Studies on the Biotransformation of Chromium (VI) by Methane Oxidising Bacteria

By

Bubaker M B Hamad

Thesis submitted in partial fulfilment for the requirements of the Degree of Doctor of Philosophy in Sheffield Hallam University, England

2009

Summary

Bioremediation is a promising and cost-effective technology widely used to clean up waste containing organic or inorganic contaminants. Cr (VI), the highest oxidation state of the metal chromium, is widely used in various industries, and is extremely toxic to animals and humans. Utilisation of chromium (Cr) reducing microbes and their products can enhance the efficiency of the process of detoxification of Cr (VI) to Cr (III).

This study was conducted to investigate the microbial reduction of chromium [Cr (VI)] in general and the potential for biological treatment of Cr (VI)-containing wastes in particular. Cr (VI) was transformed to non-toxic Cr (III) by *Escherichia coli* ATCC 33456. It was observed for the first time that the type I methanotrophic bacterium *Methylococcus capsulatus* (Bath) is able to reduce chromium (VI) to chromium (III), using methane as the carbon and energy source. The reaction occurred over a wide range of chromium (VI) concentrations (10-1,000 mg/l). The reaction was studied by a variety of techniques, including the diphenyl carbazide assay for chromium (VI), ion chromatography coupled to inductively coupled plasma atomic absorption spectroscopy (ICP-MS), transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDSX), X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS). The results indicate that chromium (VI) reduction to chromium (III) is wholly or partly associated with the cells via coordination to phosphate moieties.

BLAST searches using known chromate (VI)-reducing enzymes from other bacteria indicated the presence of five potential chromate reductases in the genome sequence of *M. capsulatus* (Bath). Detailed bioinformatic analysis, including molecular modelling, indicates a possible mode of binding of chromium to two of these.

The model type II methanotroph *Methylosinus trichosporium* OB3b did not reduce chromate (VI) in pure culture, and the future availability of the genome sequence of this organism may give additional clues to the origin of chromate (VI) reduction in *M. capsulatus*, via comparative genomics. Interestingly, however, mixed cultures of *M. trichosporium* and *E. coli* were able to reduce chromate (VI) using methane as the only source of reductant, presumably because the *E. coli* can scavenge nutrients from the *M. trichosporium*.

Table of Contents

List of Figures

List of Tables

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Dedication

To my father's heart which looks after me; my mother's soul which watches me from the heavens of Aden, and the eyes of my family (my wife and children) that always being eager for my success.

Table of Contents

List of Figures

List of Tables

1.1 Chromium Definition, Sources and Uses

Chromium (Cr) is a heavy metal which is naturally found in a variety of different forms but mainly in form of chromate, e.g. $FeCr_2O_4$ and $PbCrO_4$. It is a transition metal located in group VI-B of the Periodic Table and is a highly toxic metal which is not essential for the growth of microorganisms and plants. Chromium is the seventh commonest element on Earth and the twenty-first most common element in the Earth's crust. Amounts of chromium in the Earth's crust range from 100 to 300 mg kg^{-1}. The level of chromium in soils is generally between 5 and 3000 mg kg^{-1} (Cervantes *et al.*, 2001).

The total world production of Cr compounds is approximately 10^6 tons per annum; 60–70% of such production is applied to the production of alloys, such as stainless steel, while 15% is used in industrial chemical processes, mainly for leather tanning, pigment production and electroplating (McGrath, and Smith, 1990). Leather tanning can result in particularly recalcitrant chromium (VI) pollution because pentachlorophenol and related biocides used in the leather tanning processes also inhibit the growth of microorganisms and thus also tend to reduce the removal of chromium from tannery effluent by natural bioremedition (Srivastavaa and Thakur, 2006).

The widespread use of chromium has resulted in the metal becoming a serious air and water pollutant (Amienta-Hernández and Rodríguez-Castillo, 1995). The amount of chromium ranges from 0.1 to 0.5 μgml^{-1} in freshwater, and from 0.0016 to 0.05 mg/l in the ocean (De Filippis, and Pallaghy, 1994), but levels as high as 80 mg/l have been observed in effluents from paper mills (Sudhakar *et al.*, 1991).

Despite the fact that chromium exists in several oxidation states, the zero, trivalent, and hexavalent states are the most important in both industry and in relation to the environment. The bulk of naturally occurring chromium is in the trivalent state, generally combined with oxides of iron or other metals. Chromium compounds are widely used in industry; for example, the "stainless" component

of stainless steel results from the chemical properties of the chromium oxides. Similarly, the protective properties of chrome plating of metals, chromated copper arsenate (CCA) treatment of wood, and chrome tanning of leather all depend upon the chemistry of hexavalent chromium. Chromium has oxidation states ranging from -2 to +6. The most stable are +6 and +3. Both are widely released into the environment; Cr (VI) is common in effluents produced by tanneries and pigment-production plants. Sources of Cr (VI) pollution include: metallurgy, mining, fossil fuel combustion and wood preservation. Depending upon its oxidation state and concentration, chromium can be both beneficial and toxic to animals and humans. At low concentration, Cr (III) is required for maintaining the health of animals (Katz and Salem, 1994). Cr (VI), unlike Cr (III) is extremely toxic and may result in death when ingested in substantial doses; it is also a carcinogen. (Syracuse Research Corporation, 1993). The ingestion of Cr in food materials such as vegetable crops, provides a major proportion of the daily intake of Cr. It is therefore essential to determine which forms of Cr are present in plant tissues as well as the extent to which they are accumulated (Zayed and Terry, 2003). Because of its widespread industrial use, chromium has become a serious pollutant in a wide variety of environments (McGrath and Smith, 1990; Ackerley *et al.*, 2004 a & b).

The chemistry of environmentally-released Cr compounds is complicated (Ksheminska *et al.*, 2005). Concern about Cr as an environmental pollutant has increased due to its build up to toxic levels in some environments (Zayed and Terry, 2003). Therefore the transformation of hazardous Cr (VI) into less hazardous and less mobile, Cr (III) is desirable (Lee *et al.*, 2003).

1.2 Properties of Cr (III)

This form of chromium has a very low water solubility and low reactivity resulting in low mobility in the environment and low toxicity towards living organisms (Barnhart, 1997). Chromium (III) is also biologically essential to mammals as a requirement for the maintenance of efficient protein metabolism (Shrivastava *et al.*, 2002). Chromium deficiency causes disturbances in the

metabolism of glucose and lipid in both humans and animals (Zayed and Terry, 2003). However, low concentration of Cr can increase the growth of plants; excess Cr on the other hand is highly toxic to animals and plants and may lead to the development of cancers (Shanker *et al.,* 2005).

Cr (III) has also been shown to be harmful to cellular structures (Cieślak-Golonka, 1995; Cervantes, *et al.*, 2001; Raspor, *et. al.* 2000). However, its toxicity *in vivo* is much less than that of Cr (VI). This fact can be accounted for by the presence of complexes (positively charged) that predominate in trivalent Cr reactions; these are much less soluble and therefore less easily transported inside cells. Organically-bound Cr (III) derivatives might cross cell membranes by some, as yet unknown, mechanism (Srivastava *et al.*, 1999).

In contrast to Cr VI, Cr (III) as oxides, hydroxides or sulphates, is much less mobile in the environment because it exists mostly bound to organic matter, both in soils and in aquatic environments. However, Cr (III) can be oxidized to Cr (VI) in the presence of excess oxygen, and, as a result, become more toxic (Jackson *et al.*, 1999).

1.3 Forms of Cr (VI)

Cr (VI) is regarded as the most toxic form of Cr. It is associated with oxygen, as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions which display quite different chemical properties. Chromate is toxic, even when present at low concentrations; it is a major environmental pollutant as it is used extensively in leather tanning, electroplating and metal finishing (Horitsu *et al.*, 1987). The major sources of chromium (VI) are tannery, paint, ink, dye, and aluminium based industries. In urban areas, about two thirds of the airborne chromium comes from the emission of hexavalent chromium during the combustion of fossil fuels and from steel production; the remaining chromium in the air is in the form of trivalent chromium. The residence time of chromium in air is <10 days. Hexavalent chromium irritates both the skin and mucous membrane, and produces an allergic contact dermatitis, which is characterized by eczema (Barceloux, 1999).

Hexavalent chromium is has been identified as a pulmonary carcinogen (Park et al., 2000) and this has been recognised by the International Agency for Research on Cancer and by the US Toxicology Program. Browning (1969) found that long-term exposure to Cr (VI) causes cancer of the digestive tract and lungs and may cause epigastric pain, nausea, vomiting, severe diarrhoea and haemorrhage. An increased risk of lung cancer occurs in workers exposed to hexavalent chromium dust during the refining of chromites and the production of pigments made of chromate.

The action of chromium (VI) species as strong mutagens and carcinogens (Cieślak-Golonka, 1995; Costa, 1997) results from negatively charged hexavalent Cr ion complexes which can readily cross cellular membranes by means of sulphate ionic channels (Cervantes, 2001), and then undergo immediate reduction reactions. These intermediates cause harm to cell organelles, proteins and nucleic acids (Cieślak-Golonka, 1995; Lay and Levina, 1998). As chromium (III) is significantly less toxic and much less soluble than Cr (VI), it is essential that Cr (VI) be removed from wastewater by converting it to Cr (III) before disposal.

1.4 Exposure to Chromium in the UK Environment

In the UK, chromium emissions to the environment mainly originate from public power production, combustion of fuels, the incineration of wastes and various other industrial processes. A total of 60 tonnes of chromium was emitted in the UK in 1995 (Salway *et al.,* 1997). The most important chromium industries in the UK include the ferrochromium, chromium metal, chemical and refractory works (Papp, 1994; Ayres and Ayres, 1999). The fate and behaviour of chromium from anthropogenic sources in the UK is summarised in Figure (1.1), and the general population chromium exposures are given in Table (1.1).

In the UK, as elsewhere in the world, food is a major source of chromium exposure to most people, predominantly as Cr (III). The calculated daily oral intakes of chromium from a combination of food (based on the Ministry of Agriculture Fisheries and Food (MAFF) 1994 Total Diet Survey (MAFF, 1997)),

drinking water (based on a level in drinking water of 2 μg/l, derived by WHO, 1996) and soil ingestion (based on soil ingestion rates and levels of chromium in soils (IPEH, 1997), for infants (1 year), children (11 years) and adults, are respectively, 33–45, 123–171 and 246–343 $\mu g.person^{-1}\ day^{-1}$. Soil ingestion is an important route of oral exposure to the element in young children. Living near a point source such as a contaminated land site can increase oral intake. For example, intakes of chromium of 1046 $\mu g\ day^{-1}$ in children under 6 years and 262 $\mu g.day^{-1}$ in older children and adults have been estimated, based on levels of chromium in soil measured in public areas around a contaminated site in Glasgow, prior to 1991 (Rowbotham, *et al.*, 2001; Leonard, *et al.*, 2000). However, the consumption of foods grown locally near a chromium chemical manufacturing plant in the UK have been found not to increase daily intake of chromium above the average levels reported in the MAFF survey (IPEH, 1997). As can be seen from Table 1.1, exposure to chromium species varies according to place of residence; perhaps not surprisingly, the greatest levels of exposures occur around industrialised sites. Active (or passive) inhalation, of tobacco smoke is an additional means chromium exposure.

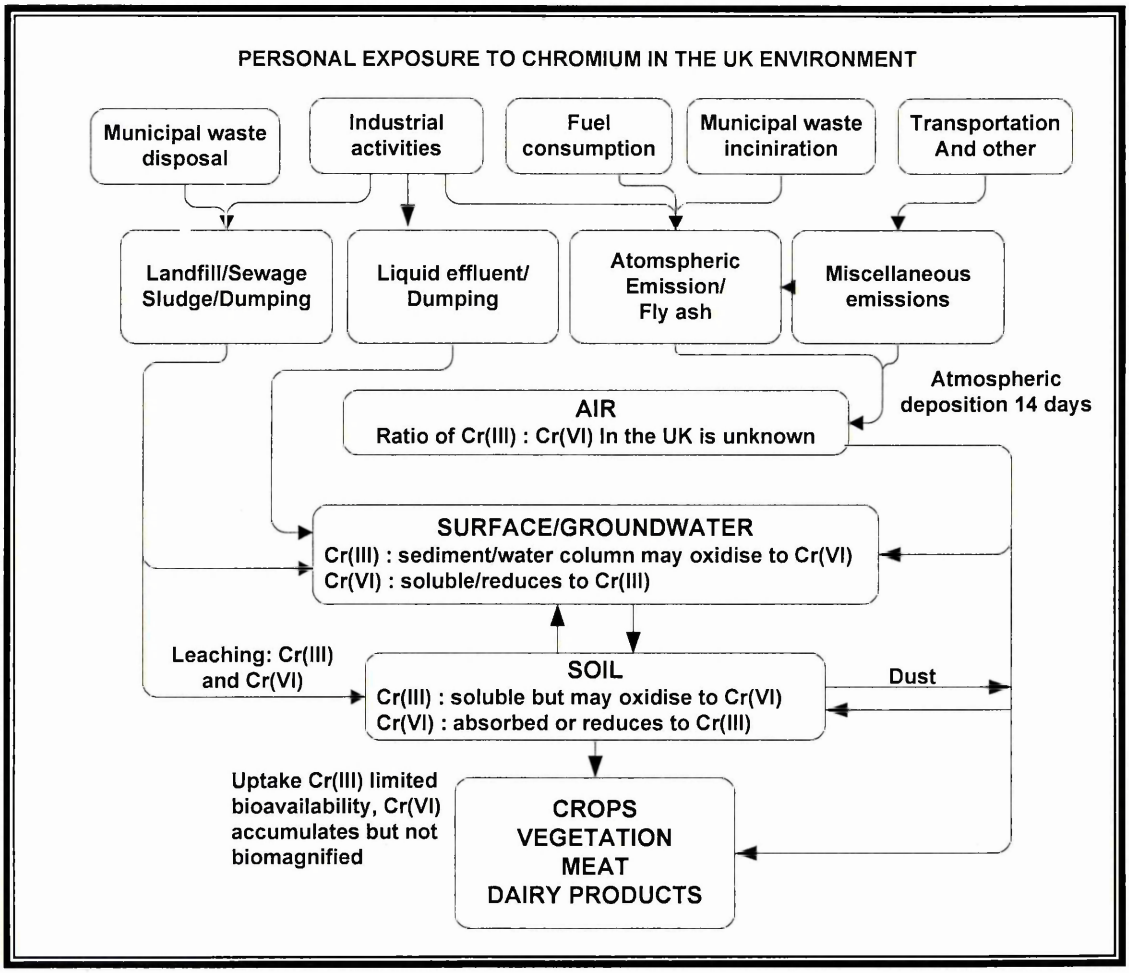


Figure 1.1: Anthropogenic source of Cr emissions in the environment

(Reproduced from Rowbothman et al., 2000)

Table 1.1: Summary of estimated intakes of total chromium for infants, children and adults using different exposure scenarios (Rowbothan *et al.*, 2000)

Exposure Scenario	Intake via inhalation[a] (µg/day)	Intake via ingestion[b] (µg/day)	Overall daily intake[c] (µg/day)	Daily intake[d] (µg/kg body weight)
Rural infant	**0.004-0.05**	**32.5-44.6**	**33-45**	**3.6-4.9**
Urban infants	**0.02-0.08**	**Ditto**	**Ditto**	**Ditto**
Urban infants, "passive smokers"	**0.03-0.14**	**Ditto**	**Ditto**	**Ditto**
Point source infant	**0.3**	**1076.9-1089**	**1077-1089**	**118-120**
Point source infant, "passive smokers"	**0.5**	**Ditto**	**1077-1090**	**Ditto**
Rural child	**0.01-0.1**	**122.9-171.4**	**123-172**	**3-4.2**
Urban child	**0.04-0.015**	**Ditto**	**Ditto**	**Ditto**
Urban child, "passive smokers"	**0.06-0.3**	**Ditto**	**Ditto**	**Ditto**
Point source child	**0.5**	**384.5-433**	**385-434**	**9.4-10.6**
Point source child, "passive smokers"	**0.9**	**Ditto**	**Ditto**	**Ditto**
Rural adult	**0.02-0.24**	**246.4-343.4**	**246-344**	**4-5.6**
Urban adult	**0.08-0.34**	**Ditto**	**Ditto**	**Ditto**
Urban adult, "passive smokers"	**0.14-0.6**	**Ditto**	**Ditto**	**Ditto**
Urban adult smoker	**0.14-10.6**	**Ditto**	**246-354**	**4-5.8**
Point source adult	**1.1**	**508-605**	**509-606**	**8.3-9.9**
Point source adult, "passive smokers"	**1.9**	**Ditto**	**510-607**	**Ditto**
Point source adult smoker	**1.9-11.9**	**Ditto**	**510-617**	**8.3-10**

a Intakes via inhalation from rural, urban, and point source (i.e., BCC chemical plant), taking into account the contribution of active and passive smoking, are based on values presented in Table 5.

b Intakes via ingestion represent the combined contribution of food, soil (contaminated soil for point source scenario, i.e., at levels reported around the Glasgow region), and drinking water based on values presented in text (the contribution from cooking utensils has not been included and is assumed to be negligible). It has been assumed that infants and children consume 12.1 and 50% that of adults (ICRP, 1975).

c The overall intake gives the combined intakes via inhalation, ingestion, and dermal routes.

d Assumes that the average infant (1 yr) weighs 9.1 kg, the average child (11 yr) weighs 41.1 kg, and the average adult (male or female) weighs 61.5 kg..

Lung cancer is a disease that has been associated with occupational exposure to chromium (VI) (e.g. IARC, 1990); however, the majority of the general population is unlikely to be exposed to the critical levels of the metal. The uptake of chromium occurs via its inhalation from rural, urban and point sources (i.e. chromium chemical plant), as well as active and passive smoking (Bertorelli and Derwent, 1995; IPEH, 1997; Layton, 1993; Smith *et al.,* 1997). The overall intake of the element comprises the combined intakes via inhalation and ingestion. No data are available to assess intake via the dermal route for the UK general population, but such intake is likely to be negligible. In contrast, Cr (III) is an essential element with a broad safety range and low toxicity. The human body has effective detoxification mechanisms that can reduce ingested or inhaled Cr (VI) to Cr (III) (Paustenbach *et al.*, 1991; De Flora *et al.*, 1997). However, there is evidence that Cr (III) can accumulate in human tissues (Stearns *et al.,* 1995). A worst case scenario for the oral consumption of chromium intake, based on available UK data, results when individuals are exposed to contaminated soil (such as that reported in Glasgow), in which case, possible daily oral intakes of up to 1089, 433 and 605 μg for infants, children and adults, respectively. Health studies in Glasgow suggested a resultant small increase in upper digestive tract disorders following chromium adsorption (Eizaguirre-Garcia, 1996). Although such levels exceed the estimated safe daily human dietary intake for Cr (III) of 50–200 $\mu g\ day^{-1}$ (ASTDR, 1993) (Table 1.1), they translate into daily intakes per kilogram of bodyweight which is well below the RFD for Cr (III) of 1000 $\mu g.kg^{-1}.day^{-1}$ (derived from a US Environmental Protection Agency). A 'no-observed-adverse-effect-level' for Cr (III) of 1468 mg kg^{-1} bodyweight has been determined from experimental studies (Duggan and Strehlow, 1995).

Currently, insufficient data is available from the UK to evaluate how much of the general population is exposed to chromium in the environment. However, there appears to be no clear indication that environmental levels of chromium are associated with adverse health effects in the total UK population, or subgroups that are exposed to higher levels of chromium around industrialised or contaminated sites. The significance of possible long-term chromium

accumulation in the body as Cr (III) is not well understood and requires to be further investigated.

The stable form of dissolved Cr is hexavalent (Cr VI), but a significant proportion of trivalent Cr (III) is often present as an organically-bound component (Nakayama *et al.,* 1981). Chromium has been found in barnacles, *Balanus* sp. by Van Weerelt *et al.* (1984) and in the polychaete, *Neanthes arenaceodentata,* (Oshida *et al.*, 1981). The trivalent form is often precipitated in seawater and is rapidly scavenged by particles, such as oxides of Fe, or by surfaces. In the crab, *Xantho hydrophilus,* for example, uptake of labelled Cr (III) has been found to be largely confined to the body surface, whereas the hexavalent form tends to be absorbed by the tissues where it is reduced to Cr (III) and organically complexed (Peternac and Legovic, 1986).

Treatment of sediment with 1 M HCl releases 90% of the total Cr from the most contaminated sediments, compared with 5% from relatively uncontaminated materials. In many estuaries, anthropogenic Cr is often associated (adsorbed or co- precipitated as Cr (III) with oxides of Fe). Attempts to relate body Cr levels in deposit-feeding species to total and HCl-extractable Cr concentrations showed that in both the clam *Scrobicularia plana* and the polychaete *Nereis diversicolor* chromium concentrations were correlated with the total sediment levels (Bryan and Langston, 1992). It seems probable that sedimentary Cr is the main source of the element in *S. plana* and *N. diversicolor.* How sediment-bound Cr reaches animal tissues is uncertain and uptake may occur from ingested sediment or from tidal water.

The discharge of Cr (VI) to surface water is regulated to below 0.05 mgL^{-1} by the US EPA (Baral and Engelken, 2002) and the European Union, while total Cr, including Cr (III), Cr (VI) and its other forms, is regulated to below 2 mg/l (EC-Official Journal of the European Communities, 1998).

1.5 Bioremediation

Bioremediation is the use of living or dead biomass, primarily microorganisms, to degrade or transfer environmental contaminants into less toxic forms. Bioremediation employs naturally occurring bacteria and fungi, or plants,

in order to degrade, or detoxify, chemicals that are hazardous to human health and/or the environment. Contaminant compounds are transformed by living organisms via reactions which take place as a part of their normal metabolic processes. As such, it uses relatively low-cost, low-technology approaches, which generally have a high public acceptance. Bioremediation can obviously only be effective where environmental conditions allow for microbial growth and activity. Bioremediation techniques are generally found to be more cost effective than traditional methods, which include incineration, and some pollutants can be treated on site, thereby reducing exposure risks to personnel involve in clean-up, or potentially wider exposure as a result of transportation. The majority of bioremediation systems operate under aerobic conditions, although running a system under anaerobic conditions may allow microorganisms to degrade otherwise recalcitrant molecules.

Bioremediation is not entirely suitable, as the range of contaminants on which it can operate effectively are limited, the time scales involved are relatively long, and the levels of residual contaminants achievable may not always be desirable. Considerable experience and expertise may be needed to design and implement a successful bioremediation program. Bioremediation has been used at a number of sites worldwide, including in the UK and Europe, with varying degrees of success.

1.6 Remediation of Chromium Contamination

The conventional remediation processes for areas contaminated with Cr (VI) involves the use of both physical and chemical technologies. These technologies are expensive and tend to result in the production of secondary waste streams that need additional treatment (Viamajala *et al.*, 2002).

Biological treatment approaches engender considerable interest because of their lower impact on the environment. Recent studies have shown that bacteria can transform hexavalent chromium, Cr (VI), into the much less toxic and less mobile trivalent form, Cr (III) (Camargo *et al.,* 2005; Pal and Paul, 2005). There are also reports of microorganisms being used to remediate Cr (VI) contaminated

soils and waters (Kratochvil *et al.*, 1998). Bacteria can protect themselves from the toxic effects of chemicals in the environment by transforming toxic compounds via oxidation, reduction or methylation, and form more volatile, less toxic or readily precipitating forms.

The use of bioremediation, including phytoremediation, to detoxify chromium-contaminated areas continues to receive interest from researchers worldwide and several methods have been suggested and experimentally evaluated with varying degrees of success (Zayed and Terry, 2003). The bioremediation of Cr (VI) contaminated environments was carried out by Puzon, *et al.,* (2005). They used a bacterial enzyme system, that employed NADH as the reductant, converting Cr (VI) to soluble NAD^+- Cr (III) complex, and cytochrome c-mediated Cr (VI) reduction producing cytochrome c – Cr (III).

The potential of Cr bioremediation is also reported by Ksheminska *et al.*, (2005), who suggested that the presence of Cr in the environment selects microbial and plant variants capable of tolerating high levels of Cr compounds. The diverse Cr-resistance mechanisms displayed by microorganisms, and probably also by plants, include biosorption, diminished accumulation, precipitation, reduction of Cr (VI) to Cr (III), and chromate efflux. Many of these systems have been evaluated as potential biotechnological tools for the bioremediation of Cr pollution.

In conclusion, bioremediation, especially using microorganisms, offers an attractive alternative treatment to chemical methods, because the technology is cost-effective and environmentally compatible.

1.7 Other Methods of Chromium Remediation

Chromium can be effectively 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). However, the relatively high costs of the chemical reagents and resultant problems of secondary pollution make these methods somewhat limited in application.

Chemical precipitation is, at the moment, the most commonly used technology for treating heavy metals in wastewaters. Conventional chemical treatment involves the reduction of Cr (VI) to Cr (III) by a reducing agent at low-pH and the subsequent adjustment of solution pH to near neutral in order to precipitate Cr (III) as hydroxides (Wang and Shen, 1997). This method generates large amounts of secondary waste products.

Physicochemical methods, including electrochemical treatment, ion exchange, precipitation (with hydroxides, carbonates or sulphides), reverse osmosis, evaporation, and sorption (e.g. onto biomass or activated carbon) (Kadirvelu *et al.*, 2001; Kadirvelu *et al.*, 2002) have been applied to the removal of heavy metals, including chromium, from waste–streams. However, such methods tend not to be cost-effective (Kadirvelu *et al.*, 2001; Volesky and May-Phillips, 1995) and can result in generalised pollution (Kratochvil *et al.*, 1998).

In recent decades, interest has been particularly focused on the use of a range of biosorbents to remove metal ions (Munoz and Guieysse, 2006, Pena-Castro *et al.*, 2004 and Davis *et al.,* 2003) and biological approaches have been considered as a means of remediating heavy metal contamination. Recently, microbial systems involving the use of fungi, bacteria and algae have been successfully used as adsorbing agents to remove heavy metals (Munoz *et al.*, 2006). Microbial populations in metal polluted environments are known to adapt well to toxic concentrations of heavy metals and become resistant to polluting metals (Prasenjit and Sumathi, 2005). Various species of *Aspergillus*, *Pseudomonas*, *Sporophyticus*, *Bacillus*, *Phanerochaete etc.*, have been reported as efficient chromium and nickel reducers (Yan and Viraraghavan, 2003, Gopalan and Veeramani, 1994). Biotransformation and biosorption are emerging technologies, which use the potential of microorganisms to transform or adsorb metals (Chen and Hao, 1998). Intact microbial cells live or dead and their products can be highly efficient bioaccumulators, both of soluble and metal-particulates (Kratochvil *et al.*, 1998). In addition, microbial cell surfaces are negatively charged because of the presence of various anionic structures, thereby enabling them to bind metal cations (Chen and Hao, 1998).

1.7.1 Microbial Mechanisms to Reduce Cr (VI) to Cr (III)

Mechanisms involving Cr (VI) reduction are of particular interest relating to the technological and biological use of microorganisms, because by this route, toxic metals can be converted into less toxic, immobile reduced forms (Daulton *et al.*, 2007). Hexavalent chromium can be converted to Cr (III) in reduced environments; this ion is far less toxic and less soluble. A number of microorganisms possess the enzyme, chromate reductase, which can facilitate such bioremediation. However, high levels of Cr (VI) may overcome the reducing capacity of the environment and thus persist as a pollutant. In addition, Cr (III) may be oxidized to Cr (VI) in the presence of an excess of oxygen, which results in it being re-converted to the more toxic form (Ishibashi *et al.*, 1990).

