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Factors affecting chromium(VI) reduction by *Thiobacillus ferrooxidans*

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Abstract

The ability of *Thiobacillus ferrooxidans* cultures to reduce chromium(VI) to chromium(III) was evaluated under different conditions. In *T. ferrooxidans* cultures with sulphur as energy source, the capacity for chromium(VI) reduction was related to the generation of sulphur compounds (sulphite, thiosulphate and polythionates) with high reducing power. In contrast with other chromium(VI)-reducing microorganisms, *T. ferrooxidans* showed higher chromium(VI) reduction at low pH. The reduction of chromium(VI) also increased with the age of the culture. A *T. ferrooxidans* cells were capable of growing under anaerobic conditions with chromium(VI) as the terminal-electron acceptor.

Keywords: *Thiobacillus ferrooxidans*; Chromium(VI); Bioreduction; Reducing compounds; Sulphur; Anaerobic; Aerobic conditions

1. Introduction

Chromium compounds have many industrial uses, such as chromite ore processing, electroplating, leather-tanning processes amongst others [1–3]. As a result of unregulated application and inappropriate waste-disposal practices, chromium is incorporated into the environment (mainly into soils and streams of water) [1]. The main aqueous species of chromium are Cr^{3+} (pH < 3.6), $Cr(OH)_4^-$ (pH > 11.5), CrO_4^{2-} (pH > 6.5) and CrO_4H^- and $Cr_2O_7^{2-}$ (pH < 6.5). Hexavalent chromium is classified as a primary contaminant because of its mobility in soil and groundwater and its reported harmful effects on organisms including humans. Within living cells, chromium(VI) compounds can induce cancer and mutation. As reduction of toxic chromium(VI) leads to the formation of stable and non-toxic chromium(III), this reduction may be implemented so as to achieve detoxification, and therefore, environmental cleanup. Chromium(VI) reduction or chromium immobilisation can be produced abiotically by different substances [4–6] but recent reports have demonstrated the feasibility of using biological reduction for the treatment of chromium(VI) containing wastes [2,3,7–15].

Thiobacilli are a group of gram-negative chemoautothotrophic bacteria that can obtain energy for growth from the oxidation of a variety of inorganic sulphur compounds. Two species of Thiobacilli, *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans*, are involved especially in bacterial leaching of sulphide ores for the recovery of several metals [16–19]. The oxidation of such compounds, particularly that of elemental sulphur, generates a series of sulphur compounds (sulphite, thiosulphate and polythionates) with high reducing power [20]. This activity has been used in cultures of *T. thiooxidans* to catalyse the reduction of manganese(IV) [21], iron(III) [22] and vanadium(V) [23]. Recently, we reported that cells of these bacteria are capable of chromium(VI) reduction in aerobic conditions through a similar mechanism [24,25] although high chromium concentrations produced partial or total inhibition. It has also been suggested that the reducing capability may be in the colloidal sulphur appearing in cultures. Results showed certain advantages using these microorganisms in relation to others used before because they are able to reduce greater concentrations of chromium (more than 100 mg l^{-1}) and no organic compounds should be added due to *Thiobacillus* cells being autotrophic.

For our purpose, colloidal sulphur in *T. ferrooxidans* cultures on elemental sulphur was separated through microfiltration. Reduction ability of *T. ferrooxidans* cultures was evaluated by passing chromium(VI) through filtration membranes with colloidal sulphur. Percentages of chromium(VI) reduction were determined in relation to culture conditions, culture method (shake flasks or fermentation vessel) and solution pH. The ability of *T. ferrooxidans* to reduce

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chromium(VI) in the presence of elemental sulphur under anaerobic conditions was also investigated.

2. Materials and methods

2.1. Microorganisms

A *T. ferrooxidans* strain (DSM 11477) was used in the experiments. Cells were routinely sub-cultured in iron-free 9 K medium [26] with powdered sulphur (10 g l^{-1}) as energy source. Cells were used as inocula in cultures carried out in shake flasks or in a fermentation vessel.

