Characterisation of the Bioremediation of Chromate by *Methylococcus capsulatus*

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Abstract

Chromate (VI) is an oxidising pollutant that is harmful to humans and the environment. Reduction of chromate (VI) produces chromium (III), which is less toxic, less soluble and less bioavailable. *Methylococcus capsulatus* Bath is an example of a diverse group of methane oxidising bacteria that are widespread in the environment and have potential for bioremediation of a wide range organic and inorganic pollutants, including reduction of chromium (VI) to chromium (III).

Cells of *Mc. capsulatus* were broken and centrifugally fractionated during the bioremediation reaction. HPLC-ICP-MS analysis showed that the concentration of chromium (VI) in the culture supernatant progressively declined and there was a corresponding increase in the concentration of chromium (III) in the cytoplasm + membranes fraction. Further fractionation showed that the distribution of chromium (III) was approximately two thirds in the cell membrane fraction and one third in the cytoplasm fraction. The cellular distribution and speciation of chromium were further investigated using transmission electron microscopy- energy dispersive X-ray (TEM-EDX), high-angle annular dark-field-scanning transmission electron microscopy (HAADF-STEM), Electron energy loss spectroscopy (EELS) and X-ray photoelectron spectroscopy (XPS) imaging of whole and sectioned cells.

Mc. capsulatus Bath took up Cr (III) that has been added to the culture medium via a process that was promoted by the growth substrate methane and inhibited by the metabolic inhibitor sodium azide. Accumulation of chromium (III) within

the *Mc. capsulatus* cells may reduce its bioavailability and help to prevent its re-oxidation to the toxic chromium (VI).

The cell fractions of *Mc. capsulatus* Bath were studied for their chromium (VI) removal activity at various temperatures and the optimum temperature of the fractions was found at 30 °C. All cellular fractions reduced chromium (VI) to chromium (III); reduction activity was greatest in the cytoplasm fraction. The chromium (VI)-removing activity, which was enhanced by the presence of NADH, was purified from the cytoplasmic fraction by means of DEAE Anion exchange and Capto Blue Dye Affinity chromatography to yield fractions that contained a single 17-kDa protein.

Three new methanotroph strains were isolated from the Leeds and Liverpool canal and a railway location near Doncaster. One of these (isolated from canal sediment via enrichment at 45 °C) is another strain of *Mc. capsulatus* that is capable of Cr (VI) removal.

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Declaration

I declare that the work described herein to study “Bioremediation of hexavalent chromium by methane oxidizing bacteria” in achievement of the requirements for the award of the Degree of Doctor of Philosophy, Biomolecular Science Research Centre, Sheffield Hallam University, was conducted by me under the supervision of Prof Tom Smith and Dr Philip Gardiner. The work in this thesis has not been submitted previously for a research degree.

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List of abbreviations

BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
ChrR	Cytoplasmic chromate reductase enzyme
Cr	Chromium
CV	Column volum
DEAE	Diethylaminoethyl
DPC	Diphenylcarbazide
EDXS	Energy dispersive X-ray spectroscopy
EELS	Electron energy loss spectroscopy
EPA	Environmental Protection Agency
EPR	Electron Paramagnetic Resonance
FAD	Flavin adenine dinucleotide
HAADF	High-angle annular dark-field
HPLC	High-performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
Mb	Methanobactin
MDH	Methanol dehydrogenase
MMO	Methane monooxygenase

MOPS	3-(N-morpholino)propanesulfonic acid
NADH	Nicotinamide Adenine Dinucleotide (reduced form)
NADPH	Nicotinamide Adenine Dinucleotide phosphate (reduced form)
Na-EDTA	Ethylenediamine-tetraacetic acid disodium salt dihydrate
NMS	Nitrate minimal salts
NPL	National Priorities List
PB	Binding buffer
PCR	Polymerase chain reaction
PKS	PolyKetide synthetase
pMMO	Particulate methane monooxygenase
pMMOH	Particulate methane monooxygenase hydroxylase
pMMOR	Particulate methane monooxygenase reductase
ROS	Reactive Oxygen Species
RuMP	Ribulose monophosphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sMMO	Soluble methane monooxygenase
TCE	Trichloroethylene
TEM	Transmission electron microscopy
XPS	X-ray photoelectron spectr

Chapter 1 Introduction and literature review

1.1 Introduction

Heavy metals (and metalloids) of environmental concern include chromium, cobalt, nickel, copper, zinc, arsenic, selenium, silver, cadmium, antimony, mercury, thallium and lead which can contaminate soils, ground water, sediments and surface waters. Heavy metal pollution is a very important global problem needing urgent attention (Faisal & Hasnain 2004).

The presence of high concentration of these pollutants in the environment, along with their different chemical forms, mobility and their availability to other life forms can result in adverse effects on crop cultivation and ultimately human and animal health. About 15 elements present in rocks and the soil at low concentrations and are essential for plant and animal nutrition. Of these, boron, copper, iron, manganese, molybdenum, selenium and zinc are essential for animal health. Furthermore, some researchers have also highlighted the essential roles played by arsenic, fluorine, nickel, silicon, tin and vanadium in animal nutrition (Sharma & Agrawal 2005; Kampa & Castanas 2008). A majority of the trace metal elements can also be toxic to plants and animals when present at high concentrations. Elements such as arsenic, cadmium, chromium, lead, mercury, molybdenum, nickel, selenium and zinc, when present at high concentrations in the foodstuffs, can make these items unfit for human consumption. These trace elements enter the environment either naturally or through anthropogenic activities. Many anthropogenic activities are responsible for introducing high concentration of elements, which far exceeds that due to natural geological activities. The human activities that lead to metal contamination in the environment include mining and smelting activities, industrial emissions, discharge effluents, vehicle emissions, urban

development, contaminated dust and rainfall, sewage sludge, composted town refuse, dumped waste materials, fertilisers, pesticides, pig slurry (it was seen to be the major source of animal waste contamination and therefore transported in the form of nutrient particles into the soil/ water or as an organic effluent) and soil ameliorant (Lepp 1981). Typical frequencies of the distribution of these pollutants are given in Figure 1-1.

Chromium (Cr) is toxic to the environment and biological systems. The removal of toxic metals (such as toxic forms of chromium) from the environment by means of microorganisms is a possible method of remediating heavy metals waste (Klaus-Joerger *et al*. 2001).

Figure 1-1 The frequencies of the most common contaminants at NPL sites (Based on data available for 976 National Priorities List (NPL) Sites with fiscal year 1982–2003. A site may contain one or more of these contaminants. Taken from (Dhal *et al*. 2013).

Bacterial cells are frequently exposed to stressful conditions and the ability to resist such pressures is fundamental for their survival. Many microorganisms have resistance mechanisms that allow them to grow at concentrations of metal ions that would otherwise be toxic. Such mechanisms include efflux systems, changes of chromium speciation. Such mechanisms can result in changes in the redox state of the metal ions, extracellular complexation and the precipitation of metals (Rouch *et al*. 1995; Beveridge *et al*. 1996).

1.2 Chromium definition, properties and uses

Chromium is a transition metal first discovered in Siberian red lead ore (crocoites) in 1798 by the French chemist, Louis-Nicolas Vaquelin (Fendorf 1995). The word chromium originates from the Greek word Chroma, meaning colour. This is because many chromium compounds have characteristic colours that cover most areas of the visible spectrum. Chromium (Cr) is one of the most common elements on earth and is a highly abundant element in the crustal rocks. The amount of chromium present in the Earth's crust can range between 100 to 300 mg kg^{-1}, and 5 to 3000 mg kg^{-1} in the soil (Cervantes *et al*. 2001). Chromium is a heavy metal that belongs to the d-block of the periodic table with an atomic number of 24. Chromium in pure form is a shiny metal that is steel grey, hard and malleable, with a density of 7.1 g cm^{-3}, boiling point of 2642 °C, it is also tasteless, and odourless. The most stable and common forms of chromium are the trivalent Cr (III) and the hexavalent Cr (VI) species (Cervantes *et al*. 2001). Chromium is naturally found in diverse forms, however, the most important is chromate, e.g. Na_2CrO_4, $FeCr_2O_4$ and $K_2Cr_2O_4$.

Chromium also exists in I, II, IV and V oxidation states (Zayed & Terry 2003). In soil trivalent Cr (III) is the most common and stable form. However, hexavalent Cr (VI) is associated with oxygen in the dichromate ($Cr_2O_7^{2-}$) and chromate (CrO_4^{2-}) oxyanions and displays chemical properties that have different effects on organisms. Whilst Cr (VI) is more toxic than Cr (III), Cr (III) tends to be absorbed through the soil surface more readily than Cr (VI) or it can precipitate as chromium hydroxide in slightly acidic or alkaline environmental conditions. Cr (VI) is water soluble in the full pH range. Therefore, Cr (VI) has a higher environmental contamination potential than Cr (III) (Fendorf 1995).

Chromium (III) is only slightly water soluble and as result it has low mobility within the environment and low toxicity to organisms in water (Barnhart 1997). Chromium (III) is a biological necessity to mammals because it is involved in the maintenance and efficiency of protein metabolism (Shrivastava *et al*. 2002). Low concentrations of Cr (III) can also increase plant growth. Surplus hexavalent chromium is strongly toxic to plants and animals and has been shown to be involved in the development of some cancers (Shanker *et al*. 2005).

Sources of environmental hexavalent chromium can be found in various industries such as mining, wood preservation, cement, metal plating, textile manufacture, inks, leather tanning, automobile manufacturing, steel, fungicides, production of paints, and pigments (Zayed & Terry 2003; Dhal *et al*. 2013).

1.3 Chromium toxicity

Since chromium may cause health problems at higher concentrations, its concentration in drinking water is regulated by the U.S. Environmental Protection Agency (EPA) (Cohen *et al*. 1993). The Environmental Protection Agency (EPA) has classified chromium as a group 'A' carcinogenic to humans, and therefore a major environmental pollutant (Figure 1-1). In both of its common forms, chromium (III) and chromium (VI), it may cause allergic contact dermatitis. The pollutants from soil, water and air can move and be transported by various biogeochemical cycles (Dhal *et al*. 2013).

Chromium (III) is less bioavailable and has less mobility in the environment compared to Cr (VI) which is highly soluble and bioavailable thus making its toxicity a greater environmental hazard, this is because chromium (VI) exists generally bound to organic materials, together in aquatic and soil environments. However, Cr (III) could oxidised to Cr (VI) in the presence of surplus oxygen, so it becomes more toxic (Jackson *et al*. 1999). Cr (VI) toxicity may be traced to the ease with which it permeates in the Eukarya, Bacteria and Archaea cell membranes with resultant respective reduction of Cr (VI) and formation of free radicals that can damage DNA and cause other adverse effects (Dhal *et al*. 2013; Xia *et al*. 2014).

Research has shown that chromium (III) is also harmful to cellular structures (Cieślak-Golonka 1996; Raspor *et al*. 2000; Cervantes *et al*. 2001). However, the chromium (VI) is more much toxic than chromium (III) in vivo. Since chromium (III) is generally less soluble it is less easily transported into cells (Srivastava *et al*. 1999).

1.4 Chromium uptake

The most common mechanisms used for bioremediation are intracellular sequestration and detoxification, which involves the chemical modification of the toxic metal form to its non-toxic form. The toxic metal Cr (VI) ion may show similar chemical and structural characteristics as nutrient ions, and hence, can be erroneously assimilated by cells. This is true of chromium, where the chromate (VI) ion (CrO_4^{2-}) has similar charge and structure to sulfate (SO_4^{2-}). In one study, Pepi & Baldi (1992) isolated two Cr (VI)-resistant microorganisms (*Candida* spp. and *Rhodosporidium* sp.) from industrial waste. They observed that these strains exhibited chromium resistance, not by the mechanism of Cr (VI) reduction, but by the reduced uptake of the Cr (VI) ions. When sulfur-containing amino acids (methionine and cysteine) were added to the culture media used for cultivating the microbes, the two yeast strains became less hypersensitive. *Candida* sp. could assimilate sulfur from sulfate more effectively from amino acids. Hence, these organisms showed a higher resistance. On the other hand, *Rhodosporidium* sp. possessed an ineffective sulfate transport process and could derive sulfur only from the amino acids. Thus, they required a very high concentration of methionine in order to tolerate Cr (VI) toxicity. In their study, de María Guillén-Jiménez *et al*. (2008) cultivated *Candida* sp. FGSFEP strain with varying sulfate salt concentration (from 0 to 23.92 mM), with and without Cr (VI) ions. In the absence of the Cr (VI) ions, the yeast did not display a significant difference in its specific growth rate, when it was grown on different sulfate concentrations. These results indicate that the presence of sulfate did not stimulate or inhibit the yeast growth. On the other hand, in the presence of the two different Cr (VI) ion concentrations (i.e., 1.7 and 3.3 mM), the specific growth rate of the yeast was significantly increased at higher sulfate

concentrations. Also, higher sulfate concentrations led to a significant reduction in Cr (VI) uptake by *Candida* sp. FGSFEP. The yeast was able to completely reduce 1.7 mM of Cr (VI). Thus, the changes in the cell permeability to Cr (VI) ions were seen to be a major factor which helped in determining the chromium resistance in the microbes. Shen & Wang (1993) investigated the chromate reductase enzyme in *Escherichia coli* ATCC 33456 and noted that this enzyme was soluble and exclusively localised within the cytoplasm.

1.5 Resistance by impermeability

As indicated in the preceding section, chromium (VI) resistance is linked to cell permeability and trans membrane nutrient uptake systems in bacterial and other cells.

Many microorganisms develop chromium-resistance by adapting to various mechanisms such as exclusion with the help of permeability barriers, or removal of the intracellular chromium ions by the active transport process or intracellular biosorption, wherein the chromium ions get sequestered by intracellular proteins. The bacteria could alter their cell wall or cell membrane characteristic by increasing the expression of some specific components thereby decreasing the permeability. A high cell membrane polysaccharide expression decreased the permeability to the toxic chromium (VI) ions. Horitsu *et al*. (1983) noted that the breadth of the cell membrane in *Pseudomonas ambigua* G-1 was directly related to the chromium permeability and resistance. The fungus showed several physiological changes, such as an increase in the cell surface roughness, elasticity and cell wall rigidity. X-ray photoelectron spectroscopy and Fourier transform infrared analysis also showed that the Cr (VI) ions that were

bound to the microbial cell wall were reduced to Cr (III) by the various cell wall components (Das *et al*. 2008). The cell wall-Cr (III) ions were able to electrostatically attract a higher concentration of chromate ions, which led to the development of a layered cell wall structure.

Cr (VI) ions easily diffuse through the bacterial cell membranes, unlike the Cr (III) ions, as shown in figure 1-2 (Nishioka 1975; Petrilli & De Flora 1977). A small proportion of the Cr (VI) ions that infiltrate into the cell are reduced to Cr (III), by reducing agents such as sulfite, ascorbate, reduced-glutathione, NAD(P)H (Petrilli & De Flora 1978). Cr (III) ions were more mutagenic compared to the Cr (VI) ions when they were within the cell, as they had a tendency to form complexes with nucleic acids. Cheung & Gu (2007) stated that there was no evidence suggesting the binding of the Cr (III) to DNA. Reducing agents such as those stated above, are one electron reducers, reducing Cr (VI) to intracellular Cr (V) and then Cr (IV) ions, which can then undergo continuous free radical redox cycles to generate Cr (III) ions. The Cr (IV) ions could bind to the different cellular components and affect their function. Thus, the Cr (VI) ions are very toxic, as they can easily enter the human cells and get reduced to yield Cr (III) ions, which are very mutagenic (Arakawa *et al*. 2000).

The presence of plasmid encoding genes was responsible for the chromium resistance in a majority of bacterial species, such as *Streptococcus lactis* (Efstathiou & McKay 1977), *Pseudomonas* sp. (Summers & Jacoby 1978), *P. fluorescens* (Ohtake *et al*. 1987) and *Cupriavidus necator* (Peitzsch *et al*. 1998). When Bopp *et al*. (1983) removed this plasmid in *P. fluorescens*, the

organism became more Cr (VI)-sensitive. Also, a bacterial strain which lacked the “chromium resistance” plasmid was seen to be chromium-sensitive.

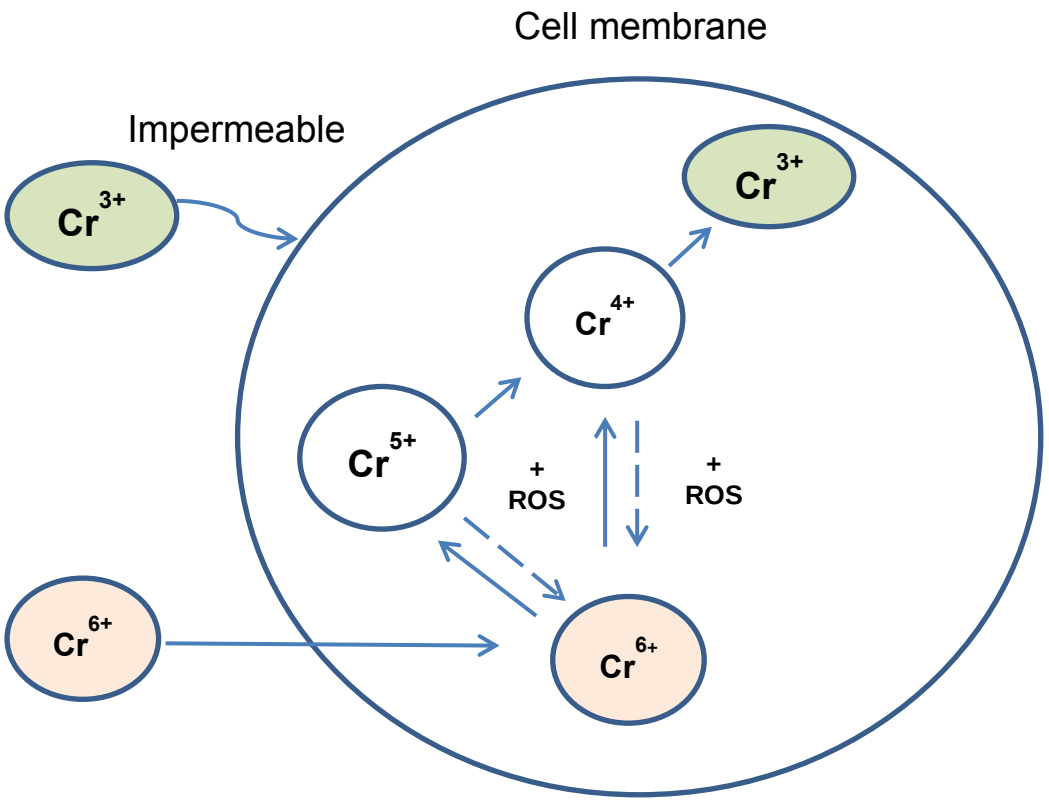


Figure 1-2 Modified from Cheung & Gu (2007) Schematic diagram of toxicity and mutagenicity of Cr (VI). The intracellular Cr (VI) reductants naturally available are frequently obligatory one-electron reducers, which generate Cr 5+ and a large amount of ROS that causes the deleterious effects of Cr (VI).

1.6 Methanotrophs

Methanotrophs are Gram-stain-negative Bacteria that were first detected by Sohnagen in 1906 and are distinguished from other organisms by the ability to utilise methane (CH_4) as their sole source of carbon and energy. Sohnagen named this first methanotroph isolated *Bacillus methanicus*. This strain has been lost and there were several further attempts to isolate methane-oxidizing bacteria until Foster's Texas Laboratory successfully isolated methanotrophic bacteria from different sources. These included a methane oxidizing bacterium,

Methylococcus capsulatus. Other attempts to isolate pure colonies had not been successful because they were contaminated with heterotrophic bacteria that can grow on their excreted products. The contaminating bacteria were difficult to separate from the methanotrophs (Bissett *et al*. 2012; Rasigraf *et al*. 2014); however, Whittenbury *et al.* (1970) isolated pure colonies of methanotrophic bacteria uncontaminated by heterotrophic bacteria (Foster & Davis 1966; Dalton 2005).

Methanotrophs have been investigated for bioremediation of a variety of organic pollutants owing to the presence of broad-spectrum methane monooxygenase enzymes (MMO), which perform the oxidation of methane to methanol and is the main defining metabolic feature of methanotrophs. Methanotrophs are a highly specialized group of aerobic bacteria and have a versatile capacity for oxidation of certain types of organic pollutants such as alkanes, aromatics, halogenated alkenes (Jiang *et al*. 2010). Most methanotrophs discovered to date show optimal growth at moderate pH (5-6) and temperature ranges (20 - 35 °C) and can grow at temperatures as low as 4 °C or as high as 72 °C. The versatility of growth temperatures allows methanotrophs to be isolated from multiple environments (Smith & Dalton 2004). Other methylotrophic bacteria can utilise different one-carbon compounds, including methylated amines, methanol, and halomethanes and methylated compounds containing sulfur for their source of carbon (Semrau *et al*. 2010; Jiang *et al*. 2010; Boden & Hutt 2018).

The oxidation of methane by methanotrophs makes an important contribution in minimising the effects of global warming. In addition to their ability to oxidise methane as the growth substrate of methanotrophs, methane monooxygenase enzymes produced by methanotrophs also have the ability to co-oxidise

hydrocarbons and halogenated organic compounds, including aromatics and trichloroethylene. The application of methanotrophs in bioremediation of such compounds has been widely investigated (Smith & Dalton 2004).

The most notable of methanotrophs that have been studied and which form the best of our general understanding of methanotrophs are *Methylomonas methanica*, *Methylosinus trichosporium*, and *Methylococcus capsulatus* Bath, representing the three classes (I, II and X) of typical methanotrophs (Chistoserdova *et al*. 2009). Three types of the enzyme methane monooxygenase (MMO) exist within methanotrophic bacteria. These are soluble methane monooxygenase (sMMO), particulate methane monooxygenase (pMMO) and a divergent form of pMMO known as pXMO. In addition to oxidising methane these enzymes, most notable (sMMO) can oxidise a large number of compounds that include diphenyl, methane, naphthalene and trichloroethylene (TCE). Some of these substrates of (MMO) are highly toxic, and so the (MMO) enzymes are potentially useful for the bioremediation of environments contaminated with these pollutants in breaking them down. The wide substrate ranges of the (MMO) enzymes have led to numerous potential opportunities as industrial biocatalysts. One possibility is to use (MMO) to oxidise the potent greenhouse gas methane and thus minimise global warming (Dalton 2005 & Tavormina *et al*. 2011).

1.7 Classification of methanotrophs

The methanotrophs that (Whittenbury *et al*. 1970) isolated as pure cultures and established the basis for the current classification of methanotrophs. The bacteria were divided into five genera (Figure 1-3), depending on the type of resting stages, carbon metabolism pathways, intercellular composition, and

morphological differences. These genera are: *Methylomonas, Methylobacter, Methylococcus, Methylocystis*, and *Methylosinus*, which are the same as the modern classification apart from addition of a number of new genera, including *Methylomicrobium* (Whittenbury *et al*. 1970; Bowman *et al*. 1995; Smith & Murrell 2009). (Hanson & Hanson 1996 & Dedysh *et al*. 2000) The methanotrophic bacteria have been grouped into three types, Types I, II, and X (Table 1.1) based on the same conditions as mentioned above.

Figure 1-3 Two distinct arrangement of intercellular membrane showed by electron microscope (Dalton 2005) of types I, X, and II methanotrophs.

Table 1-1 Characteristics of Types I, II, and X methanotrophs. Adapted from (Hanson & Hanson 1996)

Characteristic	Type I	Type II	Type X
Cell morphology	Short rods, usually occur single; some cocci or ellipsoids	Crescent- shaped rods, rods, pear shaped cells, sometimes occur in rosettes	Cocci, often found in pairs
Growth at 45 °C	No	No	Yes
G+C content (mol%)	49-60	62-67	59-65
Membrane arrangement	Yes	No	Yes
Bundles of vesicular disks paired membranes aligned to periphery of cells	No Stacks	Yes Peripheral	No Stacks
Nitrogen fixation	No	Yes	Yes
Resting stages formed	No	Some strains	No
Exospores or cysts	Some strains	Some strains	Some strains
RuMP pathway present	Yes	No	Yes
Serine pathway present	No	Yes	Sometimes
Ribulose-1,5-biphosphate carboxylase present	No	No	Yes
Major PLFAs	C14.0,C16:1ω 7c,C16:1ω5t	C18:1ω8c	C16:0,C16:1ω7c
Class	Gamma proteobacteria	Alpha proteobacteria	Gamma proteobacteria
Phylogenetic signature probe(S)	1041(5'-CTCCGCTATCTCTAACAGATT-3'), 1035(5'-GATTCTCTGGATGTCAAGGG-3'), MM650(5'-CCTCTACTCAACTCTAGT-3'),MM850(5'-TACGTTAGCTCCACCACTAA-3')	1034(5'-CCATACCGGACATGTCCAAAGC-3')	No specific probe has been tested.

Type I methanotrophs belong to the γ subdivision include the genera *Methylococcus, Methylomonas, Methylomicrobium, Methylobacter, Methylocaldum* and *Methylosphaera*, which can use the product from formaldehyde to form biomass through the ribulose monophosphate (RuMP) pathway. The Type I organisms contain mainly 16-carbon fatty acids and have bundles of intracytoplasmic membranes.

Type II methanotrophs belong to *Alphaproteobacteria* and include the genera *Methylosinus*, *Methylocystis* and *Methylocella. Methylocella* have sMMO but not pMMO and are facultative methanotrophs. They use the serine pathway as their primary means for converting formaldehyde into biomass. They also have 18-carbon fatty acids within their membrane (Hanson & Hanson, 1996; Whittenbury & Dalton, 1981).

Figure 1-4 Pathway for oxidation of methane and assimilation of formaldehyde. Adapted from (Hanson & Hanson 1996)

Type X was added to include *Methylococcus capsulatus*; these are similar to Type I in that they utilise the RuMP cycle as the formaldehyde assimilation pathway, but they differ in that they possess low levels of enzymes of the serine pathway, an indication that Type X can use both pathways (Whittenbury & Dalton 1981; Smith & Murrell 2009).

The pMMO and sMMO are produced by both *Methylococcus capsulatus* and *Methylosinus trichosporium*. However, methanotrophs such as *Methylomonas methanica* and *Methylomicrobium album* produce only pMMO. The more identified facultative methanotrophs e.g. *Methylocella silvestris,* produce only sMMO (Dedysh *et al*. 2005). *Methyloferula* is a genus of sMMO - only methanotrophs that are obligately methanotrophic (Knief *et al*. 2003; Vorobev *et al*. 2011).

1.8 Metabolic flexibility

The methanotrophs are seen to express metabolic flexibility. These organisms are able to thrive in conditions with a low oxygen tension and can oxidise chemolithotrophic hydrogen and sulfur (Ward *et al*. 2004; Kelly *et al*. 2005). Some methanotrophs are able to fix atmospheric nitrogen. *Mc. capsulatus* displays significant versatility in nitrogen conversion reactions, such as nitrification and denitrification. Ammonia gets oxidised to form nitrite by the pMMO and sMMO. This reaction occurs because of an absence of their substrate specificity. The MMO enzymes possess a related reductase enzyme. The organisms must possess a reducing activity for fixing atmospheric nitrogen. This reducing power is generally possessed by the flavodoxin or ferredoxin

molecules in the aerobic bacteria, which are usually reduced by the NADH/NADPH (Ward *et al*. 2004; Kelly *et al*. 2005).

1.9 Methanobactin

Methanobactin (Mb) is a chalkophore, a copper-binding, extracellular chromopeptide which is produced and secreted by several methanotrophs (Kim *et al*. 2004; Choi *et al*. 2005; Yoon *et al*. 2010; Yoon *et al*. 2011; Bandow *et al*. 2011). This peptide is involved in copper acquisition by methanotrophs.

The Cu complex in Mb has a pyramid-like structure, wherein a single Cu^{+} ion was coordinated by the N_2S_2 donor set, at the pyramid base. Mb has the following proposed structure: 1-(N-[mercapto-{5-oxo-2(3-methylbutanoyl) oxazol-4-ylidene} methyl]-Glyl-Ser2-Cys3-Tyr4) pyrrolidin-2-yl- (mercapto- [5-oxo-oxazol-4-ylidene] methyl)-Ser5-Cys6-Met7 (Kim *et al*. 2004; Behling *et al*. 2008; El Ghazouani *et al*. 2011) (Figure 1-5).

Figure 1-5 Structure of the copper (I) complex of Mb from *Methylosinus trichosporium*. Taken from (Behling *et al*. 2008).

Mb may be a multi-functional compound, which mediates the Cu uptake in the Mb-producing methanotrophs. When methanotrophic organisms such as *Mc. capsulatus* and *Ms. trichosporium* are cultivated in a low-Cu media Mb is

detected in the cell-free supernatant. The Mb level is significantly decreased after the addition of Cu to the medium (Zahn & DiSpirito 1996; DiSpirito *et al*. 1998; Dassama *et al*. 2017). Additionally, Mb was found to be related to pMMO, and when it dissociates from Mb, pMMO became inactive. All these observations indicated that Mb plays a major role in pMMO activity, though its mechanism of action is still unknown (Choi *et al*. 2003; Choi *et al*. 2005). Furthermore, the Mb peptide, extracted from *Mc. capsulatus, Ms. trichosporium* and *Methylomicrobium album* displayed several other enzymes activities like oxidase, superoxide dismutase and hydrogen peroxide reductase (Choi *et al*. 2008).

In their review, Kenney & Rosenzweig (2011) summarised all the biological and chemical properties of Mb. One study proposed that Mb was synthesised in the ribosomes of *Ms. trichosporium* (Krentz *et al*. 2010). Mb peptide could be synthesised by the Non-Ribosomal Peptide Synthase (NRPS) or the PolyKetide Synthase (PKS) enzymes, owing to its similarity to the siderophores (Ward *et al*. 2004; Balasubramanian & Rosenzweig 2008). The siderophores are small Fe-chelating compounds which mediate the uptake of Fe in low Fe conditions. The siderophores are usually synthesised by the NRPS enzyme, such as the pyochelin in *P. aeruginosa* (Cox *et al*. 1981) and enterobactin in *E. coli* (Ehmann *et al*. 2000) and or are synthesised by PKS, like yersiniabactin in *Yersinia pestis* (Miller *et al*. 2002).

1.10 Effect of copper on methane monooxygenase

The particulate methane monooxygenase (pMMO) has been shown to have an obligate requirement for copper. Stanley *et al*. (1983) observed that the

production of sMMO and pMMO in *Mc. capsulatus* Bath cells is dependent on the amount of copper: when the copper to biomass ratio is low, the sMMO is produced instead of the pMMO, which is expressed at a high ratio of copper to biomass. This change in MMO expression in response to copper is known as the copper switch. When copper to biomass ratio equals or exceeds 1 µmol g^{-1} dry weight of cells, the switch from sMMO to pMMO transcription occurs (Theisen & Murrell 2005; Semrau *et al*. 2010; Fru 2011). In particulate fractions derived from *Mc. capsulatus* Bath cells, the MMO activity was observed to increase along with the increasing copper content of the membranes. When membranes obtained from cells that were grown at low copper levels are likewise observed, it was found out that its enzyme activity could undergo further stimulation by adding of Cu (II) ions to the assay medium (Nguyen *et al*. 1994).

In strains that express both pMMO and sMMO, copper in the growth medium inhibits sMMO gene expression. Under high copper-to-biomass conditions, pMMO is produced together with significant intracytoplasmic membrane formation (Balasubramanian & Rosenzweig 2008). The secretion of methanobactin, as well as the production of the polypeptide MmoD, has an important role in the copper switch by elevating copper bioavailability that regulates MMO expression; notably, both methanobactin and MmoD increase the importance of bacterial response to copper (Semrau *et al*. 2010; Semrau *et al*. 2013; DiSpirito *et al*. 2016). The copper deficiency of the medium triggers the production of methanobactin, which may result in different transition and near transition metals that have a possible effect on the metals found in soils and aquatic systems (Semrau *et al*. 2013).