Depending on the microbial species employed, Cr reduction can occur under aerobic and/or anaerobic conditions. Although many bacteria can conserve energy to support growth (i.e., respire) through anaerobic metal (e.g. Fe, Mn) reduction, there are only a few reports that bacteria can conserve energy through anaerobic Cr (VI) reduction (Tebo and Obraztsova, 1998). Nevertheless, evidence indicates that Cr reduction is catalyzed by enzyme reactions (Chen and Hao, 1998). However, little is known about the electron-transport pathways involved. Microbial Cr reduction is complex, involving numerous potential pathways and unstable redox intermediates. Evidence for the involvement of two Cr redox intermediates, Cr (IV) and Cr (V), in the microbial reduction of Cr (VI) has been reported (Suzuki *et al.*, 1992, Myers *et al.*, 2000, Neal *et al.*, 2002 and Kalabegishvili *et al.*, 2003).

The means by which microorganisms interact with toxic metals which leads to their removal/and recovery are bioaccumulation, biosorption and enzymatic reduction (Srinath *et al.*, 2002). Heavy metal accumulation is often made up of two phases. There is an initial rapid phase involving physical adsorption or ion exchange at cell surface and then a subsequent and slower phase, involving active metabolism-dependent transport of metal into bacterial cells (Gadd, 1990). Biosorption is known to be a metabolism-independent process and thus can be achieved by both living and dead microbial cells. Such adsorption is based on mechanisms like complexation, ion exchange, coordination,

adsorption, chelation and microprecipitation; processes which, may be synergistically or independently involved (Hu *et al.*, 1996). However, the enzymatic reduction of Cr (VI) into Cr (III) is thought to be one of the defence mechanisms which microorganisms employ when living in Cr (VI)-contaminated environments (Chirwa and Wang, 1997).

The mechanism by which Cr (VI) is removed may be complex and depends on the properties of the biosorbents involved; previous studies suggest that the removal of Cr (VI) by biomass mainly involves exchange and binding on functional groups (Donmez and Aksu, 2002). Various mechanisms including ion exchange-redox reaction, parallel biosorption and bioreduction, direct reduction and a sequential three-step are involved. The mechanism of Cr (VI) removal by the microbial species which have been isolated from waste material may be different from other biosorbents, as the result of differences in biomass composition (Han *et al.,* 2006) and (Park *et al.*, 2005).

1.7.2 Bacterial Reduction of Chromium

One of the earliest reports on bacterial Cr (VI) reduction was by Romanenko and Korenkov (1977). Cr (VI) has been shown to serve as the final electron acceptor in the respiratory chains of: *Aeromonas* (Kvasnikov *et al.*, 1985), *P. aeruginosa* (Gvozdyak *et al.,* 1986), *B.subtilis* (Gvozdyak *et al.,* 1986), *P. fluorescens* (Bopp and Ehrlich, 1988), and *Enterobacter cloacae* (Wang *et al.,* 1990). In the absence of oxygen and nitrate, Cr (VI) was also shown to be quantitatively transformed to Cr (III), largely due to the activity of soluble reductase in *E. coli* ATCC 33456 (Shen and Wang,1993). Chromate reduction has been reported in many types of bacteria, under both aerobic and anaerobic conditions, where chromate can be used as a terminal electron acceptor (Romanenko and Korenkov, 1977; Bopp and Ehrlich, 1988; Wang *et al.*, 1989), and aerobically (Bopp and Ehrlich, 1988; Ishibashi *et al.,* 1990. In these studies, while the chromate-resistant strain was resistant to chromate under both aerobic and anaerobic conditions, only anaerobic chromate reduction occurred (Wang *et al.,* 1989).

The Cr (VI)-reducing capacity of chromate-resistant bacteria (CRB) has been widely reported (Cervantes *et al.*, 2001), especially in isolates from tannery effluent (Basu *et al.*, 1997 and Shakoori *et al.*, 2000), tannery waste (Pattanapipitpaisal *et al.*, 2001), discharge water (Campos *et al.*, 1995), electroplating effluent (Ganguli and Tripathi, 2002), activated sludge (Francisco *et al.*, 2002), evaporation ponds (Losi and Frankenberger, 1994) and *in vitro* (Park *et al.*, 2005).

Attempts to study the diversity of a population of chromium-resistant and chromium-reducing bacteria were made by Francisco *et al.* (2002). These authors characterized the microbial community that was cultureable in a chromium-contaminated industrial site. They used the MIDI system to identify six species never reported as Cr (VI)-resistant and/or Cr (VI)-reducing strains, with the predominant species identified being *Ochrobactrum antropi* (eight isolates). However, these isolates did not show Cr (VI) resistance and only two identified isolates (*Aureobacterium estearomaticum* and *Cellulomonas flavigena*) resisted and reduced Cr (VI).

In recent studies, a number of bacterial species have been identified as being capable of reducing Cr (VI). *Streptomyces griseus* strain has been found to be capable of reducing a 50 mg L^{-1} Cr (VI) standard solution over a period of 72 h (Laxman and More, 2002). *Arthrobacter* sp. and *Bacillus* sp., isolated from a long-term contaminated tannery waste soil, have also been found to reduce Cr (VI) concentrations up to 50 mg L^{-1} (Megharaj *et al.*, 2003). Chromium-resistant bacteria from Cr (VI) contaminated soils can also reduce Cr (VI) concentrations up to 1500 mg L^{-1} (Food and Agriculture Organisation, FOA, Camargo, 2003). Finally, Cr (VI) was also found to be reduced by *Shewanella oneidensisv* (Daulton *et al.*, 2007) and a metal reducing bacteria, *Shewanella alga* (BrY-MT) ATCC 55627 (Guha *et al.*, 2003). Sulphate-reducing bacteria from marine sediment were capable of almost completely reducing 0.6 mM Cr (VI*), in vitro*, within 168 hours. (Cheung and Gu, 2003). Progress toward the development of a useful means of utilising chromate reductase activity has been made by Ohtake and Silver (1994) using an anaerobic membrane respiratory chain system from a newly isolated *Enterobater cloacae*. The resistance to, and ability to reduce,

chromate were both lost during anaerobic growth on nitrate; this suggests that the nature of the anaerobic respiratory pathway is critical for chromate reduction, the reduced chromate being precipitated as insoluble chromium hydroxide. Initial levels of 1 or 2 M CrOi- were rapidly detoxified, and reduction of up to 10 mM CrOi- was achieved by cells which had been previously grown on chromate (Ohtake and Silver, 1994). Sub-cellular right-side-out membrane vesicles from *E. cloacae* were found to contain the cellular chromate reductase activity (Wang *et al.*, 1990). Efforts to develop the *E. cloacue* system to detoxify chromate-containing effluents at the pilot plant level have been made by Ohtake *et al.*, (1990).

It has sometimes proved necessary to dilute the chromate containing waste before it was exposed to the bacteria, presumably in order to lower the levels of other toxic materials present. More studies are needed using bacteria with semi-permeable membranes on cells immobilized on columns, which may lead to the development of a more useable chromate detoxification technology (Cervantes and Silver, 1992).

The enzymatic reduction of Cr (VI) has been reported. A membrane-associated, constitutive enzyme has also been found to induce the transfer of electrons from NADH to chromate (Bopp and Ehrlich, 1988).

Batch studies have been conducted to determine the environmental factors involved in the rate of Cr (VI) reduction by *Bacillus* sp. (a new isolate), and *Pseudomonas fluorescens* LB 300 (Wang and Xiao, 1995). Both bacteria use glucose during Cr (VI) reduction under aerobic conditions. Higher Cr (VI) reduction rates were found with higher initial cell concentrations, but the specific rate (normalized against initial cell concentration), was higher when lower initial cell concentrations were used. The rate of Cr (VI) reduction by *Bacillus* sp. increased with initial Cr (VI) concentrations ranging from 20 to 70 mg/l and then decreased when higher concentrations were used. Further studies showed that neither sulphate nor nitrate had an effect on Cr (VI) reduction. Additionally, there was no correlation between the rate of Cr (VI) reduction and the redox potential of the culture media. Experiments with cell-free extracts of *Bacillus* sp. showed

that soluble enzymes were responsible for Cr (VI) reduction in this organism (Wang and Xiao, 1995).

Chromium (VI) is reduced by *Escherichia coli,* strain ATCC 33456, under both aerobic and anaerobic conditions using various electron donors, such as glucose, acetate, propionate, glycerol and glycine. In addition to the type of electron-donor involved, the initial cell concentration, Cr (VI) concentration, pH, and temperature all had major effects on the rate of Cr (VI) reduction (Shen & Wang, 1994). Cr (VI) reduction was not significantly affected by levels of phenolic compounds. The concentrations that caused a 50% reduction in the rate of Cr (VI) reduction (IC_{50}) were 986, 1,526, and 5,263 mg/l for *p*-cresol, 2-chlorophenol, and phenol, respectively, in anaerobic cultures. Higher IC_{50} values were noted in aerobic cultures. Cr (VI) reduction by this strain was more sensitive to heavy metals (Zn^{+2} and Cu^{+2}) than to phenolic compounds. The reduction of Cr (VI) by *E. coli* ATCC 33456 involved enzymes, and was not affected by the redox potential of the culture media as well as other possible electron acceptors, including sulphate and nitrate (Shen and Wang, 1994). Strains of *Acinetobacter* sp. have been used for the removal of chromium from tannery effluent in sequential bioreactor; a 80% reduction in chromium being achieved after 15 days (Shrivastava and Thakur, 2003). Such a long period for the removal of chromium may however, lead to the generation and accumulation in the environment of metal and toxic compounds. The effective and efficient removal of chromium in short time should be a basic necessity of any chromium removal process. *Pseudomonas putida* is able to reduce chromate (VI) aerobically using the soluble chromate (VI) reductase ChrR (Ackerley *et al.*, 2004b). Additionally, there have been independent reports of other *Pseudomonas* strains that can grow anaerobically with chromium (VI) as the terminal electron acceptor (Kvasnikov *et al.*, (1985); Lebedeva and Lyalikova (1979); Romanenko and Korenkov (1977); Gvozdyak *et al.*(1986)) this bacterium can also reduce chromate under both aerobic and anaerobic conditions (Bopp and Ehrlich, 1988). *Escherichia cloacae* HO1, a chromate-resistant strain, is the most studied example of Cr (VI)-reducing bacteria. *Bacillus* sp. QC1-2, a strain isolated from a Cr-polluted zone, can also tolerate chromate and reduce Cr (VI) to Cr (III). Cr (VI)-reducing activity was also found to be present in both soluble and membrane fractions of the anaerobic

bacterium *Desulfovibrio vulgaris*. Cytochrome *C3* was reported to catalyze Cr (VI) and uranium [U (VI)] reduction in *D. vulgaris*, suggesting that this cytochrome may function as both U (VI) and Cr (VI) reductase. A *Bacillus* strain *QC1-2*, isolated from a chromium-polluted zone, was selected for its high ability to both tolerate and reduce hexavalent chromium [Cr (VI)] to less-toxic trivalent chromium [Cr (III)]. Cell suspensions of strain QC1-2 rapidly reduced Cr (VI), under both aerobic and anaerobic conditions, to Cr (III) which remained in solution. Cr (VI) reduction was found to depend upon the addition of glucose but not sulphate, an inhibitor of chromate transport. A strain of *Enterobacter cloacae* (HO1) capable of reducing hexavalent chromium (chromate) has been isolated from activated sludge. This bacterium resists chromate under both aerobic and anaerobic conditions but only the anaerobic culture of the *E. cloacae* isolate shows chromate reduction. Under anaerobic conditions, the yellow colour turned white with chromate and the turbidity increased with reduction, suggesting that insoluble chromium hydroxide was formed. *Enterobacter cloacae* is likely to utilize chromate as an electron acceptor anaerobically because (i) the anaerobic growth of *E. cloacae* HO1 was associated with a decrease in the chromate in the culture medium, (ii) the chromate-reducing activity was rapidly inhibited by oxygen, and (iii) the reduction occurred more quickly in glycerol- or acetate-grown cells than in cells grown in glucose. Chromate reduction in *E. cloacae* HO1 was observed at pH 6.0 to 8.5 (optimum pH, 7.0) and at 10 to 40°C (optimum, 30°C) (Wang *et al.*, 1989). Washed cell suspensions of *Desulfovibrio vulgaris* were shown to rapidly reduce Cr (VI) to Cr (III) with H_2 as the electron donor, with the c3 cytochrome of this organism functioning as a Cr (VI) reductase. *Desulfovibrio vulgaris* may turn out to have advantages over previously described Cr (VI) reducers for use in bioremediating Cr (VI)-contaminated waters. Individual cell microanalysis by transmission electron microscopy (TEM) using electron energy-loss spectroscopy (EELS) and energy dispersive X-ray spectroscopy (EDXS) demonstrated that Cr (II) is concentrated near the cytoplasmic membrane, thereby suggesting that the terminal reduction pathway is located within the cell. Further, estimated cellular Cr (II) concentrations are relatively high, at 0.03–0.09 g Cr g^{-1} bacterium (Daulton *et al.*, 2007). *Shewanella oneidensis* was also studied by Myers *et al.* (2000), using

cytoplasmic-membrane-bound Cr (VI) reductases (under anaerobic conditions where Cr (VI) was the only available terminal electron acceptor, forming Cr (III) precipitates on their outer surface; the results showed that *Shewanella oneidensis* can reduce Cr (VI) to Cr (III).

Numerous factors influence the microbial reduction of Cr (VI); for example, the presence of either aerobic or anaerobic conditions (Cheung and Gu, 2003, Chen and Hao, 1998 and Wang and Shen, 1995), or the availability of energy sources such as sulphur (for *Acidithiobacillus thiooxidans*) (Viera *et al.*, 2003) and glucose (for *P. fluorescens* LB300) (Bopp and Ehrlich, 1988), or other suitable organic materials. Natural organic matter (NOM) can also play a major role in microbial Cr (VI) reduction (Gu and Chen, 2003). The addition of organic carbon to soil has been shown to accelerate Cr (VI) reduction (Tokunaga *et al.*, 2003).

Komori *et al.* (1989) investigated the factors which affect chromate reduction by cultures of *Enterobacter cloacae HO1*. They found that reduction was sensitive to oxygen stress and that *E. cloacae HO1* could reduce chromate only anaerobically. Rates of reduction of chromate were proportional to cell number, the optimal pH being between 7.0 and 7.8, and the optimal temperature, 30°C–37°C. High rates of reduction were observed at concentrations of 1–2 mM potassium chromate, but concentrations above 5 mM killed growing cells and prevented the reduction. Substrates like acetate, ethanol, malate, succinate and glycerol all proved to be effective electron donors for chromate reduction. Glucose, citrate, pyruvate and lactate supported anaerobic growth, but only limited amounts of Cr reduction were observed. Chromate reduction by strain HO1 was inhibited by molybdate, vanadate, tellurate and manganese oxide, even at concentrations where the cell viability was not significantly affected. Metabolic poisons, notably carbonylcyanide-m-chlorophenyl hydrazone, sodium cyanide, formaldehyde and zinc sulphate also inhibited chromate reduction.

Bacteria can reduce chromate to the insoluble and less toxic Cr (III), and thus chromate bioremediation is of interest, and genetic and protein engineering of suitable enzymes might lead to improvements in bacterial bioremediation. Many bacterial enzymes catalyze one-electron reduction of chromate, generating Cr (V),

which redox cycles, generating excessive reactive oxygen species (ROS). These enzymes are not however, useable in bioremediation, since they harm the bacteria and their primary end product is not Cr (III).

The reduction of hexavalent chromium (chromate) to less-toxic trivalent chromium was studied by using cell suspensions and cell-free supernatant fluids from *Pseudomonas putida* PRS2000. Chromate reductase activity was associated with soluble protein and not with the membrane fraction. The crude enzyme activity was heat labile and showed a Km of 40 μM CrO_4^{2-}. Neither sulphate nor nitrate affected chromate reduction either *in vitro* or with intact cells (Fude *et al.*, 1994).

Most of the published studies on biological reduction of Cr (VI) have been conducted in batch reactors (flasks), using pure cultures. For example, Wang and Xiao (1995), studied several factors which affect Cr (VI) reduction in bacteria, while Wang and Shen (1997), studied the kinetics of Cr (VI) reduction by pure bacterial *in vitro.* Shakoori *et al.,* (2000), also isolated a dichromate-resistant Gram-positive bacterium from tannery effluent using flasks as batch reactors. Fein *et al.*, (2002), used pure bacterial cultures to study the non-metabolic reduction of Cr (VI) by bacteria under nutrient-absent conditions. Srinath *et al.* (2002), studied Cr (VI) biosorption and bioaccumulation by pure cultures of chromate resistant bacteria *in vitro.* Finally, Megharaj *et al.* (2003) studied hexavalent chromium reduction in flasks, by bacteria isolated from soil contaminated with tannery waste.

Recently, continuous-flow and fixed-film bioreactors have been used for the biological reduction of Cr (VI). Shen and Wang (1995) demonstrated Cr (VI) reduction in a two-stage, continuous-flow suspended growth bioreactor system. *Escherichia coli* cells grown in the first-stage completely mixed reactor were pumped into the second-stage plug-flow reactor to reduce Cr (VI). Chirwa and Wang (1997) also demonstrated the potential of fixed-film bioreactors for Cr (VI) reduction. This proved to be the first report on Cr (VI) reduction through biological mechanisms, using a continuous-flow laboratory-scale biofilm reactor which was not constantly re-supplied with fresh Cr (VI)-reducing cells; *Bacillus* sp. was used in this work for the transformation of Cr (VI) into Cr (III).

Pure cultures, and also modified consortia were used by Molokwanne and Chirwa, (2006) to reduce Cr (VI). A high Cr (VI) reduction rate was found in cultures which had been modified by substituting terminal organisms in the consortium with a known Cr (VI)-reducing organism (*Escherichia coli A*TCC 33456), and, in the pure culture, using the Cr (VI)-reducing bacteria (*Bacillus* sp.).

A consortium of bacteria with high tolerance to high concentrations of Cr (VI) (up to 2,500 ppm) and other toxic heavy metals has been isolated in China from metal- refinishing wastewaters (Fude *et al.*, 1994). This consortium involved various gram-positive and gram-negative rods and could reduce Cr (VI) to Cr (III) giving an amorphous precipitate associated with the bacterial surfaces. An endospore-producing, gram- positive rod and a gram-negative rod were found to accumulate the most metallic precipitates, and, over time, 80 to 95% of Cr was removed from solutions containing concentrations ranging from 50 to 2,000 ppm (0.96 to 38.45 mM).

Essentially all the previously reported studies on biological reduction of Cr (VI) have been conducted using laboratory scale apparatus (reactors), using sterilized conditions and pure cultures. The first report concerning Cr (VI) biological reduction in a pilot-scale trickling filter, which used a mixed culture of microorganisms from an industrial sludge, was done by Dermoua (2005), showed that the trickling filter as a Sequencing Batch Reactor (SBR) with recirculation produced significantly higher Cr (VI) reduction rates, thereby pointing to a feasible technological solution to what has become a serious environmental problem.

Electron paramagnetic resonance (EPR) measurements, sensitive to non-integer spin transition metals such as Cr (I), Cr (III), and Cr (V), indicate that Cr (V) is a possible redox intermediate for Cr (VI) reduction in *Pseudomonas ambigua* (G-1) (Suzuki *et al.*, 1992), *Arthrobacter oxydans* (Kalabegishvili *et al.*, 2003), and *Shewanella oneidensis* (Myers *et al.*, 2000). The use of X-ray photoemission spectroscopy in studies of batch cultures of *S. oneidensis* with chromate have been unable to detect evidence for Cr (V) reduction intermediates.

However, evidence for Cr (IV) intermediates at cell surfaces has been suggested (Neal *et al.*, 2002).

It is widely believed that reduction to Cr (III) is the final step in the microbial Cr (VI) reduction chain, essentially because during this process, bacterial cells become encrusted with Cr-rich precipitates (Wang *et al.*, 1990 and Fude *et al.*, 1994).

The assumed termination of the microbial reduction pathways at Cr (III) has important implications for the mechanisms of chromate reduction particularly for Cr (VI)-reducing bacteria with membrane-bound reductases. For example, Cr (VI) reductases are reported to be localized to the cytoplasmic membrane of *S.oneidensis* (Myers *et al.*, 2000), as well as being associated with membranes in *Enterobacter cloacae* (Wang *et al.*, 1990), *Pseudomonas fluorescens* (Bopp and Ehrlich, 1988), and *Pseudomonas maltophilia* (Blake *et al.*, 1993).

It is not clear that cellular uptake of toxic Cr (VI) occurs with reduction localized to the cytoplasm or periplasm, and/or electrons are transferred outside of the cells to extracellularly reduce chromium. Since Cr (III) compounds are relatively soluble only under very acidic or very basic conditions (Rai *et al.*, 1987 and Rai *et al.*, 1989) and their soluble organo-complexes (Puzon *et al.*, 2005) are large molecules that are not easily transported across the outer membrane. Bacteria with membrane-bound reductases have problems reducing Cr (VI) to Cr (III) by processes that are extracellular (Wang *et al.*, 1990), e.g. by using electron-shuttling compounds which are coupled to membrane reductases. Alternatively, a mechanism of Cr (III) mobilization and transport must be present in order to remove Cr (III) from the cell; it is assumed that such a process would involve the expenditure of cellular metabolic energy (Daulton and Little, 2006).

Both NADH and endogenous cell reserves may serve as electron donors for Cr (VI) reduction under anaerobic conditions, electron transport systems containing cytochromes seem, to be involved in Cr (VI) reduction. High cell densities are necessary in order to obtain a significant rate of Cr (VI) reduction. Cr (VI) reduction by bacteria is often inhibited by Cr (VI), oxygen, heavy metals, as well as various phenolic compounds. The optimum pH and temperature observed for Cr (VI) reduction in the main coincides with the optimal growth

conditions of cells; the optimum redox potential for Cr (VI) reduction has yet to be established.

1.7.3 Fungal Reduction of Chromium

The fact that fungi can be employed to remove chromium from leather tanning effluent was recently demonstrated by Srivastava and Thakur, (2006). Five morphologically different fungi were isolated, and *Aspergillus* sp. and *Hirsutella* sp. were shown to have the highest potential to remove chromium. The ability in *Aspergillus* sp. was evaluated *in vitro* under varying pH, temperature, inoculum size, carbon, and nitrogen source. The maximum amount of chromium was removed at pH 6, a temperature of 30 °C, sodium acetate (0.2%) as carbon, and yeast extract (0.1%) as nitrogen sources, and some 70% of the chromium was removed after 3 days. Synthetic wastewater containing Cr (VI) (Park *et al.*, 2005) was placed in contact with the dead fungal biomass of *Aspergillus niger*, and the Cr (VI) was completely removed from aqueous solution; Cr (III), which was not initially present, then appeared in aqueous solution. Park *et al.* (2005), found that four fungal strains – *Aspergillus niger*, *Rhizopus oryzae*, *Saccharomyces cerevisiae* and *Penicillium chrysogenum* completely removed Cr (VI) from aqueous solutions, with *R. oryzae* being the most effective. Cr (VI) was removed from aqueous solutions by the reduction to Cr (III) when in contact with the biomass. The removal rate of Cr (VI) increased as pH decreased and with increases in Cr (VI) and biomass. This suggests that Cr (VI) is removed via a redox reaction. Such abundant and inexpensive waste fungal biomass could be used for the conversion of toxic Cr (VI) into less toxic or nontoxic Cr (III) on a practical scale. Prasenjit and Sumathi, (2005), reported that *Aspergillus foetidus* can take up chromium during the stationary phase of growth and under growth-non-supportive conditions. They found that a 97% decrease in hexavalent chromium (initial concentration 5μg g^{-1}) occurred at the end of 92 hours of growth, which may be due to its reduction to Cr (III) and/or complexation with organic compounds which was released as a result of the organism's metabolic activity. They also noted that replacement culture studies under growth-non-supportive conditions revealed that the maximum uptake of Cr (VI) at pH 7.0 is

20 mg g^{-1} of dry biomass. Finally, they noted that at low or high pH values, Cr (VI) uptake is significantly reduced; additionally, the initial rate of total chromium uptake is also increased when higher biomass concentrations are use in the presence of glucose. It is possible therefore that *Aspergillus foetidus* could be used to treat waste effluents containing Cr (VI).

Yeasts can be used as a useful means of bioremediating environmental chromium (Pepi and Baldi, 1992; Volesky and May-Phillips, 1995; Engl and Kunz, 1995; Krauter *et al.*, 1996; Stoll and Duncan, 1996; Batic and Raspor, 1998; Haq and Shakoori, 1998). Yeasts have also been found to be very efficient at obtaining chromium-enriched biomass used for balanced nutrition of mammals and humans (Raspor *et al.*, 2000; Batic and Raspor, 2000).

1.7.4 The Reduction of Chromium by Green Algae and Higher Plants

The reduction of Cr (VI) by green algae and higher plants has been investigated using a low-frequency EPR spectrometer equipped with an extended loop gap resonator (Liu *et al.*, 1995). Incubation of algae (*Spirogyra* and *Mougeotia*) with Cr (VI) generated both Cr (V) and Cr (III). The maximum Cr (V) signal was observed in about 10 minutes. Incubation of Cr (VI) with oat, soybean, and garlic generated Cr (V) (Liu *et al.*, 1995). The maximum Cr (V) peak appeared after more than 10 hours of incubation, and Cr (V) was located predominantly in the roots. The Cr (V) peak exhibited hyperfine splittings of about 0.79 Gauss, which is typical of Cr (V) complexes with diol-containing molecules. The results suggest that the reduction of Cr (VI) to lower oxidation states by living plants can provide a means of detoxifying Cr (VI) in natural systems. The results also show that low-frequency EPR can be used to study the metabolism of paramagnetic metal ions in intact plants (Liu *et al.*, 1995). In another study Gupta *et al.* (2001), evaluated the biosorption capacity of the algae *Spirogyra* sp. (a green filamentous alga) for Cr (VI) from aqueous solutions. This alga absorbed 1.47 g of chromium/kg of dry weight biomass at a pH of 2.0 in 120 min, from an initial chromium (VI) concentration of 5 mg/l.

1.8 Introduction to Methanotrophs

Methanotrophs metabolise methane as their sole source of carbon and energy and so must oxidise methane to methanol as the initial step in their metabolic pathway. The unique defining enzyme activity of methanotrophs, which catalyses the oxidation of methane is methane monooxygenase (MMO). Methanotrophs have been extensively studied because of their role in global methane oxidation, for bioremediation of organic and chlorinated organic pollutants and as potential biocatalysts for synthesis of bulk and fine chemicals synthesis (as detailed below). However, the potential of this environmentally widespread group of bacteria for bioremediation of heavy metal pollution has not been extensively studied.

A very effective review was presented by Murrell and Radajewski (2000) for the detection and characterisation of methanotrophs in the natural environment. Also McDonald *et al* (2005) analysed both phylogenetic and functional genes to determine methanotroph diversity. Murrell (1992) summarised the genetics and molecular biology of methanotrophs. Methanotrophs are a group of gram-negative bacteria which grow aerobically, and have been isolated from various environments, including soils, sediments and freshwater. The oxidation of methane in anaerobic environment is also recognized, but little information has been published on this topic. Some methanotrophs take up and metabolise different one-carbon compounds, such as methanol, methylated amines, halomethanes and methylated compounds. Facultative methanotrophs, able to grow on multicarbon substrates in addition to methane, have now also been isolated (Dedysh et al., 2005).

