CONF-950587--2

Enhancement of Fe (III), Co (III), and Cr (VI) Reduction at Elevated Temperatures and by a Thermophilic Bacterium†

Chuanlun Zhang[1], Shi Liu[1], John Logan[1], Raja Mazumder[1], and Tommy J. Phelps*, [2]

[1]Oak Ridge Associated Universities, Oak Ridge, TN 37830; and [2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831

ABSTRACT

An unusual thermophilic bacterium has been isolated from deep subsurface sediments and tested for its ability to enhance Fe (III), Co (III), and Cr (VI) reduction. Without the bacterium, abiotic metal reduction was insignificant at temperatures below 45°C but became a major process at 75°C. Addition of the bacterium enhanced the reduction of these metals up to fourfold. This study demonstrates abiotic and biotic metal reduction under organic-rich thermic conditions and suggests that thermally and/or biologically enhanced metal reduction may provide an alternative for remediating metal contamination.

Index Entries: Thermophilic bacterium; metal reduction; ferric citrate; Co (III)-EDTA; chromate.

*Author to whom all correspondence and reprint requests should be addressed.

INTRODUCTION

Bacterial reduction of Fe (III), Mn (III, IV), and other metals is well documented (*1-4*). Most studies have focused on metal reduction by mesophilic bacteria (growth temperature less than 45°C). For example, Lovley and Phillips (*5*) isolated an obligately anaerobic bacterium from freshwater sediments, *Geobacter metallireducens*, that reduces amorphic Fe (III) oxide to magnetite in a reaction coupled to acetate oxidation. A facultative bacterium, *Shewanella putrefaciens*, uses diverse electron acceptors (O_2, NO_3^-, NO_2^-, $S_2O_3^=$, and S^0) and reduces Fe (III) or Mn (IV) anaerobically when growing on formate or lactate (*6*). Recent summaries of the knowledge on metal reduction by bacteria can be found in Lovley (*3*) and in Nealson and Saffarini (*4*).

Geological evidence suggests that bacteria may play an important role in metal reduction under thermic conditions. For example, Machel and Burton (*7*) found that authigenic magnetite, which can be formed by bacterial reduction of ferric iron (*8*), co-occurred with thermally generated hydrocarbons. Information on metal reduction by thermophilic anaerobic bacteria is scarce, however Boone et al. (*9*) reported anaerobic Fe (III) and Mn (IV) reduction by a thermophilic bacterium that grows on formate or lactate as an energy source. Examination of metal reduction at higher temperatures and by thermophilic bacteria is needed to elucidate the roles of biotic and abiotic processes in natural geothermal environments. Furthermore, information from such studies may lead to alternative techniques for abiotic and/or biotic remediation of metal contamination in thermal environments.

During a 1992 drilling operation in the Taylorsville Triassic Basin in Virginia, thermophilic bacteria were recovered from low-porosity sandstones and shales at depths of about 2.7 km below land surface. Most of these bacteria are gram-negative, rod-shaped cells capable of fermentation, denitrification, sulfate reduction, and Fe (III) or Mn (IV) reduction (*10*). Enrichments for these types of microorganisms from drilling fluids or surface sediments were not successful, indicating that these microorganisms likely originated in the deep subsurface.

Geological and hydrological evidence suggests that these microorganisms may have survived *in situ* for a long time. In this study, we examined anaerobic reduction of Fe (III), Co (III), and Cr (VI) by an anaerobic fermentative thermophilic bacterium isolated from the Taylorsville samples and compared the biotic (bacterial) and abiotic metal reduction under similar conditions.

MATERIALS AND METHODS

Medium Preparation for Abiotic and Biotic Metal Reduction

Experiments for abiotic and biotic metal reduction were performed using 15-mL pressure tubes (from Bellco Glass Inc., Vineland, New Jersey) that contained a medium having the following ingredients (g/L): NaCl, 5–70; $MgCl_2 \cdot 6H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.1; NH_4Cl, 1; 3-(N-morpholino)-propanesulfonic acid, 0.1; $NaHCO_3$, 2.5; yeast extract, 1.0–1.5; peptone, 1.0; and trace mineral and vitamin solutions (*11*). The medium was prepared anaerobically under a nitrogen gas atmosphere.