It has been reported that the regulation of sMMO by copper takes place at the transcriptional gene level (Nielsen *et al*. 1996; Nielsen *et al*. 1997), and this transcription has been confirmed by later experiments (Choi *et al*. 2003). However, a discrepancy exists regarding the level at which pMMO is regulated. Some studies suggested that pMMO is regulated at the gene transcription level (Nielsen *et al*. 1996; Nielsen *et al*. 1997) while others proposed that pMMO is regulated at a different level of regulation; post-transcriptional level (Choi *et al*. 2003).

1.11 Methane monooxygenase (MMO)

As mentioned earlier, methanotrophs oxidise methane to methanol with the enzyme methane monooxygenase (MMO). Three kinds of MMO have been established; a membrane associated or particulate methane monooxygenase (pMMO) exists in most well characterised methanotrophs and is situated in cytoplasmic membrane. The soluble form or soluble methane monooxygenase (sMMO) exists in some methanotrophs, and is situated in the cytoplasm. As detailed above, there also exist some sMMO-only methanotrophs. As stated above there is also a divergent form of pMMO known as pXMO.

Although both of sMMO and pMMO can utilize methane and convert it to methanol, there are clear differences in the amino acids sequences for sMMO and pMMO, in their quaternary structure, and metals in the active site. sMMO is soluble in the cytoplasm while pMMO is cell associated exactly membrane associated (Dalton 2005). There are many other differences between pMMO and sMMO, which suggest that they are entirely different enzymes rather than pMMO being simply a derivative of sMMO with membrane adherence (Burrows

et al. 1984). To distinguish between pMMO and sMMO a colorimetric naphthalene oxidation test is used (Brusseau *et al*. 1990), Oxidation of naphthalene shows expression of sMMO by the bacteria, since this enzyme can oxidize naphthalene while pMMO cannot oxidise this substrate (Smith & Dalton 2004).

1.11.1 Particulate methane monooxygenase (pMMO)

pMMO is a multiple copper containing enzyme for which some researchers have proposed that the absence of oxygen is necessary to purify pMMO without losing enzymatic activity (Choi *et al*. 2003) while others have found that aerobic conditions are acceptable (Nguyen *et al*. 1998) or helpful (Basu *et al*. 2003). Among many detergents examined it was found that the most effective for solubilising pMMO is dodecyl-β-D-maltoside and all reports of purified pMMO to this date relied on it (Smith & Dalton 2004).

There is still debate about the number and roles of copper ions in pMMO, as well as the possibility of the involvement of iron in the metal centres of the enzyme (Lieberman & Rosenzweig 2005; Martinho *et al*. 2007). sMMO has proven easier to study than pMMO because the pMMO loses activity when solubilised (Nguyen *et al*. 1994; Semrau *et al*. 1995; Nguyen *et al*. 1996; Lieberman & Rosenzweig 2005; Myronova *et al*. 2006). The active pMMO complex consists of two components, firstly the hydroxylase (pMMOH). This consists of three polypeptides or three subunits (α, β, γ) with molecular masses of approximately 45,000, 26,000, 23,000 Da, encoded by the *pmoBAC* genes, secondly a putative reductase (pMMOR) consisting of 63,000 and 8,000 Da

proteins (Basu *et al*. 2003; Semrau *et al*. 2010). X-ray crystallographic study demonstrated that the pMMO has an $(\alpha\ \beta\ \gamma)_3$ structure in the cell, pMMO may complex with MDH (Methanol Dehydrogenase) which acts as electron donor to pMMO (Figure 1-6) (Myronova *et al*. 2006; Smith & Murrell 2009).

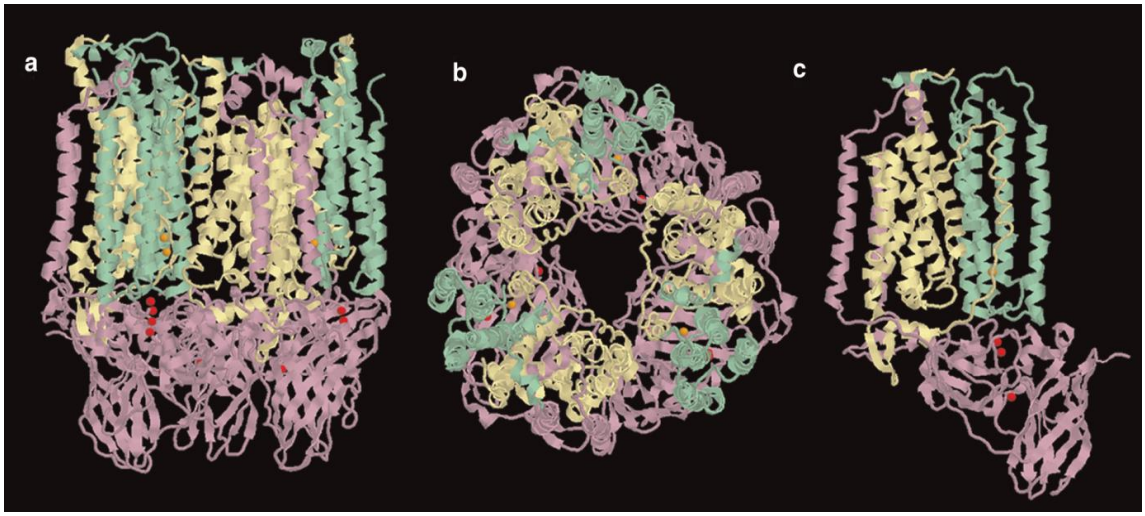


Figure 1-6 Structure of pMMO. The 49-, 27-, and 22-kDa subunits, are coloured lilac, yellow, and green, respectively. Metal atoms shown as spheres, copper red, and zinc orange. (a) $(\alpha, \beta, \gamma)_3$ enzyme; (b) view looking down on (a) from above; (c) one promoter showing the mononuclear and di-nuclear copper and zinc (Lieberman & Rosenzweig 2005 ; Smith & Murrell 2009).

1.11.2 Soluble methane monooxygenase (sMMO)

This enzyme has a wide range of substrates, which makes the bacteria that express it more useful in bioremediation and biotransformation processes (Murrell *et al*. 2000). The sMMO enzymes from two methanotrophs have been characterised in detail. *Methylosinus trichosporium* (Fox *et al*. 1989) has optimal activity at 30 °C, while *Methylococcus capsulatus* Bath (Colby & Dalton 1978) effectively works at 45 °C. In general, sMMO from *Mc. capsulatus* and *Ms. trichosporium* are similar in catalytic characteristics, structure, and substrates that they can oxidise (Smith & Dalton 2004). There is also evidence to suggest

that copper precludes sMMO activity. sMMO can incorporate molecular oxygen to methane as well as oxidising a range of other substrates (Woodland *et al*. 1986).

sMMO has three protein components, a hydroxylase (protein A) (MmoH), a regulatory (protein B) (MmoB), and a reductase, (protein C) (MmoR), all of which are needed for the activity of sMMO in the oxidation of methane to methanol (Colby & Dalton 1978; Colby & Dalton 1979; Semrau *et al*. 2010).

As shown in (Figure 1-7), the hydroxylase (protein A) (MmoH) is the main part of the sMMO complex. It consists of three polypeptide subunits: α, β and γ (α2, β2, γ2 complex), with molecular masses of approximately 60,000 Da (α-subunit), 40,000 Da (β-subunit) and 25,000 Da (γ-subunit) (Fox *et al*. 1989; Murrell *et al*. 2000). It is part of a family of enzymes consisting of active multiple hydrocarbon oxygenases with a binuclear iron centre, as well as hemerythrin, the R2 subunit of type I ribonucleotide reductases and purple acid phosphatase (Que Jr & True 1990; Rosenzweig *et al*. 1993). There are some differences in the active site in diverse cases of redox states as established by (Elango *et al*. 1997).

The coupling/regulator (protein B) (MmoB) is a single polypeptide of 16,000 Da that controls electron transfer from protein C to protein A and is encoded by *mmoB* in order to regulate the role of the enzyme in substrate oxidation (Colby & Dalton 1978; Green *et al*. 1985; Cardy *et al*. 1991; Buzy *et al*. 1998; Chang *et al*. 2001). In addition it alters the regioselectivity of the enzyme with certain substrates (Lee *et al*. 1993; Paulsen *et al*. 1994; Kazlauskaite *et al*. 1996).

The reductase (protein C), is composed of one polypeptide with a molecular mass of 39,000 Da which is encoded by mmoC and transfers electrons from NADH to the hydroxylase (protein A) MmoH (Colby & Dalton 1978; Green *et al*. 1985; Fox *et al*. 1989; Cardy *et al*. 1991; Buzy *et al*. 1998; Smith & Dalton 2004). It appears that the activity of the protein C in electron mobilisation is almost ten times the hydroxylase activity in hydroxylation (Fox *et al*. 1991). The sMMO reductases from *Mc. capsulatus* Bath and *Ms. trichosporium* OB3b are inactivated by Cu^{2+} ions in vitro (Green *et al*. 1985; Fitch *et al*. 1993). The protein C from *Mc. capsulatus* has flavin adenine dinucleotide (FAD) and Fe_2S_2 prosthetic groups (Colby & Dalton 1979). Copper ions inactivate protein C by causing loss of the Fe_2S_2 centre, thus preventing the electron transfer from the MmoR to the MmoH (Green *et al*. 1985).

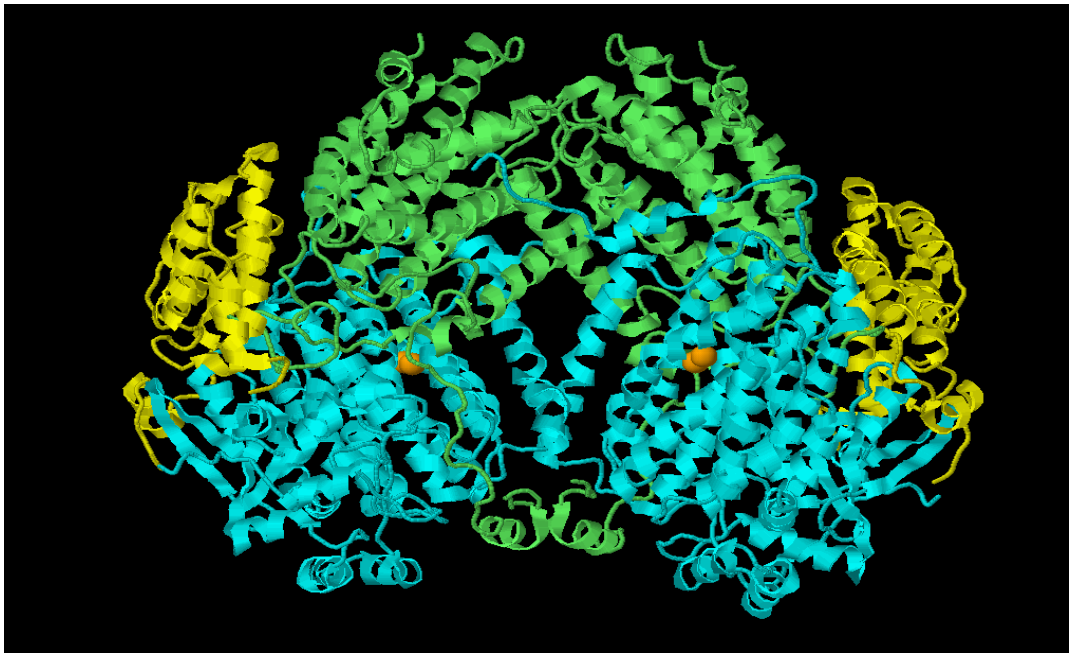


Figure 1-7 The structure of the hydroxylase component of sMMO; Blue: α subunit, Green: β subunit, Yellow: γ subunit and Orange balls: diiron centre (Smith & Murrell 2009).

1.12 Bioremediation of chromium

Certain unique challenges confront the remediation of sites that are contaminated by chromium. At present, soils contaminated by heavy metals are being cleaned up through the use of numerous technologies, the most common of which include soil removal and land filling, physico-chemical extraction, stabilisation/solidification, flushing, soil washing, and phytoremediation. Among these techniques, none are completely accepted as the best treatment option. This is because they provide only a temporary solution, or simply immobilise the contaminant, or entail costly financial outlays when used in large areas (Laxmipriya *et al*. 2010).

Bioremediation, is the process in which environmental pollution [e.g. Cr (VI)] are detoxified from polluted environments, by utilising microorganisms, green plants, fungi, or their enzymes (Thatoi *et al*. 2014). As indicated above, detoxification of Cr (VI) involves reducing it to Cr (III), which is less bioavailable, for example becoming immobilised in the soil matrix. In addition to the elimination of the toxicity of Cr (VI) by reducing it to Cr (III), the chromium may then form $Cr(OH)_3$ that is particularly insoluble within the pH range of 6–9. This results in a severe limitation of Cr (III)'s ability to migrate to ground water. Bioremediation is an inexpensive process requiring less energy input compared to other physical and chemical processes. Thus, a bacterial system with the capability to reduce chromate is possibly a powerful tool in remediating contaminated effluents and environments (Xia *et al*. 2014).

The more toxic Cr (VI) can be biologically reduced to the less toxic Cr (III). This can be done using microorganisms as a tool of biotechnology towards remediating chromate-polluted wastewater. As established by Barnhart (1997),

the process of bioremediating toxic chromium compounds is an area of special interest, not only because these metals cause serious environmental problems affecting living organisms but because it has found various uses in industrial applications. Consequently, a considerable number of bacterial strains have undergone evaluation related to the reduction of Cr (VI) to Cr (III) (Gadd & White 1993; Camargo *et al*. 2005; Pal *et al*. 2005).

Numerous reports exist regarding microorganism being utilised to remediate waters and soils contaminated with Cr (VI) (Kratochvil *et al*. 1998). As stated above, bacteria can protect themselves from the toxic substances in the environment by converting toxic compounds via methylation, oxidation, and reduction. They create less toxic, more volatile, or readily precipitating forms. The utilisation of bioremediation, including phytoremediation, to detoxify areas that are contaminated with chromium continues to gain interest from researchers around the world, and numerous methods have been suggested and evaluated experimentally with varying levels of success (Zayed & Terry 2003). Bioremediation of environments contaminated with Cr (VI) was facilitated by (Puzon *et al*. 2005). A bacterial enzyme system that utilised NADH as the reductant was used to allow the conversion of Cr (VI) into a soluble and generally safe NAD^+ - Cr (III) complex.

Generally, the operation of heavy metal bioremediation involves the metal ions' conversion into insoluble forms through specific enzyme-mediated reactions, which move them away from the aqueous phase (Park *et al*. 2000). Some bacteria have chromate reductase activity, which leads to the conversion of Cr (VI) to Cr (III). The latter metal is a lot less toxic and less soluble; therefore,

reducing these enzymes offers a way to do chromate bioremediation (Gadd & White 1993).

Previous studies of methanotrophs have suggested their application for bioremediation of a range of organic pollutants. The soluble form of Methane monooxygenase (sMMO) has the remarkable oxidisation ability to treat a wide range of substrates (George *et al*. 1996; Elango *et al*. 1997). As a consequence, attention has been called to this enzyme's capability in bioremediation and synthetic chemistry. Chloroform and trichloroethylene (TCE) are indeed two of the groundwater's most halogenated hydrocarbons pollutants; this makes them appear difficult to use as a unique carbon and energy source by bacteria (Hanson & Hanson 1996).

Al Hasin *et al*. (2009) demonstrated that *Mc. capsultatus* Bath is capable of converting chromium (VI) at different concentrations (1.4-1,000 mg L^{-1}) (0.0233-16.667 mM) into chromium (III). The initial rate of chromium (VI) removal increased with increasing concentration. The metabolic inhibitor sodium azide caused a 57% decrease in chromate (VI) removal. Reduction of chromate (VI) by whole cells of *Mc. capsulatus* Bath was dependent on the presence of the growth substrate methane. Whole cells of pure *Ms. trichosporium* cultures did not detectably reduce chromium (VI).

More recent studies of methane-driven chromium (VI) bioremediation, performed by other research groups, have focused on mixed communities of microorganisms containing methotrophs. Work using a consortium of microorganisms in a membrane biofilm reactor showed methane-driven removal of 3 mg L^{-1} (0.050 mM) of chromium (VI) (Lai *et al.* 2016). Further work in a similar reactor system showed competition between chromium (VI) and sulfate

for electrons ultimately derived from methane (Lv *et al.* 2018). Another polymicrobial biofilm reactor system showed simultaneous reduction of chromium (VI) (up to 2 mg L^{-1}) (up to 0.033 mM) to and removal of dissolved nitrate from synthetic wastewater (Long *et al.* 2017) . Chromium isotope fractionation has also been used to infer reduction of chromium (VI) rather than solely biosorption in a polymicrobial system driven by methane(Lu *et al.* 2018)

1.13 Mechanism of reduction of chromium (VI) by bacteria

In general, chromium reduction can be carried out by bacteria in two ways, enzymatic (direct chromium reduction) or non-enzymatic (indirect chromium reduction).

1.13.1 Enzymatic reduction of chromium (VI) by bacteria

Gram positive bacteria are shown to have significant tolerance to Cr (VI) toxicity at relatively high concentration, whereas Gram negative bacteria are more sensitive to Cr (VI) (Thatoi *et al*. 2014). The enzymatic reduction of chromium takes in two ways; aerobic process and anaerobic process.

A number of bacterial species have been identified as being capable of reducing Cr (VI). For example, a *Streptomyces griseus* strain has been found to be capable of reducing a 50 mg L^{-1} (0.833 mM) Cr (VI) standard solution over a period of 72 h (Laxman & 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),

Cr (VI) was also found to be reduced by *Shewanella oneidensis* (Daulton *et al*. 2007).

A membrane-associated, constitutive enzyme has also been found to induce the transfer of electrons from NADH to chromate (Bopp & Ehrlich 1988). The rate of Cr (VI) reduction by *Bacillus* sp., increased with initial cell concentrations ranging from 20 to 70 mg L^{-1} (0.333 to 1.166 mM) and then decreased when higher concentrations were used. Chromium (VI) is reduced by *Escherichia coli* 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. 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 sulfate and nitrate (Shen & Wang 1994). Strains of *Acinetobacter* sp. have been used for the removal of chromium being achieved after 15 days (Shrivastava & Thakur 2003). *Pseudomonas putida* is able to reduce chromate (VI) aerobically using the soluble chromate (VI) reductase (Ackerley *et al*. 2004).

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 electron donor. 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 (Daulton *et al*. 2007).

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 catalyse one electron reduction of chromate, generating Cr (V), which redox cycles, generating excessive reactive oxygen species (Fude *et al*. 1994).

The previously reported studies on biological reduction of Cr (VI) have been conducted using laboratory scale apparatus, 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 (Dermou *et al*. 2005). 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; 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 reductase. Cr (VI) reductase are reported to be localized to the cytoplasmic membranes of *S. oneidensis* (Myers *et al*. 2000), as well as being associated with membranes in *Enterobacter cloacae* (Wang *et al*. 1990), *Pseudomonas fluorescens* (Bopp & Ehrlich 1988), and *Pseudomonas maltophilia* (Blake *et al*. 1993).

1.13.1.1 Membrane-bound reduction system

Two different types of chromate reduction mechanisms have been reported in the literature, i.e., soluble enzyme systems and a membrane-bound system. The role of the reductase enzyme in the transfer of electrons from NADH to chromate was (EC 1.6.5.2; www.brenda-enzyme.org) first described by (Bopp *et al*. 1983). The membrane-bound reduction system in *Enterobacter cloacae* H01 was investigated by (Wang *et al*. 1990), who noted that an insoluble form of reduced chromate ions was precipitated on the cell surfaces. They also carried out a Transmission Electron Microscopy (TEM) analysis and observed that a majority of the electron scattering particles were present on the outer membranes of the pelleted H01 cells. Furthermore, they also carried out Energy-dispersive X-ray analysis, which indicated that the particles on the cell surface were Cr precipitates. They determined the location of the chromium reductase enzyme by disrupting the cells and separating the cell organelles into different fractions. This experiment indicated that the enzyme activity was localised in the right-side outer membrane vesicles.

In the case of the chromium-resistant *E. aerogenes*, the chromium reductase showed a similar expression as the nitrite reductase. Glycerol and fumarate were required for activating the chromate reductase activity. Furthermore, nitrite was seen to increase the enzyme activity, while nitrate decreased the activity. A similar mechanism was expressed by the formate-linked nitrite reductase enzyme system. Interestingly, the parent strain of the Cr-resistant *E. aerogenes* showed no significant difference in their Cr (VI) to Cr (III) reduction levels, indicating that the chromate reductase activity was not responsible for the Cr (VI) resistance, which was presumed earlier (Clark 1994).

1.13.1.2 Cytoplasmic reduction system

The anaerobic, Cr (VI)-resistant *Shewanella putrefaciens* bacteria expressed chromate reductase activity (EC 1.6.5.2), which was localised in their cytoplasm (Myers *et al*. 2000). NADH and formate were seen to be the main electron donors for this enzyme. The chromate reductase activity was not detectable if NADPH was provided as the main electron donor. In another study, Myers *et al*. (2000) observed that an addition of flavin mononucleotide (10 μmol L^{-1}) to the medium induced the formate-dependent Cr (VI) reductase activity. Also, this activity was completely inhibited when the sample was incubated in the presence of diphenyliodonium, a known flavoprotein inhibitor. The chromate reductase activity was also inhibited by azide, p-chloromercuriphenylsulfonate (pCMPS), antimycin A and 2-heptyl-4-hydroxyquinolone-N-oxide (HQNO), indicating that the enzyme gets reduced by the multi-component electron transport chain. The researchers stated that this process indicated the involvement of quinones and cytochromes.

In another study, electron paramagnetic resonance (EPR) spectroscopy was used for detecting the presence of Cr (VI), indicating that the primary step in the Cr (VI) reduction mechanism includes a one-electron reduction step, which is followed by the 2-electron transfer. Unlike *S. putrefaciens*, NADPH was the electron donor for the chromate reductase activity in *Pseudomonas putida* (Park *et al*. 2000). Both the membrane and the cytoplasmic reductase enzymes were seen to be involved in the electron transfer system; however, different bacterial strains use different enzymes for reducing Cr (VI) to Cr (III), using different enzymatic mechanisms. For instance, the ChrR cytoplasmic chromate reductase enzyme from *P. putida* MK1 was seen to reduce Cr (VI) via a one-electron shuttle, to form Cr (V), followed by a 2-electron transfer process to yield

Cr (III) (Ackerley *et al*. 2004). Furthermore, the Cr (V) intermediate could be spontaneously reoxidised to yield the reactive oxygen species (ROS), which causes significant oxidative stress (Ackerley *et al*. 2004). On the other hand, the cytoplasmic chromate reductase from *E. coli*, i.e., YieF, mediated a 4-electron shuttle for reducing Cr (VI) ions to Cr (III) in one step, while the remaining electrons were transferred to oxygen (Cheung & Gu 2007).

1.13.2 Non enzymatic reduction of chromium by bacteria

Non enzymatic Cr (VI) reduction to Cr (III) can be attributed to different chemical compounds, produced during the bacterial metabolic process. The most potential non enzymatic chromate reductants could be ascorbic acid, glutathione, cysteine or hydrogen peroxide in microbial cells. Reduction of Cr (VI) may also occur via chemical reactions that are present in intra/extra cellular locations such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione associated compounds (Thatoi *et al*. 2014).

1.14 Aims

The aims of the work described in this thesis were:

To identify and characterise the chromate (VI) reductase of *Methylococcus capsulatus* Bath.

To characterise the chromium (VI) bioremediation reaction at the cellular and molecular level.

To isolate novel methanotrophs from the environment which are both chromium (VI) resistant and can reduce chromium (VI), for possible future use in remediation of high concentrations of this pollutant.

Chapter 2 Materials and methods

2.1 Materials

All chemicals were supplied by Sigma, Fisher and Beckman Coulter and all reagents used were of analytical grade. All solutions and media were prepared with high-purity deionized water.

2.1.1 Media

Two types of media were initially proposed to be used for bacteria cultivation and investigation. Their compositions are described and all solutions and growth media were prepared with de-ionized water and sterilised by autoclaving at 15 psi for 15 min at 121 °C.

2.1.1.1 NMS medium

Nitrate minimal salts (NMS) liquid medium and agar medium containing 15 g of bacteriological agar (Oxoid) per litre were used for cultivation of methanotrophs. 10 mL sterilised phosphate buffer was added after the medium had cooled to about 60 °C, just before pouring the agar media in sterile plates. The recipe of NMS is as follows:

The NMS media contained (per L of deionized water) KNO_3, 1000 mg; $MgSO_4.7H_2O$, 1000 mg; $CaCl_2.2H_2O$, 200 mg; $NaMoO_4.2H_2O$, 0.5 mg; Fe-EDTA, 3.8 mg; $CuSO_4.5H_2O$, 0.1 mg; $FeSO_4.7H_2O$, 0.5 mg; $ZnSO_4.7H_2O$, 0.4 mg; H_3BO_3, 0.15 mg; $CoCl_3.6H_2O$, 0.05 mg; Na_2EDTA, 0.25 mg; $MnCl_2.4H_2O$, 0.02 mg; $NiCl_2.6H_2O$, 0.01 mg; Na_2HPO_4, 497 mg; KH_2PO_4, 390 mg.

The NMS phosphate buffer contained (per L of buffer): 49.7 g of Na_2HPO_4 and 39 g of KH_2PO_4. The pH was 6.8 without adjustment.

2.1.1.2 Nutrient agar medium

Nutrient agar is a general purpose medium for the cultivation of microorganisms. It was used to test for contamination of methanotroph cultures with non-methanotrophs that could grow on this medium. The composition of this medium (supplied by Sigma Aldrich UK) is 0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl. A suspension 28 g of this medium in 1000 ml of distilled water (pH 7.4) was sterilised by autoclaving at 121 °C for 15 min.

The purity of *Mc. capsulatus* Bath was routinely checked by plating cultures onto nutrient agar plates, which were incubated at 37 °C for 24 h.

2.1.3 Equipment

- Orbital incubators: Stuart Scientific S150 and Gallenkamp, UK.
- Spectrophotometer: 6715 UV/Vis. spectrophotometer JENWAY single holder, supplied by Bibby Scientific Ltd., UK.
- Autoclave: Classic Prestige Medical, UK.
- Optima ultracentrifuge Beckman Coulter made in USA.
- French press cell Disruptor Thermo Electron Corporation, UK.
- Inductively coupled plasma-mass spectrometer (ICP-MS) PerkinElmer NexION 350X, USA.
- 3505 pH meter JENWAY designed and manufactured in UK, by Bibby Scientific Ltd.
- Fermentor (Bioflo 110): New Brunswick Scientific, USA.
- Modulyod 230 Freeze Dryer: Thermo Electron Corporation, UK.
- Sorvall RT 6000D Centrifuge: Thermo Electron Corporation, UK.

- RC6 centrifuge Thermo Scientific Sorvall RC6 plus centrifuge Thermo Electron Corporation, UK.

2.2 Methods

2.2.1 Bacterial strains and growth conditions

The methanotrophic bacteria *Mc. capsulatus* Bath were grown and propagated aerobically in sterile nitrate mineral salt (NMS) media, or NMS agar plates inside an airtight jar (Smith & Murrell 2011) using methane (1:4 v/v in air) as the carbon and energy source. The experiments were performed in 50 mL liquid cultures in 250 mL conical Quickfit flasks capped with Suba-Seals (Sigma-Aldrich) to prevent methane loss while allowing the addition and removal of material using syringes. 50 cm^3 of air was removed and then the air was replaced aseptically inside the hood to avoid contamination with 60 cm^3 of methane. The *Mc. capsulatus* Bath cultures were incubated at the optimum growth temperature of 45 °C on a shaker at 180 rpm and allowed to grow to an OD_{600} of 0.6-0.9. Under the conditions used in these experiments, the *Mc. capsulatus* Bath strain reaching an OD_{600} of 0.8 typically at 40-48 h.

2.2.2 Large scale cultivation of methanotrophs

Fermentation was carried out using a Bioflo 110 fermentor/ Bioreactor (New Brunswick). NMS media (5 L) was placed inside the fermentor and the whole apparatus including tubing was autoclaved. 50 ml sterile NMS phosphate buffer (section 2.1.1.1) was added to the media. According to the published method (Pilkington & Dalton 1990), the fermentor conditions used to grow *Mc. capsulatus* Bath were as follows; agitation at 200 rpm, incubation temperature at 45 °C, 1:4 methane: air at approximately flow rate of 1 litre/min. 250 ml culture of *Mc. capsulatus* Bath were prepared in NMS and incubated at 45°C for 48 h. This culture was used to inoculate the fermentor. Culture were harvested via centrifugation (11,000 × *g*, 10 min, 4 °C) and fractionated into (cytoplasm, cell wall and cell membrane), then the fractions were immediately frozen at -80 °C.

2.2.3 Optical density (O.D) measurements

Throughout the experimentation process, the optical density (for growth curve experiments) was measured at a wavelength of 600 nm using a spectrophotometer.

2.2.4 Investigation into the role of sMMO/pMMO in *Mc. capsulatus* Bath culture

In order to investigate the involvement of MMOs (sMMO and pMMO), the *Mc. capsulatus* Bath cultures expressing sMMO activity were identified using the naphthalene oxidation assay reported by (Brusseau *et al*. 1990). The

naphthalene oxidation assay is a well-known biochemical assay for identifying and quantifying sMMO activity. sMMO oxidises naphthalene to a mixture of 1-naphthol and 2-naphthol. The naphthols are detected by reaction with tetrazotized -o-dianisidine to form purple diazo dyes with large molar extinction coefficients Naphthalene is not a substrate for pMMO and cells expressing pMMO do not oxidise naphthalene.

2.2.5 Preparation of potassium chromate stock solution

Chromate solution (10 mg L^{-1}) (0.166 mM) was prepared by dissolving 37.3 mg of K_2CrO_4 and 10 ml of concentrated sulfuric acid was added slowly with mixing into 500 ml distilled water. Then, the solution was made up to 1000 ml with distilled water.

2.2.6 Preparation of chromium (VI) standard

Six flasks were labelled (blank, 1, 2, 3, 4 and 5). Various quantities of $K_2Cr\ O_4$ and sulfuric acid (H_2SO_4) (0.18 M) were added to each tube as shown in Table 2-1 below.

Table 2-1 Amounts of K_2CrO_4 and H_2SO_4 used for the standard curve

Tube no.	**Blank**	**1**	**2**	**3**	**4**	**5**
Cr (VI) Solutions (ml)	0.00	0.4	1.0	2.0	4.0	10
0.18M (H_2SO_4) (ml)	10	9.6	9.0	8.0	6.0	0.0
Final concentration of Cr (VI) (mg L^{-1})	0	0.4	1	2	4	10

The content of each test tube was then mixed by shaking. 0.5 ml of diphenylcarbazide solution (0.5 g in 200 ml of acetone) was added to each tube and incubated for 5 min at room temperature for colour development. The absorbance was then measured at 540 nm using a spectrophotometer (Herrmann 1994). A standard curve was then plotted with absorbance vs concentration. The standard curve showed a high degree of linearity. Figure 2-1 shows a representative standard curve in water, which showed a correlation coefficient of 0.999.