1.8.1 Classification of Methanotrophs

Methanotrophs were first isolated in 1906, but it was not until 1970 when Whittenbury and co-workers (1970) isolated and characterised some 100 different strains of methane oxidising bacteria. These were separated into five groups depending on their morphology, intracytoplasmic membrane, types of resting stage, as well as other physiological and morphological traits. Methanotrophs can

be divided into two distinct groups, namely Type I and Type II, depending on various characteristics. Type I methanotrophs belong to the γ-subunit of the Proteobacteria and include the genera *Methylococcus*, *Methylomonas*, *Methylomicrobium*, *Methlobacter*, *Methylocaldum* and *Methylosphaera* (Table 1.2). They assimilate formaldehyde produced from the oxidation of methane via the ribulose monophosphate (RuMP) pathway (Table 1.2). This compound is an important metabolic branch point between dissimilation to produce energy and assimilation to make new multi-carbon compounds for proteins, DNA and lipids.

Table 1.2: Characteristics of type I and type II methanotrophs phospholipid fatty acid – Murrell *et al.* (1999)

Genus	Phylogeny	Type	Pathway	Type
Methylobacter	Gamma Proteobacteria	Type I	RuMP	pMMO
Methylocaldum	Gamma Proteobacteria	Type I	RuMP/Serine	pMMO
Methylococcus	Gamma Proteobacteria	Type I	RuMP/Serine	pMMO/sMMO
Methylomicrobium	Gamma Proteobacteria	Type I	RuMP	pMMO/sMMO
Methylomonas	Gamma Proteobacteria	Type I	RuMP	pMMO/sMMO
Methylosphaera	Gamma Proteobacteria	Type I	RuMP	pMMO
Methylocystis	Alpha Proteobacteria	Type II	Serine	pMMO/sMMO
Methylosinus	Alpha Proteobacteria	Type II	Serine	pMMO/sMMO

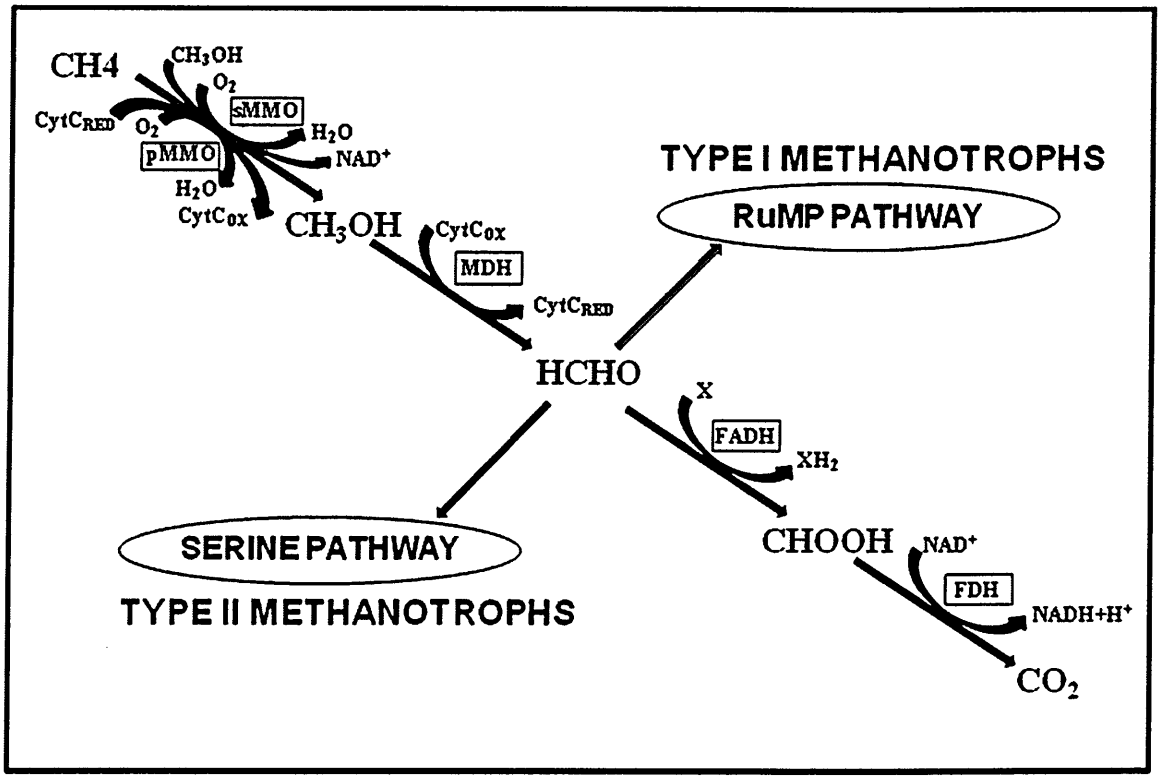


Figure 1.2: Pathways for the oxidation of methane and assimilation of formaldehyde – Hanson *et al.* (1996).

The type I organisms contain mainly 16-carbon fatty acids and have bundles of intracytoplasmic membranes. The Type II methanotrophs, on the other hand, assimilate formaldehyde by use of the serine pathway and have apparently concentric rings of intracytoplasmic membranes around the outer region of the cytoplasm. They have membranes that contain 18-carbon fatty acids in their membrane.

Type II methanotrophs belong to the α-subunit of the Proteobacteria and include the genera *Methylosinus*, *Methylocystis* and *Methylocella*. A new group of methanotroph called type X has been added; these are similar to type I in that they utilise RuMP for the formaldehyde pathway, but they differ in that they possess low levels of enzymes of the serine pathway (Figure 1.2).

These bacteria also grow at higher temperature than type I and type II and have high G + C content than most type I ; *M. capsulatus,* the principal subject of this study belongs to this group. Phylogenetic analysis of methanol dehydrogenase gene mxaF sequences show type I and type II methanotrophs to form two different clusters (McDonald and Murrell 1997a). The particulate methane monooxygenase gene pmoA as a phylogenetic marker for identifying methanotroph_specific DNA sequences is also established by McDonald and Murrell (1997b).

Two types of methane monooxygenases exist in methanotrophs. A soluble cytoplasmic enzyme complex (sMMO) is found only in a few methanotrophs, and a membrane-bound particulate enzyme complex (pMMO) is present in all known methanotrophs (Murrell *et al.*, 2000a).

1.8.2 Soluble Methane Monooxygenase (sMMO)

sMMO has been extensively characterised from *Methylococcus capsulatus* and *Methylosinus trichosporium,* although it is clear that not all methanotrophs express the sMMO enzyme. The few strains that are capable of expressing sMMO are dependent on the copper / biomass ratio, although, there is also evidence to suggest that copper inhibits sMMO activity. Unlike pMMO, sMMO has a broad substrate specificity, a fact that makes bacteria that express it attractive for use in biotransformation and bioremediation processes.

sMMO contains three protein components, a hydroxylase, a reductase and a regulatory protein B, all of which are required for the activity of sMMO in the oxidation of methane to methanol. The hydroxylase consists of three-polypeptide subunits: α, β and γ (α_2, β_2, γ_2 Complex), with sizes ~ 60, 45 and 25 KDa respectively (Figure 1.3).

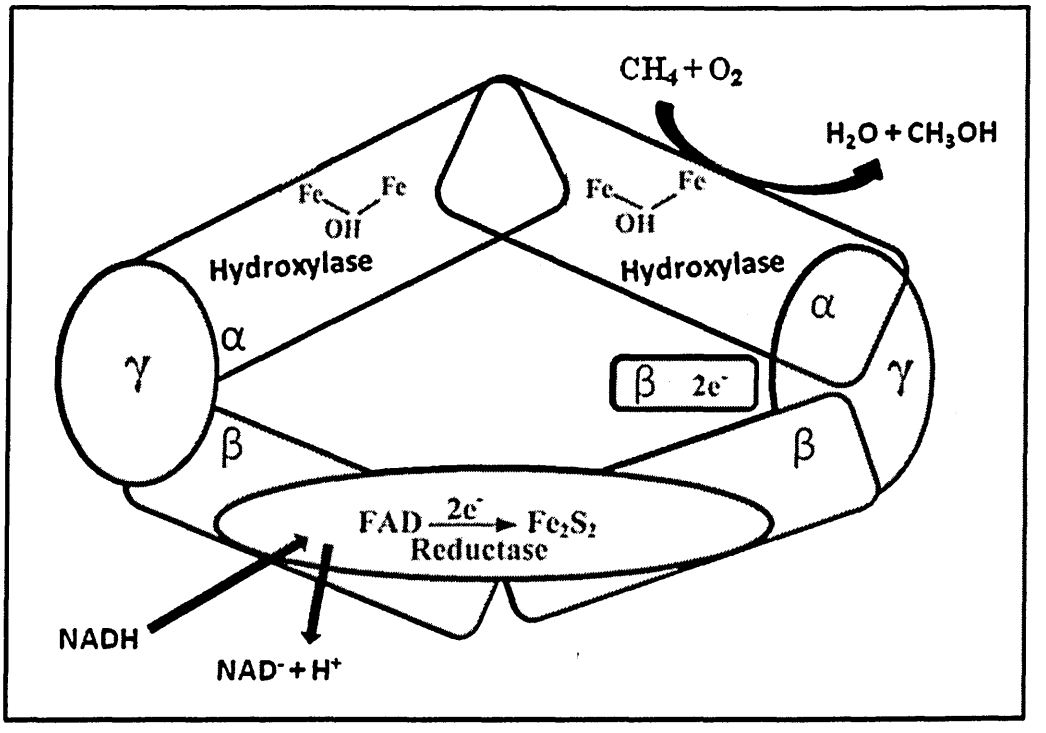


Figure 1.3: Active site of the α-subunit of the hydroxylase component *(Reproduced from Murrell et al. 2000).*

1.8.3 Environmental Application of Methanotrophs

There is an increasing interest in methanotrophs and they are now used to produce single cell protein and bulk chemicals. The fact that methanotrophs catalyse a large number of biotransformations such as the co-oxidation of a wide range of alkanes, alkenes and substituted aliphatic compounds, has led scientists to develop biochemical methods for the degradation of a variety of toxic chemicals.

There has been much speculation about the origin of the remarkably broad substrate specificity of sMMO. One possibility is that methane is a very unreactive hydrocarbon and so the enzyme must produce at its active site one of the most powerful oxidising agents in the biological world, containing iron in the ferryl (+4) oxidation state (Shu *et al.*, 1997). Hence almost any hydrophobic organic compound that can gain access to the active site will be oxidised.

1.8.4 Methanotrophs Application in Bioremediation Processes

The most successful applications of methanotrophs in bioremediation to date have been for the oxidation of trichloroethylene (TCE), which is a major pollutant in both soil and groundwater. The initial product from monooxygenation of TCE by methanotrophs is TCE-epoxide, which is very reactive intermediate (Arp, 1995). Because of its instability, TCE-epoxide will decay non-enzymatically to a number of products. First, acyl halides form, and then these then decompose further to formate, CO, glyoxlic acid and dichloroacetic acid. Glyoxylate and formate were the only aqueous intermediates found from TCE oxidation by the methanotroph *Methylomicrobium* BG8.

1.9 Biophysical Analytical Techniques

Several biophysical techniques will be used in this study to monitor the metabolic process, to determine oxidation states, to investigate the environment of chromium, and to view its distribution within the cell.

1.9.1 X-ray Absorption Spectra

X-ray adsorption spectroscopy (XAS) is a synchrotron–based technique which gives information concerning the local structure and bonding around the adsorbing element in material, via analysis of the fine structure associated with one of the element's characteristic X-ray absorption edges. This technique is capable of such determinations with a detection limit for chromium oxidation states in solids in the $\mu g.g^{-1}$ range.

1.9.2 Extended X-Ray Absorption Fine Structure

Extended X-ray absorption Fine Structure (EXAFS) is the observation of the oscillating part of the X-ray absorption spectrum. This extends ~ 1000 eV beyond the adsorption edge of the element under invertigation. The EXAFS phenomenon is observed when incident X-rays knock electron out of the metal atom. When knocked out of the atom these photoelectons are subject to interference from the wave functions of the surrounding atoms, such interference provides information on the environment of the subject atom, notably the co-

ordination environment. EXAFS is carried out on samples in solution at room temperatures. Therefore, in the case of biological samples information obtained provides data which is directly relevant to the native solution structure of the element under study. In an investigation of the effect of microorganisms on chromium contaminated soil EXAFS was used to investigate the environment of cellular chromium (Desjardin *et al.,* 2002). In this study the chromium was shown to be concentrated in a CrOOH-like environment, which is sparingly soluble.

1.9.3 Transmission Electron Microscopy

Transmission electron microscopy (TEM)–electron energy-loss spectroscopy (EELS) techniques have been used to determine the mechanisms of Cr (VI) reduction and precipitation by *S .oneidensis* under anaerobic conditions (Daulton and Little, 2006). Both TEM and scanning electron microscopy (SEM) have sufficient resolution to be useful in the study of the spatial relationship between cells and the reduction products. Energy – dispersive X-ray spectroscopy (EDXS) has been used to identify elements present in reduction products associated with bacteria and plants. Electron energy loss spectroscopy (EELS) by TEM is a direct probe of the electron configuration around atoms, and can determine the oxidation state of 3d and 4d transition metals at high spatial resolution. In addition, EELS techniques have been used to produce oxidation state maps using energy filtered imaging.

1.10 The Aims of the Work Reported in This Thesis

Chromate (VI) reductase activity, capable of reducing chromium from the +6 to the +3 oxidation state, has previously been characterised in aerobic bacteria and facultative anaerobes such as *Escherichia coli*, *Pseudomonas putida* (Ackerley *et al.*, 2004a and b; Gonzalez *et al.*, 2005; Puzon *et al.*, 2002), *Paracoccus denitrificans* (Mazoch *et al.*, 2004) and *Bacillus subtilis* (Morokutti *et al.*, 2005), as well as in anaerobic sulphate-reducing bacteria (Fude *et al.*, 1994; Smith and Gadd, 2000).

Here, the chromate-reducing properties of methane-oxidising bacteria, which are ubiquitous in the environment (Hanson and Hanson, 1996), have been

investigated for the first time. The interaction of chromate (VI) has been studied with well characterised representatives of the two major groups of methane-oxidising bacteria, the type I methanotroph *Methylococcus capsulatus* (Bath) and the type II methanotroph *Methylosinus trichosporium* OB3b. *Methylococcus capsulatus* Bath has the additional advantage that a complete genome sequence is available (Ward *et al.*, 2004).

Moreover, one of the principal purposes of this study is to employ Analytical Techniques in order to confirm the bioremediation processes for removal of Cr (VI) pollution by methanotrophs. This included use of transmission electron microscopy (TEM)–electron energy-loss spectroscopy (EELS) techniques. Furthermore it was intended to find out if these techniques are appropriate for monitoring the bioremediation processes, to determine the oxidation states, to investigate the environment of chromium, and to view its distribution in the cell. The main aims of this thesis are as set out below:

- The investigation of Cr (VI) reduction in the presence of methane-oxidising bacteria which have not previously been tested for such activities, and
- To characterise the reaction as far as possible at the molecular and cellular level.

The present work addresses bioremediation and has the following objectives:

1. To implement techniques of analysis by monitoring bioremediation of chromium (VI) as described in the literature using *E. coli*.
2. To investigate the possibility of bioremediation of chromium (VI) using *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath.
3. In the event that the methanotrophs did not demonstrate chromium (VI)-reducing activity in pure culture, to investigate the possibility of using a mixed culture of methanotrophs and a known chromium-utilising heterotrophic bacterium.
4. To investigate any newly found chromium reductase activities in the methanotrophs, both experimentally and via bioinformatics, including identification of potential chromate (VI) reductases from the genome sequence of *M. capsulatus* (Bath).

2.1 MATERIALS

2.1.1 Chemicals

Diphenylcarbazide (DPC) reagent, sodium dithosulphate, EDTA and sodium azide (Na N_3), were supplied by Sigma. Ammonium nitrate ($NH_4 NO_3$) and silica gel were supplied by Fisher scientific, UK. Sodium hydroxide (NaOH) Analar, was obtained from BDH chemicals, UK. Acetone was supplied by Sciences, UK. All other chemicals were supplied by Sigma, Fisher or BDH and were of analytical grade where possible. The water used was glass-distilled and then deionised.

2.1.2 Bacterial Strains and Growth Conditions

The following bacterial strains were used in this study:

- *Escherichia coli* ATCC 33456, obtained from (LBC Company. UK).
- *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium OB3b* were obtained from the culture collections of Howard Dalton and Colin Murrell (University of Warwick).

The methanotrophs *M. trichosporium* OB3b and *M. capsulatus* (Bath) were grown and propagated aerobically in nitrate minimal salts (NMS) medium or on NMS agar (Dalton and Whittenbury, 1976) using methane (1:4 v/v in air) as the source of carbon and energy. All cultures and chromate-reduction experiments were incubated at the optimal growth temperature of the organism concerned, 30°C and 45°C for *M. trichosporium* OB3b and *M. capsulatus* (Bath), respectively. Except where stated otherwise, chromate reduction experiments were performed in 50 mL liquid cultures in 250-mL conical Quickfit flasks sealed with a Subaseal (Fisher) to prevent loss of methane whilst allowing addition of liquids and the taking of samples using hypodermic syringes. Cultures were allowed to grow to an OD600 of 0.3-0.8 before addition of potassium chromate (VI) or potassium dichromate (VI) to give the concentration of hexavalent chromium stated for each experiment. Fermentor cultivation of *M. capsulatus* (Bath) was performed using methane as the source of carbon and energy according to the published method (Pilkington and Dalton, 1990),

in a Bioflo 110 fermentor (New Brunswick Scientific, vessel capacity 4.5 L) fitted with an additional rotameter to allow simultaneous gassing with methane and air.

2.1.3 Equipment

The pH meter Centrifuge: ALC micro-centrifuge 4214 was supplied by Fisher Scientific, Loughborough, UK.

Spectrofluorimeters: Hiatchi model 1010 (Hitachi Instruments Slough, UK), Perkin Elmer model LC-240 (PE Instruments Beaconsfield, UK) were used.

Orbital incubators: were used from Stuart Scientific S150 and Gallenkamp, UK.

Inductively coupled plasma-mass spectrometer (ICP-MS) model Hewlett Packard (HP) 4500, Yokogawa Corporation, Japan was used. Oxygen electrode, (BBC GORZ Metrawatt SG 120, UK) and Ion chromatography (IC) also used with ICP-MS system. Using a guard column type Dionex, 4×50 mm. The transmission electronic microscope used was a CM20 TEM (Philips) and fermentors (Bioflo 110 fermentor) was supplied by New Brunswick Scientific,USA.

2.1.4 Media

NMS agar medium containing 15 g of bacteriological agar (Oxoid) per litre was used. Phosphate buffer was added when cooled to 50°C, just before pouring on the sterile plates.

2.2 METHODS

2.2.1 Strain and Culture Process

The bacterial strain *Escherichia coli* ATCC 33456 was used as the chromium-reducing organism. After suspension in sterile liquid medium, 0.5 ml of *E. coli* cells were added to 0.5 ml of glycerol and kept in freezer at -20°C, and then transferred to slopes and agar plates and incubated at 30°C, as required, after checking for contamination. Bacteria were cultured for 2 days at 30°C, with shaking at 180 rpm. One ml of the inoculum was then transferred aseptically to the 50 ml of the culture-working medium and incubated for12 hours as above. A mixed culture of *E. coli* ATCC 33456 and methanotrophs of *Methylosium trichosporium* (OB3b) were

also used in some experiments. Here the inoculum was prepared by inoculating liquid NMS with colonies of both *E. coli* and *M. trichosporium* and adding methane as the sole source of carbon and energy. This reliably results in a mixed culture in which the methaotrophs grow using methane as the carbon source and the *E. coli* scavenge multi-carbon compounds from them.

The methanotrophic strain of *Methylococcus capsulatus* was sub-cultured on NMS agar plates and incubated inside anaerobic jars in the presence of methane at 45°C for 4 to 5 days cultivated using methane as the growth substrate.

2.2.2 Inoculum and Culture Process

Bacterial culture was performed in a working volume of 50 cm^3 NMS medium prepared in two conical Quikfit flasks. Flasks were inoculated with pure colonies of *M. capsulatus* and incubated at 45°C using an orbital shaker incubator at 180 to 200 rpm. Flasks were fitted with sterile bug stoppers (Bug stopper™, Erlenmeyer vent, Whatman®, Whatman Inc., Clifton, NJ, USA) and then 50 cm^3 of air were removed using a sterile plastic syringe (Terumo) fitted with hypodermic needle (Terumo), the air was then aseptically replaced with 60 cm^3 of methane.

The same process was performed following the addition of chromium (VI) to the culture, with the same concentration of the element being added to the control flasks, containing the medium plus either dead bacteria or just culture medium.

Under aseptic conditions the inoculated flasks were checked for any possible contamination using nutrient agar plates by utilising the fact that *M. capsulatus* does not grow on nutrient agar plates.

2.2.3 Analytical Measurements

2.2.3.1 Optical Density (O.D) Measurements

In all batches, the O.D level was measured using a spectrophotometer (Fisher Scientific, Loughborough, UK) at a wavelength of 450 nm. Measurements were made after 12 hours. Possible contaminations also checked during sampling time, by taking samples from the cultures and streaking them on nutrient agar plates, and were incubated for 24 hours at 45°C.

2.2.3.2 *Preparation of Chromium (VI) Stock Solution*

A stock solution containing 1.27 mg L^{-1} chromium was prepared by dissolving 36 mg of $K_2Cr_2O_7$ in10 ml of concentrated sulphuric acid and the solution was made up to 1000 ml with deionised water).

2.2.3.3 *Preparation of Chromium (VI) Standard*

A range of Cr (VI) concentrations in H_2SO_4 were prepared as follows:

a) The standard solution prepared over a range of Cr (VI) concentrations (e.g. 0-500 μgl^{-1}) with 0.18 M H_2SO_4, including a blank.

b) 0.5 ml of diphenylcarbazide solution was added to each flask, the contents mixed and then flasks allowed to stand for 5 minutes in order to develop the colour.

c) After measurement of the absorbance of these solutions, the absorbance was plotted against chromium concentration.

2.2.3.4 *Preparation of Chromium (III) Standard*

The chromium (III) standard was an aqueous solution of chromium (III) chloride that was prepared to give the same concentration of chromium as in the chromium (VI) standard in each experiment. For X-ray spectroscopy, solid chromium (III) chloride was also used as a standard.

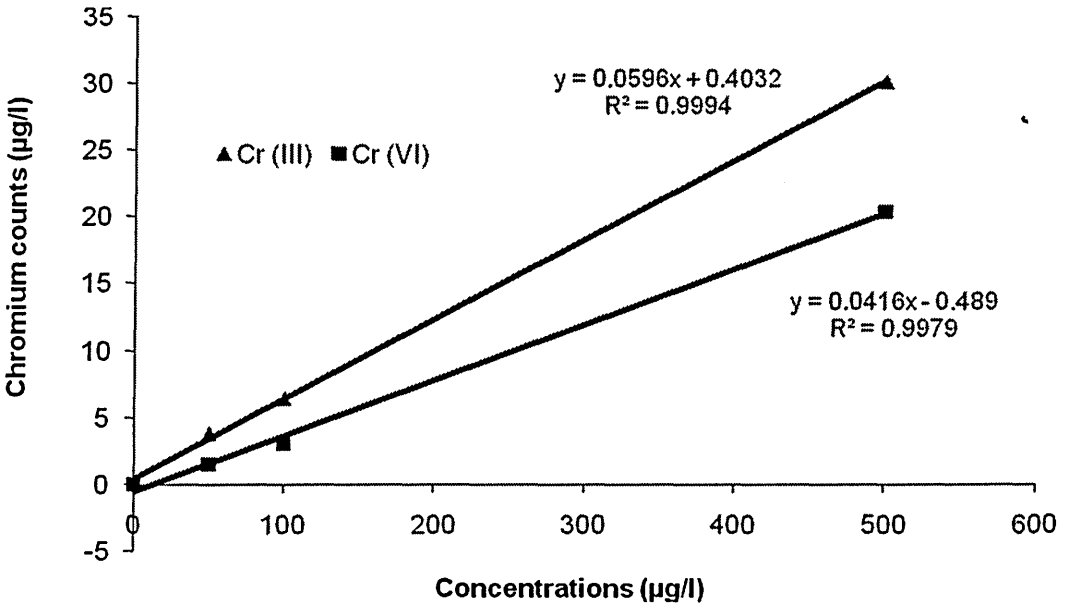


Figure 2.1: Standard curve of chromium (III) and chromium (VI) measurement by ICP-AES.

2.2.3.5 Determination of Chromium in the Samples

Measurement of chromate (VI) concentrations was performed by means of the diphenylcarbazide assay, as follows. Cells and other particulate material were removed from liquid samples by centrifugation (5,000 *g*; 5 min; room temperature) and the supernatant was acidified by addition of 3 M of H_2SO_4 to give a final concentration of 0.18 M. 1.0 mL of the acidified supernatant was then mixed with 0.05 mL of diphenylcarbazide solution (2.5 g/l in acetone) and the chromate (VI) concentration was estimated spectrophotometrically at 540 nm by reference to a standard curve of known chromate concentrations (0.05-1.27 mg/l) prepared similarly. Where necessary, samples were diluted with 0.18 M H_2SO_4 before assay to bring the absorbance within the range of the standards.

DPC reagent, 1, 5 diphenlycabozide stock solution was prepared by dissolving in acetone/water (v/v) plus H_2SO_4, with stirring. Both the stock and diluted solutions were stored at room temperature in the dark. Furthermore, the standard curve for both chromium (VI) and chromium (III) measurements via diphenylcarbazide assay is presented in Figure 2.1.

2.2.3.6 *Total Chromium Measurement*

Total chromium was analysed using Inductively Coupled Atomic Emission Spectroscopy (ICP-AES). The same procedure preparation was applied to both the sample and the standard of chromium (IV) solution, 1, 5 diphenylcarbazide reagent was added for colour developing.

2.2.3.7 *Cell Extraction*

The chromium associated with the bacterial cells was extracted by digesting the cells with 6 M of HNO_3 in a water bath at between 40°C to 45°C for 1 to 2 hours. The same total chromium assay was applied to all samples.

2.2.3.8 *Chromium (III) and Chromium (VI) Speciation*

Both chromium (III) and chromium (VI) were determined using ion chromatography inductively coupled plasma mass chromatography (IC-ICP-MS), using a regeneration suppressor based on the method recommended by Hakan and Dirk (2001).

The mobile phase was prepared using 4 g of ammonium nitrate ($NH_4 NO_3$), adjusted for pH 8 with NaOH (Fisher, UK). This was used as the chromatographic eluent. Potential interferents were separated from both Cr (EDTA)-complex and from Cr (VI).

The column used in this study possessed a guard column (Dionex, 4 × 50 mm) obtained from (Fisher, UK), as shown in Figure 2.2. An autosuppression-external water mode was placed between the analytical column and the nebulizer. The plasma mass spectrometer was used as recommended and the pump used was a Dionex IC-pump. The data obtained was analysed using integration Software using a PC computer connected to the IC-ICP-MS (Fig. 2.2).

