

## Bioremediation of Higher Amounts of Chromium (VI) by the Methane Oxidising Bacteria *Methylococcus capsulatus*

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### Abstract

Methanotrophs, a group of bacteria that uses methane as the sole energy and carbon source, ubiquitous in the environment, and oxidizes methane, helping to keep it at stable atmospheric concentration levels to effectively slow down global warming. Methanotrophs are distinguished by possessing methane monooxygenase enzymes (MMO), which oxidizes methane into methanol, and can work on an array of substances, including harmful xenobiotics, making it ideal for bioremediation efforts. Of the heavy metals currently accumulating due to increased anthropogenic action, hexavalent chromium is widely used, leading to it being accumulated in soils and aquatic bodies, posing hazardous risks to most life forms. The use of *M. capsulatus* (Bath) methanotrophic bacterium to reduce Cr from +6 oxidation state into the less harmless +3 state has been shown to be effective when used with low concentrations of Cr(VI) between 0.5 - 1.4 mg/ L. However, the ability of *M. capsulatus* (Bath) to bioremediate higher concentrations was not investigated. This study measured Cr(VI) reduction when in a relatively high concentration (5 mg/L) by this bacterium over the course of four days; The Cr(VI) levels were measured daily by taking their OD values. Results have shown that by the last day, about 75% of the initial Cr(VI) concentration have been reduced by *M. capsulatus* (Bath). Therefore, it was confirmed that this bacterium is able to bioremediate high concentration of Cr(VI), thereby suggesting this microbe as a suitable candidate for bioremediation efforts on a larger scale in the environment.

**Keywords:** Methane; Methanotrophs; MMO; Chromium; *Methylococcus capsulatus*; Bioremediation

### Introduction

Methanotrophs, are a Gram-negative group of bacteria that that can use methane as the sole source of carbon and energy, widely spread in the environment, and play an essential part in methane oxidation in the earth's biosphere [1-3]. Methanotrophs belong to the methylotrophs, a physiological group that aerobically utilize one-carbon compounds, e.g. methane, methanol, and methylated sulfur containing compounds [1].

All methanotrophs use an exclusive special type of enzymes that catalyze the oxidation of methane to methanol: methane monooxygenases (MMO). The MMO enzymes have two main types; soluble in the cytoplasm type (sMMO), and particulate type that is associated with the cell membrane (pMMO). Both share the feature of acting on a broad range of substances [4-6].

Most of the methane in today's atmosphere is produced by methanogens, methane comes second after carbon dioxide in creating the greenhouse effect; it is more effective in trapping heat that CO<sub>2</sub> in about 23 times more (mole for mole) [7,8]. Due to anthropogenic activity following the industrial revolution, the methane levels in the atmosphere have been steadily increasing over the last three hundred years [9]. Although methane is produced by large amounts, the microbial oxidation of it is what kept it in low concentration in the atmo-

sphere, mainly by methanotrophs to keep the balance of the global carbon cycle in check [2]. Although methane oxidation can occur both aerobically and anaerobically, few studies were done on the latter, with the former getting most of the attention from researchers, mainly because methanotrophs MMO have a broad specificity; Meaning aerobic methanotrophs can metabolize a large number of compounds, many are harmful or toxic to the environment [2,3].

Bioremediation is using biological means to help in the degradation of toxic compounds, a prospect that have always been a main goal when studying methanotrophs [3,6]. Methanotrophic bacteria also have many potential industrial applications; examples include the mass production of commercially relevant compounds such as single cell proteins and many others. All of these features of the harmless methanogens make this bacteria one of the most promising groups for use in environmental bioengineering [10].

One well studied methanotrophic microbe, *Methylococcus capsulatus* (Bath), has been shown by previous studies as being able to bioremediate chromium (VI) over a broad range of concentrations in the environment [3,6]. Cr(VI) is widely used in the industry and continuously released into the environment to which it has hazardous effects [11,12], which is why it is important to investigate the best methods to employ *M. capsulatus* (Bath) in reducing its harmful effect of neutralizing it. However, our knowledge of Cr(VI) bioremediation is still limited and requires further study. This study aims to further investigate the Chromium (VI) bioremediation reaction in methanotrophs.