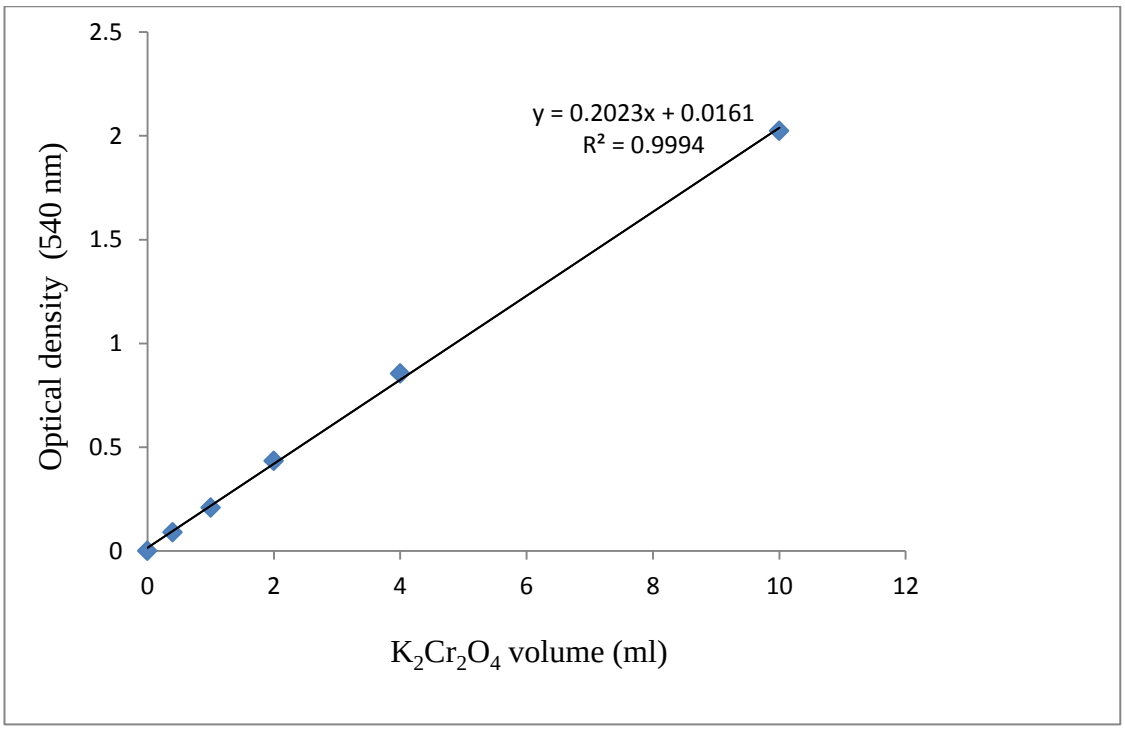


Figure 2-1 Typical standard curve of chromium (VI) concentration at OD 540 nm using the DPC assay

2.2.7 Determination of chromium in the samples

Measurement of chromium (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 supernate was acidified to 0.18 M. with $H_2SO_4$1.0 ml of the acidified supernatant was then mixed with 0.05 ml of diphenylcarbazide solution (2.5 g L^{-1} in acetone) and the chromate (VI) concentration was estimated spectrophotometrically at 540 nm by reference to a standard curve of known chromate concentrations prepared and detailed above.

2.2.8 Cell fractionation

In order to determine the location of chromium (VI) reductase activity, 5 litres culture of *Mc. capsulatus* Bath were grown on methane, the cells were harvested (11,000 × *g*, 4 °C, 10 min) to obtain a pellet. The pellet was washed with ice-cold 25 mM MOPS (pH 7), and re-suspended in 40 ml of the same buffer. The suspension was passed twice through a French pressure cell (8.3 MPa, 4 °C) in order to break the cell walls. The lysate was then fractionated by a modification of the method reported by (Smith & Foster 1995), as follows: the whole procedure was performed at 0 to 4 °C to minimize sample degradation. The lysate was centrifuged (3,000 × *g*, twice for 2 min each) to remove debris before being centrifuged (27,000 × *g*, 20 min) to sediment cell wall fragments. The cell walls were washed twice by resuspension in 25 mM MOPS (pH 7) and then resuspended in the same buffer, to give fraction F1 (cell wall-associated proteins).

The supernatant fraction was centrifuged again (27,000 × *g*, 20 min) to remove remaining wall material, and then membrane fragments were sedimented by centrifugation (105,000 × *g*, 60 min). The pellet was washed in 25 mM MOPS (pH 7), centrifuged again under the same conditions, resuspended in the same buffer to give fraction F2 (cell membranes).

The supernatant from the first ultracentrifugation was centrifuged again under the same conditions to remove remaining membranous material. This gave fraction F3 (cytoplasm).

All fractions were kept on ice throughout the procedure and care was taken not to allow any of them to become warm, by never removing them from ice for more than a few seconds and holding the tubes well above part that contained

the liquid. At the end of the procedure, all three fractions were divided into five equal aliquots and flash frozen in Eppendorf tubes in liquid nitrogen, before being stored at –80 °C. Care was taken not the heat above 100 °C, to prevent denaturing and inactivating the enzymes. A protease inhibitor benzamidine 1mM was also added prior to breaking the cells in the French press.

2.2.9 Gram stain procedure

Gram staining (Bartholomew & Mittwer 1952) was used to distinguish between Gram-positive and Gram-negative bacteria, which have distinct and consistent differences in their cell walls. The Gram stain was prepared as follows:

The slide was immersed with crystal violet solution for one minute, and washed for 5 s with tap water. Immersion with Gram's Iodine solution then followed for one minute to act as a mordant, and washed for 5 s with water. The slide was then blotted to remove excess water, and decolourised in 95% ethanol for 10 s. The slide was then counterstained by immersion in safrannin solution for 30 s. Excess stain was washed away with tap water, and bacterial slides were viewed using microscopy with 100x magnification with oil immersion lens.

2.2.10 Template DNA extractions for polymerase chain reaction (PCR)

Genomic DNA extractions were performed using Qiagen Genomic tips 20/g and buffers supplied by Qiagen. For DNA extraction a 1 ml suspension of an overnight culture of bacteria was placed into a 1.5 ml micro-centrifuge (Eppendorf) tube and centrifuged (3,000 ×*g* for 5 min). The supernatant was removed and 180 µl of a tissue lysis buffer (ATL) was added. 20 µl of 20mg/ml

proteinase K was added and the content were mixed by vortexing and incubated at 56 °C for 1 h. Tubes were mixed 2 to 3 times during incubation period to ensure efficient lysis. After, brief centrifugation followed. Then a 200 µl lysis buffer (AL) was added, the content was mixed by pulse-vortexing for 15 s and incubated at 70 °C for 10 min followed by brief centrifugation.

Ethanol(200 µl) (95 - 100%) was added and the contents of the tube were mixed by pulse-vortexing for 15 s followed by centrifugation. The mixture was carefully applied (including the precipitate) to the QIAamp spin column and centrifuged at 3,600 ×*g* for 1 min. The QIAamp spin column was placed in a clean 2 ml collection tube. The QIAamp spin column was carefully opened and 500 µl washing buffer 1 (AW1) was added and then centrifuged at 3,600 ×*g* for 1min. The QIAamp spin column was placed in a clean 2 ml collection tube. The column was carefully opened and 500 µl washing buffer 2 (AW2) was added and the tube was centrifuged at 105.000 ×*g* for 3 min. The QIAamp spin column was placed in a clean 1.5 ml Epindorf tube. The column was carefully opened and 200 µl elution buffer (AE) was added and the tube was incubated at room temperature for 1 min, and then centrifuged at 3,600 ×*g* for 1 min. The previous step was repeated with incubation for 5 min at room temperature before centrifugation, 3,600 ×*g* for 1 min. To assess the quality of the DNA yield, a portion of 10 µl was run on a 0.8% agarose gel with ethidium bromide.

2.2.11 Amplification of 16S rRNA gene

In order to identify prokaryotic cells by using extracted genomic DNA as a template, the 16S rRNA gene (1550 bp) was amplified using the primers f27 and rl492 (Bodrossy *et al*. 1997) (Table 2-2). PCR was performed in a total

volume of 50 µl by using Maxima Hot Start PCR Master Mix (2X) (400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 4 mM $MgCl_2$.) The PCR was carried out under the following amplification conditions: 95 °C for 10 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, followed by a final elongation step at 72 °C for 9 min.

Table 2-2 16S rRNA gene primers

f27	AGAGTTTGATCMTGGCTCAG
rl492	TACGGYTACCTTGTTACGACTT

2.2.12 Purification of PCR products

A PCR clean-up kit was used to purify DNA for sequencing, and The QIaquick PCR purification kit and associated protocol were used as follows:

To start 5 volumes of Binding buffer (PB) were added to 1 volume of PCR reaction mixture and pH adjusted accordingly. The mixture was then placed into a QIAquick column and the DNA allowed to bind to the filter by centrifuging for 60 s. The flow through was then discarded, and 750 µl of Wash buffer (PE) were added to each tube, and each tube centrifuged for 1 min. The flow through was then discarded and the filters were placed into a fresh 1.5 ml micro-centrifuge tube. Finally, to elute the DNA 50 µl of Elution Buffer (EB) (10 mM Tris Cl, pH 8.5) was added to the filter, and centrifuged for 1 min. The purified DNA was then analysed on an agarose gel, with 1 volume of loading dye to 5 volumes of purified DNA solution.

2.2.13 Sequencing of 16S rRNA genes

After the PCR amplification of 16S rRNA gene from the purified bacterial DNA from the isolated organisms from the Leeds and Liverpool Canal sediments, the amplified DNA was visualised by gel electrophoresis and the samples were cleaned up with the Qiagen PCR kit. The PCR product was sent for dye termination Sanger sequencing using the same primers that were used for the PCR.

2.2.14 Bioinformatics to analyse sequences

BLAST is the Basic Local Alignment Search Tool is an algorithm for comparing biological sequences such as amino acid sequences and nucleotide sequences of different proteins or NAD. A BLAST search compares the query sequence above certain threshold limit. The result that was obtained from the sequencing was compared with database of sequences and identified the organism with nearest similarity in terms of the sequence of its 16S rRNA gene.

2.2.15 Reduction of hexavalent chromium by *Mc. capsulatus* Bath

Potassium chromate or potassium dichromate (Sigma-Aldrich, Dorset, UK) was added from filter-sterilised stock solutions to *Mc. capsulatus* Bath cultures to give the desired chromium (VI) concentration towards the end of the logarithmic growth phase. The initial chromium (VI) concentrations used were 3, 5, 10, 20, 30, 40 and 50 mg L^{-1} (0.050, 0.083, 0.166, 0.333, 0.50, 0.666 and 0.833 mM). Three controls were set up for each experiment, from which bacterial inoculum, methane and the chromium (VI) species, respectively, were omitted. In order to determine the cellular location of the chromium (VI)-reducing activity, cultures

were grown to an OD_{600} of 0.8-0.9. Fractionation was performed as previously described by (Smith & Foster 1995). Where the membranes and cytoplasm were not separated from one another, the ultracentrifugation steps were omitted.

2.2.16 Measurement of the growth of the cells of *Mc. capsulatus* Bath exposed to chromium (VI) by Protein assay (BCA)

Optical density was not used to follow growth of cultures in these experiments because the chromium (VI) would contribute to the OD_{600} measurements, which would therefore not be an accurate measure of growth. Therefore, the total protein content was measured at different time intervals, and used as a measure of the growth of the microorganisms. Protein concentration in bacterial cell extracts was determined using the bicinchoninic acid (BCA) assay method (Pierce ^{TM} BCA Protein Assay Kit, Thermo Scientific, 23227). A 1.5 mL aliquot of bacterial culture was collected at different time intervals of bacterial growth and was centrifuged at 11,000 × *g* for 10 min. The pellet was washed and resuspended in 200 µl of extraction buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4; pH 7) containing protease inhibitor (1% v/v). The resulting suspension was sonicated on ice for 4 x 10 s (Sonics VCX-750 Vibra Cell Ultra Sonic Processor) and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was collected and measured for protein content using the BCA assay kit according to the manufacturer's instructions. Flasks with inoculum without the addition of chromium served as controls.

2.2.17 Acid digestion for heavy metal analysis

A known amount of each sample (0.1 g) was taken into a digestion tube and 2 ml of 50% (wt/wt) HNO_3 was added to the tube in fume hood and was left for 15 minutes for pre-digestion. The tubes were then sealed and digested in a microwave digestion apparatus for 10 min. After the samples had cooled a further 2 ml of 50% HNO_3 was added and the mixture was filtered through Whatman filter paper. The filtered supernatant was stored in capped bottles in the cold room until further analysis on ICP-MS. The tubes were washed in soap solution after filtration and then soaked in 10% HNO_3 overnight and washed with distilled water. The tubes were air dried and re-used.

2.2.18 Aqueous phase quantitation and characterization of chromium

The chromium (VI) concentrations were determined by using the diphenylcarbazide (DPC) assay or a high performance liquid chromatography (HPLC) - inductively coupled plasma mass spectrometry (ICP-MS) system. Measurement of chromium (VI) concentrations was performed by means of the diphenylcarbazide assay, as follows. Cells and other particulate material were removed from liquid samples by centrifugation (11,000 × *g*; 10 min; room temperature), and the supernatant was acidified by addition of 3M 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^{-1} in acetone) and the chromate (VI) concentration was estimated spectrophotometrically at 540 nm by reference to a standard curve of known chromate concentrations.

Inductively coupled plasma mass spectrometry (ICP-MS), illustrated in Figure 2-2, is an alternative technique where the sample is ionized by ICP and the

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

Figure 2-2 Schematic diagram of ICP-MS

Aliquots (5 mL) of the methanotroph cultures were collected at intervals and centrifuged (11,000 ×*g*; 10 min; room temperature), to remove the cells and other debris. An aliquot of the supernate (20 µL) was injected via a PerkinElmer LC Flexar autosampler into a PerkinElmer Flexar HPLC pump attached to a Hamilton PRP-X100 column, 4.6 × 250 mm, and coupled to a PerkinElmer ICP-MS NexION 350X. This column, which is an ion exchange HPLC column separated the chromium (VI) and chromium (III). The column was run a flow

rate of 1.2 mL min^{-1} using a mobile phase made up of 0.5 mmol L^{-1} ethylenediamine tetraacetic acid disodium salt dihydrate (Na-EDTA) buffer containing nitric acid (HNO_3) (0.875 mL) and adjusted to pH 7 with ammonia solution.

Standard solutions of trivalent chromium ($Cr(NO_3)_3.9H_2O$) and hexavalent chromium (K_2CrO_4) (Figure 2-3) were prepared at different chromium concentrations of 3, 5, 10, 20, 30, 40 and 50 mg L^{-1} (0.050, 0.083, 0.166, 0.333, 0.50, 0.666 and 0.833 mM) (Derbyshire *et al*. 1999).

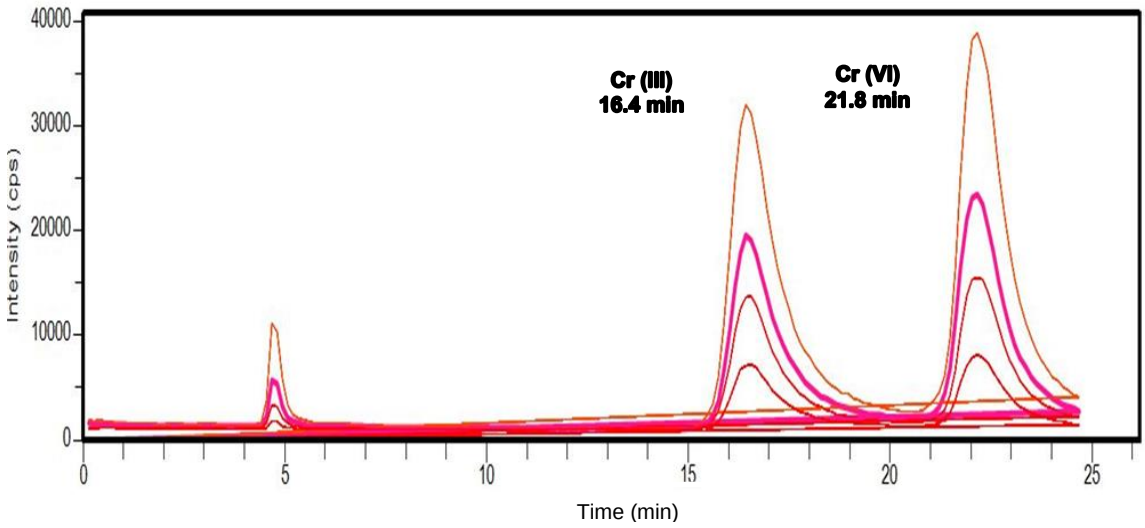


Figure 2-3 Representative separation of trivalent chromium and hexavalent chromium (each at 10 mg L^{-1}) (0.166 mM) via HPLC and ICP-MS as performed as detailed in the text.

2.2.19 Calibration of ICP-MS

Typical HPLC-ICP-MS calibration curves of standard chromium (III) and chromium (VI) are shown in Figures 2-4 and 2-5.

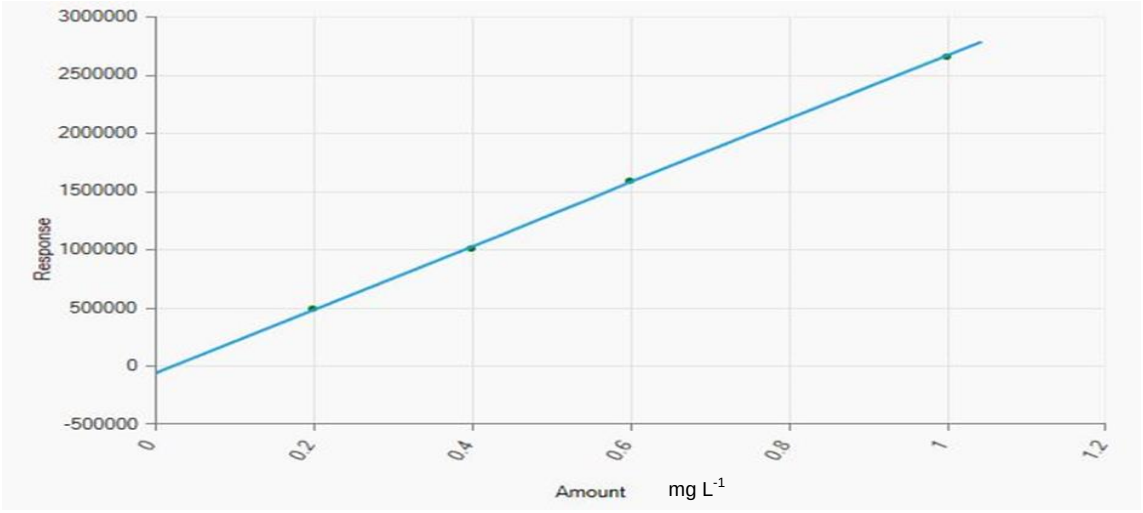


Figure 2-4 Typical calibration curve of standard chromium (III) concentration using HPLC-ICP-MS

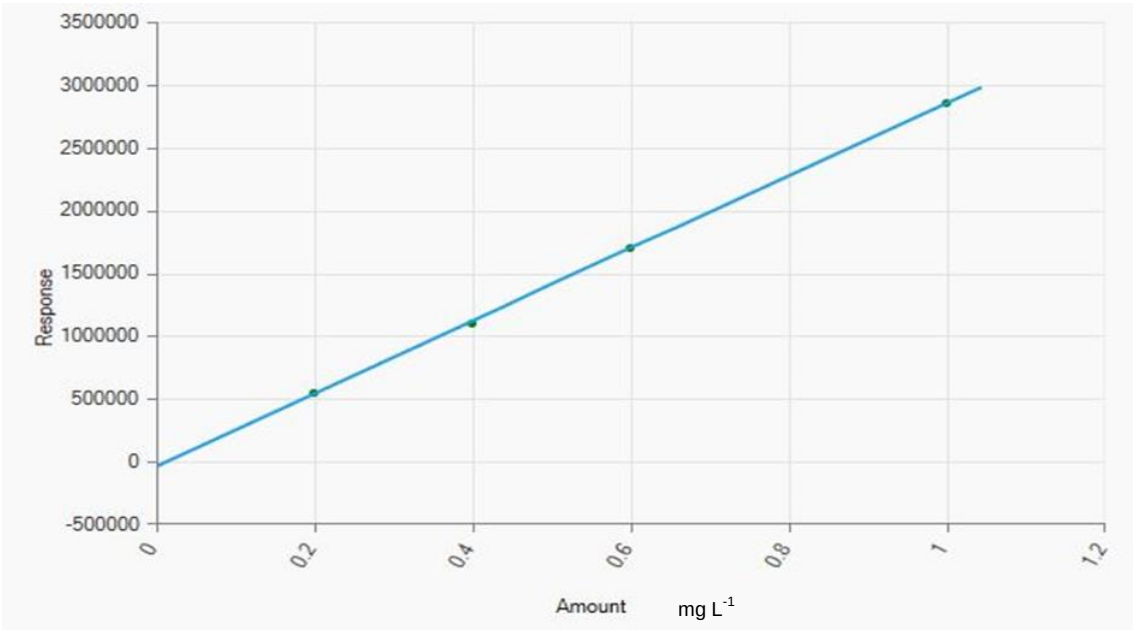


Figure 2-5 Typical calibration curve of standard chromium (VI) concentration using HPLC-ICP-MS

2.2.20 Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) spectrometry/high-angle annular dark-field (HAADF) scanning TEM (STEM) analysis

Samples of chromium culture (1.5 mL) were pelleted by centrifugation (11,000 × *g*; 10 min; room temperature), and washed with 0.1 M sodium phosphate buffer (pH 7.4). The specimens were then fixed in 3% glutaraldehyde in the same buffer overnight at room temperature and washed again in the same buffer by centrifugation and resuspension under the same conditions. The cell suspension was centrifuged again and secondary fixation was carried out by resuspending the pellet in 1% w/v aqueous osmium tetroxide and incubating for 1 hour at room temperature followed by the same wash step. The pellet of fixed cells was dehydrated through a graded series of ethanol dehydration steps (75%, 95% and 100% v/v), and then placed in a 50/50 (v/v) mixture of 100% ethanol and 100% hexamethyldisilazane for 30 min followed by 30 min in 100% hexamethyldisilazane. The specimens were then allowed to air dry overnight. A small sample of the fixed sample was crushed and dispersed in methanol, with a drop placed on a holey carbon coated copper grid (Agar Scientific). The samples were examined in an FEI Tecnai F20 field emission gun (FEG)-TEM operating at 200 kV and fitted with a Gatan Orius SC600A CCD camera, an Oxford instruments X-Max SDD EDX detector and a high angle annual dark field (HAADF) scanning TEM (STEM) detector. For thin section analysis, after the ethanol dehydration steps, the cells were embedded in EM bed 812 epoxy resin and cut into thin sections (90 nm, using a diamond knife on a Reichert Ultracut S ultramicrotome). The sections were supported on copper grids and coated with carbon. TEM specimen holders were cleaned by plasma prior to TEM analysis to minimize contamination. Samples were examined with a high-

resolution Philips CM 200 transmission electron microscope at an acceleration voltage of 200 kV under standard operating conditions with the liquid nitrogen anticontaminator in place. The thin sectioned samples were prepared at Sheffield University by Dr. Chris Hill, and the TEM analysis was done at Leeds University by Dr. Nicole Hondow.

2.2.21 X-ray photoelectron spectroscopy (XPS) analysis

Harvested samples were deposited on silicon wafer, left to dehydrate in the load lock of the XPS instrument overnight. The analyses were carried out using a Kratos Axis Ultra DLD instrument with the monochromated aluminium source. The XPS spectra were collected at Swansea University. Survey scans were collected between 1200 to 0 eV binding energy. High - resolution C 1s, N 1s and O 1s spectra were collected over an appropriate energy range at 20 eV pass energy and 0.1 eV intervals. The analysis area was 700 µm by 300 µm. Two areas were analysed for each sample, collecting the data in duplicate. Charge neutralisation was used with intention of preventing excessive charging of the samples during analysis. The data collected were calibrated in intensity using a transmission function characteristic of the instrument (determined using software from NPL) to make the values instrument independent. The data was calibrated for binding energy by making the main carbon peak C1s at 285.00, and correcting all data for each sample analysis accordingly.

Chapter 3 Speciation and distribution of chromium species

3.1 Introduction

Recent increases in the availability of methane from biogas and fracking sources has renewed interest in biotechnological applications of methanotrophs.

Work in recent years has highlighted the key environmental role played in the environment by aerobic methane-oxidising bacteria, with a much greater diversity of these organisms being present than was previously realised. These extremely diverse microorganisms, some of which can operate under effectively anaerobic conditions, are becoming increasingly recognised as significant players in transformation of heavy metals in the environment. Since the first report of chromium (VI) bioremediation by cultures of *Mc. capsulatus* Bath it has been shown that a mixed culture containing methanotrophs is able to reduce chromium (VI). We were interested to determine the response of the cells to a range of chromium concentrations and to locate the chromium on the microscopic level within the cells in order to assess how methanotrophs might be developed for practical bioremediation of heavy metals.

3.2 Growth curve of *Mc. capsulatus* Bath

The growth of *Mc. capsulatus* Bath in NMS media in the presence of methane as sole source of carbon and energy and the culture density was determined by measuring the absorbance at 600 nm and the graph was plotted of optical density vs time and the readings are shown in Figure 3-1. The growth curve exhibited a lag phase of less than 48 hours, followed by faster growth (putative logarithmic growth phase), after 96 hours of growth, the culture entered the

stationary phase. The time taken to reach the maximum OD was about 96 hours.

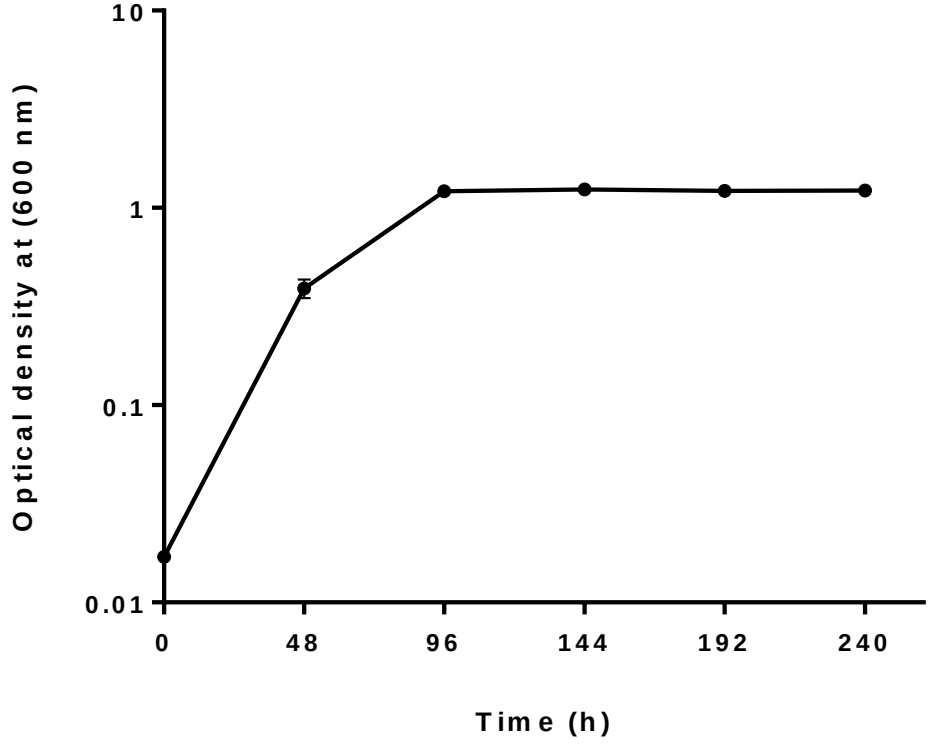


Figure 3-1 Typical growth curve of *Mc. capsulatus* Bath level at 600 nm. Data shown is from three independent cultures. Results plotted as mean ± 1 standard deviation (n=3).

3.3 Bacterial growth under chromium (VI) stress

In order to determine the toxicity of Cr (VI) to the methanotrophs, the growth of the bacteria was studied by addition of different concentrations of Cr (VI) (0, 5, 10, 20, 30, 40, 50 and 100 mg L^{-1}) (0.00, 0.083, 0.166, 0.333, 0.50, 0.666, 0.833 and 1.66 mM) to the cultures. The growth of *Mc. capsulatus* Bath in NMS media in the presence of different concentrations of Cr (VI) are shown in Figure 3-2.

Optical density was not used to follow growth of the cultures in these experiments because the Cr (VI) would contribute to the OD_{600} consequently the results would not an accurate measure of growth. Therefore, the total protein content was measured at different time intervals and used as a measure of the growth of bacterium.

At the concentration of Cr (VI) was increased, the amount of growth progressively decreased. At 40 mg L^{-1} (0.666 mM) of Cr (VI), negligible growth was observed. At higher chromium (VI) concentrations, the optical density concentration of protein actually decreased with time, suggesting cell death, lysis and proteolysis. Some toxic agents show a stimulatory effect at low concentrations, known as hormesis (Calabrese *et al*. 2007). Within the range of chromium (VI) concentrations tested there was no evidence of hormesis in the response of *Mc. capsulatus* Bath to Cr (VI).

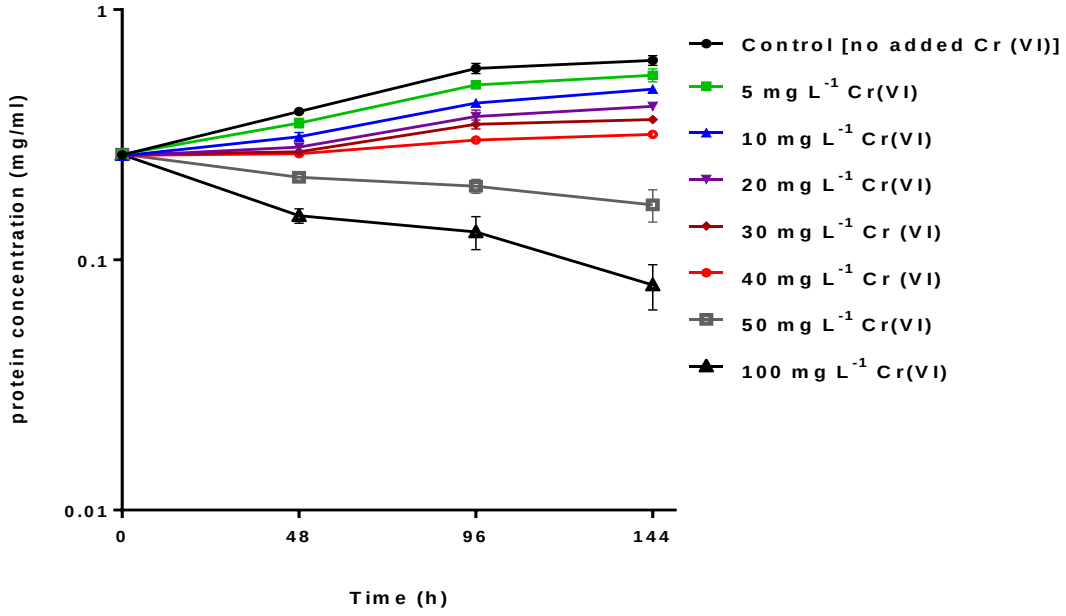


Figure 3-2 Bacterial growth of *Mc. capsulatus* Bath under different Cr (VI) concentrations. Bacterial growth was measured via the quantification of total cell protein using BCA kit, and expressed as total protein per mL culture. Data shown is from three independent cultures at each Cr (VI) concentration. Error bars show standard deviation (n=3).

Chromium (VI) reduction was not observed in the absence of methane, but in the presence of methane the Cr (VI) reduction was observed. This indicates that *Mc. capsulatus* Bath needs methane to complete the reduction of Cr (VI) (Figure 3-3).