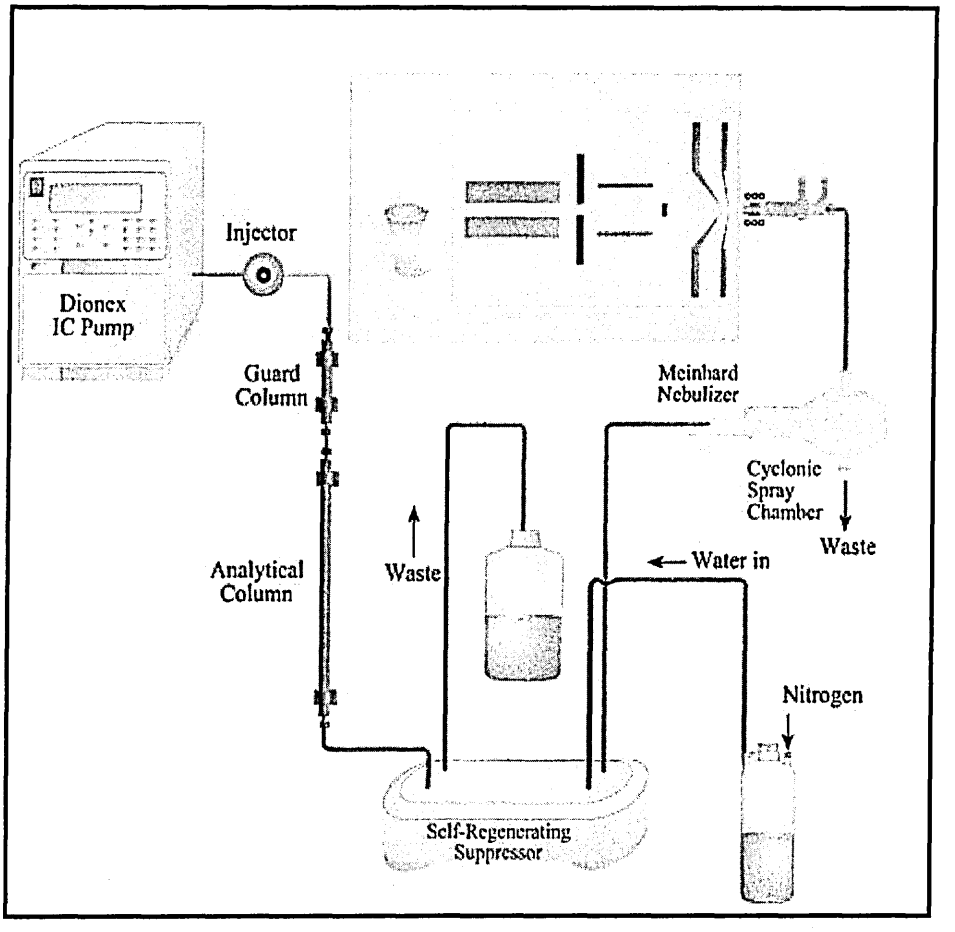


Figure 2.2: Schematic Diagram of the IC-ICP-MS System

2.2.3.9 Chromium (III) and Chromium (VI) Preparation

Chromium (III) and chromium (VI) were dissolved in 100 ml of deionised water according to the preparation instructions. The same method was conducted for the preparation of chromium III and VI standard, where the original concentration in both standards was 10 mg l^{-1}. A series of chromium concentrations were prepared from 0 - 200 µg l^{-1}, with addition of 0.4 ml EDTA at each concentration. 100 ml volumetric flasks were then made up to volume with deionised water and shaken. Approximately 10 ml of each solution was then transferred into a clean test tube and incubated in a water bath at 70°C for 1 hour. The same method was applied to all samples, except those containing bacterial cells which were modified as follows: A few drops of EDTA were added to the remaining cells, with gentle shaking in order to completely digest the cells. Then 100 µg l^{-1} was transferred into a 10 ml volumetric flask with the addition of 0.4 ml EDTA; the volume was then made up

with deionised water. The samples were then placed in the water bath for 10 minutes to ensure all cells were digested. Finally, all samples were incubated in a water bath at 70°C for 1 hour before analysis.

2.3 BIOPHYSICAL ANALYTICAL TECHNIQUES

2.3.1 Oxygen Concentration Determination

The ability of *M. capsulatus* (Bath) to metabolise methane was determined by measuring the soluble oxygen concentration under various conditions using a standard oxygen electrode. The oxygen electrode was calibrated and used as described by Robinson and Cooper (1970). The zero calibration was achieved by removing all the oxygen from a sample of water in the electrode chamber, by adding a crystal of sodium dithionite. After this, the chamber was rinsed with deionised water and the sample was then applied to the electrode and allowed to equilibrate, and the endogenous rate was determined. Methane usage was observed after the addition of 100 micro-litres of methane saturated water. Experiments were carried out using a variety of different samples including those containing sodium azide and chromate. Methane estimated oxygen uptake was determined from rates observed for samples containing only *M. capsulatus*, and those containing *M. capsulatus* and sodium azide and/or chromate.

2.3.2 Fermentation

Fermentation was carried out using a Bioflo 110 fermentor/Bioreactor (New Brunswick). NMS media (5L) was placed inside the fermentor and the whole apparatus including tubing was autoclaved. 50 ml of sterile phosphate buffer pH 7.0 was added to the media. The fermentor conditions used to grow *M. capsulatus* were as follows; agitation at 300 rpm, incubation temperature at 45°C, 1:4 methane:air at approximately flow rate of 1 liter/minute. Three 50mls cultures of *M. capsulatus* were prepared in NMS and incubated at 45°C for 72-96 hours. These cultures were used to inoculate the fermentor. Twenty four hours after inoculation, sterile chromate was added to give a final chromate concentration of 1000 mg/l. Samples were taken at 0, 24, 48, 72 and 96 hours following chromate addition, and immediately cells

were harvested via centrifugation (10 mins at 9000 g) and both cells and supernatant were frozen at –70°C.

2.3.3 Plate Dilution

Plate dilution studies were used to follow the effect of formate on the chromate reduction process and cell viability. *M. capsulatus* was grown in the presence of chromate and formate at 2mM and 100 mgL^{-1} respectively. After 48 hours growth in NMS media plus chromate and formate, serial 1:100 dilutions of the culture were made into six test tubes of fresh NMS media. 20 micro litres of each dilution was plated onto individual section of the same NMS agar plate. The plate were incubated at 45°C in the presence of methane for 16 hours. The number of colonies in each section was counted visually and the CFU/ml calculated.

2.3.4 Transmission Electronic Microscope (TEM)

Samples (1.5 ml) of cells were pelleted by centrifugation for 5-10 minutes, and the cells were fixed in 1 to 2 ml of aqueous solution of acetone (Fisher) to distribute all cells, which were embedded in this solution. The embedded specimens were then sectioned and coated on Cu TEM grids.

2.3.5 TEM and EDAX Operating Conditions

A CM20 TEM (Philips) transmission electron microscope was used, operating at 200 KeV. The instrument is equipped with an EDAX system; Samples were tilted 20 degrees to meet the horizontal X-ray detector type of (SiLi-silicon lithium detector). The method was similar to that recommended by Mclean and Bevridge (2001).

2.3.6 Extended X-Ray Fine Structure (EXFAS)

The X-ray absorption spectra were measured at room temperature and at 77 K on station 7.1 of the Synchrotron Radiation Source at CLRC Daresbury Laboratory, UK. The low temperature data were obtained by mounting the sample on the cold finger of a simple cryostat containing liquid nitrogen, both being enclosed in

a vacuum chamber fitted with mylar windows. The synchrotron was running at energy of 2 GeV and beam currents were between 100 and 150 mA 1 during all experiments. Station 7.1 uses a harmonic-rejecting, sagitally-focussing double crystal 5i (111) monochromator. The X-ray flux before and after the sample was measured using gas-filled ion chambers, so that data could be obtained in transmission mode if they were sufficiently concentrated. A chromium metal foil placed in front of a third ion chamber was used to determine the edge energies precisely. In addition, the fluorescence signal from the sample was measured using a nine-element monolithic Ge detector fitted with XPRESS electronics. This has a maximum count rate of about 150 kHz per channel. The solid samples (standard crystals) were made up of finely ground powders held between Sellotape layers. The liquid samples were held in 0.5 mm thick perspex sample holders fitted with mylar windows. Two to four scans, each taking about 30 min of beamtime, were averaged for the standards, which were all measured in transmission mode. Other samples were measured in fluorescence mode, with twelve scans being measured and averaged. In this case, spectra from the start and end of a run were visually compared to check for radiation damage. No indication of such damage was found. The data were calibrated; background subtracted and analyzed using the standard Daresbury packages EXCALIB, EXBACK and EXCURV98, respectively (srs.dl.ac.uk/xrs/). EXCURV98 uses the fast curved-wave theory (Gurman *et al.*, 1984) with electron scattering parameters calculated within the program. These have been extensively proved in numerous applications and were also checked by analyzing the data from the Cr monitor foil (which also gave a value for the amplitude parameter S0 2) and several standard crystalline samples. EXCURV98 includes a statistical test of the goodness of fit through a fit index. This identifies both the best fit parameters for a given shell of atoms and also whether the inclusion of further shells of scattering atoms improves the fit significantly (Joyner *et al.*, 1987). All shells described here improved the fit significantly.

The *M. capsulatus* culture used for obtaining X-ray absorption data was grown on methane in a fermentor as described earlier in Section 2.3.2 and chromium (VI) was added to 1000 mg/l and incubation was continued at 45 °C in the presence

of methane and air. Samples of culture were harvested for X-ray analysis after 24 and 96 hours.

In addition to analysing the experimental samples from cultures containing 1000 mg/l chromium (VI) and M. capsulatus cells, aqueous standards at the same concentration of chromium were also analysed in order to allow identification of the chromium environment in the experimental samples. These aqueous standards were: $K_2Cr_2O_7$ in water, $CrCl_3$ in water and $CrCl_3$ in the phosphate buffer component of NMS medium. Since the chromium (III) precipitated as its phosphate salt in the presence of the phosphate buffer, an additional standard of solid $CrPO_4.6H_2O$ was also analysed.

For collection of X-ray absorption data, the solid samples were made up of finely ground powders held between Sellotape layers. The liquid samples were held in 0.5 mm thick perspex sample holders fitted with mylar windows.

2.3.7 Bioinformatics Methods Used to Search for Chromate Reductases in *Methylococcus capsulatus*

The *M. capulatus* genome sequence (Ward *et al.* 2004) was accessed via the Genbank database, using the NCBI website (www.ncbi.nlm.nih.gov). BLAST (Altschul, 1990 and 1993) from the online interface at NIH-NCBI (http://www.ncbi.nlm.nih.gov/) was used to identify *M. capsulatus* proteins that may be involved in reduction of chromate. Initially, flavin reductase (Fre) from *E. coli* (Puzon *et al.*, 2002; accession no. AAA91058) was used as query to screen potential homologs in *M. capsulatus* (Bath). Sequences representing putative, partial, precursor and fragment of Chromate reductase protein were excluded from the study. Hence, 3 unique proteins homologue were retrieved from *Methylococcus. capsulatus* and considered for this study (Table 5.1 and Figure 5.1). The putative Chromate reductase protein sequences were retrieved in FASTA format and used for further analysis. Each of these identified proteins was used as query for searching similar sequence in other organisms (Figures 5.3, 5.4 and 5.5).

Multiple sequence alignments were performed by using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

2.3.8 Prediction of Physico-chemical Properties

Theoretical isoelectric point (pI), molecular weight, extinction coefficient, instability index, aliphatic index and grand average hydropathy (GRAVY) were computed using the Expasy's ProtParam server (http://us.expasy.org/tools/protparam.html/). Amino acid composition of the protein sequences can reveal their nature; SOSUI SYSTEM (ver. 1.0/10, Mar., 1996) was used to calculate the hydrophobicity.

2.3.9 Comparative Protein Structure Modelling

The amino acid sequences of the Q60Y84 (NADH quinone oxidoreductase), Q60CN1 (Glutathione-S-transferase) and Q605J4 (Nitroreductase family protein) comprised of 182, 230 and 202 amino acid residues was retrieved from Expasy.11, modeled structures using comparative homology modeling. Sequence alignments of these with the target proteins was created with the Insight/ Homology module and adjusted to align key conserved residues. Protein data bank entry 2FUG, 2ISJ and 2GDR was used as a structural template. Based on the optimized alignment comparative models of the target sequence were built by MODELLER, applying the default model building routine 'model' with fast refinement. This procedure is advantageous because one can select the best model from several candidates. Furthermore, the variability among the models can be used to evaluate the reliability of the modelling. Energy minimization was performed using the consistent valence force field and the Discover program.

2.3.10 In Silico Docking

The Docking study with various prosthetic group of these three modelled Q60Y84 (NADH quinine oxidoreductase), Q60CN1 (Glutathione-S-transferase) and Q605J4 (Nitroreductase family protein) determined by comparative homology modelling. Distance-dependent function of the dielectric constant was used for the calculation of the energetic maps and all other parameters were used by default value.16. Several independent docking processes were carried out for each complex.

CHAPTER 3

BIOREMEDIATION OF CHROMIUM

BY BACTERIA

3.1 Introduction

Chromate (VI) reductase activity, capable of reducing chromium from the +6 to +3 oxidation state, has been previously characterised in aerobic bacteria and facultative anaerobes such as *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis* and others. In order to understand key issues raised in the literature, it was considered necessary to undertake the experimental work with *E.coli* before studying the methanotrophs; the latter being the main focus of this thesis. In this way experiments with non-methanotrophs and the analytical detection techniques of chromate bioreduction could be implemented before commencing work on the methanotrophs. This chapter presents experiments investigating the bioremediation of Cr (VI) using *E .coli* and a mixed culture of *E. coli* and a methanotroph. Finally, a number of methanotrophs are tested for their suitability for bioremediation of Cr (VI).

3.1.1 Chromium Assay Using the Diphenyl carbazide Assay

Chromium (VI) was estimated colorimetrically by reaction with diphenylcarbazide, in comparison with a standard curve that was repeated for each set of assays. Full details can be found in the Materials and Methods (Section 2.2.3.5).

3.1.2 Culture Growth

Cells numbers were estimated by measuring the absorbance at 450 nm. This showed reasonable cell growth of *E. coli* ATCC33456 with time as illustrated at figure 3.1.

`3.1.3 Bioremediation of Cr (VI) Using *E.coli*

Cr (VI) reduction under aerobic conditions was investigated in the presence of *E. coli*. Reduction of Cr (VI) was nearly complete after 8 h of incubation under optimal conditions for bacterial growth (Figure 3.2).

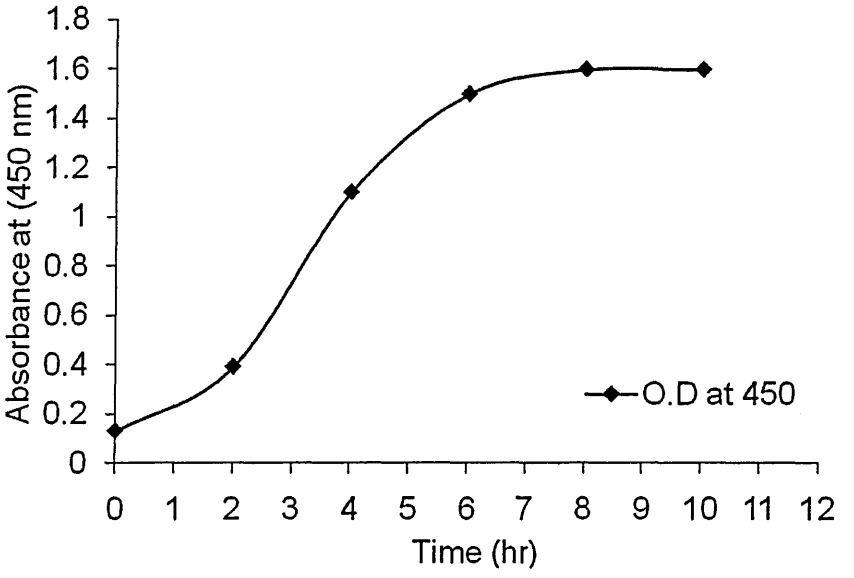


Figure 3.1: Typical growth curve of *E.coli* ATCC33456 level at 450 nm.

Chromium (VI) reduction was not observed in the control, a cell-free growth medium, indicating no a biotic Cr (VI) reduction. Figure 3.2 shows that the reduction rate was rapid for the first 4 hours and then less so until hour 8. No decrease was observed at all in the amount of the total chromium (using ICP.AS; Figure 3.2). Furthermore, Table 3.1 demonstrated that Cr (VI) was significantly reduced to Cr (III) by *E. coli.*

Whilst Figure 3.2 clearly shows the reduction of chromium (VI) by the culture, the total chromium in the experimental incubation measured by ICP-AES is substantially higher than the chromium (VI) concentration at the beginning of the experiment. The most likely reason for this inconsistency was considered to be that the concentration of chromium (VI) in the samples was outside the range of the standards used to calibrate the diphenylcarbazide assay. To address this problem, the experimental incubation and associated chromium (VI) assays were repeated (Figure 3.3), with samples diluted to fall within the range of the standards. This gave comparable initial chomium concentrations as measured by both techniques, and also confirmed the reduction of chromium (VI) by *E. coli.* In all further experiments, samples for the diphenylcarbazide assay were diluted where necessary to bring them within the range of the standards.

The amount of chromium present as chromium (III) in the whole culture (cells and culture medium) at the end of the experiment was estimated as 87.1%, based on the difference between the total chromium quantified by ICP-AES and the amount of chromium (VI) measured via the diphenylcarbazide assay. These measurements confirm the previously established effectiveness of *E. coli* for reduction of chromium (VI) to chromium (III) (Shen and Wang, 1994).

Ion chromatography and ICP-AES analysis of the chromium associated with the cells showed that there was a substantial amount of cell-associated chromium and that it was primarily in the +3 oxidation state (Table 3.2). These data suggests that chromium (VI) is taken up by *E. coli* and reduced to chromium (III). Cell density was determined by measuring the absorbance at 450 nm, the data shows the normal cell growth of *E. coli* ATCC 33456 with time figure 3.1.

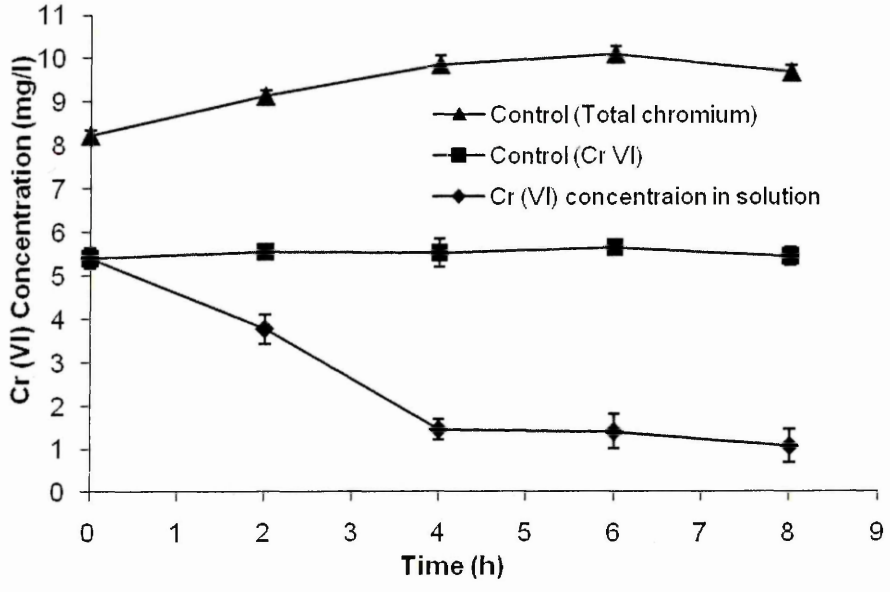


Figure 3.2: Time course of *E. coli* ATCC 33456 against (10 mg/l) chromate concentration and chromium control (experiment without cells) using ICP-AS. Chromium (VI) was measured in the culture supernatant.

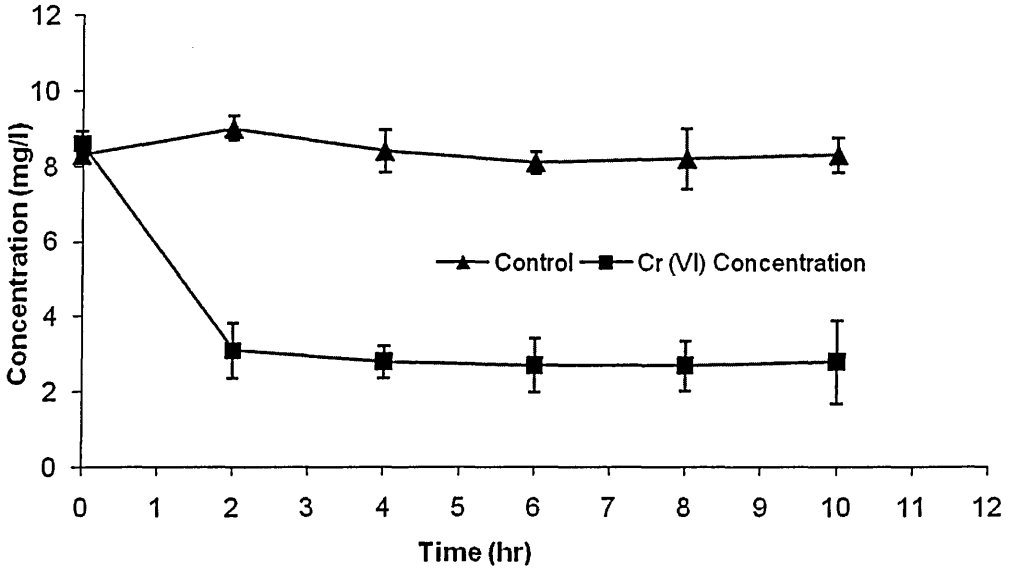


Figure 3.4: Pattern of chromium (VI) reduction with total chromium (10 mg/l) in the presence of *E. coli* ATCC 33456.

Table 3.2: Comparison of the chromate reduction by *E. coli* ATCC 33456 obtained using ICP-AES with chemical method.

Time (h)	Chromium (VI) control ($\mu g\ L^{-1}$) *	Chromium (VI) ($\mu g\ L^{-1}$)*	Chromium (III) ($\mu g\ L^{-1}$)*	Total Chromium ($\mu g\ L^{-1}$)**	Total Chromium with cells ($\mu g\ L^{-1}$)**	OD level
0.0	5.77	6.10	2.20	8.30	1.03	0.13
0.5	6.29	4.40	3.02	7.42	0.90	0.14
1.0	7.13	3.51	4.89	8.40	1.22	0.18
1.5	8.59	3.42	4.78	8.20	0.80	0.39
2.0	6.48	3.60	5.10	8.70	1.64	1.10
4.0	6.50	3.10	5.32	8.42	1.23	1.57
6.0	7.20	3.00	5.27	8.27	1.91	1.61
8.0	7.02	2.86	5.44	8.30	1.92	1.65
10.0	6.70	2.90	5.32	8.22	1.26	1.64

*Chemical Methods (Spectrophotometric).

** ICP-AES Method.

3.2 Bioremediation Using Methanotrophic Bacteria (*Methylosinus trichosporium* OB3b)

In order to investigate the potential of methanotrophic bacteria for chromate bioremediation, chromate reduction trials were performed using the methanotroph strain *Methylosinus trichosporium* OB3b, in the presence of 10 mg/l chromate with methane as the carbon and energy source. However, it was found that this concentration of chromate inhibited the growth of cells and no significant chromate reduction was observed during 200 hours at 30°C, (the optimum growth temperature for *M. trichosporium).*

3.2.1 Bioremediation of Chromium Using a Mixed Culture of *Escherichia coli* ATCC 33456 and *Methylosinus trichosporium* OB3b

It has previously been observed that a mixed population of *E. coli* and *M. trichosporium* can grow using methane as the only supplied carbon and energy source presumably because *E. coli* can derive nutrients from the methanotroph (T. J. Smith and S. L. Slade, unpublished data). It therefore seemed reasonable that methane driven chromate reduction might be achieved using *E. coli* and *M. trichosporium* grown together as a mixed culture. There was evidence that this approach might be successful because in a previous study a mixed culture of *E. coli* and a toluene-utilising *Pseudomonas* strain was found to reduce chromate using toluene as the sole carbon source while the toluene utiliser when grown alone did not reduce chromate (Megharaj *et al.*, 2004)

The data shown in Figure 3.5 suggests that some chromium reduction is occurring in the presence of the mixed culture (*E. coli* ATCC 33456 and *Methylosinus trichosporium* OB3b) where in the range of 3-4 mg/l of chromium (VI) was removed from the culture medium. The increase in cell-associated chromium mirrored the observed decrease in chromate concentration in the medium (Figure3.5), suggesting that the decrease in medium chromate (VI) concentration was accompanied by association of a roughly equal amount of chromium with the cells, possibly due to adsorption of chromium (III) by the cell walls after the reduction reaction had occurred.

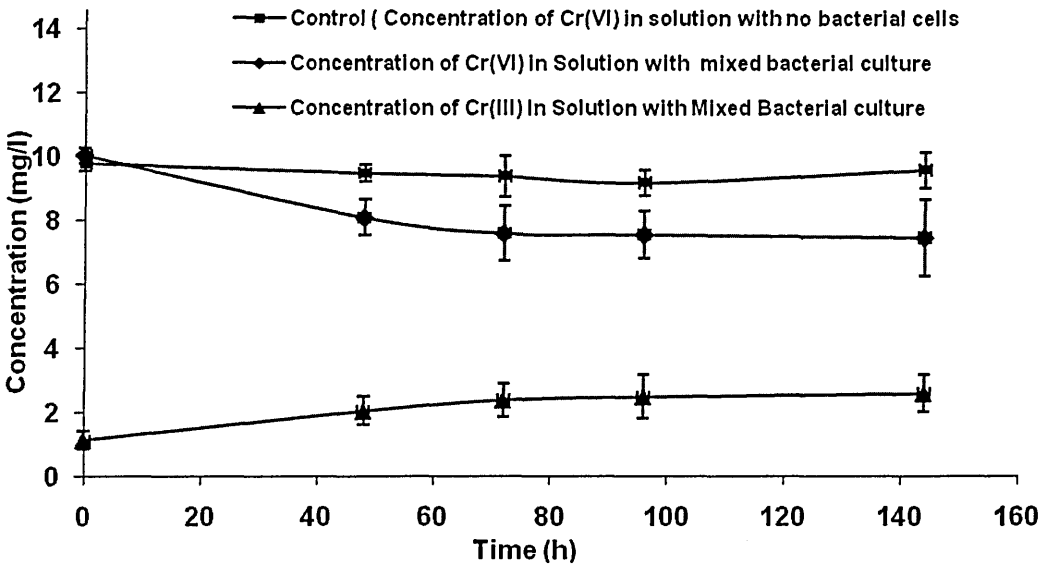


Figure 3.5: Time course of the growth of *E. coli* ATCC33456 and *Methylosinus trichosporium* OB3b (mixed culture) in the presence of chromium (VI) (concentration 10 mg/l).

