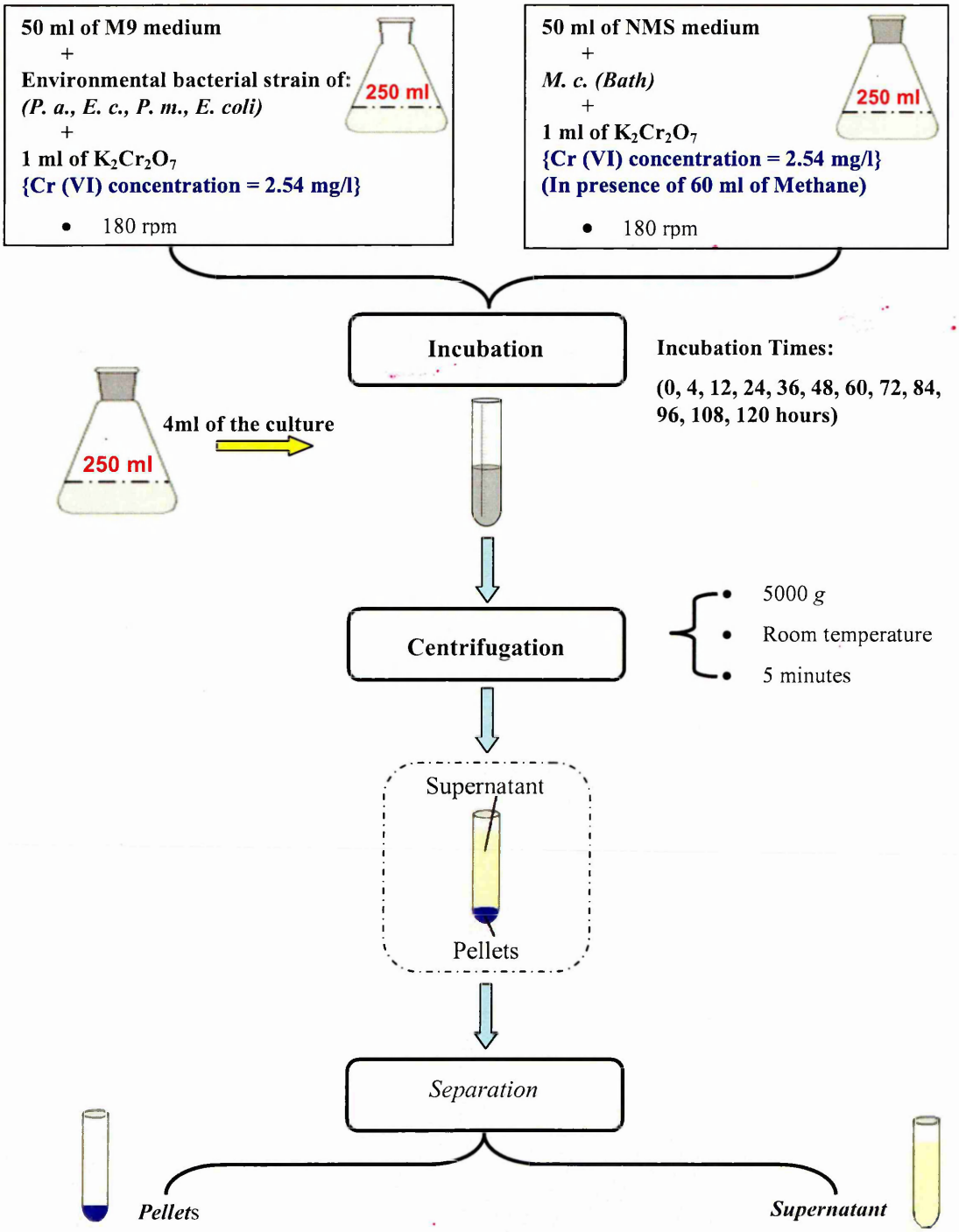


Figure 2.3: Schematic diagram of the bioremediation experiment and sampling procedure

2.2.4 *Measurement of Cr (VI) concentration removal using diphenylcarbazide (DPC) assay*

Cr (VI) concentration was measured using DPC assay analysis. Cells and other particulate material were removed from liquid samples by centrifugation at 5000 *g* for 5 minutes at room temperature. The pellets were solubilised using lysozyme solution (0.5 mg/ml of fresh lysozyme in 20 mM tris-HCl and 20 mM NaCl at pH7.5) incubated at 37 ^{o}C for one hour in order to release cytoplasmic contents. The supernatant was acidified by addition of 3 M H_2SO_4 to give a final concentration of 0.18 M. The acidified supernatant was then mixed with 0.2 ml of diphenylcarbazide solution (2.5 g per litre in acetone) and the chromate (VI) concentration was estimated spectrophotometrically at 540 nm by reference to a standard curve of known chromate concentration prepared in the same medium as described above.

2.2.5 *Measurement of total chromium concentration using inductively coupled plasma-optical emission spectrometry (ICP-OES)*

Inductively coupled plasma (ICP) is a type of plasma, which is a state of matter similar to gas in which a certain portion of the particles are ionized. The ICP-OES (optical emission spectrometry) plasma is an effective source of atomic and ionic emission, which can in principle be used for the determination of all the elements other than argon and helium. The ICP-OES technique, illustrated in Figure 2.4, works on the principle that if an atom is provided with energy, electrons within that atom will move from their ground state to higher energy levels. As those electrons return to the ground state, energy is released in the form of light. The wavelengths of light that are emitted are unique to each element and the intensity of light is directly proportional to the number of atoms (or concentration) of the element. Within the instrument, that light is separated into its component wavelengths by a diffraction grating before passing to a photomultiplier, which acts as the detector.

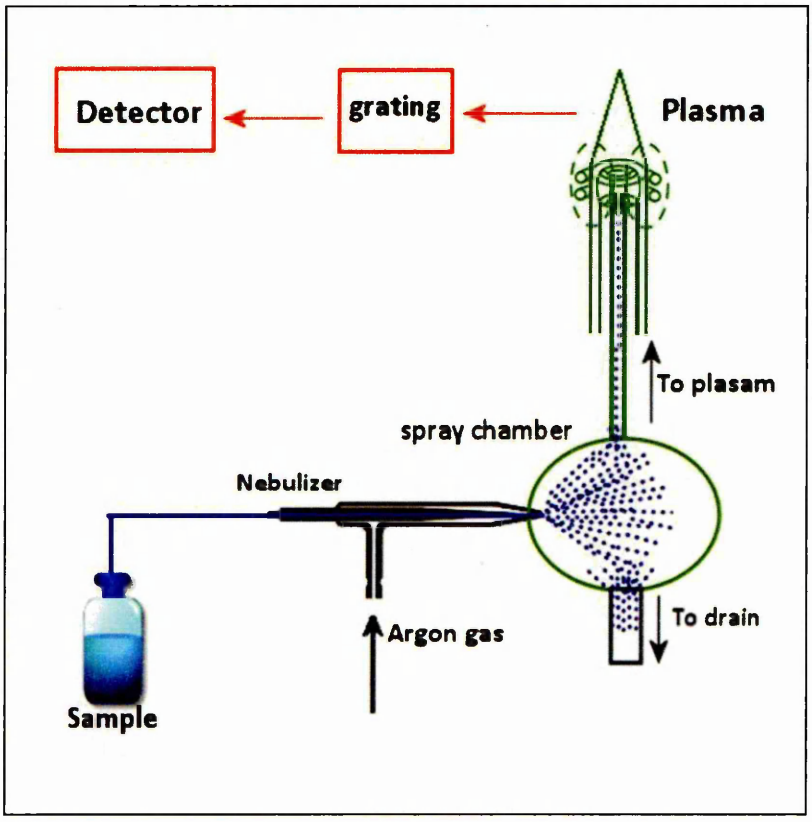


Figure 2.4: Schematic of ICP-OES (adapted from http://www.chemiasoft.com/chemd/node/52)

In this work, the analysis of total chromium concentration during the bioremediation process was monitored using ICP-OES (Figure 2.5), made using equipment manufactured by Activa Horib Jobin Yvon Gen Co., France. Cells and other particulate material were removed from liquid samples by centrifugation at 5000 *g* for 5 minutes at room temperature. Then, 4.0 ml of the supernatant was analysed and the total chromium concentration of chromium in the pellets was measured after solubilisation with lysozyme solution, as described in section 2.2.4.

Figure 2.5: ICP-OES (Activa Horib Jobin Yvon Gen Co., France)

2.2.5.1 Calibration of ICP-OES

1 ml of standard chromium for ICP-OES (1000 mg/l), supplied by Sigma Chemicals UK, was diluted with deionised water to give 1, 5 and 10 mg/l standard chromium solution.

The typical calibration curve of standard chromium concentration using ICP-OES is shown in Figure 2.6.

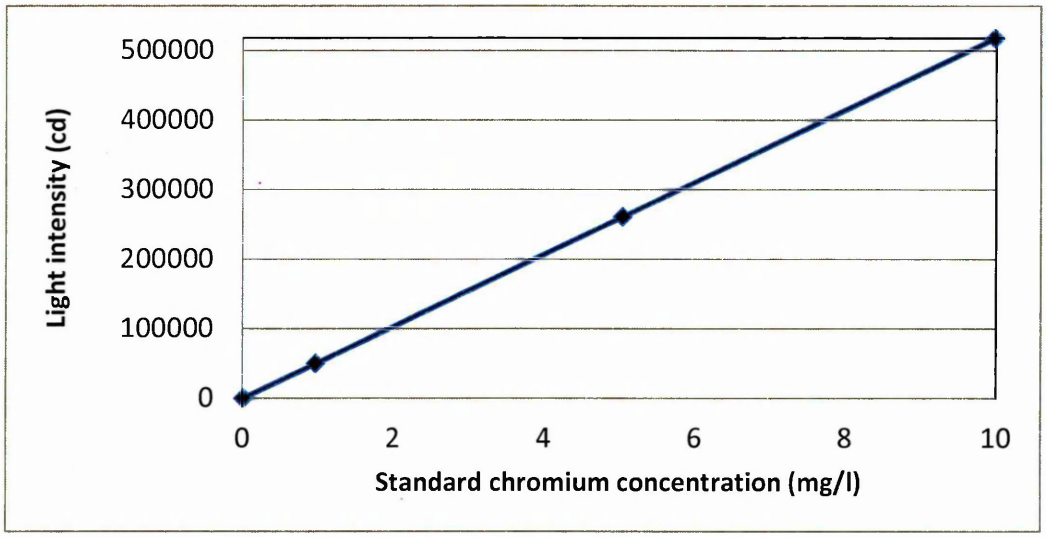


Figure 2.6: Typical Calibration curve of standard chromium concentration using ICP-OES

2.2.6 Identification of chromium species using ion chromatography, inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (ICP-MS), illustrated in Figure 2.7, is an alternative technique where the sample is ionized by ICP, but the elements of interest are detected via mass spectrometry. ICP-MS is a highly sensitive technique and capable of the determination of a range of metals and several non-metals at concentrations below one part in 10^{12} (part per trillion). It is based on coupling together inductively coupled plasma as a method of producing ions (ionization) with a mass spectrometer as a method of separating and detecting the ions. ICP-MS is also capable of monitoring isotopic speciation for the element of choice.

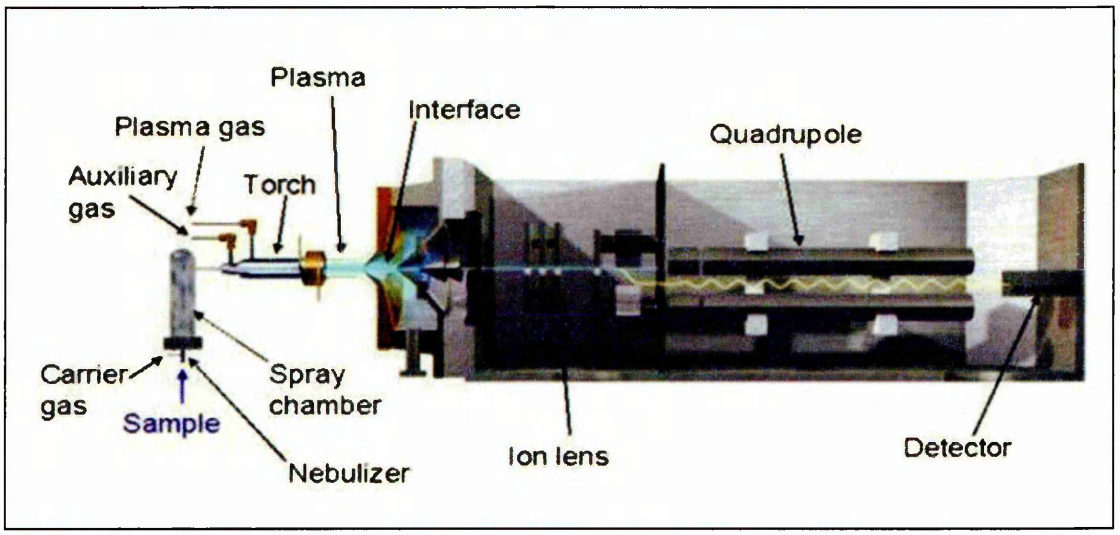


Figure 2.7: Schematic diagram of ICP-MS

2.2.6.1 Determination of Cr (III) and Cr (VI) using IC-ICP-MS

In this study, both Cr (III) and Cr (VI) were determined using ICP-MS, connected with ion chromatography column Dionex Ionpac-CG5A Guard 4 x 50 mm and Ionpac-CG5A Analytical 4 x 250 mm. The mobile phase was prepared using 0.6 mol/l KCl potassium chloride and 0.001 mol/l EDTA, flow rate of 1.00 ml/min.

Standard solutions of trivalent chromium Cr $(NO_3)_3.9H_2O$ and hexavalent chromium $K_2Cr_2O_7$ (Figures 2.8 and 2.9) were prepared at different concentrations of 1, 5 and 10 mg/l (Dionex 1991, and Derbyshire *et al.*, 1999).

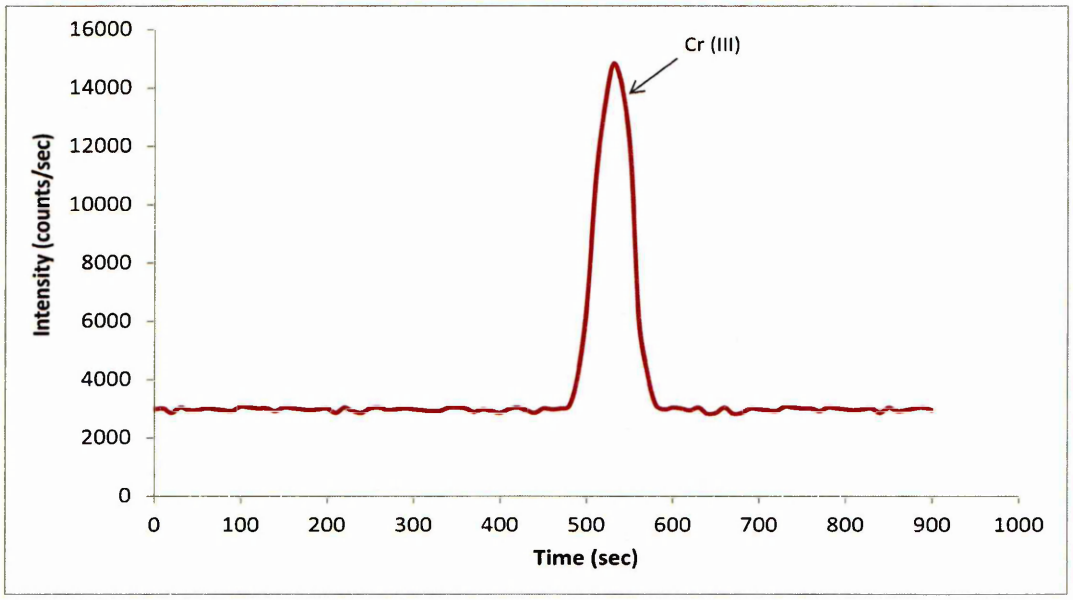


Figure 2.8: Trivalent chromium chromatogram using ICP-MS as a detector

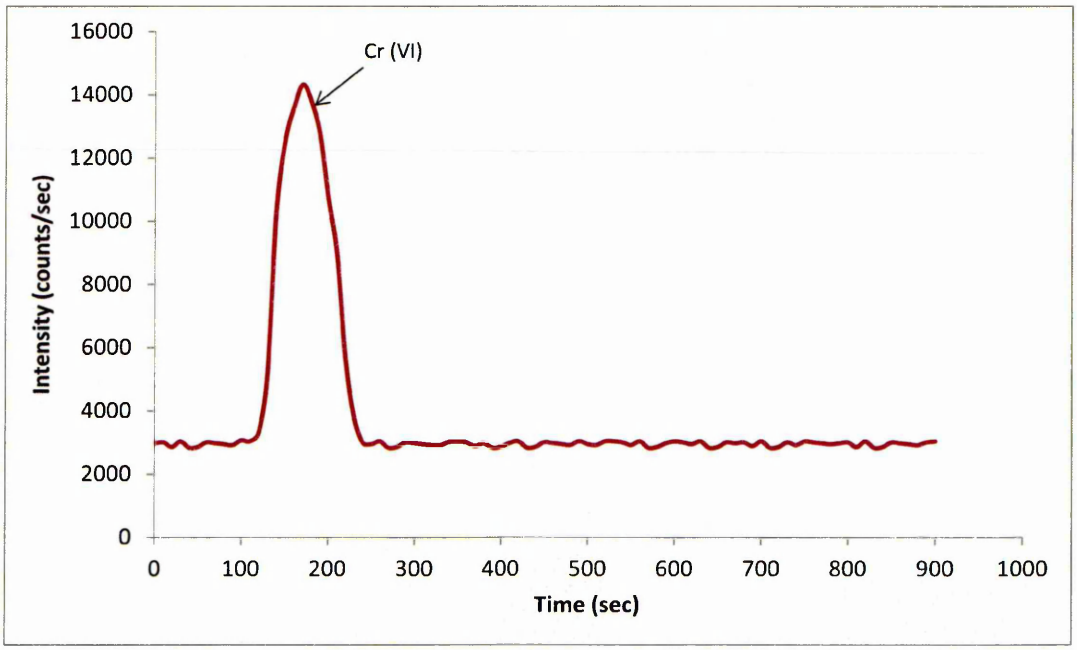


Figure 2.9: Hexavalent chromium chromatogram using ICP-MS as a detector

2.2.7 Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared (FTIR) spotlight 300 spectroscopy, depicted in Figure 2.10, is used to identify unknown materials, determine the quality or consistency of a sample or determine the amount of components in a mixture. FTIR spectroscopy is a multiplexing technique, where all optical frequencies from the source are observed simultaneously over a period of time known as *scan time*.

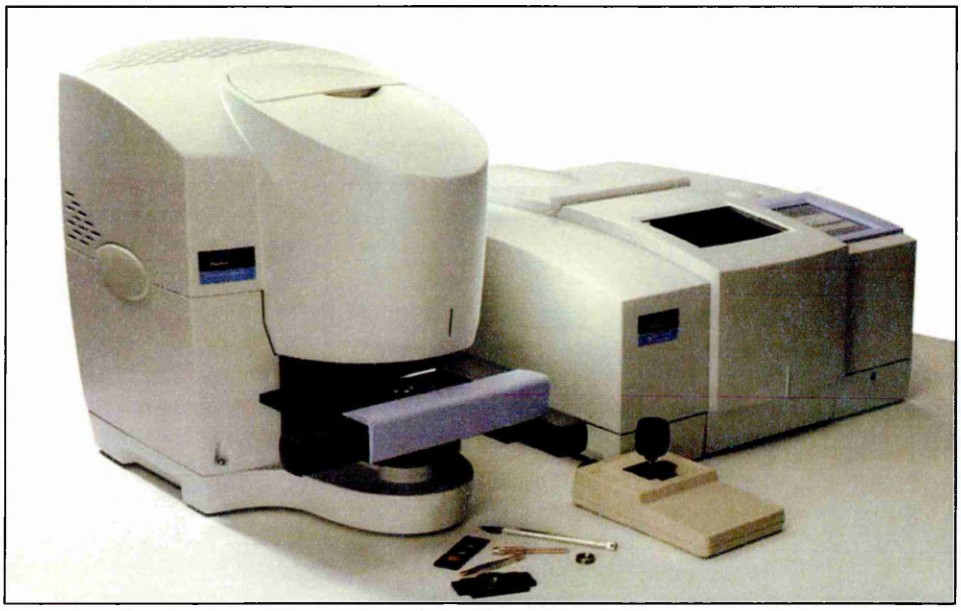


Figure 2.10: Fourier Transform Infrared Spectroscopy (FTIR) spotlight 300

The spectrometer measures the intensity of a specially-encoded infrared beam after it has passed through a sample, as illustrated in Figure 2.11. Some of the infrared radiation is absorbed by the sample and some of it is passed through. The resulting signal, which is a time domain digital signal, is called an *Interferogram* and contains intensity information about all frequencies (absorbed and transmitted) present in the infrared beam (Thermo Nicolet Corporation, 2001). This information can be extracted by switching this signal from a time domain digital signal to a frequency domain digital signal, which is accomplished by applying a Fourier transform over the interferogram and producing what

is called a *Single Beam Spectrum* (molecular fingerprint of the sample). Like a fingerprint no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy useful for several types of analysis.

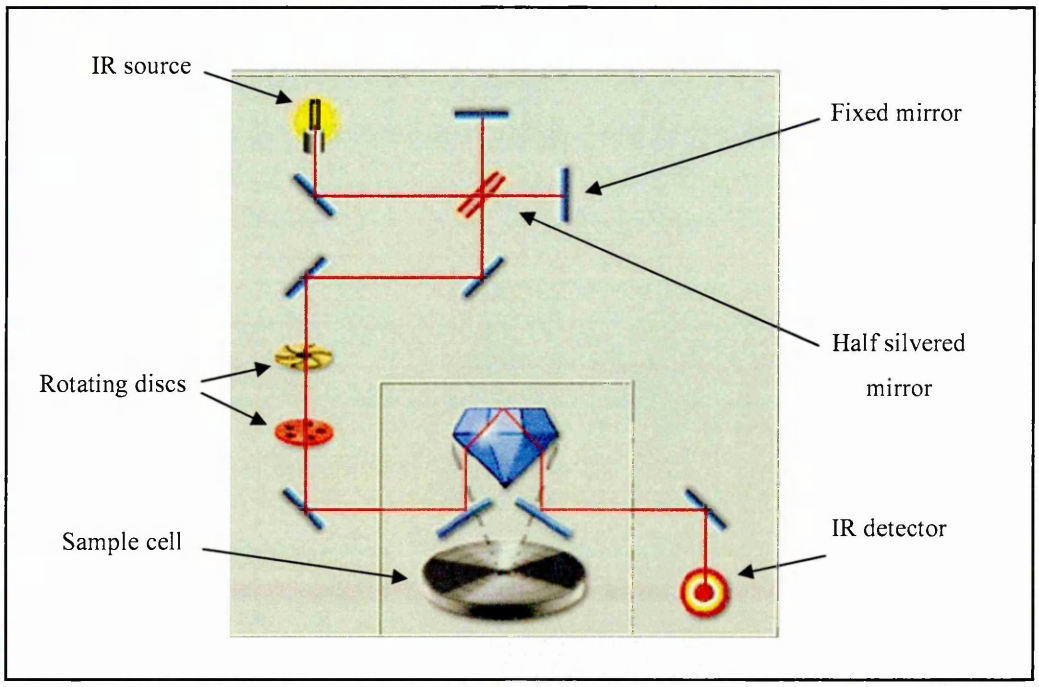


Figure 2.11: Light path in FTIR Spectroscopy

2.2.7.1 Sample preparation for FTIR experiment

- 2 L of medium (M9 medium for *P. mirabilis* and NMS for *M. capsulatus*) were prepared and inoculated with 2 ml of liquid culture of *P. mirabilis* or *M. capsulatus* (Bath), as appropriate.
- The culture was then incubated in an incubator shaker at 37 °C and 45 °C, for *P. mirabilis* and *M. capsulatus* (Bath), respectively at 180 rpm until the optical density of the culture reached between 0.5 – 1.0 at 600 nm.
- The mixture was centrifuged and washed with 20 mM NaCl three times.

- 10 ml of $K_2Cr_2O_7$ (Cr (VI) concentration is 2.54 mg/l) was added to the pellets of bacterial biomass for *P. mirabilis* and *M. capsulatus* (Bath) for the period time 0, 24 and 48 hours.
- The samples were taken at different incubation times (0, 24, and 48 hours).
- The sample was frozen at -80 °C, and then lyophilised using a freeze dryer.
- After freeze drying, the mixture was incubated again at 45 °C, in the incubator, to get a fully dried sample ready for FTIR experiment.

CHAPTER 3

3 INTERACTION OF CHROMIUM (VI) WITH GRAM-NEGATIVE BACTERIA

3.1 Introduction

In this chapter Gram-negative bacteria were investigated as a means of hexavalent chromium remediation. Five strains namely *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Proteus mirabilis*, *Escherichia coli* and *Methylococcus capsulatus (Bath)* were used to remediate hexavalent chromium. The potential of live and autoclaved (dead) bacterial strains was investigated for Cr (VI) removal. The principal reason for investigating autoclaved bacteria in parallel with live bacteria was to see whether the Cr (VI) removal reaction required the bacteria to be alive – i.e. was it catalysed by enzymes produced by the cells or was it a simple chemical reaction between the Cr (VI) and components of the cells that was not dependent on the cells' being alive.

As detailed in the introduction, several studies have reported that *Ps. aeruginosa* was able remediate chromate (Brock and Gustafson, 1976; Hardoyo and Ohtake (1991); Pimentel *et al.*, 2002; Summers and Jacoby, 1978). The ability of *E. cloacae* HO1 to reduce hexavalent chromium into soluble low valence form Cr (III) has been reported in several studies (Deleo and Ehrlich, 1994; Hardoyo and Ohtake (1991); Faisal and Hasnain, 2004; Frederickson *et al.*, 2000; Mclean and Beveridge, 2001; Wang *et al.*, 1989). One previous report, from Alhasin *et al.* (2010) working in this research centre, has described reduction of chromate (VI) by *M. capsulatus* (Bath) from the initial concentration (1.4 – 1000 mg/l). *E. coli* was also reported to reduce Cr (VI) into Cr (III) (Puzon *et al.*, 2002; Rafiqullah *et al.*, 2009).

The measurement of bacterial growth was conducted prior to and during the bioremediation process and sodium azide, which inhibits the final step of oxidative phosphorylation (reduction of O_2 to water by cytochrome *c* oxidase) was also used in order to test the involvement of oxidative phosphorylation in the chromium (VI) reduction or removal reactions. All strains were able to decrease Cr (VI) concentration at different incubation times (in most cases significant removal was observed after 48 hours).

3.2 Bacterial growth during the laboratory-scale bioremediation reaction

Ps. aeruginosa, *E. cloacae*, *P. mirabilis* and *E. coli* bacterial strains were cultivated in 50 ml M9 medium in 250 ml conical flask, using 2 % wt/vol glucose as the carbon and energy source, while *M. capsulatus (Bath)* was cultivated in 50 ml NMS medium in the presence of methane (99.95 %) as a source of energy and carbon. Cultures were allowed to grow to an OD_{600} of 0.3 – 1.0 before addition of 1 ml of filter sterilised potassium dichromate (equivalent to a Cr (VI) concentration of 2.54 mg/l) and, where stated, 1 ml of filter sterilized sodium azide solution (0.05% w/v). In order to study the chromium (VI) removal reaction, each bacterial strain was incubated under three sets of conditions:

- Pure culture of bacteria (cells with media).
- Bacterial culture with potassium dichromate (cells with media and $K_2Cr_2O_7$).
- Bacterial culture in presence of potassium dichromate with sodium azide (cells with media and $K_2Cr_2O_7 + NaN_3$).

Cultures were incubated in a shaking incubator at 37 °C and 180 rpm. For each set of conditions (pure bacterial culture, bacterial culture with $K_2Cr_2O_7$ and bacterial culture with $K_2Cr_2O_7$ + NaN_3), three independent experiments were set up in 250 ml conical flasks. Then, three separate samples (one from each flask) were collected at specified times and the corresponding growth was measured as the OD_{600} using spectrophotometer. Thus, an independent mean value was calculated.

3.3 Chromium assay using DPC and ICP-OES techniques

When liquid bacterial culture had grown in M9 medium to a sufficient extent (OD_{600} 0.3 - 1.0), 1 ml of filter sterilised $K_2Cr_2O_7$ was added to the bacterial culture (live and autoclaved bacteria) to give a final Cr (VI) concentration of 2.54 mg/l. At zero time of incubation 4 ml samples were taken and centrifuged at room temperature at 5000 *g* for 5 minutes. Supernatant samples were then separated from the pellets. 4 ml of lysozyme solution (0.5 mg/ml of fresh lysozyme in 20 mM tris-HCl and 20 mM NaCl) was added to each pellet (comprising bacteria and any particulate debris) in order to digest cell-wall

peptidoglycan for measuring Cr (VI) inside the cells. Then, the pellets were incubated with the lysozyme solution at 37 °C and 180 rpm for 1 hour in an orbital shaking incubator. The bacterial cultures were autoclaved to kill the bacteria and denature the enzymes by exposing them to a high pressure saturated steam at 120 °C.

Subsequent samples were taken at 4, 12, 24, 36, 48, 60, 84, 96, 108 and 120 hours after the start of the experiment and were processed in the same way.

Measurement of Cr (VI) and total chromium was carried out for supernatants and pellets samples using the DPC assay and ICP-OES technique, respectively. Cell-free medium plus $K_2Cr_2O_7$ and was used as a negative control. Full details of the DPC and ICP-OES methods can be found in sections: 2.3 and 2.4 of Chapter 2 (Materials and Methods).

3.4 Use of sodium azide as inhibitor during Cr (VI) removal reaction

When liquid bacterial culture had grown in M9 medium to a sufficient extent (OD_{600} 0.3 - 1.0), 1 ml of filter sterilised $K_2Cr_2O_7$ was added to the bacterial culture to give a final Cr (VI) concentration of 2.54 mg/l. At the same time, sodium azide (NaN_3) also was added (to a final concentration 0.05% w/v) to the mixture of initially live and autoclaved bacteria.

The aim of adding NaN_3 was to investigate the effect of oxidative phosphorylation enzymes in the bioremediation process. NaN_3 is a well-known inhibitor of the terminal segment of the electron transport chain. It has been reported that NaN_3 has several effects on the growth of bacterial cells. For example, it was shown to interfere with DNA and RNA synthesis (Ciesla *et al.*, 1974; Yura and Wada, 1969) and to induce mutations (Owais and Kleinhofs, 1988; Fortin *et al.*, 1990).

3.5 Results

3.5.1 Bacterial growth

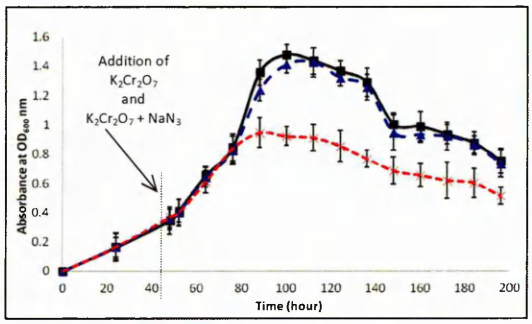
The opacity of the cultures was monitored in parallel with measurement of chromium (VI) concentration in order to assess the impact that the presence of the hexavalent chromium had on the growth of the cells. Figure 3.1 shows the bacterial growth curves of the employed bacterial strains during their cultivation period. Each curve describes the growth of the pure culture of bacteria in M9 medium without additions (indicated by solid black line), bacterial culture with $K_2Cr_2O_7$ (to give a hexavalent chromium concentration of 2.54 mg/l; represented by dashed blue line) and bacterial culture in presence of the same concentration of $K_2Cr_2O_7$ + 0.05% w/v of NaN_3 (indicated by dotted red line). The results obtained revealed that all the bacterial strains studied continued to grow after chromium (VI) or chromium (VI) plus sodium azide were added, and that the decreases in growth that these compounds caused were modest.

The chromium (VI) and sodium azide were added to each culture at the point indicated in Figure 3.1, when the OD_{600} was between 0.2 and 0.4 and it was judged that the cultures were in the logarithmic phase of growth. The experiment was continued so that the stationary phase and at least the beginning of death phase could be observed.

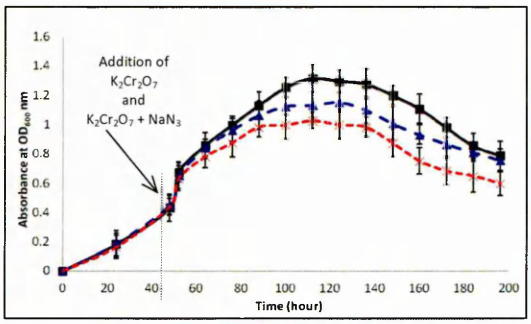
Ackerley *et al.* (2006) reported that *E. cloacae* and *E. coli* can grow in the presence of chromate. Wang *et al.* (1989), Pimentel *et al.* (2002) and Faisal and Hasnain (2004) have also studied and confirmed the growth of *E. cloacae* HO1 in the presence of potassium chromate under aerobic and anaerobic conditions. In the current study, the highest growth rate was observed with the pure bacterial culture for all employed strains, followed by the bacterial culture with $K_2Cr_2O_7$. However, it was noted that the slowest bacterial growth rate occurred in the pure bacterial culture in the presence of $K_2Cr_2O_7$ + the inhibitor of oxidative phosphorylation NaN_3, which was used in this study as an inhibitor that binds and decreases the activity of oxidative phosphorylation enzymes. This can be clearly seen in *Ps. aeruginosa* strain as shown in Figure 3.1 (a).

The results obtained revealed that sodium azide at 0.05 % exerts a relatively small inhibitory effect on the growth of all strains of bacteria tested. Forget and Fredette (1961)

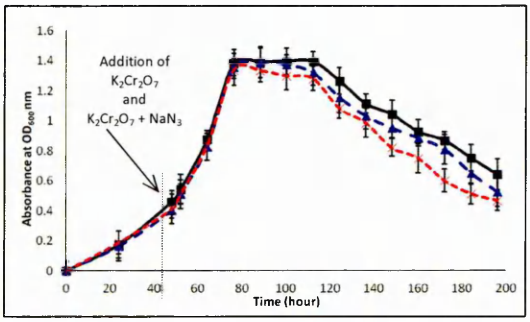
studied different NaN_3 concentrations ranging from 0.01 to 1% added to Trypticase soy broth to grow many types of bacteria including *E. coli*, *Ps. aeruginosa* and *P. vulgaris*. They noted that up to a concentration of 0.05% NaN_3 most of the bacterial strains were still able to grow. In another study conducted in 2009, Levy and Chevion showed that even at concentrations of sodium azide up to 0.07% (10 mM) there was no effect on cell growth.



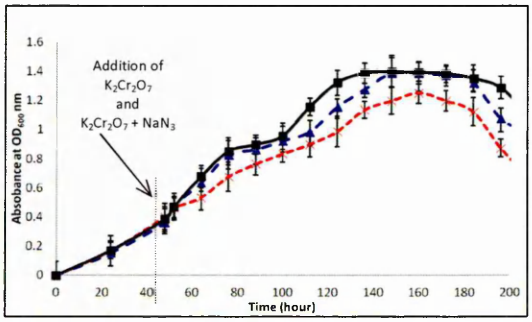
a) *Ps. aeruginosa*



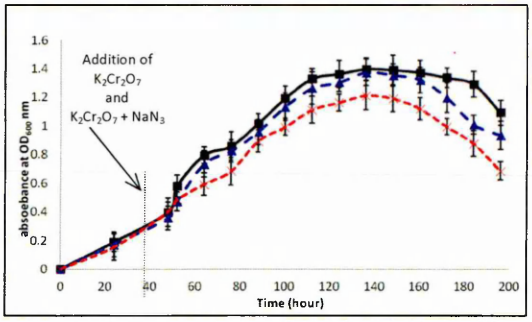
b) *E. cloacae*



c) *P. mirabilis*



d) *E. coli*



e) *M. capsulatus*

(—■—) Bacterial culture (medium + cells).

(··▲··) Bacterial culture + $K_2Cr_2O_7$ (2.54 mg/l).

(··✱··) Bacterial culture + $K_2Cr_2O_7$ + NaN_3.

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.1: Growth curves of the employed bacterial strains

3.5.2 Bioremediation of Cr (VI) using employed bacterial strains

Cr (VI) concentration in the supernatant and pellet fractions was monitored at specified intervals of incubation time (see section 2.2.3), diphenylcarbazide (DPC) assay. Measurement of total chromium was also carried out for supernatants and pellet samples using ICP-OES. Cell-free medium plus $K_2Cr_2O_7$ and was used as a negative control.

3.5.2.1 Bioremediation of Cr (VI) using live bacterial strains

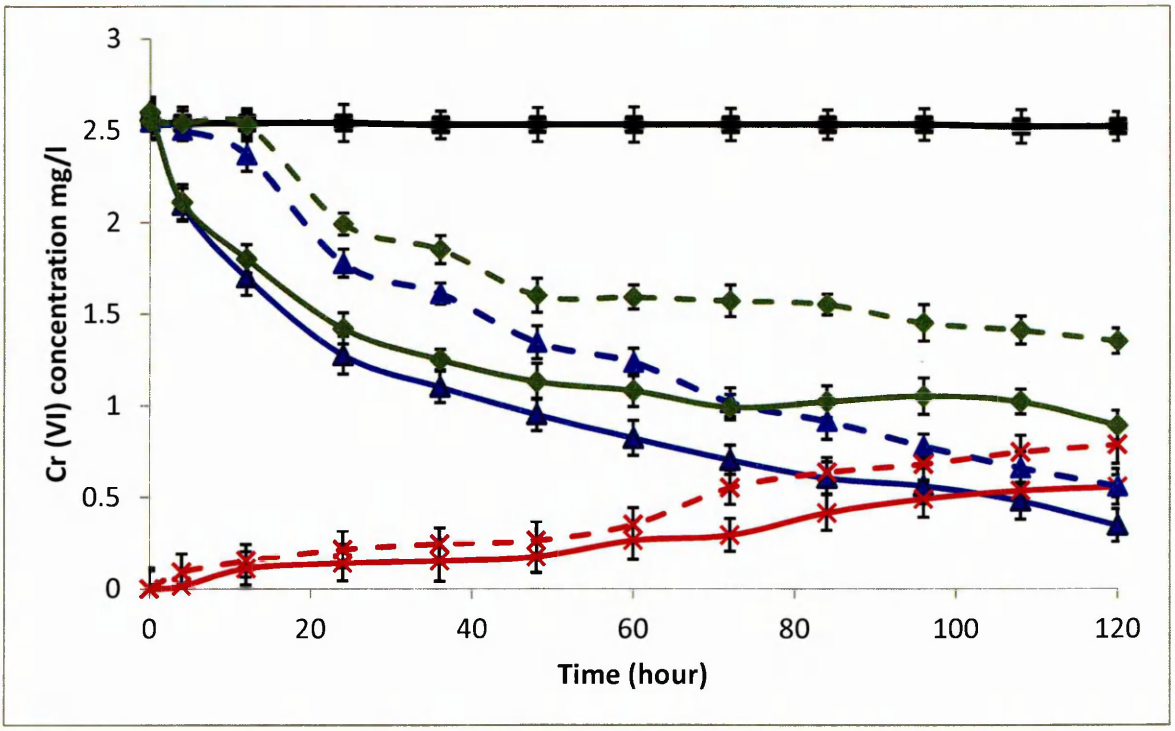
3.5.2.1.1 Measurement of Cr (VI) concentration using the DPC assay

Figures 3.2 – 3.6 depict the removal of Cr (VI) by all bacterial strains, with and without addition of NaN_3, as measured using the DPC assay. Before performing the chromium (VI) assays, pellets were resuspended in the same volume of solution as the culture from which the cells were originally derived, so that concentrations can be directly compared between supernatant and pellet samples. Removal of Cr (VI) by all five strains was nearly complete after 120 hours of incubation. It was found that Cr (VI) removal was not observed in the control samples; (i) media and $K_2Cr_2O_7$ (without cells) and (ii) media and $K_2Cr_2O_7$ + NaN_3 (without cells). A significant drop of Cr (VI) concentration from its initial value of 2.54 mg/l was observed in all supernatant samples, which are indicated by solid and dashed blue lines for without and with NaN_3, respectively. In contrast to the supernatants, the amount of Cr (VI) in the pellet fractions (represented by solid and dotted red lines for without and with NaN_3 samples) increased over the incubation period.

Figure 3.2 shows Cr (VI) removal using *Ps. aeruginosa*. The percentage of Cr (VI) removal from the supernatant was 20% at 4 hours of incubation time, and increased to reach 87% at the end of incubation period (120 hours). At the end of the incubation period Cr (VI) concentration had decreased to 0.34 mg/l. Unlike in the supernatants, Cr (VI) concentration in the pellet-derived fractions increased over the incubation period. It was 0 mg/l at the start of incubation, and then gradually increased to reach 0.56 mg/l at 120 hours.

When NaN_3 was added at the same time as Cr (VI), the decline in Cr (VI) concentration in the *Ps. aeruginosa* supernatant samples with NaN_3 (denoted by dashed blue line) was very slow at the beginning of the incubation time. Then, it slowly increased throughout the incubation time. The percentage of Cr (VI) removal from the supernatant (with NaN_3) was 2% at 4 hours and reached 78% after 120 hours. It can also be seen that more Cr (VI) was removed when sodium azide was absent (Figure 3.2). This is consistent with general inhibition of energy metabolism, where the sodium azide partially inhibited chromate reductase activity. Cr (VI) concentration in the pellet fraction with NaN_3, which is indicated by the dotted red line, gradually increased over time. At 4 hours of incubation time the amount of Cr (VI) in the pellet fraction was 0.09 mg/l then it increased gradually to reach 0.79 mg/l at the end of the incubation time.

The chromium (VI) concentration in the pellets increases during the experiment even in the presences of NaN_3, suggesting biosorption of chromium (VI) to the cells. At the same time, the chromium (VI) concentration in the supernatant decreases, and the total concentration of chromium (VI) in the system decrease as well. This suggests that some loss of chromium (VI) is occurring as well as adsorption. The decrease in total chromium (VI) over the 120 hours of the experiment is (65%) greater with the live culture than with the culture that was inhibited by NaN_3 (47%). If NaN_3 completely inhibits cellular metabolism, this suggests that 27.7% of the removal of chromium (VI) is due to the metabolism of the cells and 62.3% is due to chemical reaction between the chromium (VI) and cellular components.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✕··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

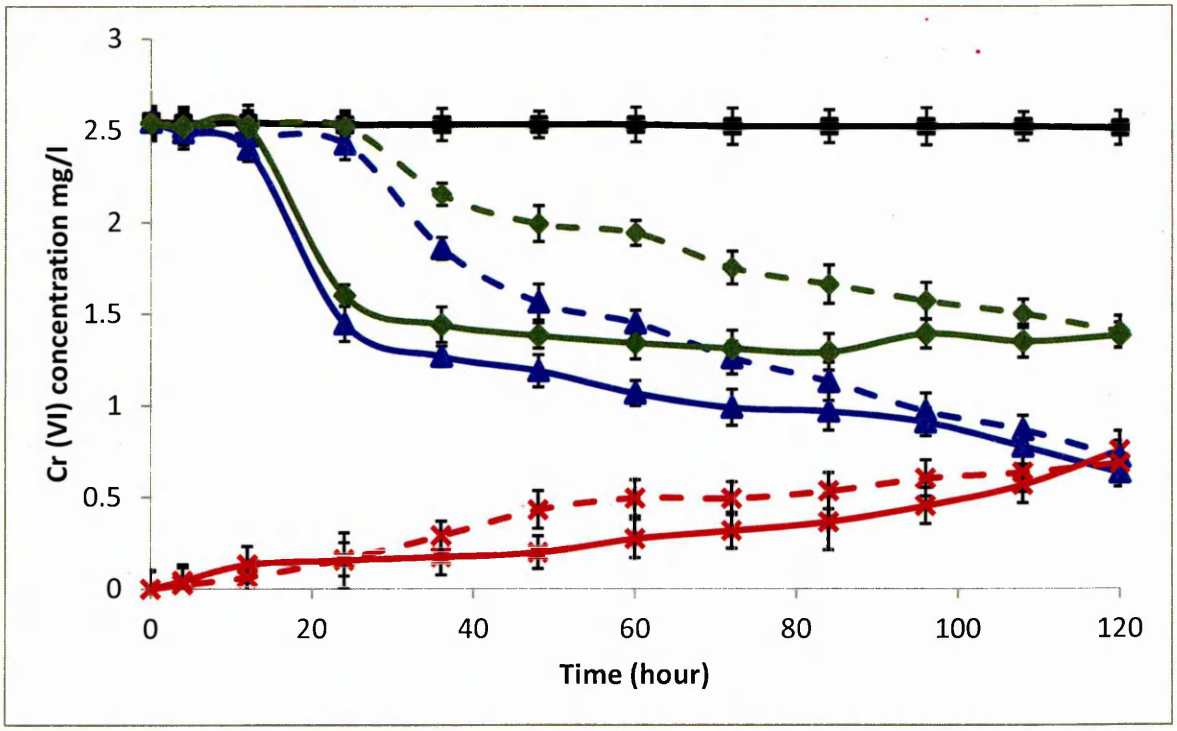
Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.2: Measurement of Cr (VI) concentration in Ps. aeruginosa bacterial strain (with and without sodium azide) using DPC assay

As shown in Figure 3.3, a small amount of Cr (VI) removal (2%) was observed with *E. cloacae* at the start of incubation period. Then, the concentration of Cr (VI) sharply decreased to reach (1.44 mg/l, 43% drop) at 36 hours and continued decreasing to record 0.63 mg/l (75% drop) at 120 hours. Cr (VI) concentration in the pellet fraction increased over the incubation period. It was 0 mg/l at the start of incubation, and then gradually inclined to 0.75 mg/l after 120 hours of incubation.