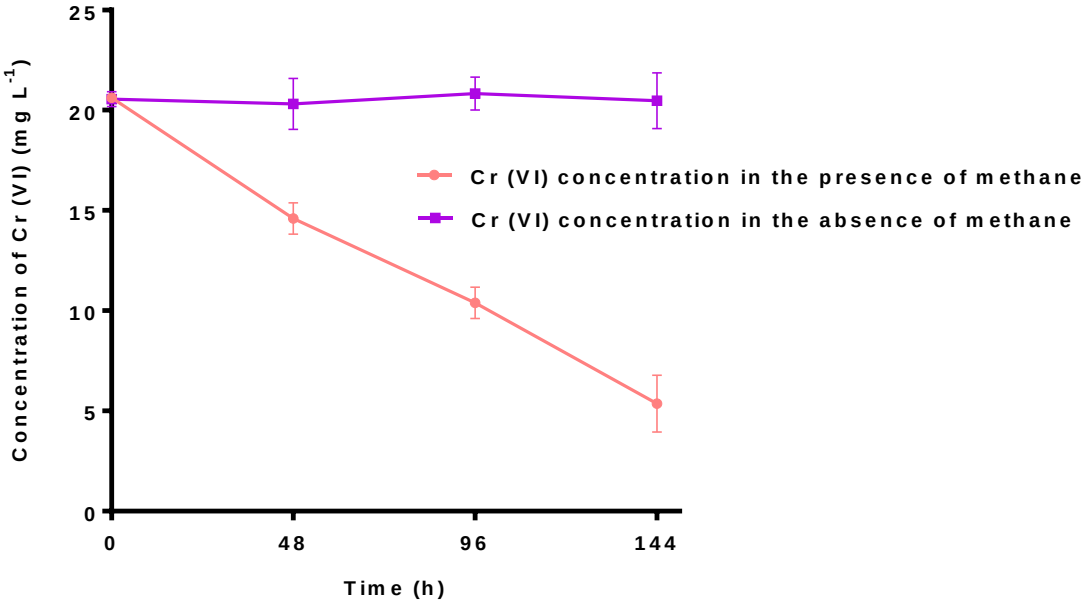


Figure 3-3 Pattern of live culture incubated with chromate in the presence and absence of methane. Initial chromate concentration is 20 mg L^{-1} (0.333 mM) of Cr (VI). Data shown is from three independent cultures. Error bars show standard deviation (n=3).

In order to determine whether the removal of chromium from the supernatant was an active process, chromium (VI) removed by live cells was compared with a control of cells killed by autoclaving. The Cr (VI) concentration in the culture supernatant with live cells decreased with time, but with the dead cells the removal of Cr (VI) was completely abolished. No Cr (VI) appeared in the cell pellets of either the live or dead cells (Figure 3-4).

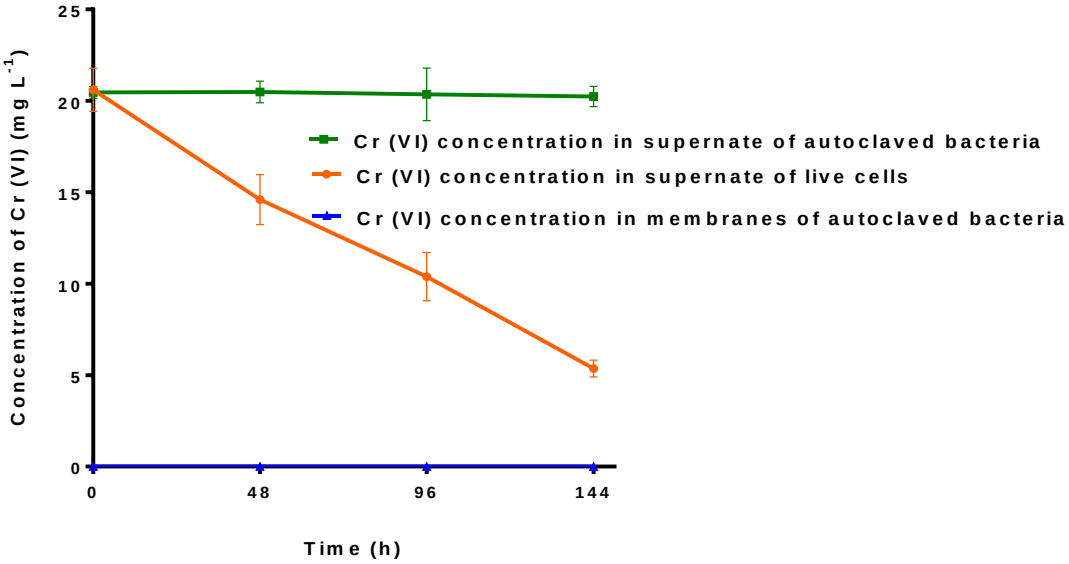


Figure 3-4 Pattern of chromium (VI) reduction in the presence of live and dead (supernatant - pellets) *Mc. capsulatus* Bath. Initial chromate concentration is (20 mg L^{-1}) (0.333 mM). Data shown is from three independent cultures. Results plotted as mean ± 1 standard deviation (n=3).

3.4 Bioremediation using methanotrophic bacteria *Mc. capsulatus* Bath

Chromate reduction trials were performed by adding Cr (VI) at various concentrations to cultures of *Mc. capsulatus* Bath (OD_{600} of 0.7- 0.9) and incubating the cultures at 45 °C in the presence of methane and air. Chromium species in the supernatant were quantified by using HPLC-ICP-MS (Figure 3-5). *Mc. capsulatus* Bath was able to remove chromate (VI) over a wide range of concentration no other detectable chromium species were observed in the culture supernatant. With starting concentrations of hexavalent chromium of 3 mg L^{-1} (0.05 mM) and 5 mg L^{-1} (0.083 mM), complete removal of chromate was achieved within 48 and 120 h, respectively. As the with concentration of hexavalent chromium was increased, a progressively smaller proportion of the chromium was removed, so that from a starting concentration of 40 mg L^{-1} (0.66 mM), only 15% of the chromium (VI) was removed during 144 h. With 50 mg L^{-1}

(0.833 mM), no detectable change in the concentration of hexavalent chromium was observed during the same period.

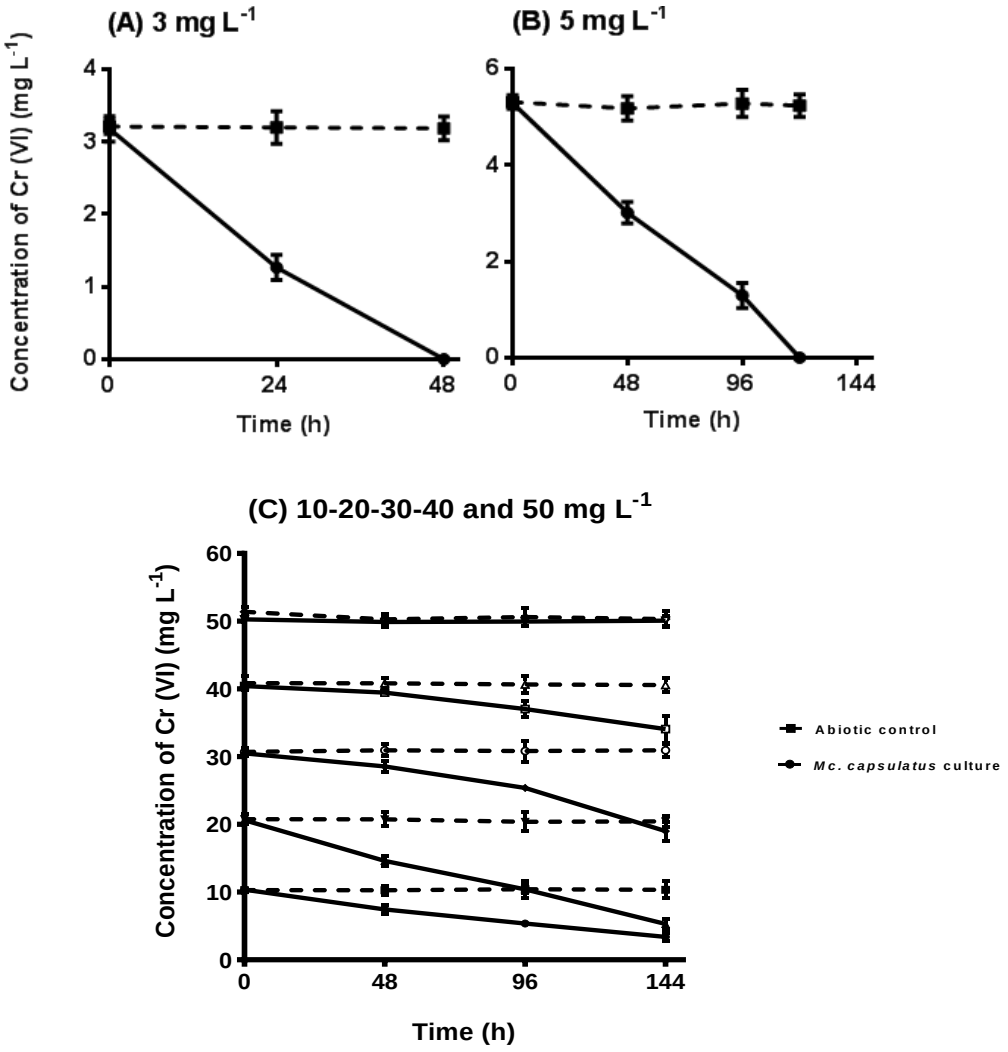


Figure 3-5 Effect of *Mc. capsulatus* Bath cultures on Cr (VI) at various concentrations. Experiments were biological triplicates. Results are shown as mean ± SD.

3.5 Reduction and accumulation of chromium species within cellular fractions of *Mc. capsulatus* Bath.

In order to determine the fate of the chromium (VI) that was removed from the medium by *Mc. capsulatus* Bath, samples of culture treated with an initial concentration of chromate (VI) of 20 mg L^{-1} (0.33 mM) were taken over a time

course of 144 h of incubation in the presence of methane and air. Cells were harvested from the samples and then broken and separated into two fractions (cell walls and a combined fraction of membranes and cytoplasm) as detailed in the Materials and Methods. Concentrations of chromium species in the culture supernatant and each cellular fraction were quantified via HPLC-ICP-MS connected with Ion exchange column HPLC (Figure 3-6).

Over the period of the experiment, the concentration of chromium (VI) in the culture supernatant declined and there was a corresponding increase in the concentration of chromium (III) in the cytoplasm+membranes fraction. No other chromium species were detected in significant concentrations in any of the samples. The constant total chromium (the sum of the chromium detected in all fractions) shown in Figure 3-6 indicates that the appearance of chromium (III) in the cytoplasm+membranes fraction accounted exactly for the decrease in chromium (VI) in the culture supernatant. These results showed that the cells of *Mc. capsulatus* Bath were able not only to reduce chromium (VI) to chromium (III) but also to accumulate all the chromium (III) within the biomass.

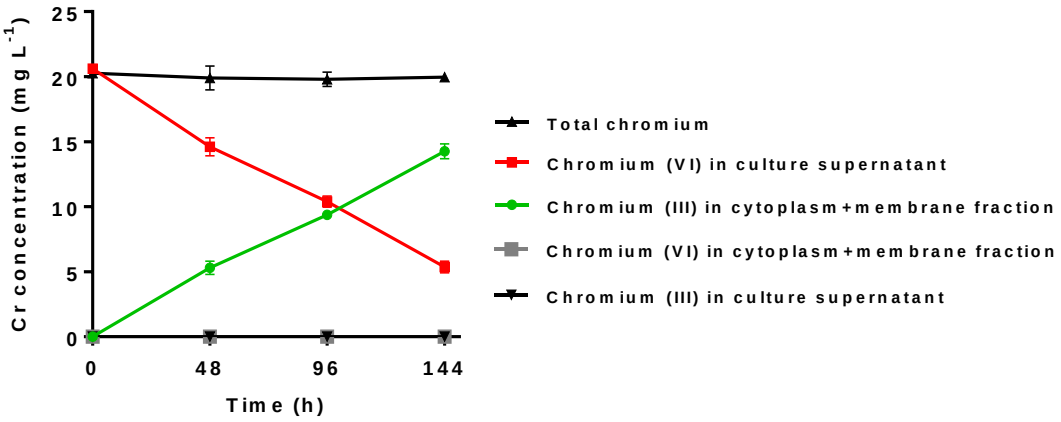


Figure 3-6 Reduction and accumulation of chromium species by *Mc. capsulatus* Bath after addition of Cr (VI) to 20 mg L^{-1} (0.33 mM). Values are the means from biological triplicates and are shown as mean ± SD. Concentrations in each of the fractions were normalised to the volume of the original culture.

3.6 Uptake of Cr (III) by *Mc. capsulatus* Bath

The fact that all of the cell-associated chromium was in the +3 oxidation state, even though the cells had been exposed to chromium in hexavalent form, raised the question of whether reduction and uptake of chromium had to be linked, or whether they could take up trivalent chromium directly. When exposed in exactly the same way to 20 mg L^{-1} (0.33 mM) of chromium (III) in the presence of methane and air, the *Mc. capsulatus* cells took up the chromium (III) completely into the cytoplasm+membranes fraction within 1 h (Figure 3-7A), much more quickly than >144 h taken for reduction and accumulation of the same amount of chromium (IV). Previous work has shown that the reduction of chromium (VI) to chromium (III) by *Mc. capsulatus* Bath is an active process requiring the presence of the carbon and energy source methane (Al Hasin *et al*. 2009). In order to investigate whether the uptake of chromium (III) was also an active process, cultures were exposed to 20 mg L^{-1} (0.33 mM) of chromium (III) aerobically though in the absence of methane. If the cells were grown to OD_{600} 0.7 in the presence of methane and then methane was removed and chromium (III) added immediately, all the chromium (III) was taken up by the cells within 1 h (Figure 3-7B). If, however, the cells were starved of methane overnight (16 h) before addition of the chromium (III), only 23.6% of the chromium (III) was taken up into the cells (Figure 3-7C). Addition of the metabolic inhibitor sodium azide (to 0.05% w/v) in the presence of methane (Figure 3-7D) abolished approximately half of the uptake of chromium (III) within a 1 h period. When the cells were both starved of methane overnight and sodium azide was added at the same time as the chromium (III), uptake of chromium (III) was completely abolished (Figure 3-7E). These results indicate that uptake of chromium (III) is an active process, but that when methane is removed from the culture, it has

sufficient reserves of energy to take up a substantial amount of chromium (III). Consistent with this, autoclaved cells were unable to take up chromium (III) (Figure 3-7F).

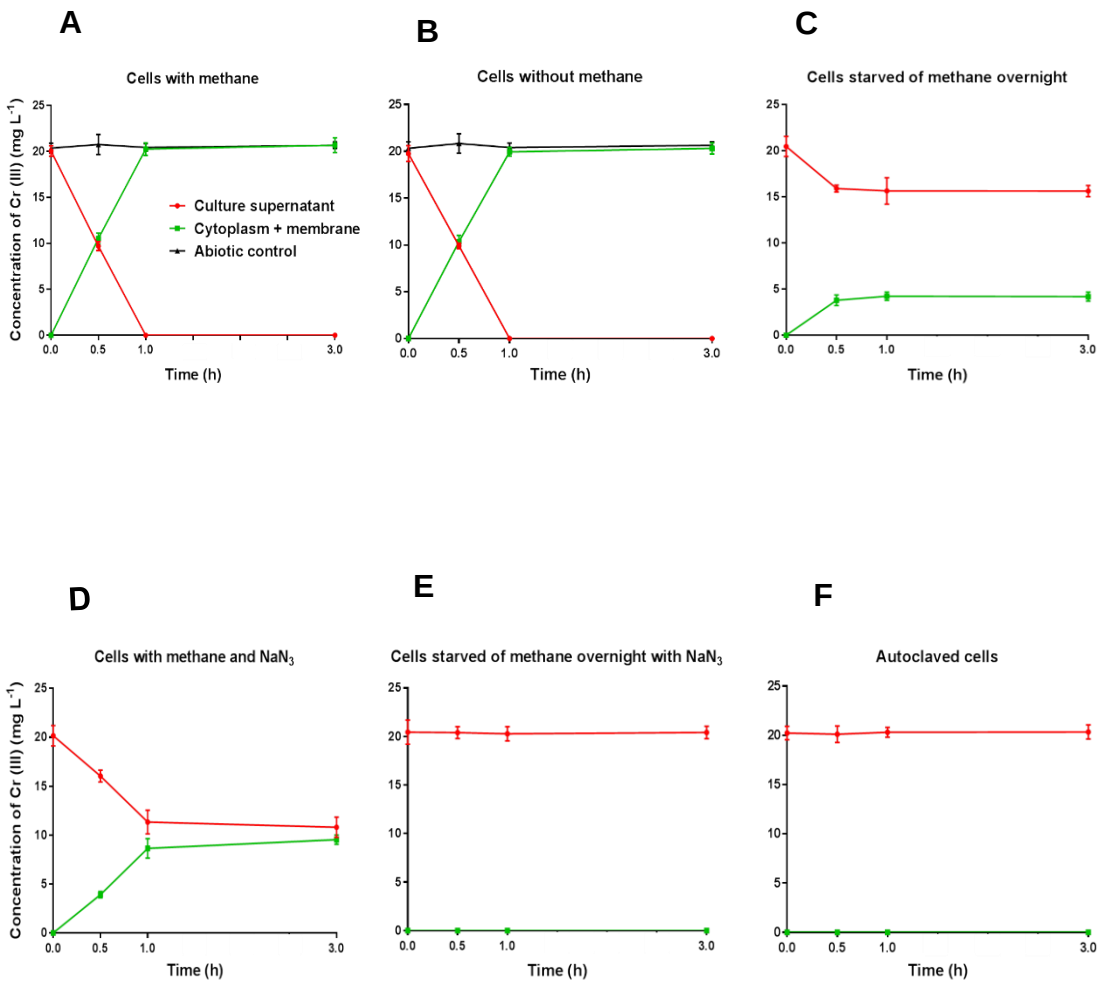


Figure 3-7 Effect of adding 20 mgL^{-1} (0.33 mM) of Cr (III) to *Mc. capsulatus* Bath cultures with and without methane. Error bars show the standard deviation of three biological replicates.

One possible explanation for the ability of the cells actively to take up some Cr (III) after starvation in the absence of methane is that the cells may still contain an energy storage compound such as volutin, which can supply energy in the form of ATP. If this is the case, such stored energy is clearly not available when the starved cells are also inhibited with NaN_3 (Pallerala *et al*., 2005).

3.7 Locating chromium species within the cells of *Mc. capsulatus* Bath

In order to find more precisely the location of chromium species within the cells, cultures were exposed to chromium (VI) at 20 mg L^{-1} (0.33 mM) and the cells in the cells were fractionated as previously, except that the fractionation protocol was extended to produce separate membrane and cytoplasm fractions. The results confirmed the reduction of chromium (VI) and accumulation of chromium (III) into the cytoplasm and membranes, and showed that the distribution of chromium between the two fractions was approximately two thirds in the cell membrane fraction and one third in the cytoplasm fraction (Figure 3-8).

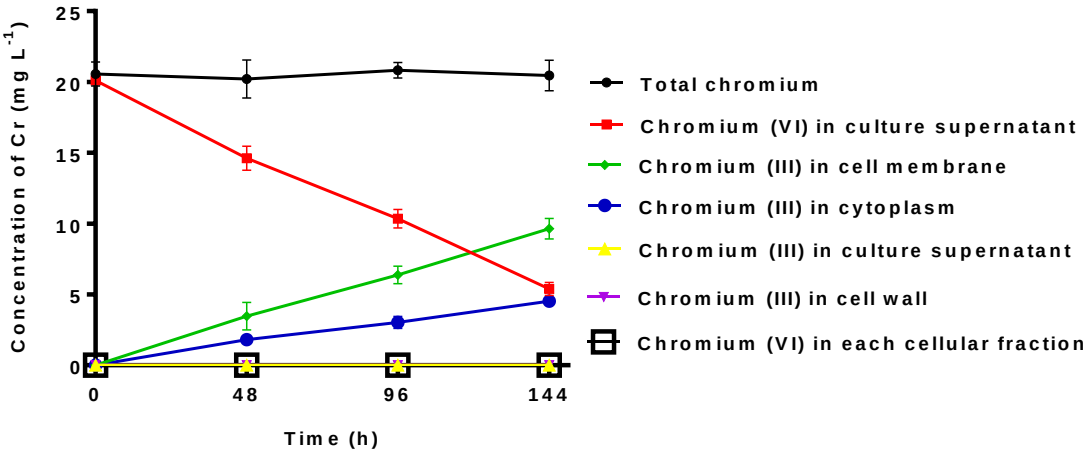


Figure 3-8 Speciation and distribution of chromium species analysed after fractionation of cells into separate cell wall, cytoplasm and membrane fractions. Initial Cr (VI) concentration was 20 mg L^{-1} (0.33 mM). Error bars show the standard deviation of three biological replicates.

Electron microscopy and associated techniques were then used to gain additional information about the speciation and distribution of the chromium at the microscopic level. Electron energy loss spectroscopy (EELS) coupled with transmission electron microscopy (TEM) of whole *Mc. capsulatus* Bath cells exposed to 20 mg L^{-1} (0.33 mM) of chromium (VI) for 96 h or 144 h confirmed,

via comparison with spectra of chromium standards, that the cell-associated chromium was in the +3 oxidation state (Figure 3-9).

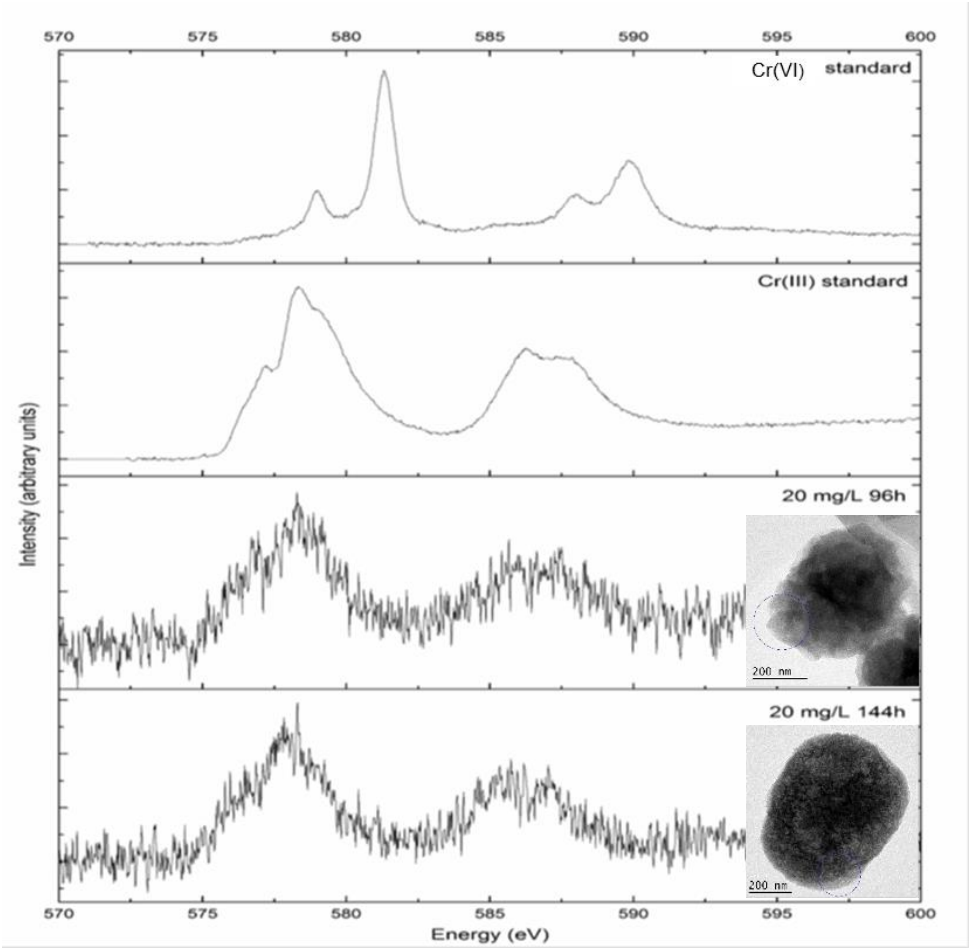


Figure 3-9 EELS spectra of *Mc. capsulatus* Bath cells compared with chromium standards. Inserts show the areas of the samples (circled) that were analysed by EELS. Initial Cr (VI) concentration was 20 mg L^{-1} (0.33 mM).

Chromium (VI)-exposed cells were also prepared as thin sections to see how chromium and other metals were distributed within the cells. HAADF-TEM-EDX of sections of cells exposed to 20 mg L^{-1} (0.33 mM) of chromium (VI) for 144 h showed the presence of chromium in the chromium-treated cells (Figure 3-10A and B) and its absence from control (chromium-untreated cells; Figure 3-10C and D). The spatial distribution of chromium (Figure 3-10E) indicated that the chromium was largely cell-associated with possibly greater amounts of

chromium toward the edges of the cell than in the interior. This is consistent with the approximate 40:60 distribution of the chromium between the cytoplasm and membrane fractions, when it is born in mind that *Mc. capsulatus* Bath under the relatively high-copper pMMO expressing conditions of these experiments is expected to have intracellular as well as peripheral membranes.

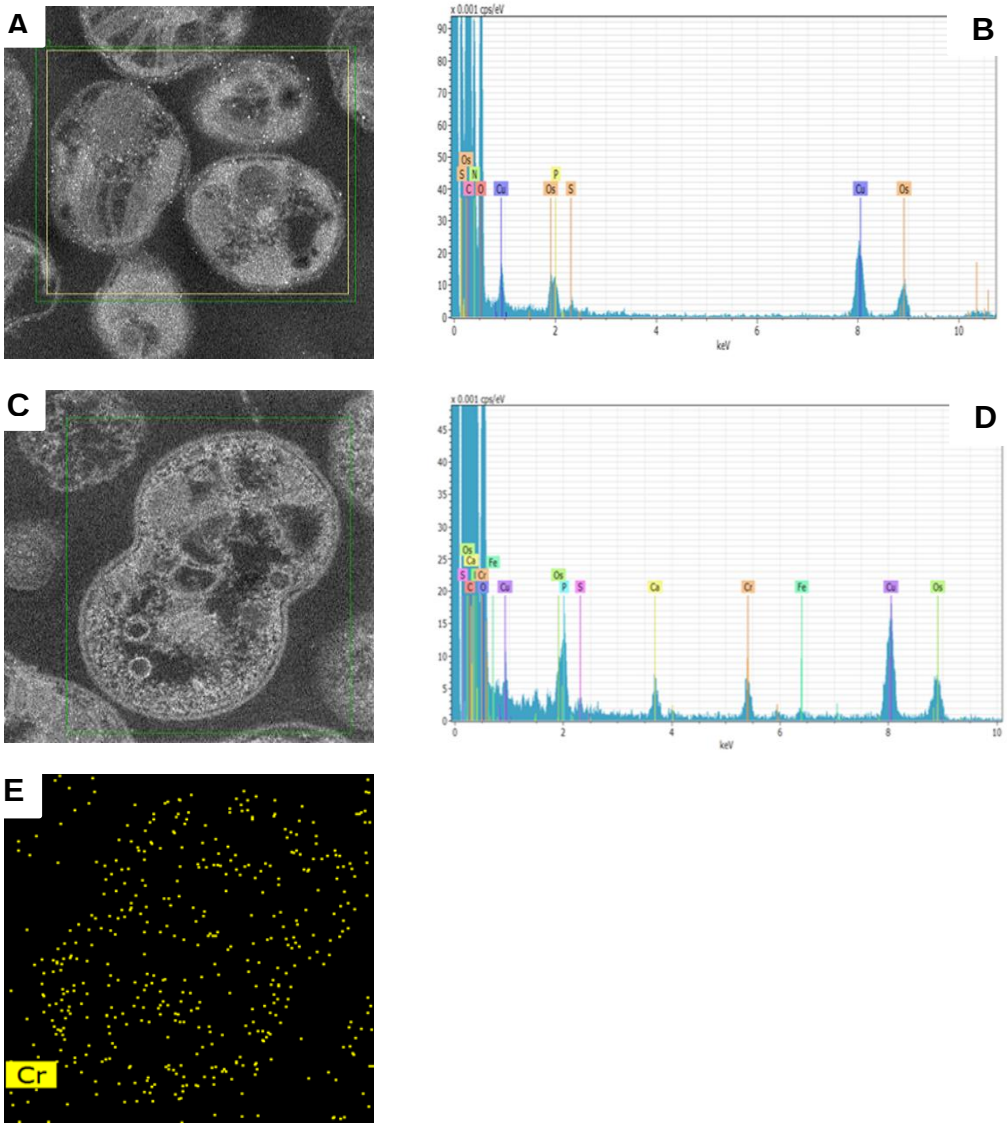


Figure 3-10 EDX of sectioned cells showing the distribution of chromium. (A) No-chromium control; (C) cells exposed to 20 mg L^{-1} (0.33 mM) after 144h; (E) spatial distribution of chromium using HAADF-STEM imaging in the sample shown in C. Green and yellow boxes on the micrographs in parts A and C show the areas of the sample analysed in the EDX spectra shown on the right (B and D).

The distribution of a number of other elements (carbon, phosphorous and oxygen) was also determined within whole *Mc. capsulatus* cells via EDX (Figure 3-11 and Figure 3-12) and EELS imaging (Figure 3-13 and Figure 3-14) coupled to TEM.

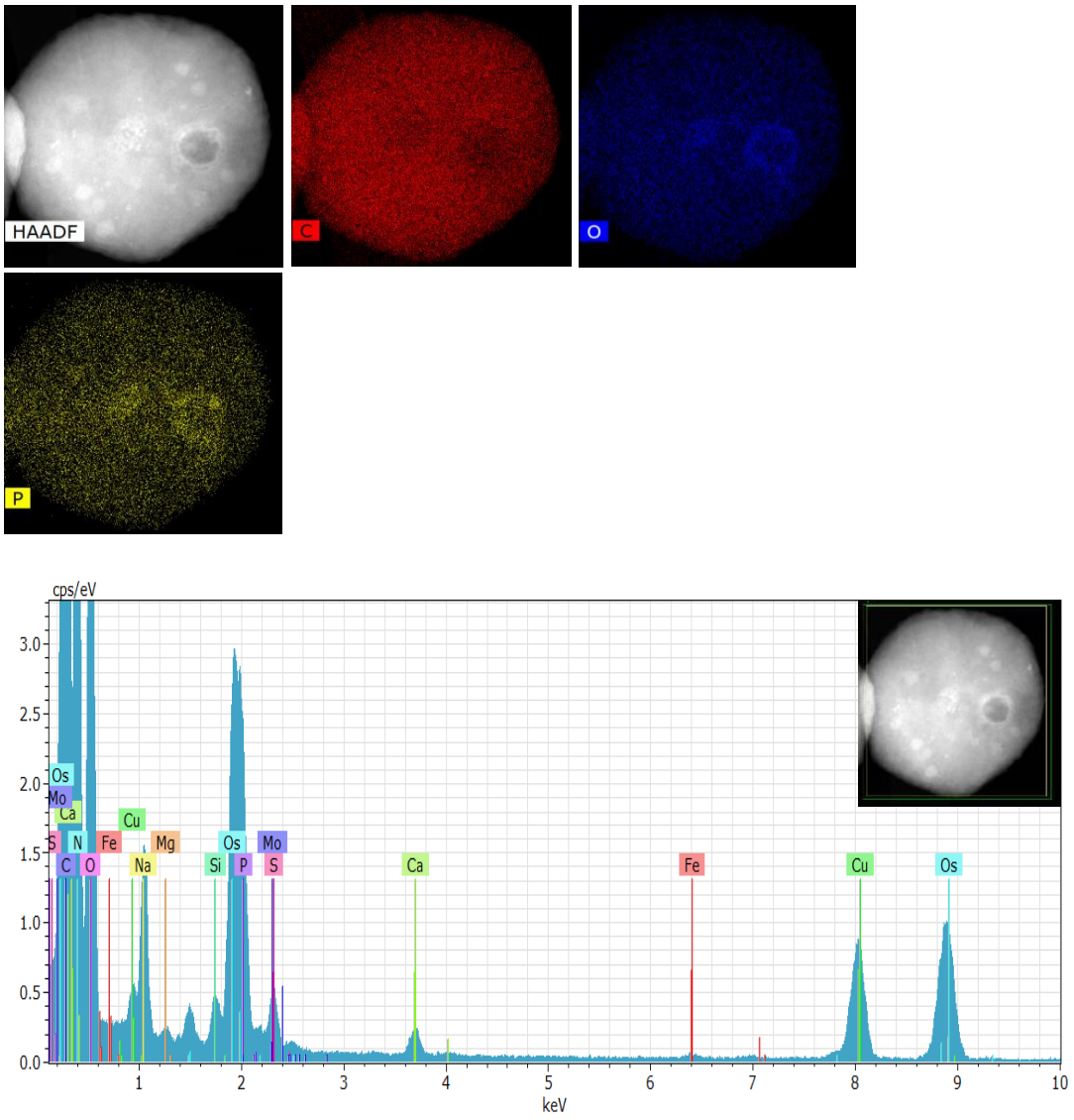
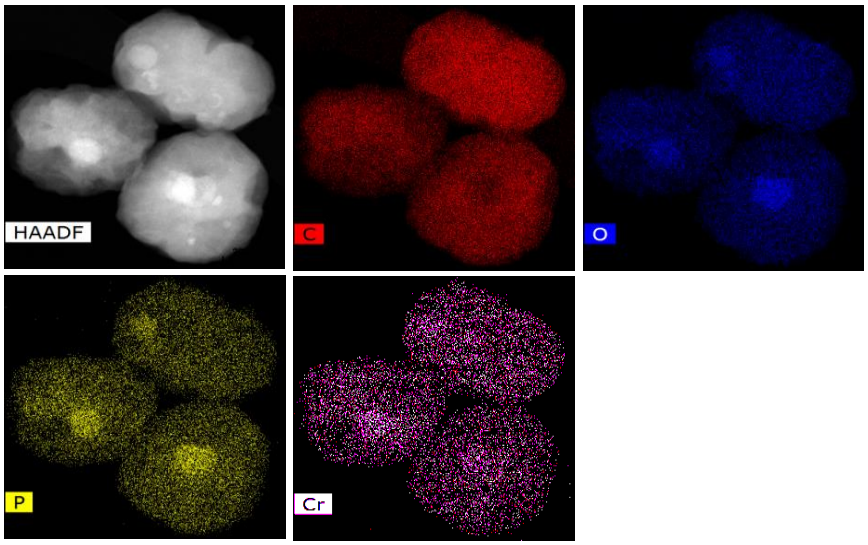


Figure 3-11 Electron micrographs with corresponding EDX spectra of whole *Mc. capsulatus* Bath cells, showing the distribution of carbon, oxygen and phosphorous after incubation of the culture in the presence of methane for 144 h at 45 °C without added chromium. The EDX spectrum was generated from data collected from the area indicated by the box in the insert.