## Materials and Methods

### Plotting the standard curve-graph for Chromium (VI) against concentration

Four flasks were prepared as shown in table 1 with different known volumes of Cr(VI) (conc. 1.27 mg/L) and 0.18 sulfuric acid, contents were mixed thoroughly. After which, an amount of 0.5 ml of diphenylcarbazide (conc. 2.5 g /L) was added in each of the flask, mixed, and left for five minutes on the bench to allow the colour to develop. Finally, Absorbency was recorded by taking 1 ml of each of the samples in semi-micro disposable cuvette, and placing them in a spectrophotometer (model number: Cecil CE 1021) which was set to measure absorbance on 540 nm. The absorbency readings were later used to plot the graph shown in figure 1 against concentration.

	Flask No. 0 (Blank)	Flask No. 1	Flask No. 2	Flask No. 3	Flask No. 4
Volume of added Cr(VI) (conc. 1.27mg/L)	0ml	0.4 ml	1 ml	2 ml	4 ml
Volume of added sulfuric acid 0.18M	10ml	9.6 ml	9 ml	8 ml	6 ml
Final Cr(VI) concentration	0.0000 mg/L	0.0484 mg/L	0.1210 mg/L	0.2419 mg/L	0.4838 mg/L

**Table 1:** The different known Cr(VI) concentrations used to calculate their OD reading.

### Bacterial samples, and growth on the media

The bacterium species, *M. capsulatus* (Bath) was used in this study, the isolate was obtained from a culture collection of the biomedical research center of Sheffield Hallam University, originally obtained from the hot spring in the Roman baths in the city of Bath in England. It was kept in petri dishes containing Nitrate Mineral Salts (NMS) agar media when it was provided prior to our work.

To prepare the *M. capsulatus* (Bath) for subsequent steps, single colonies were selected from the supplied NMS agar plates, using a loop, it was inoculated into several 250 ml conical flasks containing 50 ml of NMS liquid media, then covered with rubbery lids. After that, a syringe and needle was used to aspire 50 ml of the air from inside the conical flasks, and then 60 ml of methane was injected inside. All of the flasks were then incubated with shaking at 450C, for 6 days. Every 2 - 3 days, 50 ml of air was removed and 60 ml of methane was added in the same way as before.

### Addition of Chromium (VI) to the *M. capsulatus* (Bath) bacterium in NMS liquid medium

After six days of incubating the *M. capsulatus* in NMS liquid media, one ml of broth was taken from each flask after shaking, and placed in a glass cuvette. Optical density (OD) for growth was measured in spectrophotometer at 600nm, the blanks used for the readings were taken using 1 ml of un-inoculated NMS medium.

After taking the readings for all of the flasks, Chromium (VI) would be added for each flask in the concentration of 5 µg/ml and mixed thoroughly. After that, 1 ml would be taken up again from each flask into an Eppendorf tube, centrifuged at 13000rpm for one minute. After that, the supernatant was transferred to another Eppendorf tube while disregarding the microbial pellet, and stored in the refrigerator at 4°C until needed later.

Next, a syringe and needle was used to remove 50 ml of the air inside the flasks, and 60 ml of methane was pumped in, the flasks were then incubated to 45°C. Every day 1 ml was taken from the flasks, centrifuged and stored same as before, and the air and methane was also replaced as before. The process was repeated every day along the duration of four days. The incubation temperature 45°C was selected instead of 37°C because previous studies indicated that the former is the optimum growth temperature for *M. capsulatus* (Bath) [6], also, because using higher incubation temperatures will reduce the chances of contamination by ambient temperature microbes that usually reside at room temperature.

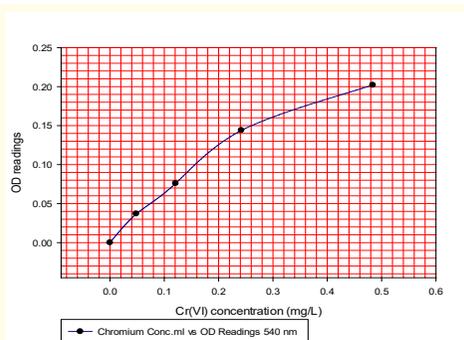
The control for this experiment was an NMS medium which had Cr(VI) but not inoculated with the bacterium, addition of methane, conditions for incubation and taking of readings were taken in the exact same way as in the other samples.