Before each experiment, the tubes were incubated at 65°C for 5–10 days. During this period, yeast extract and peptone in the medium consumed trace oxygen and decreased the redox potential. No other reductant was added. The tubes were cooled to room temperature before other chemical components or the bacterial inoculum was added. Sterile phosphate was added to give final concentration of 2 mM and glucose was added to give final concentrations of 5 or 10 mM. Sterile solutions of ferric citrate, Co(III)-EDTA, and potassium chromate were added to give final concentrations of 14 mM, 0.5–10 mM, and 0.5–2.5 mM, respectively. A 0.5 mL inoculum of freshly grown culture was used. The final pH was 7.5–8.0. Abiotic metal reduction was examined at temperatures ranging from 25 to 75°C. Bacterial metal reduction was examined at 55 and 65°C.

Bacterial Strain Selection and Growth

The bacterial strain (TOR 39) used for this study is an anaerobic, gram-negative, rod-shaped fermentative bacterium that can ferment glucose and other carbohydrates (Liu et al., manuscript in preparation). TOR 39 grows at temperatures from 50 to 70°C and at NaCl concentrations from 0.1 to 5% (wt/v).

Cell growth during the reduction of Fe (III), Co (III), and Cr (VI) was monitored by acridine-orange direct count method using an epifluorescence microscope. Subsamples (0.5 to 1 mL) were diluted with sterile phosphate buffer (pH 7), filtered onto a black Nuclepore filter (0.2-μm pore diameter), stained with 1 mL of a particle-free acridine orange solution for 2 min, and observed microscopically.

Spectrophotometric Analysis of Fe (III), Co (III), and Cr (VI) Reduction

Reduction of Fe (III) to Fe (II) was measured as the increase in Fe (II) concentration by the ferrozine method (*12*). Subsamples (0.1 mL) were withdrawn with a syringe and needle and diluted tenfold to 40-fold with anaerobic water before reacting with ferrozine. After 20 sec of mixing, samples were filtered (using a 0.2 μm-pore-diameter PVDF filter from Whatman, Clifton, New Jersey) and the concentration of Fe (II) in the filtrate was measured at A_{562}. The anaerobic water/ferrozine extraction method used here underestimated the amount of Fe (II) produced, because it did not extract solid Fe (II) compounds such as $FeCO_3$; consequently, the mass balances of iron were typically < 50%.

Reduction of Cr (VI) to Cr (III) was measured as the decrease in Cr (VI) concentration by the diphenylcarbazide method (*13*) according to Lovley and Phillips (*14*). Subsamples (0.1 mL) were withdrawn with a syringe and needle and diluted tenfold to 600-fold before reacting with diphenlycarbazide reagent (0.025 g of sym-diphenylcarbazide in 10 mL of acetone). Samples were filtered as before, and the concentration of Cr (VI) in the filtrate was measured at A_{540}. Reduction of Co (III) to Co (II) was measured as the decrease in Co (III) concentration.

Subsamples (0.5 to 1 mL) were diluted with 2 mL of distilled water and filtered as before. The concentration of Co (III) in the filtrate was measured at A_{548}.

RESULTS

Temperature Effect on Fe (III), Co (III), and Cr (VI) Reduction

The abiotic reduction of Fe (III) [measured as production of Fe (II)], Co (III), and Cr (VI) increased as a function of temperature, with the most reduction occurring at 75°C (Figures 1A, 1B, and 1C). No significant reduction (< 3%) of these metals was detected at 75°C in pure anaerobic water (data not shown). The initial Fe (II) concentration was about 0.3 mM when Fe (III)-citrate (final concentration of 14 mM) was added. This Fe (II) probably resulted from reduction of Fe (III)-citrate during autoclaving of the stock solution.