The formation of cell associated structure composed of precipitate metal ions has been observed previously, for example in the production of extracellular fibers and stalks of Fe (III) oxides by *Gallionella* (Hallberg & Ferris 2004).



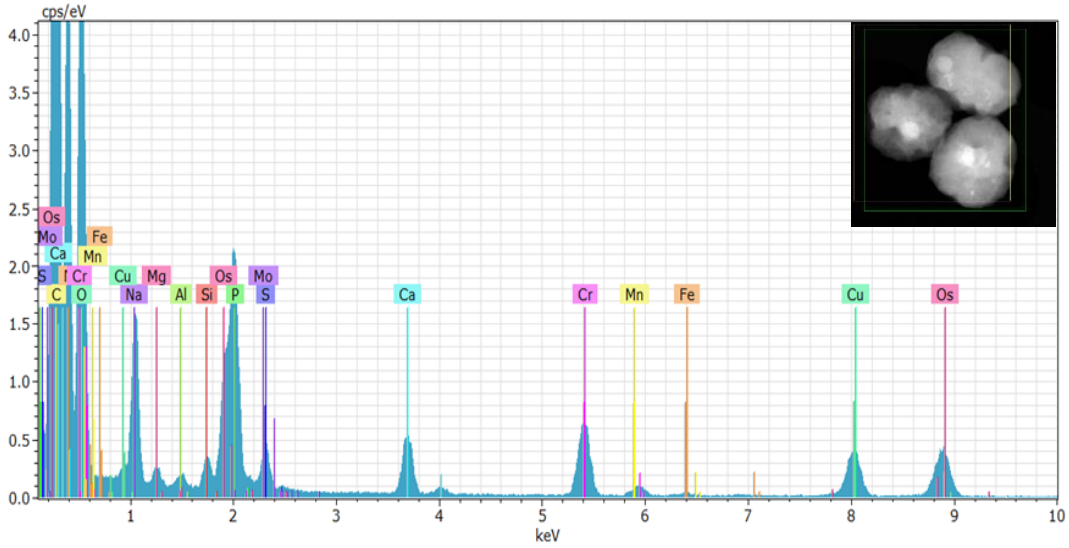


Figure 3-12 Electron micrographs with corresponding EDX spectra of whole *Mc. capsulatus* Bath cells, showing the distribution of carbon, oxygen and phosphorous after incubation of the culture in the presence of methane for 144 h at 45 °C after addition of chromium (VI) to 20 mg L^{-1} (0.33 mM). The EDX spectrum was generated from data collected from the area indicated by the box in the insert.

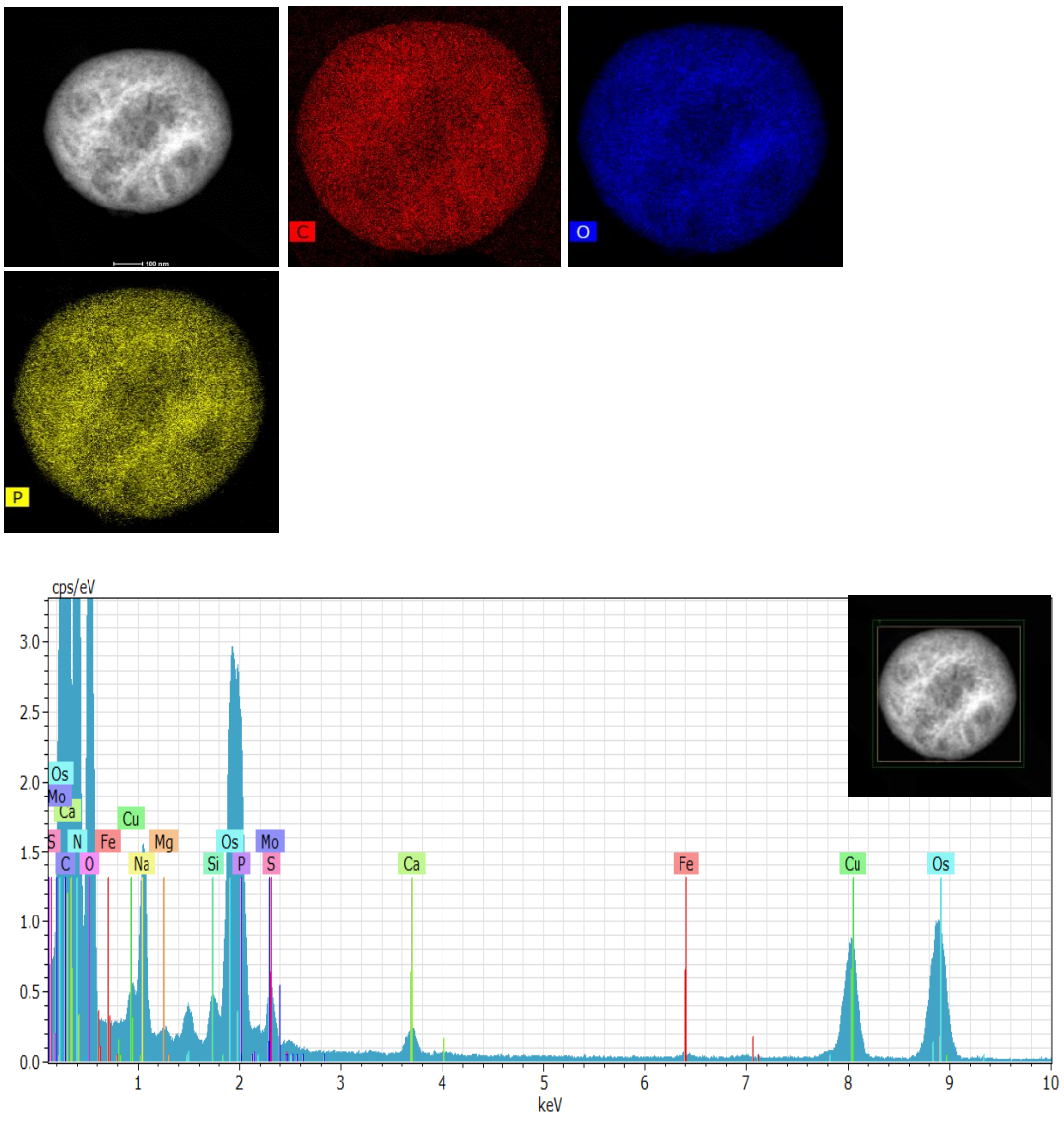


Figure 3-13 Electron micrographs showing the distribution of elements via EELS imaging of whole *Mc. capsulatus* Bath cells, after incubation of the culture in the presence of methane for 144 h at 45 °C without added chromium. The EELS spectrum was generated from data collected from the area indicated by the box in the insert.

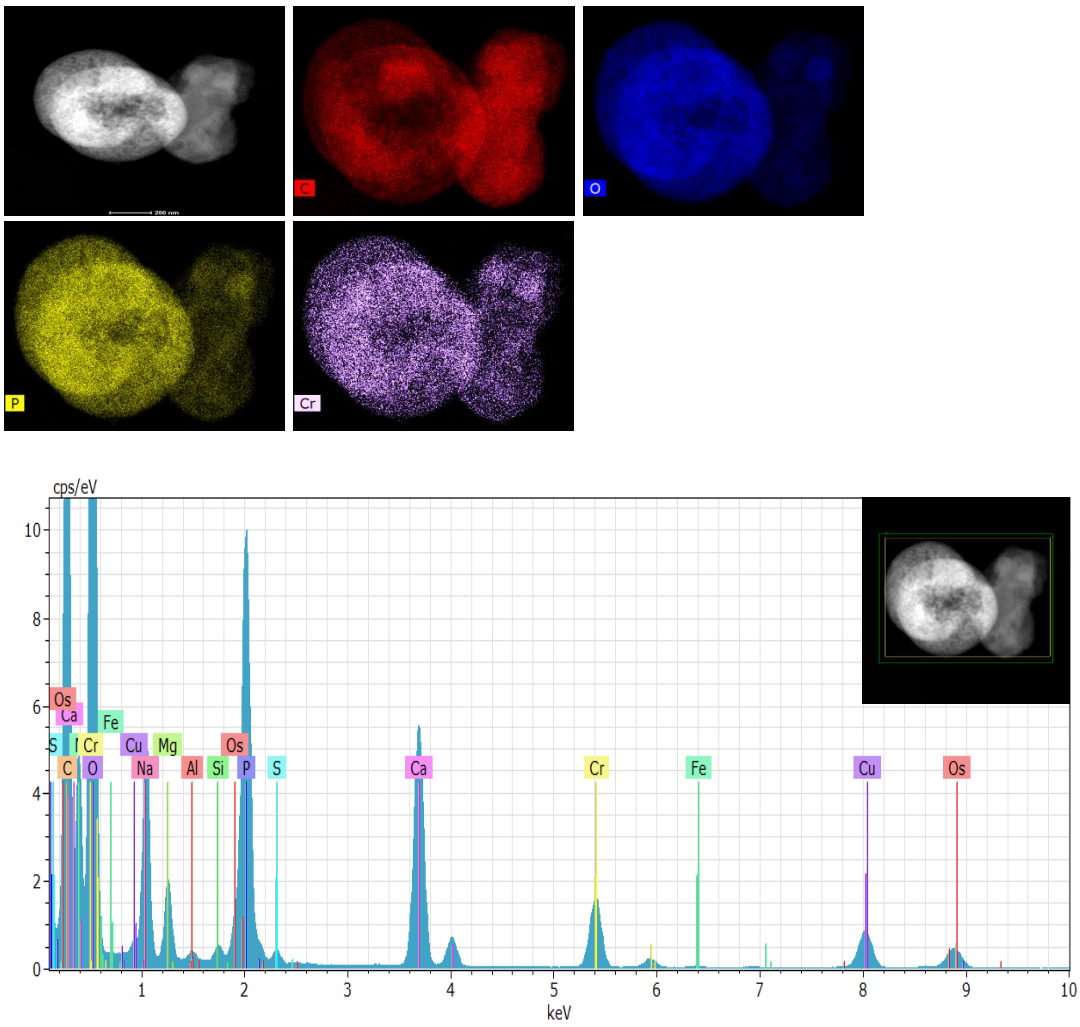


Figure 3-14 Electron micrographs showing the distribution of elements via EELS imaging of whole *Mc. capsulatus* Bath cells, after incubation of the culture in the presence of methane for 144 h at 45 °C after addition of chromium (VI) to 20 mg L^{-1} (0.33 mM). The EELS spectrum was generated from data collected from the area indicated by the box in the insert.

These results indicated that there was inhomogeneity in the distribution of all five elements, which appeared to correlate with features on the surface of the cells visible in the electron micrographs. These feature showed elevated concentrations of chromium, phosphorous and oxygen and decreased concentrations of carbon. The co-localization of chromium, phosphorous and oxygen may be due to precipitation of insoluble Cr (III) phosphate upon or within the cells. The reduced amount of carbon within these areas may simply be due to absence of carbon within this precipitated inorganic material.

3.8 X-ray photoelectron spectroscopy analysis

X-ray photoelectron spectroscopy analysis (XPS) is an analysis technique used to obtain chemical information about the surfaces of a wide range of materials. Both composition and the chemical state of surface elements can be measured by XPS. The XPS measurements were performed to further investigate the composition and functional groups on the surface of the chromium exposed cells. Regarding to the sample of chromium added to the *Mc. capsulatus* Bath, the wide scan XPS spectra are shown in Figure 3-15A while high resolution spectra for C 1S and O 1S are shown in Figure 3-15B band C respectively. However, low resolution spectra shown for N 1s and Cr 2p as in Figure 3-15D and E. The data processing and deconvolution of photoelectron peaks were obtained using a commercial software package (Casa XPS v2.3.16PR1, Casa Software Ltd., UK). Elemental chromium is generally observed between 574 and 578 eV. In our case, the deconvolution of low-resolution Cr 2p spectra, which exist at binding energy of 577.8 eV. The peak corresponds to elemental chromium.

Figure 3-15B shows strong emission due to C 1s. Several species of C 1s from different functional groups constitute this strong emission. The XPS peak at binding energy of 284.99 eV is attributed to C-H in amino acid chain. The XPS peak for C 1s at binding energy of 286.55 eV can be assigned to C-OH in alcohol group or possibly C-OR in ether group. The XPS peak at binding energy of 288.03 eV is assigned to C=O in carbonyl group. While the peak at 288.86 eV assigned to COOR on found in lipids.The O 1s spectra were deconvoluted into two component peaks. The O 1s peak at 532.90 eV is attributed to C-OH, C-O-C in alcohol groups, ester group and P-OH found in phosphate moieties

which are widely found in biomolecules including proteins, nucleic acids and carbohydrates. The second O 1s peak at 531.60 eV is attributed to C=O, P=O in carbonyl and phosphate moieties, respectively.

The N 1s spectra produce a peak at 400.1 eV which is commonly found in amino acids and amino sugars (Kaur *et al*. 2009).

This peak assigned to elemental chromium is only marginally above the surrounding noise in the spectrum. In the absence of a corresponding expanded view of the same region of the spectrum of the sample not exposed to chromium (Figure 3-16), it is not possible unambiguously to conclude that this peak is due to the chromium added to the sample.

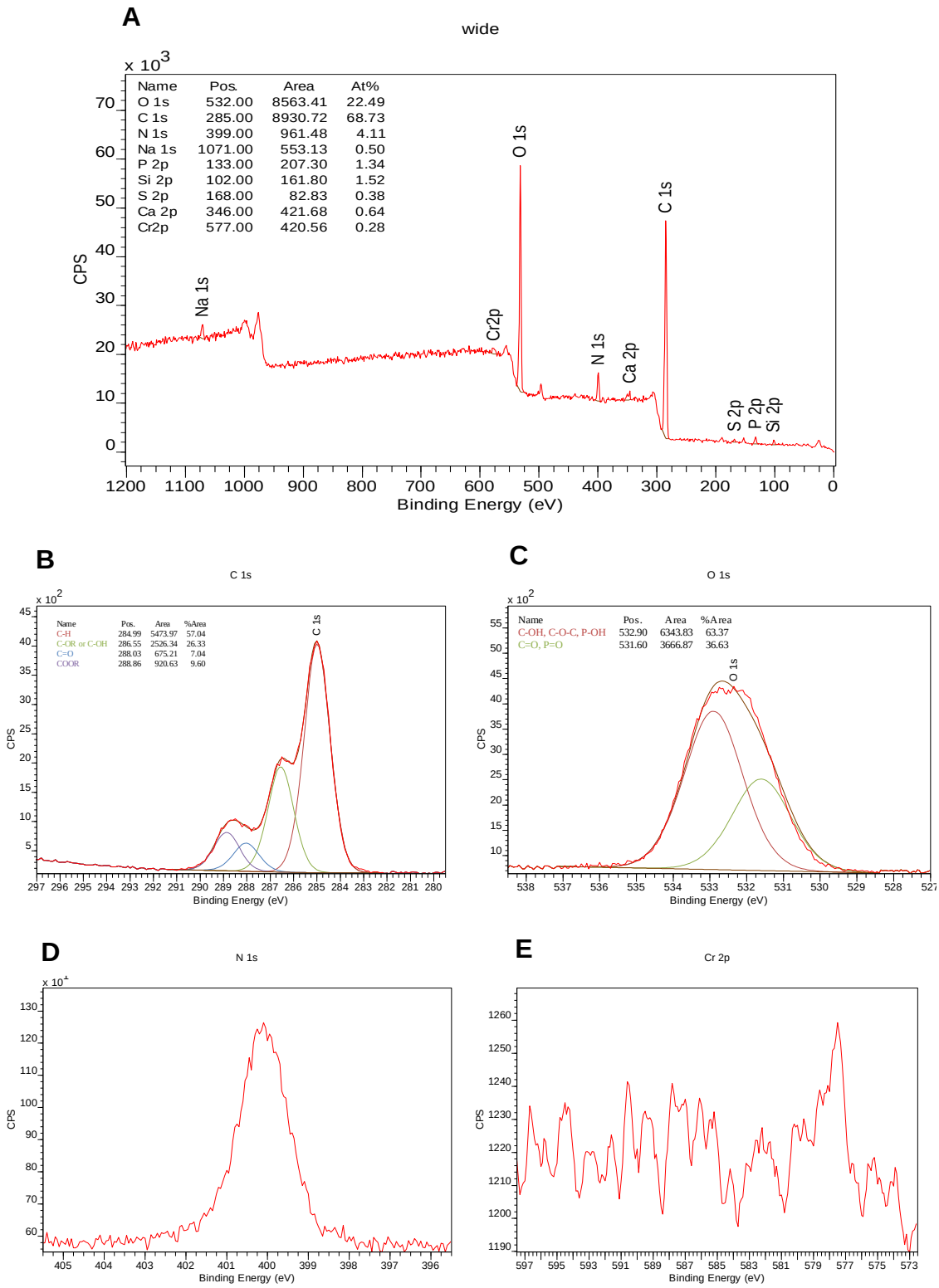


Figure 3-15 Wide scan X-ray photoelectron spectra of chromium sample exposed to *Mc. capsulatus* Bath (A) and high resolution spectra for C 1s and O 1s are shown in B and C. The low resolution spectra for N 1s and Cr 2p are shown in D and E, respectively.

On the other hand, the control sample that was not exposed to the chromium, the wide scan XPS spectra are shown in Figure 3-16A while high resolution spectra for C 1S and O 1S are shown in Figure 3-16B and C respectively. However, low resolution spectra shown for N 1S as in Figure 3-16D.

Figure 3-16B shows strong emission due to C 1s. The XPS peak for C 1s at binding energy of 284.87 eV can be assigned to C-H in amino acid group. The XPS peak at binding energy of 286.38 eV is attributed to C-OR or C-OH in ether group or alcohol group. The XPS peak at binding energy of 287.85 eV is assigned to C=O in carbonyl group. While the peak at 288.79 eV assigned to COOR on found in lipids.

The O 1s spectra were deconvoluted into two component peaks. The O 1s peak at 532.77 eV is attributed to C-OH, C-O-C, P-OH in alcohol group, ester group and phosphate moieties. The second O 1s peak at 531.50 eV is attributed to C=O, P=O in carbonyl and phosphate moieties.

The N 1s spectra produce a peak at 399.8 eV which is commonly found in amino acids and amino sugars (Kaur *et al*. 2009).

The XPS for Cu was not observed in the wide scan spectra of chromium sample that added to *Mc. capsulatus* Bath. While the control sample did not show any signal of Ca 2p, Cr 2p and Cu.

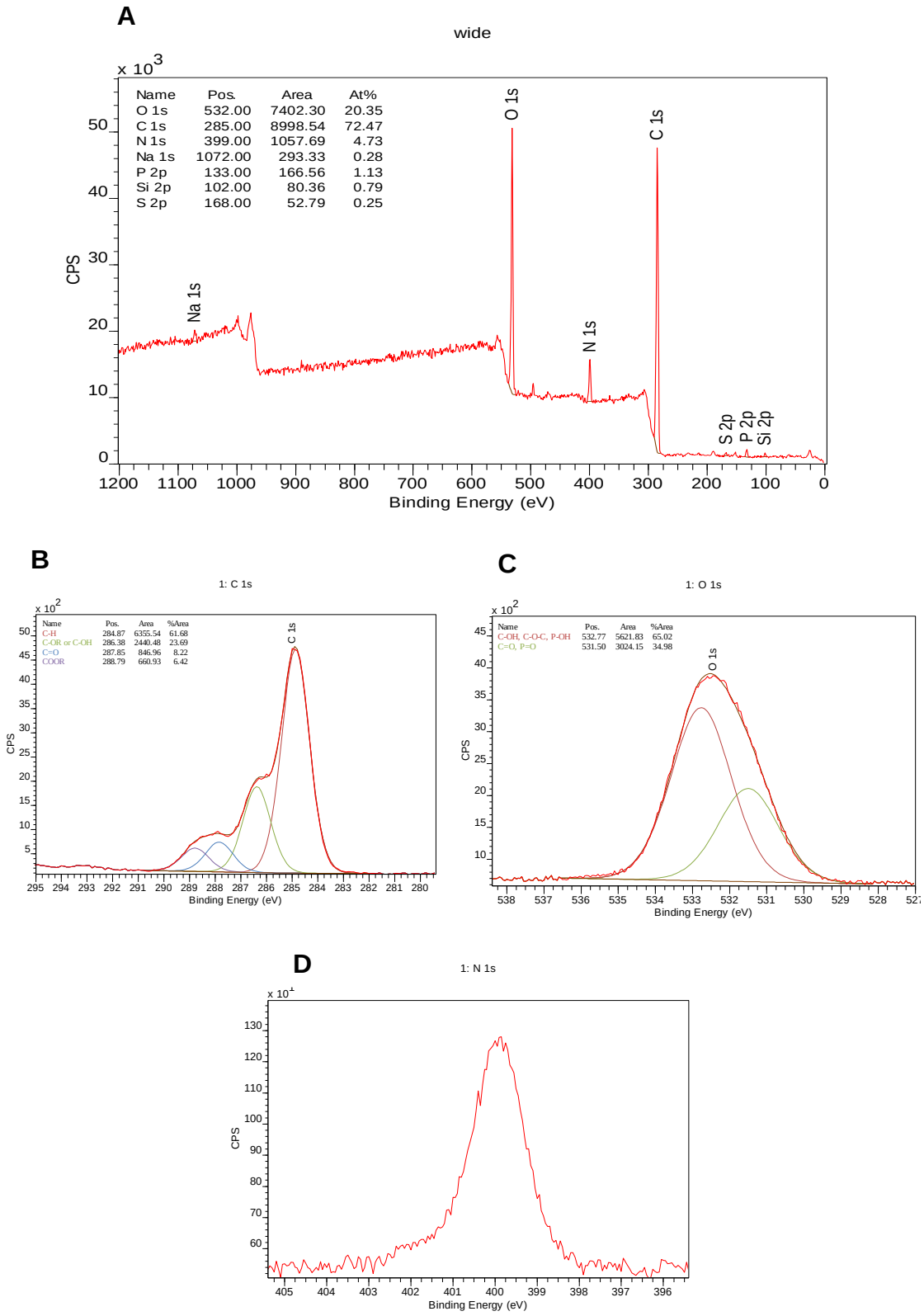


Figure 3-16 Wide scan X-ray photoelectron spectra of control sample to *Mc. capsulatus* Bath that not exposed to chromium (A) and high resolution spectra for C 1s and O 1s are shown in B and C. The low resolution spectra for N 1s is shown in D.

Table 3-1 Results of curve-appropriate C 1s spectra of *Mc. capsulatus* Bath of (control sample compared with chromium sample).

Control sample			Chromium sample		
Name of group	B. E. (eV)	% Area	Name of group	B. E. (eV)	% Area
C - H	284.87	61.68	C - H	284.99	57.04
C- OR or C - OH	286.38	23.69	C- OR or C - OH	286.55	26.33
C = O	287.85	8.22	C = O	288.03	7.04
COOR	288.79	6.42	COOR	288.86	9.60

Table 3-2 Results of curve-appropriate O 1s spectra of *Mc. capsulatus* Bath of (control sample compared with chromium sample).

Control sample			Chromium sample		
Name of group	B. E. (eV)	% Area	Name of group	B. E. (eV)	% Area
C - H, C - O - C, P - OH	532.77	65.02	C - H, C - O - C, P - OH	532.90	63.37
C = O, P = O	531.50	34.98	C = O, P = O	531.60	36.63

3.9 Conclusion

It has been confirmed that the pure strain of the methanotrophic bacterium *Mc. capsulatus* Bath is able to detoxify chromate (VI) over a wide range of concentrations and the product is chromium in the relatively nontoxic +3 oxidation state. Cr (VI) was also found to be reduced by *Shewanella oneidensis*. In the case of *S. oneidensis*, some Cr (II) as well as Cr (III) was found in association with the cells (Daulton *et al*. 2007), although it is more widely found that (as is the case with *Mc. capsulatus*) reduction to Cr (III) is the final step in the microbial Cr (VI) reduction chain (Cervantes *et al.* 2001; Wang *et al*. 1990; Fude *et al*. 1994).

In the work described in this chapter, the speciation and distribution of the chromium species in the methanotroph *Mc. capsulatus* Bath were identified by HPLC-ICP-MS, TEM-EDX, HAADF-STEM, EELS and XPS. Increase the appearance of chromium (III) in the cytoplasm+membranes fraction was accompanied by a deficiency of chromium (VI) in the culture supernatant. No chromium (III) was detected in culture supernatant; in contrast, there is no chromium (VI) in the cytoplasm+membranes fraction. A number of previous studies have used similar techniques, though with different kinds of microorganisms. Chardin *et al*. (2006) used HPLC coupled to ICP-MS to examine the kinetics of Cr (VI) reduction by sulfate reducing bacteria, which showed an increase of Cr (III) concentration during 6 hours of incubation with the bacteria. Sandar *et al*. (2011) used SEM-EDX to show that chromium associated with cells of *Bacillus* strain during Cr (VI) removal. Neal *et al*. (2002) used EELS and XPS to show the chromium associated with *S. oneidensis* of Cr (III) oxidation state.

To more precisely locate the chromium species within the cells, cells of *Mc. capsulaus* Bath were fractionated into separate cell wall, cytoplasm and membrane fractions. The results showed that the distribution of chromium (III) was considerably more in the cell membrane fraction than in the cytoplasm fraction, and this result also confirmed by HAADF-TEM-EDX of thin sections of cells. Similar individual cell microanalysis in *S. oneidensis* by transmission electron microscopy (TEM) using electron energy loss spectroscopy (EELS) and energy dispersive X-ray spectroscopy (EDXS) demonstrated that Cr (III) is concentrated near the cytoplasmic membrane in this organism (Daulton *et al*. 2007), in constrast to the more widespread distribution of chromium within the cells observed in *Mc. capsulatus* Bath.

The results presented in this chapter strongly suggest that the *Mc. capsulatus* Bath cells accumulate the chromium concomitant with reducing it. Reduction of Cr (VI) and the uptake of the Cr (III) are active processes. All of the chromium becomes associated with the cells, which may be a useful property for bioremediation applications.

Chapter 4 Purification of Cr (VI) reductase of *Mc. capsulatus* Bath

4.1 Introduction

In the previous chapter it was shown that when the cells of *Mc. capsulatus* Bath were exposed to chromium (VI), the cell-associated chromium was found exclusively in the Cr (III) oxidation state. Cr (III) was found in the cytoplasm and membrane fractions in the approximate proportion 2:1. In order to characterise the bioremediation of chromium (VI) by *Mc. capsulatus* Bath it was necessary to characterise the chromium (VI) reducing activity, to determine whether or not it is enzymatic and where in the cell it was located.

In order to achieve this, the cells were fractionated and the individual fractions assayed for chromium (VI) reducing activity and the optimal temperature for the reaction was determined. In an attempt to determine the source of electrons for the reduction reaction, a range of electron donors was tested. To enable this, the abiotic reaction between the electron donors and chromium (VI) was investigated and conditions were found that minimised the abiotic reaction to enable the biological reaction to be studied. The cytoplasm, which was found to contain the greatest chromium (VI) reducing activity, was chosen for purification of the active component. A two-step procedure was then used to purify the chromium (VI) reducing activity, comprising Anion exchange chromatography followed by Capto Blue Dye Affinity chromatography.

4.2 Methods

4.2.1 Cr (VI) reduction by cell fractions

Cells of *Mc. capsulatus* Bath were grown on methane, harvested, broken using the French press and fractionated into cytoplasm, membrane and cell wall-associated fractions as described by Smith and Foster (1995) and detailed in Chapter 2 (Materials and Methods). Fractions were assayed for chromium (VI)

reduction activity and a range of biological electron donors (NADH, NADPH as electron donors for sMMO, duroquinol as electron donor for pMMO and benzyl viologen as efficient electron donor) were investigated to determine the coenzyme requirement of the enzyme(s). The duroquinol was prepared as follows (Zahn & DiSpirito 1996): 50 µl of concentrated HCl was added to 20 ml of ethanol (3 mM HCl) and then 0.2 g of duroquinone was added with stirring, after that 0.28 g sodium hydrosulfite (sodium dithionite) was added to the solution with continuous stirring for a minimum of 3 min, then the solution was diluted with 150 ml H_2O. The solution was filtered through a Watman no. 1 filter. The water insoluble duroquinol remained colorless (white) and was collected on the filter. The duroquinol was rinsed/washed on the filter with approximately 200 ml H_2O and air dried by suction filtration for at least 5 min. The duroquinol was removed from the filter and then stored in a foil covered serum vial at -20 °C for no longer than 2 days.

Chromium (VI) reduction by each of the fraction was assayed in triplicate with each of the electron donors. For each fraction, triplicate controls were performed with no added electron donor. Each assay contained potassium chromate to give a starting concentration of chromium (VI) of 2.6 mg L^{-1} (0.043 mM), 10 µl of the electron donor solution (0.2 mM) where appropriate and 980 µl of the cellular fraction or fraction from column chromatography. The purpose of the reactions without electron donor was to measure the background rate of reduction of chromium (VI).

Chromium concentration was determined colorimetrically by the diphenylcarbazide method taking 300 µl of sample and acidifying by adding 700

µl 0.18 M sulfuric acid and adding 50 µl diphenylcarbazide (DPC) solutions (2.5 g L^{-1} in acetone) to produce the colour change. Initial samples of culture were taken when potassium chromate was added and then further readings at 48 and 96 hour time points.