By the end of the incubation period, all the Eppendorf tubes that were kept at 4°C were taken out. And 400 µl was taken from each of the samples into a sterile flask, later; 9.6 ml of sulfuric acid (0.18 M) was also added and mixed. Finally, to each flask Diphenylcarbazide (0.5 ml) was added and mixed thoroughly and left at the bench for five minutes to allow for the colour to develop. Later, 1 ml was taken from each flask, and transferred to a new Semi-micro disposable cuvette. Absorbency (OD) was measured and recorded using the spectrophotometer at 540 nm. The blanks (1 ml) used for taking the readings were taken from a flask containing 10 ml sulfuric acid (0.18 M) with Diphenylcarbazide (0.5 ml).

## Results and Discussion

### Standard curve for Chromium (VI) concentrations

The graph shown in figure 1, drawn using the Sigmaplot software, was made by matching the known concentrations of Cr(VI), to their OD readings. The created curve was later used to determine the unknown Cr(VI) concentrations in other samples, by only using the OD values at 540nm. The spectrophotometer was set-up to measure absorbance at 540 nm because this was found to be the optimum wavelength to measure Cr(VI) concentration levels [13].



**Figure 1:** The standard curve that was plotted to show the OD readings for known Cr(VI) concentrations.

Diphenylcarbazide is used routinely as an analytical color developing agent; the higher the Cr(VI) concentration used in the samples, the stronger the developing purple color will be, due to the chemical reaction between both which results in chromium-diphenylcarbazone as shown in figure 2 [14].

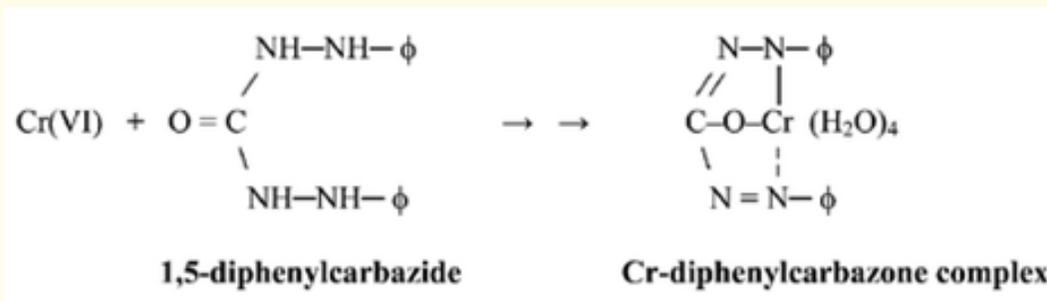
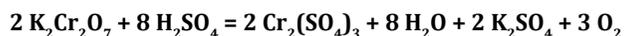


Figure 2: The chemical reaction between chromium (VI) and diphenylcarbazide, which results in chromium-diphenylcarbazone.

Also, the chemical formula for the interaction of Cr(VI) in potassium chromate with sulfuric acid is shown in the following equation:



#### Identification of Cr(VI) reducing activity within *M. capsulatus* (Bath)

After the standard curve of known Cr(VI) concentration was determined, it was used as a reference point to calculate the Cr(VI) concentration decline within the four days' samples using their OD values. Figure 3, which shows the Cr(VI) concentration in each of the three samples and the control along the course of 4 days.

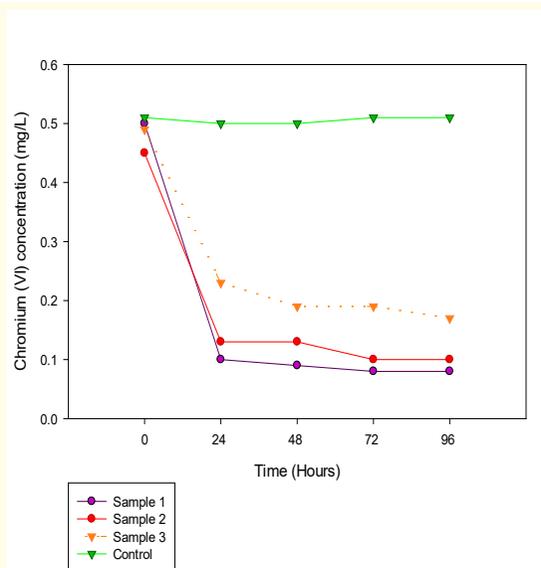


Figure 3: Chromium(VI) concentration within each of the samples as calculated using OD540nm measurements every 24 hours for four days.