As shown in Figure 1A, no significant increase in Fe (II) was detected at 25 and 45°C [< 1% of initial Fe (III) concentration], and only a slight increase was observed at 55 and 65°C (< 3% of initial Fe (III) concentration), after 6 h of incubation; however, there was a dramatic increase in Fe (II) [about 13% of initial Fe (III) concentration] at 75°C during this period. While the Fe (II) production rate remained slow (< 0.02 mM h^{-1}) at 25 and 45°C, the rate increased > twofold at 65°C. The production rate of Fe (II) at 75°C was 0.22 mM h^{-1} before reaching an Fe (II) plateau at 24 h. At the end of the experiment, the amount of Fe (II) produced was less than 5% of initial Fe (III) concentration at 25 and 45°C, 9% at 55°C, 16% at 65°C, and ~ 50% at 75°C.

Figure 1B shows decreased Co (III) concentrations for all temperatures after 6 h of incubation. Again, similar to Fe (II) production profiles, the fastest reduction rate was at 75°C (0.67 mM h^{-1} versus 0.13 mM h^{-1} at 25°C). The reduction of Co (III) increased from 14% at 25°C to 82% at 75°C.

Abiotic reduction of Cr (VI) at 65 and 75°C (Figure 1C) was rapid and linear, in contrast to the variable results obtained at lower temperatures. At 45 and 55°C, Cr (VI) reduction showed

the same trend with a general decrease in Cr (VI) concentration between 0 and 22 h. Reduction of Cr (VI) at 25°C showed a less regular trend. Final reduction for Cr (VI) was 92% at 75°C, 52% at 65°C, and less than 10% between 25 and 55°C.

Among the three metals with initial concentrations indicated in Figures 1A, 1B, and 1C, Co (III) had the highest reduction rates for all temperatures, ranging from 0.13 mM h^{-1} at 25°C to 0.67 mM h^{-1} at 75°C (Figure 1C). Fe (III) and Cr (VI) reduction rates were less than 0.05 mM h^{-1} at temperatures below 65°C and increased to 0.3 and 0.08 mM h^{-1} at 75°C, respectively. All three metals showed a significant correlation between reduction rate and temperature ($r^2 > 0.82$, $P < 0.01$) when data were fitted to an exponential curve (Figure 2).

Microbial Enhancement of Fe (III), Co (III), and Cr (VI) Reduction by TOR 39

The potential enhancement of Fe (III), Co (III), and Cr (VI) reduction by bacterial strain TOR 39 was examined after freshly grown inocula were transferred into the medium containing the oxidized metals. Figure 3A shows the production of Fe (II) reduced from 14 mM ferric citrate in the medium in the presence or absence of the bacterium. Also shown are cell counts during biotic Fe (II) production at 65°C. After a lag phase of 6 hours, production of Fe (II) at 65°C increased dramatically in the presence of TOR 39 with a rate of 0.29 mM h^{-1} between 6 and 24 h, while the abiotic production rate was only 0.05 mM h^{-1} during this period. The amount of Fe (II) produced in the presence of TOR 39 reached the maximum value at 34 h, which was about 3.5 times higher than in the absence of the bacterium.

Bacterial production of Fe (II) at 55°C followed the trend observed at 65°C (Figure 3A); however, a dramatic increase in Fe (II) at 55°C was observed after 24 h of incubation, and the maximum amount of Fe (II) produced at 34 h was less (5.8 mM versus 7.4 mM) than at 65°C. Similarly, abiotic Fe (II) production at 55°C was slower (0.02 mM h^{-1} versus 0.05 mM h^{-1}) and less (1.5 mM versus 2.6 mM) than at 65°C.

TOR 39 density increased from 6.9×10^7 cells mL^{-1} at 0 h to 4.4×10^8 cells mL^{-1} at 12 h during the early stage of biotic Fe (II) production at 65°C (Figure 3A); however, the cell

numbers decreased to 4.1 x 10^7 cells mL^{-1} by the end of experiment. Bacterial cells were not counted during Fe (II) production at 55°C.

Figure 3B shows abiotic and biotic reduction of Co (III) (initial concentration of 10 mM) and bacterial cell counts at 65°C. Rates of abiotic and biotic reduction of Co (III) were similar (0.39 mM h^{-1} versus 0.46 mM h^{-1}) after 6 h incubation, suggesting that the lag phase of bacterial growth was similar to that seen during Fe (II) production. After 6 h, the Co (III) reduction rate was about twice as fast in the presence of TOR 39 than in its absence. By the end of the experiment, the amount of Co (III) reduced was 1.5 times higher in the presence of TOR 39 than in its absence (Figure 3B). Cell numbers also increased during bacterial reduction of Co (III).