2.2. Cultures in shake flasks

Experiments were carried out in flasks incubated in a shaker at 180 rpm and 30°C. The 1.0 g of analytical grade powdered sulphur and 100 ml of iron-free 9 K medium inoculated at 10% v/v with *T. ferrooxidans* were added in all flasks. The initial pH was 2.0.

2.3. Cultures in a fermentation vessel

Cultures were also carried out in stirred and aerated baffled LKB fermenter (fermentation vessel made of glass, length 50 cm, i.d. 25 cm; 400 rpm; air flow 0.6 l min^{-1}) containing 6 l of iron-free medium at pH 2.0 inoculated at 10% v/v with *T. ferrooxidans* and 120 g of analytical grade powdered sulphur. Cultures were maintained at 30°C.

2.4. Preparation of filters with colloidal sulphur

The total amount of 100 ml of cultures from shake flasks or from the fermentation vessel were filtered through blue ribbon filter paper (pore size 3 μm) to eliminate sulphur particles larger than 3 μm. Then the medium was filtered through a 0.45 μm filter. Filtration membranes with both retained biomass and colloidal sulphur (strictly speaking, sulphur particles with size less than 3 μm) were used in the following experiments.

2.5. Chromium(VI) reduction procedure using filters

The total amount of 5 ml of potassium dichromate solution (pH 2.0 and chromium(VI) 10 mg l^{-1}) were filtered through the filtration membranes described above, using an accessory vacuum filter. Contact time between filter and solution was about 5 min. In some experiments, the procedure was repeated twice, each time with 5 ml of potassium dichromate solution. Each filtering procedure will be called, from now on "reduction step". After the reduction step(s), an iron-free medium of pH 2.0 was used to flush chromium possibly adsorbed in the filter. All reduction experiments were carried out in duplicate using analytical-grade potassium dichromate.

2.6. Chromium(VI) reduction in cultures under different conditions

Experiments of chromium(VI) reduction in three reduction steps were carried out using colloidal sulphur and cells (see above) from cultures at different conditions and with shake flasks or the fermentation vessel. Sulphur oxidation by *Thiobacillus* cells produces sulphuric acid; that is why, the condition of the culture where samples were taken from, was correlated with proton concentration reached in this culture. A potassium dichromate solution (10 mg l^{-1} and pH 2.0) was used in these experiments.

2.7. Chromium(VI) reduction at different pH

Experiments of chromium(VI) reduction in three reduction steps were carried out using colloidal sulphur and cells (see above) from a culture in a fermentation vessel when proton concentration was about 200–250 mmol l^{-1}. A solution with 10 mg l^{-1} chromium(VI) at different pH (2.0, 4.0, 6.5 and 8.5) was used in the experiments.

2.8. Chromium(VI) reduction under anaerobic conditions

Experiments were carried out in hermetic flasks with two valves allowing gaseous circulation through the solution. The flasks were incubated in a shaker at 180 rpm and 30°C. The total 1.0 g of elemental sulphur and 100 ml of a iron-free 9 K medium containing 12.5 mg l^{-1} of chromium(VI) inoculated at 10% v/v with *T. ferrooxidans* were added to all flasks. The initial pH was 2.0. In order to reach anaerobic conditions, a stream of N_2 with 2.7% v/v of CO_2 (without oxygen by previous passage through pyrogalol) was circulated through the medium. Valves were closed when oxygen was not detected in the gaseous stream coming from the medium. Samples were regularly taken from flasks and N_2/CO_2 was recirculated to achieve anaerobic conditions.

When chromium(VI) concentration lowered significantly, more chromium(VI) was added avoiding concentrations higher than 15 mg l^{-1}. This was done four times, so that final chromium concentration would have been about 50 mg l^{-1}.