After addition of NaN_3, *E. cloacae* strain extended its ability to remediate Cr (VI) but with slower rate at the beginning of bioremediation reaction. The percentage of Cr (VI) removal from the supernatant with NaN_3 was 1% at 4 hours of the incubation and increased to reach 72% at 120 hours. It was noticed that Cr (VI) removed was less than that from supernatant without sodium azide. This is consistent with a model in which sodium azide fully inhibited reduction of chromium (VI) until 36 hours of the incubation. Then, it partly inhibited the chromium (VI) removal (after 48 hours) relative to the control sample. Cr (VI) concentration in the pellet fractions with NaN_3, labelled with dotted red line, gradually increased from 0 mg/l at the start of bioremediation process to reach 0.68 mg/l at the end of incubation period.

The concentration of total chromium (VI) in the supernatant and pellets decreased from its initial value by 45% and 46% at 120 hours of the experiment in the live culture with and without NaN_3 respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

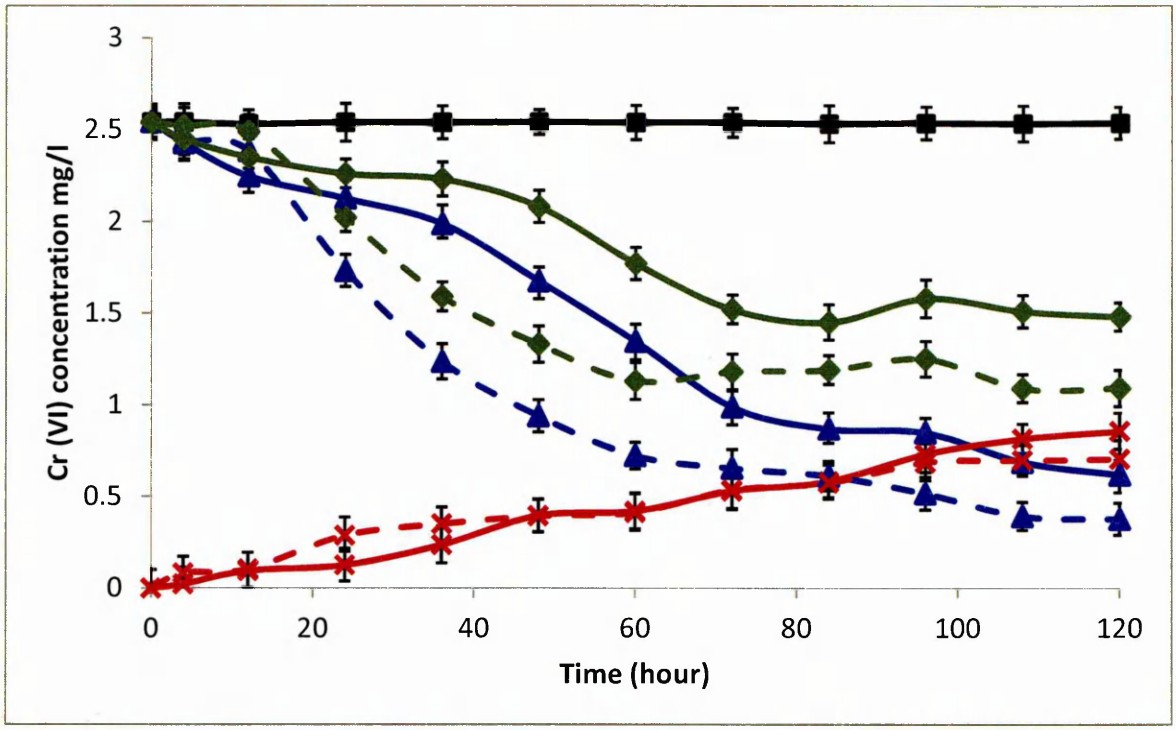
Figure 3.3: Measurement of Cr (VI) concentration in E. cloacae bacterial strain (with and without sodium azide) using DPC assay.

Figure 3.4 shows Cr (VI) removal by *P. mirabilis*, which has significantly decreased Cr (VI) concentration in supernatant samples. At the end of the incubation time Cr (VI) concentration declined to 0.62 mg/l. The percentage of Cr (VI) removal was 5% at 4 hours of incubation time, and extended to reach 76% at the end of incubation period (after 120 hours). Cr (VI) concentration the pellets, however, increased over incubation

period. It was 0 mg/l at the start of incubation, and then gradually inclined to record 0.86 mg/l after 120 hours of incubation.

Cr (VI) removal from the supernatant sample with NaN_3 (denoted by dashed blue line) was very slow at the beginning of the incubation time (up to 12 hours of incubation). Then, the removal was dramatically increased between 24 and 60 hours. It can be noticed that Cr (VI) removal was greater than from the supernatant without sodium azide. The removal of Cr (VI) increased until the Cr (VI) concentration reached its lowest level of 0.38 mg/l at 120 hours. The percentage of the Cr (VI) removal was 85% at the end of the incubation. This suggests that the sodium azide stimulated the enzymes responsible for Cr (VI) reduction rather than inhibiting them. This reveals that these enzymes are not responsible of Cr (VI) reduction. Cr (VI) concentration in the pellet fractions with NaN_3, labelled with dotted red line, gradually increased over time to reach 0.71 mg/l at the end of incubation period.

At the end of experiment, the total chromium (VI) concentration in the supernatant and pellets samples decreased 41% and 57% for the samples without and with NaN_3, respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

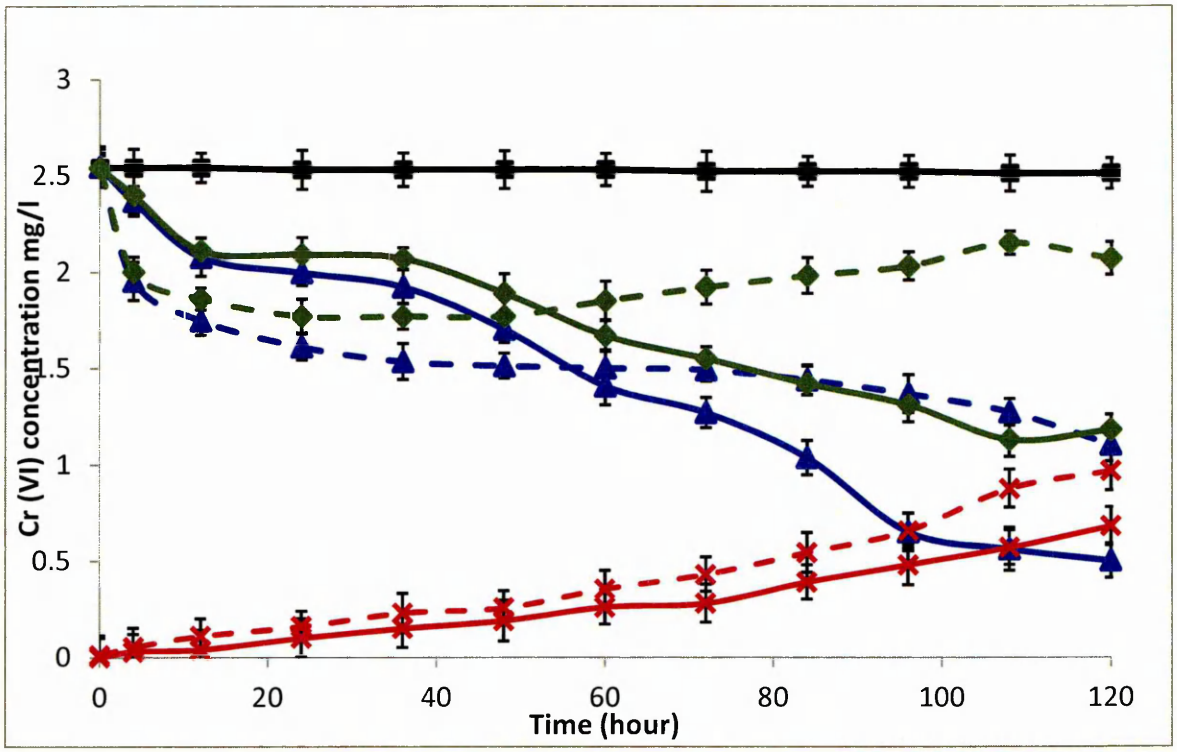
Figure 3.4: Measurement of Cr (VI) concentration in P. mirabilis bacterial strain (with and without sodium azide) using DPC assay

When using *E. coli*, Cr (VI) concentration in the supernatant samples slowly declined from 2.54 mg/l at zero time to record 0.50 mg/l at 120 hours, as shown in Figure 3.5. The percentage of Cr (VI) removal was 7% at 4 hours of incubation time, and extended to reach 80% at the end of incubation period. However, Cr (VI) concentration in the pellets increased from zero mg/l at the start of incubation, and then gradually increased to reach 0.68 mg/l after 120 hours of incubation.

Cr (VI) removal from the supernatant sample with NaN_3 was very sharp at the beginning of bioremediation process (between 0 and 12 hours). After 12 hours Cr (VI) concentration was 1.75 mg/l. After that it further decreased to reach 1.1 mg/l at the end of the incubation time. The percentage of the Cr (VI) removal from the supernatant with NaN_3 was 23% at 4 hours of the incubation time. After 12 hours the reduction was 31%. Then, the drop in the Cr (VI) gradually increased to 57% at the end of bioremediation process. Cr (VI) removal was less than from the supernatant without sodium azide. It seems that sodium azide stimulated the enzymes responsible for Cr (VI) reduction between zero time and 60 hours. Then, the drop in Cr (VI) concentration slowly increased till the end of incubation time.

Cr (VI) concentration in the pellet fraction with NaN_3 was 0.05 mg/l at 4 hours, then, gradually increased with respect to incubation time to reach 0.97 mg/l at the end of bioremediation process.

The total chromium (VI) concentration in the supernatant and pellets decreases during the experiment even in the presences of NaN_3. This suggests that some Cr (VI) removal is occurring by biosorption of chromium (VI) to the cells. The decrease in total chromium (VI) over the 120 hours of the experiment was 35% comparing with the live culture that was inhibited by NaN_3 (19%).



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✕··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

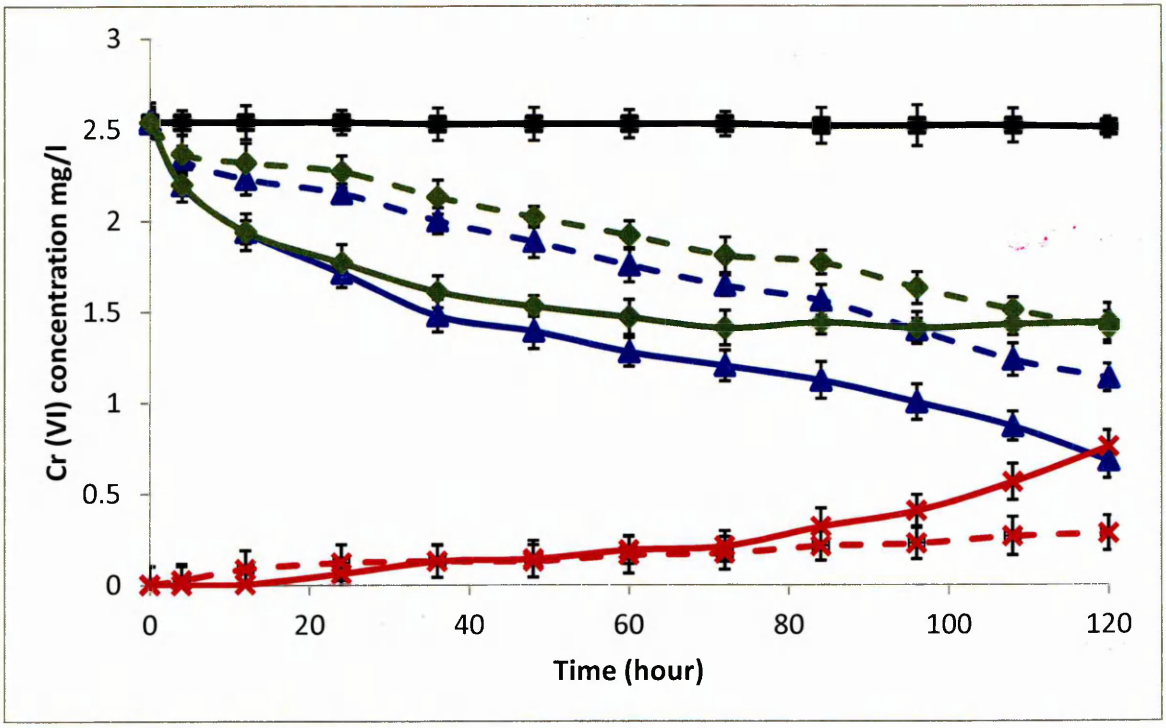
Figure 3.5: Measurement of Cr (VI) concentration in E. coli bacterial strain (with and without sodium azide) using DPC assay

Similar to other bacterial strains *M. capsulatus* significantly decreased Cr (VI) concentration in supernatant samples, as illustrated Figure 3.6. At zero time Cr (VI) concentration in the supernatant samples was 2.54 mg/l. Then, it gradually declined to record 0.68 mg/l at 120 hours. The percentage of Cr (VI) removal was 13% at 4 hours of incubation and reached 73% at the end of incubation time. While, Cr (VI) concentration

in the pellets gradually increased over incubation period to record 0.76 mg/l after 120 hours of incubation. As mentioned earlier, similar result by Alhasin *et al.* (2010) reported that *M. capsulatus* (Bath) was able to lower chromate (VI) concentration by 76% from the initial 2.54 mg/l.

With NaN_3, Cr (VI) removal by *M. capsulatus* was slower than the one without NaN_3. Cr (VI) concentration in the supernatant decreased in the presences of bacteria from its initial value of 2.54 mg/l decreased to 2.34 mg/l (8% of removal) after 4 hours. At the end of the incubation time Cr (VI) concentration was 1.13 mg/l, which corresponds to 56% of reduction. In this case the sodium azide partly inhibited the enzymes of the oxidative phosphorylation. As in the other cultures Cr (VI) concentration in the pellets gradually increased to reach 0.28 mg/l at the end of incubation period.

With *M. capsulatus* (Bath), the total chromium (VI) concentration in the supernatant and pellets together decreased by 43% and 44% for the culture without and with NaN_3, respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.6: Measurement of Cr (VI) concentration in M. capsulatus bacterial strain (with and without sodium azide) using DPC assay

3.5.2.1.2 Measurement of total chromium using ICP-OES technique

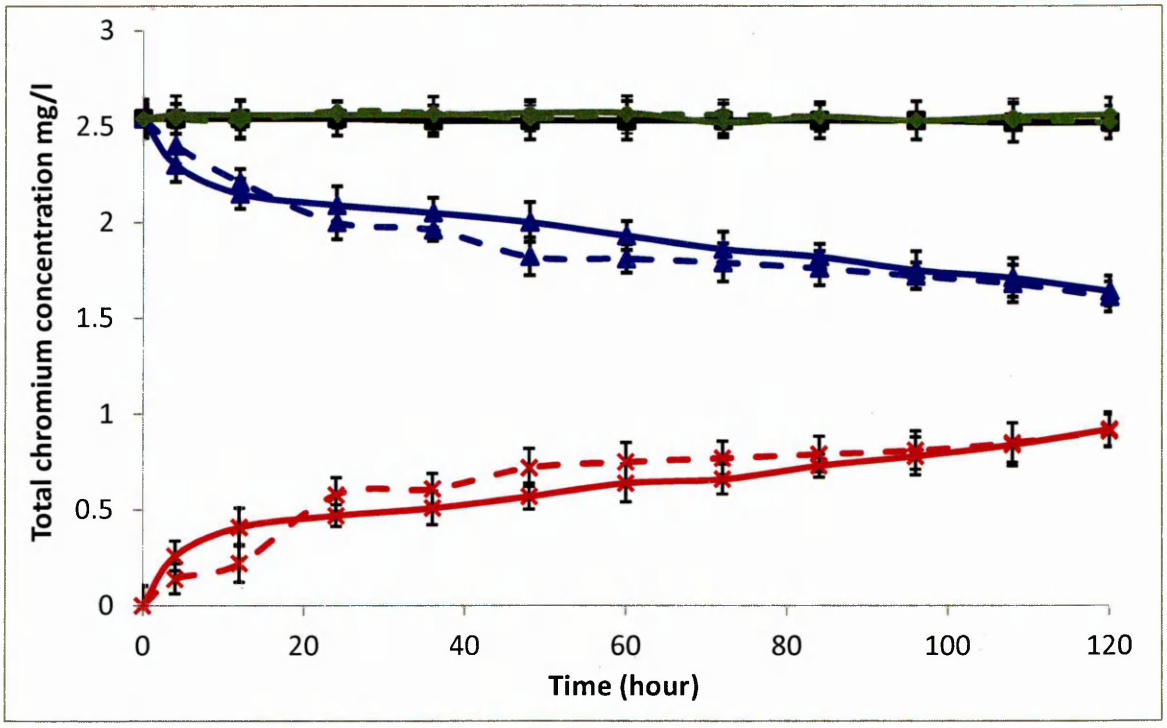
Figures 3.7 – 3.11 show the total chromium concentration using ICP-OES technique. Similar to the measurement of Cr (VI) concentration using DPC assay, there are two control samples (without cells, denoted by black solid lines); M9 medium with $K_2Cr_2O_7$ and M9 medium with $K_2Cr_2O_7$ + NaN_3. Total chromium concentration in the

supernatant samples (without and with NaN_3) is represented by solid and dashed blue lines, respectively. While, total chromium concentration in the pellet fraction (without and with NaN_3) is represented by solid and dotted red lines, respectively.

Figure 3.7 shows the total chromium concentration profile during the bioremediation of Cr (VI) with *Ps. aeruginosa*. Total chromium concentration in the supernatant fell from 2.54 mg/l at the start of the reaction time (zero time) to 1.64 mg/l after 120 hours of incubation time. In contrast, the total concentration in the pellet fraction increased from 0 mg/l at zero time to reach 0.92 mg/l at 120 hours.

Measurement of total chromium was also carried out after addition of sodium azide. At the beginning of the incubation time, total chromium concentration in the supernatant slowly decreased to reach 1.61 mg/l the end of the incubation period. The components of the bacteria in the pellet fractions showed an increase of total chromium concentration throughout the bioremediation process. It started with 0 mg/l at the beginning of the incubation time, and slowly increased to record 0.91 mg/l after 120 hours.

The effect of NaN_3 upon the measurement of chromium from the supernatant to the pellet fraction was mostly with the margin of experimental period. The total chromium in both fractions throughout all experiments was as expected equal to the total chromium concentration at the start of the experiment. The figure also shows that the total chromium in the supernatant and the pellets samples overlapped.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✖—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✖··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

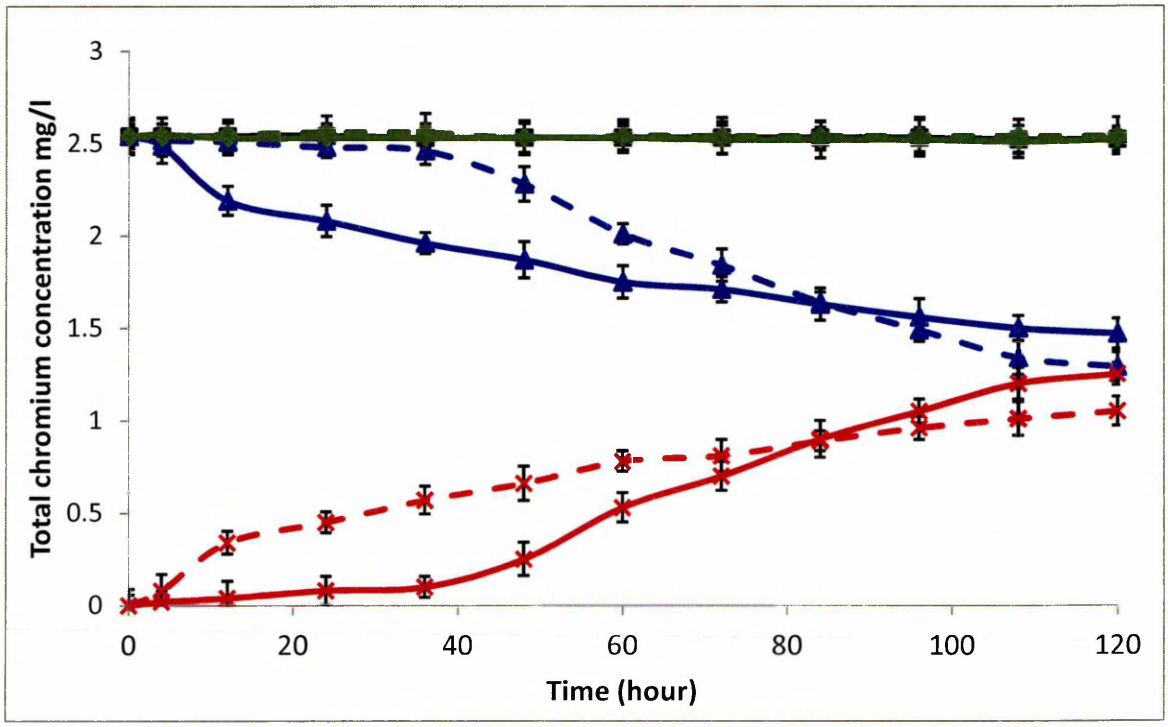
$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.7: Measurement of total chromium concentration in P. aeruginosa bacterial strain (with and without sodium azide) using ICP-OES

In Figure 3.8 the total chromium concentration in the supernatant samples, using *E. cloacae*, decreased from 2.54 mg/l at the start of the reaction time (zero time) to 1.47 mg/l after 120 hours of incubation time. While, the total concentration in the pellet sample (solid red line) increased from 0 mg/l at zero time to reach 1.05 mg/l at 120 hours. After addition of sodium azide the reduction of total chromium concentration in the supernatant was insignificant at the beginning of the incubation time (between zero time

and 36 hours). Then, the concentration of total chromium gradually decreased to reach 2.28 mg/l at 48 hours and continued decreasing to record 1.29 mg/l the end of the incubation period. The components of the bacteria in the pellet fractions showed an increase of total chromium concentration throughout the incubation time. It started with 0 mg/l at the beginning of the incubation time, and slowly increased to record 1.25 mg/l after 120 hours.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

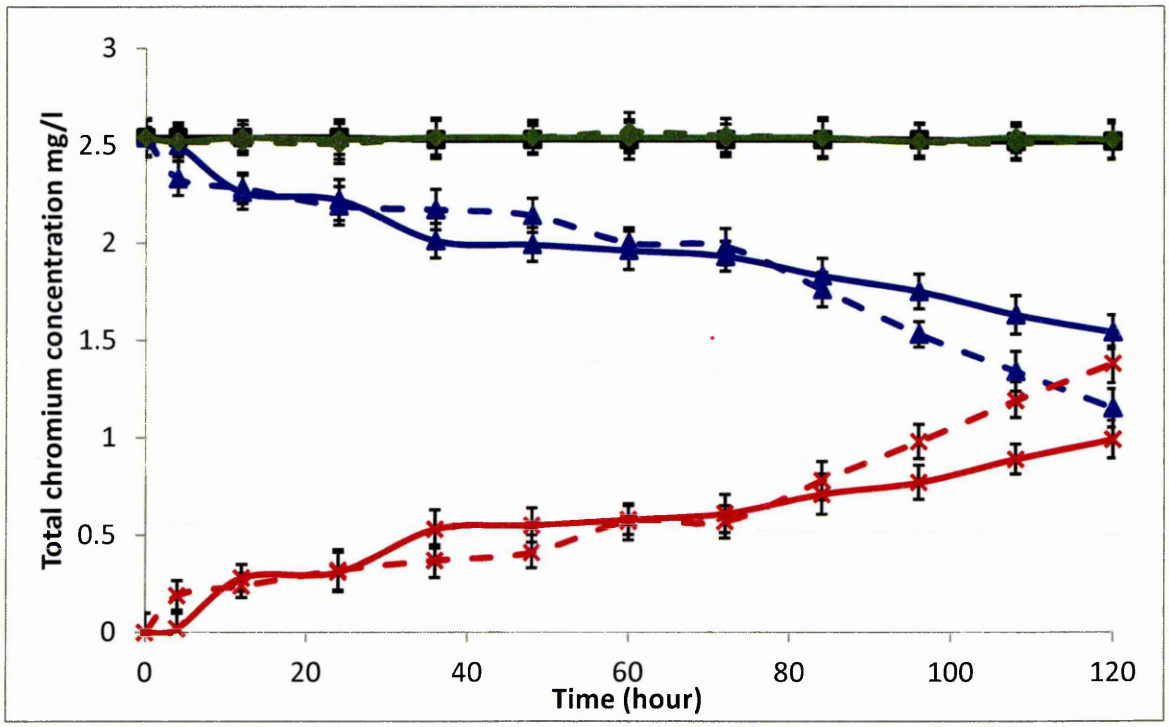
(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.8: Measurement of total chromium concentration in E. cloacae bacterial strain (with and without sodium azide) using ICP-OES

In the case of *P. mirabilis*, shown in Figure 3.9, the total chromium concentration in the supernatant fell from 2.54 mg/l at the start of the reaction time (zero time) to 1.54 mg/l after 120 hours. In contrast, the total concentration in the pellet sample (solid red line) increased from 0 mg/l at zero time to reach 0.97 mg/l at 120 hours. While in sodium azide samples the total chromium concentration in the supernatant slowly decreased to reach 1.15 mg/l the end of the incubation period. The components of the bacteria in the pellets showed an increase of total chromium concentration throughout to record 1.38 mg/l after 120 hours.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

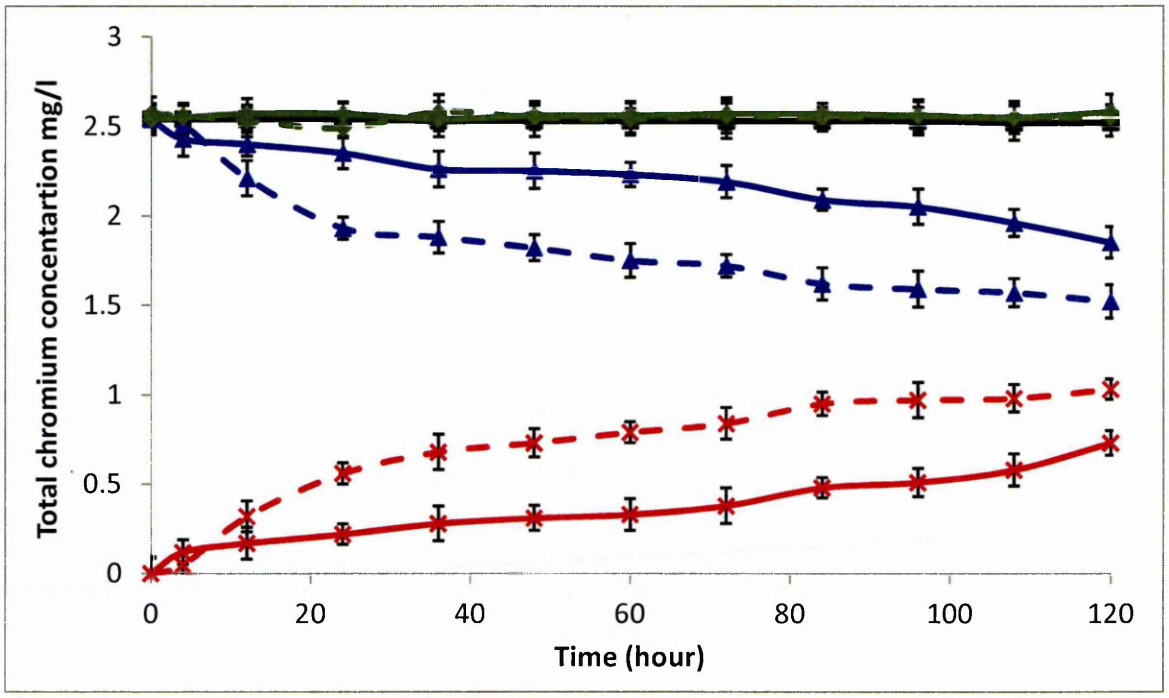
(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 3.9: Measurement of total chromium concentration in P. mirabilis bacterial strain (with and without sodium azide) using ICP-OES

Figure 3.10 shows the total chromium concentration using *E. coli* with and without sodium azide. The total chromium concentration in the supernatant (without sodium azide) gradually decreased from 2.54 mg/l at the start of the reaction time (zero time) to 1.85 mg/l after 120 hours of incubation time. The total chromium concentration in the supernatant with sodium azide slowly decreased from 2.54 mg/l to 1.52 mg/l at the end of reaction time. However, the total concentration in the pellet sample without and with sodium azide increased from 0 mg/l at zero time to reach 0.73 mg/l and 1.03 mg/l after 120 hours, respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

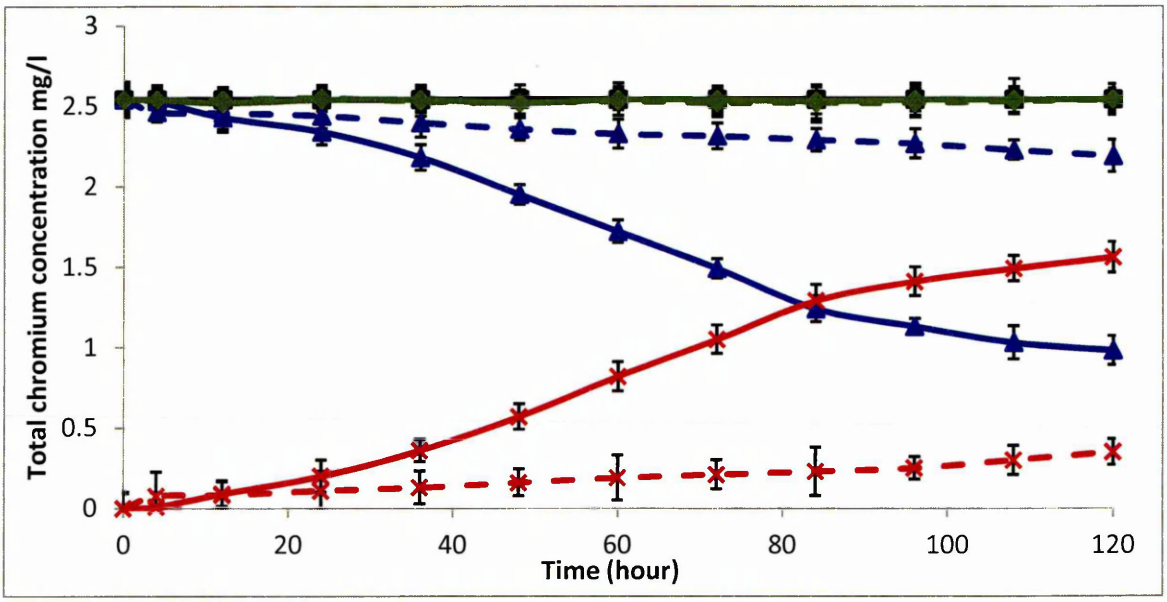
(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 3.10: Measurement of total chromium concentration in E. coli bacterial strain (with and without sodium azide) using ICP-OES

Figure 3.11 shows the measurement of total chromium using *M. capsulatus*. As mentioned earlier, the control sample for *M. capsulatus* (Bath) represents NMS medium and $K_2Cr_2O_7$, which is denoted with a solid black line. The total chromium concentration in the supernatant decreased from 2.54 mg/l to 0.85 mg/l after 120 hours of incubation time. In contrast, the total concentration in the pellet sample increased from 0 mg/l to 1.56 mg/l at 120 hours. After addition of sodium azide, total chromium concentration in the supernatant slowly decreased to record 2.12 mg/l after 120 hours. While, the chromium concentration in the bacterial pellet fractions increased to reach 0.35 mg/l after 120 hours further comparisons of these and other chromium reduction data are given later in the chapter.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 3.11: Measurement of total chromium concentration in M. capsulatus bacterial strain (with and without sodium azide) using ICP-OES

3.5.2.2 Bioremediation of Cr (VI) using autoclaved bacterial cells

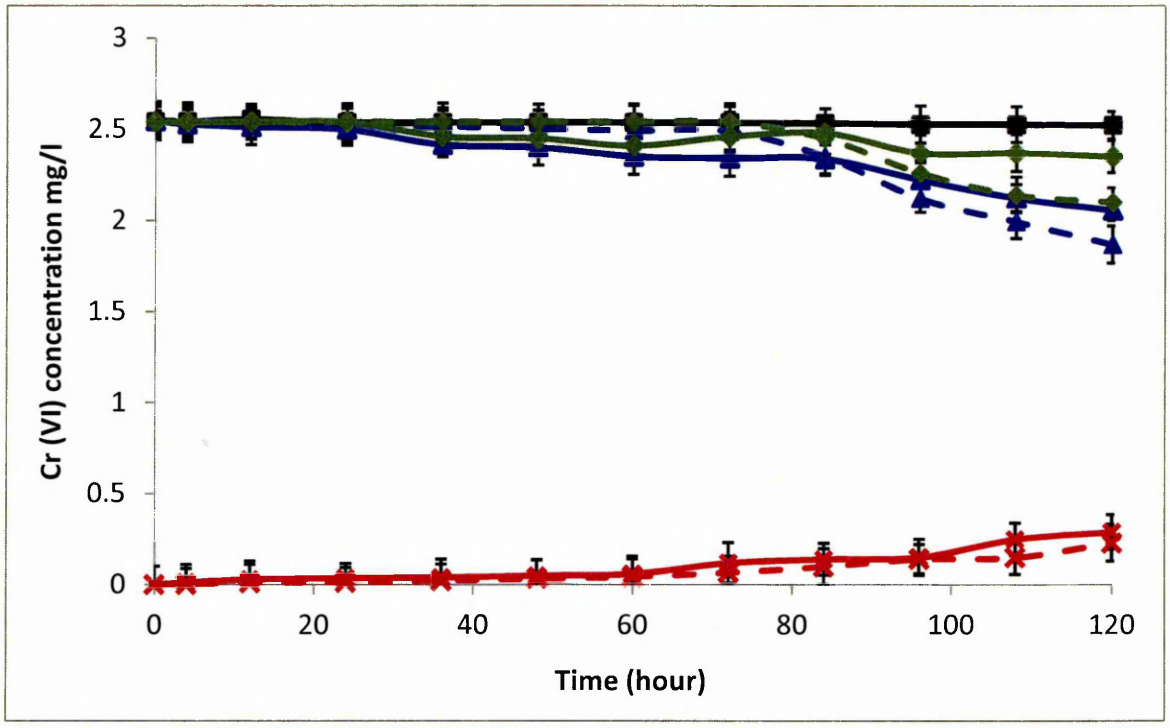
As explained above, autoclaved bacteria were also tested for bioremediation of Cr (VI) to determine whether the reaction was dependent upon metabolism of the cells. The bacterial culture (M9 or NMS medium and bacteria cells OD_{600} = 0.675 nm) was firstly autoclaved to kill the bacteria (denature the enzymes and damage the bacteria's components) by exposing them to a high pressure saturated steam at 120 °C, then used in the bioremediation process.

3.5.2.2.1 Measurement of Cr (VI) using DPC assay

Figures 3.12 – 3.16 show Cr (VI) removal activity during the bioremediation process using all autoclaved bacterial strains with and without NaN_3.

Figure 3.12 shows the Cr (VI) removal using *Ps. aeruginosa*. There was some change in Cr (VI) concentration in the supernatant samples (without and with sodium azide). The percentage extent of the removal at the beginning (between 0 and 24 hours) of reaction time was less than 1% for both without and with sodium azide samples. At the end of reaction time Cr (VI) concentration was 2.06 mg/l and 1.87 mg/l in both supernatant samples (without and with sodium azide respectively), which corresponds to a removal of 19% and 27%, respectively. Cr (VI) concentration in the pellets was very low at the beginning of the reaction time and started elevating after 48 hours and continued with slow down to reach 0.29 mg/l and 0.23 mg/l after 120 hours of reaction for the two samples without and with sodium azide respectively.

The addition of NaN_3 to the autoclaved culture increased the apparent removal of chromium (VI) in the system from 7.5% (without NaN_3) to 17% (with NaN_3). Hence, it is possible that addition of NaN_3 to the autoclaved culture increases the rates of chemical removal of Cr (VI).



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✖—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✖··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(--◆--) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

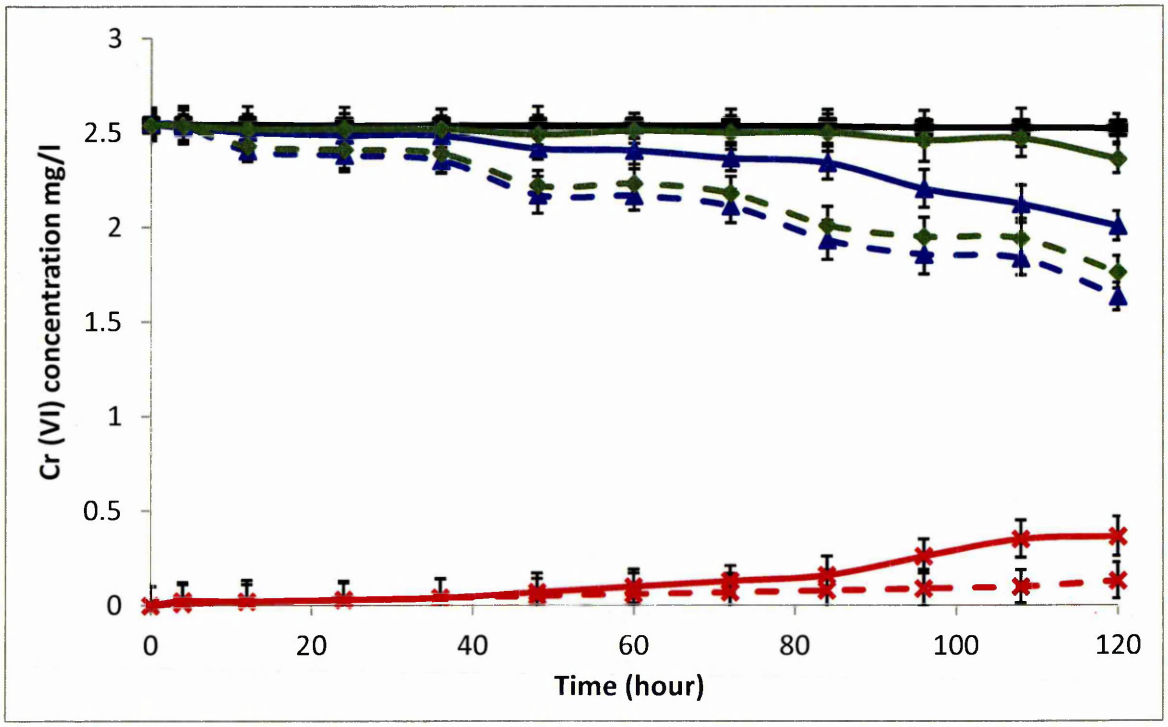
Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.12: Measurement of Cr (VI) concentration in autoclaved Ps. aeruginosa bacterial strain (with and without sodium azide) using DPC assay

Cr (VI) removal activity using *E. cloacae* is shown in Figure 3.13. It was found that the decline of Cr (VI) concentration in the supernatant sample with sodium azide was higher than without sodium azide. After 120 hours of reaction time Cr (VI) concentration was 1.63 mg/l (fell by 35%) in the supernatant with sodium azide, while it was 2.01 mg/l (decreased by 21%) without sodium azide. On the other hand, the increment of Cr (VI)

concentration in the pellets samples was almost the same. It was 0.36 mg/l and 0.13 mg/l for the samples without and with sodium azide at 120 hours, respectively.

When adding Cr (VI) concentration in the supernatant with Cr (VI) in the pellets samples together yields 7% and 31% reduction of total chromium (VI) in without and with NaN_3 respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✕··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

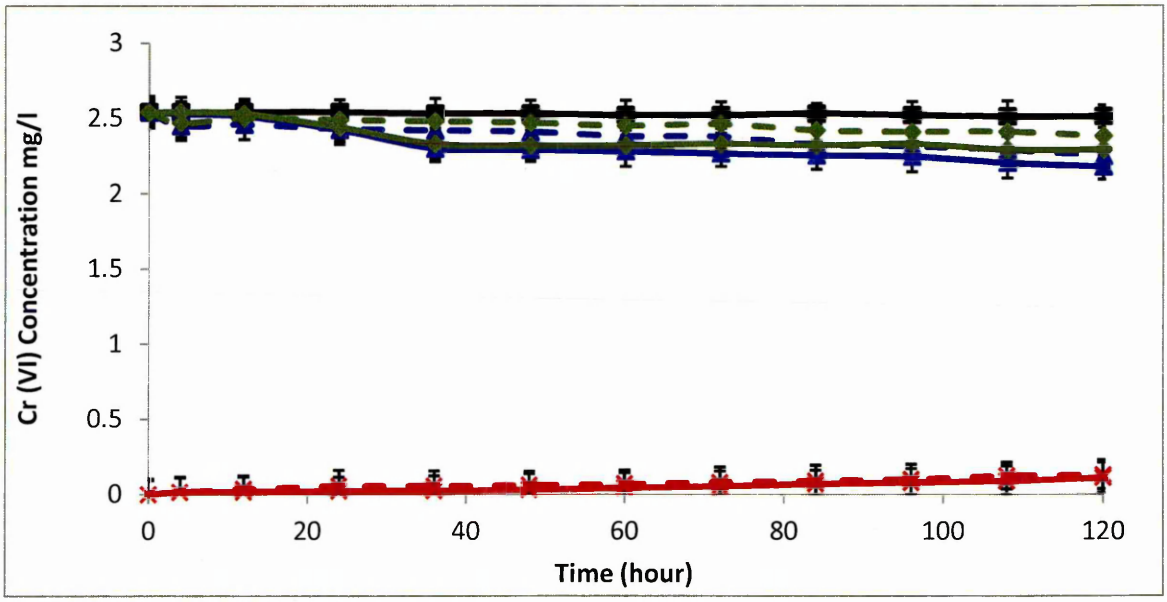
$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.13: Measurement of Cr (VI) concentration in autoclaved E. cloacae bacterial strain (with and without sodium azide) using DPC assay

Figure 3.14 shows Cr (VI) concentration was decreased using autoclaved *P. mirabilis*. Similar to *E. cloacae*, Cr (VI) concentration loss in the supernatant sample with sodium azide was greater than the supernatant samples without sodium azide. At the end of reaction time, Cr (VI) concentration was 2.25 mg/l (decreased by 12%) in the supernatant with sodium azide, while it was 2.17 mg/l (dropped by 15%) in the one without sodium azide. In the pellets samples Cr (VI) concentration gradually increased to reach 0.11 mg/l at the end of incubation time in the sample without sodium azide and 0.13 mg/l in the pellets with sodium azide.

In autoclaved *P. mirabilis*, the total chromium (VI) decrease was 10% for the samples without sodium azide and 6% for the samples with sodium azide. Consistent with the previous results, this suggests that NaN3 increases the chemical reaction of Cr (VI) in autoclaved culture.

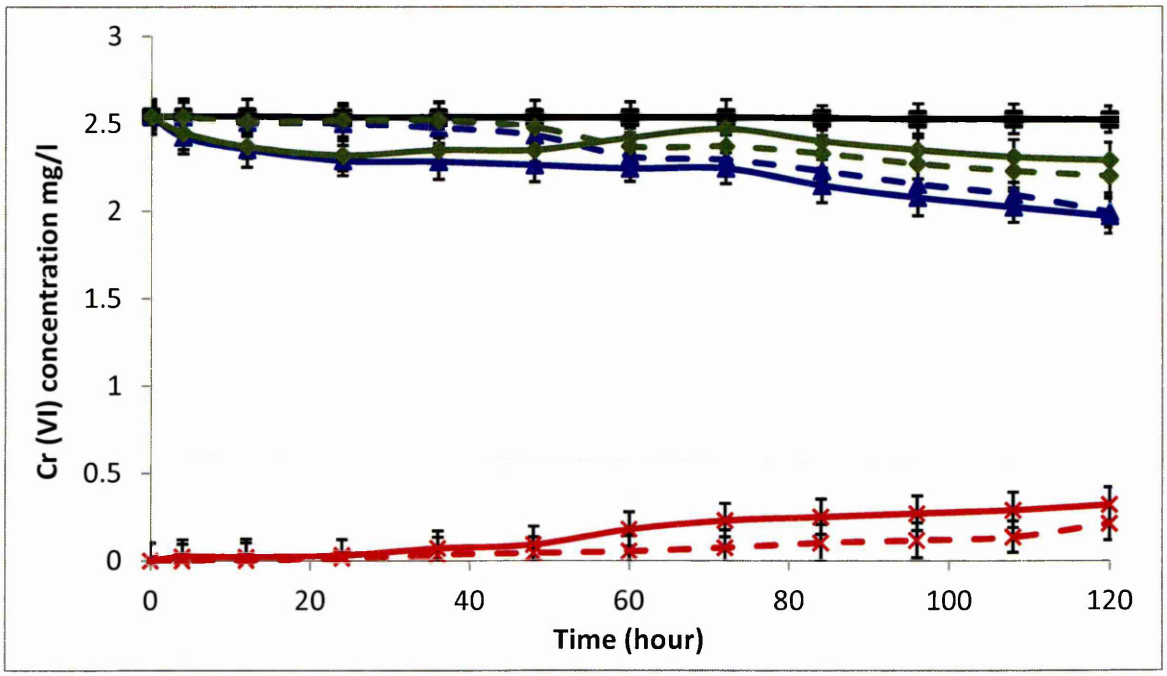


(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.
(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.
(···▲···) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).
(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.
(···✕···) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.
(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).
(···◆···) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 3.14: Measurement of Cr (VI) concentration in autoclaved P. mirabilis bacterial strain (with and without sodium azide) using DPC assay

Figure 3.15 shows Cr (VI) removal activity using autoclaved *E .coli*. Cr (VI) concentration decrease was very slow at the beginning of reaction time, and then it gradually increased to record 1.97 mg/l (fell by 23%) for the supernatant sample without sodium azide and 1.99 mg/l (22% drop) in the supernatant with sodium azide. In contrast, Cr (VI) concentration in the pellets increased and reached 0.32 mg/l for the sample without sodium azide and 0.21 mg/l with sodium azide sample at the end of reaction time.