3.3 Bioremediation of Chromium (VI) Using *Methylococcus capsulatus.*

This section gives results on the reduction of chromium (VI) to chromium (III) using *Methylococcus capsulatus*. Table 3.3 gives the analysis of both total chromium and chromium (VI) during chromium reduction by *M. capsulatus* in the presence of methane. The data show a reduction in chromium (VI), while the total detectable chromium in the system remains almost constant. Figures 3.7, 3.8, 3.9 and 3.10 show chromate reduction by *M. capsulatus* in concentrations up to 100 mg l^{-1}. It can be seen that maximum chromate reduction by this bacterium in the presence of methane, as a sole and energy source is in the first 48 hours under these conditions. Moreover, *M. capsulatus* revealed high resistance to various levels of chromium (VI) concentrations as shown in Figure 3.6.

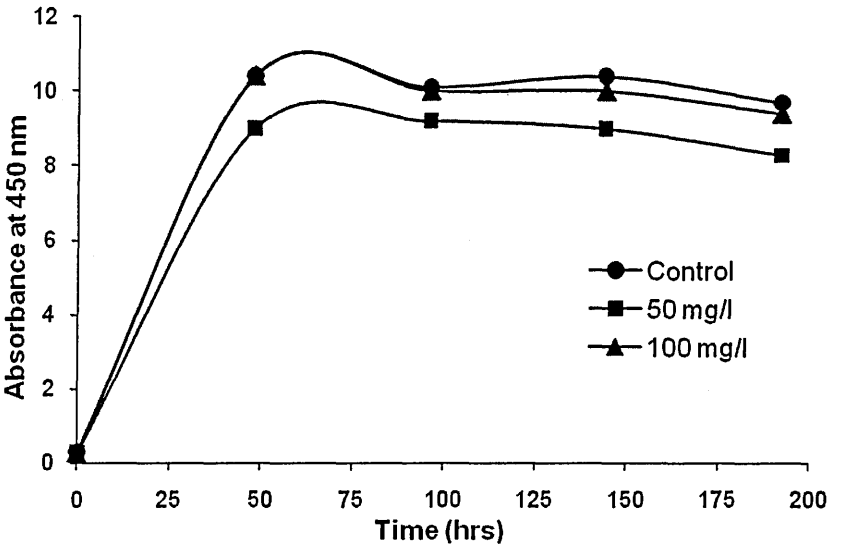


Figure 3.6: The OD levels of *M. capsulatus* at various levels of chromate concentration.

Table 3.3: Reduction of chromium (VI) by *M. capsulatus* (Bath) with initial chromium (VI) concentration (mg/l)

Time (h)	Total chromium (III) mg/l	SEM Standard Error Mean	Total chromium (VI) mg/l	SEM Standard Error Mean	Probability Score
0	9.140	0.19	11.32	0.19	0.0043
48	8.240	0.51	5.020	0.50	0.004
96	8.620	0.11	3.370	0.57	0.003
144	8.720	0.63	2.62	0.15	0.001

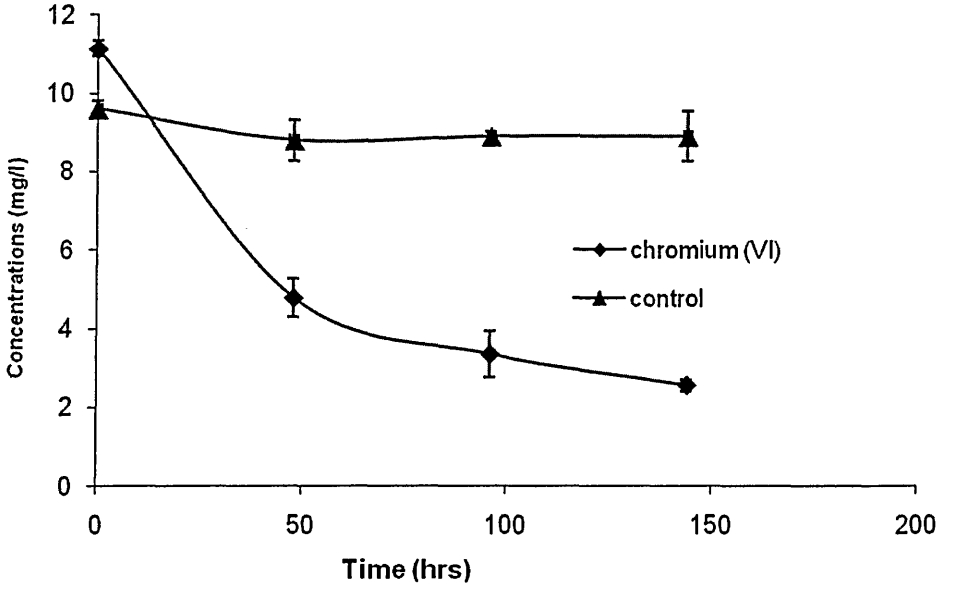


Figure 3.7: Pattern of chromium (VI) reduction in the presence of *M. capsulatus.* Initial chromate concentration is (10 mg/l).

In order to quantify the concentration of both chromium (VI) and chromium (III) in the medium, inductively coupled plasma mass spectroscopy was used, connected on the downstream from a dionex column that separated the two form of the metal by suppressed ion chromatography (SIC-ICP-MS).

Full analytical details are given in the Materials and Methods (Chapter 2). Typical calibration curves for chromium (VI) and chromium (III) are shown in Figure 2.1.

In addition to the change in chromium oxidation state described above, there is also a change in distribution of chromium from supernatant to cells (Figure 3.9). As shown in Figure 3.10 chromate reduction was also observed at 50 mg/l initial chromate concentration and here, since both chromium (VI) and chromium (III) levels were monitored by using SIC- ICP-MS, it is possible to see that the loss of chromium (VI) is accompanied by a corresponding increase in chromium (III).

3.4 Conclusion

The potential of *E. coli* ATCC 33456 for the reduction of chromium (VI) into less toxic, and less soluble chromium (III) has been confirmed, Figures 3.2 and 3.3. The strain used showed reasonable cell growth over time. The total chromium recovery achieved 83.1%. Total chromium remained effectively constant during growth of *E. coli* ATCC 33456 as well as in the cell-free controls (Table 3.2). Mixed cultures of *E. coli* and *M. trichosporium*, grown using methane as the sole source of carbon and energy, showed a small but significant amount of reduction of chromium (VI) (Figure 3.9), as confirmed by ICP-AES measurements after ion chromatograpy, as well as diphenyl carbazide assays for chromium (VI). Most importantly, chromium (VI)-reducing activity was observed in pure cultures of the methanotrophic bacterium *M. capsulatus* at a range of chromium (VI) concentrations. As detailed in the Introduction, *M. capsulatus* has shown considerable promise as a system for bioremediation of diverse organic pollutants. The results presented in this chapter indicate its potential for bioremediation of the toxic heavy metal species chromium (VI).

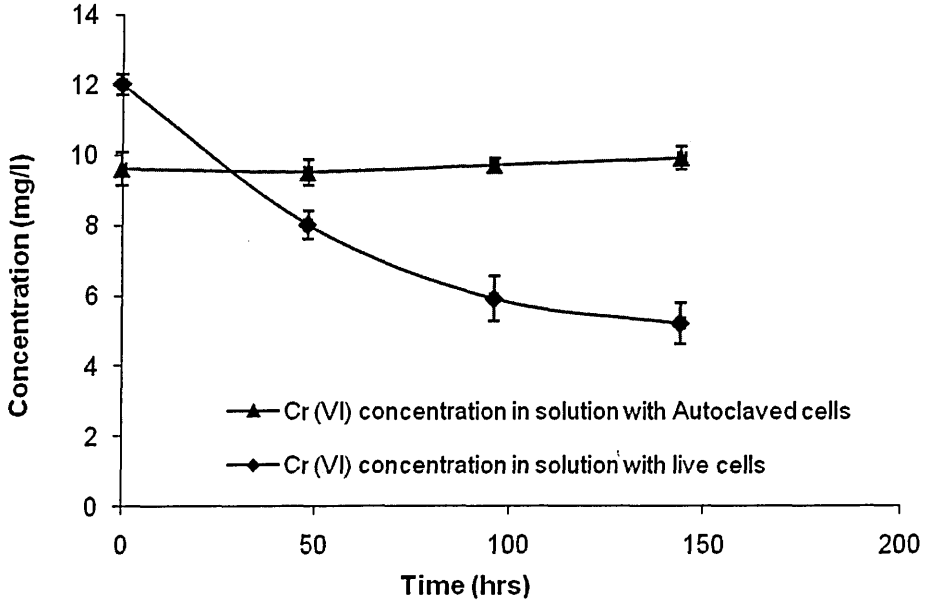


Figure 3.8: Pattern of chromium (VI) reduction in the presence of live and dead *M. capsulatus*. Initial chromate concentration is (10 mg/l).

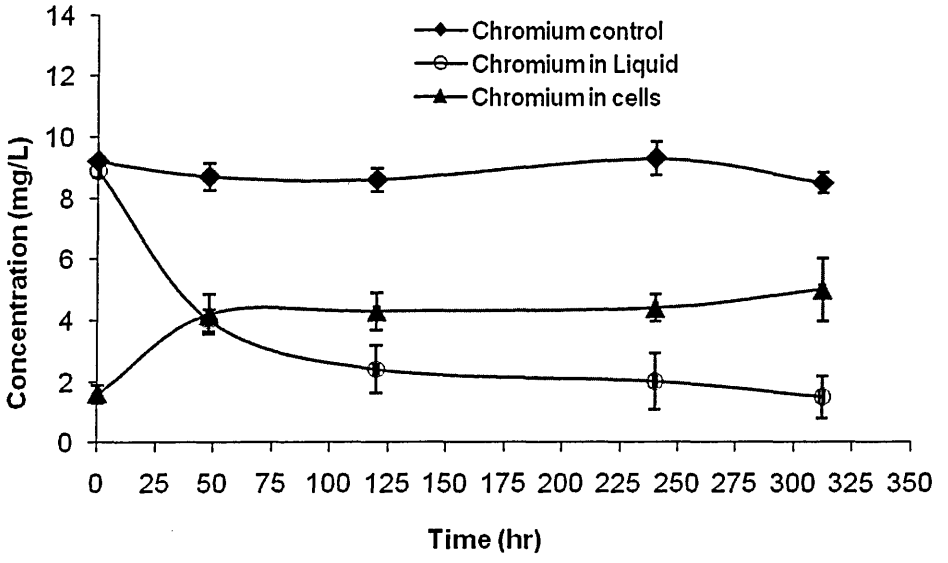


Figure 3.9: Pattern of total chromium (mg/l) distribution throughout the incubation with *M. capsulatus* in the presence of methane, measured by ICP-AES.

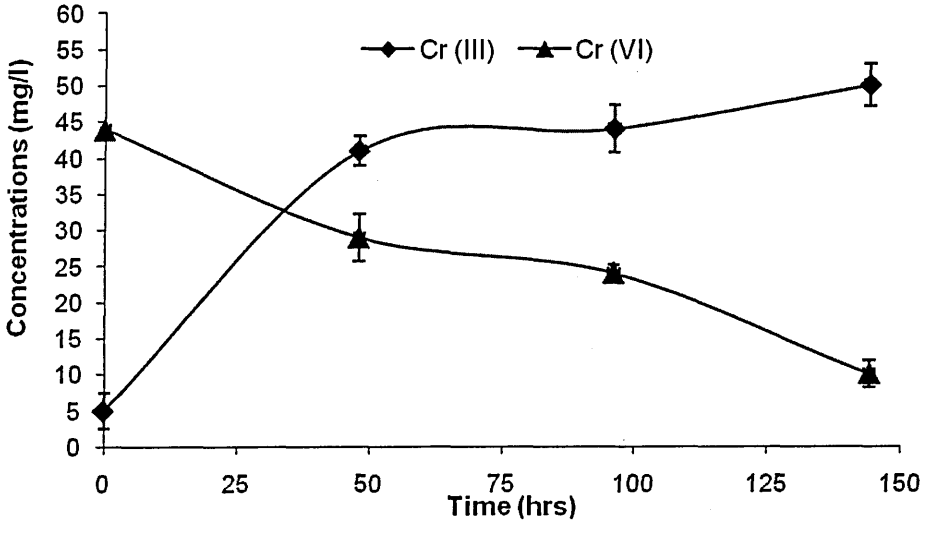


Figure 3.10: Chromium (III) and Chromium (VI) levels during chromate reduction by *M. capsulatus* in the presence of methane, and an initial chromate concentration of 50 mg/l.

3.5 Methane Oxidation Activity

Oxygen uptake rates are given as a percentage of the observed rate over the 24 hours inhibitor culture, (30.1 μ mol of O_2 min^{-1} L^{-1}). The effect of chromate and the metabolic inhibitor, sodium azide, on the methane-stimulated oxygen uptake by *M. capsulatus* was investigated (Table 3.4) to see whether chromate was toxic to the cells at the levels used. It was found that the addition of the chromium (VI) to 100 mg/l almost completely inhibited cellular metabolism as judged by the rate of methane-stimulated oxygen uptake. The data also show that sodium azide (0.02%) further inhibited methane – stimulated O_2 uptake when added to cells that had already been exposed to chromium (VI), which leads to the question of whether the metabolic inhibitor azide would interfere with the chromium (VI) reduction itself. This was explored by examining the effect of sodium azide on the reduction of chromium (VI), as described in methodology (Chapter 2, Section 2.3.1).

Table 3.4: Time course of the rate of oxygen uptake in the presence of *M. capsulatus* with Na N_3 as inhibitor.

Time	Culture			Culture + Chromium VI 100 mg l^{-1}			Culture + Chromium VI + Na N_3 100 mg l^{-1}		
	%	μ (mol.min^{-1})	OD	%	μ (mol.min^{1})	OD	%	μ (mol.min^{-1})	OD
24	100.0	30.1	1.046	0.4	0.96	0.57	0.5	1.2	0.63
48	63.0	18.7	1.035	0.1	0.28	0.55	0.0	0.0	0.62
100	57.4	17.0	0.995	0.0	0.0	0.52	0.0	0.0	0.57

CHAPTER 4

Study of Location and Speciation of Chromium in *Methylococcus capsulatus* (Bath)

4.1 Introduction

The most widely used method for following the chromate reduction process is the diphenylcarbazide colorimetric method and Ionic Coupled Plasma Mass Spectroscopy (ICP-MS) and Ionic Coupled Plasma Atomic Emission Spectrometry (ICP-AES). Some other techniques can provide more information at the subcellular level of distribution of chromium in the cell, and these techniques are important for understanding microbial activity in relation to chromium. Transmission Electron Microscopy (TEM) assay can be used to identify the metals present in or associated with the bacterial cells.

4.1.1 TEM and EDXS Results

The electron micrographs demonstrate the typical spherical morphology of *Methylococcus capsulatus* cells and the cells were full of electron-dense particles (Figure. 4.1), especially when the chromate concentration was 100 mg l^{-1}. This observation is consistent with a report in the literature where chromium species led to electron dense staining associated with bacterial cells that could be seen via TEM (McLean and Beveridge, 2001). Hence, in the absence of large amounts of other heavy metals, it was concluded that the particles and other elecron-dense material in Figure 4.1 was due to the presence of chromium species. Because of the observed electron dense particles within the cells, it is presumed that it is precipitated within the cytoplasm either as hydroxide or as a phosphate or bound to the protein. The images also show (Figure 4.2) that under some conditions, the cells of *M. capsulatus* are disrupted and undergo lysis. This was due to the presence of high level of chromate concentration (about 500 mg/l) and is a phenomenon which was also observed by Adel and Terry (2001).

Energy-dispersive X-ray spectroscopy (EDXS) was also used to confirm the presence of and oxidation state of chromium. The EDXS spectra of the bacterial cells with and without exposure to 100 mg/l of chromium (VI) are given in Figure 4.3. The observed EDXS results from the sample exposed to chromium (VI) show two

peaks for chromium, representing two different chromium oxidation states (Cr VI and III), which in turn indicate chromium reductase activity. If there is

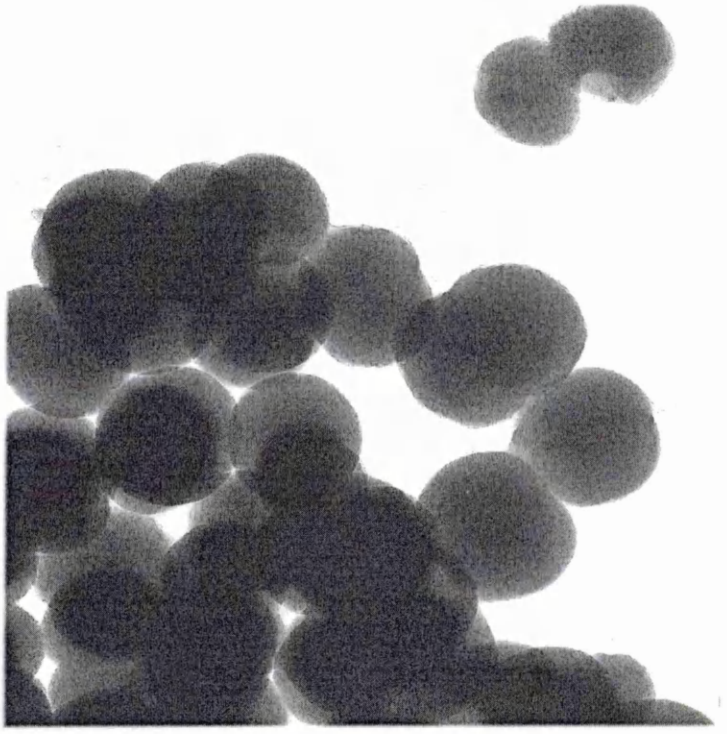


Fig. 4.1: Images of *Methylococcus capsulatus* in the presence of 100 mgL^{-1} chromate under the TEM.

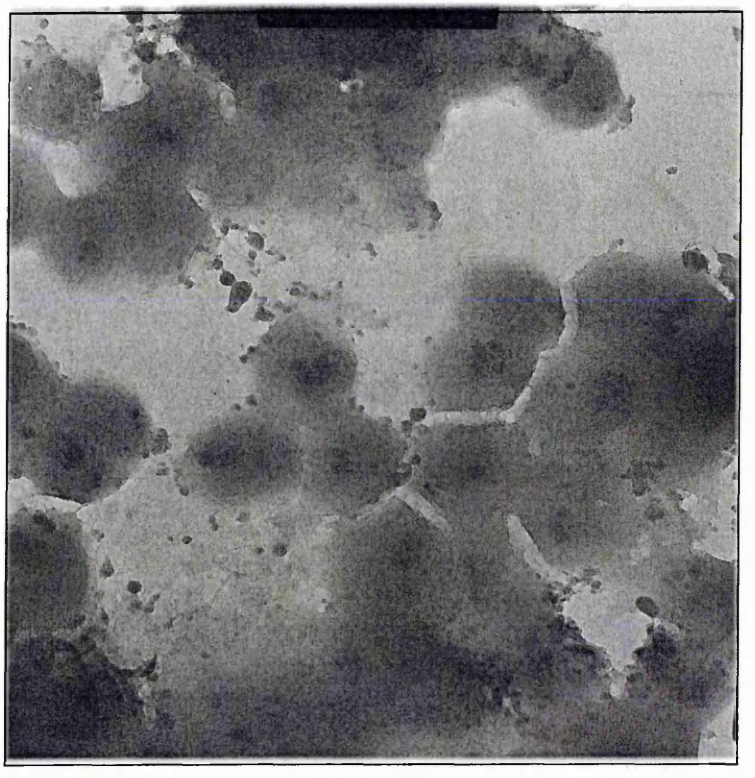
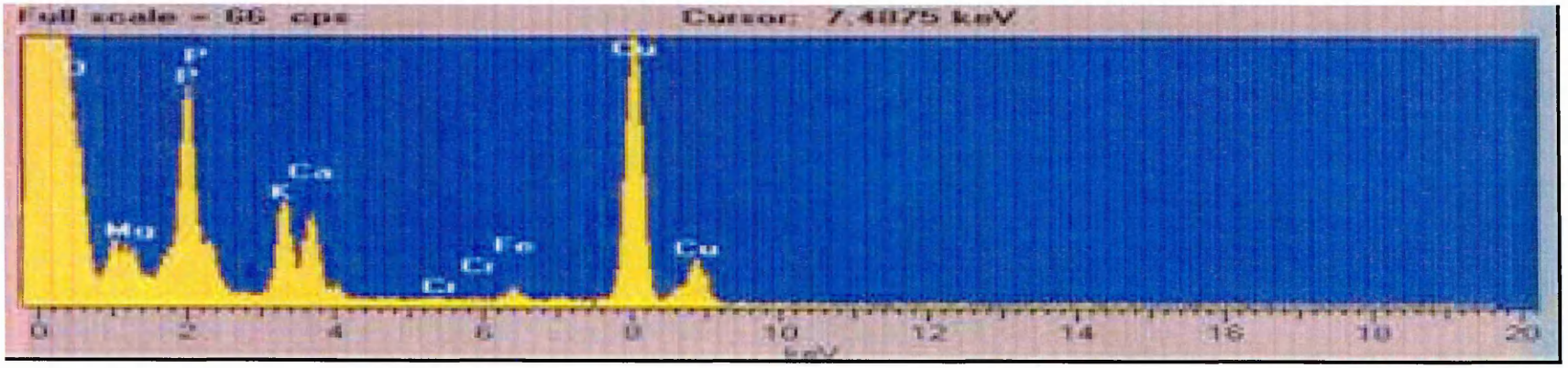


Figure 4.2: TEM images of *Methylococcocus capsulatus* in the presence of 500 mg/l chromate.

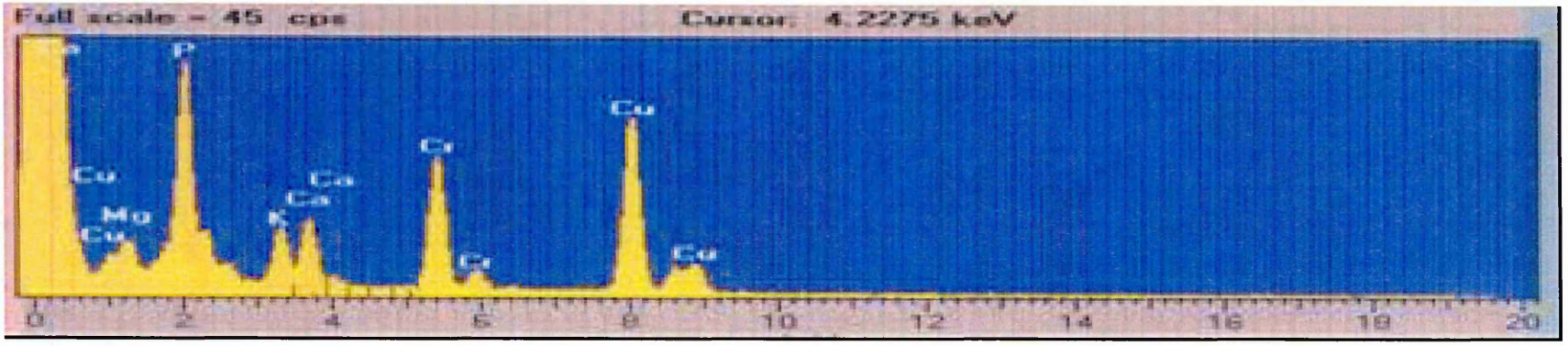
no bioremediation of chromium, only one oxidation state corresponding to Cr (VI) would have been observed in the EDXS. The almost complete absence of the chromium peaks from the no-chromium control shows that in the experimental sample the chromium associated with the cells was derived from the chromium (VI) added to the culture medium.

The bioaccumulation of precipitates by bacteria has been shown in previous conventional TEM studies. In addition to the work of McLean and Beveridg (2001) mentioned above, a consortium of sulphate–reducing bacteria, cultured in an anaerobic medium containing Cr (VI) ions, has previously been found to accumulate Cr-rich amorphous precipitates presumably on the cell surfaces (Fude *et al.*, 1994). Similarly, a species of *Aeromonas* became electron-dense after removing Cr (VI) (originally in form of Cr O_4^{-2}) from the culture medium (Hill and Cowley, 1986). These authors assumed that reduced chromium accumulated as intracellular electron-dense bodies. Electron-dense particles have also been associated with *Pseudomonas aeruginosa* after cell incubation in a CrO_4^{-2} containing medium. In this instance, evidence that precipitates were intracellular was provided by the fact that it was difficult to remove the precipitates from the cells by washing with 1mM EDTA, adjusted to pH 2 with H_2SO_4 (Marques *et al.*, 1982). For both prokaryotic organisms (such as bacteria) and eukaryotic cells, Cr (VI) can pass through the cellular membrane and form Cr (III) as a reduction product in the cytoplasm. Eukaryotic cells can additionally reduce Cr (VI) in mitochondria and nuclei (Ohtake *et al.*, 1990).

Trivalent Cr (III) is impermeable to biological membranes so that any Cr (III) generated inside the cell binds to protein and then interacts with nucleic acid. Unfortunately, no dilution state measurements were performed in published TEM studies. In such studies, the electron-dense particulates associated with the cells were merely assumed to be products of bioreduction. The reduction of Cr (VI) in the bacterium, *Shewanella oneidensis* has been studied by absorption spectrophotometry *in situ* and environmental cell– transmission electron microscopy (EC-TEM) coupled with electron energy loss spectroscopy (EELS).



(a)



(b)

Figure 4.3: Semi-quantitative chromium levels in *M. capsulatus* using Energy- Dispersive X-ray Spectroscopy (EDXS): (a) without chromium addition, and (b) with addition of 100 mg L^{-1} Cr (VI).

4.2 EXAFS Analysis

Methylococcus capsulatus cells grown in the fermentor in the presence of 1,000 ppm chromium (VI) and methane were harvested over several time points. These cells were then analysed using EXAFS to detect chromium and determine its oxidation state. The results obtained show little variation and it is concluded that chromium uptake shows no significant changes with time. Results from the EXAFS analysis are shown in Tables 4.1, 4.2 and 4.3.

Table 4.1: Results of the EXAFS analysis of samples

24 Hr rt			96 Hr rt			96 Hr 77 K		
N	**Type**	**σ^2**	**N**	**Type**	**σ^2**	**N**	**Type**	**σ^2**
5.1 ± 0.9	O	20 ± 15	5.6 ± 0.7	O	15 ± 10	7.1 ± 2.0	O	65 ± 35
5.1 ± 0.9	O	15 ± 15	5.5 ± 0.7	O	15 ± 10	7.2 ± 2.0	O	60 ± 35
6.2 ± 3.5	P	90 ± 60	4.6 ± 2.4	P	75 ± 50	4.5 ± 5.5	P	85 ± 120
5.2 ± 1.0	O	20 ± 20	5.5 ± 0.7	O	15 ± 15	5.4 ± 0.9	O	0 ± 15
5.1 ± 0.9	O	20 ± 20	5.5 ± 0.6	O	15 ± 10	5.4 ± 0.9	O	0 ± 15
6.4 ± 3.3	P	90 ± 60	5.3 ± 2.8	P	90 ± 60	4.2 ± 4.0	P	100 ± 120

Results of fitting the EXAFS:

The uncertainties quoted are 2σ errors.
The parameters are atom type; number coordinated to Cr (N); mean distance (R) and mean square variation in distance (σ^2).
kmax: is the highest value of the photoelectron wavevector used in the fit: all fits went down to the edge. The Fit Index gives an indication of the quality of fit. Values below 20 are acceptable fits. A shell significantly improves the fit if adding it lowers the Fit Index by more than one. rt (room temperature) or 77K at the head of the column indicates temperature of EXAFS data acquisition.