Sterile controls were used, replacing inoculum by sterile medium with similar conditions as in the inoculated flasks. Chromium(VI) concentration did not alter so no further additions were necessary.

2.9. Analytical methods

Bacterial population was determined by using a Petroff–Hausser counting chamber under a microscope with a contrast phase attachment and proton concentration was analysed by titration with a 0.02N NaOH.

Chromium(VI) was determined by the diphenylcarbazide method [27,28]: 0.50 ml solution prepared with 0.025 g of diphenylcarbazide in 10 ml of acetone were added to 10 ml

of sample (diluted when necessary). After 10 min incubation at room temperature, absorbance at 540 nm was determined. Total chromium concentration was determined in the same way after oxidising chromium(III) with a solution of $KMnO_4$ boiling for 10 min.

3. Results and discussion

3.1. Chromium(VI) reduction under cultures at different conditions

Fig. 1 shows chromium(VI) reduction percentage by colloidal sulphur and cells from cultures in shake flasks or in the fermentation vessel at different points in time (represented by the proton concentration which indicates bacterial growth). Cultures took approximately 250 h to reach a proton concentration of 500 mM in the fermentation vessel or 340 mM in shaken flasks.

Reduction percentage was estimated by the quotient between chromium(III) concentration (determined as the difference between total chromium and chromium(VI)) and the total chromium concentration. The ability to reduce chromium(VI) increases as the proton concentration increases.

The sulphur-oxidation mechanism has not been completely explained, yet. But, in *T. thiooxidans* and perhaps in *T. ferrooxidans* [16], reduced glutathione (GSH) is required to oxidise elemental sulphur

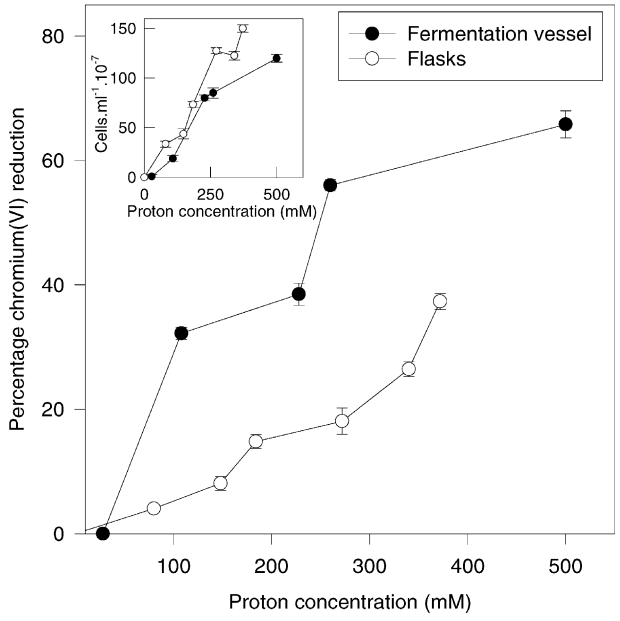

Fig. 1. Percentage chromium(VI) reduction under different culture conditions (outer graph). Relationship between free bacterial population and proton production in *T. ferrooxidans* culture (inner graph). The values are the means from two cultures. Error bars represent standard deviations. Negligible error bars indicate that results in duplicates were almost identical.

$$S_n + GSH \rightarrow GSS_nH \quad (1)$$

Later, polysulphide is oxidised to sulphite by the cells according to the following reaction:

$$GSS_nH + O_2 + H_2O \rightarrow GSS_{n-1}H + {SO_3}^{2-} + 2H^+ \quad (2)$$

Finally, the sulphite, thus produced is oxidised to sulphate by the cells:

$${SO_3}^{2-} + \tfrac{1}{2}O_2 \rightarrow {SO_4}^{2-} \quad (3)$$

If this mechanism is correct, reaction (2) produces reduction compounds (in this case, sulphite) and is responsible for protons appearing in the solution. Our results qualitatively agree with this mechanism because reduction ability is accompanied by higher proton concentration in the solution. The majority of reducing compounds produced are finally oxidised according to Eq. (3). On the other hand, when colloidal sulphur was retained in the filtration membranes, cultures did not reduce chromium(VI) substantially; moreover, elemental sulphur retained in the first filtration (sulphur particles larger than 3μm) did not cause chromium(VI) reduction either (data not shown). These results suggest that only reducing compounds associated with colloidal sulphur reduce chromium(VI) while reducing compounds concentration in solution is low due to bacterial action, as indicated in Eq. (3). Besides, the latter reaction would be essentially due to the action of unattached bacteria having those reducing compounds as energy source [29]. Reducing compounds associated with colloidal sulphur are probably joined in a labile way so as to reduce chromium(VI) but not in such a way as to be removed by successive washing. Thus, according to Steudel [30], colloidal sulphur in cultures of *T. ferrooxidans* would be present as long-chain polythionates forming micelles or globules of up to a few micrometers.

The amount of reducing compounds associated with the culture at a given moment is substantially less than that suggested by the stoichiometry in Eq. (2) taking into account the acid production. This result suggests that reaction (3) is very fast, especially in shake flasks, due to the higher unattached bacterial population at the same acid production (Fig. 1; inner graph). Probably, greater stirring in the fermentation vessel increased the exposed area and consequently the bacterial attachment to the sulphur [31]. In contrast to this, sulphur surface in flasks was saturated with cells faster than in the fermentation vessel, increasing free bacterial population and decreasing the amount of reducing compounds (Eq. (3)).

3.2. Chromium(VI) reduction at different pH

Fig. 2 shows chromium(VI) reduction percentage when circulating chromium(VI) solutions had pH values of 2.0, 4.0, 6.5 and 8.5. The outer graph also shows the amount of chromium retained by the filter (even after being washed). At pH 2.0, that amount was negligible but it increased as chromium(VI) solution pH increased and reached 60% of

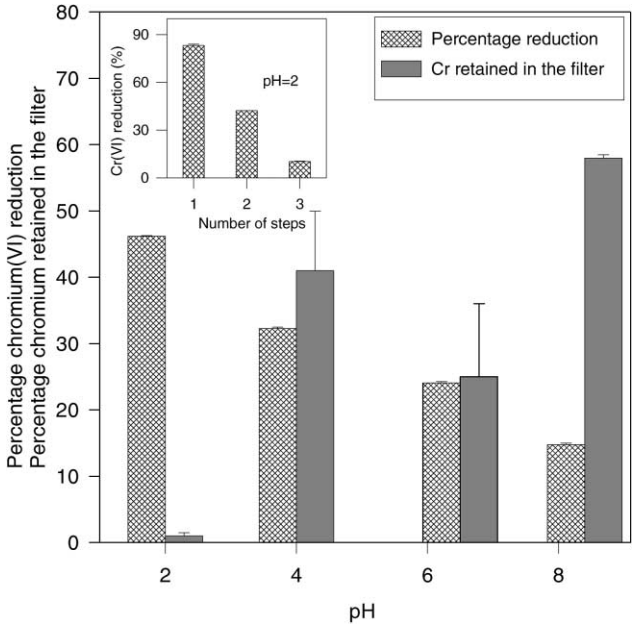

Fig. 2. Chromium(VI) reduction and chromium retained on the filter in experiments using chromium(VI) solutions at different pH (outer graph). Chromium(VI) reduction percentages for three reduction steps (inner graph). The values are the means from two cultures. Error bars represent standard deviations. Negligible error bars indicate that results in duplicates were almost identical.