Autoclaved *E. coli* recorded a similar result achieved by the autoclaved *P. mirabilis* sample, where the total chromium (VI) concentration decreased by 10% and 13% for the samples without and with NaN3 respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✕··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

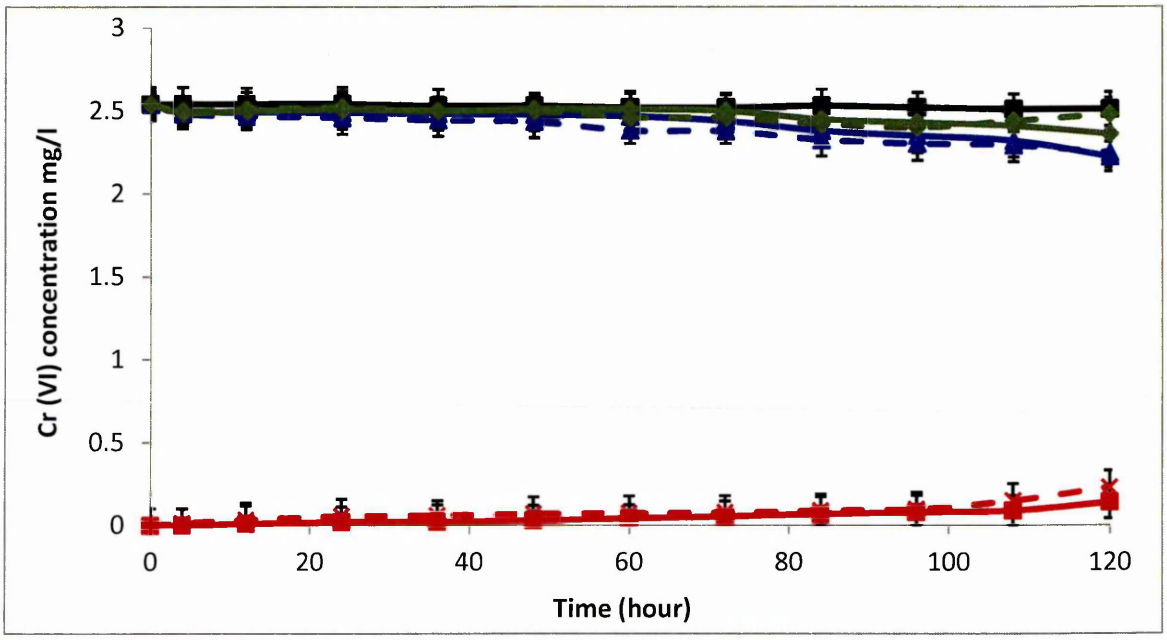
(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 3.15: Measurement of Cr (VI) concentration in autoclaved E .coli bacterial strain (with and without sodium azide) using DPC assay

The changes of Cr (VI) concentration when using *M. capsulatus*, which was grown in NMS media, were almost the same for the supernatant and pellets samples (with and without sodium azide), as shown in Figure 3.16. Cr (VI) concentration in the supernatant samples was lowered from 2.54 mg/l to 2.22 mg/l (13%) and 2.25 mg/l (11%) for both samples with and without sodium azide at the end of reaction time respectively. Cr (VI) concentration in the pellets increased with a small rate throughout the incubation time, and reached 0.14 mg/l and 0.23 mg/l at the end of reaction period.

With autoclaved *M. capsulatus* strain, the total chromium (VI) concentration without sodium azide decreased 7% over 120 hours of the reaction and 2% for the autoclaved culture with NaN_3.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✕—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✕··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

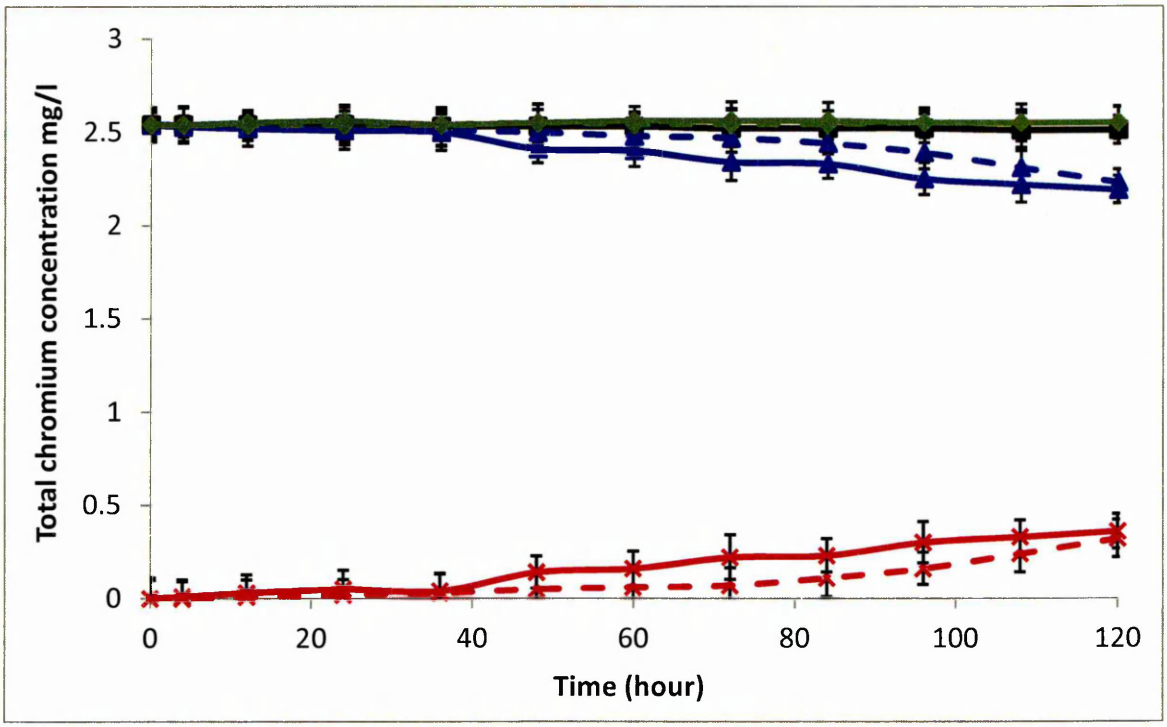
Figure 3.16: Measurement of Cr (VI) concentration in autoclaved M. capsulatus bacterial strain (with and without sodium azide) using DPC assay

All the bacterial strains studied showed measurable Cr (VI) removal even after the cells had been autoclaved. This is consistent with the study of Fein *et al.* (2002) which indicated that the nonmetabolic Cr (VI) reduction observed, in the absence of externally added electron donors, is likely a result of the oxidation of organic molecules within the cell wall, possibly the Cr-reducing enzymes responsible for metabolic enzymatic Cr (VI) reduction or cell wall cytochromes, which have large reduction potential. The same study suggested that in the absence of abundant external electron donors, there are molecules within the cell wall matrix that serve as electron donors for Cr (VI) reduction to Cr (III). In another study carried out by Faisal and Hasnain (2004), living cells showed higher chromate uptake than heat-killed cells.

3.5.2.2.2 Measurement of total chromium using ICP-OES technique

Figure 3.17 – 3.21 show the total chromium concentration, during the bioremediation process using autoclaved bacterial strains, using ICP-OES technique. Similar to the experiments with live bacteria, there are two control samples (without cells, denoted by black solid lines); M9 or NMS medium with $K_2Cr_2O_7$ and M9 or NMS medium with $K_2Cr_2O_7$ + NaN_3. Total chromium concentration in the supernatant samples (without and with NaN_3) is represented by solid and dashed blue lines, respectively. While total chromium concentration in the pellets samples (without and with NaN_3) are represented by solid and dotted red lines, respectively.

Figure 3.17 shows the total chromium concentration in the supernatant and pellet fractions using autoclaved *Ps. aeruginosa*. Chromium removal was generally low in the supernatant samples. It was 14% and 12% in the samples without and with sodium azide respectively. The corresponding total chromium concentration in the supernatant samples was 2.19 mg/l and 2.23 mg/l. However, total chromium concentration slowly increased to reach 0.36 mg/l and 0.32 mg/l in the pellets without and with NaN_3 respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

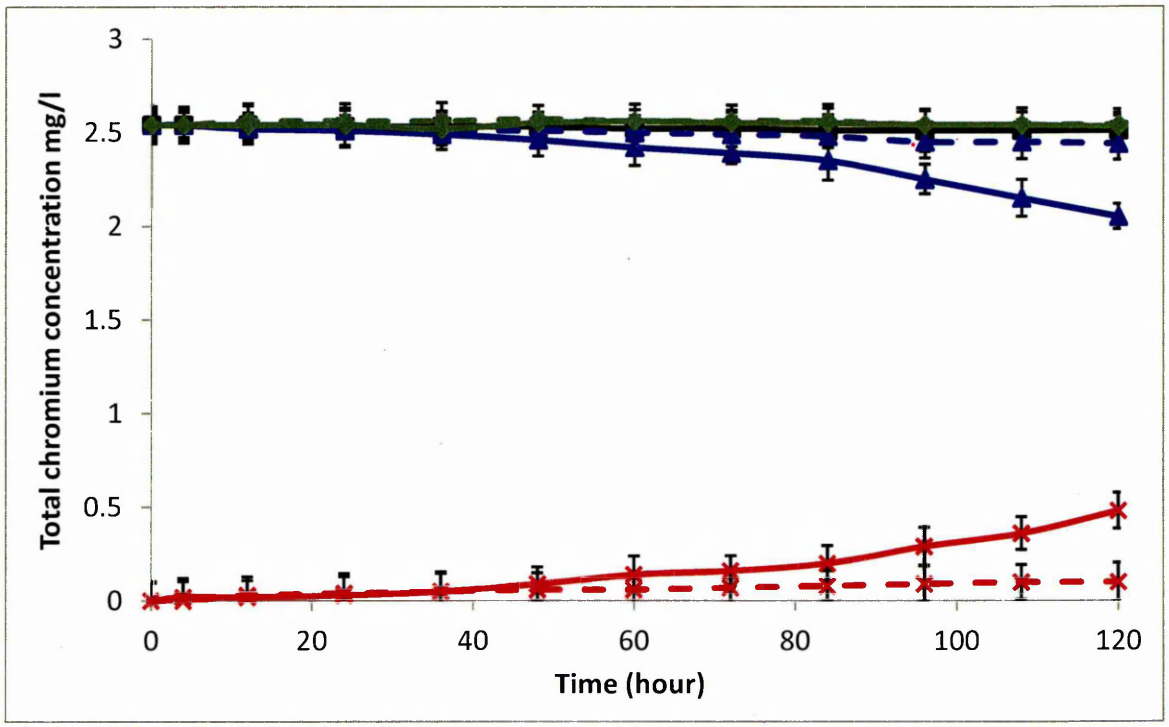
$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.17: Measurement of total chromium concentration in autoclaved Ps. aeruginosa bacterial strain (with and without sodium azide) using ICP-OES

Compared to autoclaved *Ps. aeruginosa*, the removal of chromium from the supernatant using autoclaved *E. cloacae* was not high (as shown in Figure 3.18) from its initial value of 2.54 mg/l. It was 2.05 mg/l and 2.44 mg/l, which corresponds to 19% and 4% in the supernatant samples without and with sodium azide, respectively, after 120 hours. In terms of the pellets, total chromium concentration elevated from 0.0 mg/l at the

beginning of reaction time to reach 0.46 mg/l and 0.16 mg/l at the end of the bioremediation process.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

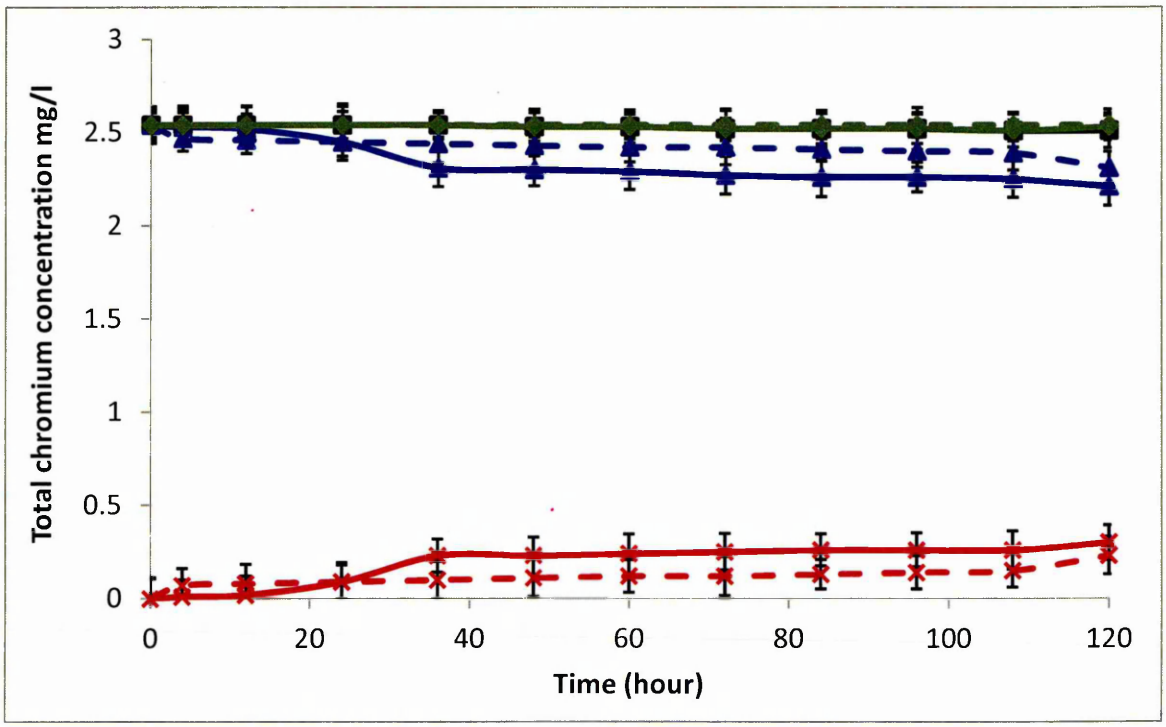
(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.18: Measurement of total chromium concentration in autoclaved E. cloacae bacterial strain (with and without sodium azide) using ICP-OES

Measurement of total chromium concentration during the bioremediation process using autoclaved *P. mirabilis* is shown in Figure 3.5 (c). Total chromium concentration in the supernatants was 2.21 mg/l and 2.31 mg/l (i.e. a removal rate of 13% and 9% for supernatant samples without and with sodium azide) at the end of reaction time. However, it increased in the pellets to reach 0.3 mg/l and 0.23 mg/l for both samples, respectively.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

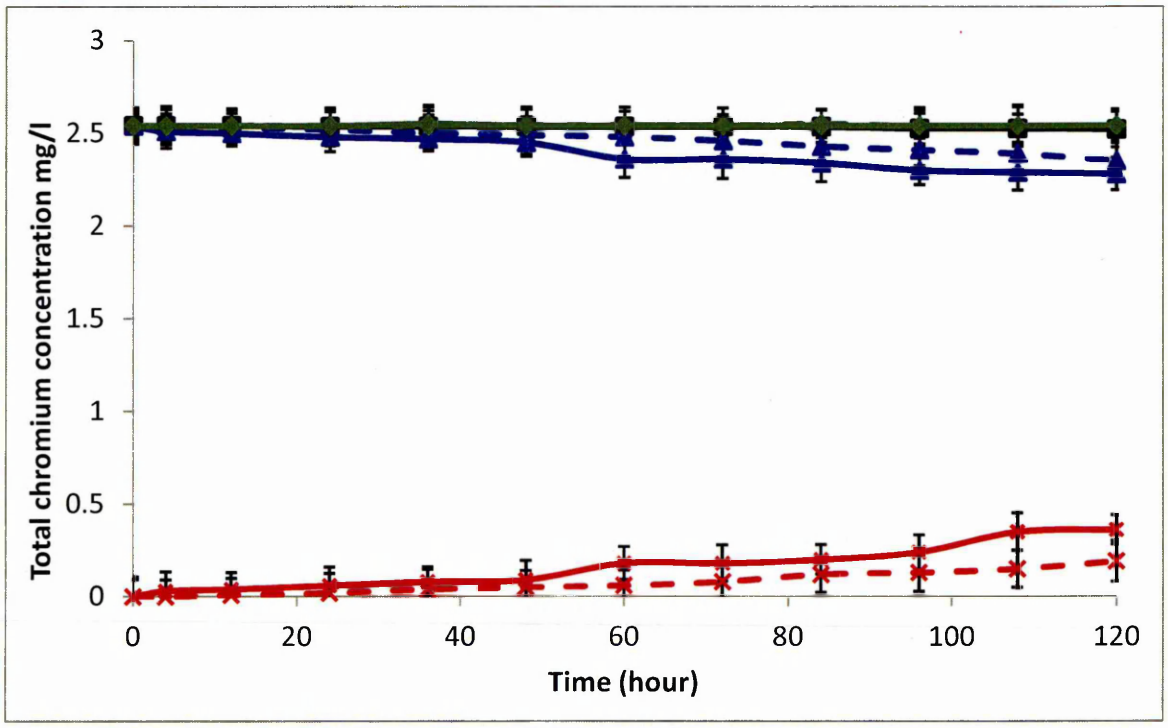
(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.19: Measurement of total chromium concentration in autoclaved P. mirabilis bacterial strain (with and without sodium azide) using ICP-OES

For autoclaved *E. coli*, total chromium concentration in the supernatant samples was 2.28 mg/l and 2.35 mg/l for without and with sodium azide, as shown in Figure 3.20. The corresponding removal rate was 10% and 7% for without and with sodium azide. A similar total chromium concentration of 0.36 mg/l was recorded after 120 hours in the pellets for without and with sodium azide.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✱··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

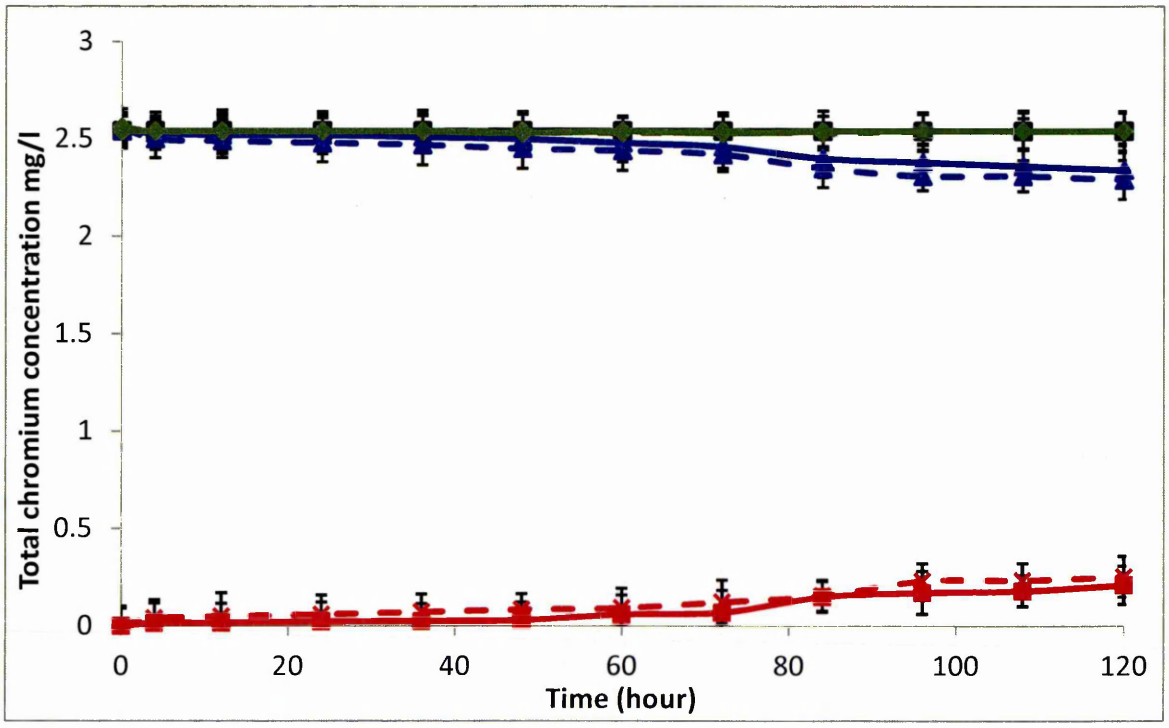
(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.20: Measurement of total chromium concentration in autoclaved E. coli bacterial strain (with and without sodium azide) using ICP-OES

Figure 3.21 shows total chromium concentration during the bioremediation process using autoclaved *M. capsulatus*. The concentration of Cr (VI) was 2.34 mg/l in the supernatant samples without sodium azide and 2.29 mg/l with sodium azide. That is corresponds to a removal of 8% and 10% for the two samples, respectively. However, total chromium concentration in the pellets was 0.21 mg/l and 0.25 mg/l after 120 hours of reaction time.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. & NaN_3 = supernatant sample (medium with cells, $K_2Cr_2O_7$ and NaN_3).

(—✖—) Pellets sample = cells with $K_2Cr_2O_7$.

(··✖··) Pellets & NaN_3 = cells with $K_2Cr_2O_7$ and NaN_3.

(—◆—) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 3.21: Measurement of total chromium concentration in autoclaved M. capsulatus bacterial strain (with and without sodium azide) using ICP-OES

3.5.3 Comparative assessment of Cr (VI) removal using the bacterial strains studied

This section presents a comparative assessment of the data presented above between *M. capsulatus* (Bath), *E. coli*, *E. cloacae*, *P. aeruginosa*, and *P. mirabilis* for Cr (VI) remediation. The comparison is conducted between live and autoclaved strains to lower Cr (VI) concentration in the supernatant samples during the incubation period up to 120 hours.

Table 3.1 summarises the percentage of Cr (VI) concentration remaining in the supernatant and pellets fractions and total Cr (VI) removal over a period of 120 hours. Among live bacterial strains, the highest percentage of total Cr (VI) removal was recorded by *Ps. aeruginosa* (65%). This is evident from the amount of Cr (VI) remaining in the supernatant samples and pellets fractions (13% and 22% respectively). After addition of NaN_3 *P. mirabilis* achieved the greatest removal of total Cr (VI) (57%), since the concentration of Cr (VI) remaining in the supernatant samples and pellets fractions was 15% and 28%, respectively. If all the Cr (VI) remaining in the system is accounted for by the concentrations measured in the supernatant and pellets fractions, this result strongly suggests that 57% of the Cr (VI) added at the start of the experiment has been converted into a different chemical form, most likely by reduction to Cr (III). NaN_3 inhibits the final step of oxidative phosphorylation (reduction of O_2 to water by cytochrome *c* oxidase). It may be that inhibition of cytochrome *c* oxidase in *P. mirabilis* leads to channelling of electrons away from central metabolism into reduction of Cr (VI).

For autoclaved bacterial strains, the percentage of total Cr (VI) drop ranged between 7% - 10%. When NaN_3 was added, the percentage of total Cr (VI) concentration increased. *E. cloacae* recorded the highest removal of total Cr (VI) (31%), as the concentration of Cr (VI) remaining in the supernatant samples and pellets fractions was 64% and 5%, respectively. The effect of autoclaving the cells clearly shows that Cr (VI) removal was due to substantial enzymatic activity.

Table 3.1: Summary of Cr (VI) concentration using DPC assay after 120 hours of bioremediation process

Bacterial strain	Amount of Cr (VI) after 120 h as a percentage of the amount of Cr (VI) added initially		The amount of Cr (VI) chemically reduced	Treatment
	Supernatant samples	Pellet fractions		
Ps. aeruginosa	13%	22%	65%	None
	22%	31%	47%	NaN_3
	81%	11%	8%	Autoclaved
	74%	9%	17%	Autoclaved with NaN_3
E. cloacae	25%	30%	45%	None
	28%	27%	45%	NaN_3
	79%	14%	7%	Autoclaved
	64%	5%	31%	Autoclaved with NaN_3
E. coli	20%	27%	53%	None
	43%	38%	19%	NaN_3
	78%	13%	9%	Autoclaved
	78%	8%	14%	Autoclaved with NaN_3
P. mirabilis	24%	34%	42%	None
	15%	28%	57%	NaN_3
	86%	4%	10%	Autoclaved
	89%	5%	6%	Autoclaved with NaN_3
M. capsulatus	27%	30%	43%	None

	44%	11%	45%	NaN_3
	87%	6%	7%	Autoclaved
	89%	9%	2%	Autoclaved with NaN_3

The percentage of total chromium concentration remaining in the supernatant and pellets fractions and Cr balance after 120 hours of reaction is shown in Table 3.2. The chromium balance ranges 99% - 102%. This indicates that all the chromium added at the start of the experiments could be accounted for by the chromium detected in the two fractions analysed at 120 hours.

Table 3.2: Summary of total chromium concentrations using ICP-OES after 120 hours of bioremediation process

Bacterial strain	**Total Cr in each fraction at 120 h as a percentage of the amount of Cr added initially (as Cr [VI])**		**Cr balance**	**Treatment**
	Supernatant samples	**Pellets fractions**		
Ps. aeruginosa	65%	36%	101%	None
	63%	36%	99%	NaN_3
	86%	14%	100%	Autoclaved
	88%	12%	100%	Autoclaved with NaN_3
E. cloacae	58%	41%	99%	None
	51%	49%	100%	NaN_3
	81%	18%	99%	Autoclaved
	96%	6%	102%	Autoclaved with NaN_3

E. coli	73%	29%	102%	None
	60%	40%	100%	NaN_3
	90%	10%	100%	Autoclaved
	93%	8%	101%	Autoclaved with NaN_3
P. mirabilis	61%	38%	99%	None
	60%	40%	100%	NaN_3
	87%	13%	100%	Autoclaved
	91%	9%	100%	Autoclaved with NaN_3
M. capsulatus	39%	61%	100%	None
	83%	16%	99%	NaN_3
	94%	8%	102%	Autoclaved
	90%	10%	100%	Autoclaved with NaN_3

Figure 3.22 compares the performance of both live and autoclaved bacterial strains. All live strains have shown the ability to decrease Cr (VI) concentration. The highest percentage of Cr (VI) removal over a period of 120 hours with a starting concentration of 2.54 mg/l of Cr (VI), was recorded by *P. aeruginosa* (87%) followed by *E. coli* with 80%, *P. mirabilis* with 76%, *E. cloacae* 75% and the lowest percentage of removal was 73% achieved by *M. capsulatus* strain. Unlike live strains, the autoclaved bacteria were less effective in reducing the concentration of Cr (VI). Among all autoclaved strains, the highest level of Cr (VI) was recorded with *E. coli* bacteria (23%), followed by *E. cloacae* 21%, *P. aeruginosa* recorded 19%, *P. mirabilis* 15% and then *M. capsulatus* with only 11%.

It can be clearly seen that the concentration of Cr (VI) was significantly decreased with the live strains during the bioremediation process when compared with the

autoclaved strains. This is due to chromate reducing enzymes, which are responsible for metabolic enzymatic Cr (VI) reduction or cell wall cytochromes, which are denatured and inactivated during autoclaving (Fein *et al.*, 2002). Presumably, the Cr (VI) reducing activity that remains after autoclaving is due to chemical reaction between Cr (VI) and denatured cellular components.

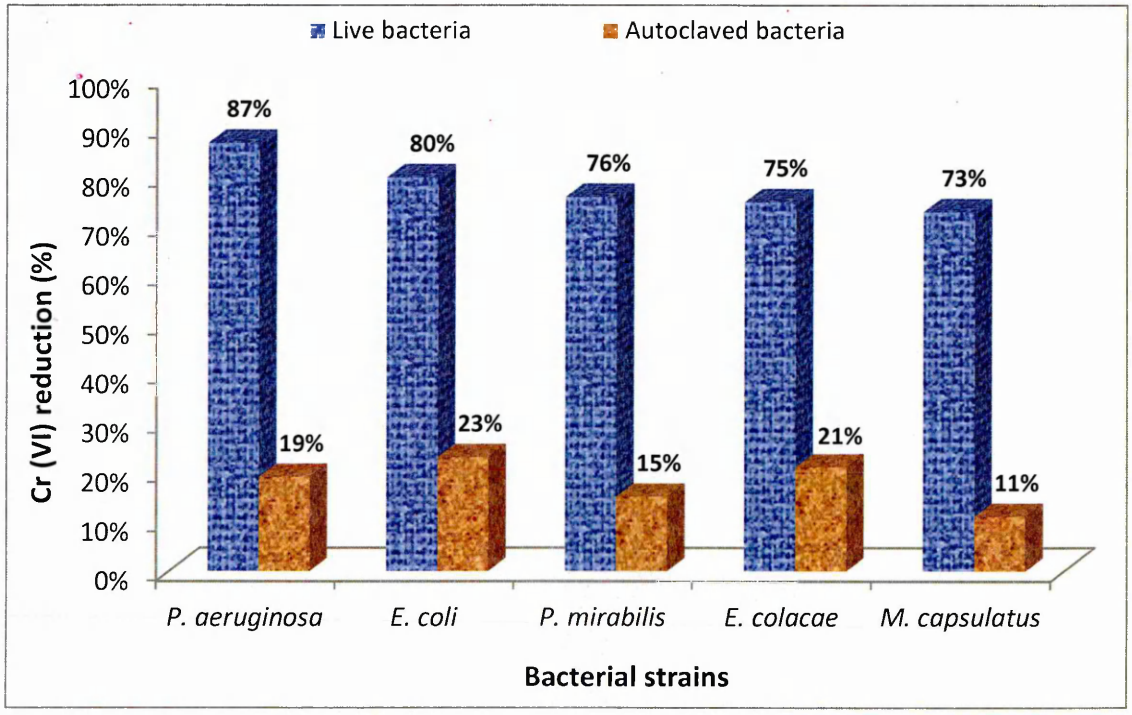


Figure 3.22: Percent Cr (VI) removal using live and autoclaved bacterial strains in the supernatant at the end of incubation period

3.6 Summary

All bacterial strains used in this study have shown satisfactory performance of Cr (VI) removal at different incubation times. No decrease in the concentration of chromate VI was detected with cell-free NMS and M9 medium (control) samples. Overall results have shown the autoclaved bacterial strains were also able to lower Cr (VI) concentration, although the removal of chromium VI occurred to a lesser extent than

using their live counterpart. Fein *et al.* (2002) suggested that in the absence of abundant external electron donors, there are molecules within the cell wall matrix that serve as electron donors for Cr (VI) reduction to Cr (III). This seems the most likely explanation because it is unlikely that substantial enzymatic activity could survive autoclaving.

The use of autoclaved bacteria in Cr (VI) removal process may under certain circumstances, offer several advantages over living cells. The use of autoclaved bacteria eliminates nutrient supply and the heavy metal toxicity problems which occur in living cells (Bai and Abraham, 2001; Zouboulis *et al.*, 1999). Besides that, killed cells could be stored or used for extended periods, and therefore, problems associated with the need to provide suitable growth conditions would not arise (Sheng *et al.*, 2004).

The growth of bacterial strains has been measured in either NMS or M9 medium containing potassium dichromate and potassium dichromate with sodium azide. All bacterial strains have been successfully grown prior to the bioremediation process. It was clear that the growth of cells with sodium azide was slow compared with the pure cells and the cells with potassium dichromate.

Since chromate is a strong oxidizing agent, it is reasonable that some of the chromate reduction could be a result of chemical redox reactions rather than biochemical activity of cells. However, inhibition of cell metabolism by adding sodium azide has a much smaller effect on Cr (VI) removal than autoclaving and as mentioned earlier in some instances sodium azide actually increased the Cr (VI) removal activity. Therefore, it can be concluded that only a part of the enzyme catalyzed Cr (VI) removal activity can be inhibited as a result of adding 0.05% NaN_3.

Hexavalent chromium removal using *P. mirabilis* strain (in both living and autoclaved cells) was selected for more detailed analysis, because Cr (VI) removal or reduction using *P. mirabilis* has not been previously reported. Similarly the chromium VI removal activity by *M. capsulatus* was chosen for further investigation because only a single study in the literature had previously focused on Cr (VI) reduction by this organism.

CHAPTER 4

4 DETAILED ANALYSES OF CHROMIUM (VI) INTERACTION WITH *PROTEUS MIRABILIS*

4.1 Introduction

In this chapter, the potential of *Proteus mirabilis* is investigated as a means of Cr (VI) removal. Bioremediation of hexavalent chromium using *P. mirabilis* bacteria is a novel method and has not been reported in the literature. *P. mirabilis* is a Gram-negative, rod-shaped bacterium. The *Proteus* group belongs to the family *Enterobacteriaceae*. According to Mckell and Jones (1976) found in O'Hara *et al.* (2000) the tribe *Proteeae* includes three genera which are *Proteus*, *Morganella* and *Providencia*. The genus *Proteus* is found in soil, water, and faecally contaminated materials (Mordi and Momoh 2009). It also may be found in the intestines of normal individuals and are widely disseminated through human communities. They may function as primary as well as secondary pathogens and their incidence in infected burns and wounds has increased in recent years. Since *Proteus* species contaminate wounds and thus cause infections, they are frequently isolated from infected wounds.

In the previous chapter, a *P. mirabilis* bacterial strain isolated from a nosocomial infection was shown capable of Cr (VI) removal. In this chapter further experimental investigations are reported on the same strain including the confirmation of the bacterial strain using the 16S rRNA-specific PCR and gene sequencing. Cell fractionation experiments were also carried out to establish in which part of the bacteria cell that the reduction or removal of Cr (VI) occurs. Identification of chromium species that exist in the supernatant samples was carried out using ICP-MS coupled with an ion chromatography column. FTIR analysis was also carried to investigate the changes in the cells after the addition of potassium dichromate.

Since *P. mirabilis* has not been reported previously in Cr (VI) bioremediation, it was decided to investigate the process using a well known *P. mirabilis* strain, to give information whether the chromate VI removal activity is common among *P. mirabilis*, and the results can be compared to results that others have obtained on divers aspects of the biology of *P. mirabilis.* Thus, the Cr (VI) bioremediation experiment was carried out using *P. mirabilis* HI4320 bacterial strain was obtained from Professor Harry L. T. Mobley Research Laboratory, Department of Microbiology and Immunology, Medical

School, University of Michigan, USA. Cr (VI) removal activity is reported in this chapter using DPC assay results.

4.2 Methods

4.2.1 Identification of Proteus mirabilis using 16S rRNA-specific PCR and gene sequencing

16S rRNA gene sequencing is a powerful tool used to trace phylogenetic relationships between bacteria and to identify bacteria from various sources, such as environmental or clinical specimens. 16S rRNA gene sequences contain hyper variable regions which can provide species-specific signature sequences useful for bacterial identification. 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacteria identification (Chakravorty *et al.*, 2007). In this investigation, a strain of *P. mirabilis*, which was clinically isolated from a wound swab, has been characterised using the 16S rRNA gene sequencing method as described below.

4.2.1.1 DNA Extraction Protocol

A 2 ml liquid culture of *P. mirabilis* grown in M9 medium (OD_{600} was 1.3) was transferred to 2 Eppendorf tubes (1 ml in each tube) and centrifuged at 13000 *g* for 2 minutes. The pellet was resuspended in 400 µl of TE+RNase.

30 µl SDS (10%) and 3 µl proteinase K (20 mg/ml) were added and the mixture was incubated at 37 °C in water bath for 1 hour. Then, 300 µl chloroform and phenol (1:1 v/v) was added to each Eppendorf tube, which was gently inverted 10 times in order to mix.

Each tube was centrifuged for 10 minutes at 13000 *g* at room temperature. The upper phase was transferred to a clean Eppendorf tube. 700 µl of isopropanol was added and mixed gently. The mixture was then centrifuged again at 13000 *g* for 15 minutes at room temperature, the upper phase was removed to a clean Eppendorf tube, and then the

pellets were washed with 1 ml of 70 % ethanol. After that the mixture was spun again in a centrifuge at 13000 *g* at room temperature for 10 minutes. After the removal of the supernatant, 50 µl of sterilised distilled water was added to the pellets and heated at 55 °C with gentle mixing.

The concentrated DNA samples were diluted with the appropriate volumes of distilled water to give a range of concentrations equivalent to 1%, 10%, 20% and 100% that of the original DNA preparation.

4.2.1.2 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed using Primus 96 plus Thermocycler (MWG Biotech). In this experiment the primers were identified to amplify the highly conserved 16S rRNA gene for the identification of bacteria.

PCR was performed using *Taq* DNA polymerase, recombinant (Invitrogen). The reaction mixture contained a total volume of 50 µl; 5 µl of 10 x PCR buffer, 0.5 µl bovine serum albumin (BSA), 2.5 µl $MgCl_2$, 1 µl of reverse and forward primer specific for the 16S rRNA gene (Bodrossy *et al.*, 1999), 0.5 µl dNTP and 0.5 µl *Taq* DNA polymerase, and 2 µl of DNA extracted from the product were used as template.

The cycling parameters for PCR were carried out on the thermocyclers conditions, where the first step consisted of 5 minutes of denaturation at 95 °C, followed by 35 cycles of denaturation for 30 seconds at 95 °C, 30 seconds of annealing at 50 °C and elongation for 60 seconds at 72 °C. The program was concluded with a final extension of 72 °C for 10 minutes.

4.2.1.3 Agarose gel electrophoresis

DNA samples were immersed into each well on the agarose gel (1%), which was stained with 2 µl of 10 mg/ml ethidium bromide, after mixing the samples with loading dye with buffer which gave the solution colour and the dye acts as colour indicator during

electrophoresis. Gene ruler 1 kb plus DNA ladder (Fermentas) was added into its well, which provided the comparison for DNA to determine the DNA size.

After the samples were transferred into the wells, the gel electrophoresis was run by applying an electrical current of 52 mA to the gel tank for 30 minutes. The gel was then removed from the tank and the DNA under photographed with ultraviolet light illumination using a transilluminator.

4.2.1.4 Purification of PCR products

PCR purification was used to purify and amplify DNA for sequencing. PCR products were purified following the manufacturer's instructions from the QIAquick spin handbook (cat. no. 28104 Qiagen Ltd), QIAquick PCR purification kit protocol, as follows;

5 volumes of buffer PB were added to 1 volume of the PCR sample and the mixture was centrifuged at 13000 *g* for 1 minute. Then, the mixture was transferred to a QIAquick column and centrifuged again for 1 minute at 11000 *g*. Then, the flow-through was discarded. 0.75 ml of buffer PE was transferred into in the QIAquick column. The contents of the column were then centrifuged for 1.5 minutes at 11000 *g* and the flow-through was discarded again.

The QIAquick column was placed in a clean 1.5 ml Eppendorf tube. 50 µl of Buffer EB was added to the centre of the QIAquick membrane and the column was centrifuged for 1 minute to collect the buffer containing the eluted purified DNA. Then, the purified DNA was analysed on a gel by adding 1 µl of dye blue and 5 µl of purified DNA. The purified PCR products were stored at -2 °C.

4.2.2 Cell fractionation

Cell fractionation is a combination of various methods used to separate cell organelles and components by cell breakage followed by centrifugation. At the end of this process cell components are separated into three fractions; cell wall, cell membrane and cytoplasm.

In this work, a standard bacterial cell fractionation method reported in Smith and Foster (1995) was adopted with slight modification, as follows. 500 ml of *P. mirabilis* bacterial culture was grown in 1 L conical flask in an orbital shaking incubator at 37 °C and 180 rpm. When the culture density reached an optimum (OD_{600} 0.5-1.0), the cells were harvested by centrifugation at 11000 *g* and 4 °C for 10 minutes. Then, they were washed with ice-cold 50 mM Tris-HCl (pH 7.5) and resuspended in 5 ml of the same buffer. The suspension was passed through a French pressure cell (180 MPa at 4 °C), which was used to breakdown the cell walls (Figure 4.1).

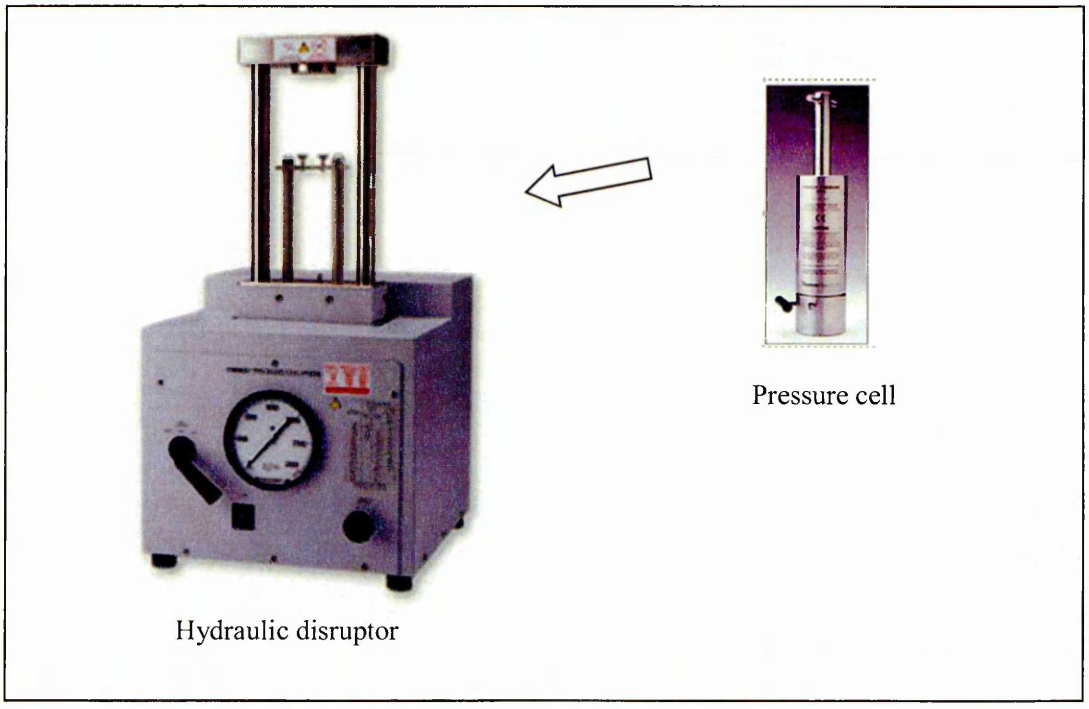


Figure 4.1: French press cell disruptor

The whole procedure was performed at 4 °C to minimize protein and peptidoglycan degradation. The lysate was centrifuged at 3000 *g* twice for 2 minutes to remove debris before being centrifuged again (27000 *g* for 20 minutes) to sediment cell wall fragments.

The cell walls were washed twice by resuspension in 50 mM Tris-HCl (pH 7.5) to give fraction F1 (cell wall-associated material). The supernatant fraction was centrifuged again at 27000 *g* for 20 minutes to remove the remaining wall material.

Membrane fragments were sedimented by centrifugation again at 105000 g for 60 minutes. The pellet was washed in ice cold 50 mM Tris-HCl (pH 7.5) and centrifuged again under the same conditions to give fraction F2 (cell membrane).

The supernatant from the first ultracentrifugation was centrifuged again under the same conditions to remove the remaining membranous material and to yield the cytoplasm fraction as the supernatant.

All fractions were stored at -70 °C and used individually for chromium VI removal using the DPC assay, explained in section 2.2.4 of Chapter 2 (Material and Methods).

4.2.3 Analysis of chromium species during the bioremediation process

4.2.3.1 Determination of Cr (III) and Cr (VI) using ICP-MS

The details of the utilized column and standard can be found in section 2.5.1 of Chapter 2 (Material and Methods).

4.2.3.2 FTIR analysis

See the method described in section 2.2.7.1 of Chapter 2 (Material and Methods).

4.2.4 Bioremediation of Cr (VI) using P. mirabilis HI4320 bacterial strain

The same method described in section 3 of Chapter 3 is used here.

4.3 Results

4.3.1 Confirmation of the bacterial isolate as Proteus mirabilis

The detection and identification of the bacteria was done on the basis of 16S rRNA analysis (Woese *et al.*, 1983; Bodrossy *et al.*, 1999). Figure 4.2 shows electrophoresis of the 16S rRNA gene of *P. mirabilis* 090321-A bacterial strain, after amplification of the whole 1500 bp gene fragment, which is specific to prokaryotic cells. PCR 16S rRNA gene sequence analysis was used for products derived from four different concentrations of bacterial DNA, namely 1/5, 1/10, 1/100 and full concentrated sample. The DNA sequences were compared with the reference database of the National Centre for Biotechnology Information (NCBI) for accurate confirmation. The sequenced portion of the 16S rRNA gene was confirmed as identical to that from the complete genome of *Proteus mirabilis* ATCC 29906T over 629 bases.