4.2.2 Effect of cell fractions on Cr (VI) without added electron donors at various temperatures

In order to determine the effect of temperature on the chromium (VI)-reducing activity of the cellular fractions of *Mc. capsulatus* Bath, the fractions were studied for their activity at various temperatures between 10 and 50 °C. Fractions were incubated for 96 hours, followed by measurement of chromate reductase activity as already described in section 4.2.1.

4.2.3 Effect of electron donors on chromium (VI) in the absence of cell fractions

In order to be able to quantify the effect of added electron donors on reduction of chromium (VI) by the methanotroph cell fractions, it was necessary to determine whether any reaction occurred between chromium (VI) and the electron donors in the absence of added cellular fractions. A range of NADH, NADPH, duroquinol and benzyl viologen concentrations were investigated in order to find the concentration that would best allow detection of enzyme-catalysed Cr (VI) reduction activity.

4.2.4 Anion exchange chromatography

Anion exchange chromatography was performed with a Hiscreen Capto diethyl-aminoethyl DEAE (CV = 4.7 ml; GE Healthcare Life Sciences) column connected to an Akta protein purification system. The flow rate of buffer was set to 2.7 ml/min throughout the preparation of the column and purification. The column pressure remained around 3 bar (0.3 MPa) throughout. All purification steps were carried out at cold room temperature with enzyme preparations stored at 4 °C.

The column was washed with 1 CV of distilled water before use. This step was used in order to pre-equilibrate the column and remove the ethanol used for storage of the column. The column was then equilibrated with 10 CV of the start buffer (20 mM Tris- HCl, pH 8.0). According to the manufacturer of the column, such washing with 10 CV of starting buffer is sufficient to stabilise the UV baseline, pH and conductivity of the eluate.

The cytoplasmic fraction of *Mc. capsulatus* Bath prepared as described in section 4.2.1. was made free of particulate material (including membrane fragments) by centrifugation in the ultra-centrifuge (105,000 × *g*; 60 min; 4 °C). The resulting supernatant (containing 300 mg total protein in 75 ml of 20 mM Tris-HCl, pH 8.0) was loaded onto the column. This was within the dynamic binding capacity of the column based on the information provided by the suppliers (dynamic binding capacity of > 90 mg of protein per ml CV, hence a 4.7 ml column can bind at least 423 mg of protein).

The column was then washed with 10 CV of start buffer until the UV trace returned to the baseline. The run through was collected in case the chromate reducing activity did not bind to the column.

The column was then eluted with a linear gradient, from 0% to 100% elution buffer (20 mM Tris-HCl, 1M NaCl, pH 8.0) (Figure 2-4). Fractions of 5 ml were collected during elution and then were assayed for chromium removing activity (as detailed in section 4.2.1.).

In order to regenerate the column for further use, it was washed as follows. The column was first washed with at least 2 CV of 2 M NaCl and washed with at least 4 CV of 1 M NaOH. Then the column washed with at least 2 CV of 2 M NaCl and washed with at least 2 CV of distilled water. Finally the column washed with at least 10 CV of start buffer.

4.2.5 Protein concentration assay

Protein concentration was measured via the BCA protein assay with bovine serum albumin (BSA) as the standard using the BCA protein assay kit (Thermo Scientific, UK, catalogue no. 23225). Proteins in samples were separated and characterised by means of analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-12% PAGE was performed using Mini-PROTEAN TGX Precast Gels (Bio-Rad, Watford, UK) were used. Samples were mixed with 4 × Laemmli loading buffer (Amresco, Solon, Ohio, USA) and heated at 95 °C for 5 min before loading onto the gel. Gels were run in at 100 V for 90 min in TGS running buffer (25mM Tris base, 192 mM glycine, 0.1% wt/vol SDS). Gels were stained with Instant Blue stain (Expedion, Cambridge, UK).

4.2.6 Purification by Capto Blue Dye Affinity Chromatography

The HiScreen Capto Blue column (4.7 mL column, GE Healthcare Life Sciences) used in this work was supplied prepacked with 4.7 ml Capto Blue coupled to the base matrix by a hydrophilic spacer immobilized with a stable amine bond. Capto Blue is more chemically stable and has a more rigid agarose base matrix and this allows the use of faster flow rates and large sample volumes, leading to higher throughput and improved process economy. The column is ideal for screening of selectivity, binding and elution conditions, as well as small scale purifications. The column is used in an optimal way with liquid chromatography systems such as AKTA.

HiScreen Capto Blue column was equilibrated with at least 5 column volume (CV) start buffer (20 mM Tris- HCl, pH 8.0), the flow rate of buffer was set to 2.3 ml/min throughout the purification. The column pressure was 3 bar (0.3 MPa) throughout the procedure. All purification steps were carried out at cold room temperature at 4 °C.

The dynamic binding capacity of the Capto Blue column matrix is reported by the manufacturer to be 25 mg/mL and the total volume for Capto Blue was 4.7 ml, so the binding capacity of the column was expected to be 25 x 4.7 = 117.5 mg of protein. Active fractions from the DEAE Anion exchange chromatography were pooled and the protein concentration was 2.31 mg/ml x 9 ml cytoplasm fraction = 20.79 mg.

The pooled sample was dialysed against 20 mM Tris-HCl pH 8.0 using a 3.5 kDa cut-off dialysis tubing with three changes of buffer at 4 °C to remove the salt used to elute the protein from the Anion exchange column and to exchange the proteins into the start buffer for the subsequent Dye Affinity purification step.

The 9 ml dialysed sample was loaded onto the Capto Blue column and then the column was washed with 5 CV of a start buffer (20 mM Tris- HCl, pH 8.0) (until the UV trace returned to near baseline), and the run through was collected in case the chromate reducing activity did not bind to the column.

The column was then eluted by linear gradient elution, from 0% to 100% elution buffer (20 mM Tris-HCl, 2 M NaCl, pH 8.0)(Figure 4-14). Fractions of 3 ml were collected during elution and then the fractions corresponding to the peaks of absorbance were tested for chromium VI removal as shown in Figure 4-15 by means of the diphenyl carbazide assay. Chromium VI reducing activity was found in tubes fractions 9 and 10.

The column was washed by several steps were; the column was washed with 4 column volumes (CV) of 0.5 M NaOH and then washed with at least 4 (CV) of 70% ethanol after that the column washed immediately with at least 5 (CV) filtered start buffer (20 mM Tris-HCl, pH 8.0).

4.3 Results

4.3.1 Effect of cell fractions on Cr (VI) without added electron donors at various temperatures

Although the fractions were active between 20 and 40 °C, activity of the fractions was found to reach a maximum at 30 °C, and was reduced at both higher and lower temperatures, thus proving that the enzyme is tolerant of temperature variations within these limits (Figure 4-1).

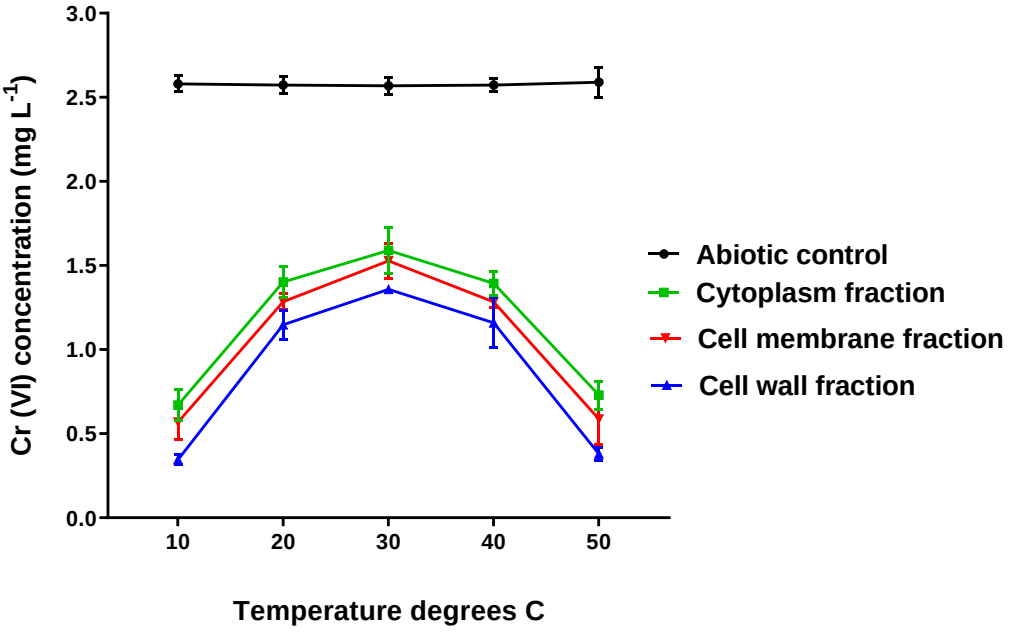


Figure 3-17 Cell fractions with Cr (VI) at 96 h in the absence of electron donors at various temperatures ranges between 10 and 50 °C. Data shown is from three independent cultures. Results plotted as mean ± 1 standard deviation (n=3).

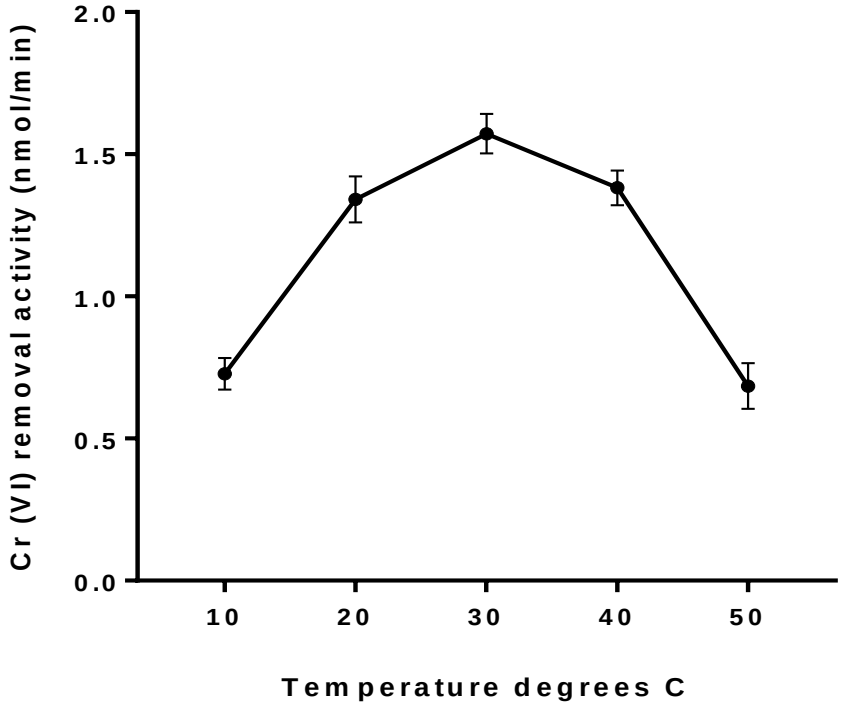


Figure 3-18 Cytoplasmic chromium (VI) removal activity at 96 h. Error bars show standard deviation (n=3).

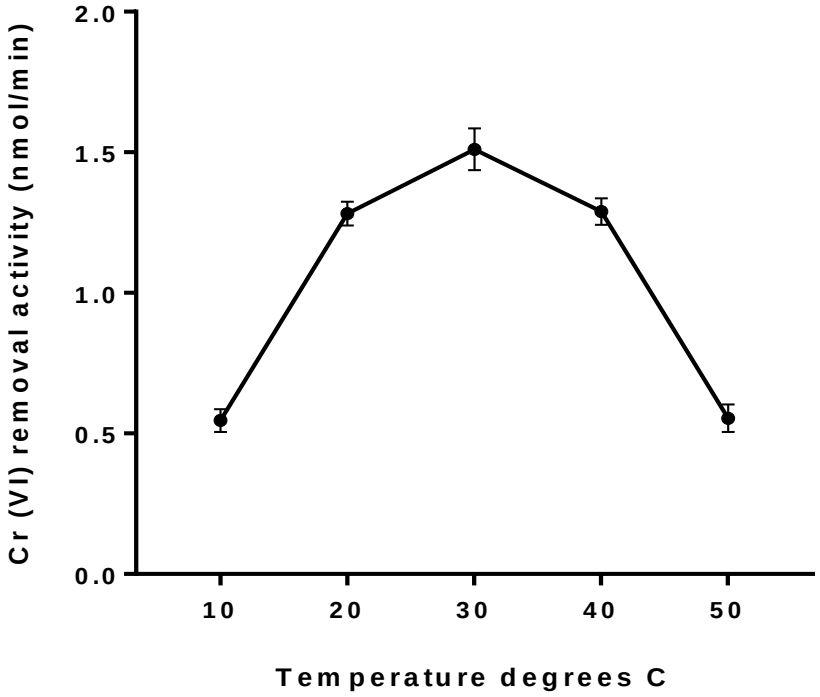


Figure 3-19 Cell membrane chromium (VI) removal activity at 96 h. Error bars show standard deviation (n=3).

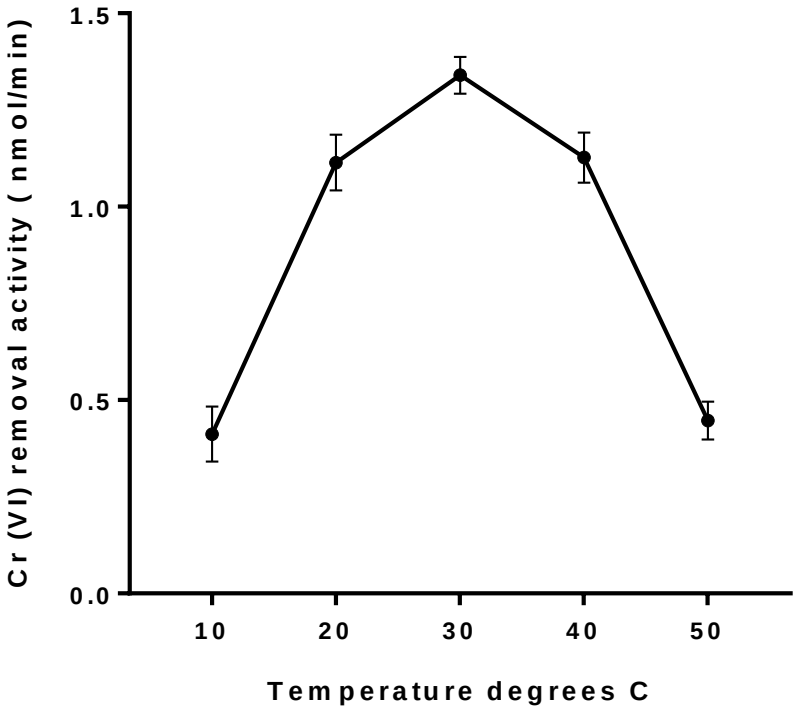


Figure 3-20 Cell wall chromium (VI) removal activity at 96 h. Error bars show standard deviation (n=3).

These results also established that the cellular fractions of *Mc. capsulatus* Bath were capable of removing chromium (VI), presumably by chemical reduction, in the absence of added reducing agents. Hence, a certain amount of the reduced coenzyme(s) or other electron donor(s) must be present in the cellular fractions to account for this reduction.

4.3.2 Effect of electron donors on chromium (VI) in the absence of cell fractions

It was found that 0.4 mM of NADH, NADPH, duroquinol and benzyl viologen reduced the concentration of Cr (VI) from 2.6 mg L^{-1} (0.043 mM) to (2.378, 2.350, 2.306 and 2.305) mg L^{-1} (0.0396, 0.0391, 0.03843 and 0.03841) mM respectively. These were only a (8.54%, 9.62%, 11.31% and 11.35%) reduction. With such a reduction rate in this condition it would be difficult to differentiate reduction by a protein and NADH, NADPH, duroquinol and benzyl viologen thus generating the possibility of false positive results (Figure 4-5). 0.2 mM of NADH, NADPH, duroquinol and benzyl viologen on the other hand, did not reduced of Cr (VI). Hence 0.2 mM concentrations of NADH, NADPH, duroquinol and benzyl viologen were used as electron donor in subsequent experiments in order to minimise the background non-biological reduction of chromium (VI).

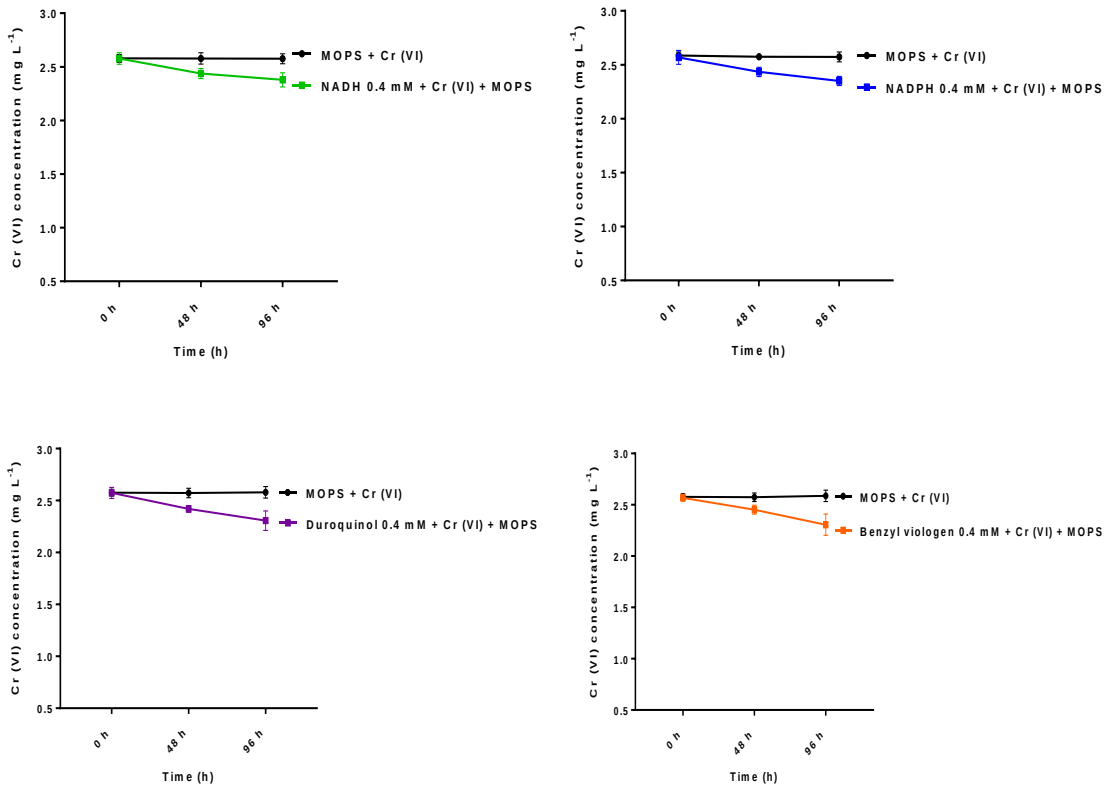


Figure 3-21 Quantification of Cr (VI) via the DPC assay, from an initial concentration of 2.6 mg L^{-1} (0.043 mM) by NADH, NADPH, duroquinol and benzyl viologen were used at concentrations of 0.4 mM and a negative control (without NADH, NADPH, duroquinol and benzyl viologen) was performed. Solutions were prepared in (25 mM MOPS, pH 7) buffer as to simulate the conditions of the cell fractions. Results plotted as mean ± 1 standard deviation (n=3).

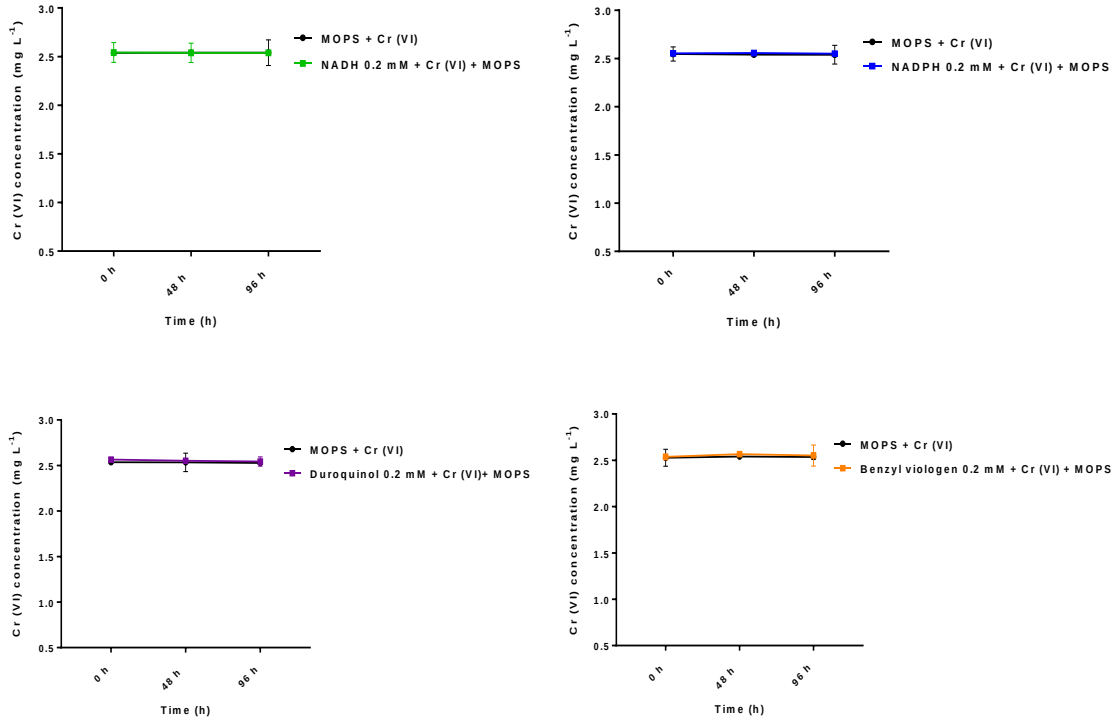


Figure 3-22 Quantification of Cr (VI) via the DPC assay, from an initial concentration of 2.6 mg L^{-1} (0.043 mM) by NADH, NADPH, duroquinol and benzyl viologen were used at concentrations of 0.2 mM and a negative control (without NADH, NADPH, duroquinol and benzyl viologen) was performed. Solutions were prepared in (25 mM MOPS, pH 7) buffer as to simulate the conditions of the cell fractions. Results plotted as mean ± 1 standard deviation (n=3).

From this experiment, it was discovered, via the DPC assay, that NADH, NADPH, duroquinol and benzyl viologen reduced Cr (VI) to Cr (III) with concentration (0.4 mM) from electron donor to some extent without reductase from the cell fractions present, so an additional control was devised is 25 mM MOPS (pH 7) added to it Cr (VI) in order to show whether direct (non-enzyme-catalysed) reactions of NADH, NADPH, duroquinol and benzyl viologen with Cr (VI) were likely to be significant, but with concentration (0.2 mM) from electron donor there are not any reduction of Cr (VI), this concentration was therefore used for Cr(VI) reduction by cell fractions.

4.3.3 Chromium (VI) reduction by cell fractions in the presence of added electron donors

As observed previously (Figure 4-1), in the absence of added electron donors the cellular fractions removed added chromium (VI). The chromium (VI) removal activity was greatest in the cytoplasm, in comparison with the cell membrane and cell wall fractions. These fractions reduced the concentration of Cr (VI) in the absence of an electron donor from 2.6 mg L^{-1} (0.043 mM) to (0.976, 1.007 and 1.184) mg L^{-1} (0.0162, 0.0167 and 0.0197) mM, respectively, over a period of 96 h (Figure 2-3A, B, C and D).

In the cytoplasm and cell membrane fractions there was a substantial reduction of Cr (VI) during the first 48 hours, removing more than two-thirds of the initial concentration. However, after 48 hours the decline continued but was slower than first 48 hours.

The cytoplasmic fraction caused a reduction of 62.46% which appears to be the maximal amount of reduction possible without adding more electron donor.

In the cell wall fraction the rate of reduction was approximately equal before and after 48 hours.

The ability of the various cell fractions to reduce 2.6 mg L^{-1} (0.043 mM) Cr (VI) was tested with 0.2 mM of NADH, NADPH, duroquinol and benzyl viologen in 25 mM MOPS (pH 7) buffer and measured by using the DPC assay. The amount of reduction performed by the cytoplasm fraction increased slightly with added NADH. There was also a slight increase in reduction by the membrane fraction upon adding duroquinol. No other significant effects of the reducing agents were seen.

The cytoplasm fraction with NADH and NADPH (green line) has further reduction compared with the cytoplasm fraction without NADH and NADPH (blue line) and the Cr (VI) removal activity was (1.868 and 1.692) mg L^{-1} (0.0311, 0.0282) mM, respectively from 2.6 mg L^{-1} (0.043 mM) as initial concentration of Cr (VI), at 96 hours (Figure 4-7A + Figure 4-8A).

The cell membrane fraction with duroquinol has further Cr (VI) removal activity (1.702 mg L^{-1}) (0.0283 mM) compared with the same fraction starved of duroquinol it was (1.566 mg L^{-1}) (0.0261 mM), at 96 hours (Figure 4-9B).

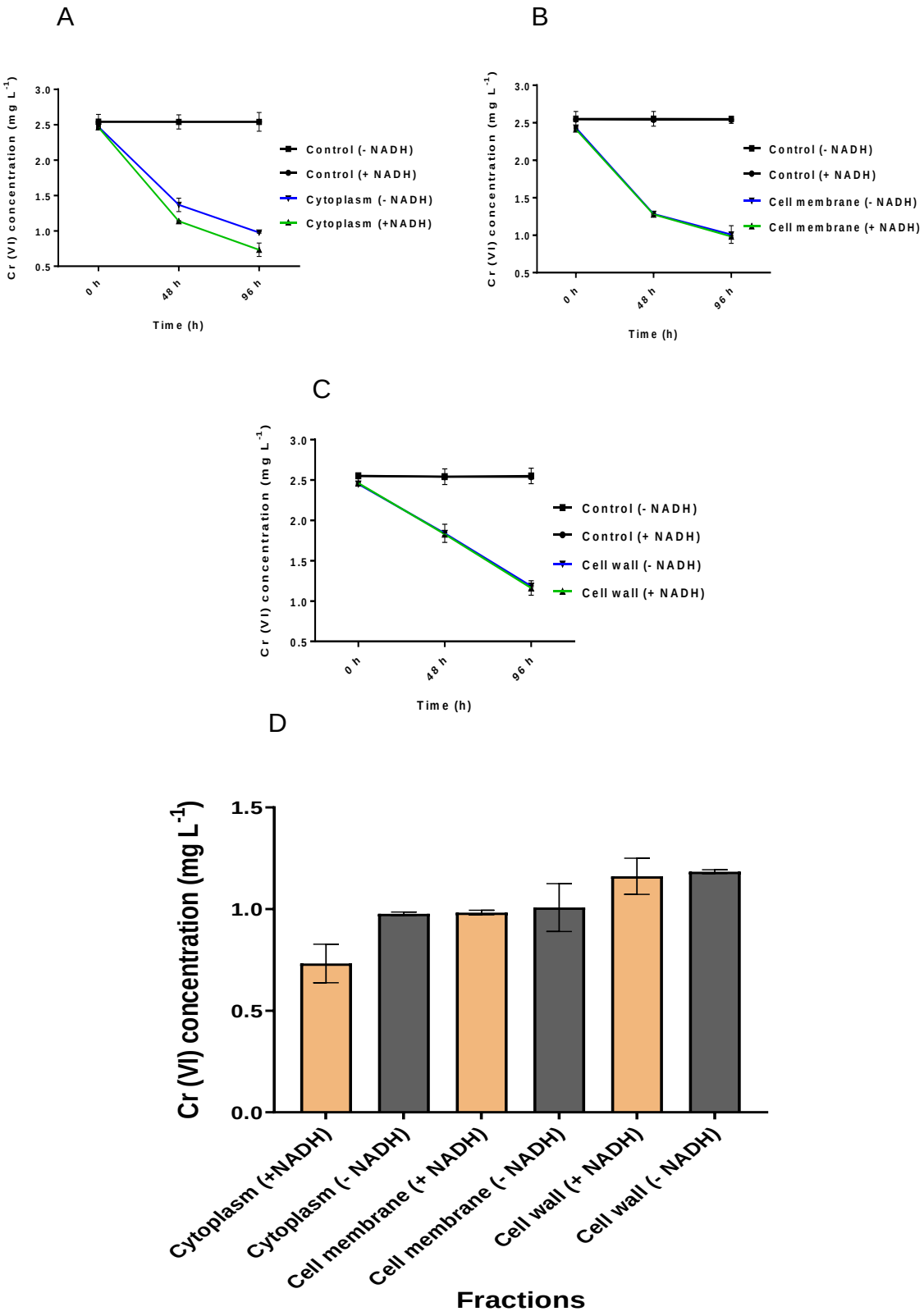


Figure 3-23 Cell fractions plus Cr (VI) with and without NADH as electron donor. Error bars show the standard deviation of three biological replicates.

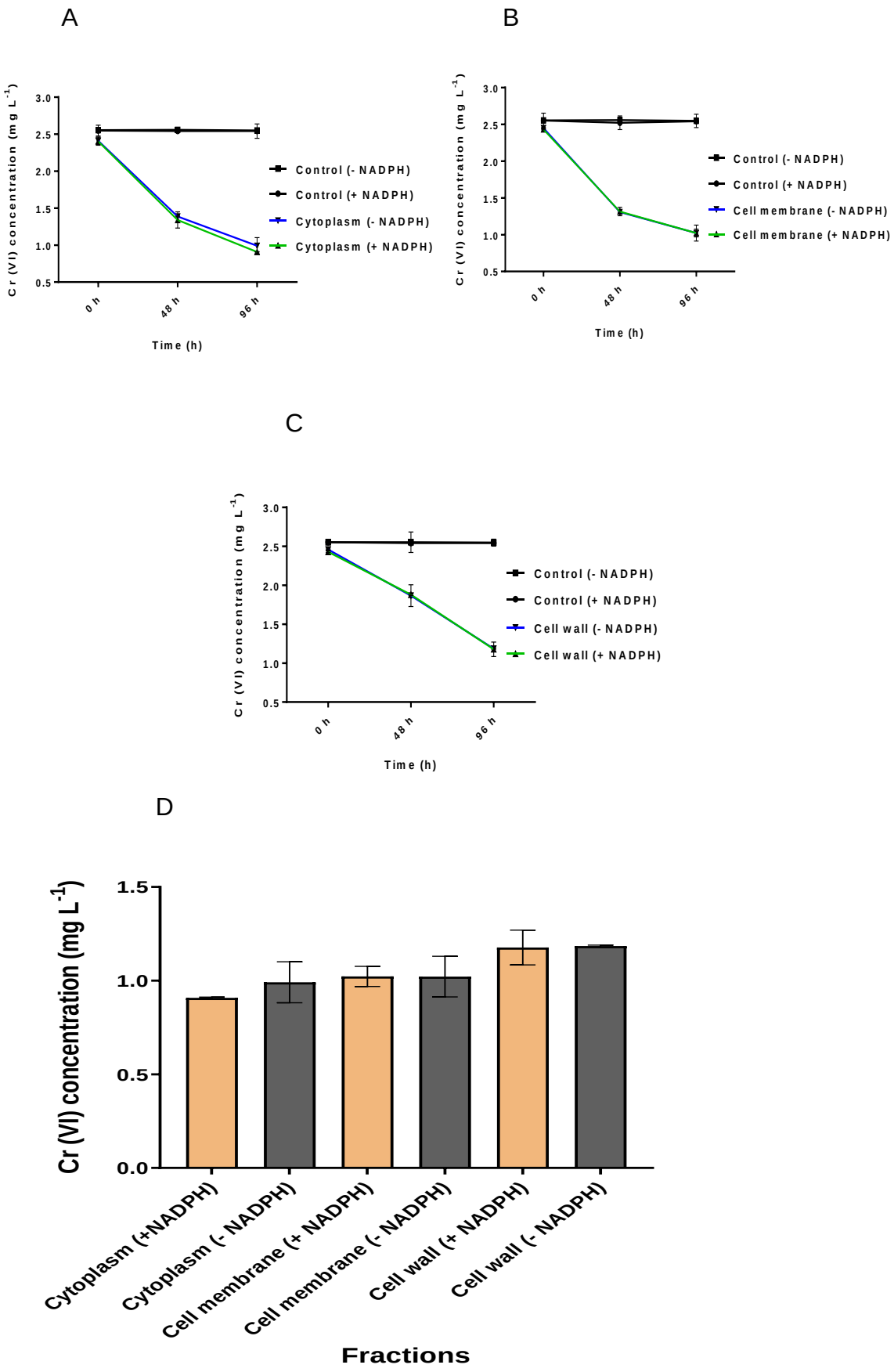


Figure 3-24 Cell fractions plus Cr (VI) with and without NADPH as electron donor. Error bars show the standard deviation of three biological replicates.