Table 4.2: Results of the EXAFS analysis of soluble standards

$K_2Cr_2O_7$ in water			$Cr Cl_3$ in water			$CrCl_3$ in phosphate		
N	Type	σ^2	N	Type	σ^2	N	Type	σ^2
2.7 ± 0.5	O	-5 ± 10	6.3 ± 0.6	O	15 ± 10	5.6 ± 0.9	O	0 ± 15
2.9 ± 0.5	O	5 ± 10	6.6 ± 0.8	O	20 ± 10	5.6 ± 0.9	O	-5 ± 10
3.6 ± 1.1	O	3.5 ± 25	6.0 ± 1.0	O	5 ± 10	5.7 ± 1.1	O	0 ± 10

N, Type, and σ^2 are as described earlier in Table 4.1.

Table 4.3: Results of the EXAFS analysis of solid standard

$CrPO_4.6H_2O$		
N	Type	σ^2
5.9 ± 0.9	O	5 ± 10
5.7 ± 0.9	O	0 ± 10
4.7 ± 3.3	P	70 ± 60
5.6 ± 0.8	O	-5 ± 10
4.0 ± 2.8	Cr	60 ± 60
5.7 ± 0.9	O	0 ± 10
4.9 ± 4.5	Cr	130 ± 90

N, Type, and σ^2 are as described earlier in Table 4.1.

From the data shown in the above tables, it can be seen that chromium (VI) has a short Cr-O distance, whereas chromium (III) has a longer Cr-O distance. Additionally, the Cr-P is significantly larger that the Cr-O distance. A comparison of the sample and standard data indicates that the chromium found in the *M. capsulatus* cells is present in the 3+ oxidation state and is bound to phosphate. This strongly suggests that the chromium in the cells exists as $CrPO_4$.

The comparision of EXAFS analysis of experimental samples as shown in Table 4.1 and with that of EXAFS of standard Cr (VI) and Cr (III) as shown in Table

4.1 and 4.2, clearly indicated that biotransformation of Cr (VI) to Cr (III) took place by the enzymatic activity of *Methylococcus capsulatus* cells. Further, it can be concluded that in the cells the Cr (III) is bound to phosphate-containing cellular components such as nucleotide enzymes (Puzon *et al.*, 2002) and DNA. It is also possible that Cr (III) inside/outside the cells is coordinated by inorganic phosphate groups.

CHAPTER 5

METHYLOCOCCUS GENOME ANALYSIS FOR PUTATIVE CHROMATE REDUCTASES

proteins that may be involved in reduction of chromate. Initially flavin reductase (Fre) from *E. coli* (Puzon *et al.*, 2002; accession no. AAA91058) was used as query to screen potential homologs in *M. capsulatus* (Bath). The result of the protein-protein BLAST search of chromate reductases proteins in *M. capsulatus* indicated the presence of three candidate proteins with high homology to *E. coli* Fre chromate reductase: (1) putative oxygenase (Accession No: YP_114919.1), (2) Na(+)-translocating NADH-quinone reductase (Accession no. YP_114800)), (3) methane monooxygenase reductase component MmoC (Accession no. YP_113665.1). The accession numbers and the corresponding E-values are presented in Table 5.1. A CLUSTAL W multiple amino acid sequence alignment of the chromate reductase from *E coli* and the three different putative homologous proteins showed the level of similarity and identity between the *E coli* protein and the three candidate proteins (Figure 5.1).

In order to gain additional information about the reductases identified from the *M. capsulatus* genome, these sequences were used as query sequences for new BLAST searches to find the closest homologues of each about which additional information was available (Figures 5.3, 5.4 and 5.6).

The *E. coli* nitroreductase NfsA, which also reduces chromate (Ackerley *et al.*, 2004a), has one significant homologue in *M. capsulatus* (accession no. YP_113831, $E = 6 \times 10^{-30}$). However, the chromate reductase ChrR of *P. putida* (Ackerley *et al.*, 2004b; accession no. Q93T20), a flavoprotein capable of chromate reduction, did not have any homologue in *M. capsulatus.*

5.2 Identification of Other Bacterial Proteins with Similarity to *M. capsulatus* oxygenase, Quinone Reductase and Methane Monooxygenase (mmoc)

In order to identify template proteins of known structure for molecular modelling studies, the three candidate chromate (VI) reductases from *M. capsulatus* were used as query sequences in BLAST searches of the UniProt database. This set of protein could then be used for homology modeling.

The three proteins identified in Section 5.1 oxygenase (YP_114919), quinone reductase (YP_114919) and methane monooxygenase subunit (YP_113665 mmoC) of *Methylococcus*

capsulatus was subjected to *blastp* (protein-protein BLAST) similarity search tool to trace the related sequences in other bacteria, as detailed in the Materials and Methods.

The *blastp* results confirm that the Oxygenase enzyme of *Methylococcus capsulatus* showed similarity to Oxygenase and ferrodoxin reductase of other bacteria. The six most relevant matches from *Mariprofundus ferrooxydans, Myxococcus xanthus, Shewanella woodyi, Legionella pneumophila* and *Shewanella sediminis* were subjected to Multiple Sequence Alignment using CLUSTALW to study the conserved regions (Fig. 5.3). Quinone reductase enzyme of *Methylococcus capsulatus* showed similarity to Quinone reductase enzyme of other bacteria. The seven most relevant matches from *Azotobacter vinelandii, Silicibacter pomeroyi, Rhodobacterales bacterium, Pseudomonas mendocina, Pseudomonas stutzeri* and *Silicibacter sp.* were subjected to Multiple Sequence Alignment to study the conserved regions (Fig. 5.4). Methane monooxygenase enzyme of *Methylococcus capsulatus* showed similarity to methane monooxygenase and oxidoreductase enzyme of other bacteria. The six most relevant matches from *Methylococcus capsulatus, Methylomonas sp., Methylomicrobium japanense , Methylocella silvestris,* and *Methylosinus sporium* were subjected to Multiple Sequence Alignment to study the conserved regions (Figure 5.5).

5.3 Predicted Physicochemical Properties of *Methylococcus capsulatus* Possible Chromate Reductases: Oxygenase, Quinone Reductase and Methane Monooxygenase Reductase Proteins

The oxygenase enzyme YP_114919, quinone reductase enzyme YP_114800 and methane monooxygenase subunit (MmoC) YP_113665 of *Methylococcus capsulatus* were considered likely to be involved in reducing chromium and hence were chosen for further analysis. *MmoC* has a known NAD(P)H-dependent reductase activity (it naturally reduces the di-iron centre of the methane monooxygenase from the diferric to the diferrous state) (Lipscomb 1994) and so this was a reasonable candidate for a chromate reductase activity.

In order to study the chromium reducing activities of these proteins, further analysis; hydrophobicity prediction, studies of physico-chemical properties, structure prediction and interaction analysis were carried out (Table 5.1).

Table 5.1: Putative Chromate reductases of *M. capsulatus* sequences considered for the study

No.	Accession	Length	Description	pI	MW	E. value
1	YP_114919.1	358	Putative oxygenase	6.70	35684.8	2×10^{-12}
2	YP_114800	324	Na(+) translocating NADH-Quinone	5.36	45639.5	3×10^{-10}
3	YP_113665.1	348	Methane monooxygenase reductase MmoC	4.69	38541.6	3×10^{-10}
4	Q 605J4	221	Nitroreductase family protein	6.25	25480.2	-
5	Q 60CN1	203	Gluathione-S-tranferase	6.31	19640.5	-
6	Q 608Y4	171	NADHQuinone oxidoreductase	4.93	22799.1	-

Amino acid composition determines the fundamental properties of the protein. Isoelectric point (pI) is the pH at which net charge existing on the protein is zero. The pI values of all protein sequences are in the range of (4.69 - 6.70) indicating that all considered Putative Chromate reductase sequences are slightly acidic. The calculated isoelectric point (pI) will be useful because at pI, solubility is least and mobility in an electro focusing system is zero. The instability index which gives clue about the stability of a protein *in vitro.* All the considered sequences were classified as stable with value ranging from 42.36 to 60.59 as a value < 40 indicates and stable protein except Q 60CN1 which has instability index 38.64 indicates unstable.

The aliphatic index (AI) which is defined as the relative volume of a protein occupied by aliphatic side chains is regarded as a positive factor for the increase of thermal stability of globular proteins. Aliphatic index for the Putative Chromate reductase

sequences ranged from 83.27- 96.16. The very high aliphatic index of most Putative Chromate reductase sequences indicates that these proteins may be stable for a wide temperature range. From the molar extinction coefficient of tyrosine, tryptophan and cystine (cysteine does not absorb appreciably at wavelengths >260 nm, while cystine does) at a given wavelength. The computed protein concentration and extinction coefficients help in the quantitative study of protein–protein and protein–ligand interactions in solution. The Grand Average hydropathy (GRAVY) value for a peptide or protein is calculated as the sum of hydropathy values of all the amino acids, divided by the number of residues in the sequence. GRAVY indices of Putative Chromate reductase are ranging from - 0.359 to - 0.1159. This low range of value indicates the possibility of better interaction with water. A set of conserved amino acid residues located in vicinity that provides clues to the functions is termed as membrane anchoring region.

The SOSUI system (ver. 1.0 / 10, Mar., 1996) is a useful tool for secondary structure prediction of membrane proteins from a protein sequence. The system deals with three types of prediction: discrimination of membrane proteins from soluble ones prediction of existence of transmembrane helices and determination of transmembrane helical regions (Hirokawa *et al.*, 1998).

SOSUI distinguishes between membrane and soluble proteins and predicts the transmembrane helices from amino acid sequences quickly with high precision. One Putative Chromate reductase from *M. capsulatus* was classified as membrane protein by SOSUI server while all other Putative Chromate reductase was predicted to be soluble proteins. The transmemebrane region predicted was found to be rich in hydrophobic amino acids and it is also evident in Kyte and Doolittle mean hydrophobicity profile generated using online tool (http://gcat.davidson.edu/rakarnik/kyte-doolittle.htm) (Figure 5.2) in which many points lie above the 0.0 line and a clear peak was observed in plot that indicates about the plausible transmembrane region. Since there is lack of experimental structures for 5 Putative Chromate reductase considered, Discovery studio MODELLER was used to predict the 3D structures of proteins.

5.3.1 Hydropathy Scale Prediction for Oxygenase, Quinone Reductase and Methane Monooxygenase

Hydrophobicity (or hydrophilicity) plots are designed to display the distribution of polar and non-polar residues along a protein sequence. Most commonly, such analysis has the goal of predicting membrane-spanning segments (highly hydrophobic) or regions that are likely exposed on the hydrophilic domains of proteins (Kyte and Doolittle, 1982). This is particularly relevant to the putative chromate reductases because the cells were able to reduce chromate (VI) when added extracellularly to intact cells, suggesting either that the chromate reductase is on the outside of the cell membrane or that chromate can gain access to the cytoplasm.

Oxygenase enzyme YP_114919 has a hydrophobicity index of -0.212037 indicative of a soluble protein. Quinone reductase enzyme YP_114800 has an index of 0.109582 with one transmembrane helix and suggesting the potential to localise to membranes. This proposed transmembrane helix has the sequence ILGVFFTAIVVALVFVILGAK, which (apart from the C-terminal lysine) is lacking in any charged or hydrophilic sidechains, in keeping with its proposed location in the hydrophobic lipid environment of the membrane. The proposed membrane association of the quinone reductase is consistent with the known role of quinones in the membrane electron transport chains of bacteria, and particularly the ability of duroquinol (a reduced quinone) to act as the electron donor for particulate methane monooxygenase (a membrane-associated enzyme) in methane-oxidising bacteria (Shiemke *et al.* 1995). Methane monooxygenase subunit (MmoC) YP_113665 has an index of -0.273851 suggestive of a soluble protein (Fig.5.2). The cytoplasmic nature of this protein is now well established from experimental studies (Lipscomb 1994). Since the 'oxygenase' YP_114919 has the similar conserved Fe_2S_2 and flavin-binding domains as MmoC (the fer2 and FNR_like superfamilies, identified via the NCBI conserved domains search engine connected to the BLAST front end at http://blast.ncbi.nlm.nih.gov/Blast.cgi), it is reasonable to assume that it acts as in a manner similar to an NADH-dependent reductase. The FNR domains are actually slightly different between the two proteins; further analysis of these differences might reveal any significance.

5.4 Structure Prediction Using the Concept of Homology Modelling

In protein structure prediction, homology modeling, also known as comparative modeling, is a class of methods for constructing an atomic-resolution model of a protein from its amino acid sequence (the "query sequence" or "target"). Almost all homology modeling techniques rely on the identification of one or more known protein structures (known as "templates" or "parent structures") likely to resemble the structure of the query sequence, on the production of an alignment that maps residues in the query sequence to residues in the template sequence (Gasteiger *et al.*, 2005). The sequence alignment and template structure are then used to produce a structural model of the target. Because protein structures are more conserved than protein sequences, detectable levels of sequence similarity usually imply significant structural similarity.

5.4.1 3-D Structure Prediction of YP_114919 and YP_114800 Using Accelrys Discovery Studio

Discovery Studio® is a single, easy-to-use, graphical interface for drug design and protein modeling research. Discovery Studio 2.0 contains both established gold-standard applications (e.g., Catalyst, MODELER, CHARMm, etc.) with years of proven results (Eswar, *et al.*, 2006). Discovery Studio 2.0 is built on the SciTegic Pipeline Pilot™ open operating platform, allowing seamless integration of protein modeling, pharmacophore analysis, and structure-based design, as well as third-party applications.

In order to explore possible interactions between Chromiun VI and the proteins believed likely to be involved in reduction of chromate in *M. capsulatus*, an attempt was made to predict the structures of these proteins using Discovery Studio. The modeling of 3D structure of YP_114919 and YP_114800 followed a stepwise procedure, starting with a template structure search from PDB (Protein Data Bank), related to the target sequence using BLASTp (DS Server). From 50 BLAST hits, a potential template structure (PDB-ID: 1TVC), representing the structure of the flavin domain of the reductase component (MmoC) of the soluble methane monooxygenase form M*ethylococcus capsulatus* with 27% identity was taken as template for

model building (Figure 5.6a). The flavin domain is likely to be the one that is most relevant to chromate reduction because the known chromate reductase Fre (from *E. coli*) has flavin domain but lacks the Fe_2S_2 domain that is present in MmoC, YP_114919 and YP_114800. From 35 BLAST hits, a potential template structure (PDB-ID: 2EIX), representing the structure of cytochrome reductase from *Physarum polycephalum* with 30% identity was taken as the template for model building (Figure 5.6b). The target template alignment was generated by Discovery Studio and used as input to generate the 3D structure of YP_114919 and YP_114800. Interaction studies were performed to study the mode of interaction of oxygenase, quinone reductase, methane monooxygenase with chromium. But no interactions were observed for these proteins in spite of metal binding ability of predicted active sites (Figures 5.7a, and 5.7b).

5.5 Search for Other Protein Involved in Chromate Reductase Activity

Three proteins as Q605J4- nitroreductase family protein - Involved in Porphyrin and chlorophyll metabolism, Q60CN1-Gluthione-S-transferase- Involved in Glutathione metabolism and drug and xenobiotic metabolism by Cytochrome P450, Q608Y4- NADH-quinone oxidireductase subunit (involved in conserving redox energy in a proton gradient oxidative phosphorylation), in ubiquinone biosynthesis were identified in *Methylococcus capsulatus* with chromate reductase activity, based on a literature search (Kwak *et al.*, 2003, Sparla *et al.*, 2002).

5.5.1 Hydropathy Scale Prediction for the Three Proteins Using SOSUI, Kyte-Doolittle

When the hydrophobic scale of the proteins was verified using SOSUI, Q608Y4 (NADH quinone oxidoreductase) gave a score of -0.335088, Q605J4 (Nitroreductase) gave a score of -0.358823 and Q60CN1 (GST) gave a score of -0.158621. All the proteins were considered to be soluble because of the low hydrophobic score and since no transmembrane regions were detected (Figure 5.8).

5.6 3-D Structure Prediction of Quinone Oxidoreductase Enzyme (Q605J4), Nitroreductase Enzyme (Q60CN1) and Glutathione-S-Transferase (Q608Y4)

In order to study the interaction between Chromium VI and the proteins assumed to be involved in reduction of chromium (VI) in *M. capsulatus*, the structures of these proteins was predicted using Discovery Studio. The modeling of 3D structure of Q608Y4 followed a stepwise procedure, starting with a template structure search from PDB (Protein Data Bank), related to the target sequence using BLASTp (DS Server). From 32 BLAST hits, a potential template structure (PDB-ID: 2FUG), representing the structure of NADH-quinone oxidoreductase from *Thermus thermophilus* with 43% identity was taken as template for model building (Figure 5.9a). From 16 BLAST hits, a potential template structure (PDB-ID: 2ISK), representing the structure of Blub Bound To Oxidized Fmn from *Sinorhizobium meliloti* with 45% identity was taken as template for model building (Figure 5.10a). From 38 BLAST hits, a potential template structure (PDB-ID: 2GDR), representing the structure of Crystal Structure of a Bacterial Glutathione Transferase with 57% identity was taken as template for model building (Figure 5.11a) The target template alignment was generated by Discovery Studio and used as input to generate the 3D structures of Q608Y4, Q605J4 and Q60CN1 respectively.

5.6.1 Structures of the NADH Quinone Oxidoreductase, Nitroreductase Family Protein, Glutathione-S-Transferase with Bound co-factors

Models of the protein component of the three putative chromate reductases were constucted by using the templates with known three-dimensional structure, as detailed above. The metals and prosthetic groups present in the native enzymes were then introduced into the modelled structures by copying their coordinates from the PDB files containing the template structures. The modelled structure of Q60Y84 (the putative NADH quinone oxidoreductase) is shown in Fig 5.9b, along with its presumed iron-sulfur cluster prosthetic groups. The structure of the protein has a ferridoxin like-fold and belongs to the ferridoxin superfamily. As can be seen in figure 5.9b, these Fe_4S_4 clusters have a tetrahedral arrangement of irons and sulfurs. Each is coordinated by the sulfur atoms of four cysteine residues.

The modelled structure of the possible chromate reductase enzyme Q605J4 (Nitroreductase family protein) shows a bound flavin mononucleotide (FMN) prosthetic group (Figure 5.11). The protein has a sandwich architecture, which produces a cleft that is proposed to bind the FMN. The bound FMN is stabilised by hydrogen bond interactions with the protein, as follows: ARG21: NH1 - FMN217:O2P, ARG22: NE - FMN217:O3P, ASP23: N - FMN217:O1P, ARG25: NE - FMN217:O2, SER155: N - FMN217:O4, FMN217: N3 - ILE97:O, FMN217:O3' - ASP23:OD2 (Figure 5.11b).

The structure of Q60CN1, the putative glutathione S-transferase from *M. capsulatus* that may also be involved in reduction of chromate (VI) shows the bound with glutathione and HPX (2Z, 4E)-2-hydroxy-6-oxo-6-phenylhexa-2, 4- dienoic acid) that were present in the template structure used for modelling. The protein has an up-down bundle and sandwich architecture (Figure 5.10b).

Possible modes for the interaction of the modelled quinone reductase and nitroreductase proteins with hexavelent chromium ion were studied *in silico* using discovery studio and the putative interacting amino acid moieties and distances are documented below (Table 5.2). There are already separate NMR-derived structures for the two domains of the reductase component of soluble methane monooxygenase (PDB accession no. 1TVC(FAD and NADH binding domine) and 1JQ4(Fe2S2 domine), therefore, this structure was not included in the modelling.

Table 5.2: Interaction of Hexavelent chromium and the distance in A° unit with associated amino acid

Molecule	**Amino acid**	**Distance (Angstrom unit)**
Q608Y4-NADH quinone oxidoreductase	Glu135: OE1-Cr	1.472
	Glu135: OE2-Cr	1.411
Q605J4- Nitroreductase family protein	Leu166: CD2-Cr	3.491
	Leu166: CD1-Cr	3.866
	Ile70: CD1-Cr	3.768
Q60CN1- Glutathione-S-Transferase	Ser160: OG-Cr	1.296
	Leu159: N- Cr	4.133
	Ala157: O- Cr	1.304

5.7 Conclusions

In studying the chromate reductase activity of *Methylococcus capsulatus* the sequence similarity search with *E. coli* confirms the presence of three chromate reductase like proteins in *Methylococcus capsulatus*: Oxygenase (YP_114919), Quinone reductase (YP_114800) and Methane monooxygenase subunit C (YP_113665). The sequence analysis and hydrophobicity index reveal that the Oxygenase enzyme is a soluble protein, Quinone reductase has one transmembrane helix and is a membrane protein and Methane monooxygenase is a soluble protein. In order to study the interaction between Chromiun (VI), and these proteins their 3D models were predicted using Discovery Studio. Interaction studies were performed to study the mode of interaction of these proteins with chromium. In order to identify other proteins in *Methylococcus capsulatus* with chromate reductase activity, a literature search was done and the following proteins were obtained: Quinone Oxidoreductase enzyme (Q608Y4), Nitroreductase enzyme (Q605J4) and Glutathione-S-Transferase (Q60CN1). Hydropathy scale prediction of Q608Y4 and Q605J4 confirms also to be hydrophobic proteins and they have a very little metal binding capability. However, the positive score for hydrophobicity of Q60CN1 indicates that it could be involved in metal binding. 3D models were constructed for these proteins to study their interaction with chromium (VI). The modelled proteins show potential binding sites for chromium (IV). Suggesting that Quinone Oxidoreductase enzyme (Q608Y4), Nitroreductase enzyme (Q605J4) and Glutathione-S-Transferase (Q60CN1) have ability to bind chromium (VI) and reduce it to chromium (III). Further, active site analysis proves that Q60CN1 of *Methylococcus capsulatus* has more hydrophobic residues in the active site and hence could have more protein to bind to chromium.

The bioinformatics studies described in this chapter have identified a small number of candidate chromate reductases in the genome sequence of *M. capsulatus* (Bath). Further biochemical and specific gene inactivation studies are needed to see which, if any, of these are responsible for the observed chromate reductase activity.

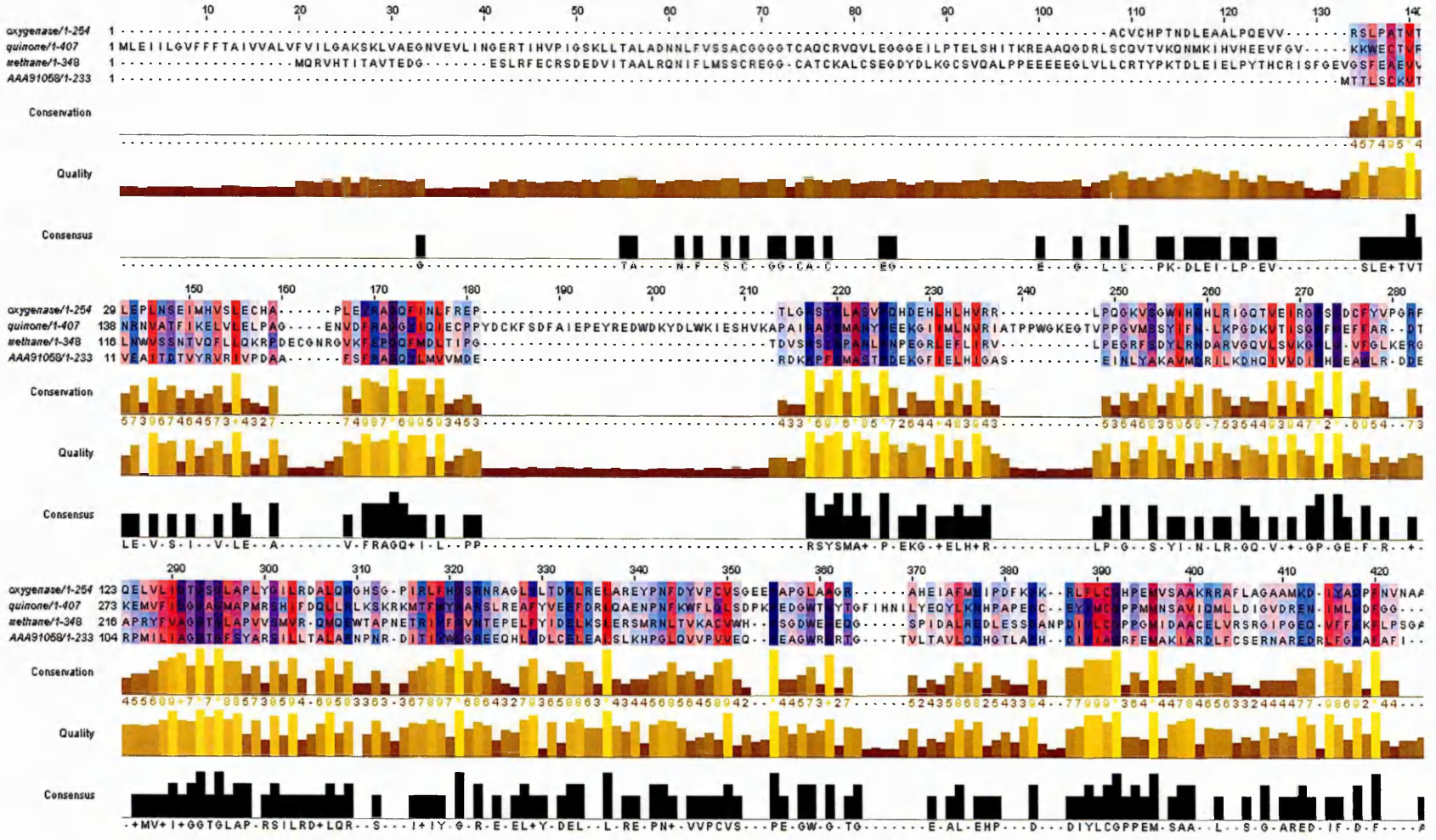


Figure 5.1: Multiple alignment (Clustal W) of the chromate reductase from E.coli (accession no. AAA91058) and the predicted Chromate reductases, oxygenase enzyme YP_114919, quinone reductase enzyme YP_114800 and methane monooxygenase subunit YP_113665 of *Methylococcus capsulatus.*

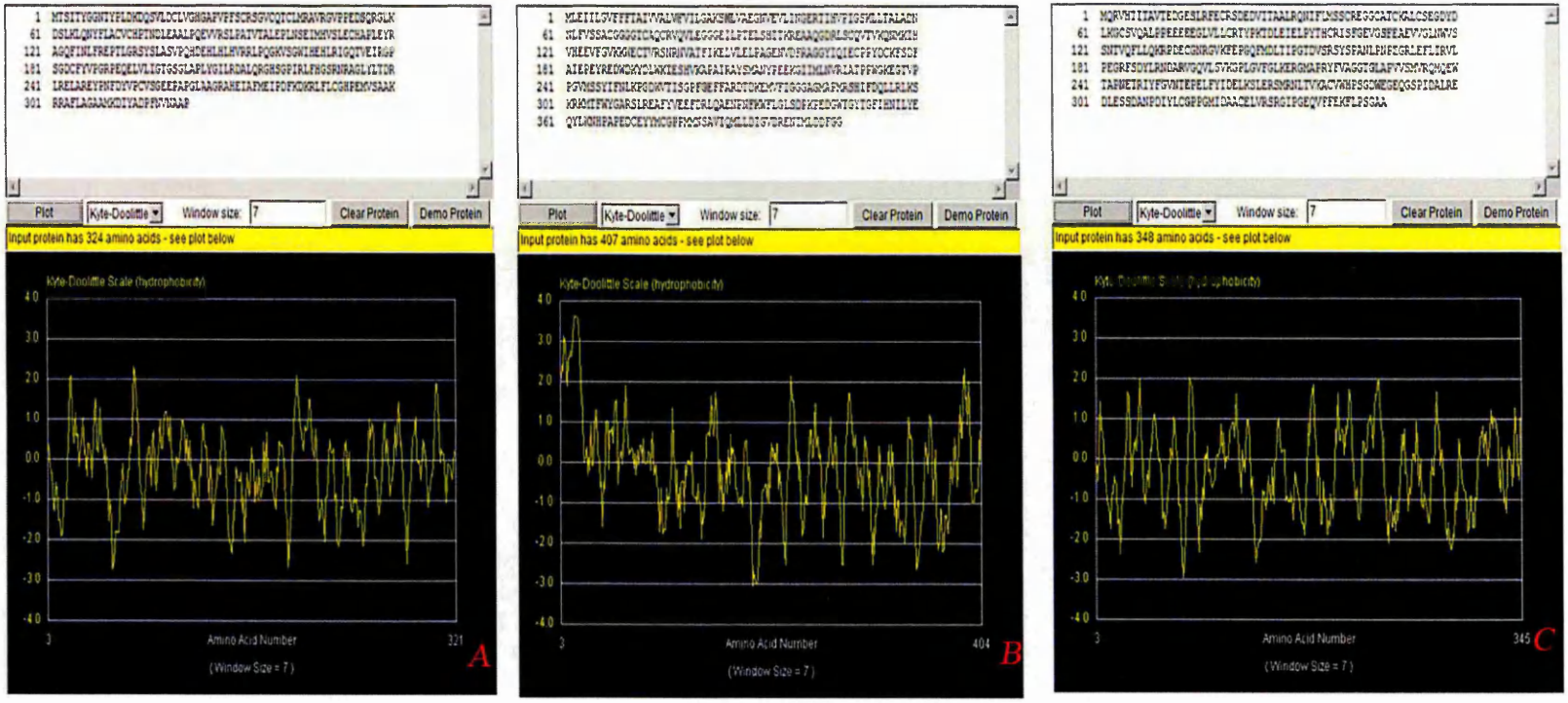


Figure 5.2: Hydrophobicity scale prediction for all the three proteins (a) oxygenase enzyme, (b) quinone reductase enzyme, and (c) methane monooxygenase using kyte-doolittle.