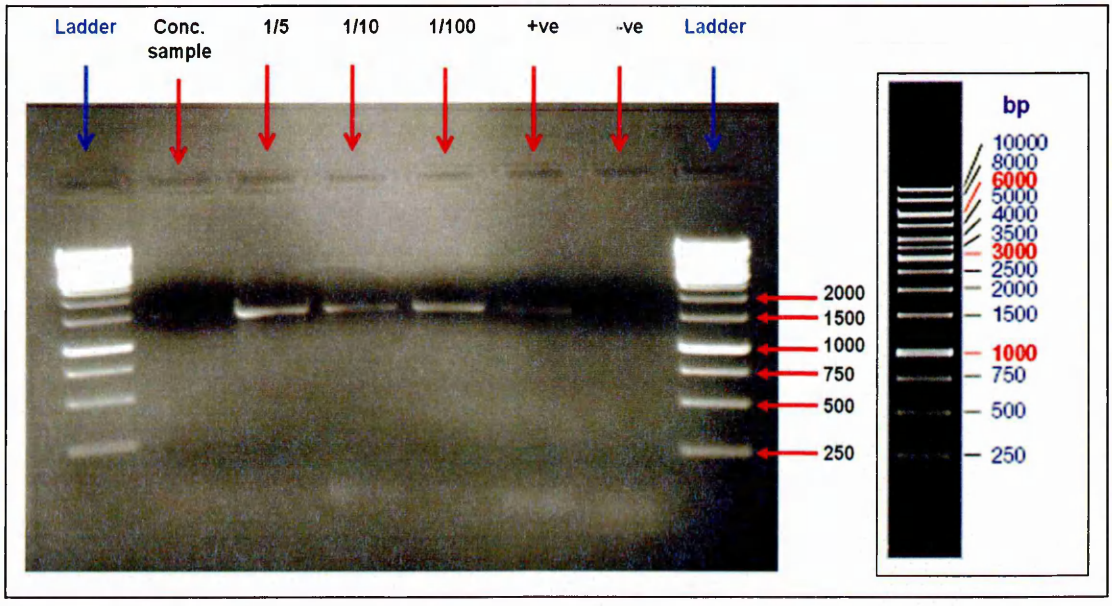


Figure 4.2: Agarose gel electrophoresis profile of P. mirabilis 090321-A strain

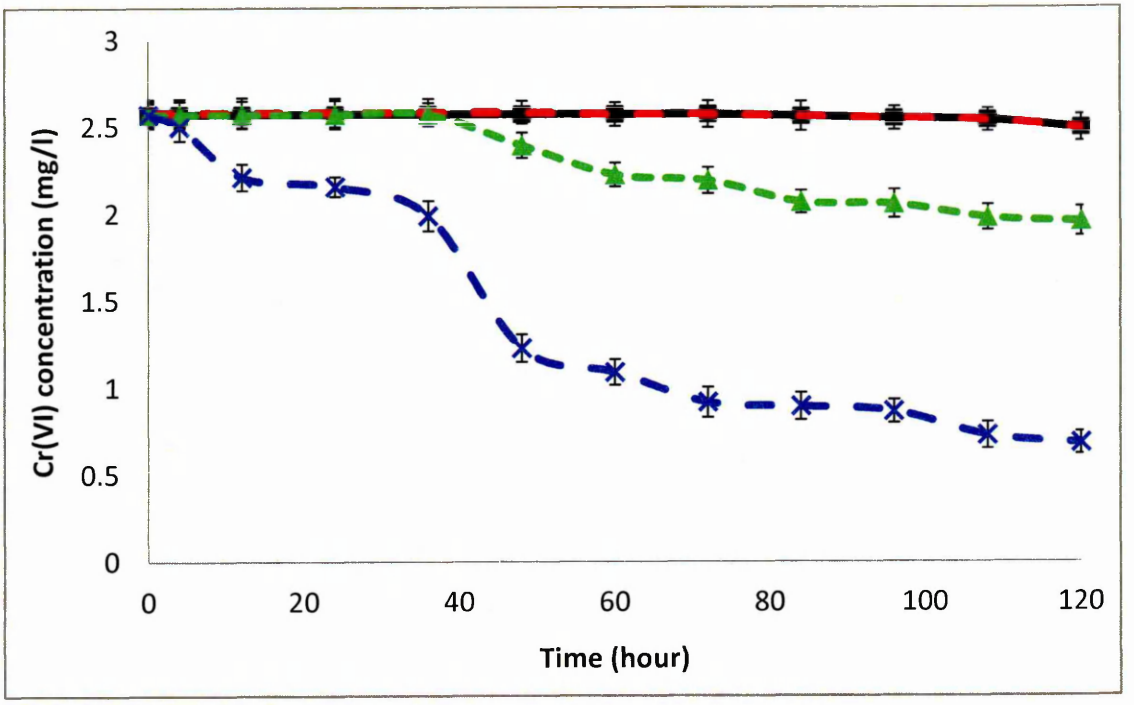
4.3.2 Cell fractionation

Figure 4.3 shows Cr (VI) removal from the cell fractions. The control sample, indicated with solid black line, contains Tris –HCl 50 mM and $K_2Cr_2O_7$. The fractions are: cell wall associated material (denoted by dashed red line), cell membrane in a dashed green line and cytoplasm, which is labelled with a dashed blue line.

As shown in the graph no removal or changes occurred in the cell wall associated material throughout the incubation. A significant removal of chromium VI in both the cell membrane and cytoplasm samples were observed. In the cell membrane, the removal of Cr (VI) began after 48 hours of incubation time. The decline in chromium VI concentration was slow and in small amounts. Cr (VI) concentration reached 1.96 mg/l at the end of the incubation time (120 hours). The chromium VI removal activity in cytoplasm started after 4 hours. Cr (VI) concentration in this portion decreased to 1.23 mg/l after 48 hours. Then, it continued decreasing until it reached its lower level (0.68 mg/l) at the end of incubation period.

These results reveal that the highest Cr (VI) removal activity resides in the cytoplasm, and there was also activity in the cell membrane. But no chromium VI removal activity was observed in the cell wall.

Since no exogenous electron donors were added in these experiments it is possible that the cell wall fraction contained enzymes capable of chromium VI reduction but lacked the necessary electron donors. To test this possibility it would be necessary to repeat the assays in the presence of likely electron donors such as NADH, NADPH, and reduced FMN.



(—■—) Control sample (Tris-HCl with $K_2Cr_2O_7$) (Cr (VI) conc. = 2.54 mg/l).

(·····) Cell wall associated material with $K_2Cr_2O_7$.

(··▲··) *P. m.* (09032-A) Pellets = pellets sample of *P. m.* (09032-A), membrane fraction.

(··✱··) Cytoplasm fraction with $K_2Cr_2O_7$.

Error bars represent triplicate measurements, standard deviation (SD) = 0.05 – 0.1mg/l

Figure 4.3: Chromate (VI) removal by cell fractions of P. mirabilis

4.3.3 Analysis of Chromium species

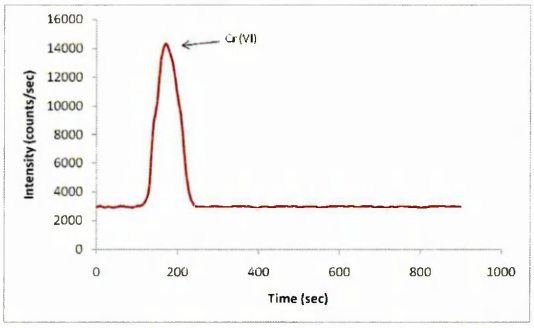
4.3.3.1 Determination of Cr (III) and Cr (VI) using ICP-MS

As described in Material and Methods chapter, the standard solution of Cr (VI) and Cr (III) are expected to elute in the regions 130-230 and 480-580 seconds, respectively.

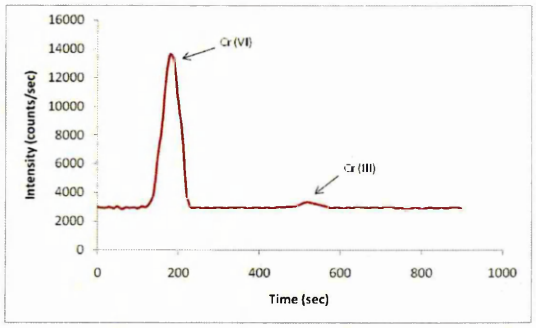
Figure 4.4 shows chromium species (Cr (VI) and Cr (III)) formed in the supernatant samples of *P. mirabilis* 09032-A with $K_2Cr_2O_7$ Cr (VI) 2.54 mg/l during the reaction period (0 to 120 hours) using ICP-MS connected with IonPac column. At the

beginning of the bioremediation processes, as shown in Figure 4.4 (a), Cr (VI) appeared as a sharp peak while Cr (III) was below the threshold of detection.

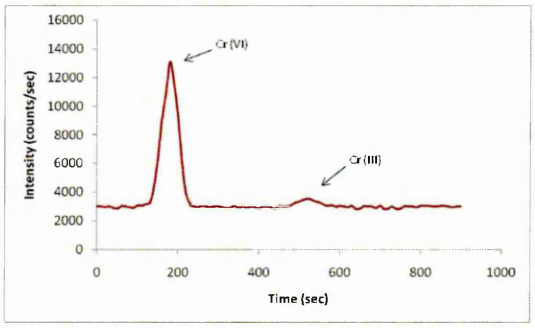
After 4 hours of incubation a very small peak of Cr (III) appeared. At the same time Cr (VI) peak decreased (Figure 4.4 (b)). The peak corresponding to Cr (III) increases while Cr (VI) decreases with the time. This result confirms that *P. mirabilis* has the potential to reduce the toxic Cr (VI) to the less toxic and less soluble Cr (III).



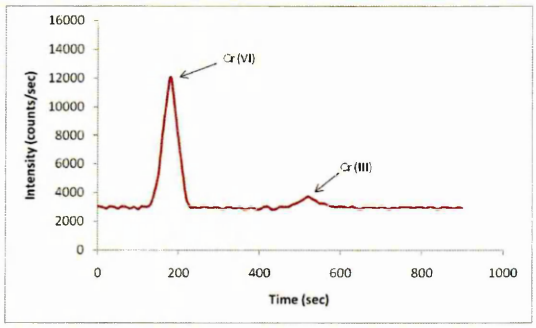
a) 0 time



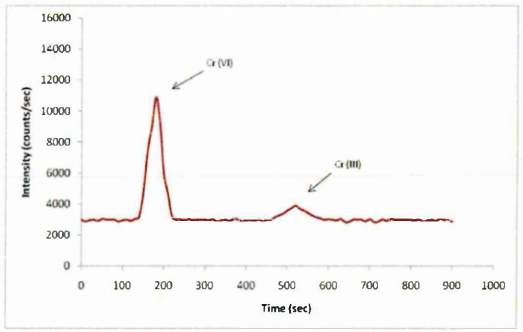
b) 4 hours



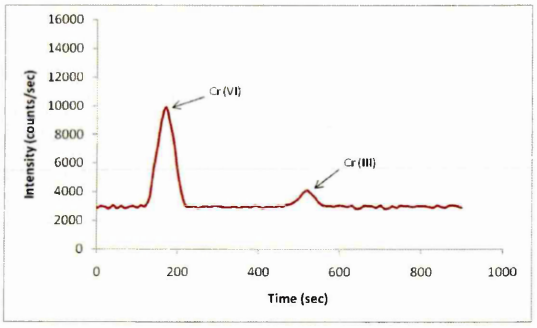
c) 12 hours



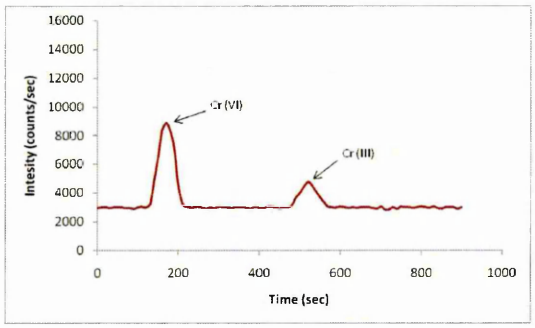
d) 24 hours



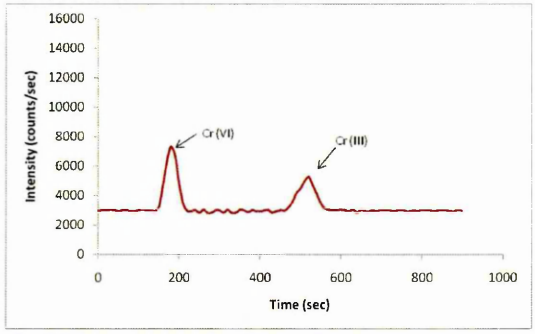
e) 36 hours



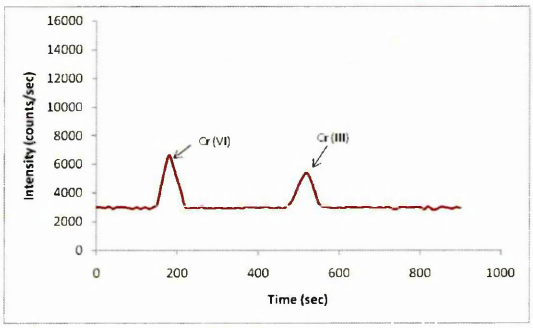
f) 48 hours



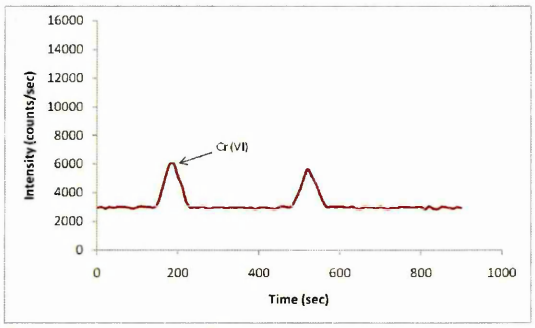
g) 60 hours



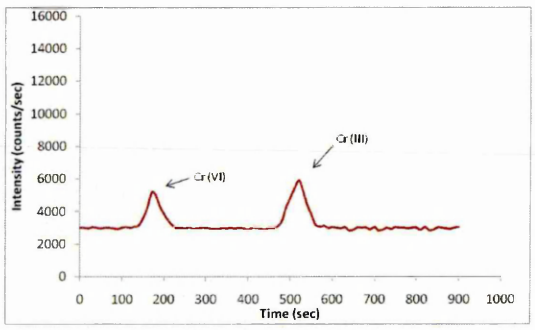
h) 72 hours



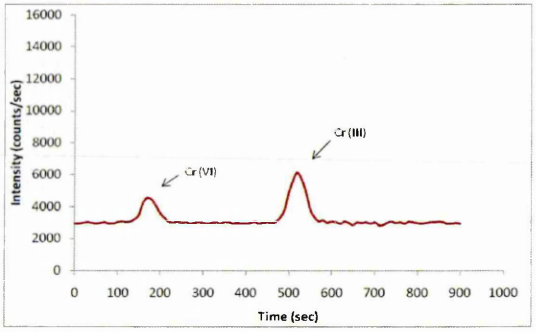
i) 84 hours



j) 96 hours



k) 108 hours



l) 120 hours

Figure 4.4: Chromatogram of Cr (VI) and Cr (III) in P. mirabilis supernatant samples using ICP-MS

In order to monitor the changes in concentrations of Cr (VI) and Cr (III) in the supernatant samples, Figure 4.5 shows the trend of the two chromium species during the reaction time. It can be seen that Cr (VI) concentration gradually decreased from its initial value (2.54 mg/l), while Cr (III) concentration increased with time. This figure shows a progressive decrease in the total chromium concentration (Cr (VI) + Cr (III)) in the supernatant, which is consistent with precipitation and biosorption of chromium species to the cells.

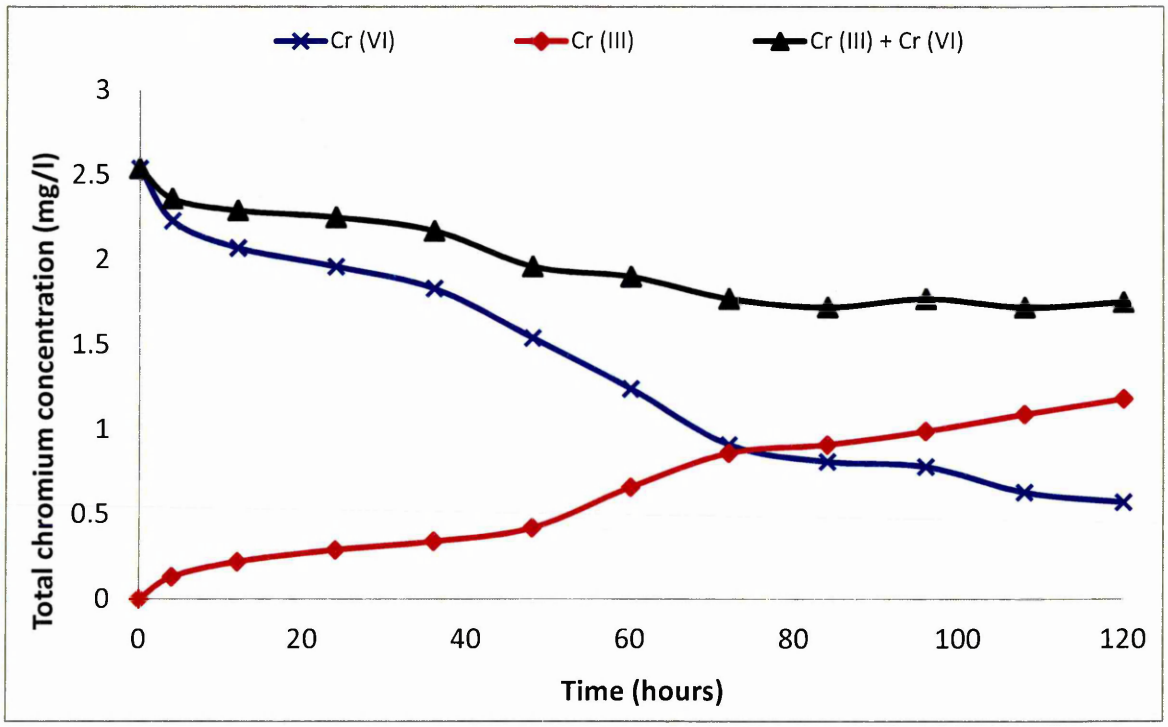


Figure 4.5: Cr (III) and Cr (VI) profiles in P. mirabilis supernatant samples during the reaction time (data of this figure were adapted from Figure 4.4)

4.3.3.2 FTIR analysis

In order to identify the changes that occur in the bacterial strain whose spectrum is being considered, it is essential to identify the functional groups present on the biomass involved in the process. FTIR spectra of the biomass show the presence of amino, carboxyl, hydroxyl, phosphate and sulfonate groups on the unabsorbed biomass (Das and Guha, 2007).

Table 4.1 summarizes the locations of the various absorption bands of some functional groups that are present in *P. mirabilis*. The functional groups, such as proteins, carbohydrates, lipids, polyphosphate groups, and other polysaccharides reported in several research studies, are identified and assigned for the infrared bands and corresponding frequencies for bacteria (Parikh and Chorover, 2006; Ojeda et *al.*, 2008). For instance, the region between 2800 – 3000 cm^{-1} presents in fatty acids and lipids (Ojeda *et al.*, 2008a). The band 1550 – 1654 cm^{-1} is attributed to amide I and II (protein) (Yee *et al.*, 2004a; Wei *et al.*, 2004). The bands at 1045 cm^{-1} correspond to polysaccharides (Yee et *al.*, 2004).

Table 4.1: List of important functional groups for bacteria and their corresponding infrared spectral bands

Functional group assignment	Wavenumber (cm^{-1})
Stretch C=O of ester functional groups from membrane lipids and fatty acids	1739 – 1725
Stretching C=O in amides (amide I)	1647 – 1660
N-H bending and C-N stretching in amides (amide II)	1548
Symmetric stretching for deprotonated COO- group	1402
Bending CH_2/CH_3	1453
Symmetric stretching of COO^- bending CH_2/CH_3	1384
Vibration C-N from amides	1305
Vibrations of C-O from esters or carboxylic acids	1300 – 1250
Vibrations of –COOH and C-O-H double bond stretching general phosphate group and phosphdiester of nucleic acids	1262
Stretching of P=O in phosphates	1225
Asymmetric and symmetric stretching of $P(OH_2)$ in phosphates, vibrations of C-OH, C-O-C and C-C of polysaccharides.	1200 – 950
Stretching P=O of phosphodiester, phosphorylated proteins, or polyphosphate products.	1085
Symmetric stretching vibration of phosphoryl group.	976
Fatty acids region	3100
$-CH_3$, CH_2, ≡CH	2800
Amide region	1800
Dominated by the amide I and amide II bands of proteins and peptides.	1500
Mixed region proteins, fatty acids and phosphate	1500 – 1200
Polysaccharide region cell wall	1200 – 900

Figure 4.6 shows three FTIR spectra of *P. mirabilis* with potassium dichromate at three different reaction times (0 hours, 24 hours and 48 hours) for an entire region 650 – 4000 cm^{-1}. The spectrum at 0 time was used as reference and conspicuous changes in the FTIR spectrum are observed after incubation for 24 and 48 hours on the biomass. In order to show the observed changes in infrared bands (appearance, disappearance and shifting of peaks), the entire region 650 – 4000 cm^{-1} was divided into sub regions. There were no changes observed in the region 650 – 900 cm^{-1}, since the region below 900 cm^{-1} is considered as a finger print region with small spectral features (Uldelhoven *et al.*, 2000).

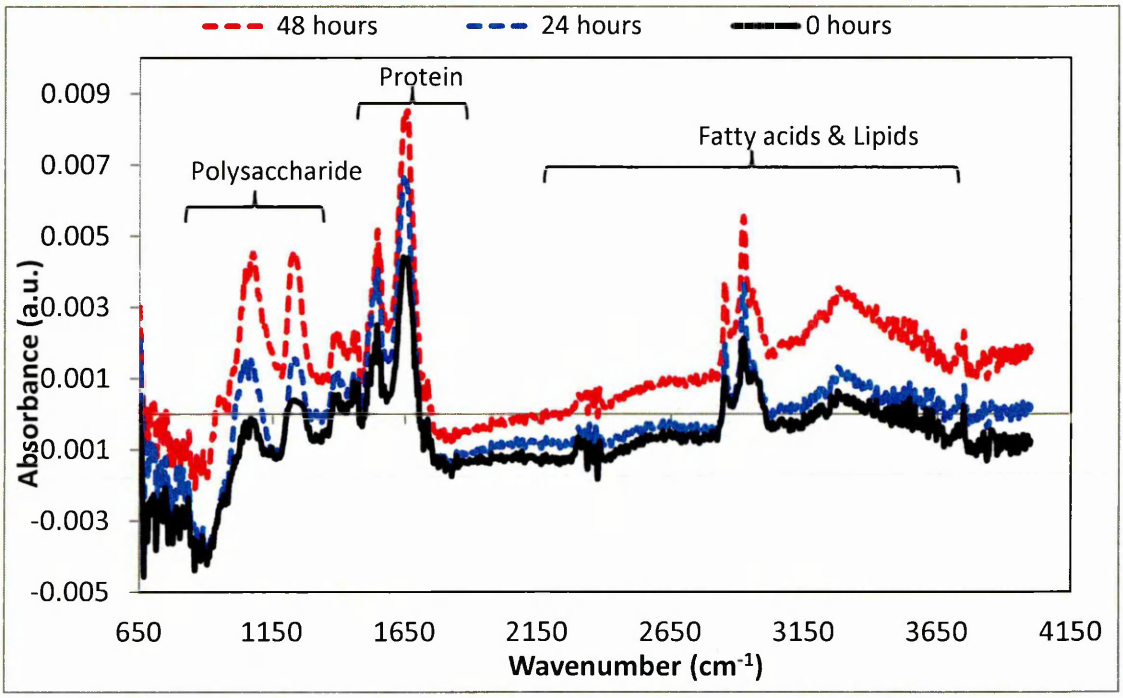


Figure 4.6: FTIR spectra of P. mirabilis with $K_2Cr_2O_7$ at different incubation times

Figure 4.7 shows expanded FTIR spectra in the region 900 - 1300 cm^{-1}. The changes in FTIR spectra appeared in the form of shoulder at 950 cm^{-1} (at 24 hours of reaction time), and converted into a small peak after 48 hours of incubation. The peak at 1066 cm^{-1} became bigger as shown on 24 and 48 hours curves. The peaks in the region of 910 – 1040 cm^{-1}, 970 – 1050 cm^{-1} and 1150 cm^{-1} for P-OH, P-O-C and P=O stretching,

respectively, depict the presence of phosphate groups in the biomass (Das and Guha, 2007). A new peak formed at 1221 cm^{-1} after 48 hours of incubation time. The region 900 - 1300 cm^{-1} belongs to saccharide and polysaccharide functional groups in which vibration is dominated by the C-O absorption bands of carbohydrates present within cell wall and membranes (Amiel *et al.*, 2001, Das and Guha, 2007; Melin *et al.*, 2001, Mariey *et al.*, 2001). The appearance of the new peak indicates phosphate bond intervention in chromium adsorption.

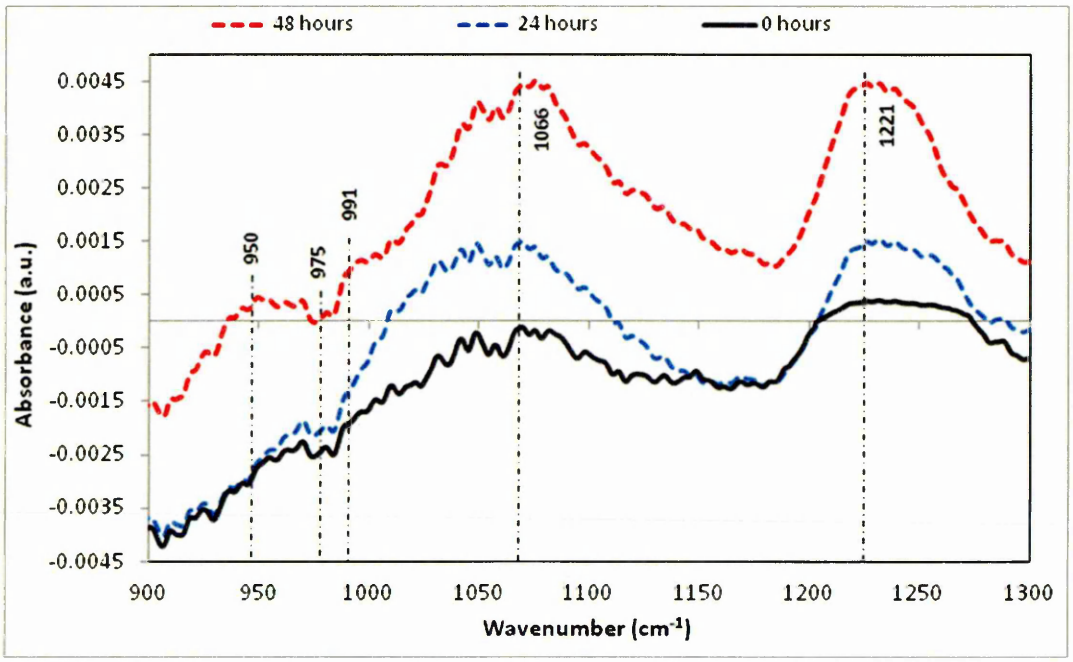


Figure 4.7: FTIR spectra (900 - 1300 cm^{-1}) of P. mirabilis with $K_2Cr_2O_7$ at different incubation times

Figure 4.8 shows FTIR spectra in the region 1350-1700 cm^{-1}. According to Melin *et al.* (2001), this region is a mixed region of protein, fatty acids and phosphate. The sub-region between 1500- 1700 cm^{-1} is dominated by amide I and amide II bands of proteins and peptides (Melin *et al.*, 2001, Tavitian *et al.*, 1986 and Udelhoven *et al.*, 2000). It was observed that the shoulder at 1552 cm^{-1} became smaller after 48 hours of incubation time. The two peaks at 1640 cm^{-1} and 1653 cm^{-1} were flat and became sharper as the reaction

time increases (i.e. at 24 and 48 hours), indicating chromium involvement in the adsorption process. The main amide I component at 1653 cm^{-1} corresponds to the stretching C=O and bending C-N vibrational modes, along with the amide II band at 1552-1566 cm^{-1}, which is assigned to the bending N-H and stretching C-N (Melin *et al.*, 2001).

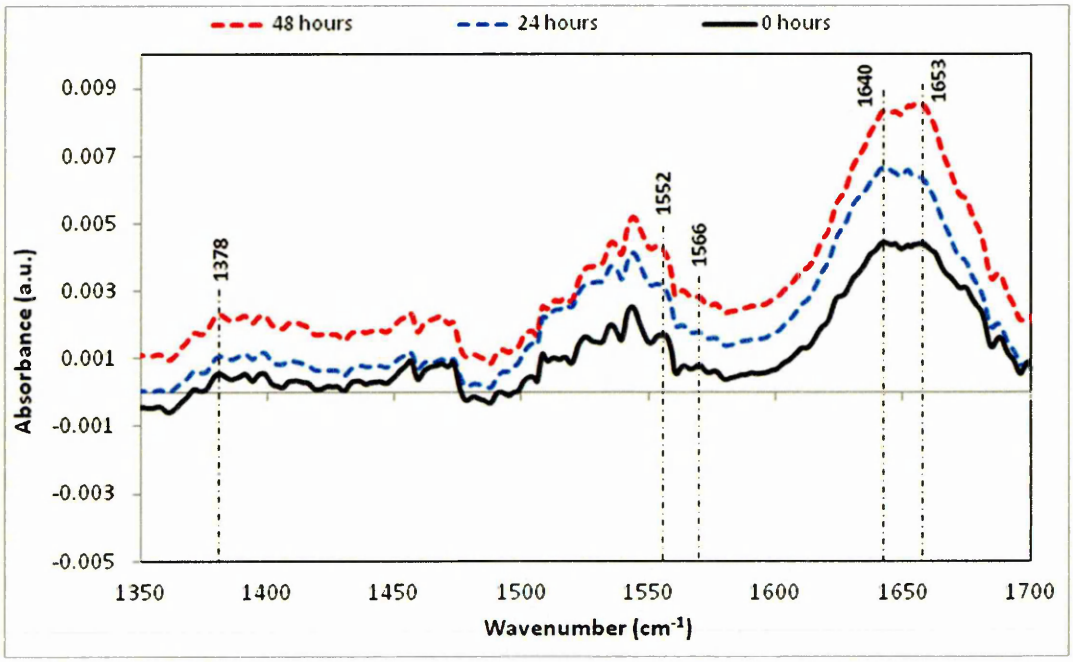


Figure 4.8: FTIR spectra (1350 - 1700 cm^{-1}) of P. mirabilis with $K_2Cr_2O_7$ at different incubation times

Figure 4.9 shows FTIR spectra between2800 and 3000 cm^{-1}. This region is the fatty acid region, which is dominated by the CH_3, $>CH_2$, and >CH- stretching vibrations of groups usually present in fatty acid components of various membranes (Melin *et al.*, 2001; Udelhoven *et al.*, 2000).The peaks at 2850 cm^{-1} and 2919 cm^{-1} became bigger and sharper, while the shoulder at 2934 cm^{-1} disappeared after 24 and 48 hours of exposure to chromium VI. Another peak appeared at 2955 cm^{-1} after 24 hours and became sharper after 48 hours of incubation time.

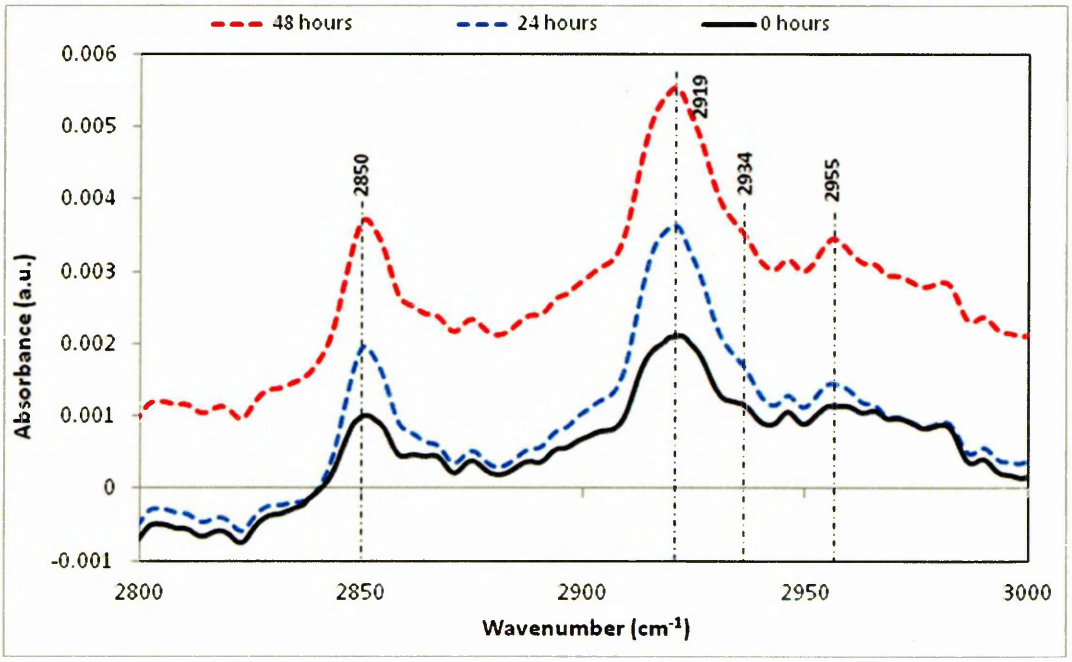


Figure 4.9: FTIR spectra (2800 - 3000 cm^{-1}) of P. mirabilis with $K_2Cr_2O_7$ at different incubation times

Figure 4.10 shows the FTIR spectra at 3700 to 3900 cm^{-1}, which is dominated by fatty acids and lipids. There were two peaks at 3725 cm^{-1} and 3758 cm^{-1} after 24 and 48 hours. The peak at 3823 cm^{-1} became sharper after 24 and 48 hours. Whereas, the peak at 3866 cm^{-1} became flat after 24 and 48 hours of the incubation time.

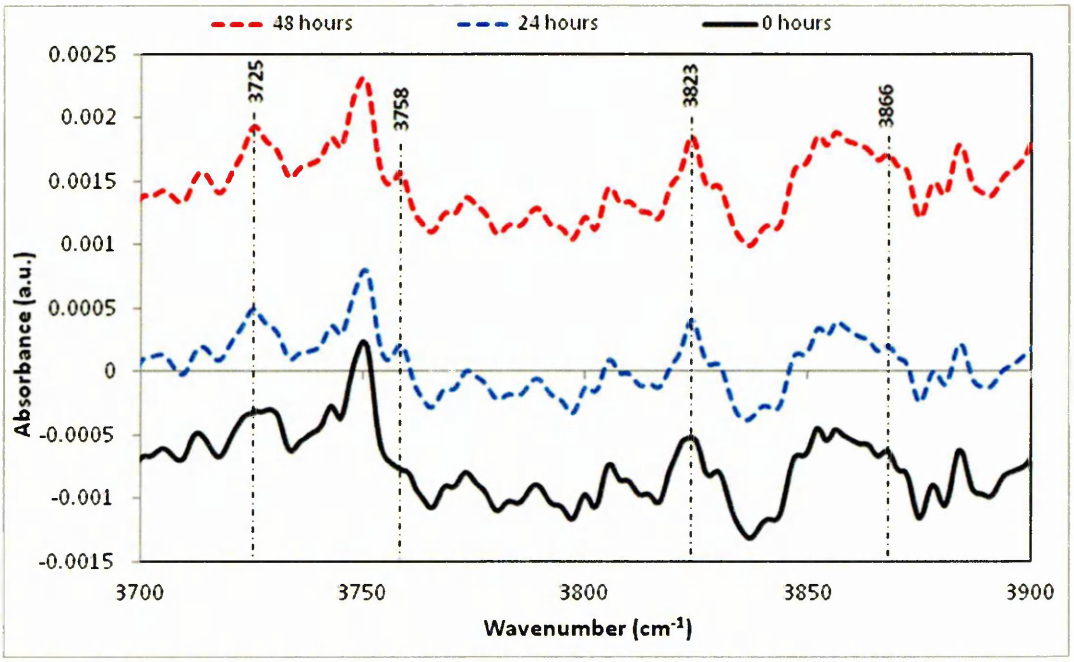
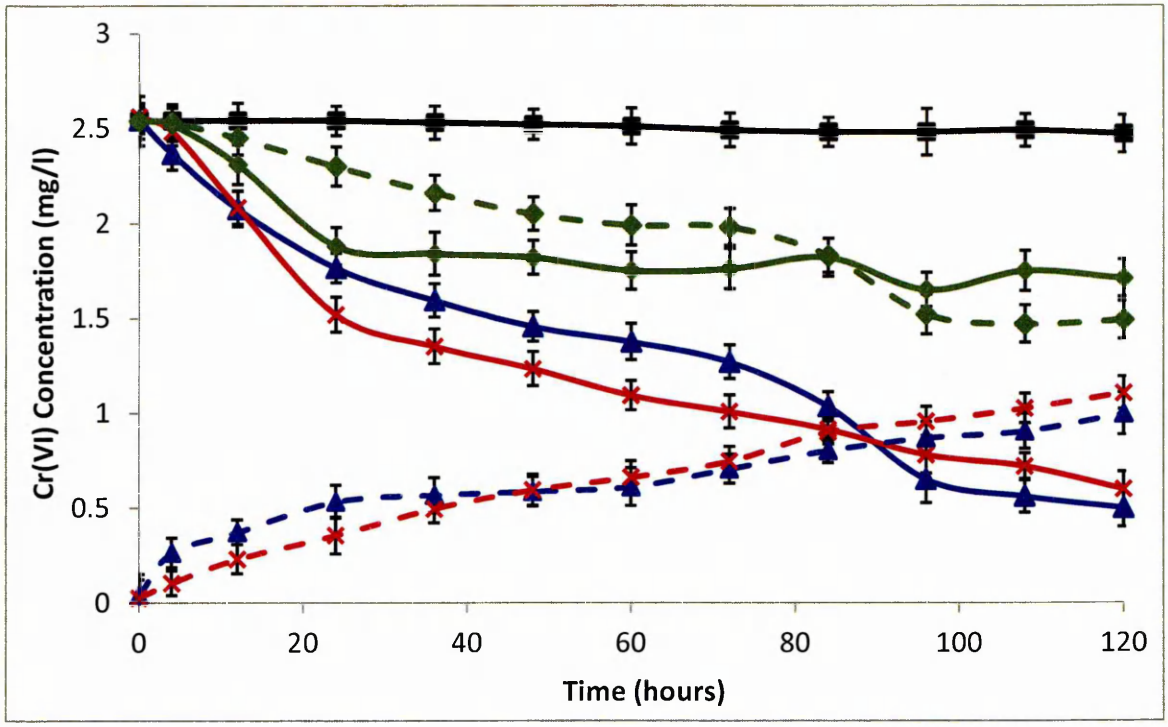


Figure 4.10: FTIR spectra (3700 - 3900 cm^{-1}) of P. mirabilis with $K_2Cr_2O_7$ at different incubation times

4.3.4 Removal of Cr (VI) using HI 4320 and 09032-A P. mirabilis bacterial strains

The aim of this experiment is to confirm the ability of *P. mirabilis* to decrease Cr (VI) concentration, as it has not been reported in the literature before. The experiment was conducted, using the two types of *P. mirabilis* bacterial strain; HI 4320 and 09032-A, using the same method described in section 3 of Chapter 3.

The results confirm the potential of *P. mirabilis* in Cr (VI) removal as shown in Figure 4.10. At the end of the incubation time Cr (VI) in the supernatant decreased by 77% and 80% for HI4320 and 09032-A bacterial strains, respectively. Hence the two independent isolates of *P. mirabilis*, including the strain HI4320 for which there is a genome sequence, showed very similar chromium (VI) removal activities. The decreases in total chromium (VI) concentration among the pellet and supernatant fractions of both strains consistent with reduction of Cr (VI) to Cr (III) by both strains.



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) *P. m.* (090321-A) Sup. = supernatant sample of 090321-A *P. m.* strain.

(···▲···) *P. m.* (090321-A) pellets = pellets sample of 090321-A *P. m.* strain.

(—✕—) *P. m.* (HI4320) Sup. = supernatant sample of HI4320 *P. m.* strain.

(···✕···) *P. m.* (HI4320) pellets = pellets sample of HI4320 *P. m.* strain.

(—◆—) Total Cr (VI) in *P. m.* 090321-A = supernatant and pellets.

(···◆···) Total Cr (VI) in *P. m.* HI4320 = supernatant and pellets.

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 4.11: Comparison of Cr (VI) removal by whole cells of HI4320 and 09032-A P. mirabilis bacterial strains [Cr (VI) concentration was monitored using the DPC assay].

4.3.5 Bioinformatics study of putative chromate reductase in P. mirabilis ATCC 29906T genome

Sequence comparison is one of the most powerful concepts in bioinformatics. It helps to uncover structural, functional and evolutionary relationships between sequences (Larkin *et al.*, 2007). In this study an attempt is made to identify proteins and genes

within the genome of *P. mirabilis* ATCC 29906T, which has primary and secondary structural properties to enable heavy metal reduction, specifically chromium (VI) to chromium (III).

Online BLAST searches (www.ncbi.nlm.nih.gov/blast) were carried out for potential homology of chromate reductase proteins in other bacterial databases against the genome information for *P. mirabilis* ATCC 29906T. The rationale of the search is to identify genes that could encode proteins with sequence similarity involved in reduction of chromate.

The search results indicated the presence of three significant homologues and two borderline homologues of the flavin/Fe_2S_2 containing *E. coli* Fre oxidoreductase accession no. M74448 (Puzon *et al.*, 2002) in *P. mirabilis* ATCC 29906T genome. The results of BLAST search are summarized in Table 4.2.

Table 4.2: Results of BLAST search with the *E. coli* Fre oxidoreductase against the *P. mirabilis* genome

No.	Accession no.	Description	No. of aligned amino acids	E value	Similarity (%)	Significant
1	EE147802.1	A protein annotated as a putative FMN reductase	233	2×10^{-119}	80	significant
2	EE146967.1	NADH-ubiquinone oxidoreductase, F subunit	204	3×10^{-9}	42	significant
3	EE148497.1	2Fe-2S iron sulfur cluster binding domain protein	232	2×10^{-6}	43	significant
4	EE149421.1	methionyl-tRNA formyltransferase	99	0.002	45	Borderline
5	EE148614.1	Globin	104	0.007	48	Borderline
6	EE149100.1	endonuclease III	101	0.45	41	No

7	EE148272.1	PRD domain protein	41	1.0	56	No
8	EE146745.1	phosphoglycerate kinase	23	4.6	60	No
9	EE149630.1	cold-shock DNA-binding domain protein	19	7.8	73	No
10	EE147629.1	DNA mismatch repair domain protein	38	9.3	57	No

The known chromate efflux system ChrA, typified by chromate efflux pump of *Pseudomonas aeruginosa* plasmid pUM505, accession no. P 14285 (Cervantes *et al.*, 1990), contributes to resistance of the *Pseudomonas aeruginosa* cells to chromate (VI), has no significant homologues in *P. mirabilis* ATCC 29906T genome (Table 4.3).

Table 4.3: Results of BLAST search with the Chromate efflux system ChrA against *P. mirabilis* genome

No.	**Accession no.**	**Description**	**No. of aligned amino acids**	**E value**	**Similarity (%)**	**Significant**
1	EE148292.1	amino acid permease	34	0.16	64	No
2	EE149291.1	glucose-6-phosphate dehydrogenase	53	1.6	39	No

Table 4.4 shows the results of BLAST search with *E. coli* nitroreducatse NfsA, which reduces chromate (Ackerley *et al.*, 2004). It has one borderline homologue in *P. mirabilis* ATCC 29906 T genome.

Table 4.4: Results of BLAST search with the *E. coli* nitroreducatse NfsA against in *P. mirabilis* genome

No.	Accession no.	Description	No. of aligned amino acids	E value	Similarity (%)	Significant
1	EE149032.1	nitroreductase family protein	177	0.001	40	Border
2	EE147316.1	type IIIsecretion apparatus needle protein	21	0.81	71	No
3	EE149876.1	tRNA uridine 5-carboxmethylaminomethyl modification enzyme GidA	139	1.3	41	No
4	EE147703.1	SCP-2 sterol transfer family protein	55	4.3	49	No
5	EE150020.1	hypothetical protein HMPREF0693_0058	46	8.4	45	No

The BLAST search with the chromate reductase from *B. subtilis* YcnD has revealed one significant homologue and seven hits that were not significant in *P. mirabilis* ATCC 29906T genome, as shown in Table 4.5.

Table 4.5: Results of BLAST search with the *B. subtilis* YcnD chromate reductase against *P. mirabilis* genome

No.	Accession no.	Description	No. of aligned amino acids	E value	Similarity (%)	Significant
1	EE149032.1	nitroreductase family protein	86	8×10^{-6}	56	significant
2	EE148943.1	hypothetical protein HMPREF0693_1011	75	0.11	42	No
3	EE146536.1	hypothetical protein HMPREF0693_3395	39	1.9	66	No
4	EE146898.1	hypothetical protein HMPREF0693_3164	86	6.3	44	No
5	EE148400.1	folate-binding protein YgfZ	17	6.8	64	No
6	EE146738.1	transporter, betaine/carnitine/choline family	27	7.8	41	No
7	EE149808.1	pyrimidine dimer DNA glycosylase	80	9.5	46	No
8	EE149016.1	carbamoyltransferase HypF	46	9.5	52	No

The cytochrome *c* from *Desulfovibrio vulgaris* (Lovley and Phillips, 1994), has six non- significant homologues in *P. mirabilis* ATCC 29906T genome. The details of the homologues are summarised in Table 4.6.

Table 4.6: Results of BLAST search with the *Desulfovibrio vulgaris* cytochrome *c* against *P. mirabilis* genome

No.	Accession no.	Description	No. of aligned amino acids	E value	Similarity (%)	Significant
1	EE147908.1	site-specific recombinase, phage integrase family	50	0.46	60	No
2	EE149732.1	ATPase/histidine kinase/DNAgyrase B/HSP90 domain protein	53	2.9	49	No
3	EE147741.1	D-alanyl-D-alanine carboxypeptidase/D-alanine-endopeptidase	116	5.1	39	No
4	EE149571.1	hypothetical protein HMPREF0693_0508	22	6.2	63	No
5	EE147540.1	thiamine/thiamine pyrophosphate ABCtransporter, thiamine/thiamine pyrophosphate-binding protein	56	6.7	39	No
6	EE146579.1	chaperone protein Dnaj	16	9.4	68	No

The bioinformatics study conducted in this chapter using online BLAST search confirmed the presence of three significant and two borderline homologues of *E. coli* Fre oxidoreductase. The results also indicated one significant homologue of *E. coli* nitoreductase NfsA. These genes may be considered possible candidates for encoding proteins involved in chromate reduction.

4.4 Summary

This investigation has demonstrated the potential of *P. mirabilis* 090321-A bacterial strain for Cr (VI) removal. The strain, which was clinically isolated from wound swab in a previous research group study, was found very efficient in the removal of hexavalent chromium. It has been identified using 16S rRNA gene sequence analysis prior to the bioremediation process. DNA sequences were compared with reference database of NCBI. On the basis of phylogenetic data the sequenced portion of the 16S rRNA gene was confirmed as identical to that from the complete genome of *Proteus mirabilis* ATCC 29906T over 629 bases.

The results obtained from the cell fractionation experiment revealed another important observation. The results show that no Cr (VI) removal activity occurs due to components of the cell wall alone. However, a significant removal by the cytoplasmic fraction and a small activity in the cell-membrane have also been observed. This suggests that the enzymes exist in the cytoplasm and cell-membrane are responsible for Cr (VI) reduction or other reaction that leads to a fall in the amount of Cr (VI). Therefore, a further investigation of this study is advantageous to identify the type of enzymes. It would be desirable to repeat the assays in the presence of NADH, NAD and reduced FMN as electron donors.