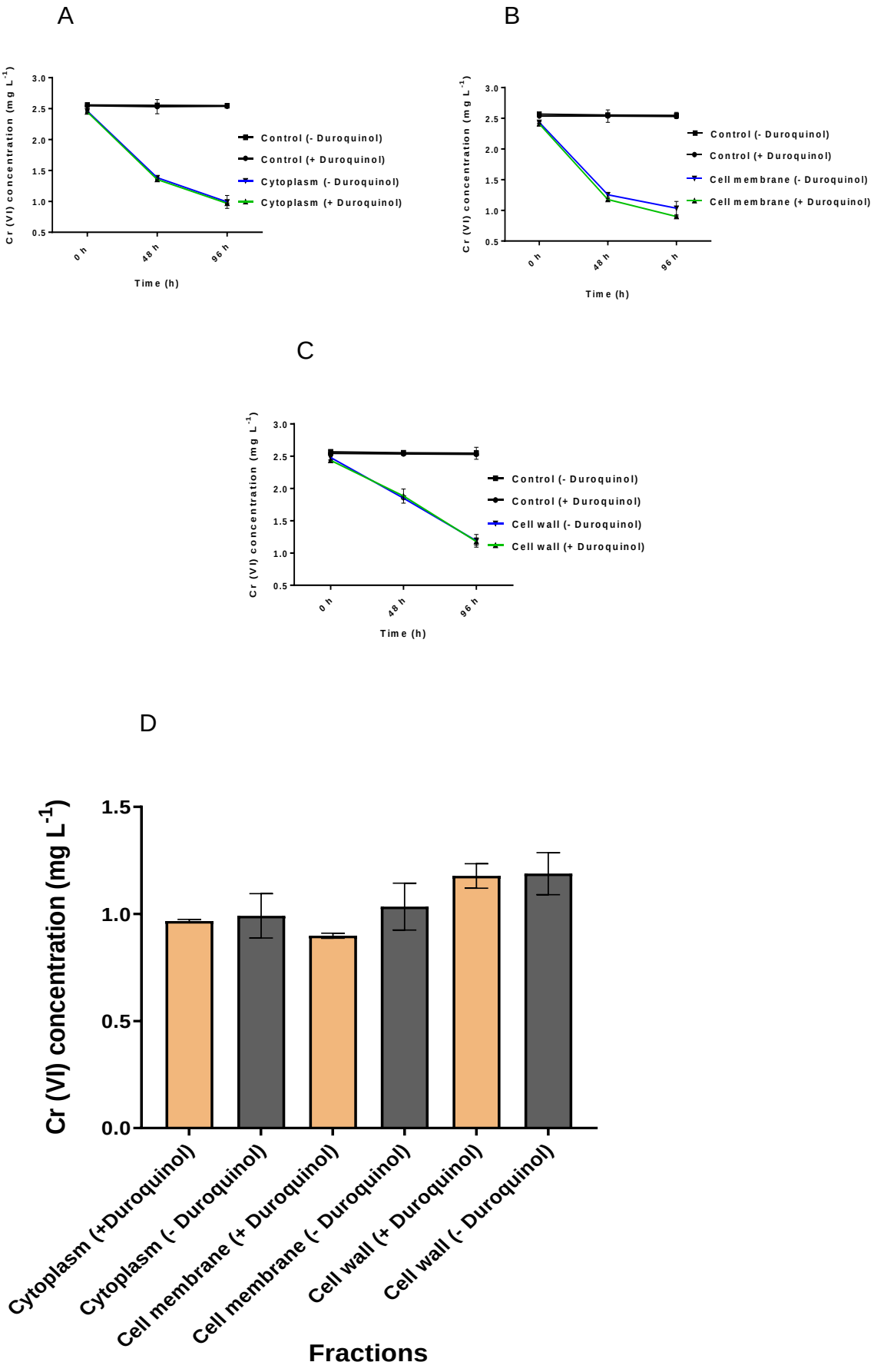


Figure 3-25 Cell fractions plus Cr (VI) with and without Duroquinol as electron donor. Error bars show the standard deviation of three biological replicates.

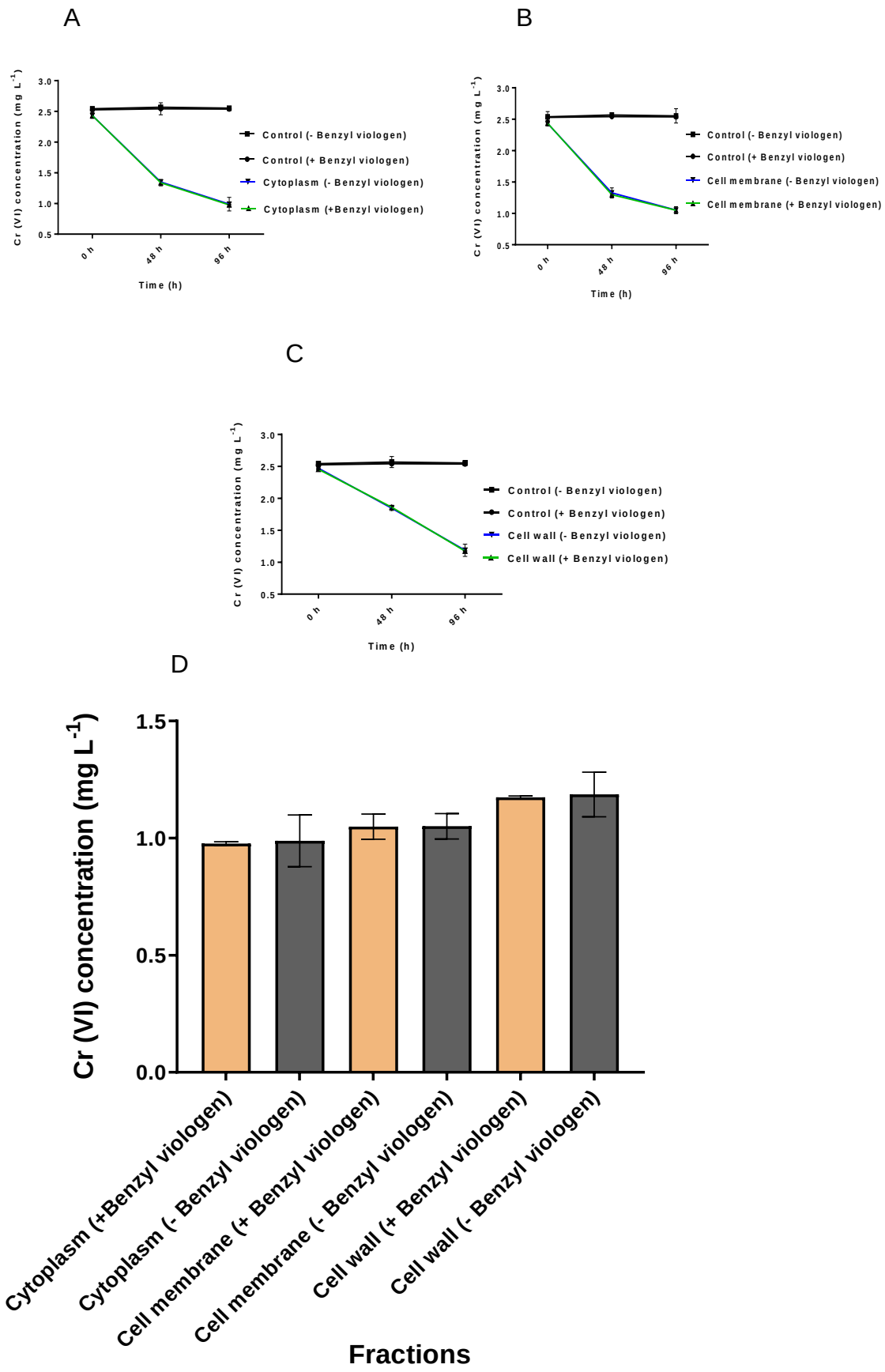


Figure 3-26 Cell fractions plus Cr (VI) with and without Benzyl viologen as electron donor. Error bars show the standard deviation of three biological replicates.

4.3.4 Separation of cytoplasm fraction by DEAE Anion exchange Chromatography

As the initial step towards purifying the chromium (VI)-reducing activity, the cytoplasm fraction was separated by means of DEAE Anion exchange chromatography. An Anion exchange step is commonly used as a first chromatographic step for protein purification protocols, because it removes a lot of unwanted proteins and it also concentrates the protein in to small volume. A number of previous studies (Park *et al*. 2000) that have purified chromate reductases have used an initial Anion exchange step and so it was decided to begin the purification of the chromate-reducing activity from the cytoplasmic fraction of *Mc. capsulatus* Bath using such a separation.

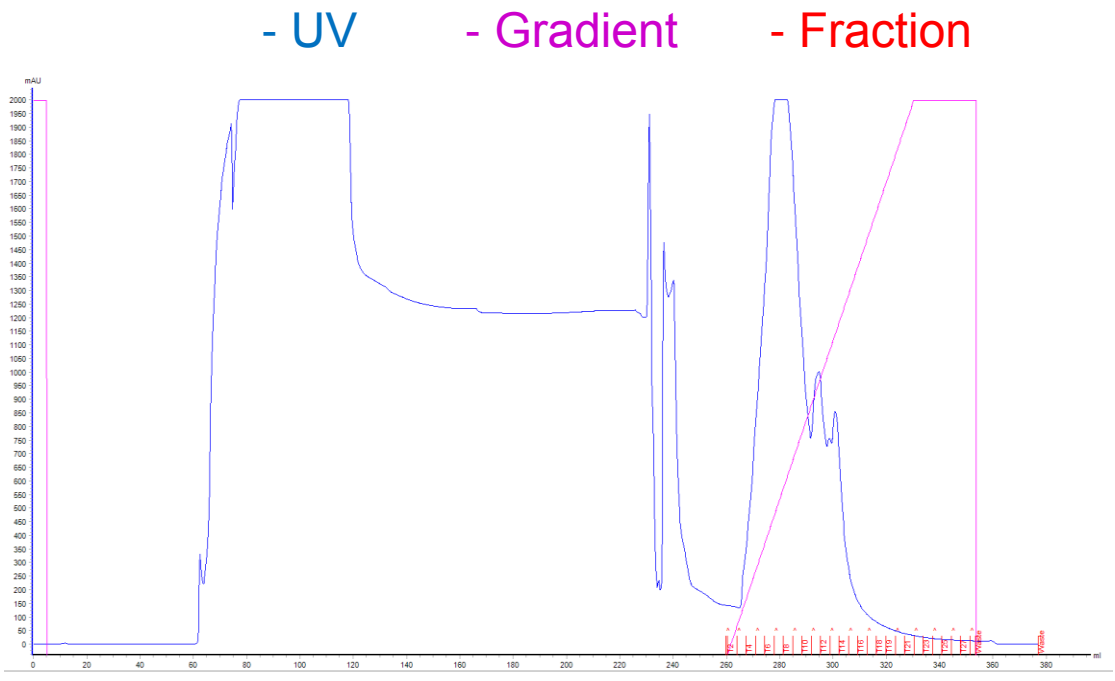


Figure 3-27 Separation of cytoplasm fraction by DEAE Anion exchange Chromatography.

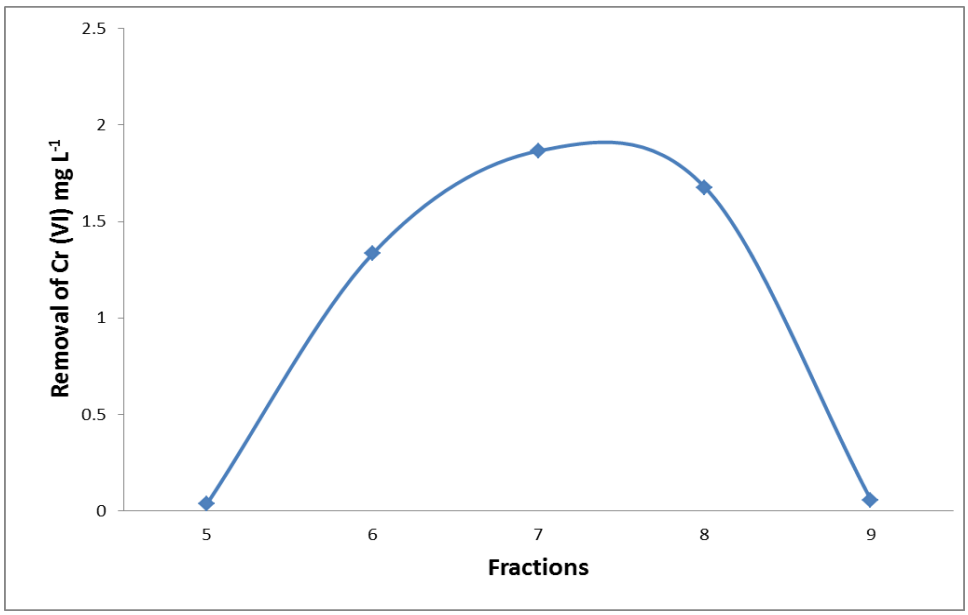


Figure 3-28 Chromate reductase assays of Anion exchange fractions. Assays were performed as described in the Methods section of this chapter. The graph shows the concentration of Cr (VI) removed at the 96 h timepoint in assays with an intial Cr (VI) concentration of 2.6 mg L^{-1} (0.043 mM).

A peak of chromium (VI) reducing activity was found in fractions 6, 7 and 8 from the Anion exchange chromatography. The fractions before and after this peak of chromium (VI) reduction activity, which were fractions 5 and 9, did not have any reduction activity (Figure 4-12). When the active fractions were analysed by means of SDS-PAGE, it was found that they contained a large number of proteins and so another purification step was needed.

Based on the possibility that the chromate reductase may have NADH as a cofactor, Capto Blue Dye Affinity chromatography was chosen as the next step. Capto Blue Dye Affinity columns are used for purification of many proteins, such as albumin, interferon, lipoproteins and blood coagulating factors. The column also binds several enzymes including kinases, dehydrogenases and most enzymes requiring adenyl-containing cofactors (e.g., NAD^+). It has been

suggested that in such Dye Affinity chromatography, the dye molecule has a similar shape and similar size and similar chemical properties to the natural co-factor of the enzyme (e.g. NAD^+ or $NADP^+$) and that it therefore binds to the protein via the same binding site to which the co-factor binds (Garg *et al*. 1996).

Also, Dye Affinity chromatography has been successfully used to purify chromate reductases previously. For example, the cytoplasmic chromate reductase was purified from the crude soluble fraction of *Thermus scotoductus* to homogeneity through DEAE-Toyopearl, phenyl-Toyopearl, Blue Sepharose Dye Affinity chromatography, and Sephacryl S100HR chromatography (Opperman *et al*. 2008).

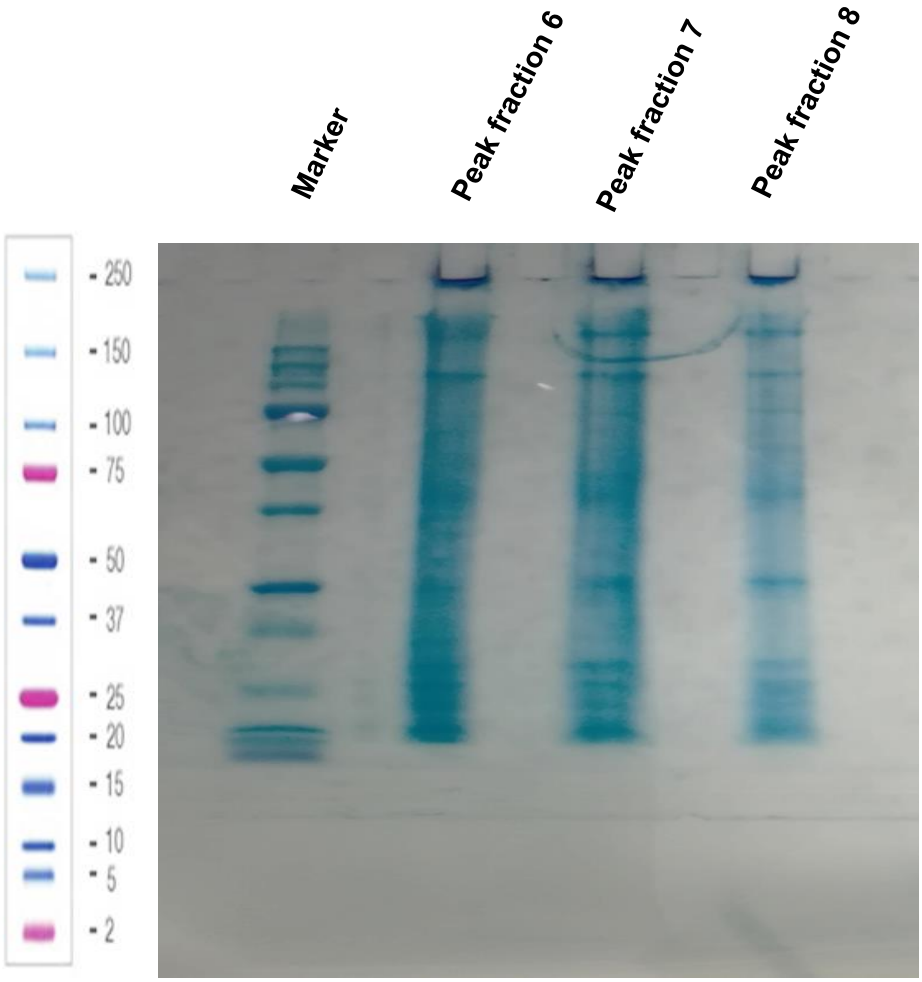


Figure 3-29 SDS-PAGE gel images of the Anion exchange fractions of *Mc. capsulatus* Bath. The amount of marker that loaded in the gel was 5 µl and the amount of each fraction that loaded in the gel was 15 µl. The image is representative of three replicates of experiments.

4.3.5 Purification by Capto Blue Dye Affinity Chromatography

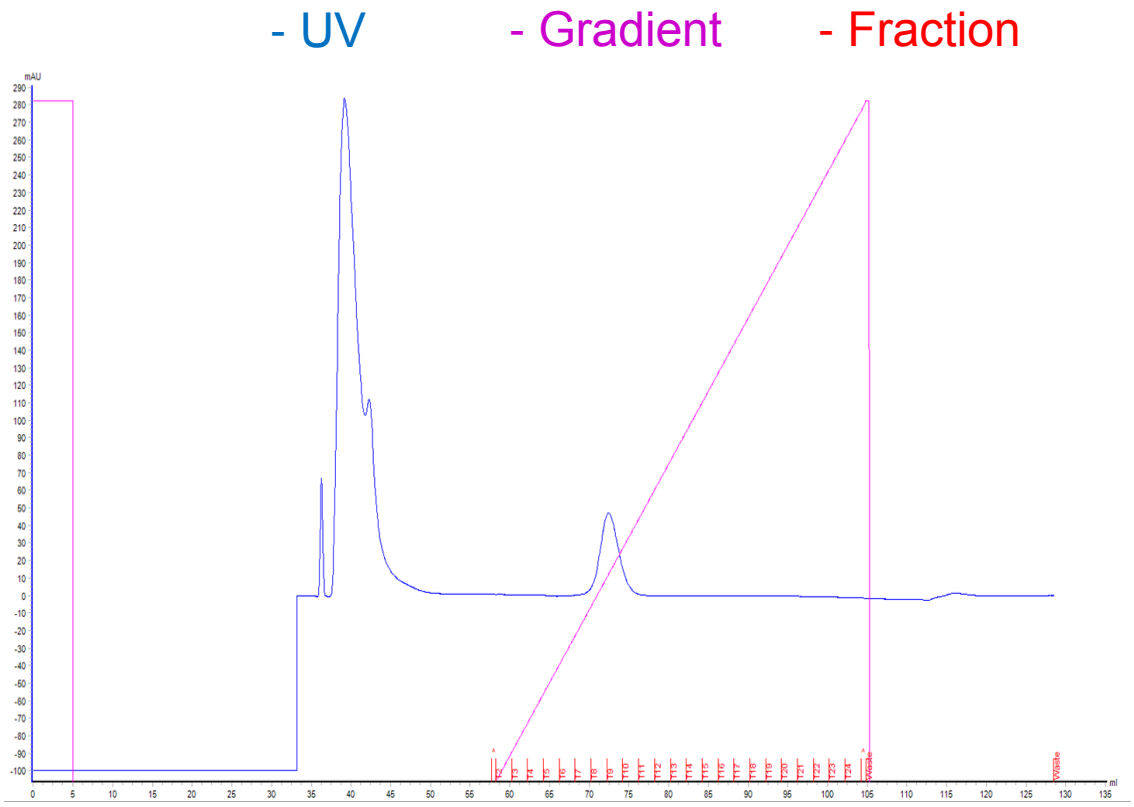


Figure 3-30 Absorbance trace from Capto Blue chromatography step during the purification of the reductase from the cytoplasm fraction. Blue line, absorbance at 280 nm; purple line, salt gradient; red line, fractions.

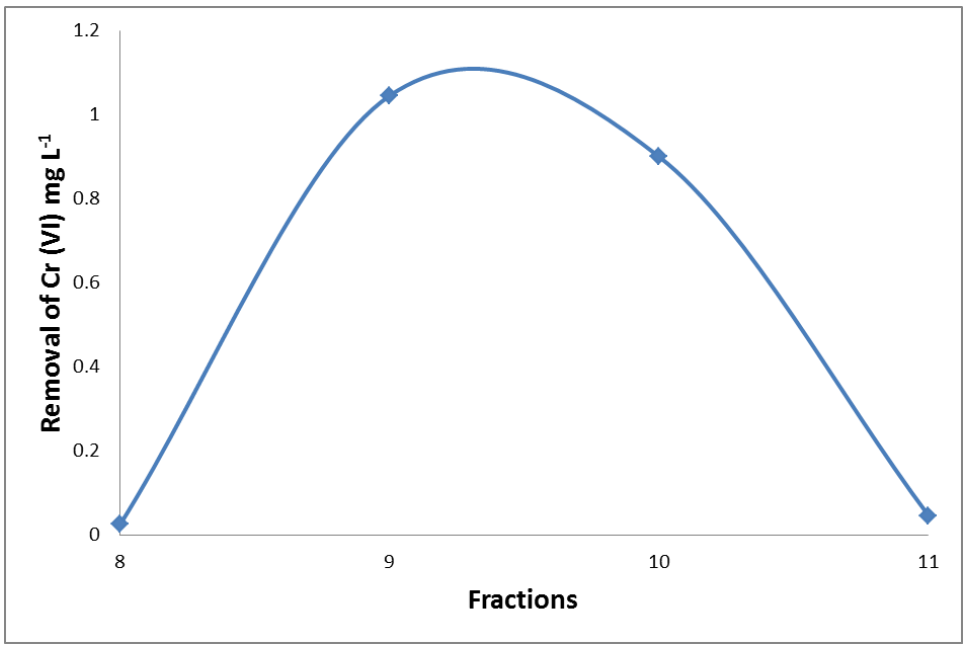


Figure 3-31 Chromate reductase assays of Capto Blue fractions. Assays were performed as described in the Methods section of this chapter. The graph shows the concentration of Cr (VI) removed at the 96 h timepoint in assays with an intial Cr (VI) concentration of 2.6 mg L-1 (0.043 mM).

After dialysis, the pooled active fractions (6, 7 and 8) from the Anion exchange column, containing 20.79 mg of protein were loaded onto the column and eluted with a salt gradient as detailed in the Methods section. A peak of chromium (VI) reducing activity was found in fractions 9 and 10 from the Capto Blue chromatography (Figure 4-14 and 4-15). The fraction before and after a peak of chromium (VI) reduction activity, which were 8 and 11, did not have any reduction activity. SDS-PAGE revealed a single protein of 17 kDa molecular mass in fractions 9 and 10 (Figure 4-16).

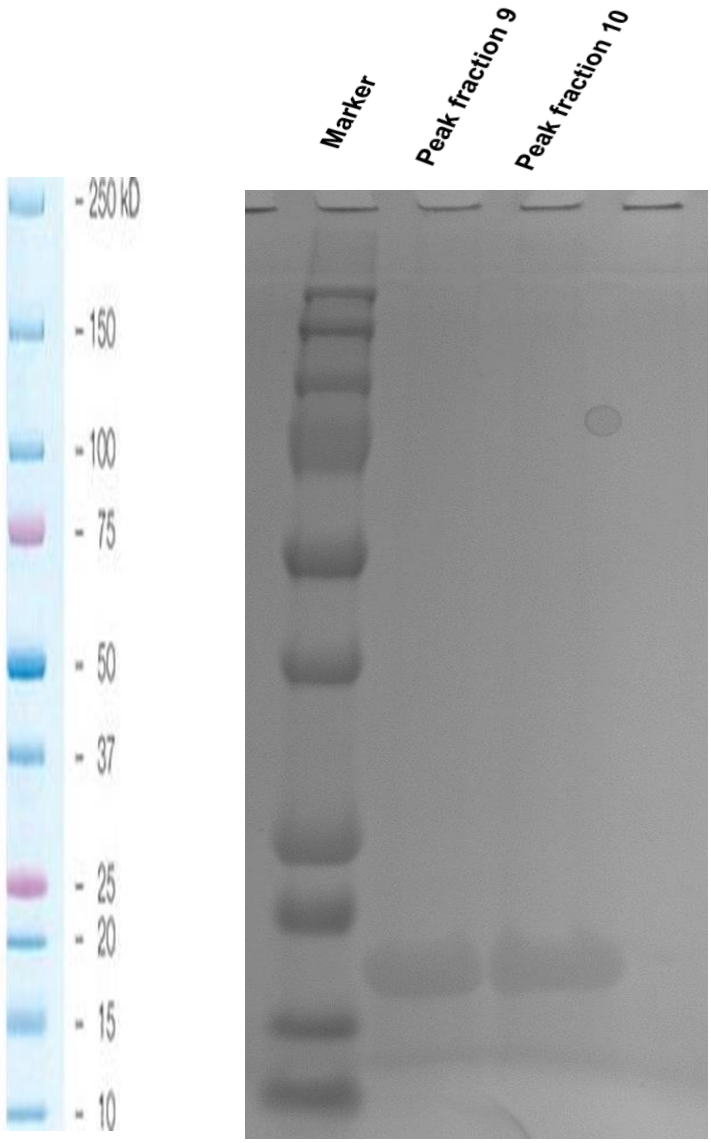


Figure 3-32 SDS-PAGE gel images of the Capto Blue fractions of *Mc. capsulatus* Bath. The amount of marker that loaded in the gel was 5 µl and the amount of each fraction that loaded in the gel was 15 µl. The image is representative of three replicates of experiments.

4.4 Conclusion

The cell fractions of *Mc. capsulatus* Bath were studied for their chromium (VI) removal activity at various temperatures. The activity of the fractions was found to reach a maximum at 30 °C, and this activity was reduced at both higher and lower temperatures. It was found that the 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).The optimum temperature of this reaction is somewhat lower than the optimum temperature (45 °C) for the growth of *Mc. capsulatus* Bath. The temperature activity profile (Figure 4-1) indicates that there is a substantial amount of activity as high as 50 °C and as low as 10 °C (for example, 30.76 and 26.09% of the maximal activity at 50 °C and 10 °C, respectively, for the cytoplasm fraction). This shows that the enzyme or other molecular species involved in chromium (VI) reduction is tolerant of temperature variations.

From the experiment with various electron donors at a concentration of 0.4 mM in 25 mM MOPS buffer (pH 7.0), it was discovered that NADH, NADPH, duroquinol and benzyl viologen reduced Cr (VI) to Cr (III) without any of the cell fractions present. In order to avoid this direct, non-biological reaction between Cr (VI) and the electron donors, the concentration of electron donors was reduced to 0.2 mM. At this concentration, there was no observable removal of Cr (VI) by any of the electron donors over a period of 96 h. This concentration of each electron donor was therefore used for Cr (VI) reduction assays with the cell fractions. All cellular fractions reduced Cr (VI) to Cr (III); reduction activity was greatest in the cytoplasm fraction.

The chromium (VI)-removing activity was partially purified from the cytoplasmic fraction by DEAE Anion exchange Chromatography as a first step and then purified by Capto Blue Dye Affinity Chromatography. Its activity was enhanced (by 1.34%) by adding the electron donor NADH, although most of the activity even in this highly purified protein-containing fraction was independent of added NADH. A previous study conducted by Manikandan *et al*. (2016) also found a substantial chromate-reducing activity in a purified chromate reductase from a *Bacillus* isolate even in the absence of added electron donors. Hence, in this previous study as well as in the work reported in this chapter, either a source of electrons copurifies with the protein or the protein itself is able to provide electrons for the reaction. The fractions from the Capto Blue Dye Affinity chromatography that showed the peak of chromium (VI)-removing activity contained a single 17-kDa cytoplasm-derived protein for the chromium (VI) removal activity of these fractions. The majority of previous studies, such as that of Manikandan *et al*. (2016), have observed that a chromate reductase enzymes are generally proteins of at least 30 kDa in size. The enzyme purified from *Bacillus sp*. DGV 019 by Manikandan *et al*. (2016) had a molecular mass of 34.2 kDa. In contrast to this, and more similar to the current study of *Mc. capsulatus*, a very recent report found that a substantially smaller protein from *S. maltophilia*, of molecular weight around 25 kDa had chromate (VI) reduction acivity (Baldiris *et al*. 2018). Another possibility that cannot be excluded without further work is that the chromium (VI) reducing activity reported in the purified protein samples this chapter may be due to a non-protein small molecule that copurifies with the 17-kDa protein. Future identification of the 17-kDa protein and subsequent bioinformatic analysis may indicate whether it is likely to be responsible for chromium (VI) reduction.

Chapter 5 Enrichment and isolation of methanotrophs from sediment samples

5.1 Introduction

The uptake of heavy metals in the environment by microorganisms is mainly by the following mechanisms, biosorption, bioaccumulation, and efflux and chemical transformation such as reduction or precipitation. Chromium reduction has been reported in several species of bacteria, fungi, yeasts and some actinomycetes to date (Mala *et al*. 2015). The different population of microbes present in different habitats have different capabilities to reduce the hexavalent chromium and transform other heavy metal pollutants.

Methanotrophic bacteria are considered as suitable organisms for bioremediation of organic pollutants because of their co-metabolic transformation of such compounds, possibility of complete compound degradation without the formation of toxic metabolites, board spectrum of compounds availability and widely available and inexpensive growth of substrate (Sullivan *et al*. 1998).

Environmental pollution can be attributed to several anthropogenic activities. When various toxic metals are introduced into the environment they can remain persistent, thereby affecting human health by entry through food chain and produce toxic effects. The metals which remain in the environment are held there by sorption, precipitation and complexation reactions. These metals can be removed from the soil by uptake with plants, leaching and volatilization process. The fate of the metals in the soil environment depends on the soil properties and environmental factors (Das & Dash 2014).

Methanotrophs or methane oxidising bacteria are capable of oxidising methane which is a very potent greenhouse gas. These organisms can remediate a

range of pollutants as well as oxidising methane and using it as sole source of energy, thus adding a beneficial effect to the environment.