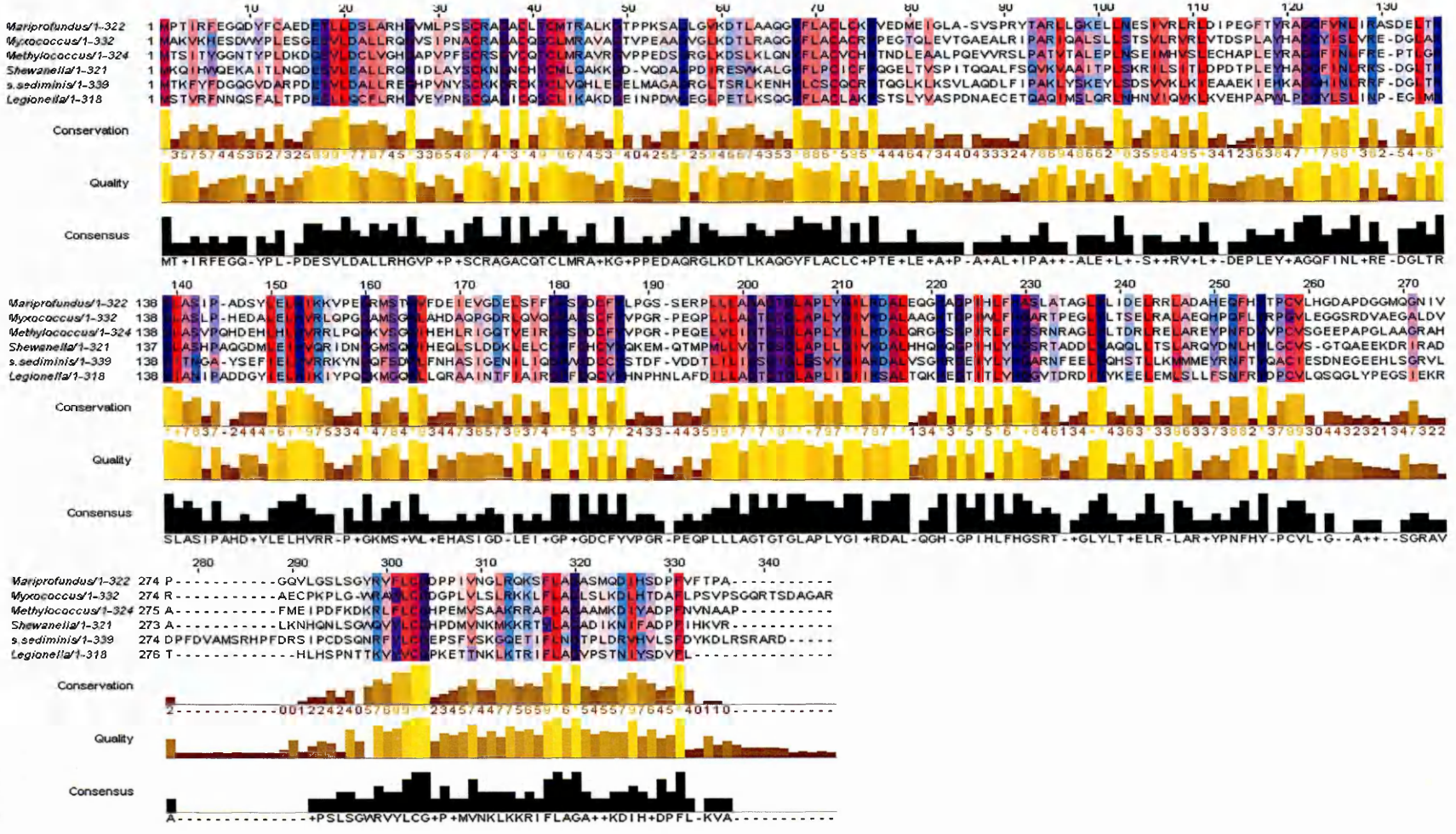


Figure 5.3: Multiple alignment (ClustW) of the enzyme YP_114919 of *Methylococcus capsulatus* showing similarity and conserved al regions to Oxygenase and ferrodoxin reductase of other Bacteria

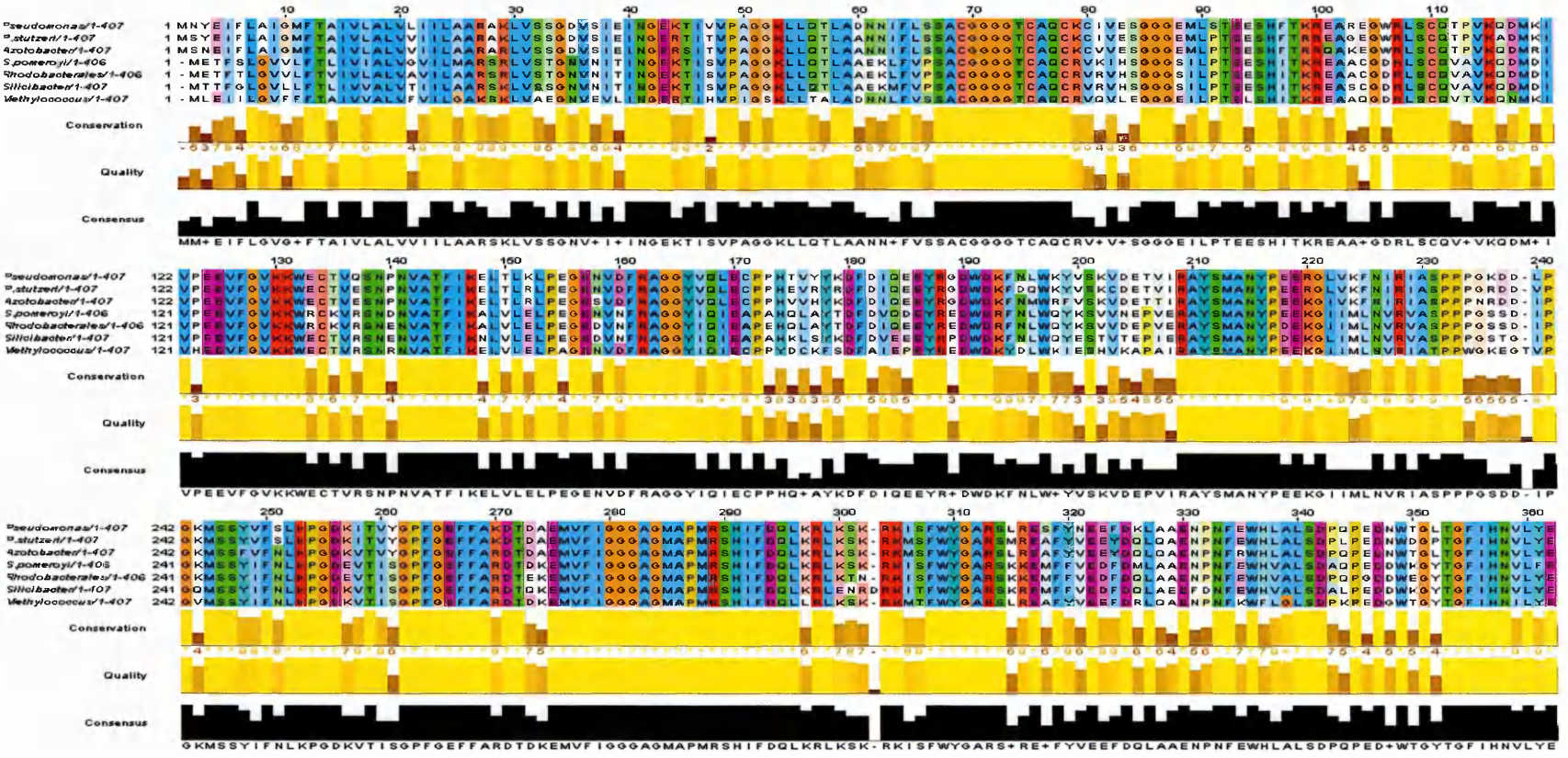


Figure 5.4: Multiple alignment (Clustal W) of the Quinone reductase enzyme YP_114919 of *Methylococcus capsulatus* showing similarity and conserved regions to Quinone reductase enzyme of other Bacteria.

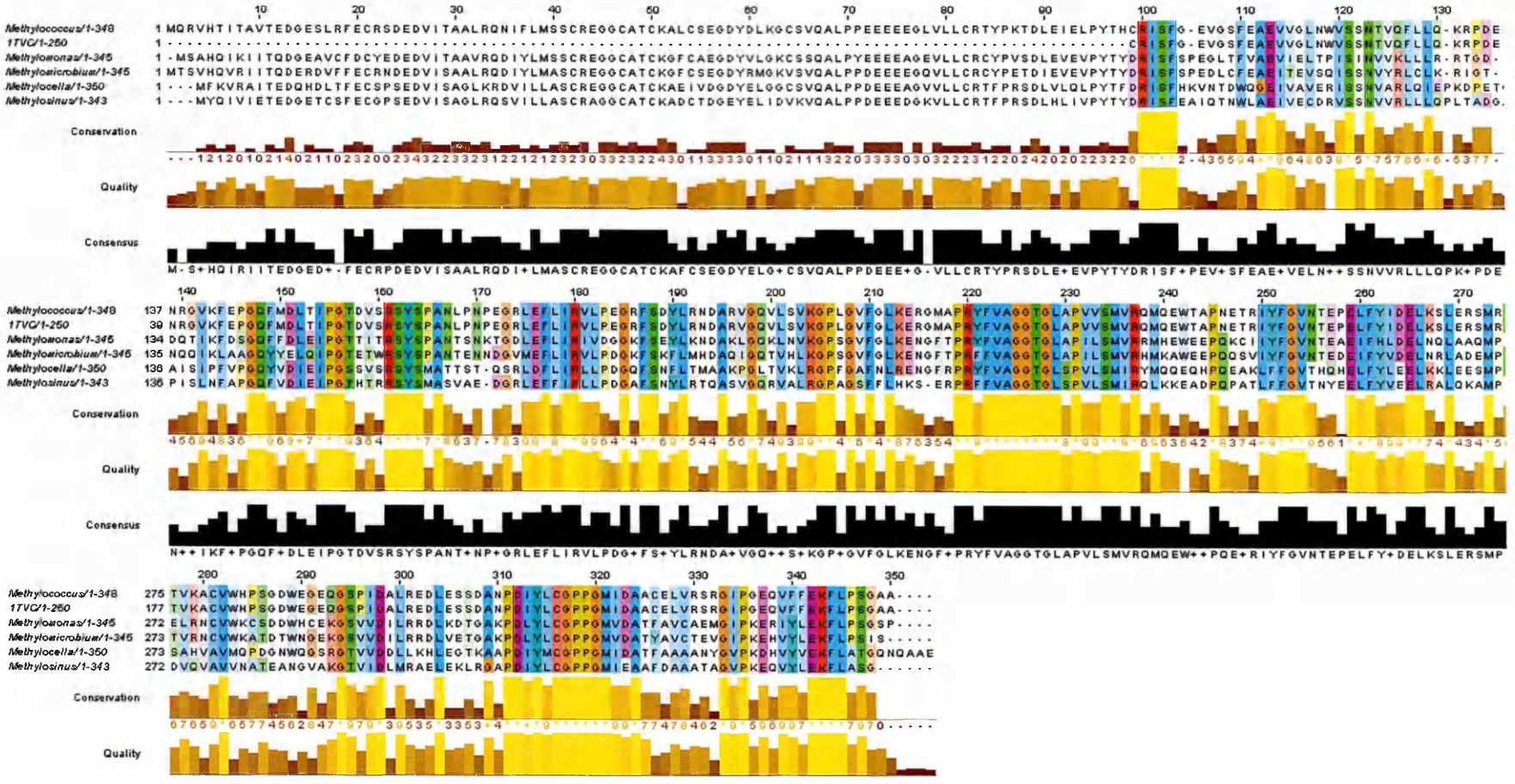
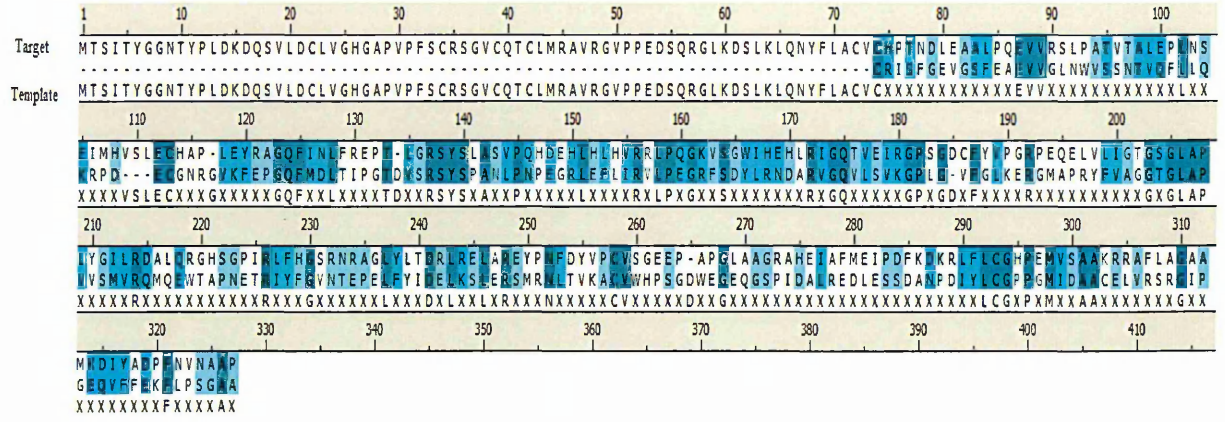
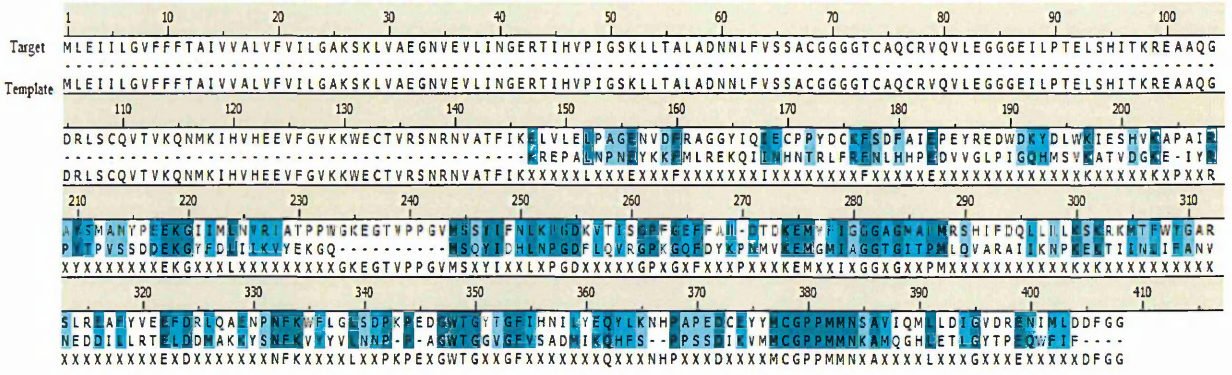


Figure 5.5: Multiple alignment (Clustal W) of the methane monooxygenase (mmoC) subunit YP_113665 of *Methylococcus capsulatus* showing similarity and conserved regions to methane monooxygenase and oxidoreductase enzyme of other Bacteria

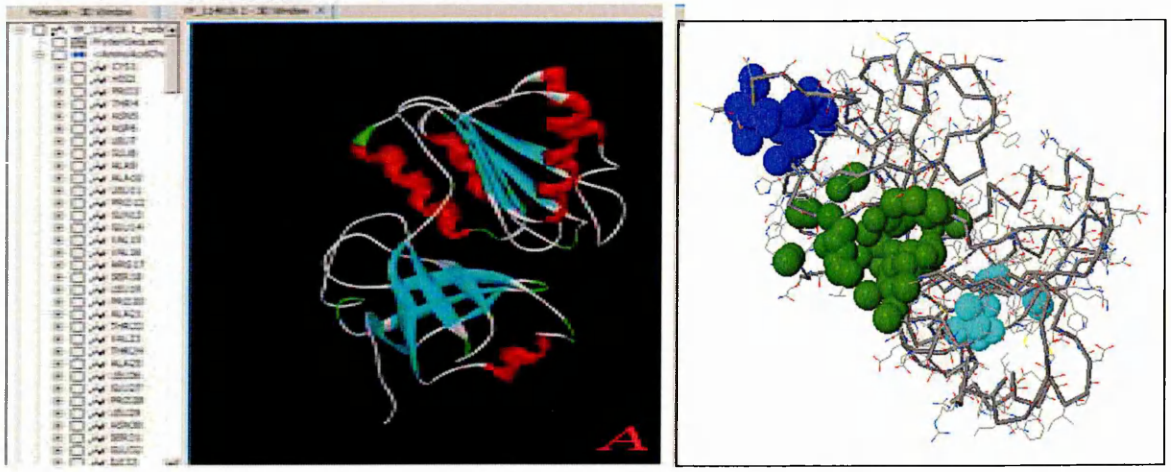


(a)

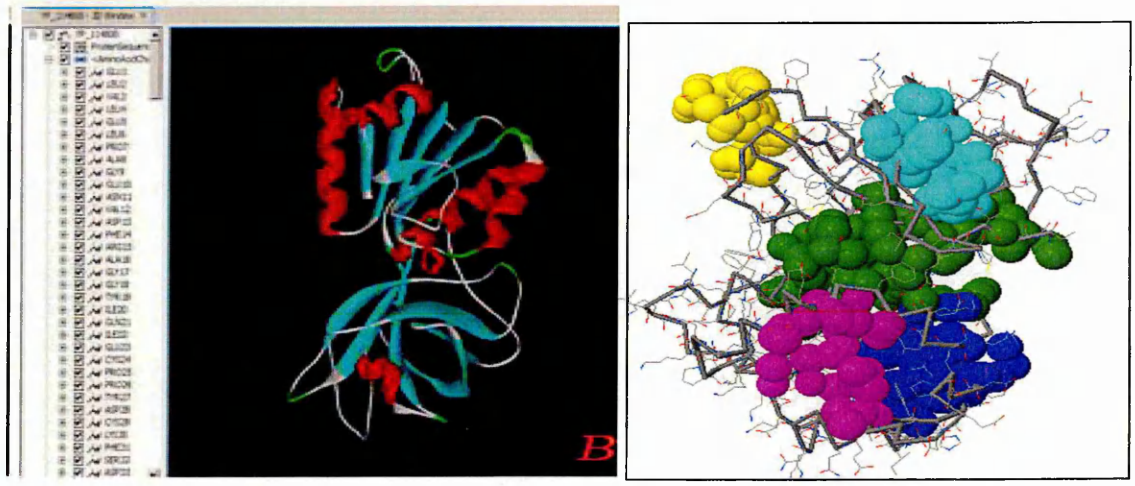


(b)

Figure 5.6: Target template alignment for (a) Oxygenase YP_114919, and (b) Quinone reductase YP_114800



(a)



(b)

Figure 5.7: Protein modelling Output with Active cite prediction (a) YP_114919, and (b) YP_114800, using Accelry's Discovery Studio. Coloured spear indicates the bound co-factor

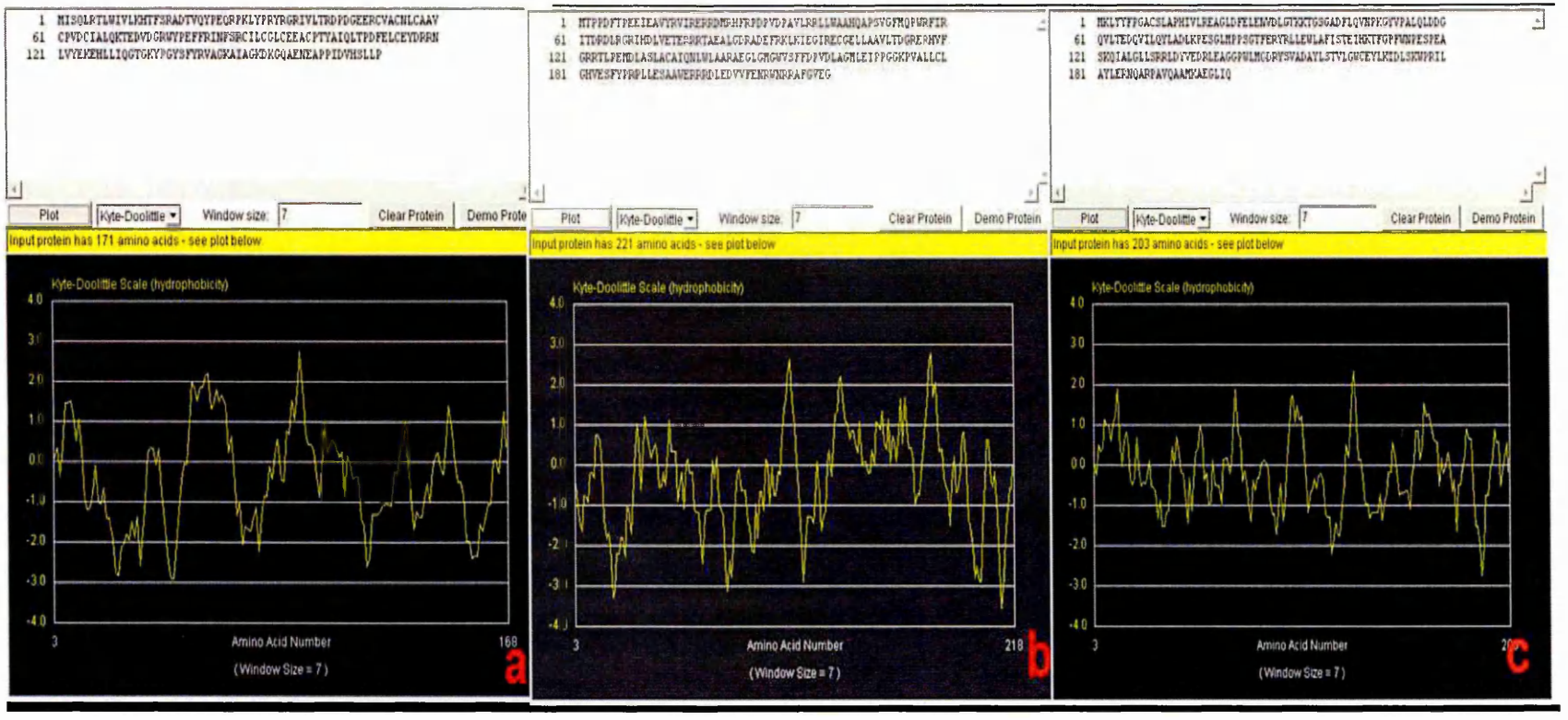


Figure 5.8: Hydropathy scale prediction performed using the Discovery Studio package, for all the three proteins(a) NADH Quinone Oxidoreductase , (b) Nitroreductase protein family (c) Glutathione-S-Transferase using SOSUI, Kyte-doolittle.

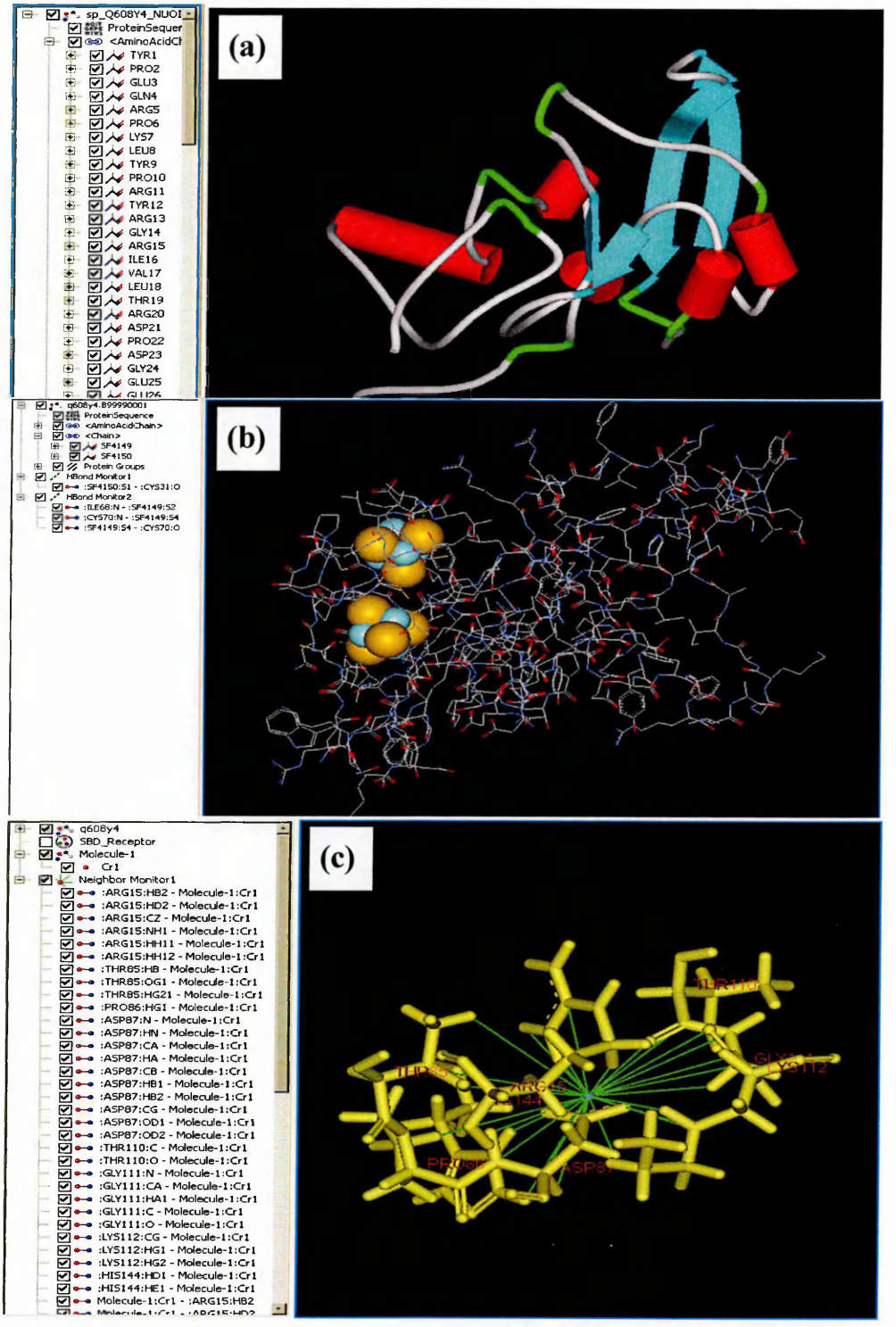


Figure 5.9: Protein modeling Output of (a) NADH quinine oxidoreductase (b) Structure of Q60Y84 (NADH quinine oxidoreductase) with SF4 (Iron/Sulfur cluster: iron blue, sulfur orange) (SF4150, SF4149) (c) interactive amino acids with Hexavelent chromium using discovery studio.