The results of ICP-MS analysis show that *P. mirabilis* has successfully reduced Cr (VI) and detoxified it to less toxic and less soluble Cr (III). This agrees with previous studies (Dermou *et al.*, 2005; Sarangi and Krishnan, 2008; Sultan and Hasnain, 2007; Thacker *et al.*, 2007) which have reported that bacteria may protect themselves from toxic substances in the environment by transforming toxic compounds through oxidation, reduction or methylation into more volatile, less toxic or readily precipitation forms.

The results from FTIR spectra showed some changes (appearance, disappearance and shifting of peaks in infrared bands) occurred to the internal structure of the bacterial strain after adding chromium (after 24 and 48 hours of reaction time). The observed changes belong to saccharides and polysaccharides, fatty acids, amide I, amide II and lipids present within cell wall, membranes and cytoplasm (Amiel *et al.*, 2001, Das and Guha, 2007; Melin *et al.*, 2001, Mariey *et al.*, 2001).

To confirm the ability of *P. mirabilis* to decrease Cr (VI) concentration, a known bacterial strain (*P. mirabilis* HI 4320) were tested and compared with *P. mirabilis* 090321-A. The two independent isolates of *P. mirabilis* showed very similar chromium (VI) removal activities. At the end of reaction time Cr (VI) dropped from its initial concentration (2.54 mg/l) by 77% and 80% in the supernatant using HI4320 and 09032-A bacterial strains, respectively.

The results obtained from the online BLAST search of bioinformatics study in *P. mirabilis* ATCC 29906T genome have confirmed the presence of four significant and three borderline homologues of *E. coli* Fre oxidoreductase and one significant homologue of *E. coli* nitoreductase NfsA. These genes could be considered possible candidates for encoding enzymes responsible for Cr (VI) reduction.

As a conclusion, the above results can be considered as a step forward for further investigation to exploit the ability of *P. mirabilis* for the bioremediation of other forms of heavy metal contamination, since it has not been investigated in this process before.

CHAPTER 5

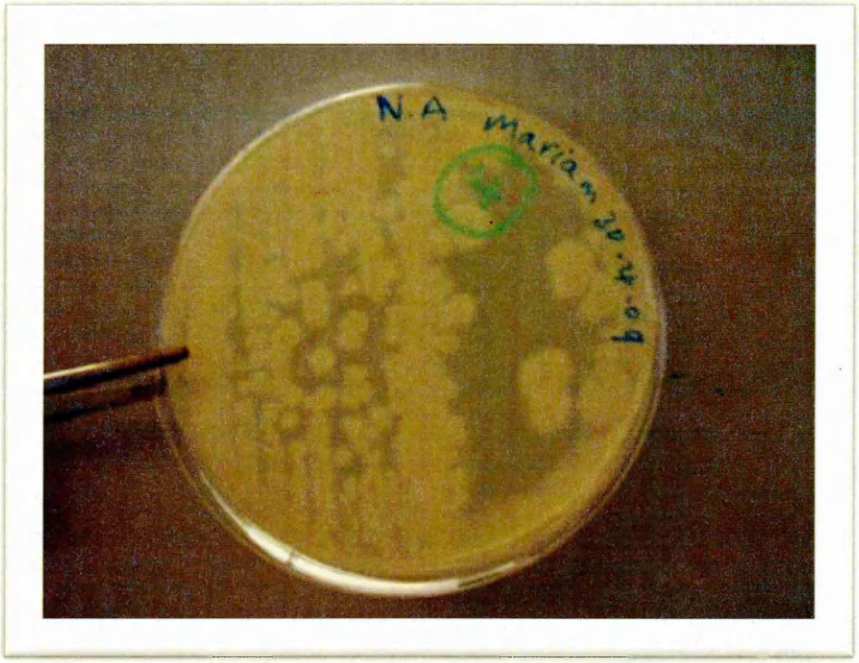
5 DETAILED ANALYSES OF CHROMIUM (VI) INTERACTIONWITH *METHYLOCOCCUS CAPSULATUS* (BATH)

5.1 Introduction

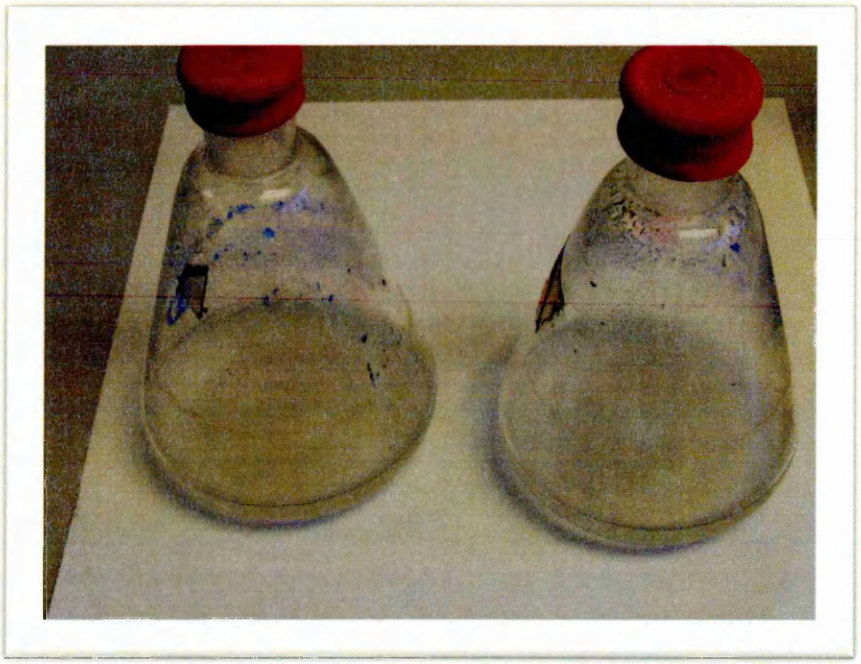
Methylococcus capsulatus (Bath), employed in this study for Cr (VI) bioremediation, is one of the methanotrophic bacteria (Hanson *et al.*, 1990). It possesses two types of methane monooxygenase (MMO), a soluble enzyme (sMMO) and a membrane-bound or particulate (pMMO), which is produced, depends on the concentration of copper sulfate during the growth of the bacterial strain in NMS media. At low copper-to-biomass ratio, sMMO is produced. Conversely, pMMO that is associated with the intracellular membranes is the dominant MMO enzyme at high copper-to-biomass ratio (Murrell *et al.*, 2000b).

Following the results from Chapter 3, this chapter investigates using high and low copper sulfate media and phenylacetylene as inhibitor for MMO enzymes to identify the enzyme (pMMO or sMMO) that is either fully or partially responsible for Cr (VI) removal. Further analyses were carried out using ICP-MS coupled with IC to determine chromium species during the bioremediation process. FTIR analysis was carried out to verify the changes in the bacterial composition during the bioremediation process.

During the growth of *M. capsulatus* (Bath) a contaminant bacterial strain was detected in some cultures, as shown in Figure 5.1. This strain was identified as *Bacillus licheniformis* using PCR 16S rRNA gene sequencing. Pure cultures of M. capsulatus were selected for use in the experiments detailed above. The mixed culture, *M. capsulatus* (Bath) and contaminant strain, was also tested in reducing Cr (VI) using the DPC assay.



a) Contaminant bacteria in nutrient agar plate



b) *M. capsulatus* with associated contaminant bacteria in NMS liquid media

***Figure 5.1: Samples of contaminant bacteria associated with M. capsulatus* (Bath)**

5.2 Materials and methods

5.2.1 Confirmation of M. capsulatus (Bath) using PCR 16S rRNA sequences

Swabs from plates of *M. capsulatus* (Bath), grown in methane on NMS agar plates, as described in section 2.1.3.1 of Chapter 2, were taken in 4 Eppendorf tubes, and 14 µl of triton X-100 (10%) was added to each tube, then spun in the centrifuge at 10000 *g* for 5 minutes. The tubes were incubated as follows; one tube at room temperature, the second tube at 45 °C, the third in a boiling water bath and the last tube in a sonicator water bath at 45 °C for 7 minutes. Each tube was then centrifuged for 5 minutes at 10000 *g*, 10 µl was taken from each tube to be used as the template for PCR.

Polymerase chain reaction (PCR) was performed using Primus 96 plus Thermocycler (MWG Biotech). 6 PCR tubes, each tube contained a total volume of 40 µl of the reaction mixture; 10 x PCR buffer, $MgCl_2$, 16 S1 forward and 16 S2 reverse primer and Taq DNA Polymerase (Bodrossy *et al.*, 1999). 10 µl of template was added to 4 PCR tubes, 10 µl of a solution of DNA from an MRSA strain from another project that was known to give 16S rRNA gene product and 2 µl of control and 8 µl of distilled water to the negative PCR tube. In this experiment the primers were chosen to amplify the highly conserved 16S rRNA gene that is used for the identification of bacteria. The cycling parameters for PCR were as follows: the first step consisted of 5 minutes of denaturation at 95 °C, followed by 35 cycles of denaturation for 30 seconds at 95 °C. Then, 30 seconds of annealing at 50 °C and elongation for 60 seconds at 68 °C with a final extension of 68 °C for 9 minutes.

5.2.1.1 Agarose gel electrophoresis protocol

Using the same method described in section 4.2.1.3 of Chapter 4, DNA samples were loaded into each well on the agarose gel (1%).

5.2.1.2 Purification of PCR products

A PCR purification kit was used to purify PCR products for sequencing. Using the procedure described in section 4.2.1.4 of Chapter 4.

5.2.2 Bioremediation of Cr (VI) using M. capsulatus (Bath) bacteria

5.2.2.1 High and low copper sulfate NMS media

M. capsulatus (Bath) was cultured in 250 conical quick fit flasks containing 50 ml of NMS medium, as previously described in section 2.1.3.1 of Chapter 2, in the presence of methane as a source of energy and carbon at a temperature of 45 °C. Low copper sulfate NMS medium was prepared for sMMO expression by omitting copper sulfate from the medium. 1.5 mg/l of $CuSO_4.5H_2O$ was added to NMS to produce high copper sulfate for pMMO expression growth conditions.

Measurement of Cr (VI) removal and total chromium concentration were carried out for supernatants and pellets samples using the diphenylcarbazide (DPC) assay and inductively coupled plasma-optical emission spectroscopy (ICP-OES), respectively. Cell-free media plus $K_2Cr_2O_7$ was used as a negative control. The aim of this experiment is to identify the enzymes that are responsible of Cr (VI) removal. Full details of the DPC and ICP-OES methods can be found in sections 2.2.4 and 2.2.5.1 of Chapter 2 (Materials and Methods).

5.2.2.2 Use of phenylacetylene as inhibitor during Cr (VI) removal reaction

When the bacterial culture had grown in the NMS medium to a sufficient extent (OD_{600} 0.3 - 1.00), 1 ml of filter sterilised $K_2Cr_2O_7$ was added to the bacterial culture to give a final Cr (VI) concentration of 2.54 mg/l. At the same time, phenylacetylene was added (to a final concentration of 200 μM). The aim of this experiment is to investigate the effect of phenylacetylene inhibitor on Cr (VI) reaction. Specifically, to confirm which MMO enzyme phenylacetylene inhibits during the bioremediation process. Cr (VI) concentration was measured using the DPC assay described in section 2.2.4 of Chapter 2.

Control samples in the absence of cells showed there was no reduction of Cr (VI) by reaction with phenylacetylene.

5.2.3 Determination of chromium species during the bioremediation process

5.2.3.1 Determination of Cr (III) and Cr (VI) using ICP-MS

The details of the column and standard can be found in section 2.2.6 of Chapter 2 (Materials and Methods).

5.2.3.2 FTIR analysis

The details of sample preparation and FTIR are given in section 2.2.7.1 of Chapter 2 (Materials and Methods).

5.2.4 Identification of contaminant bacteria associated with M. capsulatus (Bath) using PCR 16S rRNA sequences

Swabs from contaminant bacteria plates were taken in 4 Eppendorf tubes, and 14 µl of triton X-100 (10%) was added to the tubes, then spun in the centrifuge at 10000 *g* for 5 minutes. The chromosomal DNA extraction protocol was then carried out as described in section 4.2.1.1 of Chapter 4. After this, PCR was used to amplify 16S rRNA gene sequences (the cycling parameters of PCR were carried out under the same thermocyclers conditions explained in section 4.2.1.2 of Chapter 4) to identify the contaminant bacterial strain. This is followed by showing the DNA fragment in agarose gel electrophoresis. Finally the PCR products were purified using the method in section 4.2.1.4 of Chapter 4.

5.2.5 *Bioremediation of Cr (VI) using mixed culture containing M. capsulatus and B. licheniformis*

The measurement of Cr (VI) concentration using DPC assay was carried out using the same method outlined in section 2.1.2.3 of Chapter 2 (Materials and Methods).

5.3 Results

5.3.1 *Confirmation of M. capsulatus (Bath) using PCR 16S rRNA sequences*

Figure 5.2 shows electrophoresis gel of the 16S rRNA gene (1500 bp) (Woese *et al.*, 1983; Bodrossy *et al.*, 1999) for *M. capsulatus* bacterial strain after amplification via PCR at four different growth conditions; room temperature, 45 °C, boiling water bath and sonicating water bath. It can clearly seen from the figure that the only band appeared in gel belongs to the bacterial sample grown at 45 °C.

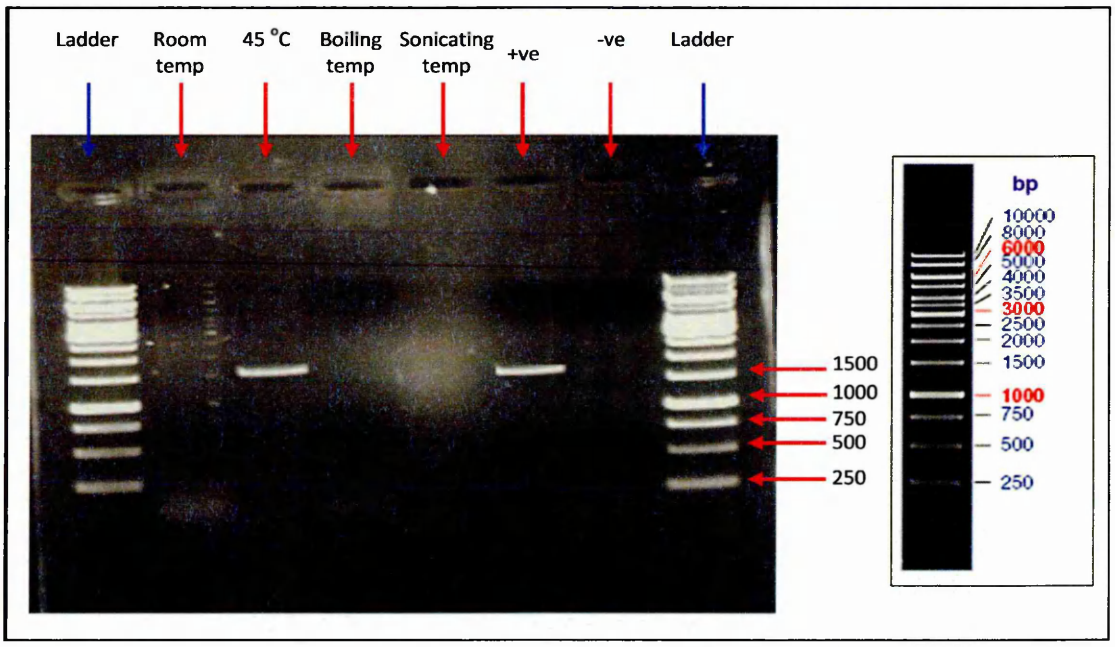


Figure 5.2: Agarose gel electrophoresis of 16S rRNA gene-specific PCR using cells prepared in various ways as the templates *(+ve and -ve indicate a positive and negative control respectively).*

Figure 5.3 shows electrophoresis gel of the 16S rRNA gene (1500 bp) for the purified PCR products of the bacterial strain at 45 °C. After amplification of the 16S rRNA gene via PCR, DNA sequence analysis was performed. The DNA sequences were compared with the reference database of the National Centre for Biotechnology Information (NCBI) using the BLAST program (www.ncbi.nlm.nih.gov/blast). The sequence was confirmed as 100% identical to the 16S rRNA gene sequence from *Methylococcus capsulatus* strain Bath over 633 bases.

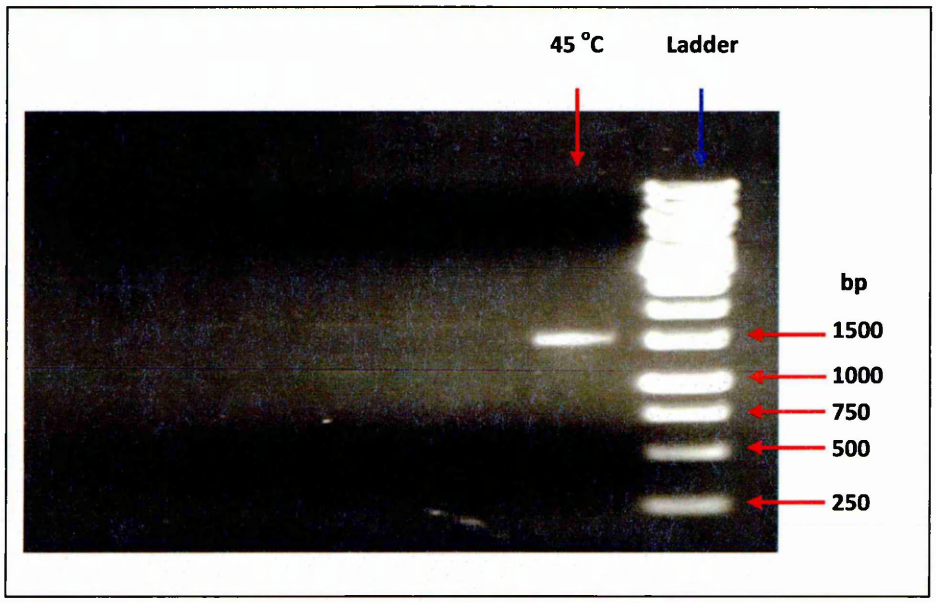


Figure 5.3: Purified PCR product in agarose gel electrophoresis

5.3.2 Bioremediation of Cr (VI) using M. capsulatus (Bath)

5.3.2.1 High and low copper sulfate media

The ability of *M. capsulatus* (Bath) to lower Cr (VI) from its starting concentration of 2.54 mg/l was tested in high and low copper sulfate NMS media. Cr (VI) concentration in the supernatant and pellets samples was monitored at intervals of incubation time using the DPC assay.

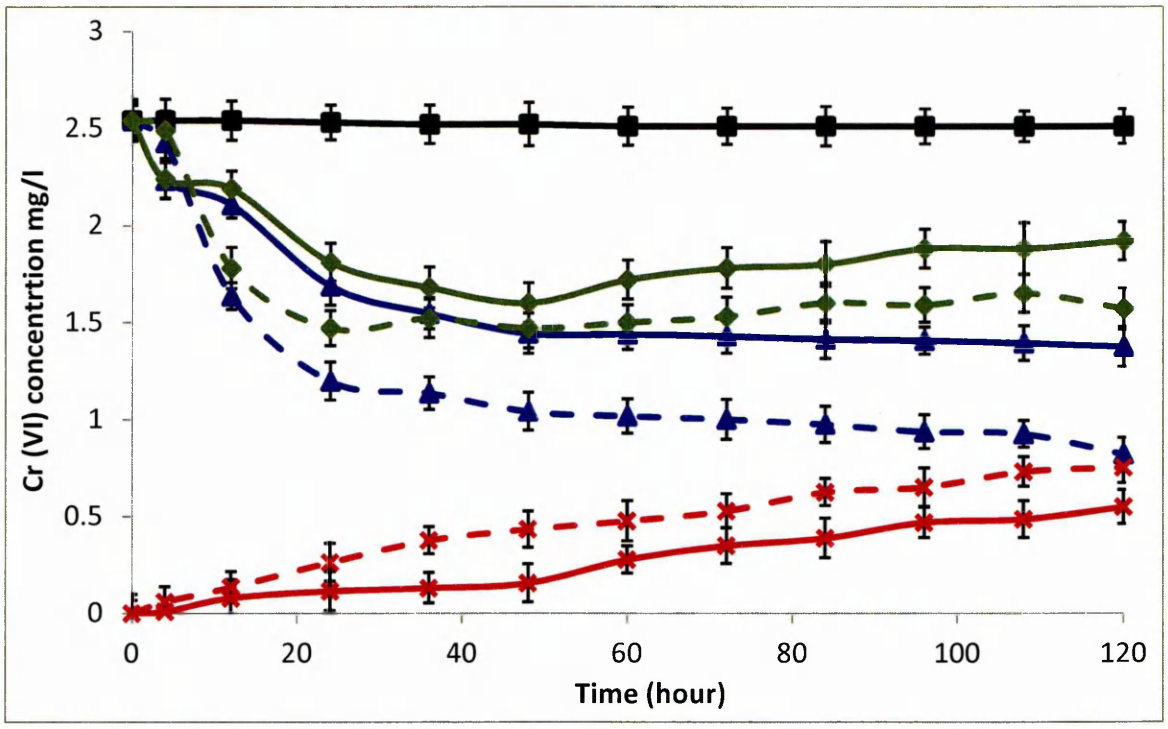
Figure 5.4 shows the bioremediation of Cr (VI) using *M. capsulatus* (Bath) in high copper (1.5 mg/l $CuSO_4.5H_2O$) and low copper (no added copper) NMS media. There are two control samples, indicated by with the solid black lines, representing $K_2Cr_2O_7$ without cells in high and low copper sulfate NMS media. Since Cr (VI) removal was not observed in either of these samples the resulting data overlap on the graph. The supernatant samples in low and high copper sulfate NMS media are denoted with solid and dashed blue lines, respectively. A significant drop of Cr (VI) from its initial concentration of 2.54 mg/l was observed in the supernatant samples. In contrast, Cr (VI) concentration in the pellet samples increased during the incubation period.

At the beginning of the reaction the percentage of Cr (VI) removal in the high copper sulfate supernatant was 4% and at the end of the incubation time was 68%. Cr (VI) concentration was 0.82 mg/l after 120 hours. On the other hand, the Cr (VI) concentration in the pellets increased gradually to reach 0.75 mg/l at the same time.

The total chromium (VI) concentration (in the supernatant and pellets) at the end of the reaction time 120 hours was 38% of the initial value.

The percentage of Cr (VI) concentration in the supernatant of low $CuSO_4$ media fell by 12% at 4 hours of incubation time, and extended to reach 46% at the end of incubation period. At the end of the incubation time Cr (VI) concentration was decreased to 1.37 mg/l. Unlike in the supernatants, the Cr (VI) concentration in the pellets increased over the incubation period. It was 0 mg/l at the start of incubation, and then gradually increased to 0.55 mg/l at 120 hours.

The percentage of the total chromium (VI) in the low copper sulfate reached 24% of the initial value at the end of the incubation time (120 hours). This suggests a greater reduction of Cr VI to Cr III by cells grow in low copper sulfate medium rather than high copper sulfate medium.



(—■—) Control sample (no cells) = High and low $CuSO_4$ NMS medium with $K_2Cr_2O_7$.

(—▲—) Sup. (Low) = supernatant in low $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. (High) = supernatant in high $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(—✕—) Pellets (Low) = pellets in low $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(··✕··) Pellets (High) = pellets in high $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(—◆—) Total Cr (VI) = supernatant and pellets (low $CuSO_4$ NMS medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr (VI) = supernatant and pellets (high $CuSO_4$ NMS medium with cells, $K_2Cr_2O_7$ and NaN_3).

$K_2Cr_2O_7$ concentration = 2.54 mg/l and NaN_3 concentration = 0.05% w/v.

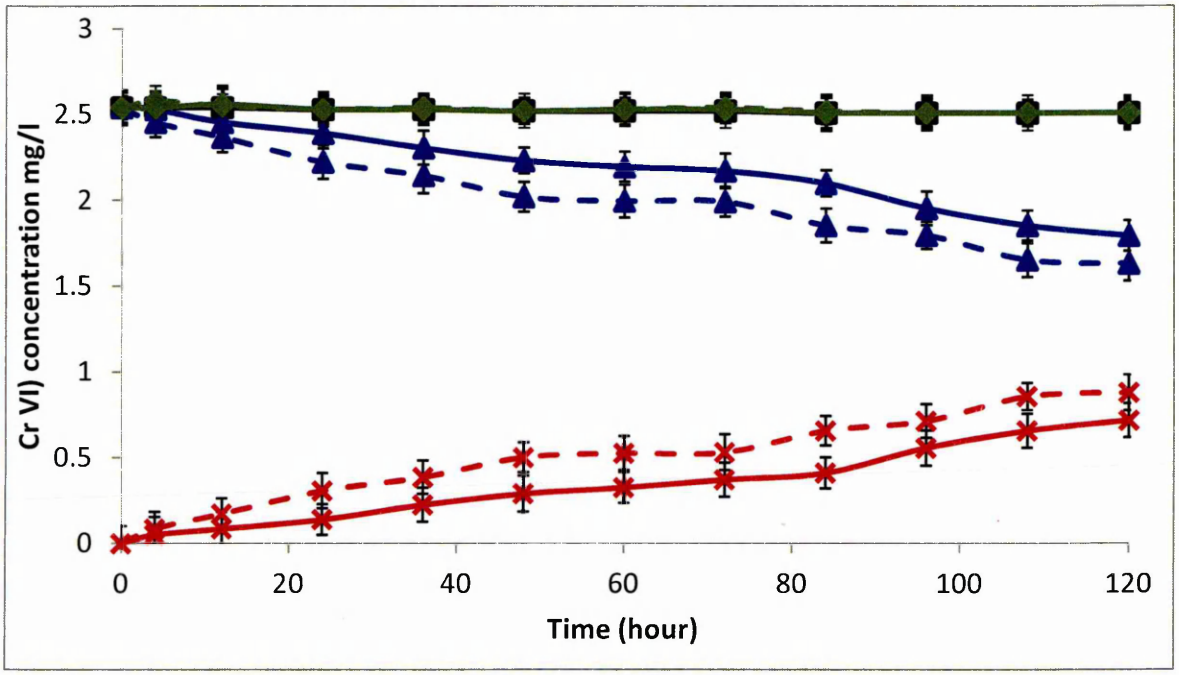
Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 5.4: Cr (VI) removal using M. capsulatus* (Bath) *in high and low $CuSO_4$ NMS media using DPC assay.

ICP-OES technique was used to measure the total chromium concentration during the bioremediation activity. Total chromium concentration in the supernatant and pellets in the presence of high and low $CuSO_4$ NMS media are shown in Figure 5.5. In the supernatant samples (in high $CuSO_4$ NMS media) the total chromium concentration was 2.54 mg/l at 0 time and start decreasing slowly to reach 1.63 mg/l at 120 hours. The concentration in the pellets was 0.0 mg/l at the beginning of incubation time and also

slowly increased to reach 0.89 mg/l after 120 hours of incubation time. Total chromium concentration in the supernatants of low $CuSO_4$ NMS media samples was 2.54 mg/l at the beginning of reaction time. Then, it decreased to reach 1.79 mg/l at the end of reaction. The total chromium concentration in the pellets increased from 0 mg/l to 0.72 mg/l at 120 hours.

The total chromium concentration in the supernatant and pellets in low and high copper sulfate NMS media are showing in Figure 5.5 was mostly with the margin of experimental period. The total chromium in both fractions throughout all experiment was as expected equal to the total chromium concentration at the start of the experiment.



(—■—) Control sample (no cells) = High and low $CuSO_4$ NMS medium with $K_2Cr_2O_7$.

(—▲—) Sup. (Low) = supernatant in low $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(··▲··) Sup. (High) = supernatant in high $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(—✱—) Pellets (Low) = pellets in low $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(··✱··) Pellets (High) = pellets in high $CuSO_4$ NMS medium with cells and $K_2Cr_2O_7$.

(—◆—) Total Cr = supernatant and pellets (low $CuSO_4$ NMS medium with cells, $K_2Cr_2O_7$).

(··◆··) Total Cr = supernatant and pellets (high $CuSO_4$ NMS medium with cells, $K_2Cr_2O_7$ and NaN_3).

Figure 5.5: Total chromium concentration using M. capsulatus* (Bath) *in high and low $CuSO_4$ NMS media using ICP-OES.

The result shown in Figure 5.4 indicates that the drop of Cr (VI) concentration in supernatant sample of high copper sulfate media was greater than low copper sulfate media. At the end of reaction time, the concentration was 68% and 46% in high and low copper sulfate NMS media, respectively. Cr (VI) concentration however increased in the pellets (fraction samples). There are large numbers of proteins (60) that are induced by copper (Kao *et al.*, 2004), including pMMO enzyme, which is possibly involved in reducing Cr (VI) since it has reductase (pMMOR) component.

In addition to the pMMO enzyme, di-heme cytochrome *c* peroxidases are generally known to be present in the periplasm and to play a role in reducing peroxides generated by oxidative metabolism. Karlsen *et al.* (2011) found that in *M. capsulatus* (Bath) redox reactions involving copper ions take place on the cell surface, and that different c-type cytochromes are induced and needed at different copper-to-biomass ratios. The type and abundance of the particular c-type cytochrome vary depending on the bioavailability of copper ions in the growth medium (Karlsen *et al.*, 2008). Di-heme cytochrome *c* peroxidase is therefore a candidate enzyme of reducing chromium (VI).

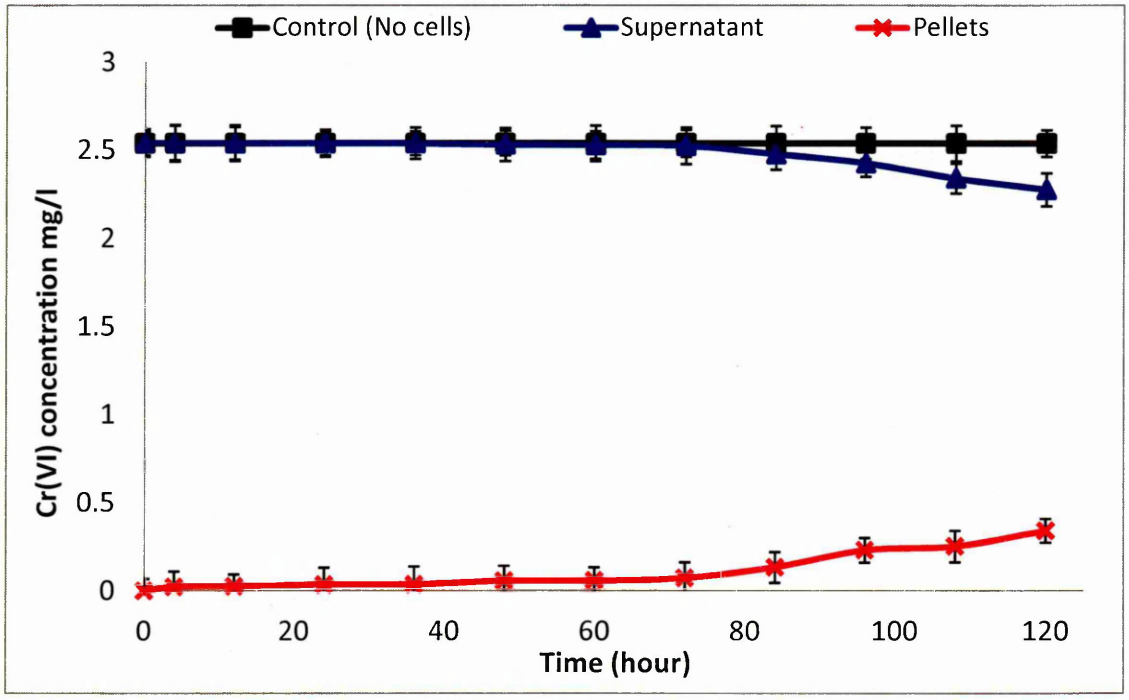
5.3.2.2 Use of phenylacetylene as inhibitor during Cr (VI) removal

Figure 5.6 shows Cr (VI) removal activity using *M. capsulatus* in the presence of phenylacetylene as an inhibitor to MMO enzymes. No removal of Cr (VI) was observed before 72 hours of reaction time. After that Cr (VI) concentration slowly decreased in the supernatant sample to reach 2.28 mg/l (fell by 10%) after 120 hours. However, the Cr (VI) concentration in the pellets increased 0.34 mg/l at the same time.

This result clearly indicates that phenylacetylene completely inhibited the enzymes that are responsible for Cr (VI) removal. This finding agrees with a similar result reported by Lontoh *et al.* (2000) who found that 100% sMMO was inhibited with 200 μM of phenylacetylene, and 52% pMMO.

Phenylacetylene can reduce enzymatic activity by either preventing other substrates from binding or forming reactive intermediates that could inactivate AMO, sMMO or pMMO (Lontoh *et al.*, 2000). It has also been suggested that phenylacetylene

might differentially inhibit whole-cell AMO, pMMO and sMMO activity (Vannelli *et al.*, 1996).



(—■—) Control sample (no cells) = NMS medium with $K_2Cr_2O_7$ and phenylacetylene.

(—▲—) Supernatant = NMS medium with cells, $K_2Cr_2O_7$ and phenylacetylene.

(--✖—) Pellets = Cells with $K_2Cr_2O_7$ and phenylacetylene.

Error bars represent triplicate measurements, standard deviation (SD) = 0.05 – 0.1 mg/l

Figure 5.6: Cr (VI) removal using Mc. capsulatus (Bath) in the presence of phenylacetylene (200 µM).

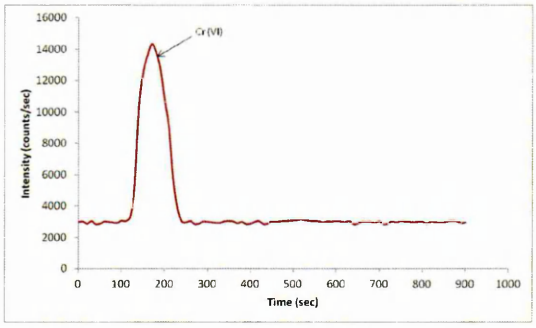
5.3.3 Analyses of chromium species

5.3.3.1 Determination of Cr (III) and Cr (VI) using IC- ICP-MS

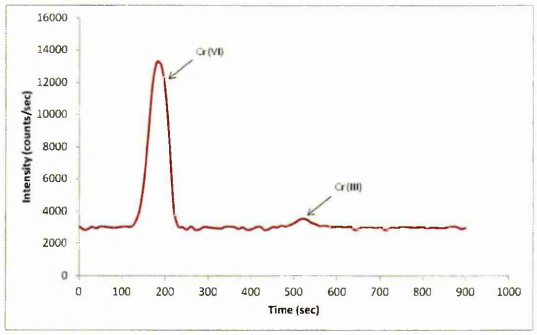
This experiment was conducted using ICP-MS coupled with IC to determine chromium species in the supernatant samples of *M. capsulatus* with potassium dichromate (Cr VI concentration 2.54 mg/l). Potassium chloride (0.6 mol/l), EDTA (0.001 mol/l) and flow rate of 1.00 ml/min were used as mobile phase. The standard

solution of Cr (VI) and Cr (III) eluted in the regions 130-230 and 480-580 seconds, respectively (as depicted in Figures 2.8 and 2.9).

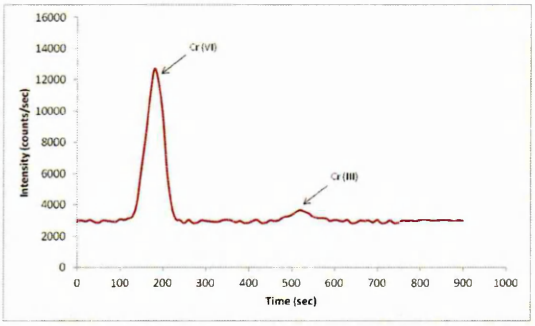
Figure 5.7 shows chromium species (Cr (VI) and Cr (III)) present in the supernatant samples during the reaction period. At the beginning of the bioremediation processes (0 time), Cr (VI) appeared as a large sharp peak and Cr (III) was below the threshold of detection. After 4 hours reaction time, a small peak of Cr (III) was noticed and this peak became larger and larger as the time passes. In contrast, Cr (VI) peak decreased. This result shows that *M. capsulatus* (Bath) has reduced Cr (VI), to the less soluble and less toxic Cr (III).



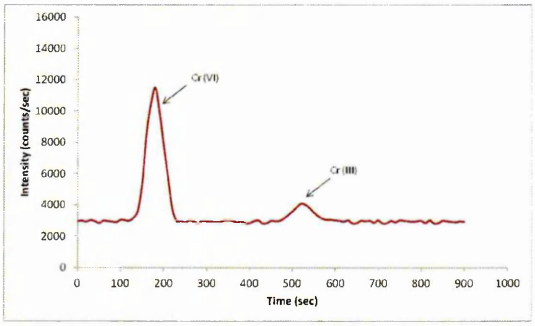
a) 0 time



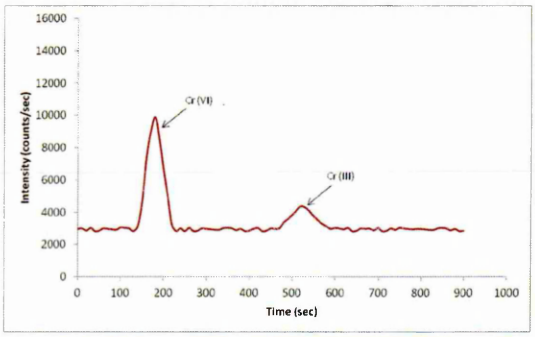
b) 4 hours



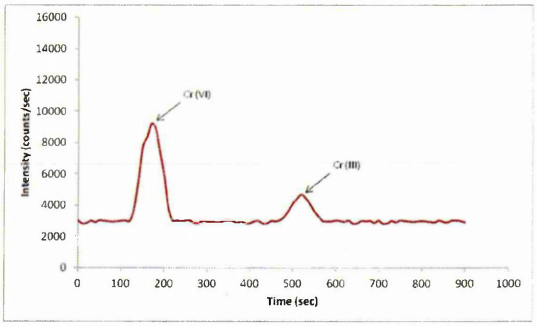
c) 12 hours



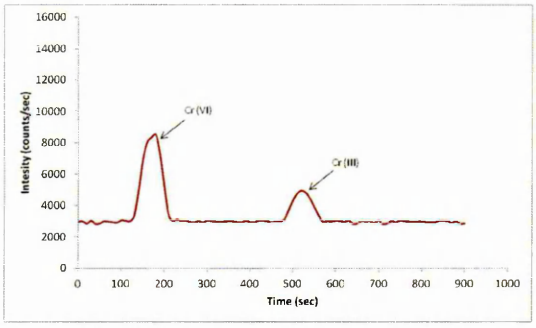
d) 24 hours



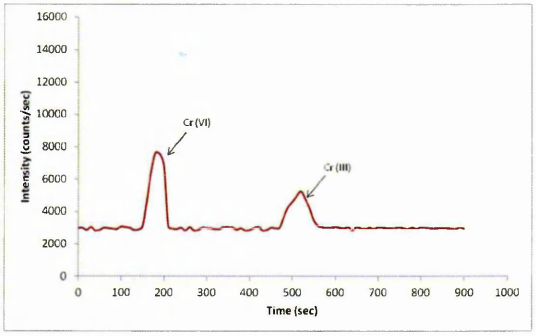
e) 36 hours



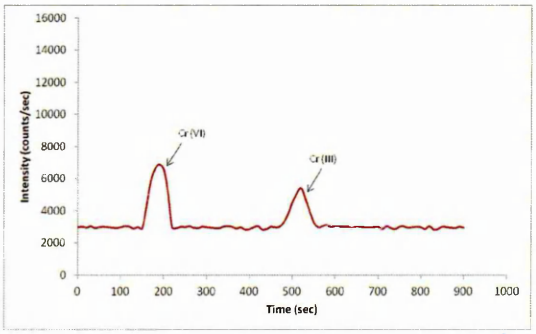
f) 48 hours



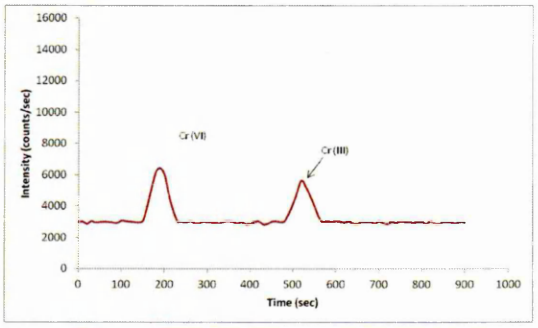
g) 60 hours



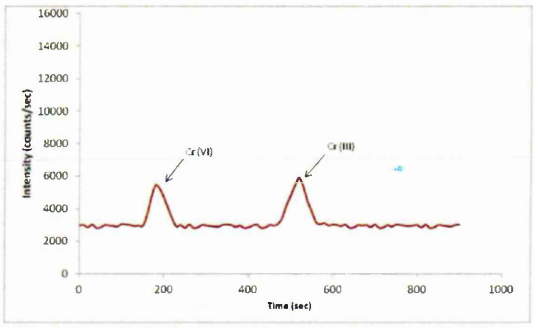
h) 72 hours



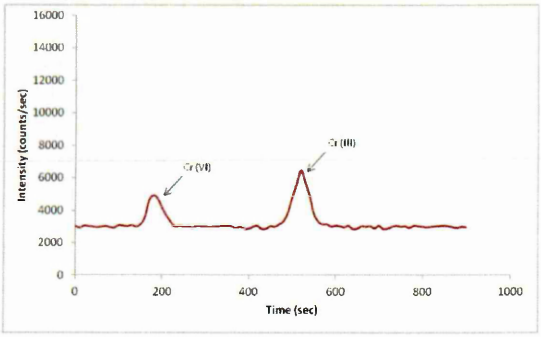
i) 84 hours



j) 96 hours



k) 108 hours



l) 120 hours

Figure 5.7: Chromatogram of Cr (VI) and Cr (III) in M. capsulatus supernatant samples using ICP-MS

Figure 5.8 shows the changes of Cr (VI) and Cr (III) concentrations in the supernatant samples during the reaction time. It can be seen that Cr (VI) concentration decreased from its initial value (2.54 mg/l), while Cr (III) concentration increased with time.

This figure shows a progressive decrease in the total chromium concentration (Cr (VI) + Cr (III)) in the supernatant, which is consistent with precipitation and biosorption of chromium species to the cells.

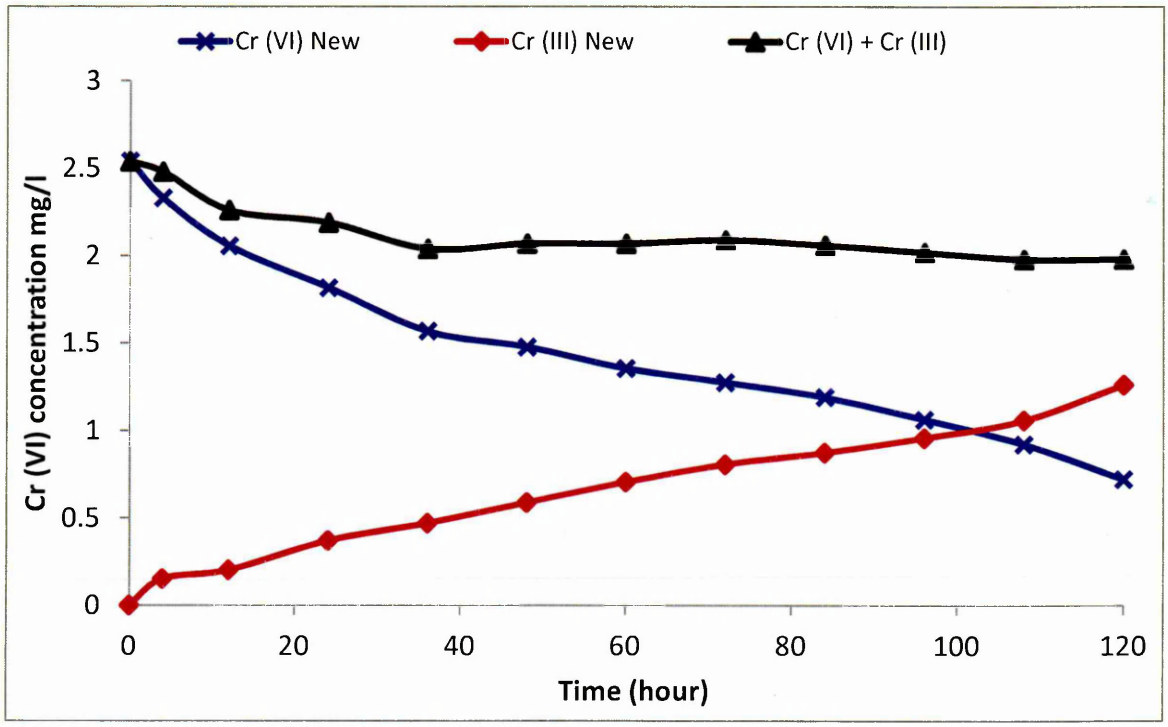


Figure 5.8: Cr (III) and Cr (VI) profiles in M. capsulatus supernatant samples during the reaction time (data of this figure were adapted from Figure 5.7)

5.3.3.2 FTIR analysis

As described earlier in section 2.2.7, the FTIR spectroscopy technique was used to measure the intensity of encoded infrared beam that passes through *M. capsulatus* (Bath) with potassium dichromate dried sample, which was prepared as described in section 2.2.7.1.

Figure 5.9 shows the spectrum of *M. capsulatus* (Bath) with potassium dichromate at three different reaction times (0 hours, 24 hours and 48 hours). Table 4.1 in Chapter 4 summarises the observed infrared bands for bacteria corresponding to the presence of functional groups. Unlike the *P. mirabilis* spectrum, there were no changes in 0 and 24 hours, but a little change in 48 hours observed in the regions between 650 – 1040 cm^{-1} and 2980 – 4000 cm^{-1}. The region below 900 cm^{-1} is typically referred to as a finger print region with small spectral features (Uldelhoven *et al.*, 2000).