Bacterial enhancement of Cr (VI) reduction was examined at Cr (VI) concentrations below 1.0 mM because Cr (VI) concentrations ≥ 1.0 mM inhibited the growth of TOR 39 (unpublished data). Figure 3C shows the biotic and abiotic reduction of Cr (VI) at 55 and 65°C with an initial Cr (VI) concentration of about 0.7 mM. The rates of abiotic reduction of Cr (VI) at 55 and 65°C in the absence of TOR 39 were about 0.01 mM h^{-1}. In contrast, initial rates of biotic Cr (VI) reduction were 3 times faster at both temperatures. Between 9 and 40 h, biotic reduction of Cr (VI) continued rapidly, whereas the abiotic reduction proceeded slowly at both temperatures. The increase in cell counts during the early stage of incubation was similar to the trend of cell counts during Fe (II) production. By the end of the experiment, the initial concentration of Cr (VI) was reduced biotically by 88% at 65°C and by 95% at 55°C. Abiotic reduction of Cr (VI) at 65 and 55°C was ≤ 20%.

Figure 4 summarizes the biotic versus abiotic reduction of Fe (III), Co (III), and Cr (VI). This figure shows that TOR 39 significantly enhances reduction of these metals at 55 and 65°C.

Salinity tests indicated that TOR 39 enhanced reduction of these metals at NaCl concentrations up to 3% (w/v), with the maximum enhancement at 1% NaCl (data not shown), while other cultures indicated enhancement of metal reduction at temperatures ≥ 70°C and NaCl concentrations ≤ 7% (w/v) (data not shown).

DISCUSSION

Temperature profiles of abiotic reduction of Fe (III), Co (III), and Cr (VI) (Figures 1A, 1B, and 1C) suggest that abiotic metal reduction was insignificant at mesophilic temperatures (<45°C), was significant above 45°C, and became a major process at 65 and 75°C. Results of Fe (III) reduction in this study support the current understanding that in many sedimentary environments of mesophilic temperatures, the potential for Fe (III) reduction coupled to organic matter oxidation by abiotic mechanisms is low (*2,15*); however, abiotic reduction of Fe (III) may play an important role in higher-temperature environments such as geothermal vents and deep aquifers where temperatures can be above 50°C.

Besides enhancing the reduction of Fe (III), Co (III), and Cr (VI), TOR 39 also enhanced the reduction of other metals such as Mn (IV) and U (VI) (data not shown). Similar thermophilic bacteria (e.g., TOR 9, TOR 35) tolerated higher temperatures (up to 75°C) and NaCl concentrations (up to 7%) and also indicate that they have the potential for reducing metals (data not shown). Fermentative bacteria at mesophilic temperatures have been shown to have low efficiency in transferring electrons from organic matter to Fe (III) or other metals (2). Little is known about electron transfer efficiency by fermentative bacteria at thermophilic temperatures. It is possible that at high temperatures, fermentative bacteria may readily transfer electrons to oxidized metals, possibly by non-specific mechanisms.

Fe (III) forms have a significant effect on Fe (III) reduction. Increasing crystallization results in less reduction of Fe (III), probably becaused of decreases in the solubility and surface area of the Fe (III) form (*1,16*). The bacteria-enhanced Fe (III) reduction in this study was probably caused by the high solubility of ferric citrate. Other studies reported that Fe (III) reduction rates were stimulated by the addition of ferric citrate (*17,18*). The ability of TOR 39 to reduce other forms of Fe (III) is being tested.

Abiotic reduction of toxic Cr (VI) by reactive organic matter such as phenol at mesophilic temperatures has been well established (*19,20*). Raising temperatures may increase the rate of Cr (VI) reduction (see Figure 1C), and adding thermophilic bacteria may enhance the reduction

even further. Bioenhancement of Cr (VI) reduction has been demonstrated at mesophilic temperatures (12) and has a potential application for removing Cr (VI) from contaminated water and waste streams.