This test aimed to investigate the ability of *M. capsulatus* to bioremediate chromate when methane is present as a growth substrate. Potassium chromate was used as the source for Cr(VI) when added to the NMS media, and was added in the final concentration of 5 mg/L to log-phase cultures of *M. capsulatus* bacterium.

Results from figure 3 clearly shown that as time progressed, the Cr(VI) concentration in the three samples went lower; meaning *M. capsulatus* (Bath) cultures caused a decline in the concentration of chromate (VI). By comparing the Cr(VI) concentrations means between the first reading (0.48 mg/L) and the last reading after 96 hours (0.12 mg/L) for the samples, a decline of 75% in Cr(VI) between the two is reported. The presence of a control, which had only the Cr(VI) in the NMS media, with no inoculated *M. capsulatus* microbes, indicates that microbes within the three samples are responsible for the decline of Cr(VI) levels. This is because the Cr(VI) concentration within the controls remained the same over the four days, with no significant decline observed within it. Another observation is that most of the Cr(VI) present within the NMS media was bioremediated by the *M. capsulatus* (Bath) microbes within the first 24 hours. After which the remaining Cr(VI) concentrations kept on declining as time passed, albeit at a lower rate. A possible explanation for this might be that within the 24 hours, the NMS media might have been still fresh and contained less toxic waste, as time progressed, the accumulation of waste products with the deprivation of some nutrient elements might have contributed to inhibit microbial growth, thus leading to a lesser Cr(VI) bioremediation rate. Other possible explanations for this might be that the chromium compounds may have killed or otherwise inhibited the cells, or that the culture may have grown to saturation and the cells ceased to be metabolically active for that reason.

Reduction of Chromium (VI) by microorganisms to the +3 oxidation state, which is insoluble and gets precipitated at high pH, has been investigated before [15-18]. However, only limited number of studies investigated Cr(VI) reduction by methanotrophs; in one particular study that is closely related to our work, Al Hasin., *et al.* [6] investigated the ability of two methanotrophs to bioremediate chromium, *M. capsulatus* (Bath) and *Methylosinus trichosporium* OB3b. This was done by the addition of Cr(VI) in various concentrations ranging between 0.5 - 1.4 mg/L to the microbes inoculated within NMS, and measuring the concentration of it during the incubation period. While *Methylosinus trichosporium* OB3b did not show signs for Cr(VI) reduction, the reduction pattern for *M. capsulatus* (Bath) was very similar to our findings; by the end of the 144 hours incubation period, a 77% fall in Cr(VI) concentration from the initial seeding one was reported, which is close to our reported 75% within 96 hours incubation time. Furthermore, their study also showed that most of Cr(VI) was reduced by the microbes during the first 24 hours of the incubation, the same result that was concluded from our study [6]. With the use of other techniques to confirm the results, such as transmission electron microscopy (TEM) and genomic analysis, Al Hasin and co-workers concluded that *M. capsulatus* (Bath) can bioremediate detoxify Cr(VI) in concentrations below 1.4 mg/L, thus converting harmful Cr(VI) into the less toxic Cr(III). However, no study has attempted to investigate *M. capsulatus* (Bath) in concentrations above 1.4 mg/L, which is why it was chosen to use the 5 mg/L concentration within our study. The reason why it is important to study the efficiency of Cr(VI) reduction in various concentration is because some studies have shown that too low or high concentration of Cr(VI) can have a harmful impact on certain microbes, as shown by Singh., *et al.* [19] studies on the methanogenic bacterium *Methanothermobacter thermautotrophicus*. The use of the control within our work proves that the reduction of the Cr(VI) is caused by a biological, cellular metabolic mechanism.

Based on the data described earlier, we have demonstrated that *M. capsulatus* (Bath) is able to bioremediate Cr(VI) and remove it when present in high concentrations (5 mg/L). Although not investigated within our study, this reduction requires methane to proceed, which imply that methane might supply electrons for Cr(VI) reduction [6].

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