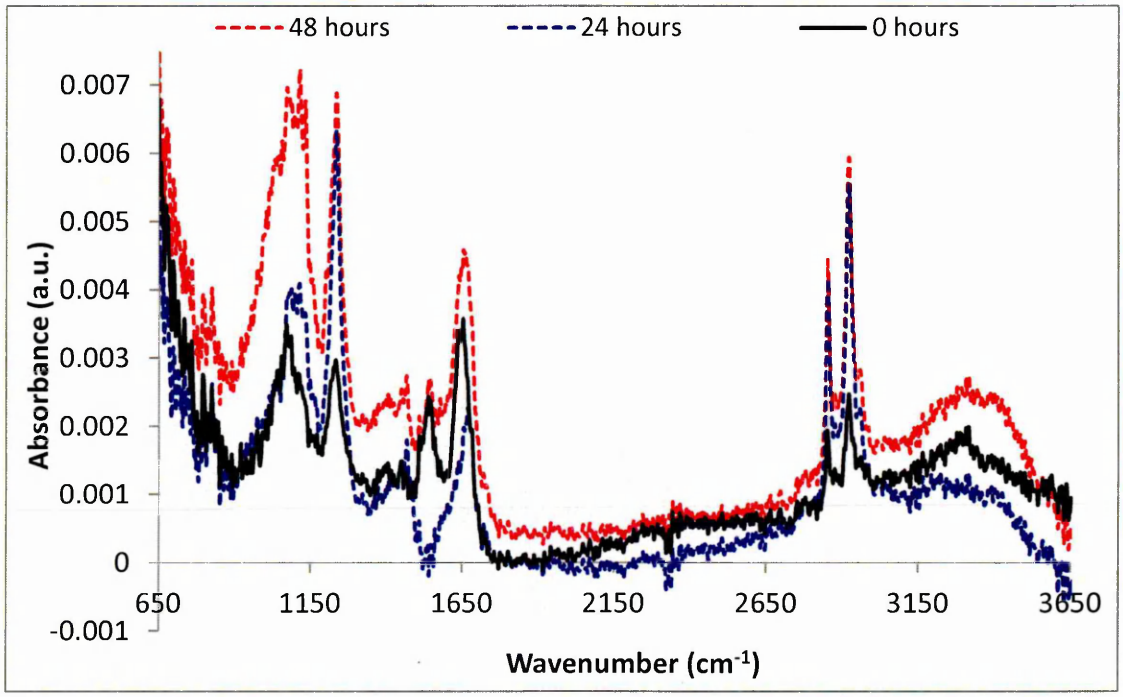


Figure 5.9: FTIR spectra of M. capsulatus with potassium dichromate at different incubation times.

The data from Figure 5.9 are shown in expanded form in Figures 5.10 – 5.13 to allow analysis of specific regions of the spectrum. As shown in the Figure 5.10, the intensity of the band at wave number 1080 cm^{-1} became straighter in 24 and 48 hours. The band at 1114 cm^{-1} increased and a new peak appeared at the wave number 1132 cm^{-1}. These bands lie in the region 900 – 1200 cm^{-1}, which corresponds to the polysaccharide group (cell wall) (Udelhoven *et al.*, 2000; Parikh and Chorover, 2006; Ojeda et *al.*, 2008).

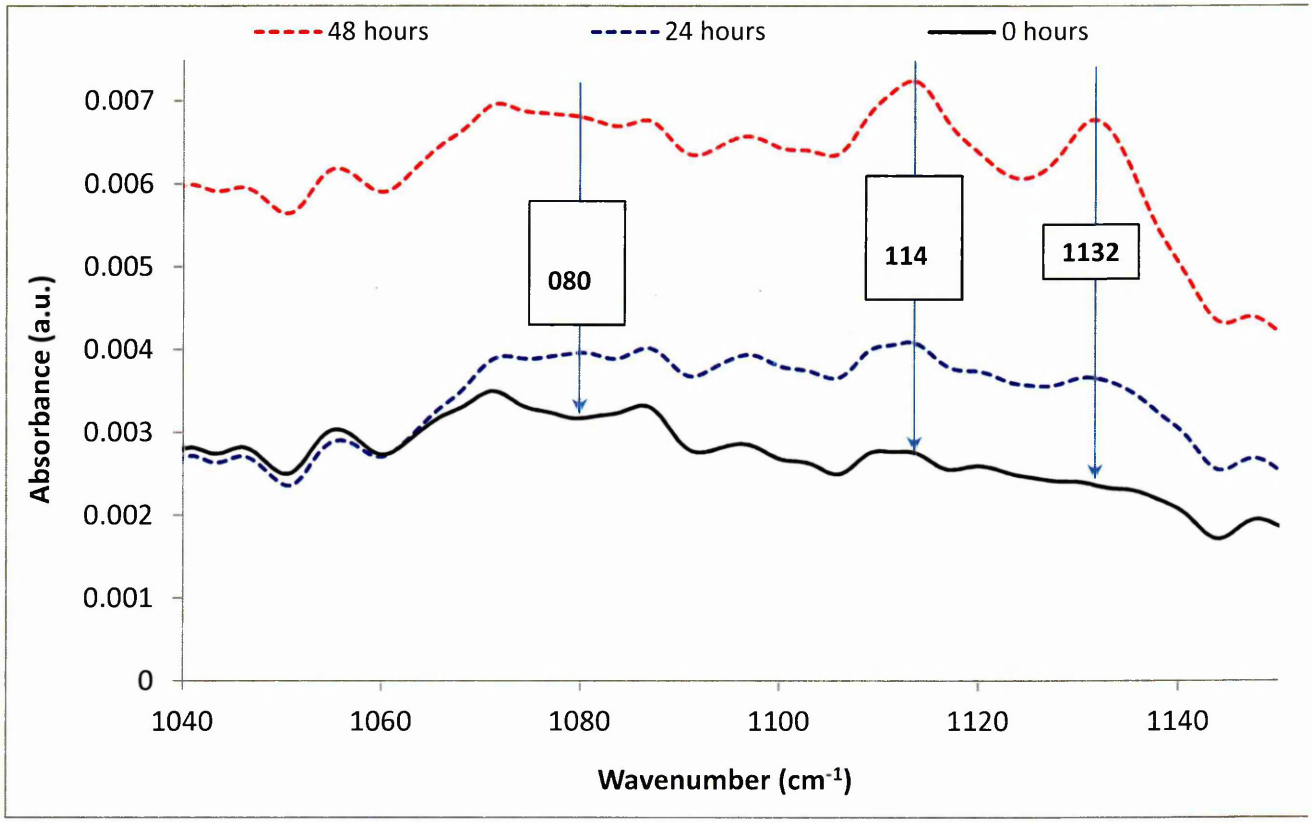


Figure 5.10: FTIR spectra (1040 – 1140 cm^{-1}) of M. capsulatus with potassium dichromate at different incubation times.

Figure 5.11 shows a new peak at 1467 cm^{-1}, the corresponding region (1200 – 1500 cm^{-1}) is a mixed region that comprises proteins, fatty acids and phosphate (Amiel *et al.*, 2001; Melin *et al.*, 2001; Mariey *et al.*, 2001). According to Udelhoven *et al.* (2000), the region from 1200 - 1450-cm^{-1} dominated by carboxylic groups of proteins, free amino acids, and polysaccharides (1400 – 1450 cm^{-1} and the region from 1200 - 1250 cm^{-1} by RNA/DNA and phospholipid content.

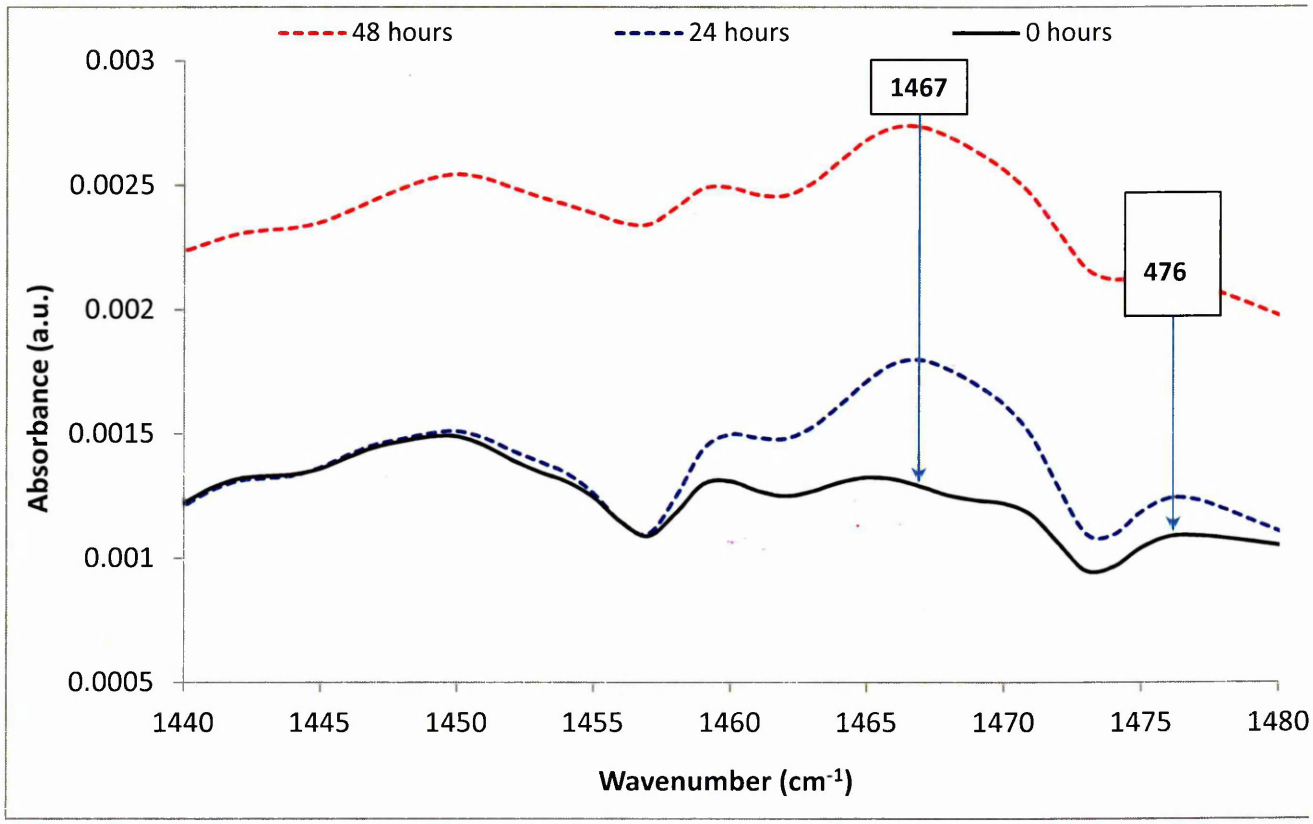


Figure 5.11: FTIR spectra (1440 – 1480 cm^{-1}) of M. capsulatus with potassium dichromate at different incubation times.

In Figure 5.12, the intensity of the major peak that lies at 2851 cm^{-1} greatly increased, while the peaks at 2841 and 2865 cm^{-1} disappeared. These peaks are located in the fatty acid compound region that covers the region 2800 - 3100 cm^{-1}.

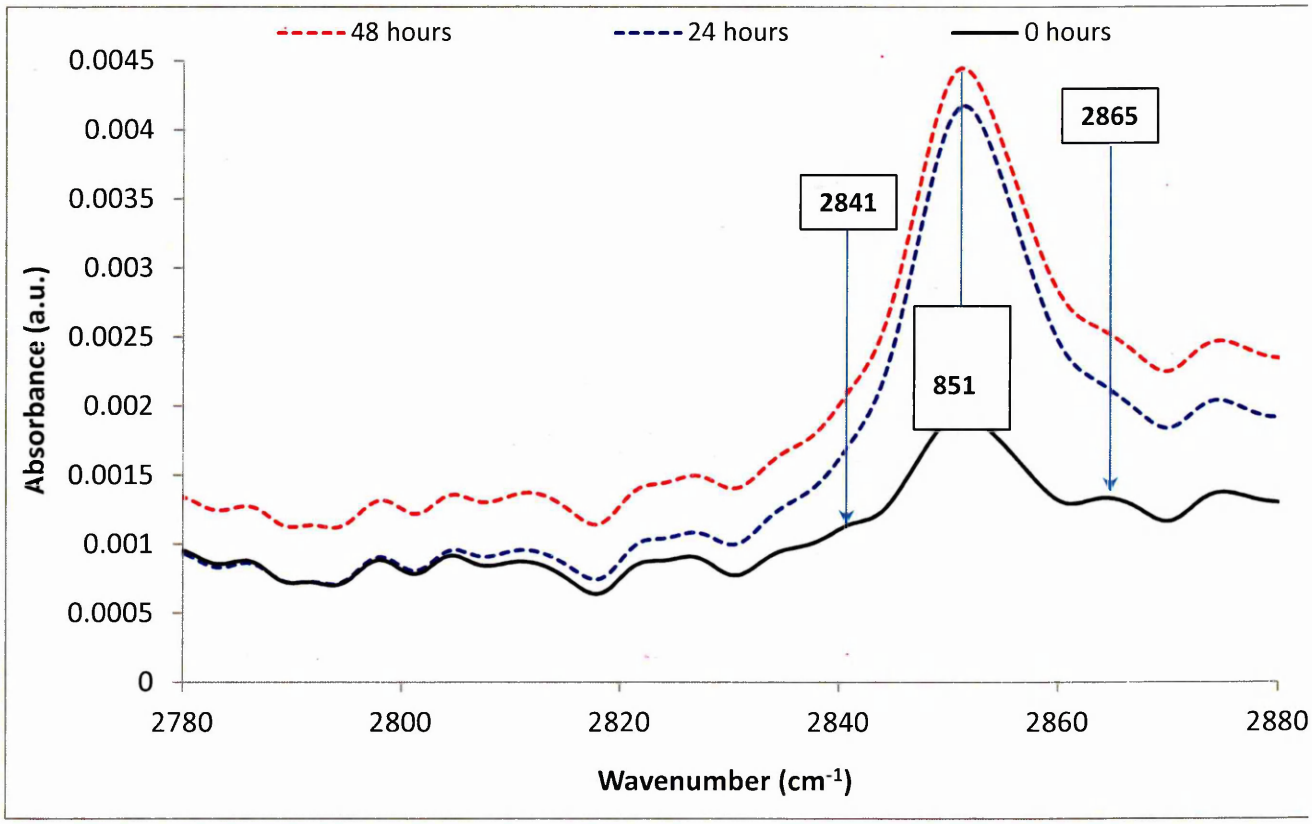


Figure 5.12: FTIR spectra (2780 – 2880 cm^{-1}) of M. capsulatus with potassium dichromate at different incubation times.

In the same band, as depicted in Figure 5.13, three original peaks at 2907, 2916 and 2928 cm^{-1} disappeared, the intensity of the peak at 2922 cm^{-1} increased, and a new peak appeared at 2958 cm^{-1}.

The above results reveal that changes in *M. capsulatus* (Bath) on exposure to potassium dichromate were observed in the cell wall, the membrane, and the cytoplasm.

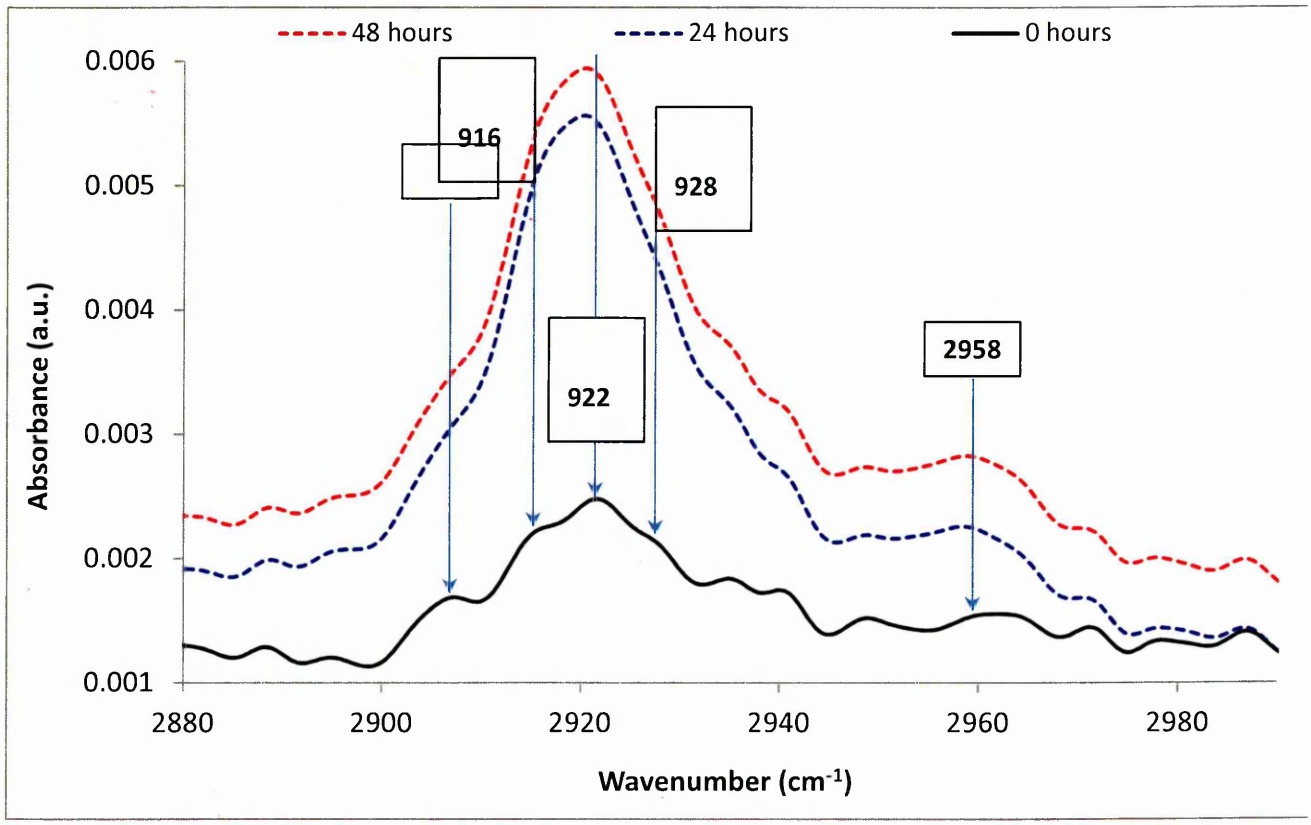


Figure 5.13: FTIR spectra (2880 – 2980 cm^{-1}) of M. capsulatus with potassium dichromate at different incubation times.

5.3.4 Identification of contaminant bacteria using PCR 16S rRNA sequences

During the growth phase of *M. capsulatus* (Bath) either in flasks or plates, purity tests were carried out. It was noticed that a contaminant bacteria has appeared in some cultures (see section 5.1). The contaminating strain was identified as the Gram-positive bacterium *Bacillus licheniformis* since, when the 16S rRNA gene was amplified by PCR (Figure 5.13) and subsequently sequenced; it was shown to be 99% identical to the sequence from *B. licheniforms* strain NJ-5 with accession number FJ 435674.1.

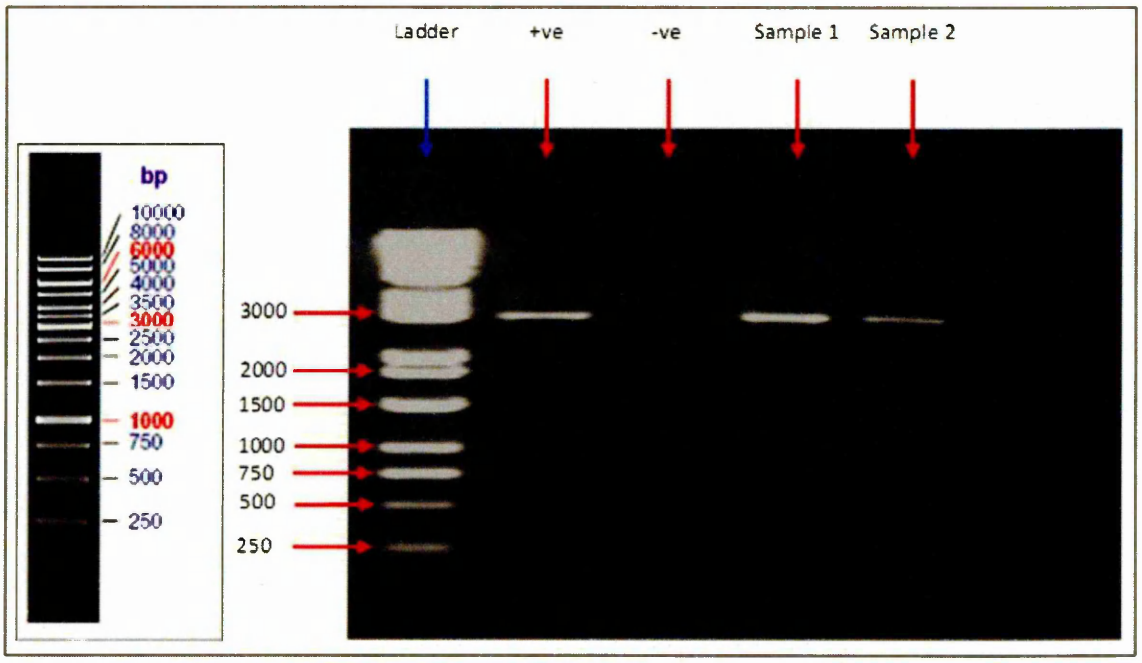
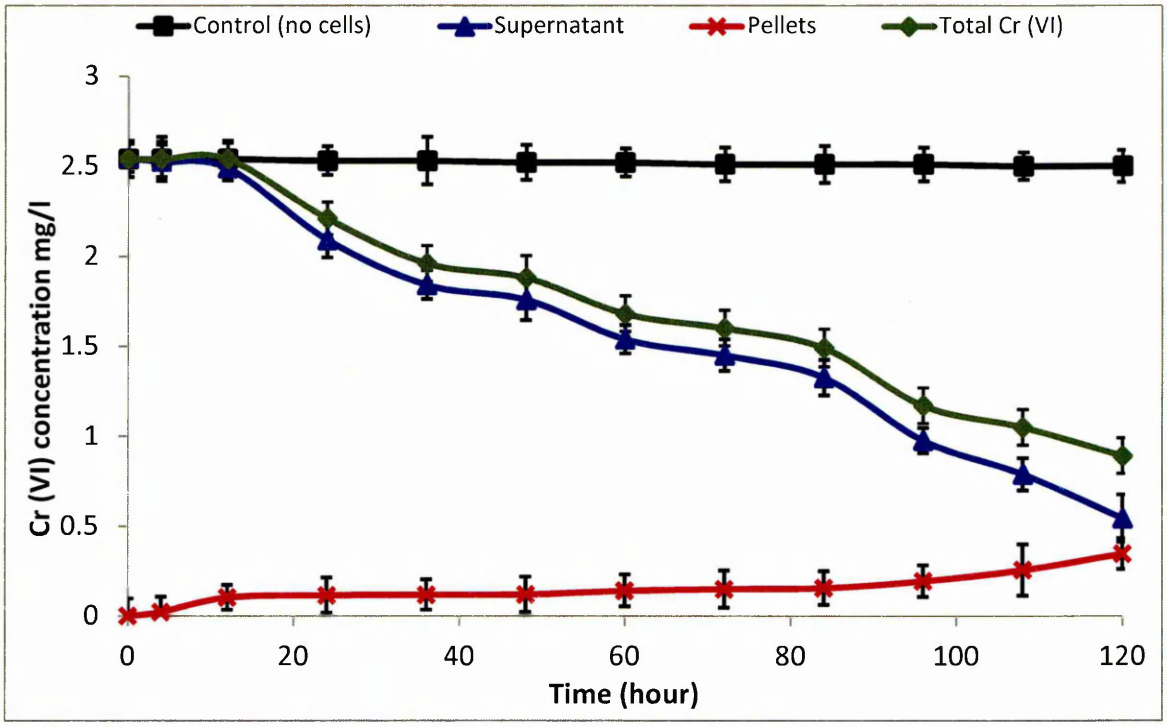


Figure 5.14: 16S rRNA gene sequence electrophoresis gel profile of contaminant bacteria

5.3.5 Bioremediation of Cr (VI) using mixed culture containing M. capsulatus and B. licheniformis

DPC assay was used to investigate the performance of *M. capsulatus* that had become contaminated with *B. licheniformis* bacteria decreasing Cr (VI) concentration. Figure 5.14 shows Cr (VI) reduction for the mixed culture containing *M. capsulatus* (Bath) and *B. licheniformis*. The removal activity was slow (less than 1%) at the beginning of reaction period. After 24 hours of reaction Cr (VI) concentration decreased and it reached 0.54 mg/l at the end of reaction. The percentage of Cr (VI) removal after 120 hours was 79%. Cr (VI) concentration in the pellets was 0 mg/l at the beginning of incubation time, then, it has gradually increased to reach 0.35 mg/l after 120 hours.

The decrease in the total Cr (VI) (supernatant + pellet) in the experiments is consistent with reduction of Cr (VI) to Cr (III).



(—■—) Control sample (no cells) = medium with $K_2Cr_2O_7$.

(—▲—) Sup. = supernatant sample = medium with cells and $K_2Cr_2O_7$.

(—✱—) Pellets sample = cells with $K_2Cr_2O_7$.

(—◆—) Total Cr (VI) = supernatant and pellets (medium with cells, $K_2Cr_2O_7$).

$K_2Cr_2O_7$ concentration = 2.54 mg/l.

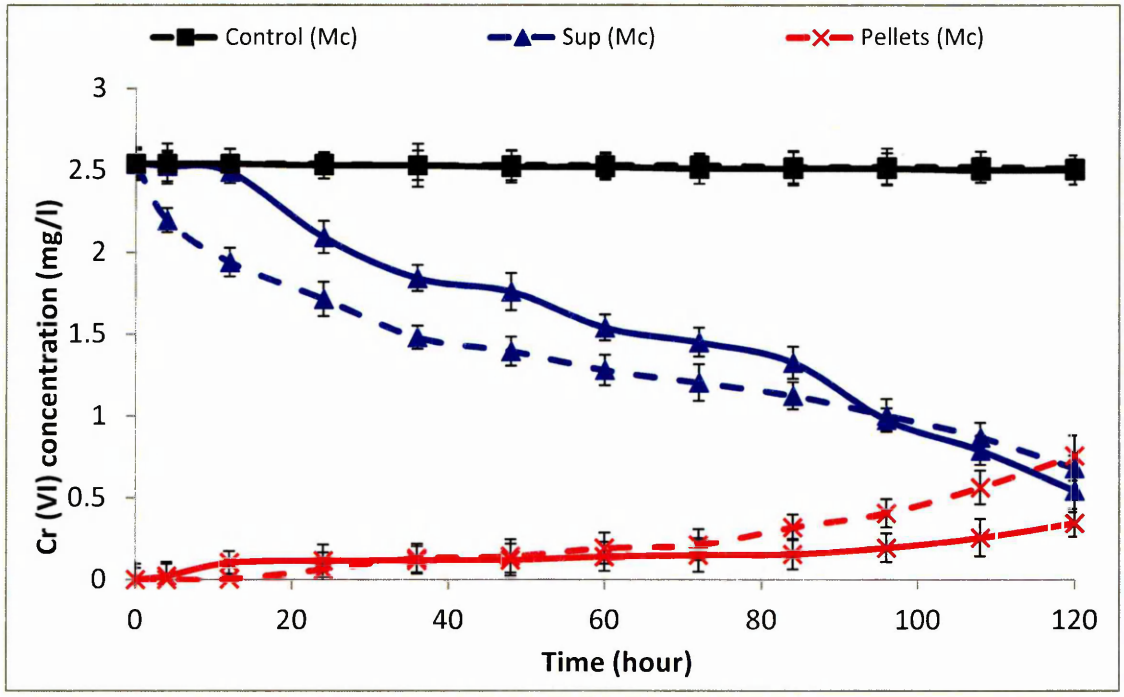
Error bars represent standard deviation (SD) of triplicate measurements (SD ranged from 0.05 to 0.1 mg/l).

Figure 5.15: Cr (VI) removal using mixed culture (M. capsulatus + Bacillus licheniformis)

5.3.5.1 Comparative assessment between pure M. capsulatus (Bath) and mixed culture in Cr (VI) removal

The aim of this experiment is to compare the performance of pure *M. capsulatus* (Bath) strain with the mixed culture that contains *M. capsulatus* and associated contaminated stain *B. licheniformis* in the removal of hexavalent chromium. Figure 5.15 shows the performance of the two bacteria strains. It can be seen that the pure *M. capsulatus* strain lowered Cr (VI) concentration in the supernatant samples from the beginning of chromium VI removal reaction, however there was no removal observed with the mixed culture during the first 12 hours. Then, Cr (VI) concentration gradually

decreased using both bacterial strains with greater removal of chromium VI in the case of the pure *M. capsulatus* bacterial strain. In contrast to the drop of Cr (VI) concentration in the supernatant samples, there was an increase in its concentration in the pellets fractions.



(—■—) Control (Mixed) = NMS medium with $K_2Cr_2O_7$ (Cr (VI) 2.54 mg/l).

(··■··) Control (*M. c.*) = NMS medium with $K_2Cr_2O_7$ (Cr (VI) 2.54 mg/l).

(—▲—) Sup. (Mixed) = NMS medium with mixed culture cells and $K_2Cr_2O_7$.

(··▲··) Sup. (M. c.) = NMS medium with *M. capsulatus* cells and $K_2Cr_2O_7$.

(—✕—) Pellets (Mixed) = Mixed cells with $K_2Cr_2O_7$.

(··✕··) Pellets (M. c.) = *M. capsulatus* cells with $K_2Cr_2O_7$.

Error bars represent triplicate measurements, standard deviation (SD) = 0.05 – 0.1 mg/l

Data for Pure *M. capsulatus curves* were taken from Chapter 3, Figure 3.2 (e)

Figure 5.16: Comparison between pure M. capsulatus (Bath) and mixed culture (M. capsulatus (Bath) + Bacillus licheniformis) in Cr (VI) removal

5.3.6 Bioinformatics study of putative chromate reductase in M. capsulatus (Bath) genome

Alhasin *et al.* (2010) reported a bioinformatics study in which they identified proteins and genes within the genome of M. capsulatus (Bath). The online BLAST search results indicated the presence of three significant homologues of *E. coli* Fre oxidoreductase, one significant *E. coli* nitoreductase NfsA and one high significant homologoues old yellow enzyme type chromate reductase of *Therus scotoductus* in *M. capsulatus* (Bath) genome. The details of these genes and other nonsignificant ones can be found in Alhasin *et al.* (2010).

5.4 Summary

In this chapter, further investigation was conducted on the ability of *M. capsulatus* to decrease Cr (VI) concentration in order to obtain additional information about the type of enzymes that are responsible for the reaction. The bacterial strain was confirmed as *M. capsulatus* (Bath), using 16S rRNA gene sequencing. Further analysis included the measurement of Cr (VI) concentration in high and low copper sulfate NMS media, use of phenylacetylene as an inhibitor during the bioremediation process and determination of chromium species using IC ICP-MS and FTIR.

It was noticed that during the growth stage of *M. capsulatus* (Bath), a contaminant bacteria has appeared in some cell cultures. Thus, further investigation was conducted to identify the associated contaminant bacteria.

The results obtained from Cr (VI) removal using *M. capsulatus* in the high and low copper sulfate NMS media has shown that the drop of Cr (VI) concentration in supernatant sample of high copper sulfate NMS media was greater than in low copper sulfate NMS media. This reveals that pMMO, sMMO and other enzymes that are induced by copper probably include the enzymes responsible for Cr (VI) reduction, because there was increase in the proportion of Cr (VI) fell from 46% to 68% in the supernatant between low and high copper medium.

During the Cr (VI) removal reaction using *M. capsulatus* in the low copper condition in the presence of phenylacetylene as an inhibitor, it was found that phenylacetylene totally inhibited the ability of the bacteria to remove Cr (VI). This confirms the results that methane monooxygenase enzymes (MMO) were responsible or at least necessary for Cr (VI) removal activity. It has been shown by Lontoh *et al.* (2000) that 100% of sMMO activity was inhibited with 200 μM of phenylacetylene (the same concentration as used in the current experiment) these results are consistent with a role for sMMO (presumably its reductase component) in reduction of Cr (VI). Alternatively, it is possible that inhibition of sMMO by phenylacetylene prevents reduction of Cr (VI) by other cellular enzymes via depletion of intracellular pools of reduced coenzymes such as NADH and NADPH when the methane oxidation pathway is inhibited.

Further analyses have been conducted to identify chromium species using IC ICP-MS and FTIR techniques. *M. capsulatus* has shown the potential of Cr (VI) and detoxified it to Cr (III), which is less toxic and less soluble. The FTIR spectra have shown some changes in the polysaccharide, fatty acids, phosphate and proteins regions.

The contaminant in the mixed culture was identified as *Bacillus licheniformis* (100%) using PCR 16S rRNA sequences. *B. licheniformis* is generally described as Gram-positive, thermophilic bacterium with an optimal growth temperature 35 ^{o}C - 50 ^{o}C (Herman et al., 1989). The mixed sample that contains both *M. capsulatus* (Bath) plus *Bacillus licheniformis* decreased Cr (VI) concentration by 79% after 120 hours of incubation period (Figure 5.15).

CHAPTER 6

6 CONCLUSIONS AND GENERAL DISSCUSION

6.1 Conclusions

In this research study, the removal of hexavalent chromium was investigated using five strains of Gram-negative bacteria; *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Escherichia coli*, *Proteus mirabilis* and *Methylococcus capsulatus* (Bath). As described in Chapter 2 section (2.1.3), three of them were nosocomial isolated bacteria, which were obtained from a previous study of hospital microorganisms. *Ps. aeruginosa* 090124-B was isolated from left leg ulcer, *E. cloacae* 090226-B was isolated from a sputum sample and *P. mirabilis* 090321-A was isolated from a wound swab. The other two strains; *E. coli* DH5α was obtained from the microbiology laboratory of the Biomedical Research Centre (BMRC) at Sheffield Hallam University and *M. capsulatus* (Bath) was obtained from NCIMB, Scotland, UK. The overall objective of this study was to investigate the potential of Gram-negative bacteria to reduce hexavalent chromium to the less toxic and less soluble trivalent chromium. The bioremediation process is considered as an effective approach to reduce risk to human health and the environment.

Three types of media, nutrient broth no. 2, M9 medium and NMS medium, were tested to investigate if a reaction occurred between Cr (VI) and the medium used in this study. It was found from the absorbance in the diphenylcarbazide assay, shown in Figure 2.2, the remaining Cr (VI) concentration in nutrient broth no. 2 medium is far lower than that observed in other media and solutions. This removal of Cr (VI) by nutrient broth medium in the absence of bacteria is attributed to its composition (meat and casein peptone) that includes molecules that could chemically reduce Cr (VI). On the other hand, M9 and NMS media (both are chemically defined media) did not react with Cr (VI), as no decline in its concentration was noticed in the absence of bacteria. Thus, nutrient broth no.2 medium was excluded from use throughout this study.

The growth of bacterial strains has been measured in either NMS or M9 medium containing potassium dichromate and potassium dichromate with sodium azide. All bacterial strains were successfully grown prior to the bioremediation process (Figure 3.1). It is clear that the growth of cells with sodium azide is slower compared with the pure cells and the ones without sodium azide. The ability of all bacterial strains to decrease chromate concentration was tested by adding potassium dichromate at a concentration of

2.54 mg/l Cr (VI) to log phase cultures of the strains and incubation times up to 120 hours. Ackerley *et al.* (2006) reported that *E. cloacae* and *E. coli* can grow in the presence of chromate. Wang *et al.* (1989), Pimentel *et al.* (2002) and Faisal and Hasnain (2004) have also studied and confirmed the growth of *E. cloacae* HO1 in the presence of potassium chromate under aerobic and anaerobic conditions. To investigate the effect of oxidative phosphorylation enzymes in the bioremediation process, sodium azide (0.05%) was added to the cell culture with potassium dichromate at the same time in the log phase cultures. The highest growth rate was observed with the pure bacterial culture for all employed strains, followed by the bacterial culture with potassium dichromate. However, it was noted that the slowest bacterial growth rate occurred in the pure bacterial culture in the presence of potassium dichromate and sodium azide. The results obtained revealed that sodium azide at 0.05 % exerts a relatively small inhibitory effect on the growth of all strains of bacteria tested.

All bacterial strains were found to be very effective in decreasing Cr (VI) concentration in the supernatant and pellet fractions. All live bacterial strains used in this study have shown notable Cr (VI) removal at different incubation times. No removal of chromate was detected with cell-free NMS and M9 medium (control) samples. This result is a step forward in utilizing these bacterial strains in the treatment of industrial effluents and other heavy metals. The highest percentage of Cr (VI) removal was recorded by *Ps. aeruginosa* (87%) followed by *E. coli* with 80%, *P. mirabilis* with 76%, *E. cloacae* 75% and the lowest percentage of removal was 73% achieved by *M. capsulatus* strain. *Ps. aeruginosa*, *E. cloacae* and *E. coli* were previously investigated for Cr (VI) removal (Brock and Gustafson, 1976; Hardoyo and Ohtake, 1991; Pimentel *et al.*, 2002; Summers and Jacoby, 1978). The ability of *E. cloacae* HO1 to reduce hexavalent chromium into the soluble low valence form of Cr (III) has been reported in several studies (Deleo and Ehrlich, 1994; Hardoyo and Ohtake, 1991; Faisal and Hasnain, 2004; Frederickson *et al.*, 2000; Mclean and Beveridge, 2001; Wang *et al.*, 1989). One previous report, from Alhasin *et al.* (2010) working in this research centre, has described reduction of chromate (VI) by *M. capsulatus* (Bath) from the initial concentration (1.4 – 1000 mg/l). *E. coli* was also reported to reduce Cr (VI) into Cr (III) (Puzon *et al.*, 2002; Rafiqullah *et al.*, 2009).

A hexavalent chromium reduction system of this nature can be economical to run, because it requires only readily available bacterial cultures and an inexpensive growth substrate that could be biological CH_4 or waste biomass material. A relatively inexpensive bioreactor system could allow re-cepting bacterial cells by encapsulatory than in kappa- kargenana beads. Remaining Cr (VI) after treatment with the bacteria, which was around 1.5 ppm in the laboratory experiments, may be lower in the condition of a bioreactor, or possibly this residual Cr (VI) could be removed by reaction with further cells, which could be tested in a future pilots study. The results obtained here suggests a more promising technology than existing chemical methods, which require expensive reagents and infrastructure, and are generally not effective at the low levels of chromium (VI) investigated in this thesis.

The autoclaved bacterial strains were also able to lower Cr (VI) concentration, although the amount of removal of Cr (VI) was lower than that for live cells. The main reason for using the autoclaved bacteria in parallel with the live cells was to investigate whether a Cr (VI) reduction reaction was catalysed by enzymes produced by the cells or it was a simple chemical reaction between Cr (VI) and components of the cells, which are not dependent on the cells' being alive. The Cr (VI) removal observed with autoclaved cells agreed with Fein *et al.* (2002), and indicates that the nonmetabolic Cr (VI) reduction observed, in the absence of externally added electron donors, is likely a result of the oxidation of organic molecules within the cell wall, possibly the Cr-reducing enzymes responsible for metabolic enzymatic Cr (VI) reduction or cell wall cytochromes, which have large reduction potential. Furthermore, in the absence of abundant external electron donors, there are molecules within the cell wall matrix that serve as electron donors for Cr (VI) reduction to Cr (III). Among all autoclaved strains, the highest level of Cr (VI) removal was recorded with *E. coli* bacteria (23%), followed by *E. cloacae* 21%, *P. aeruginosa* recorded 19%, *P. mirabilis* 15% and then *M. capsulatus* with only 11%. This result reveals that autoclaved cells could be used for the biosorption process for the removal of toxic Cr (VI) under all conditions including when the cells are no longer alive. The use of autoclaved bacteria in the Cr (VI) removal process may under certain circumstances, offer several advantages over living cells. The use of autoclaved bacteria eliminates nutrient supply and the heavy metal toxicity problems which occur in living

cells (Bai and Abraham, 2001; Zouboulis *et al.*, 1999). Besides that, killed cells could be stored or used for extended periods, and therefore, problems associated with the need to provide suitable growth conditions would not arise (Sheng *et al.*, 2004).

The analysis of total chromium concentration during the bioremediation process was monitored using the ICP-OES technique. In addition to the change in chromium oxidation state described above, there was a change in distribution of chromium in supernatant and pellet fractions. The data showed a decrease in chromium (VI) concentration, while the total detectable chromium in the system remained constant.

As shown in Table 3.1 in Chapter 3, in all instances autoclaving of the cells diminished the amount of Cr (VI), suggesting that in all strains a substantial part of reduction of Cr (VI) to Cr (III) was dependent on the presence of living cells.

Addition of NaN_3 to active cells (i.e. not autoclaved) usually either diminished the removal of Cr (VI) or had little effect on it. However, in *P. mirabilis* NaN_3 actually increased the removal of Cr (VI), which may suggest channelling of reducing equivalents from central metabolism into chromium VI reduction (see section 3.3.3, Chapter 3).

Autoclaving of the cells either alone or in combination with NaN_3 in all instances diminished the amount of chromium (VI) associated with the cells. The effect of NaN_3 on the distribution of remaining Cr (VI) between the supernatant and pellet fractions varied from strain to strain.

The fact that the Cr balance, shown in Table 3.2 in Chapter 3, for all ICP-OES data is not significantly different from 100% indicates that the Cr detected in the pellet and supernatant fractions accurately for all Cr added at the start of the experiments.

In all cases, killing of the cells by autoclaving decreased the proportion of Cr that was associated with the pellet, whether NaN_3 was present or not. This shows that the processes that led to biosorption and/or precipitation of Cr are to a certain extent dependent upon the presence intact living cells.

NaN_3 when added alone did not greatly affect the detoxification of Cr species with P. aeruginosa, *E. cloacae* and *P. mirabilis*. With *E. coli* it led to an increase of the amount of chromium associated with the pellet fractions from 29 to 40%. With *M.*

capsulatus (Bath), NaN_3 led to a decrease in the amount of chromium associated with the pellet (from 61% to 16%).

Since chromate is a strong oxidizing agent, it was thought that some of the chromate removal could be a result of chemical redox reactions rather than biochemical activity of cells. However, inhibition of cell growth by adding sodium azide (an inhibitor of oxidase phosphate enzymes) has resulted in a simultaneous loss of chromate concentration in the case of *P. mirabilis* and *E. coli* strains.

Hexavalent chromium removal by *P. mirabilis* bacterial strain (in both living and autoclaved cells) is considered as one the main contributions of this research, as Cr (VI) removal using *P. mirabilis* has not been previously reported.

The results obtained from the cell fractionation experiment conducted using *P. mirabilis* have shown a significant Cr (VI) removal activity has occurred in the cytoplasm and a small amount of removal by the cell-membrane fraction, while, no removal activity due to components of the cell wall alone has been observed. This suggests that the enzymes responsible for Cr (VI) removal exist in the cytoplasm and cell membrane. In this experiment, there was no electron donor added to the fractions, so even if there were a chromium VI reductase enzyme in the cell wall, the activity may not have been observed. It is suggested that the experiments of cell fractionation be repeated with the electron donors to check which enzymes are responsible for chromium (VI) reduction.

To confirm the ability of *P. mirabilis* to decrease Cr (VI) concentration, a well characterised bacterial strain (*P. mirabilis* HI 4320) was tested and compared with *P. mirabilis* 090321-A. The two independent isolates of *P. mirabilis* showed very similar chromium (VI) reduction activities. At the end of reaction time Cr (VI) decreased from its initial concentration (2.54 mg/l) by 77% and 80% in the supernatant using HI4320 and 09032-A bacterial strains, respectively.

Cr (VI) removal using *M. capsulatus* in the high and low copper sulfate NMS media shows that the reduction of Cr (VI) in the supernatant sample of high copper sulfate NMS media was greater than in low copper sulfate NMS media. This reveals that pMMO, sMMO and other enzymes that are induced by copper probably include the

enzymes responsible for Cr (VI) reduction or removal, because there is an increase in the proportion of Cr (VI) declined from 46% to 68% between the low and high copper media.

During Cr (VI) removal reaction using *M. capsulatus* in NMS containing 1 mg/l of $CuSO_4.5H_2O$ in the presence of phenylacetylene as an inhibitor, it was found that phenylacetylene totally inhibited the ability of the bacteria to decrease Cr (VI) concentration. This confirms the results that methane monooxygenase enzymes (MMO) were responsible or at least necessary for Cr (VI) removal or reduction activity. Phenylacetylene (200 μM) which inhibits sMMO enzymes in the low copper sulfate NMS media (Lontoh *et al.*, 2000) completely inhibited Cr (VI) removal. It suggested that, sMMO or another enzyme responsible for Cr (VI) reduction is directly inhibited by phenylacetylene. Alternatively, it is possible that inhibition of sMMO by phenylacetylene prevents reduction of Cr (VI) by other cellular enzymes via depletion of intracellular pools of reduced coenzymes such as NADH and NADPH when the methane oxidation pathway is inhibited.

Results using ICP-MS have shown the ability of *M. capsulatus* and *P. mirabilis* to reduce Cr (VI) to Cr (III), which is less toxic and insoluble. FTIR spectra have shown some changes in the polysaccharide, fatty acids, phosphate and protein regions which may indicate binding of Cr (VI) to cellular components such as the cell wall, cell membrane, cytoplasm, RNA and DNA.

The potential of *P. mirabilis* and *M. capsulatus* strains for use in the reduction of chromium (VI) to less soluble chromium (III) has been confirmed using ICP-MS coupled with dionex column ion chromatography. The technique was used to separate the two forms of the metal. The amount of Cr (VI) measured in the supernatant at the end of the experiment by ICP-MS agrees clearly with those measured by the DPC assay (for *P. mirabilis* and *M. capsulatus*). The DPC assay showed 24 and 27%, respectively of initial Cr as Cr (VI) in the supernatant. The amount of Cr associated with the pellet fractions deduced from ICP-MS (32%) agrees closely with the amount measured by ICP-OES (38%) for *P. mirabilis*. The corresponding values for *M. capsulatus* are somewhat different with the amount of pellet associated Cr being greater in the ICP-OES measurement. The culture used for ICP-MS and ICP-OES for both strains were grown on

different occasions and it may be that there was some variation in the *M. capsulatus* culture.

The results from FTIR spectra showed some changes (appearance, disappearance and shifting of peaks in infrared bands) occurred in the internal structure of the bacterial strain after adding chromium (after 24 and 48 hours of reaction time). The observed changes belong to saccharides and polysaccharides, fatty acids, amide I, amide II and lipids present within cell wall, membranes and cytoplasm.