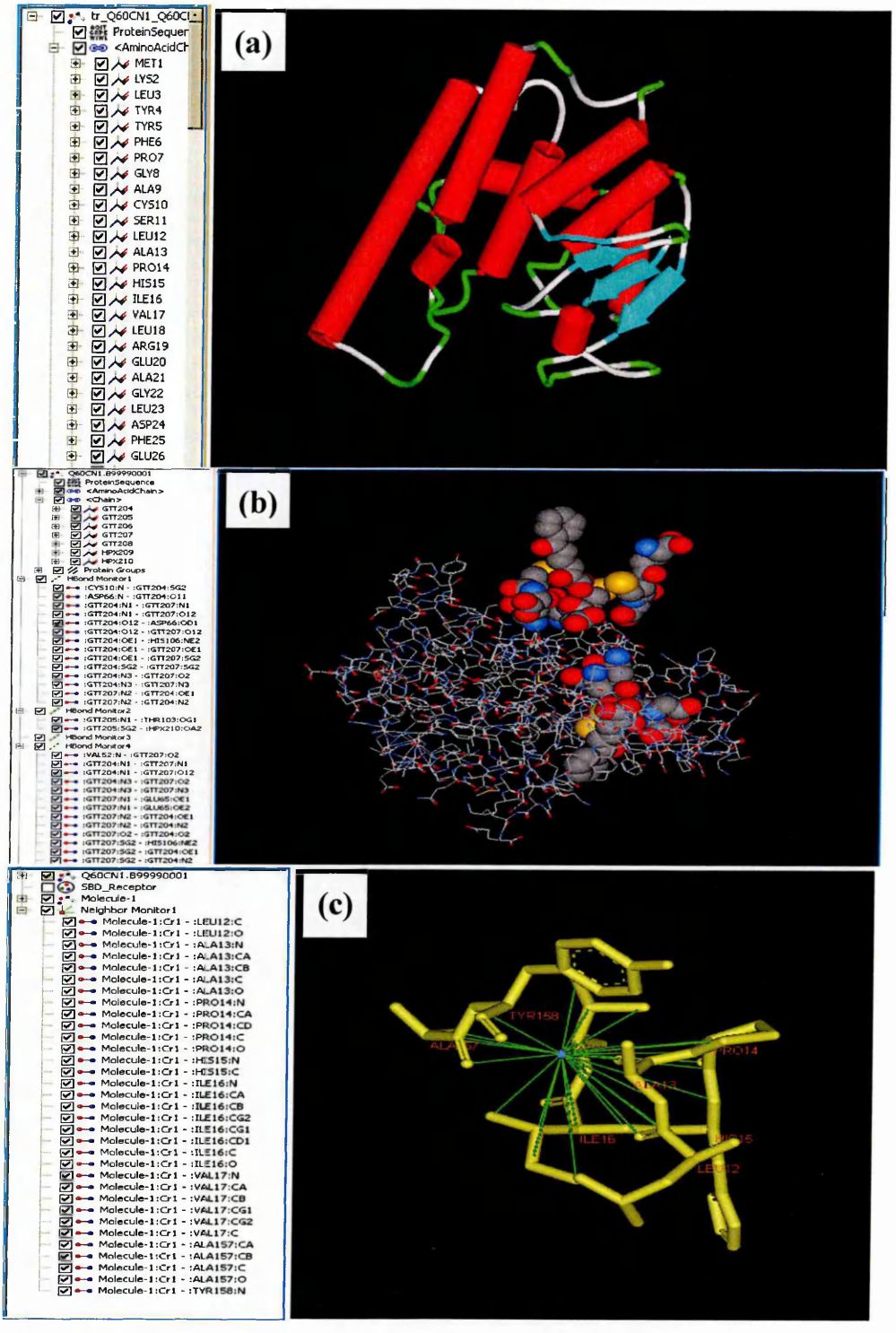


Figure 5.10: Protein modelling Output of (a) Glutahione-S- Transferase (b) Structure of Q60CN1 bound with Glutathione and HPX (2Z, 4E)-2-HYDROXY-6-OXO-6-PHENYLHEXA-2, 4- DIENOIC ACID (c) Interactive amino acids with Hexavalent chromium using Discovery Studio

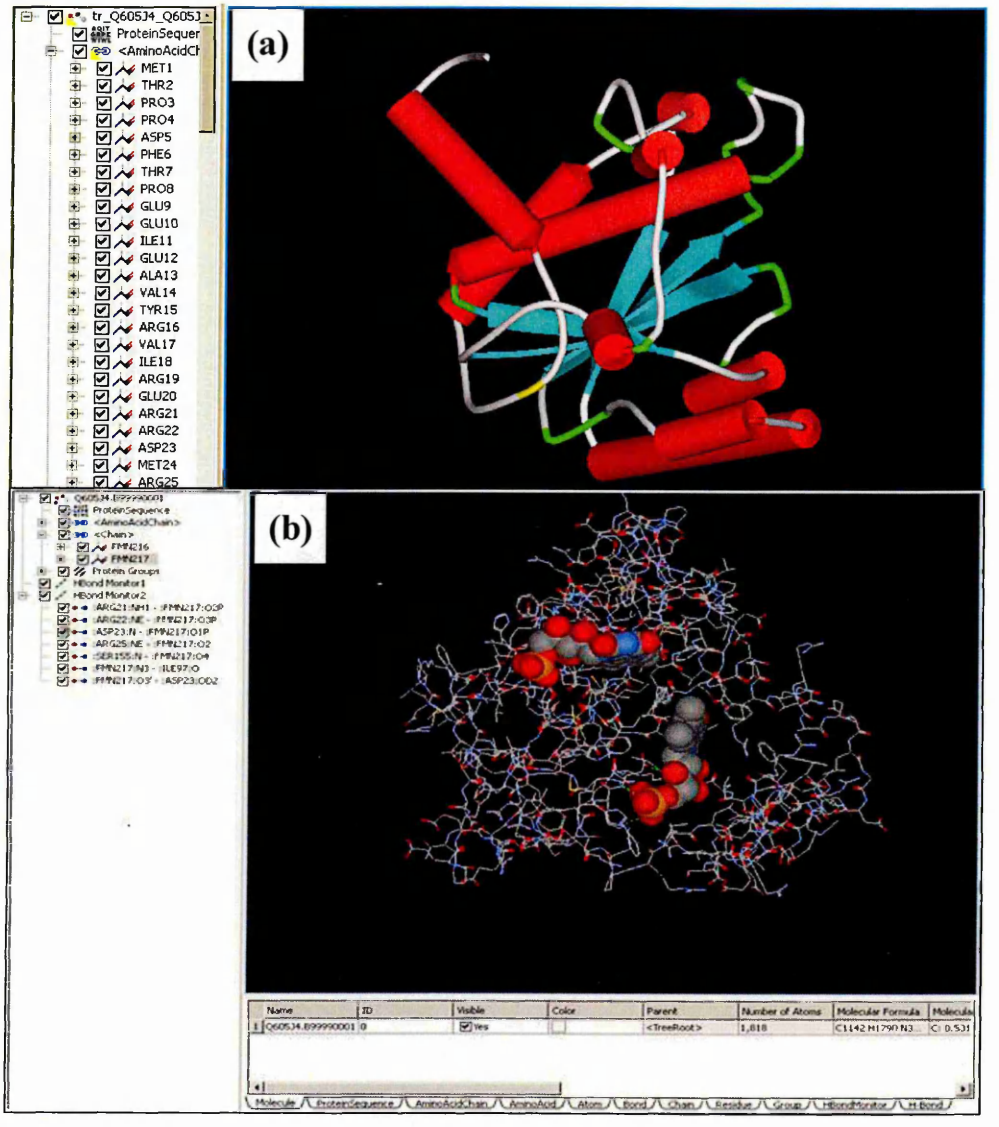


Figure 5.11: Protein modelling output of (a) Nitroreductase family protein, and (b) Structure of Q605J4 (Nitroreductase family protein) bound with FMN (FMN217, FMN216) using Discovery Studio.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 Discussion

The major achievement of this research is that for the first time, it has been shown that the methanotrophs, a major group of microorganisms with a critical role in the global methane cycle, have worked well for the biotransformation of chromate (VI) pollution. In the presence of methane as a sole carbon source, the methanotrophs were shown to reduce chromate (VI) to chromium (III)

E. coli ATCC 33456, and the methanotrophic species (*Methylosinus trichosporium* OB3b and *Methylococcus capsulatus*) were used to study the transformation of Cr (VI) into Cr (III) in vitro. While *E. coli* has previously been studied for chromium transformation, methanotrophs have been overlooked for this purpose. Initial results showed that pure cultures *M. trichosporium* did not significantly reduce chromate (IV). It was therefore decided to study the mixed culture of *E. coli* with *Methylosinus trichosporium* OB3b using methane as the only supplied carbon and energy source. It was known that *E. coli* can derive nutrients from the methanotroph and it was thought possible that such a mixed culture may be able to reduce chromium (VI) in the same way as has been observed previously using a mixed culture of *E. coli* ATCC 33456 and a toluene utilising *Pseudomonas* strain (Megharaj *et al.*, 2004). A mixed culture of *E. coli* ATCC 33456 and *Methylosinus trichosporium* OB3b showed inhibition in growth in the presence of chromium in solution. Chromium was not adsorbed greatly by dead bacterial cells. In mixed cultures, slight chromium oxidation occurred, but not in useful amounts. Previous results have been confirmed and the methodology to measure the bioreduction of chromium was established. The potential of *Esherichia coli* ATCC 33456 for use in the reduction of chromium (VI) to less toxic and less soluble chromium (III) has been confirmed. The strain showed reasonable cell growth with time and under the most favourable conditions tested, 83.1 % of the chromium (VI) added was recovered at chromium (III). This value is sufficient to confirm the usefulness of *E. coli* ATCC 33456 capabilities in the bioremediation field.

The ability of the methanotroph strains to remediate chromate was tested by adding potassium chromate (VI) (to a final concentration of 10 mg/l of Cr) to log-phase cultures of the strains and continuing incubation in the presence of the growth substrate methane, during which time the supernatant chromate (VI) concentration was monitored at intervals by using the diphenylcarbazide assay. Whilst cultures of the type II methanotroph *M. trichosporium* OB3b did not detectably reduce the chromate (IV) concentration over a 200 h incubation period (data not shown), cultures of *M. capsulatus* (Bath) caused a fall in chromate concentration of 77% from an initial value of 11.5 mg/l over 144 h (Figure 3.7). That this effect was due to the presence of active *M. capsulatus* cells was established by control experiment in which the culture was killed by autoclaving before addition of the chromate (VI), where significant decline in the supernatant chromate concentration was not observed (Figure 3.8). It was found that *M. capsulatus* (Bath) was able to reduce chromate (VI) from initial concentrations ranging from 1.4 to 1000 mg/l. The initial rate of chromate (VI) removal increased with increasing concentration, although the percentage of chromate removed in the experiment decreased (Figures 3.7 and 3.8). With a starting concentration of chromium (VI) of 1.4 mg/l, (at which complete removal of chromate was achieved), addition of the metabolic inhibitor sodium azide caused a 57% decrease in chromate (VI) removal. Omission of methane (the source of carbon, energy and reducing equivalents for the methanotrophic cells) completely suppressed the Cr (VI) removal ability.

The data shown in Figure 3.6 indicates that some chromium reduction occurred in a mixed culture of *E. coli* ATCC 33456 and *Methylosinus trichosporium* OB3b and small quantities of chromium were also removed from the culture medium. The increase in cell-associated chromate mirrored the observed decrease in chromate concentration in the medium, suggesting that the decrease in chromate concentration was due to chromate uptake by the cells. Since pure cultures of *E. coli* ATCC 33456 could reduce chromium (VI) but pure culture of *M. trichosporium* could not, it is likely that in the mixed culture experiments (where methane is the only source of carbon and energy) that the chromium reduction was effected by the *E. coli* cells, supported by multi-carbon nutrients scavenged from the methanotroph growing on methane.

In contrast to the results obtained with the type II methanotroph *M. trichosporium*, the type I methanotroph *M. capsulatus* was found to be very effective in reducing chromium (VI) to chromium (III) in pure culture. These results, initially obtained using the diphenyl carbazide assay, were confirmed by ICP-MS and Transmission Electronic Microscopy, X-ray absorption spectroscopy and EDSX.

The analysis of both total chromium and chromium (VI) during chromium reduction by *M. capsulatus* was determined in the presence of methane. The data show a reduction in chromium (VI), with the total detectable chromium in the system remains constant. Chromate was reduced by *M. capsulatus*, with the maximum chromate reduction by this bacterium occurring in the presence of methane as the sole energy source during the first 48 hours (Figure 3.7).

In order to quantify both chromium (VI) and chromium (III), inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was used, connected downstream from a dionex column that separated the two forms of the metal (chromate (VI) and chromium (III)) by suppressed ion chromatography. In addition to the change in chromium oxidation state described above, there was a change in distribution of chromium from supernatant to cells, which was shown by separate ion chromatography and ICP-AES on cell and supernatant samples. Chromate (VI) reduction by *M. capsulatus* was observed over a wide range of chromium (VI) concentrations, from 10 mg/l and 1,000 mg/l.

The effect of chromate and the metabolic inhibitor sodium azide, on methane–stimulated oxygen uptake by *M. capsulatus* was investigated to see whether chromate was toxic to the cells and to investigate whether the chromate reduction process was dependent on cellular metabolism. The metabolism of *M. capsulatus* was measured using the oxygen electrode, because oxygen-uptake was measured in response to a decline of methane saturated water. This approach could therefore be used to determine whether the chromate reduction process was metabolically dependent. The presence of sodium azide inhibits chromate reduction, and demonstrates that the reduction of chromate by *M. capsulatus* is a metabolically dependent process.

The results are corroborated by using analysis including Inductively-Coupled Plasma-Mass Spectrometer (ICP-MS), Transmission Electron Microscope (TEM),

Energy dispersive X-ray Spectroscopy (EDXS) and X-ray absorption Fine Structure (XAFS). Transmission electron micrography of cells exposed to 100 mg/l of chromate (VI) indicates that the chromate is evenly distributed within or over the spherical *M. capsulatus* cell walls. When an initial chromate (VI) concentration of 500 mg/l was used, electron absorbing material was still localised largely in or on the cells, but took the form of small particles rather than uniform staining of the cells (Figures 4.1 and 4.2).

X-ray spectroscopy (XANES and EXAFS) was used to analyse the oxidation state of chromium and its chemical environment after reduction by the *M. capsulatus* culture. The near edge region of the spectrum (i.e. the XANES region) is shown in Figure 4.3 for the cells and some standard materials. The energy zero has been set at 5989 eV, the edge energy for chromium metal. The shift in the energy of the edge as we proceed from chromium metal to Cr (III), from the $CrCl_3$ solution, to Cr (VI), from the chromate solution, is clear, as is the strong pre-edge feature on the Cr (VI) edge which may be used to identify this species. To within the resolution and stability of the monochromator, the edges from the cell spectra are identical in energy to that for Cr (III) in solution. Thus we identify the chromium in the cells as being in the Cr (III) charge state. Also, the edge spectra from the cells are essentially identical to that from chromium in a phosphate solution. The EXAFS analysis (see below) suggests that this solution contains insoluble chromium phosphate as a suspension. Thus we suggest that the cells contain chromium in the form of (insoluble) phosphate. The analysis of the EXAFS from the standard crystals gave inter-atomic distances in good agreement, generally to within 0.02 Å, with the known crystal structures. The analysis of the chromium metal monitor foil spectrum gave a value of 0.9+0.1 for the amplitude reduction factor AFAC. This is in line with many other determinations of this factor for transition metals and so 0.9 was used for the rest of the analysis. This may result in a systematic error of about 10% for the coordination numbers. With this value of AFAC the co-ordination numbers obtained for the standard crystals were in agreement with the known values to within the statistical error of about ±20% (EXAFS amplitudes are always much less precise than the distances). The mean square variations in distance were then reasonable, agreeing with values calculated from vibrational frequencies. The results from the analysis of the spectra from aqueous solutions are given in Table 4.1. The aqueous

solution of potassium chromate gave a three-fold co-ordinated chromium ion with a Cr (VI)-O distance identical to that in the crystal. The two aqueous solutions containing trivalent chromium gives a Cr (III)-O distance identical to that in crystalline $CrC_{13}.6H_2O$ and crystalline Cr (PO_4). Cr (III) in aqueous solution is six-fold co-ordinated, presumably by water molecules. Addition of phosphate to the aqueous solution of chromium chloride gave an identical Cr (III)-O co-ordination. However, a further peak is visible at about 300 pm in the Fourier transform of the EXAFS of this sample. Including an extra shell at this distance significantly lowers the fit index. Such a second shell contribution rarely, if ever, appears in solutions, unless the atom is complexed. EXAFS is not particularly good at distinguishing atoms of similar atomic number, since their electron scattering factors are very similar. This contribution could be fitted by any atom with an atomic number between about 10 and 30. The composition of the sample and the distance led us to assume that this contribution was due to phosphorus. It is then found that there are about six phosphorus atoms at a distance of 322 pm. This chromium environment is identical to that found in Cr (PO_4). Chromium phosphate is insoluble in water which suggests that this sample contains a suspension of chromium phosphate. There was no sign of a diminution of the chromium fluorescence signal during the six hours of data taking, suggesting that no settling is occurring. The suspension must therefore be very finely divided. The significance of chromium in the cells is identical to that in the solution containing phosphate. Again the inclusion of a second shell significantly lowers the fit index. The results imply that chromium in the cells is in the Cr (III) state (as shown by the Cr-O distance) and probably in a suspension (as shown by the second shell contribution). This suspension is most probably insoluble Cr (PO_4). To within the rather large uncertainties there is no change in the chromium with either time or temperature. This suggests that chromium in these cells is precipitated out within 24 hours.

In order to identify genes that can encode proteins involved in reduction of chromate by *M. capsulatus*, the database of translated open reading frames from the complete genome sequence (i.e. all the known and potential proteins of the organism) was searched for homologues to the known classes of chromate reductases from other bacteria. BLAST searches indicated the presence of three homologues of the *E. coli* Free chromate reductase (Puzon *et al.*, 2002; accession no. AAA91058) in

the *M. capsulatus* (Bath) genome all of which are known or likely flavin/Fe_2S_2 oxidoreductases: (Table 5.1). The *E. coli* nitroreductase NfsA, which also reduces chromate (Ackerley *et al.*, 2004a), has one significant homologue in *M. capsulatus* (accession no. YP_113831, $E = 6 \times 10^{-30}$). The chromate reductase ChrR of *P. putida* (Ackerley *et al.*, 2004b; accession no. Q93T20), a flavoprotein capable of chromate reduction, did not have any significant homologues in *M. capsulatus*. The known chromate efflux system ChrA, typified by the chromate efflux pump of *Pseudomonas aeruginosa* plasmid pUM505 (accession no. P14285; Cervantes *et al.*, 1990), which might have contributed to resistance of the cells to chromate (VI), did not have any significant matches in the genome of *M. capsulatus*. The hydropathy score of these proteins was found to be very low which suggest that all these proteins are soluble, although the putative NADH quinone oxidoreductase has one potential membrane-spanning helix, suggesting a largely globular protein anchored into the membrane via one helix. The hydrophobicity scores obtained using the SOSUI program were as follows: Q608Y4 (NADH quinone oxidoreductase), -0.335088); Q605J4 (Nitroreductase), -0.358823); Q60CN1 (glutathione S-transferase), -0.158621.

The result shows that the putative NADH quinone oxidoreductase has about 52.7% hydrophobicity and the amino acid leucine has the highest percent of occurrence ~11.4%. The hydrophobicity profile of the protein indicated that it was a largely soluble protein and the average hydrophobicity value for the protein was calculated to be -0.212037. This is consistent with a protein with a single transmembrane helix, where most of the protein resides in aqueous solution outside the membrane.

The structures were modeled along with the bound factors copied from their respective templates and it has been possible to suggest how the proteins may be involved in interaction with redox-active prosthetic groups and substrates that may be involved in reduction of chromium (VI).

The interaction of the proteins with chromium ion was studied using discovery studio and the interacting amino acid atom and distances are Glu135: OE1-Cr: 1.411Å, Glu135: OE2-Cr:1.472 Å for NADH quinone oxidoreductase, Leu166: CD2-Cr: 3.491 Å, Leu166: CD1-Cr: 3.866 Å, Ile70: CD1-Cr:3.768 Å for

nitroreductase, Leu159: N- Cr: 1.296 Å, Ser160: OG-Cr: 4.133 Å, Ala157: O- Cr: 1.304 Å.

6.2 Conclusions

The results presented in this Thesis show that a methanotrophic bacterium, *M. capsulatus* (Bath), is able to detoxify chromate (VI) over a wide range of concentrations and that the product is chromium in the relatively nontoxic +3 oxidation state. The observation of chromium (III) in a phosphorous/oxygen coordination environment in the particulate fraction after exposure of cells to 1,000 mg/l of hexavalent chromium is consistent with the formation of insoluble chromium (III) phosphate in the phosphate-containing growth medium. Indeed, the electron-dense particles seen in via transmission electron microscopy of cells from cultures exposed to 500 mg/l of chromium (VI) may be particles of such chromium (III) phosphate. Phosphorous/oxygen coordination environments would also be produced from association of (a proportion of) the chromium (III) with phosphate containing cellular components such as nucleotide coenzymes (Puzon *et al.*, 2002) and DNA, where phosphorus/oxygen coordination of chromium (III) may be important in the mutagenic properties of chromate (VI) (Zhitkovich *et al.*, 2001). The apparently uniform staining of the cells with electron dense material after exposure to the lower concentration of chromate (VI) of 100 mg/l suggests association of the chromium with some form of cellular material.

The effect of the metabolic inhibitor sodium azide on the chromate (VI) reduction reaction, and also the fact that the reaction is not shown by autoclaved (dead) cells, supports the conclusion that the reaction is dependent on active cellular metabolism rather than merely a reaction between the chromate and cellular constituents as described by Fein *et al.* (2002). The effect of azide is presumably an indirect one since inhibition of the reduction of dioxygen to water would not per se prevent channelling of reducing equivalents into reduction of chromate. The sequence similarity searches using known chromate-reducing enzyme sequences indicate at least four possible candidates for the chromium reducing enzyme(s) in *M. capsulatus*. One of these is the well characterized reductase-component of soluble methane monooxygenase, a component of one of the enzyme systems that is

involved in oxidation of methane to methanol (Lund *et al.*, 1985; Smith and Dalton, 2004). It is unlikely that this particular candidate is involved in reduction of chromate in these experiments, because the cells were cultivated under conditions of high copper-to-biomass ratio, when the soluble methane monooxygenase is not expressed (Stanley *et al.*, 1983). *M. capsulatus* also produces a 1,216-Da fluorescent chromopepetide metal-binding compound, methanobactin (Kim *et al.*, 2004), which is believed to be involved in uptake of copper by the bacterium. Methanobactin also binds a range of other metal ions in the +1 to +3 oxidation states (Choi *et al.*, 2006); although to our knowledge its interaction with chromium species has not been investigated. Whilst a role in interaction with chromium cannot be discounted, methanobactin is unlikely to be a significant contribution to the chromium (III) species observed by means of EXAFS spectroscopy, since its binding to copper (I), and probably to other metals, is via nitrogen and sulfur (Kim *et al.*, 2004; Choi *et al.*, 2006). The results presented here indicate that certain methane-oxidizing bacteria, which as a group are widely spread in diverse environments, are able to detoxify hexavalent chromium by reducing it to chromium (III). It is therefore possible that methane-oxidizing bacteria contribute to chromate (VI) reduction in contaminated environments and may be suitable candidates for remediating inocula for bioremediation applications when both hexavalent chromium and organic pollutants are present. In *E. coli* the major response to chromate (VI) exposure comprises expression of enzymes necessary to counteract the oxidative stress cause by the oxidizing metal and, possibly, the highly reactive chromium (V) intermediate that results from one-electron reduction of chromate (VI) (Ackerley *et al.*, 2006). The data presented here extends the bioremediation potential of methanotrophs to include heavy metals. Furthermore, bioinformatics analysis has identified five potential chromate reductases in the genome of *M. capuslatus*, whose role in chromate (VI) reduction can now be tested experimentally (see Future work below)

6.3 Further Work

It is suggested that this work should be repeated using a field scale, rather than small scale bioreactors, to determine whether the observed bioremediation of chromium can be scaled up to a practical process of industrial use.

A very large number of methanotroph strains have been cultivated, of which only two have been tested for chromium (VI) reduction 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 methanotroph species should be investigated, including the species of the genera *Methylococcus* and *Methylosinus* that have not yet been tested. The mechanism of chromium reductase of *M. capsulatus* can be better understood and it may be evidenced by using "Electron Spin Resonance" analysis for identification of transient intermediate species of Cr (V) while the in vitro experiment of Cr (VI) reduction to Cr (III) is taking place.

In order to investigate the roles played by the putative chromate (VI) reductases identified via the bioinformatics study, further biochemical and specific gene inactivation studies are needed to see which, if any, of these are responsible for the observed chromate reductase activity.

The complete annotated genome sequence for *M. trichosporium* OB3b, which did not reduce chromium (VI) under the conditions tested, will become available during April, 2010. Comparison of this new information with the genome of *M. capsulatus,* which is already in the public domain, may allow more confident prediction of the gene encoding chromate-reducing enzyme, which may be absent from *M. trichosporium.* Alternatively, if the genome sequence of *M. trichosporium* contains all of the putative chromium (VI) reductases found in *M. capsulatus*, it may be possible from their genetic context to propose modified culture conditions that might allow reduction of chromium (VI) by *M. trichosporium.*

It is hoped the results presented in this thesis, together with the results of the additional work proposed in this section, will allow widespread implementation of methane-oxidising bacteria for the remediation of chromium (VI) pollution.

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