In summary, this study demonstrated that abiotic reduction of Fe (III), Co (III), and Cr (VI) was insignificant in the presence of organic matter such as glucose, yeast extract, and peptone at mesophilic temperatures (<45°C) or in distilled water at higher temperatures (data not shown). Abiotic reduction of these metals was significant above 45°C, however, and became a major process at 75°C. On the other hand, reduction of Fe (III), Co (III), and Cr (VI) was two to four times higher in the presence of a thermophilic bacterium than in its absence, and the number of bacterial cells increased concomitantly with the reduction of these metals.

ACKNOWLEDGMENTS

We thank Philip M. Jardine and David L. Taylor for providing Co (III)-EDTA, and Anthony V. Palumbo and Susan M. Pfiffner for technical assistance. This research was supported by the U.S. Department of Energy Subsurface Science Program (Deep Microbiology Subprogram), managed by Frank Wobber, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

REFERENCES

1. Lovley, D. R. (1987), *Geomicrobiol. J.* **5**, 375–399.
2. Lovley, D. R. (1991), *Microbiol. Rev.* **55**, 259–287.
3. Lovley, D. R. (1993), *Annu. Rev. Microbiol.* **47**, 263–290.
4. Nealson, K. H. and Saffarini, D. (1994), *Annu. Rev. Microbiol.* **48**, 311–343.
5. Lovley, D. R. and Phillips, E. J. P. (1988), *Appl. Environ. Microbiol.* **54**, 1472–1480.

6. Myers, C. R. and Nealson, K. H. (1988), *Science* **240**, 1319–1321.

7. Machel, H. G. and Burton, E. A. (1991), *Geophys.* **56**, 598–605.

8. Lovley, D. R. (1990), *Iron Biominerals*, Frankel, R. B. and Blakemore, R. P., eds., Plenum Press, New York, pp. 151–166.

9. Boone, D. R., Liu, Y., Zhao, Z., Balkwill, D. L., Drake, G. R., Stevens, T. O., and Aldrich, H. C. (1995), Intnl. J. Syst. Bacteriol., in press.

10. Balkwill, D. L., Boone, D. R., Colwell, F. S., Griffin, T., Kieft, T. L., Lehman, R. M., McKinley, J. P., Nierzwicki-Bauer, S., Onstott, T. C., Tseng, H. Y., Gao, G., Phelps, T. J., Ringelberg, D., Russell, B., Stevens, T. O., White, D. C., and Wobber, F. J. (1994), *EOS* **75**, 385, 395–396.

11. Phelps, T. J., Raione, E. G., White, D. C., and Fliermans, C. B. (1989), *Geomicrobiol. J.* **7**, 79–91.

12. Stookey, L. L. (1970), *Anal. Chem.* **42**, 779–781.

13. Urone, P. F. (1955), *Anal. Chem.* **27**, 1354–1355.

14. Lovley, D. R. and Phillips, E. J. P. (1994), *Appl. Environ. Microbiol.* **60**, 726–728.

15. Lovley, D. R., Phillips, E. J. P., and Lonergan, D. J. (1991), *Environ. Sci. Technol.* **25**, 1062–1067.

16. Kostka, J. E. and Nealson, K. H. (1995), *Environ. Sci. Technol.*, in press.

17. Arnold, R. G., Dichristina, T. J., and Hoffmann, M. R. (1986), *Appl. Environ. Microbiol.* **52**, 281–289.

18. Jones, J. G., Gardener, S., and Simon, B. M. (1983), *J. Gen. Microbiol.* **129**, 131–139.

19. Elovitz, M. S. and Fish, W. (1994), *Environ. Sci. Technol.* **28**, 2161–2169.

20. Palmer, C. D. and Wittbrodt, P. R. (1991), *Environ. Health Perspect.* **92**, 25–40.

Figure Legends

Figure 1. Abiotic reduction of Fe (III), Co (III), and Cr (VI) at different temperatures. (A) Reduction of 14 mM Fe (III) measured as the production of Fe (II) with time. (B) Reduction of 10 mM Co (III) with time. (C) Reduction of 2.5 mM Cr (VI) with time. Glucose concentration was 5 mM in A and 10 mM in B and C. Yeast extract and peptone concentrations were 0.1–0.15% in A, B, and C.