The results obtained from the online BLAST search of *P. mirabilis* ATCC 29906T genome have confirmed the presence of four significant and three borderline homologues of *E. coli* Fre oxidoreductase and one significant homologue of *E. coli* nitoreductase NfsA. These genes could be considered possible candidates for encoding enzymes responsible for Cr (VI) reduction.

A contaminant bacterium appeared in some cell cultures during the growth of *M. capsulatus* (Bath). It was identified as a Gram-positive *Bacillus licheniformis* (100%) using PCR and 16S rRNA gene sequencing. The ability of the mixed culture, that contains *M. capsulatus* (Bath) plus *Bacillus licheniformis*, was also tested for Cr (VI) removal. It lowered Cr (VI) concentration by 79% after 120 hours of incubation.

6.2 Future work

Five Gram-negative bacterial strains have been cultivated and tested for chromium (VI) removal during the work reported in this thesis. In order to find new and useful organisms for bioremediation of chromium (VI), it is proposed that the interaction between chromium (VI) and other strains (Gram-negative and positive) should be investigated, including other methanotrophic bacteria, since the results obtained showed that the methanotrophic bacteria are very promising in chromium VI reduction and biosorption/precipitation of chromium species.

The bioinformatics study carried out in Chapter (IV) has identified seven candidates of chromate reductase proteins in the genome of *P. mirabilis* ATCC29906. Alhasin *et al.* (2010) conducted a bioinformatics study on *M. capsulatus* (Bath) genome.

The study identified a number of genes that could encode proteins involved in reduction of Cr (VI). In order to investigate the roles played by the reductases identified via the bioinformatics study, further biochemical and specific gene inactivation studies are worth investigating to see which of these are responsible for the observed chromate reductase activity. The reductase genes in question would be cloned in plasmids in *E. coli* and constructs made with antibiotic resistance cassettes within the cloned gene. These would then be introduced into *M. capsulatus* (Bath) or *P. mirabilis* as appropriate by means of conjugation from the *E. coli* donor strain as described in Csaki *et al.* (2003) and Martin and Murrell (1995). Subsequent recombination into the chromosome of the recipient strain and inactivation of the desired gene would be confirmed by PCR using primers specific for appropriate DNA fragments (Csaki *et al.*, 2003). A chromate assay could be carried out via the colomertric assay to identify which of the genes contributes to the chromate removal process (Caballero-Flores *et al.* 2012).

Biosorption, a property of inactive (immobilised) microbial biomass to bind and concentrate heavy metals from dilute aqueous solutions, is an alternative mechanism for the removal of toxic metals from industrial effluents. It is an environmental friendly process that could be sustainable technology for the removal of toxic metals. The development of the biosorption processes requires further investigation in the direction of modelling, of regeneration of biosorbent material and of testing immobilized raw biomasses with industrial effluents.

It is suggested that a further work is required to investigate whether it is possible to scale the small laboratory scale bioreactor to a commercial bioreactor for *P. mirabilis* and *M. capsulatus* (Bath), to determine whether the observed bioremediation of chromium can be scaled up to a practical process of industrial use.

Ackerley, D. F., Gonzalen, C. F., Park, C. H., Blake, R., Keyhan, M. and Matin, A. (2004). Chromate-reducing properties of soluble flavoproteins from pseudomonas putida and escherichia coli. *Applied and environmental microbiology,* **70** (2), 873-882.

Aguilera, S. Aguilar, M. E., Chavez, M. P., Lopoz-Meza, J. E., Pedraza-Reyes, M., Campos-Garcia, J. and Cervantes, C. (2004). Essential residues in the chromate transporter ChrA of *Pseudomonas aeruginosa. FEMS Microbiol. Lett.* **232,** 107-112.

Ahluwalia, S. S. and Goyal, D. (2007). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Bioresource Technology*, 98(12), 2243-2257.

Ahmad, W. A., Ahmad, W. H. W., Karim, N. A., Raj, A. S. S. and Zakaria, Z. A. (2013). Cr (VI) reduction in naturally rich growth medium and sugarcane bagasse by *Acinetobacter haemolyticus. Internal. Biodeter. Biodegrd.* 1-6.

Ajmal, M., Nomani, A. A. and Ahmad, A. (1984). Acute toxicity of chrome electroplating wastes to microorganisms: Adsorption of chromate and chromium (VI) on a mixture of clay and sand. *Water, air, & soil pollution,* **23** (2), 119-127.

Alhasin, A., Gurman, S. J., Murphy, L. M., Perry, A., Smith, T. J. And Gardiner, P. H. E. (2010). Remediation of chromium (VI) by methane-oxidizing bacterium. *Environ. Sci. Technol.* **44** (1), 400-405.

Allaway, B. J. (1995). Soil processes and the behavior of heavy metals. In heavy metals in soils , edited by Allaway, B. J. 11-37. Blackie Academic and Professional, London, U.K.

Allen, L. A. and Hanson, R. S. (1985). Construction of broad-host-range cosmid cloning vectors: identification of genes necessary for growth on methanol in *Methylobacterium organophilum* XX. *J. Bacteriol.*, **161**, 955–961.

Alvarez, A. H., Moteno-Sanchez, R. and Cervantes, C. (1999). Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa. Journal Bacteriol.* **181**, 7398-7400.

Amiel, C., Mariey, L., Denis, C., Pichon, P. and Traver, J. (2001). FTIR spectroscopy and taxonomic purpose: contribution to the classification of lactic acid bacteria. Lait. **81**, 249-255.

Anderson, D. J. and Lidstrom, M. E. (1988). The moxFG region encodes four polypeptides in the methanol-oxidizing bacterium methylobacterium sp. strain AM1. *Journal of bacteriology,* **170** (5), 2254-2262.

Anderson, D. J., Morris, C. J., Nunn, D. N., Anthony, C. and Lidstrom, M. E. (1990). Nucleotide sequence of the methylobacterium extorquens AM1 moxF and moxJ genes involved in methanol oxidation. *Gene,* **90** (1), 173-176.

Anderson, R. A., Bryden, N. A. and Polansky, M. M. (1992). Dietary chromium intake: freely chosen diets, institutional diets and individual foods. *Biol Trace Elem Res,* **32**, 117-121.

Anthony, C. (1982). *The biochemistry of methylotrophs.* Academic Press.

Anthony, C. (1991). Autotrophic microbiology and one-carbon metabolism. *The quarterly review of biology,* **66** (3), 341.

Anthony, C. (1986). Bacterial oxidation of methane and methanol. *Advances in microbial physiology.* **27**, 113-210.

Anthony, C. (1990). The oxidation of methanol in gram-negative bacteria. *FEMS microbiology letters,* **87** (3-4), 209-214.

Atlas, R. M. (1993). Hand book of microbiological media. In Parks, L. M (ed.). *Library of Congress- in Publication Data.* USA.

Ayres, R. U. (1992). Toxic heavy metals: Materials cycle optimization. *Proceedings of the national academy of sciences,* **89** (3), 815-820.

Bagchi, D., Bagchi, M. and Stohs, S. J. (2001). Chromium (VI) induced oxidative stress, apoptotic cell death and modulation of p53 tumor suppressor gene. *Molecular and cellular biochemistry,* **222** (1), 149-158.

Bai, R. S. and Abraham, T. E. (2001). Biosorption of Cr (VI) from aqueous solution by *Rhizopus nigricans. Bioresource technology,* **79** (1), 73-81.

Bailar, J. C. (1997). The promise and problems of meta-analysis. *New england journal of medicine,* **337** (8), 559-561.

Ballatori, N. (1994). Glutathione mercaptides as transport forms of metals. *Advances in pharmacology (san diego, calif.),* **27**, 271-298.

Barnhart, J. (1997). Chromium chemistry and implications for environmental fate and toxicity. *Soil and sediment contamination: An international journal.* **6** (6), 561-568.

Barta, T. M. and Hanson, R. S. (1993). Genetics of methane oxidation in gram-negative methylotrophic bacteria. Antonie van Leeuwenhoek, **64**, 109–120.

Bartlett, K. B. and Harriss, R. C. (1993). Review and assessment of methane emissions from wetlands. Chemosphere **26**, 261–320.

Bartlett, R. J. (1991). Chromium cycling in soils and water: Links, gaps, and methods. *Environmental health perspectives,* **92**, 17-24.

Basso, M. C., Cerrella, E. G. and Cukierman, A. L. (2002).Activated carbons developed from a rapidly renewable biosource for removal of chromium (II) and nickel (II) ions from dilute aqueous solutions. *Industrial and Engineering Chemistry Research*. **41**, 180-189.

Bastien, C., Machlin, S., Zhang, Y., Donaldson, K. and Hanson, R., S. (1989). Organization of genes required for the oxidation of methanol to formaldehyde in three type II methylotrophs. *Appl. Environ. Microbiol*, **55**, 3124–3130.

Basu, P., Katterle, B., Anderson, K. K. and Dalton, H. (2003). The membrane-associated from methane mono-oxygenase from *Methylococcus capsulatus* (Bath) is a copper/iron proton. *Biochem. Journa,* **369** (2), 417-427.

Batic, M. and Raspor, P. (2000). Effect of cultivation mode on a bioprocess for chromium yeast biomass enrichment. *Pflügers archiv european journal of physiology,* **439** (7), 73-75.

Becquer, T., Quantin, C., Sicot, M. and Boudot, J. P. (2003). Chromium availability in ultramafic soils from New Caledonia. *Science of the total environment*, **301** (1-3), 251-261.

Bedard, C. and Knowles, R. (1989). Physiology, biochemistry and specific inhibitors of CH4, NH4 and CO oxidation by methanotrophs and nitrifiers. *Microbiol. Rev.* **53**, 668–84.

Beleza, V. M., Boaventura, R. A. and Almeidas, M. F. (2001). Kinetics of chromium removal from spent tanning liquors using acetylene production sludge. *Environ.sci.technol,* **35** (21), 4379-4383.

Berven, F. S., Karlsen, O. A., Straume, A. H., Flikka, K., Murrell, J. C., Fjellbrkeland, A., Lillehaug, J. R., Eidhammer, I. and Jensen, H. B. (2006). Analysing the outer membrane subproteome of *Methylococcus capsulatus* (Bath) using proteomics and novel biocomputing tools. *Archives of microbiology,* **184** (6), 362-377.

Bhide, J. V., Dhakephalkar, P. K. and Paknikar, K. M. (1996). Microbiological process for the removal of Cr (VI) from chromate-bearing cooling tower effluent. *Biotechnology letters,* **18** (6), 667-672.

Blake II, R. C., Choate, D. M., Bardhan, S., Revis, N., Barton, L. L. and Zocco, T. G. (1993). Chemical transformation of toxic metals by *Pseudomonas* strain from a toxic waste site. *Environmental Toxicology and chemistry*, **12** (8), 1365-1376.

Bodrossy, L., Kovacs, K. L., McDonald, I. R. and Murrell, J. C. (1999). A novel thermophilic methane-oxidising γ-Proteobacterium. *FEMS Microbiology Letters*. **170**, 335-341.

Bopp, L. H., Chakrabarty, A. M. and Ehrlich, H. L. (1983). Chromate resistance plasmid in *Pseudomonas fluorescens*. *Journal Bacterol.* **155**, 1105-1109.

Bopp, L. H. and Ehrlich, H. L. (1988). Chromate resistance and reduction in *Pseudomonas fluorescens* LB 300. *Arch. Microbiol.*, **150**, 425-231.

Borodina, E., Nichol, T., Dumont, M.G., Smith, T.J. and Murrell, J.C. (2007). Mutagenesis of "leucine gate" to explore the basis of catslytic versatility in soluble methane monooxygenase. *Appl. Environ. Microbiol.* **73** (20), 6460-6467.

Bowman, J. P., Sly, L. I. and Stackbrandt, E. (1995). The phylogenetic position of the family methylococcaceae. *International journal of systematic and evolutionary microbiology,* **45** (1), 182-185.

Bowman, J. P., Skerratt, J. H., Nichols, P. D. and Sly, L. I. (1991). Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilizing bacteria. *FEMS Microbiol. Ecol.* **85**, 15–22.

Brady, D. and Duncan, J. R. (1994). Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. *Applied microbiology and biotechnology,* **41** (1), 149-154.

Butt, T. R. and Ecker, D. J. (1987). Yeast metallothionein and applications in biotechnology. *Microbiology and molecular biology reviews,* **51** (3), 351-364.

Caballero-Flores, G. G., Acosta-Navarrete, Y. M., Ramírez-Díaz, M. I., Silva-Sánchez, J. and Cervantes, C. (2012). Chromate-resistance genes in plasmids from antibiotic-resistant nosocomial enterobacterial isolates. *FEMS Microbiol. Lett.*, **327** (2), 148-154.

Camargo, F. A.O. Bento, F. M., Okeke, B. C. and Frankenberger, W. T. (2003). Chromate reduction by chromium-resistant bacteria isolated from soils contaminated with dichromate. *Journal of Environmental Quality.* **32**, 1228-1233.

Cavanaugh, C. M. (1993). Methanotroph-invertebrate symbioses in the marine environment: ultrastructural, biochemical and molecular studies, p.315–328. *In* J. C. Murrell and D. P. Kelley (ed.), Microbial growth on C1 compounds. Intercept Press, Ltd., Andover, United Kingdom.

Cervantes, C., Campos-Garcia, J., Devars, S., Gutierrez-Corona, F., Loza-Tavera, H., Torres-Guzman, J. C. and Moreno-Sanchez, R. (2001). Interactions of chromium with microorganisms and plants. *FEMS microbiology reviews,* **25** (3), 335-347.

Cervantes, C. and Silver, S. (1992). Plasmid chromate resistance and chromate reduction. *Plasmid,* **27** (1), 65-71.

Cervantes, C. and Campos-Garcia, J. (2007). Reduction and efflux of chromate by bacteria. In Nies, D. H. and Silver, S. (eds). Molecular Microbiology of Heavy Metals. Springer-verlag, Berlin. 407-420.

Chan, S. I., Nguyen, H. H. T., Shiemke, A. K. and Lidstrom, M. E. (1993). Biochemical and biophysical studies towards characterization of the membrane-associated methane monooxygenase, p. 93–107. *In* J. C. Murrell and D. P. Kelley (ed.), Microbial growth on C1 compounds. Intercept Press, Ltd., Andover, United Kingdom.

Chardin, B., Gallice, P., Sari, J. C. and Bruschi, M. (2002). Thermodynamics of cleaning upof waste waters polluted by chromium. Journal therm. Analyt. Calorimet. **70**, 475-482.

Chen, J. M. and Hao, O. J. (1998). Microbial chromium (VI) reduction. *Critical reviews in environmental science and technology,* **28** (3), 219-251.

Cheung, K. H., Lai, H. Y. and Gu, J. D. (2006). Membrane-associated hexavalent chromium reductase of bacillus megaterium TKW3 with induced expression. *Journal of microbiology and biotechnology,* **16** (6), 855-862.

Cheung, K. H. and Gu, J. D. (2002). Bacterial colour response to hexavalent chromium, Cr^{+6}. The Journal of Microbiology. **40**, 234-236.

Cheung, K. H. and Gu, J. D. (2003). Reduction of chromate Cr O_4^{2-} by an enrichment consortium and isolate of marine sulfate-reducing bacteria. *Chemosphere* **52**, 1523-1529.

Childress, J. J., Fisher, C. R., Brooks, J. M., Kennicut, M. C., Bidigare, R. and Anderson, A. E. (1986). A methanotrophic marine molluscan (Bivalvia: *Mytilidae*) symbiosis: mussels fuelled by gas. Science **233**, 1306–1308.

Cicerone, R. J. and Oremland, R. S. (1988). Biogeochemical aspects of atmospheric methane. *Global biogeochemical cycles,* **2** (4), 299-327.

Ciesla, Z., Mardarowicz, K. and Klopotowski, T. (1974). Inhibition of DNA synthesis and cell division in Salmonella typhimurium by azide. *Mol. Gen. Genet.* **135**, 339-348.

Cieslak-Golonka, M. (1995). Toxic and mutagenic effects of chromium (VI). *Rev. Polyhedron.* **15**, 3667-3689.

Cohen, M. D., Kargacin, B., Klein, C. B. and Costa, M. (1993). Mechanisms of chromium carcinogenicity and toxicity. *Crit. Rev. Toxicol.* **23**, 255-281.

Colby, J. and Dalton, H. (1978). Resolution of the methane monooxygenase of *Methylococcus capsulatus* (Bath) into three components: purification and properties of component C, a flavoprotein. *Biochem. J.* **171**,461–468.

Colby, J., Stirling, D. I. and Dalton, H. (1977). The soluble methane monooxygenase of methylococcus capsulatus (bath). *Biochem.j,* **165**, 395-402.

Collins, M. L. Bucholzl, P. and Remsen, C. C. (1991). Effect of copper on *Methylosinus albus* BG8. Appl. Environ. Microbiol. **57**, 1261–1264.

Crabtree, R. H. (1995). Aspects of methane chemistry. *Chemical reviews,* **95** (4), 987-1008.

Craig, H. and Chou, C. C. (1982). Methane- the record in polar ice cores. *Geophysical research letters,* **9**, 1221-1224.

Crutzen, P. J. (1991). Atmospheric chemistry: Methane sinks and sources. *Nature,* **350,** 380-381.

Csaki, R., Bodrossy, L., Klem, J., Murrell, J. C. and Kovacs, K. L. (2003). Genes involved in the copper-dependent regulation of soluble methane monooxygenase of in Methylococcus capsulatus (Bath): cloning, sequencing and mutational analysis. *Microbiology*, **149**, 1785-1795.

Csaki, R. Hanczar, T., Bodrossy, L., Murrell, J. C. and Kovacs, K. L. (2001). Molecular characterization of structural genes coding for a membrane bound hydrogen in Methylococcus capsulatus (Bath). *FEMS Microbiology Letters.* **205**, 203-207.

Czako-Ver, K., Batie, M., Raspor, P., Sipiczki, M. and Pesti, M. (1999). Hexavalent chromium uptake sensitive and tolerant mutants of *Schizosaccharomyces pombe. FEMS Microbiology Letters.* **178** (1), 109-115.

Dalton, H. and Leak, D. J. (1985). Methane oxidation by microorganisms, p.173–200. *In* S. R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism. Academic Press Ltd., London.

Dalton, H. and Stirling, D. I. (1982). Co-metabolism. Philos. Trams. R. Soc. Lon. B. Biol. Sci. **297**, 481-496.

Das, S. K. and Guha, A. K. (2007). Biosorption of chromium by *Termitomyces clypeatus. Elsevier.* **60**, 46-54.

Davis, T. A., Volesky, B. and Mucci, A. (2003). A review of the biochemistry heavy metal biosorption by brown algae. *Water Res.* **37**, 4311-4330.

Deleo, P. C. and Ehrlich, H. L. (1994). Reduction of hexavalent chromium by *Pseudomonas fluorescens* LB 300 in batch and continuous cultures. *Appl. Microbial. Biotechnol.* **40,** 756-759.

Derbyshire, M., Lamberty, A. and Gardiner, P. H. E. (1999). Optimization of the simultaneous determination of Cr (III) and Cr (VI) by ion chromatography with chemiluminescence detection. *Anal. Chem.* **71,** 4203-4207.

Dermou, E., Velissariou, A., Xenos, D. and Vayenas, D. V. (2005). Biological chromium (VI) reduction using a trickling filter. Journal of Hazardous Material. **126**, 78-85.

De Vries, G. E., Kues, U. and Stahl, U. (1990). Physiology and genetics of methylotrophic bacteria. *FEMS Microbiol. Rev.* **75**, 235–258.

Diels, L., Vander Lelie, N. and Bastianes, L. (2002). New developments in treatment of heavy metal contaminated soils. *Environmental Science and Bio/technology.* **1**, 75-82.

Dijkhuizen, L., Levering, P. R. and De Vries, G. E. (1992). The physiology and biochemistry of aerobic methanol-utilizing gram-negative and gram-positive bacteria. *Methane and methanol utilizers,* **5**, 149-181.

Engl, A. and Kunz, B. (1995). Biosorption of heavy metals by saccharomyces cerevisiae: Effects of nutrient conditions. *Journal of chemical technology & biotechnology,* **63** (3).

Faisal, M. and Hasnain, S. (2004). Comparative study of Cr (VI) uptake and reduction in industrial effluent by *Ochrobactrum intermedium* and *Bervibacterium sp. Biotechnology letters.* **26**, 1623-1628.

Faisal, M. and Hasnain, S. (2004). Microbial conversion of Cr (VI) in to Cr (III) in *industrial effluent. African Journal of Biotechnology.* **3** (11), 610-617.

Fein, J. B., Fowle, D. A., Cahill, J., Kemmer, K., Bovanov, M. and Bunker, B. (2002). Nonmetabolic reduction of Cr (VI) by bacterial surfaces under nutrient-absent conditions. *Geomicrobiology journal.* **19**(3), 369-382.

Fendorf, S. E, Fendorf, M., Sparks, D. L., and Gronsky, R. (1992). Inhibitory mechanisms of Cr (III) oxidation by Delta-M no2. *Journal of colloid and interface science,* **153** (1), 37-54.

Fisher, C. (1990). Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Rev. Aquat. Sci.* **2**, 399–436.

Fisher, C. R., Brooks, J. M., Vodenichar, J. S. Zande, J. M. Childress, J. J. and Burke, R. A. (1993). The cooccurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. *Mar. Ecol.*, **114**, 277–289.

Forget, A. and Fredetta, V. (1962). Sodium azide selective medium for the primary isolation of anaerobic bacteria. *Journal of Bacteriology.* **83** (6), 1217-1223

Fortin, Y., Phoenix, P. and Drapeau, G. (1990). Mutations conferring resistance to azide in Escherichia coli occur primarily in the secA gene. *Journal of Bacteriology.* **172** (11), 6607 – 6610.

Fox, B. G., Froland, W. A., Dege, J. E. and Lipscomb, J. D. (1989). Methane monooxygenase from *Methylosinus trichosporium* OB3b, purification and properties of a three-component system with high specific activity from a type II methanotroph. J. *Biol. Chem.* **264**, 10023-10033.

Fox, B. G., Borneman, J. G., Wackett, L. P. and Lipscomb, J. D. (1990). Haloalkene oxidation by the soluble methane monooxygenase from methylosinus trichosporium OB3b: Mechanistic and environmental implications. *Biochemistry,* **29** (27), 6419-6427.

Francis, C. A., Obraztosova, A. Y. and Tebo, B. M. (2000). Dissimilatory metal reduction by facultative anaerobic *Pantoea agglomerans* spi. *Applied Environmental Microbiology.* **66**, 543-548.

Fredrickson, J. K., Kostandarithes, H. M., Li, S. W., Plymale, A. E. and Daly, M. J. (2000). Reduction of Fe(III), Cr (VI), U (VI) and Te (VII) by *Denococcus radiodurans* R1. *Appl. Environ. Microbial.* **66** (5), 2006-2011.

Fude, L., Harris, B., Urrutia, M. M. and Beveridge, T. J. (1994). Reduction of Cr (VI) by a consortium of sulfate reducing bacteria (SRBIII). *Applied and Environ. Microbiol.* **60** (40), 1525-1531.

Fukai, R. (1967). Valency state of chromium in seawater. Nature (Lond.) 213, 901.

Gadd, G. M. and White, C. (1993). Microbial treatment of metal pollution-a working biotechnology? *Trends in biotechnology (United Kingdom),* **11**, 353-359.

Ganguli, A. and Tripathi, A. K. (2002). Bioremediation of toxic chromium from electroplating effluent by chromate-reducing pseudomonas aeruginosa A2Chr in two bioreactors. *Applied microbiology and biotechnology,* **58** (3), 416-420.

Green, J. and Dalton, H. (1985). Protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). *The Journal of Biological Chemistry.* **260** (29), 15795-15802.

Green, P. N. (1992). Taxonomy of methylotrophic bacteria, p. 23–84. *In* J. C. Murrell and D. P. Kelley (ed.), Microbial growth on C1 compounds. Intercept Press, Ltd., Andover, United Kingdom.

Gonzalez, C. F., Ackerley, D. F., Lynch, S. V. and Matin, A. (2005). ChR, a soluble quinine reductase of *Pseudomonas putida* that defends against H_2O_2. *J. Biol. Chem.* **280**, 22590-22595.

Gopalan, R. and Veeramani, H. (1994). Studies on microbial chromate reduction by pseudomonas sp. in aerobic continuous suspended growth cultures. *Biotechnology and bioengineering,* **43** (6).

Guha, H., Jayachandran, K. and Maurrasse, F. (2001). Kinetics of chromium (VI) reduction by a type strain *Schewanella alga* under different growth conditions. *Environ. Pollut.* **115** (2), 209-218.

Gupta, A. Phung, T. L., Taylor, D. E. and Silver, S. (2001). Diversity of silver resistance genes in incH incompatibility group plasmids. *Microbioligy* **147**, 3393-3402.

Gvozdyak, P. I., Dmitrenko, G. N. and Kulikov, N. I. (1985). Treating industrial waste waters by fixed microorganisms. *Soviet journal of water chemistry and technology,* **7** (1), 93-98.

Hagendorfer, H. and Goessler, W. (2008). Separation of chromium (III) and chromium (VI) by ion chromatography and an inductively coupled plasma mass spectrometer as element-selective detector. *Talanta.* **76** (3), 656-661.

Hanczar, T., Csaki, R., Bodrossy, L., Murrell, J. C. and Kovac, K. L. (2002). Detection and localization of two hydrogenase in *Methylococcus capsulatus* (Bath) and their potential role in methane metabolism. *Arch. Microbiol.* **177**, 167-172.

Hanson, R. S., Netrusov, A. I. and Tsuji, K. (1992). The obligate methanotrophic bacteria methylococcus, methylomonas, and methylosinus. *The prokaryotes,* 2352–2364.

Hanson, R. S. and Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiology and molecular biology reviews,* **60** (2), 439.

Haq, R. and Shakoori, A. R. (1998). Microbiological treatment of industrial wastes containing toxic chromium involving successive use of bacteria, yeast and algae. *World journal of microbiology and biotechnology,* **14** (4), 583-585.

Hardoyo, J. K. and Ohtake, H. (1991). Effects of heavy metal cations on chromate reduction by *Enterobacter cloacae* strain HOI. *J. Gen. Appl. Microbiolo.* **37**, 519-522.

Herrmann, M, S. (1994). Testing the waters for chromium. *Journal of Chemical Eduction.* **71** (4), 323-332.

Harms, N. (1993). Genetics of methanol oxidation in *Paracoccus denitrificans*, p. 235–244. *In* J. C. Murrell and D. P. Kelley (ed.), Microbial growth on C1 compounds. Intercept Press, Ltd., Andover, United Kingdom.

Hogan, K. B., Hoffman, J. S. and Thompson, A. M. (1991). Methane on the greenhouse gas agenda. *Nature,* **354**, 181-182.

Hovland, M. and A. G. Judd. (1988). Seabed pockmarks and seepages. Impact on geology, biology and the marine environment. Grahm & Trotman, London.

Institute of Medicine Food and Nutrition Board. (2001). Dietary reference intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. *National Academy Press*, Washington, DC.

Ishibishi, Y., Cervantes, C. and Silver, S. (1990). Chromium reduction in pseudomonas putida. *Applied and environmental microbiology,* **56** (7), 2268-2270.

Jahng, D. and Wood, T. K. (1996). Metal ions and chloramphenicol inhibition of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. *Appl. Microbiol. Biotechnolog.* **45**, 744-749.

Jan, T. K. and Young, D. R. (1978). Determination of microgram amounts of some transition metals in sea water by methyl isobuthyl ketone-nitric acid successive extraction and flameless atomic absorption spectrophotometry. *Analytical chemistry,* **50** (9), 1250-1253.

Janssen, D., Grobben, B. G. and Witholt, B. (eds.) (1987). Toxicity of chlorinated aliphatic hydrocarbons and degradation by methanotrpohic consortia *Proceedings of the 4th european congress on biotechnology.* Elsevier Biomedical Press Amsterdam, The Netherlands. 515-518.

Kadiiska, M. B., Xiang, Q. H. and Mason, R. P. (1994). In vivo free radical generation by chromium (VI): an electron resonance spin-trapping investigation. *Chem. Res. Toxicol.* **7**, 800-805.

Kadirvelu, K., Thamaraiselvi, K. and Namasivayam, C. (2001). Removal of heavy metals from industrial wastewaters by adsorption onto activated carbon prepared from an agricultural solid waste. *Bioresource Technology.* **76**, 63-65.

Kadirvelu, K., Senthilkumar, P., Thamaraiselvi, K. and Subburam, V. (2002). Activated carbon prepared from biomass as adsorbent: elimination of Ni (II) from aqueous solution. *Bioresource Technology.* **81**, 87-90.

Karlsen, O. A., Lillehaug, J. R. and Jensen, H. B. (2008). The presence of multiple *c*-type cytochromes at t he surface of methanotrophic bacterium *Methylococcus capsulatus* (Bath) is regulated by copper. *Mol. Microbiol.* **70**, 15-26.

Karlsen, O. A., Larsen, Q. and Jensen, H. B. (2011). The copper responding surfacecome of *Methylococcus capsulatus* Bath. *FEMS. Microbiology letters.* **323** (2), 97-104.

Kapoor, A. and Viraraghavan, J. (1995). Fungal biosorption an alternative option for heavy metal bearing wastewater: a review. Bioresour. Technol. **53** (3), 195-206.

Kato, H. J. and Ohtake, H. (1991). Effects of heavy metal cations on chromate reduction by *Enetrobacter cloacae* strain HO1. *Journal of General and Applied microbiology.* **37,** 519-522.

Katz, S. A. and Salem, H. (1994). The biological and environmental chemistry of chromium. VCH Publishers, New York.

Khattar, J. I.S., Sarma, T. A. and Singh, D.P. (1999). Removal of chromium ions by agar immobilized cells of the cyanobacterium anacystis nidulans in a continuous flow bioreactor. *Enzyme and microbial technology,* **25** (7), 564-568.

Kim, C., Zhou, Q., Deng, B. Thornton, E. C. and Xu, H. (2001). Chromium (VI) reduction by hydrogen sulfide in aqueous media: stoichiometry and kinetics. *Environ. Sci. Technol.* **35** (11), 2219-2225.

Kimbrough, D. E., Cohen, Y., Winer, A. M., Creelman, L. and Mabuni, C. (1999). A critical assessment of chromium in the environment. *Critical reviews in environmental science and technology,* **29** (1), 1-46.

Knowles, C. B. (1989). Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+ and co-oxidation by methanotrophs and nitrifiers, Microbiology and Molecular Biology Review. **53** (1), 68-84.

Koh, S. C., Bowman, J. P. and Sayler, G. S. (1993). Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, methylomonas methanica G 8-1. Apllied Enviro. Microbiolo. **59** (4), 96-967.

Kotas, J. and Stasicka, Z. (2000). Chromium occurrence in the environment and methods of its speciation. *Environ. Pollut.*, **107**, 263-283.

Krauter, P., Martinelli, R., Williams, K., and Martins, S. (1996). Removal of Cr (VI) from ground water by saccharomyces cerevisiae. *Biodegradation,* **7** (4), 277-286.

Kratochvil, D. and Volesky, B. (1998). Advances in the biosorption of heavy metals. *Trends in biotechnology,* **16** (7), 291-300.

Krishna, K. R. and Philip, L. (2005). Bioremediation of Cr (VI) in contaminated soils. *Journal of Hazardous material.* **121** (1-3), 109-117.

Krishnamurthy, S., and Wilkens, M. M. (1994). Environmental chemistry of chromium. *Northeastern geology,* **16** (1), 14-17.

Ksheminska, H., Fedorovych, D., Babya, L., Yanovych, D., Kaszycki, P. and Koloczek, H. (2005). Chromium (III) and (VI) tolerance and bioaccumulation in yeast: a survey of cellular chromium content in selected strains of representative genera. *Process Biochem.* **40**, 1565-1572.

Kuo, H. W., Lai, J. S. and Lin, T. J. (1997). Nasal spetum lesion and lung function in workers exposed to chromic acid in electroplating factories. *International Archive of occupational and Environmental Health.* **70**, 272-276.

Kwak, Y. H., Lee, D. K. And Kim, H. B. (2003). Vibrio harveyi Nitroreductase is also a chromate reductase. *Applied Environmental Microbiology.* **69**, 4390-4395.

Lai, K., C. and Lo, M., C. (2008). Removal of chromium (VI) by acid-washed zero-valent iron under various groundwater geochemistry condition. *Environ. Sci. Technol.*, **42** (4), 1238-1244.

LaRock, P. A., Hyun, J. H. and Bennison, B. W. (1994). Bacterioplankton growth and production at the Louisiana hydrocarbon seeps. *Geo. Mar. Lett.* **14**, 2–3.

Large, P. J. And Bamforth, C. W. (1988). Methylotrophy and biotechnology. John Wiley and sons, New York.

Leak, D. J., and Dalton, H. 1(986). Growth yields of methanotrophs. 1. Effect of copper on the energetics of methane oxidation. *Appl. Microbiol. Biotechnol.* **23**, 470–476.

Leak, D. J., Stanley, S. H. and Dalton, H. (1985). Implications of the nature of methane monooxygenase on carbon assimilation in methanotrophs, p. 201–208. *In* R. K. Poole and C. S. Dow (ed.), Microbial gas metabolism, mechanistic, metabolic, and biotechnological aspects. Academic Press Ltd., London.

Lee, S. K., Nesheim, J. C., and Lipscomb, J. D. (1993) *J. Biol. Chem.* **268**, 21569–21577.

Lee, S. J. and Lipscomb, S. J. (1999). Oxygen activation catalysed by methane monooxygenase hydroxylase component proton delivery during O-O bond cleavage steps. *Biochemistry*. **38,** 4423-4432.

Lelieveld, J., Crutzen, P. J. and Bruhl, C. (1993). Climate effects of atmospheric methane. *Chemosphere,* **26** (1-4), 739-768.

Lidstrom, M. E. (1991). The aerobic methylotrophic bacteria. *The prokaryotes.springer-verlag, new york,* 431–445.

Lidstrom, M. E. (1992). The genetics and molecular biology of methanolutilizing bacteria, p. 183–206. *In* J. C. Murrell and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York.

Lidstrom, M. E. and Stirling, D. I. (1990). Methylotrophs: Genetics and commercial applications. *Annual reviews in microbiology,* **44** (1), 27-58.

Lieberman, R. L. and Rosenzuweig, A. C. (2005). Crystal structure of a membrane-bound metallonzyme that catalyses the biological oxidation of methane. Nature **434**, 177-182.

Lin, C. J. (2002). The chemical transformations of chromium in natural waters-a model study. *Water, Air, and Soil Pollution*, **139**, 137-158.

Lipscomb, J. D. 1994. Biochemistry of the soluble methane monoxygenase. *Annu. Rev. Microbiol.*, **48**, 371–399.

Lontoh, S. and Semrau, J. D. (1998). Methane and trichloroethylene degradation by *Methylosinus trichosporium* OB3b expressing particulate methane monooxygenase. *Applied and environmental microbiology,* **64** (3), 1106-1114.

Lontoh, S., Dispirito, A. A., Krema, C. L., Whittaker, M. R., Hooper, A. B. and Semrau, J. D. (2000). Differential inhibition in vivo of ammonia mononxygenase, soluble methane monooxygenase by phenylacetylene. *Environmental Microbiology*. **2** (5), 485-494.

Liu, Y., Nesheim, J. C., Lee, S. K., and Lipscomb, J. D. (1995) *J. Biol. Chem.* **270**, 24662–24665.

Lloyd, J. S., Bhambra, A., Murrell, J. C. and Dalton, H. (1997). Inactivation of regulatory protein B of soluble methane monooxygenase from *Methylococcus capsulatus* (Bath) by proteolysis can be overcome by a Gly to Gln modification. *Eur. J. Biochem.* **248**, 72-79.

Llovera, S., Bonet, R., Simon-puiol, M. D. and Congregado, F. (1993). Chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916. *Applied Environmental Microbiology*. **59**, 3516-3518.

Lovely, D. R. (1993). Dissimilatory metal reduction. *Ann. Rev. Microbiol.* **47**, 263-290.

Lovely, D. R. (1995). Bioremediation of organic and metal contaminant with dissimilatory metal reduction. *Journal of Industrial Microbiology*. **14**, 85-93.

Lovley, D. R. and Coates, J. D. (1997). Bioremediation of metal contamination. *Current opinion in biotechnology,* **8** (3), 285-289.

Lovley, D. R. and Phillips, E. J. P. (1994). Novel processes for anaerobic sulfate production from elemental sulfur by sulfate-reducing bacteria. *Applied and environmental microbiology,* **60** (7), 2394-2399.

Lovley, D. R. and Phillips, E. J.P. (1994). Reduction of chromate by *Desulfovibrio vulgaris* and its c_3 cytochrome. *Applied and environmental microbiology,* **60** (2), 726-728.

Lytle, C. M., Lytle, F. W., Yang, N., Qian, J. H., Hansen, D., Zayed, A. and Terry, N. (1998). Reduction of Cr (VI) to Cr (III) by wetland plants: Potential for in situ heavy metal detoxification. *Environ.sci.technol,* **32** (20), 3087-3093.

Machlin, S., Tam, P., Bastien, C. and Hanson, R. S. (1988). Genetic and physical analyses of *Methylobacterium organophilum* XX genes encoding methanol oxidation. *J. Bacteriol.* **170**, 141–148.

Mazoch, J. Tesarik, R., Sedlacek, V., Kucera, I. and Turanek, J. (2004). Isolation and biochemical characterization of two soluble iron (III) reductases from *Paracoccus denitrificans*. *Eur. J. Biochem.* **271**, 553-562.

Mahajan, S. P. (1985). Pollution control on process industries. *Tata McGraw-Hill* publishing company limited 7 West Patel Nager, NewDelhi.

Marison, I.W. and Attwood, M. M., (1982) *J. Gen. Microbiol.* **128,** 1441-1446.

Martinho, M., Choi, D-W, DiSpirito, A. A., Antheoline, W. E., Semrau, J. D. and Muncle, E. (2007). Mossbauer studies of the membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath), evidence for a diiron centre. *J. Am. Chem. Soci.* **129**, 15783-15785.

Mann, H. (1990). Biosorption of heavy metals by bacterial biomass. *Biosorption of heavy metals.*

Mariey, L., Signolle, J. P., Amiel, C. and Travert, J. (2001). Discrimination, classification, identification of microorganisms using FTIR spectroscopy and chemometric. *Vibrational Spectroscop.* **26**, 151-159.

Marques, M. J., Salvador, A., Morales-Rubio, A. and Guardia, M. (2000). Chromium speciation in liquid matrices: A survey of the literature. *Fresenius journal of analytical chemistry,* **367** (7), 601-613.

Martin, H. and Murrell, J. C. (1995). Methane monooxygenase mutants of *Methylosinus trichosporium* constructed by marker-exchange mutagenesis, *FEMS Microbiol. Lett.*, **127** (3), 243-248.

Masaru, N., Kazuaki, M., Chieh-Chen, H., Zen'ichiro, K. and Ginro, E. (2004). Dissemination of Tn*MERI*1-like mercury resistance transposons among *Bacillus* isolated from worldwide environmental samples. *FEMS Microbial.* Ecol. **48**, 47-55.

McCullough, J., Hazen, T. C., Benson, S. M., Metting, F. B. and Palmisano, A. C. (1999). Bioremediation of metals and radionuclides: what it is and how it works. A NABIR primer. Booklet prepared for Natural Research program. Office of biological and environmental Research, Office of science, U.S. Department of Energy.

McDonald, I. R., Kenna, E. M. and Murrell J. C. (1995). Detection of methanotrophic bacteria in environmental samples with the PCR. *Appl. Environ. Microbiol.* **61**, 116–121.

McGrath, S. P. and Smith, S. (1990). Chromium and nickel. In Heavy metals in soils (Edited by Alloway, B. J.) 125-146. Blackie, London.

McLean, J., Beveridhe, T. J. and Phipps, D. (2000). Isolation and characterization of chromium reducing bacterium from a chromate copper arsenate contaminated site. *Environ. Microbiol.* **2**, 611-619.

McLean, J. and Beveridge, T. J. (2001). Chromate reduction in *Pseudomonad* isolated from a site contaminated with chromate copper arsenate. *Appl. Environ. Microbio.* **67**, 1076-1084.

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** (1), 51-54.

Melin, A-M., Perromat, A. and Deleris, G. (2001). Fourier-transform infrared spectroscopy: a pharmacotoxicologic tool for in vivo monitroring radical aggression. *Canadian Journal of Physiology and Pharmacology.* **79** (2), 158-165.

Michel, C., Brugna, M., Aubert, C., Bernadac, A. and Bruschi, M. (2001). Enzymatic reduction of chromate: comparative studies using sulfate-reducing bacteria. *Applied Microbiology and Biotechnology.* **55** (1), 95-100.

Middleton, S. S., Latmani, R. B., Mackey, M. R., Ellisman, M. H., Tebo, B. M. and Criddle, C. S. (2003). Coetabolism of Cr (VI) by *Shewanella oneidenisis* MR-1 produces cell-associated reduced chromium and inhibits growth. *Biotechnology and Bioengineering.* **83** (6), 627-637.

Moore, J. W., Ramamoorthy, S. and Ballantyne, E.E. (eds.) (1984). *Heavy metals in natural waters; applied monitoring and impact assessment.* Springer-Verlag.

Munoz, R. and Guieysse, B. (2006). Algae bacteria process for the treatment of hazardous contaminants: a review. Water Res. **40** (15), 2799-2815.

Murrell, J. C. (1992). Genetics and molecular biology of methanotrophs. *FEMS Microbiol. Rev.* **88**, 233–248.

Murrell, J. C. (1994). Molecular genetics of methane oxidation. *Biodegradation* **5**, 145–160.

Murrell, J. C., Gilbert, B. and McDonald, I. R. (2000). Molecular biology and regulation of methane monooxygenase. *Arch. Microbiol.* **173**, 325-332.

Murrell, J. C., McDonald, I. R. and Gilbert, B. (2000). Regulation of expression of methane monooxygenases by copper ions. *Trends Microbiol.* **8**, 221-225.

Myers, C. R., Carstens, B. P., Antholine, W. E. and Myers, J. M. (2000). Chromium (VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanela putrefaciens* MR-1. *Applied Microbiology* **88**, 98-106.

Myronova, N., Kitmitto, A., Collins, R. F., Miyaji, A. And Dalton, H. (2006). Three-dimensional structure determination of a protein super complex that oxidizes methane to formaldehyde in *Methylococcus capsulatus* (Bath). *Biochemistry.* **45**, 11905-11914.

Nakajma, T., Uchiyama, H., Yagi, O. and Nakahara, T. (1992). Purification and properties of a soluble methane monooxygenase from *Methylocystis sp. M. Biosci. Biochem.* **56**, 736-740.

National Research Council, Food and Nutrition Board. (1989). *Recommended Dietary Allowances*. 10th Edition. National Academy Press, Washington, DC.

Nethercott, J., Paustenbach, D., Adams, R., Fowler, J., Marks, J., Morton, C. and Taylor, J. (1994). A study of chromium induced allergic contact dermatitis with 54 volunteers: implications for environmental risk assessment. *Occupational and Environmental Medicine*, **51**, 371-380.

Nies, D. H. (1999). Microbial heavy-metal resistance. *Applied microbiology and biotechnology,* **51** (6), 730-750.

Nguyen, H. H. T., Shiemke, A. K., Jacobs, S. J., Hales, B. J., Lidstrom, M. E. and Chan, S. I. (1994). The nature of copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J. Biol. Chem.* **269**, 14995-15005.

Nguyen, H. H. T., Zhu, M., Elliott, S. J., Nakagawa, K. H., Hedman, B., Costello, A. M., Peeples, T. L., Wilkinson, B., Morimoto, H., Williams, P. G., Floss, H. G., Lidsrom, M. E., Hodgson, K. O. and Chan, S. I. (1996). The biochemistry of the particulate methane monooyxgenase. in: Lidstrom, M. E., Tabita, F. R. (eds). Microbial growth on C1 compounds. Kluwer Academic, Dordrect. 150-158.

Nriagu, J. O. and Pacyna, J. M. (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace elements. *Nature,* **333** (6169), 134-139.

Nucifora, G., Chu, L., Misra, T. K. and Silver, S. (1989). Cadmium resistance from *Staphylococcus aureus* plasmid PI258 Cad A gene results from cadmium-efflux ATPase. *Proceedings of National Acaemy of Sciences of the USA*, **86**, 3544-3548.

Nunn, D. N. and Lidstrom. M. E. (1986). Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *J. Bacteriol.* **166**, 581–590.

Nyer, E. K. (1992). Treatment methods for inorganic compounds. Ground water treatment technology. New York Van Nostrand Reinhold.

O'Connor, M. L., and Hanson, R. S. (1978). Linkage relationships between C-1 mutants of *Methylobacterium organophilum. J. Gen. Microbiol.* **104**, 105–111.

Ohtake, H., Cervantes, C. and Silver, S. (1987). Decreased chromate uptake in *Pseudomonas fluorecens* carrying a chromate resistance plasmid. *Journal Bacteriol.* **169**, 3853-3856.

Ohtake, H., Fuji, E. and Toda, K. (1990). Bacterial reduction of hexavalent chromium; Kinetic aspects of chromate reduction by Enterobacter cloacae HO1. Biocatalysis, **4**, 227-235.

Ohtake, H. and Silver, S. (1994). Bacterial detoxification of toxic chromate. *Biological degradation and bioremediation of toxic chemicals*, 403-415.

Ojeda, J. J., Romero-Gonzalez, M. E., Pouran, H. M. and Banwart, S. A. (2008). In situ monitoring of the biofilm formation of *Pseudomonas putida* on hematite using flow-cell ATR-FTIR spectroscopy to investigate the formation of inner-sphere bonds between the bacteria and the mineral. *Mineralogical Magazine.* **72**, 101-106.

Oldenhuis, R. and Janssen, D. B. (1993). Degradation of trichloroethylene by methanotrophic bacteria. *Microbial growth on C,* **1**, 121–133.

Oliver, D., Cabelli, R., Dolan, K. and Jarosik, G. (1990). Azide-resistant mutants of Escherichia coli alter the SecA protein, an azide-sensitive component of the protein export machinery. *Proc. Nati. Acad. Sci.* **87**, 8227 - 8231.