Methanotrophs have been used in the present study because of their potential and promising results with the metal remediation (as detailed in the literature review chapter) and also these organisms which are widespread in the environment has not been extensively explored in terms of metal remediation compared to the other microbes.

Methanotrophs are common in the environment and well suited to biotechnological processes. The isolation and characterization of pure cultures has led to the discovery of several new genera and species of extremophilic/tolerant methanotrophs (Trotsenko & Khmelenina 2002). Only two methanotrophs have been shown able to reduce Cr (VI) which are *Mc. capsulatus* Bath (Al Hasin *et al*. 2009) and *Methylomonas koyamae* (SHU 1) (Challa 2015). One substantial limitation on the application of methanotrophs for remediation of chromium (VI) is that the strains that are currently available are sensitive to Cr (VI). As detailed in Chapter 3, there is little or no growth or chromium (VI) reduction by *Mc. capsulatus* Bath at more than 40 mg L^{-1} (0.666 mM) of chromium (VI). Hence, it would be an advantage to isolate chromium (VI) reducing methanotrophs that are more resistant to chromium (VI).

Previous work has shown the diversity of methanotrophs (reviewed in the Introduction chapter) and the ability to isolate heavy metal resistant strains (De Marco *et al*. 2004). Other work has identified extremophilic methanotrophs, including thermophiles, acidophiles and alkaliphiles (Trotsenko & Khmelenina 2002; Dunfield *et al*. 2007; Pol *et al*. 2007). Here, enrichments have been set up from environmental samples to isolate new methanotrophs, including

enrichments in the presence of chromium (VI) with the intention of finding chromium (VI)-resistant organisms.

Environmental samples were taken from canal sediment from the Leeds and Liverpool canal and incubated in enrichment culture with methane and in the presence of a range of chromium VI concentrations.

5.2 Methods

Enrichment of microbes from the study sites were carried out to ensure isolation of various pure strains of methanotrophs to be cultivated to employ in the experiment. Sediment samples collected from the Leeds and Liverpool canal and a railway location near Doncaster that has a history of chromium (VI) exposure from chromium (VI) usage as a wood preservative for railway sleepers were used for the enrichment and isolation of methanotrophs. 0.5 g of sediment samples collected from the Leeds and Liverpool canal and a railway location near Doncaster were enriched with 50 ml of NMS media (nitrate minimal salts) in 250 ml Erlenmeyer flasks, with or without addition of chromium (VI) over a range of concentrations (0 - 100 mg L^{-1})(0 - 1.66 mM). Methane gas was introduced at 1:4 v/v with air into the culture flasks at regular intervals using hypodermic syringes and the flasks were sealed with subseals (Fisher) to prevent methane loss. The flasks were incubated at (30 and 45 °C) on a rotary incubator for 1 week to 10 days for the growth of methanotrophs in the flask. The growth of methanotrophs was observed by monitoring the turbidity of the enrichments in flasks.

The cultures in the flasks were sub cultured into fresh NMS medium and incubated at (30 and 45 °C) on a rotary incubator for 1 week to 10 days of

growth. After 1 week to 10 days of growth in fresh flasks, a loopful of culture was streaked on fresh NMS plates and incubated at (30 and 45 °C) in a methane air atmosphere until single colonies of the isolate were obtained. The plates were then put into a gas tight jar and methane gas was introduced into the air in the jar as the sole source of carbon and energy and incubated at (30 and 45 °C) for 1-3 weeks. Depending on the physical appearance and morphological features of colonies, colonies of the same appearance were streaked on fresh NMS plates and incubated at (30 and 45 °C) for 1-3 weeks for appropriate growth of methanotrophs on the plates.

A pure colony of the same type that was obtained from the plates described above was grown on a fresh NMS plate to produce cells for the DNA preparation, and then the 16S rRNA gene was amplified by PCR (the methods for DNA purification and PCR are described in detail in chapter 2).

The PCR product of the 16S rRNA gene from the cultivated methanotrophs was subjected to gel electrophoresis (method is described in chapter 2) to identify the presence of a product of the expected size. Later the gel electrophoresis product was cleaned with the Qiagen kit and then subjected to sequencing.

5.3 Results

5.3.1 Enrichment cultures

A range of chromium (VI) concentrations (2, 3, 4, 5, 10, 20, 30, 50 and 100 mg L^{-1}) (0.033, 0.050, 0.066, 0.083, 0.166, 0.333, 0.50, 0.833 and 1.66 mM) enrichments were used for environmental samples from both sampling sites in

the presence of methane gas at two temperatures (30 and 45 °C) to attempt to isolate new strains of methanotrophs. Organisms that were derived from these enrichments did not grow well in NMS with methane even in the absence of chromium (VI) and were found on subsequent analysis not to be methanotrophs.

Enrichments that were set up in the absence of chromium (VI) were successful in culturing methanotrophs. One strain isolated from the Leeds and Liverpool Canal samples and two from the railway site are described below.

5.3.1.1 Methanotroph isolate from the Leeds and Liverpool Canal

5.3.1.1.1 Microscopic examination of isolated colonies

A strain isolated at 45 °C from the Leeds and Liverpool Canal samples was characterised in detail. The isolated organism was streaked several times on fresh NMS plates and, prior to sequencing of the 16S rRNA genes, and was also examined under the microscope to observe features such as shape (Figure 5-1).

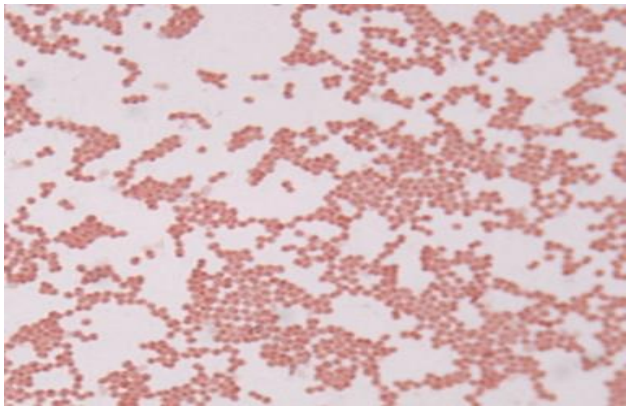


Figure 0-1 Microscopic examination colonies shows the bacteria have a coccal shape. *Mc. capsulatus* under the light microscope at 100x magnification.

The results from the 16S rRNA gene-specific PCR and subsequent sequencing showed the type of organism that was been isolated from the sediments of the canal sediment from the Leeds and Liverpool canal and was identified as *Methylococcus capsulatus.*

5.3.1.1.2 Bioremediation of chromium (VI) using the new isolate of *Methylococcus capsulatus*

The new strain (*Methylococcus capsulatus*) was tested at a wide range of Cr (VI) concentrations from (2 to 5) mg L^{-1} (0.033 to 0.083) mM where the bacteria was capable of reducing Cr (VI) to Cr (III), (OD_{600} of 0.7- 0.9) and incubating the cultures at 45 °C in the presence of methane and air. The chromium was quantified in the supernatant by using HPLC-ICP-MS.

Figure 5-2 shows the removal of Cr (VI) at 2 mg L^{-1} (0.033 mM) from the supernatant. After 48 h, 20% of Cr (VI) had been removed, and this increased to reach 85% Cr (VI) removal at the end of incubation period (144 h). At the end

of the incubation period Cr (VI) concentration had decreased to 0.32 mg L^{-1} (0.0053 mM). Figure 5-3 shows the removal of Cr (VI) at 3 mg L^{-1} (0.050 mM). After 48 h, 15% of Cr (VI) had been removed, and this reached 70% Cr (VI) removal after 144 h.

With 4 mg L^{-1} starting concentration, 5% loss of chromium (VI) occurred within 48 h. After 144 h the percentage remaining was 20% as shows in Figure 5-4. When the concentration of hexavalent chromium was increased to 5 mg L^{-1} (0.083 mM), a smaller proportion of the chromium was removed; only 10% of the chromium (VI) was removed during 144 h (Figure 5-5). The amount of Cr (VI) that was removed decreases as the initial concentration increases.

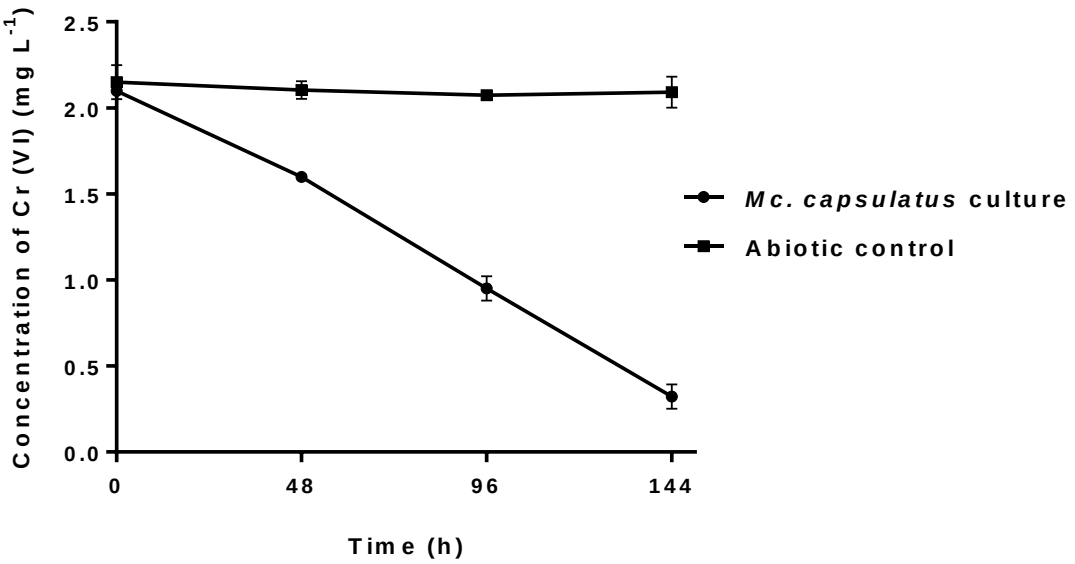


Figure 0-2 Reduction of chromium (VI) by *Methylococcus capsulatus* after addition of Cr (VI) to 2 mg L^{-1} (0.033 mM). Data shown is from three independent cultures. Results plotted as mean ± 1 standard (n=3).

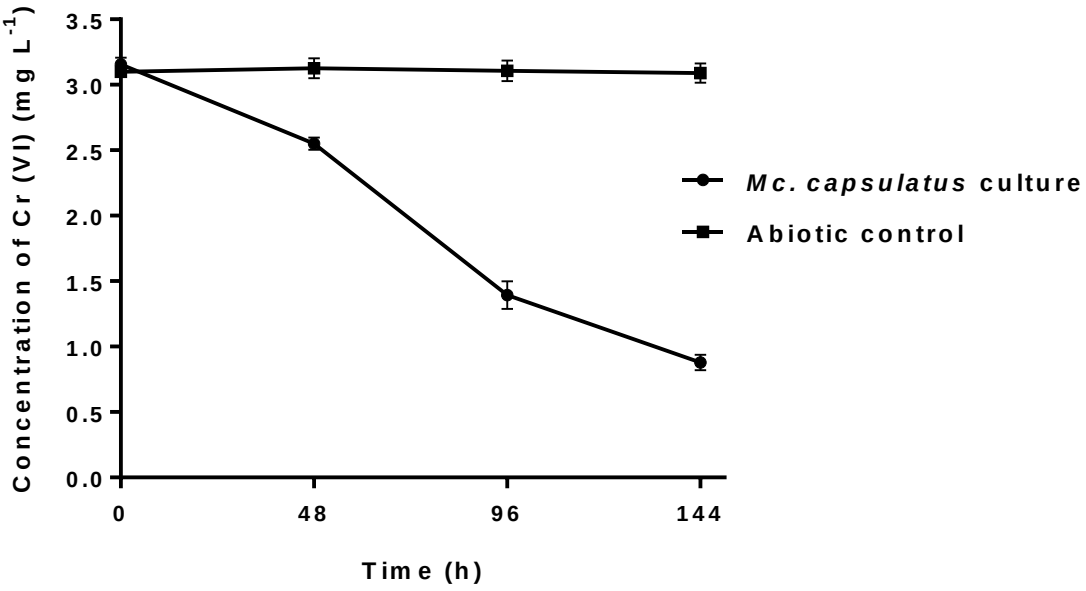


Figure 0-3 Reduction of chromium (VI) by *Methylococcus capsulatus* after addition of Cr (VI) to 3 mg L^{-1} (0.050 mM). Error bars show the standard deviation of three biological replicates.

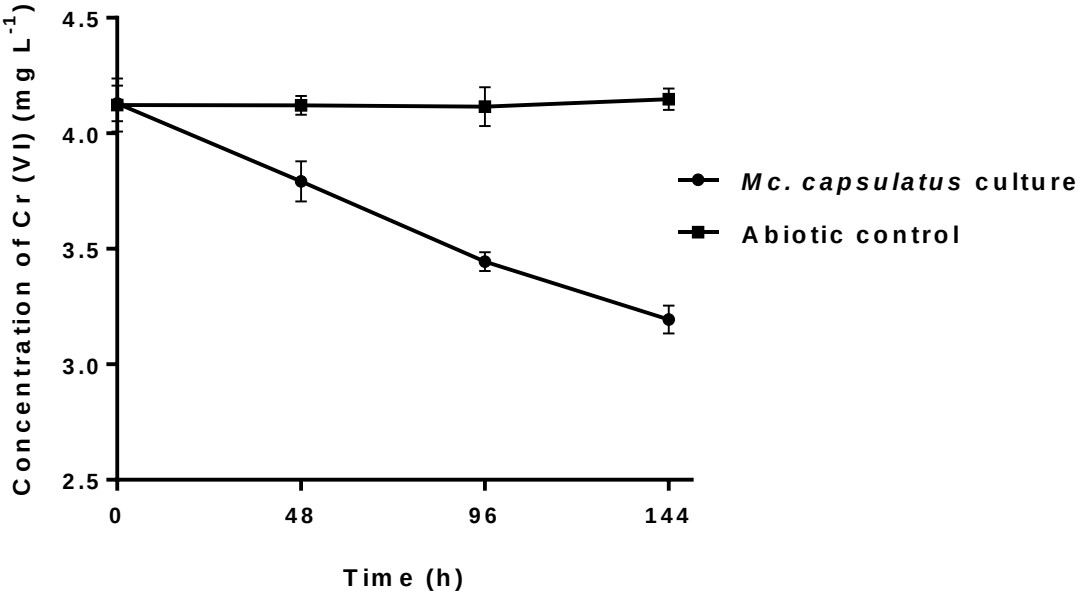


Figure 0-4 Reduction of chromium (VI) by *Methylococcus capsulatus* after addition of Cr (VI) to 4 mg L^{-1} (0.0666 mM). Error bars show the standard deviation of three biological replicates.

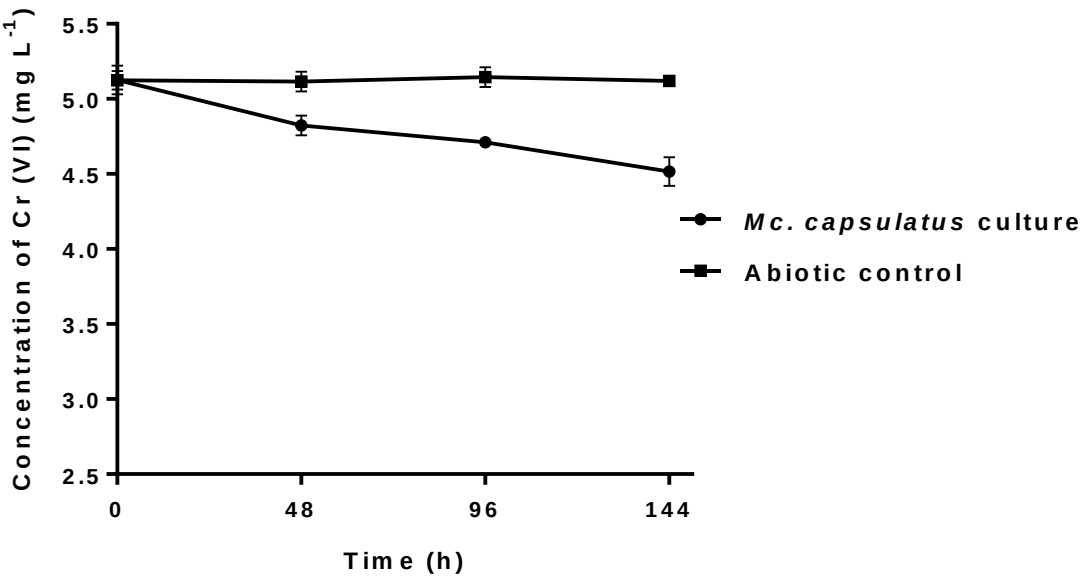


Figure 0-5 Reduction of chromium (VI) by *Methylococcus capsulatus* after addition of Cr (VI) to 5 mg L^{-1} (0.083 mM). Data shown is from three independent cultures. Results plotted as mean ± 1 standard (n=3).

5.3.1.2. Isolating new strain of methanotrophs at 30 °C

Two new methanotroph strains were isolated from enrichment cultures of the railway site samples grown on methane at temperature (30 °C) with the same method used above. The purity of strains was checked by plating cultures onto nutrient agar plates, which were incubated at 37 °C for 24 h and the strains did not grow on nutrient agar, which is consistent with their being pure methanotroph strains.

5.4 Conclusion

In the present study when the samples of the Leeds and Liverpool canal and a railway location near Doncaster were enriched with methane as sole source of carbon and energy, three new methanotroph strains were isolated. The sediment samples collected from the Leeds and Liverpool canal were enriched at 45 °C with methane as the carbon and energy source. The isolated methanotrophs was identified, based on the sequencing of its 16S rRNA gene, as *Mc. capsulatus*. Previously characterised methanotrophs in this group use the product from formaldehyde to form biomass through the ribulose monophosphate (RuMP) cycle as the main pathway and so it is reasonable to presume that the newly isolated *Mc. capsulatus* strain fixes carbon via the same principal pathway. This organism was tested for the removal of chromium (VI) and it was found the organism could reduce the concentration of chromium in the range of 2 - 5 mg L^{-1} (0.033 - 0.083 mM) in the presence of methane as sole source of carbon and energy. The removal of Cr (VI) from the culture supernatant at initial concentrations of 2 and 3 mg L^{-1} (0.033 and 0.050 mM) reached 85 and 70%, respectively, at 144 h. When the concentration of hexavalent chromium was increased, the removal of Cr (VI) at concentration of 4 and 5 mg L^{-1} (0.066 and 0.083 mM) reached 20 and 10%, respectively, at 144 h. These results showed that as the initial concentration of Cr (IV) increased, the amount of Cr (VI) that was removed decreased. This promising strain for bioremediation applications should be characterised further. Also, the two new methanotroph isolates should be identified and their bioremediation potential characterised.

Chapter 6 General discussion and future directions

6.1 General discussion

This study confirmed that the methanotrophs, which are a major microbial group that plays a vital role in maintaining the global methane cycle, can carry out the biotransformation of the chromate (VI) ions, thereby decreasing chromium pollution. One lab-strain of the methane-oxidising microorganisms, which used methane as a sole carbon and energy source, i.e., *Mc. capsulatus* Bath could reduce the chromate (VI) ions to chromate (III), as shown earlier (Al Hasin *et al.* 2009). Furthermore, it was also able to reduce the mercuric ions to metallic mercury, which confirmed some earlier reports (Boden & Murrell 2011). *Mc. capsulatus* Bath showed an ability to remove various concentrations of the chromate (VI) ion (ranging between 1.4 and 1000 mg L^{-1})(0.0233 and 16.667 mM), which was consistent with a previous study (Al Hasin *et al*. 2009).

Though several studies have investigated the microbial transformation of chromium in the environment, none of the reports investigated the chromium (III) species distribution in the biomass. A number of recent studies of the transformation of chromium (VI) by mixed populations of microorganisms are encouraging in terms of the use of methane to drive chromium (VI) bioremediation but do not give unambiguous information about the role of methanotrophs in the reduction of chromate itself. The results presented in this thesis, using pure cultures of *Mc. capsulatus* Bath have allowed the transformation and accumulation of chromium species by the methanotroph itself to be investigated (Lai *et al*. 2016; Long *et al.* 2017; Lu *et al.* 2018; Lv *et al.* 2018).

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In Chapter 3, it was noted that there was a decrease in the chromium (VI) ion concentration in the culture supernatant, with a subsequent increase in the chromium (III) concentration in the cytoplasm and membrane fractions. This phenomenon was investigated with biochemical and biophysical techniques, namely cell fractionation, Anion chromatography ICP-MS, XPS, EDX and the EELS imaging of the whole or sectioned cells. Electron Energy Loss Spectroscopy (EELS)-coupled with the Transmission Electron Microscopy (TEM) of the whole cells was used to derive additional information regarding the speciation of the chromium ions after comparing them with the chromium standard spectra. The cell associated chromium ions were present in a +3 oxidation state.

For determining the precise location of the chromium ions in the cells, the cells were fractionated in order to separate the cell membranes and cytoplasmic fractions. The results showed the distribution of the chromium ions between these fractions and showed that the membrane fraction contained 66.6% of the total chromium ions while the remaining 33.3% was present in the cytoplasmic fraction. These results were confirmed by sectioning the cells and investigating the chromium distribution using the ICP-MS and HAADF-TEM-EDX techniques. The distribution of other important elements such as oxygen, phosphorus and carbon, in the whole *Mc. capsulatus* cells was investigated using the EDX and TEM-coupled EELS imaging techniques. The results showed that these elements were non-homogenously distributed. The *Mc. capsulatus* Bath cells were also able to take up Cr (III) ions from the cell culture medium. Chromium uptake was an active process which reduced the chromium bioavailability. This would prevent the re-oxidisation of Cr (III) ions to their toxic Cr (VI) form, which

is a significant problem where chromium (III) becomes associated with soluble organic molecules such as amino acids (Varadharajan 2017), rather than (as in the methanotroph) being trapped within the biomass. Under a range of conditions tested, the removal of chromium (VI) from the medium was exactly matched by the appearance of cell-associated chromium (III) (Figure 3-7). This suggests that *Mc. capsulatus* Bath could be used for complete removal of chromium (VI) and its immobilisation within the biomass, over the conditions tested. The ability of *Mc. capsulatus* to take up chromium (III) contrasts with reports of other bacteria (Nishioka 1975; Petrilli & De Flora 1977), where it was found that the Cr (VI) ions could easily diffuse through the microbial cell membranes, but the Cr (III) ions could not (Figure 1-2).

As observed previously by Al Hasin *et al*. (2009) the Cr (VI) ion reduction was not detected in the absence of methane, which indicated that the *Mc. capsulatus* Bath requires methane for effectively reduce the Cr (VI) ions (Figure 3-3).

Metal ions play a vital role in many cellular processes. Some metal ions such as Co, Ca, Cu, Cr, Mg, Zn, K and Na are essential nutrients which are needed in regular metabolic activities. Generally, the presence of metal ions at toxic high concentrations at polluted sites is harmful to microorganisms, although it also selects for organisms resistant to and able to detoxify such pollutants, thereby enabling bioremediation (Srivastava & Thakur 2006). When the microbes come in contact with the heavy metal ions, they interact with these ions in the following manners: 1) They utilise these trace metal ions for metabolic processes; 2) They can tolerate the metal ions till their threshold limit; 3)

Detoxify the metal ions; 4) They offer resistance to the toxic metal ion levels. Furthermore, the uptake of the heavy ions by the microbial cells is based on the mechanisms such as bioaccumulation, biosorption, efflux and the chemical transformation processes like precipitation or reduction. The chromium reduction has been noted in many bacterial, fungal, yeast and actinomycetes species (Mala *et al*. 2015). The different microbial populations in various habitats show a differing capacity for reducing the Cr (VI) ions and transforming other heavy metal ions. The reduction of the Cr (VI) to Cr (III) is an important research area. The Cr-reducing microbes were first discovered in the 1970s (Romanenko & Korenkov 1977), and a majority of the Cr (VI)-resistant microbial species belonged to the *Bacillus, Ochrobactrum, Pseudomonas, Lysinibacillus, Arthobacter, Shewanella* and *Cupriavidus* genera (Pei *et al*. 2009). These species become resistant to the Cr (VI) ions, detoxify them, and survive in their presence. The Cr (VI) reduction is a co-metabolic process or would display respiratory or dissimilatory properties under anaerobic conditions (Pei *et al*. 2009). The Cr (VI) ion reduction is either plasmid-borne as seen in the *Pseudomonas* sp. or located in the chromosomal DNA as seen in the *Enterobacteriaceae* or *Bacillus* sp. (Pei *et al*. 2009).

In the current study of *Mc. capsulatus* Bath a sufficiently high concentration of chromium (VI) was toxic to the microorganisms, shown by the lack of chromium (VI) removal when the initial concentration of chromium (VI) was greater than 40 mg L^{-1} (0.666 mM). Likewise, with chromium (VI) concentrations greater than 40 mg L^{-1} (0.666 mM), the protein concentration within the culture actually decreased with time (Figure 3-2).

Characterisation of the chromium (VI) removal reaction by ion chromatography and ICP-MS (Chapter 3) confirmed previous X-ray spectroscopy analysis (Al Hasin *et al*. 2009), which showed that the product of chromium (VI) transformation was chromium (III). In this thesis it is also clearly shown that the cells are able to accumulate chromium (III) derived from extracellular chromium (VI) and also to take up chromium (III) from the medium.

Amongst the chromium-reducing bacteria (CRB), the Gram-negative microbes generally show a lower resistance to high Cr (VI) concentrations, in comparison to the Gram-positive bacteria (Coleman 1988). The microbial species use various resistance mechanisms for overcoming the chromium toxicity in environments, such as the extracellular Cr (VI) reduction, decreased uptake of the Cr (VI) ions, detoxification of Reactive Oxygen Species (ROS), a reduction of the detoxifying enzymes/ intracellular Cr (VI) concentration, presence of DNA repair enzymes, and the efflux of the Cr (VI) ions from cells (Thatoi *et al*. 2014). It may be that the ability of *Mc. capsulatus* Bath which is a Gram-negative bacterium, to reduce chromium (VI), is a mechanism to protect the cells against this toxic species. Consistent with this, hexavalent chromium ions in the range between 3 to 40 mg L^{-1} (0.050 to 0.666 mM) are effectively detoxified and no chromium (VI) has been observed in any part of the cell (cell wall, cell membrane and cytoplasm fraction) (Figure 3-8).

The Cr (VI) transportation across the cell membrane occurs via the sulfate uptake pathway since the chromate and sulfate ions are oxyanions with similar structures (Ramírez-Díaz *et al*. 2008). The data presented in this thesis do not

directly address whether the sulfate transporter of *Mc. capsulatus* is involved in uptake of chromium (VI).

The transportation of the Cr (III) ions into the cells is generally considered to be very slow due to the insolubility of Cr (III). Nonetheless, Cr (III) complexes may be transported using siderophores, which are also known as iron-uptake mediators. Siderophores are seen to be a structurally-diverse group consisting of biogenic chelating agents that are related to the uptake of the iron and similar other metal ions. Siderophores bind to several types of metal ions, solubilise the metal complexes and improve the mobility of the toxic heavy metal ions (Duckworth *et al*. 2014). In the present study, *Mc. capsulatus* Bath cells took up chromium (III) from the medium completely into the cytoplasm + membranes fraction within 1 h and the results indicate that uptake of chromium (III) is an active process. The involvement of transmembrane transporters or molecules such as siderophores in chromium (III) uptake by *Mc. capsulatus* has not been investigated. More work is needed particularly with *Mc. capsulatus* Bath to determine the reactions and genes involved in chromium (VI) reduction and chromium (III) accumulation.

Heat-killing is known to be an effective enzyme inactivation process. Optimal temperatures are a significant criterion, which plays a vital role in microbial growth and also affect the Cr (VI) reduction. The temperature variations generally affect cell viability and may lead to cell death. At lower temperatures, in whole-cell systems the membrane fluidity decreases and enzyme activity decreases, and this affects the functioning of the transport system. Thus, metabolic reactions become slower, substrates cannot enter the cell, and so the rate of growth and other cellular reaction decreases. Higher temperatures cause

the irreversible thermal denaturation of the proteins and damage to membranes and small molecules (Narayani & Shetty 2013).

The cell fractions of *Mc. capsulatus* Bath were studied for their chromium (VI) removal activity at various temperatures. The activity of the fractions was found to reach a maximum at 30 °C. This is 15 °C lower than the optimal growth temperature of *Mc. capsulatus* and the temperature at which the whole-cell chromium (VI) reduction experiments were performed. The chromium (VI) removing activity of the cytoplasm faction had 42.03% of its maximum activity at 45 °C, which may account for the reduction observed in the whole-cell experiments. It is also interesting that the optimum temperature for reduction of selenite to elemental selenium by fractions of *Mc. capsulatus* Bath is also 30 °C (Abdurrahman Eswayah, personal communication).

When measuring the effect of electron donors on the chromium reduction reaction, concentrations of electron donor of 0.4 mM led to measurable direct reaction between the electron donor and the chromium (VI). 0.2 mM concentration of each electron donor (NADH, NADPH, duroquinol and benzyl viologen), at which concentration the direct reaction with chromium (VI) was not observed, was therefore used for Cr (VI) reduction assays with the cell fractions. All cellular fractions reduced Cr (VI) to Cr (III); reduction activity was greatest in the cytoplasm fraction. The chromium (VI)-removing activity was purified from the cytoplasmic fraction by adding the electron donor NADH. The fractions from the Capto Blue Dye Affinity chromatography that showed the peak of chromium (VI)-removing activity contained a single 17-kDa cytoplasm-derived protein for the chromium (VI) removal activity of these fractions. Future

identification of this protein and subsequent bioinformatic analysis may indicate whether it is likely to be responsible.

One of the three newly isolated methanotrophs was identified, based on the sequencing of its 16S rRNA gene, as *Mc. capsulatus* which is belongs to type X methanotrophs. This organism was tested for the removal of chromium (VI) and it was found the organism could reduce the concentration of chromium in the range of (2 - 5) mg L^{-1} (0.033 - 0.083) mM in the presence of methane as sole source of carbon and energy. The removal of Cr (VI) from the culture supernatant at initial concentrations of (2 and 3) mg L^{-1} (0.033 and 0.050) mM reached 85 and 70%, respectively, at 144 h. When the concentration of hexavalent chromium was increased, the removal of Cr (VI) at concentration of 4 and 5 mg L^{-1} (0.066 and 0.083) mM reached 20 and 10%, respectively, at 144 h. These results showed that as the initial concentration of Cr (VI) increased, the amount of Cr (VI) that was removed decreased.

6.2. Future directions

The results obtained from this study provide the basis for understanding the growth characteristics of this group of bacteria in the presence of chromium (VI) and their ability to detoxify and immobilise this harmful pollutant. This study has extended understanding of the speciation and distribution of chromium species as well as that the uptake of the Cr (III) by *Mc. capsulatus* Bath is an active processes. Also the chromium (VI)-removing activity has been purified from the cytoplasmic fraction.

There are still many areas identified in this study that need to be explored. An area of potential interest is the identification of enzyme(s) responsible for Cr (VI) reduction from cell wall and cell membrane fractions, since the results reported in this study have concentrated on the cytoplasmic fraction. The conversion of Cr (VI) to Cr (III) needs to be investigated in greater detail. For example, it may be that there are other chromium species such as Cr (IV) and Cr (V) as intermediates during chromium (VI) reduction.

Use of the new strain that has been isolated might be investigated for remediation of other potential heavy metal pollutants or detoxification of lead, mercury and arsenic. Organisms which can reduce hexavalent chromium also have potentiality to reduce several organic compounds. The organisms could be tested in the bioremediation of the organic pollutants.

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 methanotrophs species should be investigated.

The results presented in this study suggest that this work should be repeated using a field scale investigation, rather than small scale (under laboratory conditions), to determine whether the observed bioremediation of Cr (VI) and immobilisation of the resulting Cr (III) within the bacterial biomass can be scaled up to a practical process of industrial use.

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