Figure 2. Abiotic reduction rates of Fe (III) [measured as increase in Fe (II) production], Co (III), and Cr (VI) as a function of temperature. Fe (III) and Co (III) data were from Figures 1A and 1B, respectively. Cr (VI) data were from Figure 1C and other experiments. All data were fitted into exponential curves with r^2 indicating correlation coefficients.

Figure 3. Microbially enhanced reduction of Fe (III), Co (III), and Cr (VI) at 55 and/or 65°C. (A) Time course measurement of abiotic and biotic production of Fe (II) and cell counts. (B) Time course measurement of abiotic and biotic reduction of Co (III) and cell counts. (C) Time course measurement of abiotic and biotic reduction of Cr (VI) and cell counts. See Figure 1 for concentrations of glucose, yeast extract, and peptone in the medium. Results shown are the average of duplicate measurements ± one standard deviation (SD) except for cell numbers at 0 h (A, B, and C) and 40 h (B), which represent a single measurement. For those points at which SD's are not visible, the error bars were smaller than the points as plotted.

Figure 4. Ratio of biotic versus abiotic metal reduction at 55 and 65°C with the indicated initial metal concentrations. Fe (III) reduction was measured as the increase of Fe (II). Results are the mean ± standard error (SE) for two to four parallel experiments except for Cr (VI) reduction at 55°C, which represents the average ± one SD of a single experiment (Figure 3C). For those bars in which the error bars are not visible, the SE is < 0.1.