Oremland, R. S. and Culberston, C. W. (1992). Importance of methane-oxidizing bacteria in the methane budget as revealed by the use of a specific inhibitor: Nature, **356**, 439-423.

Ortiz, D. F., Kreppel, L., Speiser, D. M., Scheel, G., McDonald, G. and Ow, D. w. (1992). Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *EMBO journal* **11** (10), 3491-3499.

Owais, W. M. and Kleinhofs, A. (1988). Metabolic activation of the mutagen azide in biological systems. *Mutation Res.* **197**, 313-323.

Palmer, C. D. (2000). Precipitates in a Cr (VI) contaminated concrete. *Environ. Sci. Technol.* **34** (19), 4185-4192.

Palmer, C. D. and Wittbrodt, P. R. (1991). Processes affecting the remediation of chromium-contaminated sites. *Environmental health perspectives,* **92**, 25-40.

Pena-Castro, J. M., Martinez-Jeronimo, F., Esparza-Garcia, F. and Canizares-Villanueva, R. O. (2004). Heavy metals removal by the *Microagla Scenedesmus Incrassa Tulus* in continuous cultures. Bioresource Technology. **94** (2), 219-222.

Parikh, S. J. and Chorover, J. (2006). ATR-FTIR spectroscopy reveals bond formation during bacterial adhesion to iron oxide. *Longnuir.* **22** (20), 8492-8500.

Park, D., Yun, Y. -S. and Park, J. M. (2006). Comment on the removal mechanism of hexavalent chromium by biomaterials or biomaterial-based activated carbons. *Industrial Engineering Chemistry Reseaserch.* **45**, 2405-2407.

Park, C. H. Keyhan, M., Wielinga, B., Fendrof, S. and Matin, A. (2000). Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbio.* **66**, 1788-1795.

Park, K. Y., Inamori, Y., Mizuochi, M. and Ahn, K. H. (2000).Emisson and control of nitrous oxide from a biological wastewater treatment system with intermittent aeration. *Journal of Bioscience and Bioengineering.* **90** (3), 247-252.

Park, S., Brown, K. W. and Thomas, J. C. (2002). The effect of various environmental and design parameters on methane oxidation in a model biofilter. *Waste Management and Research.* **20**, 434-444.

Patel, R. N., Hou, A. I., Laskin, A. I., Felix, A. and Derelank, P. (1979). Microbial oxidation of gaseous hydrocarbons: hydroxylation of *n*-alkanes and epoxidation of

n-alkenes by cell-free particulate fractions of methaneutilizing bacteria. *J. Bacteriol.* **139**, 675–679.

Patel, R. N., Hou, C. I., Laskin, A. I. and Felix, A. (1982). Microbial oxidation of hydrocarbons: Properties of a soluble methane monooxygenase from a facultative methane-utilizing organism, *Methylobacterium sp.* strain CRL-26. *Applied and environmental microbiology,* **44** (5), 1130-1137.

Patnaik, P. R. (2003). Oscillatory metabolism of *Saccharomyces cerevisiae*: An overview of mechanisms and models. *Biotechnology advances,* **21** (3), 183-192.

Patterson, J. W. (1985). *Industrial wastewater treatment technology.* (2nd). Butterworth-Heinemann, London.

Pattanapipitpasial, P., Brown, N. L. and Macaskie, L. E. (2001). Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr (VI)-contaminated site. *Applied Microbiological Biotechnology.* **57**, 257-261.

Peitzsch, N., Eberz, G. and Nies, D. H. (1998). *Alcaligenes cutrophus* as a bacterial chromate sensor. *Applied Environ. Microbiol.* **64**, 453-458.

Pepi, M. and Baldi, F. (1992). Modulation of chromium (VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes, *Biometals.* **5** (3), 179-185.

Prior, S. D. and Dalton, H. (1985). Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS microbiology letters,* **29**, 105-109.

Pilkington, S. J. and Dalton, H. 1990 Soluble methane monooxygenase from *Methylococcus capsulatus* (Bath). in: Lidstrom M E (ed). Hydrocarbons and methylotrophy. Academic press, San Diego (Methods in Enzymology) **188**, 181-190.

Pimentel, B. E., Moreno-Sanchez, R. and Cervantes, C. (2002). Efflux of chromate by cells of *Pseudomonas aeruginosa* expressing the ChrA protein. *FEMS Microbiol. Lett.* **212**, 249-254.

Prior, S. D. and Dalton, H. (1985). Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS microbiology letters,* **29**, 105-109.

Pugazhenthi, G., Sachan, S., Kishore, N. and Kumar, N. (2005). Separation of chromium (VI) using modified ultrafiltration charged carbon membrane and its mathematical modeling. *Journal of membrane Science*. **254**, 229-239.

Puzon, G. J., Petersen, J. N., Roberts, A. G., Kramer, D. M. and Xun, L. (2002). A bacterial flavin reductase system s reduces chromate to a soluble chromium (III) - NAD complex. Biochem. Biophys. Res. Commun. **294**, 76-81.

Quayle, J. R. (1980). Aspects of the regulation of methylotrophic metabolism. *FEBS Letters*. 117, K16-K27.

Quilter, J. A. and Jones, C. W. (1984). The organisation of methanol dehydrogenase and c-type cytochromes on the respiratory membrane of *Methylophilus methylotrophus*, *FEBS Lett.* **174**, 167–175.

Rafiquallah, I. M., Hossain, A., Llias, M. and Haq, M. M. (2009). Chromium (VI) reduction native microorganisms for remediation of chromium eco-toxicity in environment of Bangladesh. *Bangladesh journal of scientific and industrial research*. **43** (4), 455-466.

Ramirez-Diaz, I. M., Diaz-Perez, C., Vargas, E., Riveros-Rosas, H., Campos-Garcia, J. and Cervantes, C. (2008). Mechanisms of bacterial resistance to chromium compounds. *Biometals*, **2**, 321-332.

Raport, A. I. and Muter, O. A. (1995). Biosorption of hexavalent chromium by yeasts. *Process biochemistry,* **30** (2), 145-150.

Raspor, P., Batic, M., Jamnik, P., Josic, D. J., Milacic, R., Recek, M., Rezic-Dereani, V. and Skrt, M. (2000). The influence of chromium compounds on yeast physiology (a review). *Acta microbiologica et immunologica hungarica,* **47**, 143-173.

Richard, F. C. and Bourg, A. C. M. (1991). Aqueous geochemistry of chromium: a review. *Water Res*. **25**, 807-816.

Riley, R. G., Zachara, J. M. and Wobber, F. J. (1992). Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research. Report DOE/ER-0547T. US Department of Energy, Washington, D. C.

Rock, M. L., James, B. and Helz. G. R. (2001). Hydrogen peroxide effects on Cr oxidation state and solubility in four divers, Cr enriched soils. *Environ Sci. Technol*. **35** (20), 4054-4059.

Rodhe, H. (1990). A comparison of the contribution of various gases to the greenhouse effect, *Science,* **248** (4960), 1217-1219.

Romanenko, V. I. and Korenkov, V. N. (1977). A pure culture of bacteria utilizing chromates and dichromate as hydrogen acceptors in growth under anaerobic conditions. *Microbiologiya.* **46** (3), 414-417.

Rosenzweig, A. C., Frederick, C. A., Lippard, J. and Nordlun, P. (1993). Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* **366**, 537–543.

Rosenzweig, A. C., Nordlun, P., Takahara, P. M., Frederick, C. A. and Lippard, J. (1995). Geometry of the soluble methane monooxygenase catalytic diiron centre in two oxidation states. *Chem. Biol.* **2**, 409–418.

Rossin, A. C., Sterritt, R. M. and Lester, J. N (1982). The influence of process parameters on the removal of heavy metals in activated sludge. *Water, air, & soil pollution,* **17** (2), 185-198.

Sarangi, A. and Krishnan, C. (2008). Comparison of in vitro Cr (VI) reduction by CFEs of chromate resistant bacteria isolated from chromate contaminated soil. *Bioresource Technology.* **99** (10), 4130-4137.

Seishima, M., Oyama, Z. and Oda, M. (2003). Cellular phone dermatitis with chromate allergy. *Dermatology.* **207** (1), 48-50.

Semrau, J. D., Christoserdov, A., Lebron, J., Costello, A., Davagnino, J., Kenna, E., Holmes, A. J., Finch, R., Murrell, J. C. and Lidstrom, M. E. (1995). Particulate methane monooxygenase genes in methanothrophs. *J. Bacteriol.* **177**, 3071-3079.

Shakoori, A. R., Tahseen, S. and Haq, R.U. (1999).Chromium-tolerant bacteria isolated from industrial effluent and their use in detoxication of hexavalent chromium. *Folia* M*icrobiologic.* **44** (1), 50-54.

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 and Biotechnology,* **53** (3), 348-351.

Shanker, A. K., Cervantes, C., Loza-tavera, H. and Avudainayagam, S. (2005). Chromium toxicity in plants. *Environment international,* **31** (5), 739-753.

Shen, H. And Wang, Y. T. (1994). Biological reduction of chromium by *E. coli*. *Journal of Environmental Engineering*. **120** (3), 560-572.

Shi, X. and Dalal, N. S. (1990). On the hydroxyl radical formation in the reaction between hydrogen peroxide and biologically generated chromium (VI) species. *Arch. Biochem. Biophys*. 277, 342-350.

Silver, S., Budd, K., Leahy, K. M., Shaw, W. V., Hammond, D., Novick, R. P., Willsky, G. R., Malamy, M. H. and Rosenberg, H. (1981). Inducible plasmid-determined resistance to aresnate, aresenite and antimony (III) in *Escherichia coli* and *Staphylococcus aureus. J. Bacteriol*. **146**, 1983-1996.

Silver, S. and Phung, L. T. (2005). Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl. Environ. Microbial.* **71** (2), 599-608.

Sikaily, A. E., Nemr, A. E., Khaled, A. And Abdelwehab, O. (2007). Removal of toxic chromium from wastewater using green alga *Ulva lactuca* and its activated carbon. *Journ. Hazard. Mater.* **148**, 216-228.

Smith, D. D. S. and Dalton, H. (1989). Solubilization of methane monooxygenase from *Methylococcus capsulatus* (Bath). *Eur. J. Biochem*. **182**, 668–671.

Smith, T. J. and Foster, S. J. (1995). Characterization of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. Journl of bacteriology. **177** (13), 3855-3862.

Smith, W. A., Apel, W. A., Petersen, J. N., and Peyton, B. M. (2002). Effect of carbon and energy source on bacterial chromate reduction. *Bioremediation journal,* **6** (1), 205-215.

Smith, T. J. and Murrell, J. C. (2009). Methanotrophy/methane oxidation. In: Moselio Schaechter (ed.). *Encyclopedia of microbiology.* Oxford, Academic Press, 293-298.

Stainthorpe, A. C., Salmond, G. P. C., Dalton, H., and Murrell, J. C. (1990) *FEMS Microbiol. Lett.* **70**, 103–108.

Stanley, S. H., Prior, S. D., Leak, D J. and Dalton, H. (1983). Copper stress underlies the fundamental change in intracellular location of methane mono-oxygenase in methane-oxidizing organisms: Studies in batch and continuous cultures. *Biotechnology letters,* **5** (7), 487-492.

Stauffer, B., Fischer, G., Neftel, A. And Oeschger, H. (1985). Increase of atmospheric methane recorded in Antarctic ice core. *Science,* **229** (4720), 1386-1388.

Sultan, S. and Hasnain, S. (2007). Reduction of toxic hexavalent chromium by *Ochrobactrum intermedium* strain SDCr-5 stimulated by heavy metals. *Bioresource Technology.* **98** (2), 340-344.

Summers, A. O. and Jacoby, G. A. (1978). Plasmid-determined resistance to boron and chromium compounds in *Pseudomonas aeruginosa. Antimicob. Agent Chemother.***13**, 637-640.

Suzuki, T., Miyata, N., Haritsu, H., Kawai, K., Takamizawa, K., Tai, Y. and Okazaki, M. (1992) NAD(P)H-dependent chromium (VI) reductase of *Pseudomonas ambiga* G-1: a Cr (VI) intermediate is formed during the reduction of Cr (VI) to Cr (III). *J. Bacteriol.* **174**, 5340-5345.

Tavitian, B. A., Nebedryk, E.. Mantele, W. and Breton, J. (1986). Light-induced Fourier Transform Infrared (FTIR) spectroscopic investigation of primary reaction in photosystem and photosystem II. *FEBS Lett.* **201**, 151-157.

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**(8), 1541-1547.

Thermo Nicolet Corporation. (2001). Introduction to Fourier Transform Infrared Spectrometry.

Topp, E., Hanson, R. S., Ringelberg, D. B., White, D. C. and Wheatcroft, R. (1993). Isolation and characterization of an N-methylcarbamate insecticide-degrading methylotrophic bacterium. *Applied and environmental microbiology,* **59** (10), 3339-3349.

Turpeinen, R., Kairesalo, T. and Haggblom, M. (2004). Microbial community structure and activity in arsenic, chromium and copper contaminated soil. *FEMS Microbiology Ecology.* **47**, 39-50.

Udelhoven, T., Naumann, D. and Schmitt, J. (2000). Development of a Hierachical classification system with artificial neural networks and FTIR spectra for the identification of bacteria. *Applied Spectroscopy.* **54**, 1471-1479.

Valls, M., Victor, D. E. L., Roser, G. D. and Silvia, A. (2000). Engineering outer membrane proteins in *Pseudomonas putida* for enhanced heavy metal bioadsorption. *Journal Inorg. Biochem*, **79**, 219-223.

Viamajala, S.,Peyton, B. M. and Petersen, J. N. (2003). Modelling chromate reduction in *Shewunella oneidensis* MR-1 development of a novel dual-enzyme kinetic model. *Biotechnology and Bioengineering.* **83**(7), 790-797

Viti, C. and Giovannetti, L. (2001). The impact of chromium contamination on soil heterotrophic and photosynthetic microorganisms. *Annals of microbiology,* **51** (2), 201-214.

Vidali, M. (2001). Bioremediation. An overview. *Pure Appl. Chem.* **73** (7), 1163-1172.

Volesky, B. and May-Philips, H. A. (1995). Biosorption of heavy metals by *saccharomyces cerevisiae. Applied microbiology and biotechnology,* **42** (5), 797-806.

Volesky, B. and Naja, G. M. (2007). Biosorption technology: starting up an enterprise. *Int. J. Technol. Trans. Com.*, **6**, 196-211.

Vorholt, J. A. (2002). Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. *Arch. Microbiol.*, **178**, 239-249.

Vulkan, R., Zhao, F-J., Barbosa-Jeleson. V., Preston, S., Paton, G. I., Tipping, E. and McGrath, S. P. (2000). Copper speciation and impacts on bacterial biosensors in the pore water of copper-contaminated soils. *Environ. Sci. Technol.*, **34**, 5115-5121.

Wang, Y. T. and Shen, H. (1995). Bacterial reduction of hexavalent chromium. *Journal of industrial microbiology and biotechnology,* **14** (2), 159-163.

Wallar, B. J. and Lipscomb, J. D. (2001).Methane monooxygenase component B mutants alter the kinetics of steps throughout the catalytic cycle. *Biochemistry.* **40** (7), 2220-2233.

Wang, P. Mori, T., Toda, K. and Ohtake, H. (1990). Membrane-associated chromate reductase activity from *Enterobacter cloacae. Journal Bacteriol.* 172, 1670-1672.

Wang, P. C., Mori, T., Komori, K., Sasatsu, M., Toda, K. and Ohtake, H. (1989). Isolation and characterization of *Enterobacter cloacae* strain that reduces

hexavalent chromium under anaerobic conditions. *Applied Environ. Microbiol.* **55** (7), 1665-1669.

Wang, Y. T. and Shen, H. (1997). Modeling Cr (VI) reduction by pure bacterial cultures. *Pergamon.* **31**, 727-732.

Wang, Y. T. and Xiao, C. (1995). Factors affecting hexavalent chromium reduction in pure cultures of bacteria. *Water Res.* **29** (11), 2467-2474.

Ward N., Larsen, O., Sakwa, J., Bruseth, L., Khouri, H. et al., (2004). Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLOS Biology.* **2**, 1616-1628.

White, C., Sayer, J. A. and Gadd, G. M. (1997). Microbial solubilization and immobilization of toxic metals: Key biogeochemical processes for treatment of contamination. *FEMS microbiology reviews,* **20** (3-4), 503-516.

Whittenbury, R. and Dalton, H. (1981). The methylotrophic bacteria. *The prokaryotes.* **1**, 894–902.

Whittenbury, R. and Krieg, N. R. (1984). Family IV. methylococcaceae. *Bergey's manual of systematic bacteriology (krieg, NR and holt, JG, eds.),* **1**, 256-262.

Whittenbury, R., Phillips, K. C. and Wilkinson, J. F. (1970). Enrichment, isolation and some properties of methane-utilizing bacteria. *Microbiology,* **61** (2), 205.

Williams, J. W. and Silver, S. (1984). Bacterial resistance and detoxification of heavy metals. *Enzyme and microbial technology,* **6** (12), 530-537.

Willison, T. W., O'Flaherty, M. S., Tlustos, P., Goulding, K. W. T. and Powlson, D. S. (1997). Variations in microbial populations in soils with different methane uptake rates, *Nutrient cycling in agroecosystems,* **49** (1), 85-90.

Woese, C. R., Gutell, R., Gupta, R. and Noller, H. R. (1983). Detailed higher order structure of 16S- like ribosomal ribonucleic acids. *Microbiological Reviews.* **47**, 621-669.

Wolf, H. J. and Hanson. R. S. (1978). Alcohol dehydrogenase from *Methylobacterium organophilum. Appl. Environ. Microbiol.* **36**, 105–114.

Wong, P. T. S. and Trevors, J. T. (1988). Chromium toxicity to algae and bacteria. Chromium in the natural and human environments .edited by JO nriagu and E.nieboer.john wiley & sons, New York, 305–315.

Yura, T. and Wada, C. (1969). Phenethyl alcohol resistance in *Escherichia coli*. I. Resistance of strain C600 and its relation to azide resistance. *Genetics.* **59**, 177-190.

Zahoor, A. and Rehman, A. (2009). Isolation of Cr (VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater, *Journal of environmental sciences,* **21** (6), 814-820.

Zahn, J. A. and DiSpirito, A. A. (1996). Membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath). *Journal Bacteriol.* **178** (4), 1018-1029.

Zayed, A., Lytle, C. M., Qian, J. H., Terry N. and Qian, J. H. (1998). Chromium accumulation, translocation and chemical speciation in vegetable crops, *Planta,* **206** (2), 293-299.

Zayed, A. M. and Terry, N. (2003). Chromium in the environment: Factors affecting biological remediation, *Plant and soil,* **249** (1), 139-156.

Zetic, V. G. Stehil-Tomas, V., Grba, S., Lutilsky, L. And Kozlek, D. (2001). Chromium uptake by *saccharomyces cerevisiae* and isolation of glucose tolerance factor from yeast biomass. *Journal of biosciences,* **26** (2), 217-223.

Zhao, S. J. and Hanson, R. S. (1984). Isolate 761M: A new type I methanotroph that possesses a complete tricarboxylic acid cycle. *Applied and environmental microbiology,* **48** (6), 1237-1242.

Zhao, S. J. and Hanson, R. S (1984). Variants of the obligate methanotroph isolate 761M capable of growth on glucose in the absence of methane. *Applied and environmental microbiology,* **48** (4), 807-812.

Zhitkovitch, A. and Costa, M. (1992). A simple, sensitive assay to detect DNA-protein cross-links in intact cells and in vivo. *Carcinogenesis* **13**, 1485–1489.

APPENDIX A: STANDARD CURVES

Chromium (VI) standard curves using diphenylcarbazide (DPC) assay

Preparation of potassium dichromate stock solution

Chromium (VI) solution (1.27 mg/l) was prepared by dissolving 3.6 mg of $K_2Cr_2O_7$ and 10 ml of concentrated sulfuric acid into 500 ml distilled water. Then, the solution was made up to 1000 ml with distilled water.

Six test tubes were obtained and labelled (1, 2, 3, 4, 5 and 6). The quantities of $K_2Cr_2O_7$ and H_2SO_4 (0.18 M) were added to each test tube as shown in Table A.1:

Table A.1: Amounts of $K_2Cr_2O_7$ and H_2SO_4 used for the standard curve

Tube no.	1	2	3	4	5	6
$K_2Cr_2O_7$ (ml)	0.00	0.4	1.0	2.0	4.0	10
H_2SO_4 (ml)	10	9.6	9.0	8.0	6.0	0.0

The content of each test tube was mixed by shaking. Then, 0.5 ml of diphenylcarbazide solution (0.5 g in 200 ml of acetone) was added to each test tube. The contents of each test tube were mixed and let stand for 5 minutes for colour development. The absorbance of each sample was measured using spectrophotometer at wave length of 540 nm (Herrmann, 1994). The corresponding standard curve was plotted as shown in Figure A.1.

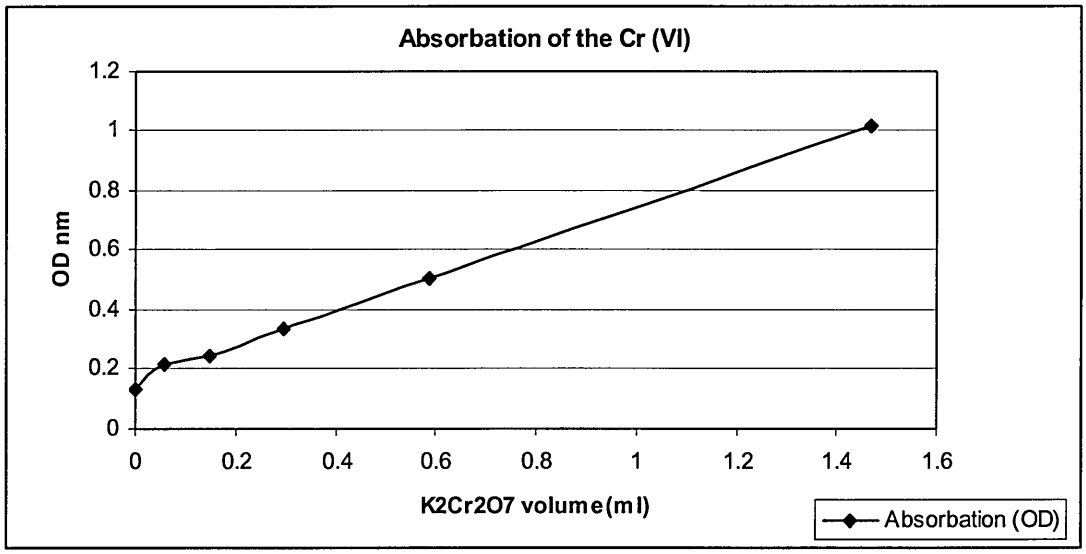


Figure A.1: Typical standard curve of chromium (VI) concentration at OD_{540} nm using DPC assay

Then, the calculation of the Cr (VI) concentration in the solution stock using a standard method reported by (Herrmann, 1994) given as follows:

$$Cr(VI)\ concentration = (weight\ of\ K_2Cr_2O_7) \times \left(\frac{molecular\ wieght\ of\ Cr_2}{molecular\ weigth\ of\ K_2Cr_2O_7}\right) (1)$$

Where, the weight of $K_2Cr_2O_7$ is 3.6 mg/l, the molecular weight of $K_2Cr_2O_7$ is 294.18 and the molecular weight of Cr is 51.99. Equation (1) can be written as:

$$Cr(VI)\ concentration = 3.6\frac{mg}{l} \times \frac{51.99}{294.18} \times 2 = 1.272\ (mg/l) \quad (2)$$

The obtained value of Cr (VI) concentration in the solution stock is used to calculate Cr (VI) concentration for every $K_2Cr_2O_7$ volume in each test tube is given by:

$$\text{Cr(VI) con. in test tube} = \left(\frac{\text{K2Cr2O7 vol in the test tube}}{\text{total volume}}\right) \times \text{Cr(VI) conc. in solution stock} \quad (3)$$

The values of $K_2Cr_2O_7$ volume, optical density and Cr (VI) concentration are summarised in Table A.2. These values are used to create the standard curve as shown in Figure A.2.

Table A.2: Amounts of Cr (VI) concentration for every $K_2Cr_2O_7$ volume

$K_2Cr_2O_7$ volume (ml)	0	0.4	1.0	2.0	4.0	10.0
Optical Density (nm)	0.135	0.216	0.244	0.334	0.504	1.016
7 Cr (VI) concentration (mg/l)	0	0.05	0.127	0.254	0.508	1.272

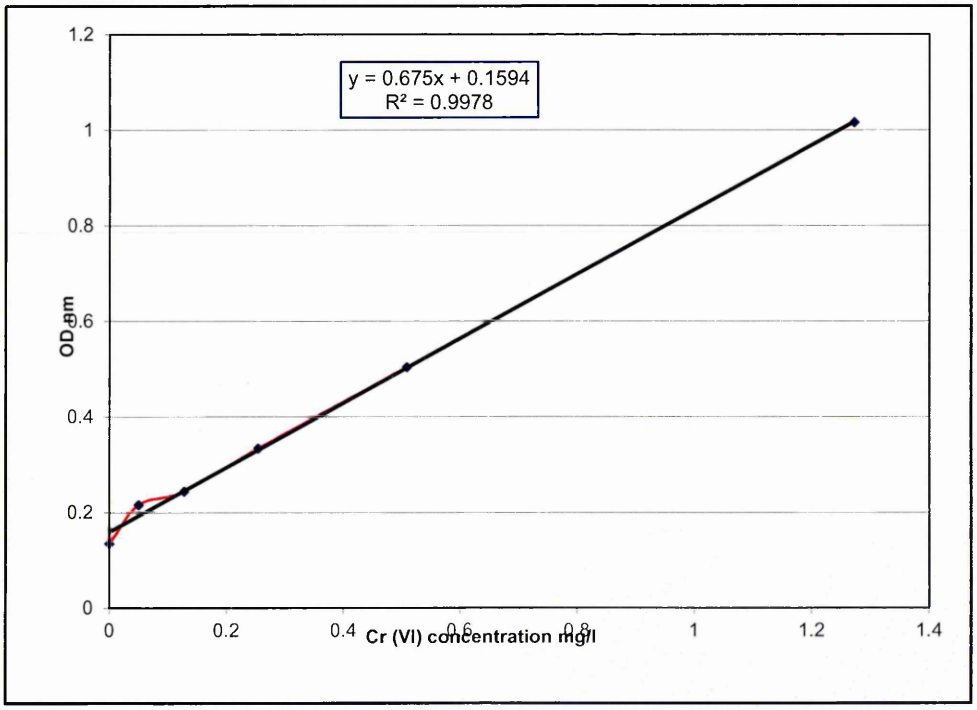


Figure A.2: Standard curve of chromium (VI) concentration for every $K_2Cr_2O_7$ volume

From the standard curve developed in Figure A.2, a straight line equation was derived as follows:

$$y = 0.675x + 0.1594 \quad (3)$$

Where, y represents the optical density and x represents the Cr (VI) concentration. Therefore, equation (3) can be re-arranged to obtain the Cr (VI) concentration as follows:

$$x = (y - 0.1594)/0.675 \quad (4)$$

The general form of above equation was used for calculating the Cr (VI) concentration in the bioremediation experiments.

Standard curve for Cr (VI) concentration in M9, NMS and nutrient broth no.2 media, lysozyme and tris-HCl solutions

Materials

Cr (VI) solution (1.27 mg/l) was prepared using 3.6 mg of $K_2Cr_2O_7$, 10 ml of concentrated sulphuric acid and distilled water to about volume of 1000 ml. 1, 5-Diphenylcarbazide solution (0.50 g in 200 ml acetone). 3 M sulphuric acid solution (0.6 ml).

5 ml of media, lysozyme solution or Tris-HCl 50 mM were used for the measurement of Cr (VI) concentration in each case.

Chromate assay

Six test tubes was obtained and labelled (1, 2, 3, 4, 5 and 6). 5 ml of utilised media and 0.6 ml of 3 M H_2SO_4. The quantities of $K_2Cr_2O_7$ and deionised water was added to each test tube according to the Table A.3 below:

Table A.3: Amounts of $K_2Cr_2O_7$ and deionised H_2O

Tube no.	1	2	3	4	5	6
$K_2Cr_2O_7$ (ml)	0.00	0.4	1.0	2.0	4.0	0.0
H_2O (ml)	4.4	4.0	3.4	2.4	0.4	0.0

The content of each test tube was mixed by shaking. 0.5 ml of Diphenylcarbazide solution was added to each test tube. The contents of the test tubes were mixed and let stand for 5 minutes for colour development. The absorbance of each sample was measured at 540 nm.

Cr (VI) concentrations and their corresponding optical densities in each case are summarised in Tables A.4, A.5, A.6, A.7 and A.8. Their standard curves for Cr (VI) concentration in each media were plotted in Figures A.3, A.4, A.5, A.6 and A.7 respectively.

Table A.4: Cr (VI) concentration vs optical density of nutrient broth no.2 medium

Cr (VI) concentration	Nutrient broth no.2 medium
0	0.024
0.0508	0.037
0.127	0.042
0.245	0.072
0.508	0.155

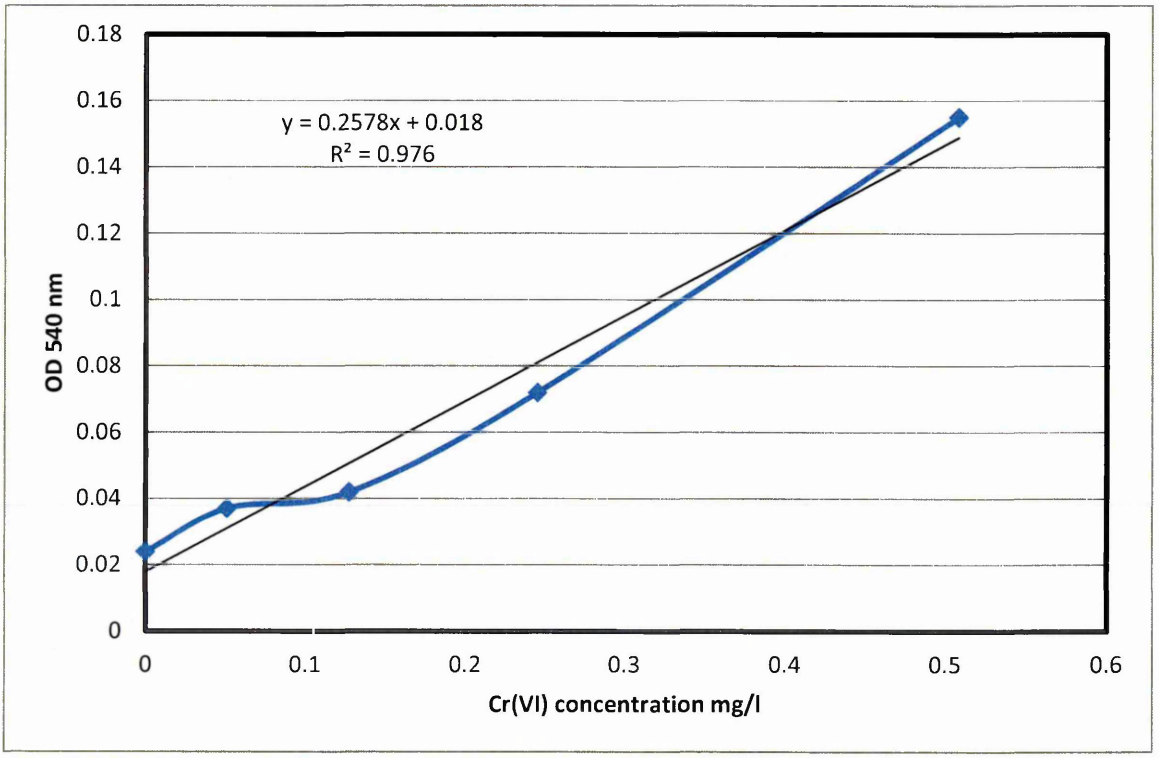


Figure A.3: Standard curve of chromium (VI) concentration in nutrient broth No.2 medium

Table A.5: Cr (VI) concentration vs optical density of M9 medium

Cr (VI) concentration	M9 medium
0	0.02
0.0508	0.068
0.127	0.130
0.245	0.256
0.508	0.471

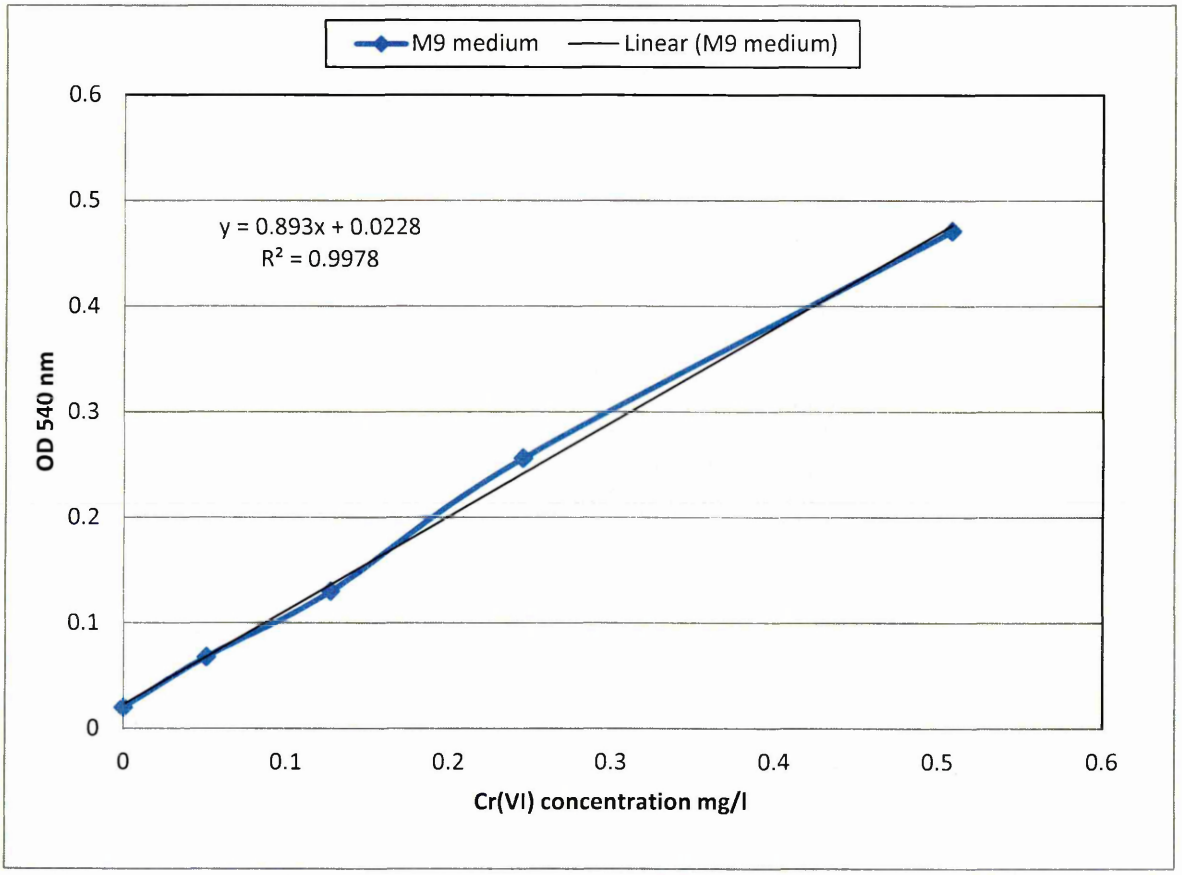


Figure A.4: Standard curve of chromium (VI) concentration in M9 medium

Table A.6: Cr (VI) concentration vs optical density of NMS medium

Cr (VI) concentration	NMS medium
0	0.018
0.0508	0.067
0.127	0.140
0.245	0.255
0.508	0.485

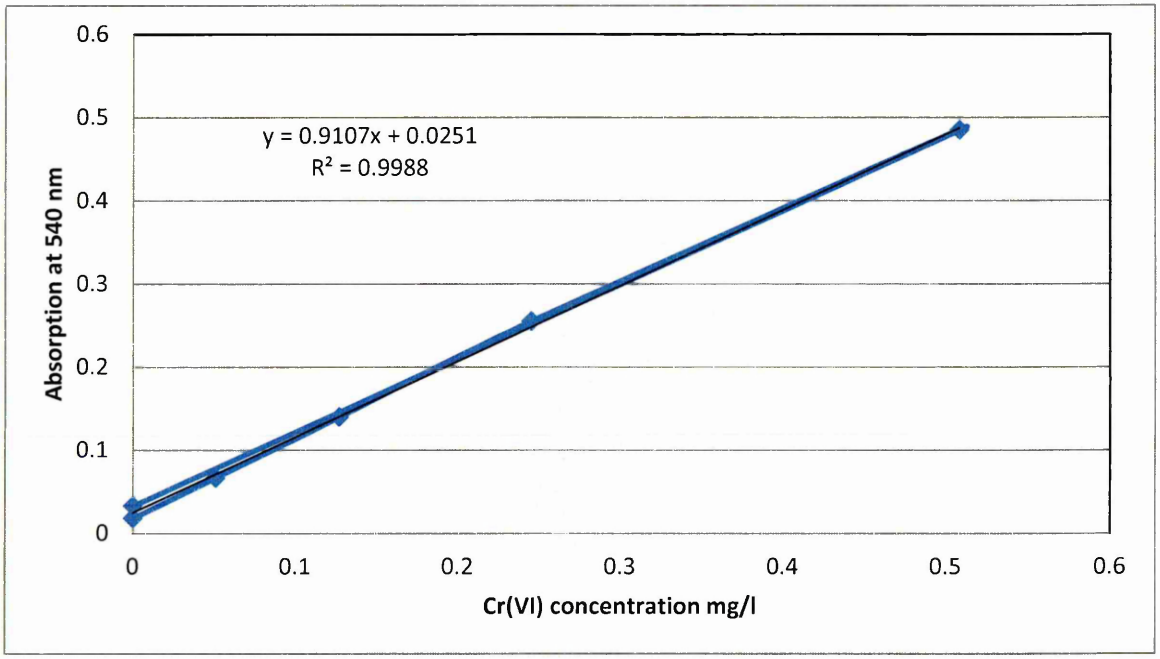


Figure A.5: Standard curve of chromium (VI) concentration in NMS medium

Table A.7: Cr (VI) concentration vs optical density of lysozyme solution

Cr (VI) concentration	OD of lysozyme solution.
0	0.017
0.0508	0.065
0.127	0.128
0.245	0.241
0.508	0.456

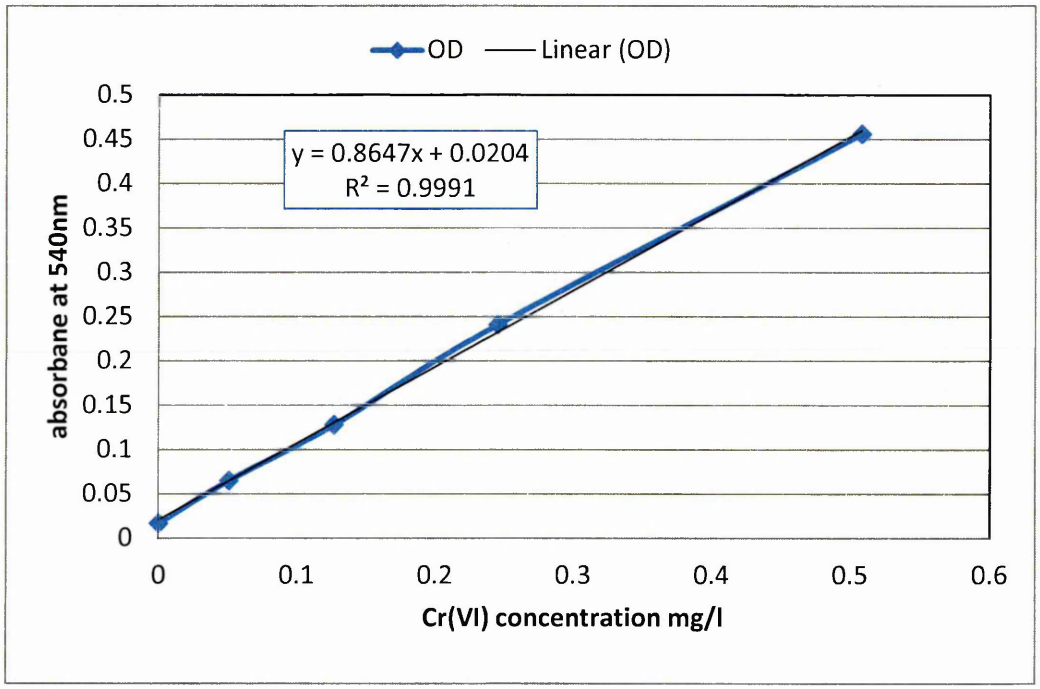


Figure A.6: Standard curve of chromium (VI) concentration in lysozyme solution

Table A.8: Cr (VI) concentration vs optical density of Tris-HCl 50mM solution

r (VI) concentration	OD of Tris-HCl
0	0.027
0.0508	0.069
0.127	0.146
0.245	0.253
0.508	0.442

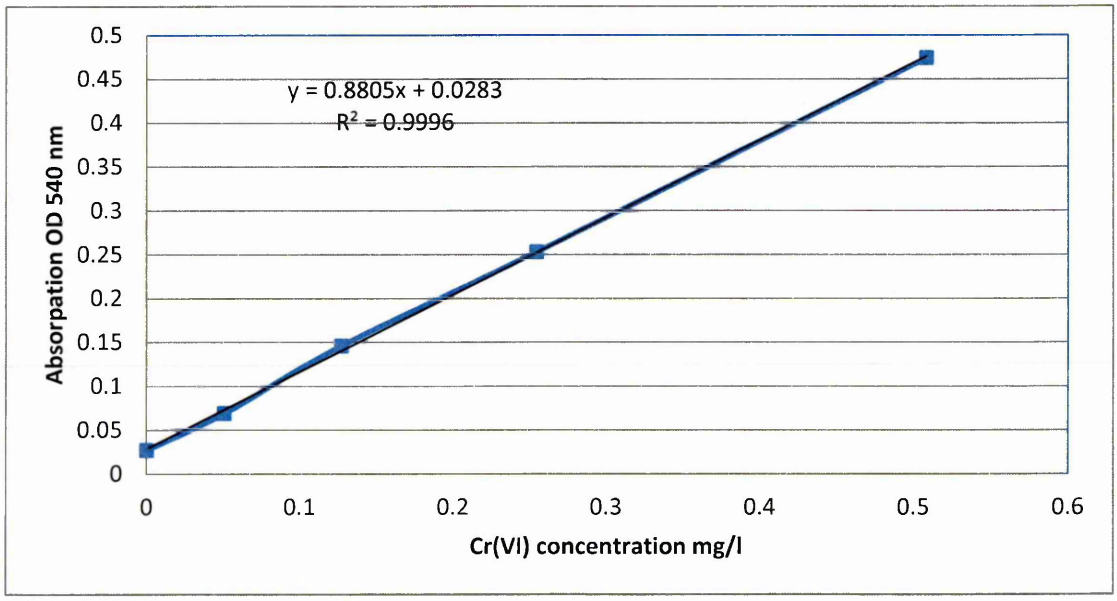


Figure A.7: Standard curve of chromium (VI) concentration in Tris-HCl 50 mM

APPENDIX B: FTIR SPECTRA OF POTASSIUM DICHROMATE

FTIR spectroscopy reveals bond formation during bacterial bioremediation of Cr (VI). Figure B.1 shows the spectrum of potassium dichromate ($K_2Cr_2O_7$). The identified peaks in the spectrum are 556 & 566 and 764 & 769 indicate Cr-O-Cr. The peaks 885, 891, 902, 908, 924, 934, 940 & 946 are for CrO_3 and the peaks 950, 956 & 966 denote O_3Cr-O-CrO_3.

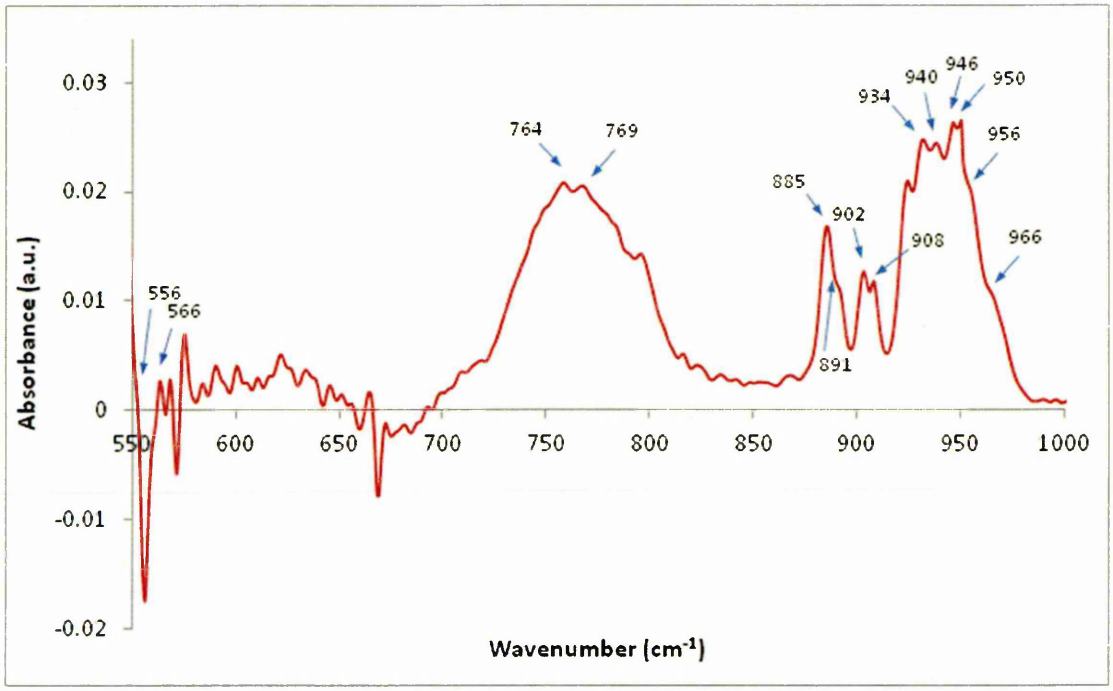


Figure B.1: FTIR spectra of potassium dichromate