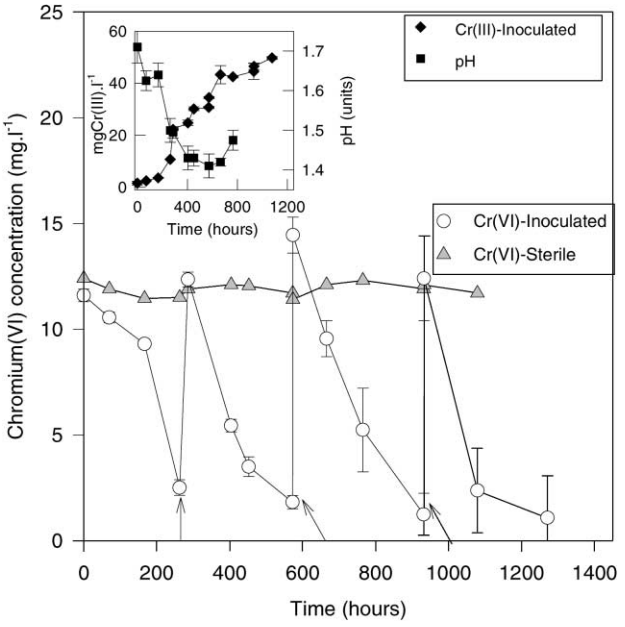

Fig. 3. Changes in culture pH and chromium(III) concentration (inner graph) and in chromium(VI) concentration (outer graph) during *T. ferrooxidans* culture under anaerobic conditions. The arrows indicate new additions of chromium(VI). The values are the means from two cultures. Error bars represent standard deviations. Negligible error bars indicate that results in duplicates were almost identical.

added chromium when pH was 8.5. Probably, chromium was retained (as chromium(III) hydroxide which is insoluble at high pH) by the filter [32]. This would indicate greater reduction on the filter than is suggested by the figure.

The inner graph shows chromium(VI) reduction percentage for three reduction steps when chromium(VI) solution pH was 2.0; reduction power lowered critically, nearly disappearing in the third stage. Under other pH values, chromium solution behaviour was similar.

The reason why reduction is higher when pH is lower may be related to the greater oxidation power of chromium(VI) compounds as pH lowers (dichromate has a standard electrode potential of 1.33 V while the value for chromate is −0.12 V). Although greater immobilisation-reduction was found at higher pH values, reduction percentage detected was maximum when pH was 2.0.

3.3. Chromium(VI) reduction under anaerobic conditions

Fig. 3 illustrates chromium(VI) reduction under anaerobic conditions. Chromium reduction was not observed under sterile conditions. Neither was it observed in an inoculated system which was boiled during 20 min before adding dichromate (data not shown). The latter served to discard dichromate reduction due to the organic matter of the bacteria. The inner graph shows chromium(III) evolution in cultures confirming chromium(VI) reduction. In the same graph, it can be seen that pH is also slightly reduced during the experiment.

These results indicate that chromium(VI) has been the last electron acceptor. Constant decrease of free bacterial population was also observed in these cultures, suggesting the possibility that chromium(VI) reduction catalysed by cells is not coupled to their growth. Chromium(VI) reduction under anaerobic conditions was slower than that under aerobic conditions [24].

Summarising, the present paper suggests that in *T. ferrooxidans* cultures on elemental sulphur, certain reducing compounds are generated, which are able to reduce chromium(VI); these reducing compounds are basically located on colloidal sulphur. The reduction ability of colloidal sulphur takes place within a wide range of pH values in the chromium(VI) solution, though it increases when pH decreases. Cultures taken from fermentation vessels present greater reducing power than cultures from shake flasks, though in both cases, the reducing power is greater if the growth phase is more advanced. Besides, *T. ferrooxidans* cells showed their ability to use chromium(VI) as electron acceptor in the absence of oxygen. In this way, the use of *T. ferrooxidans* cells immobilised in elemental sulphur or colloidal sulphur coming from *T. ferrooxidans* cultures, becomes an adequate process to be used in the detoxification of chromium(VI) polluted effluents because it can be used under diverse pH values, aerobic and even anaerobic conditions.

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