Figure 1A

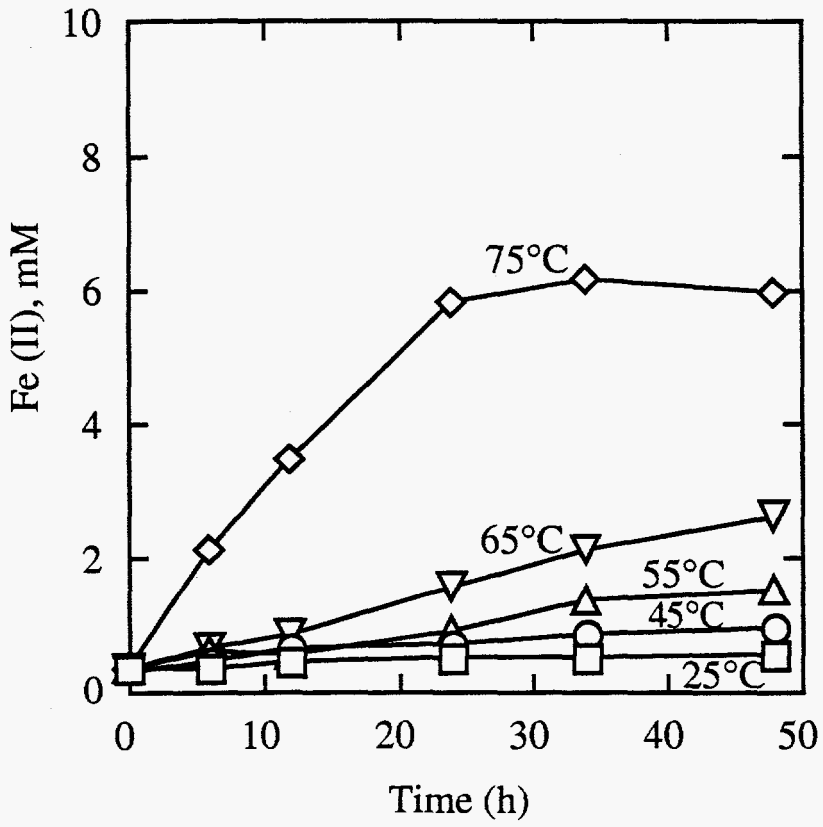


Figure 1B

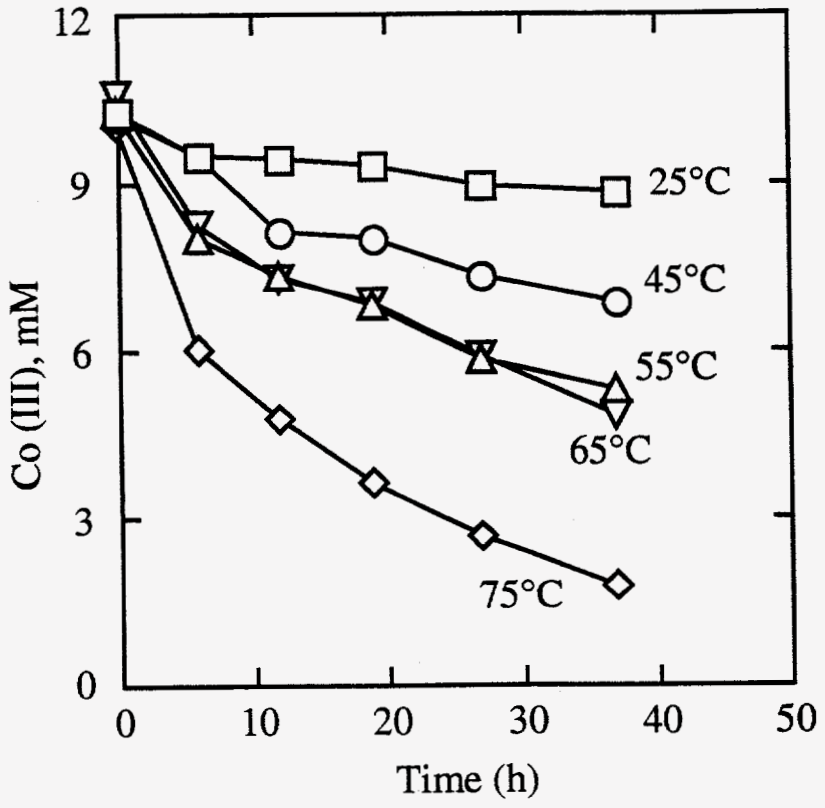


Figure 1C

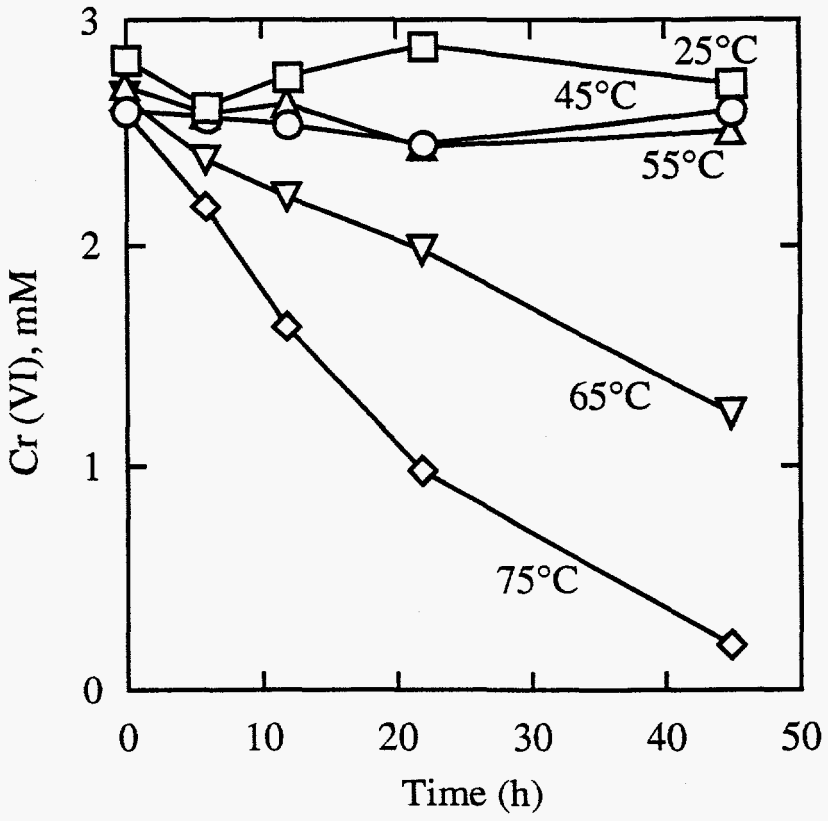


Figure 2

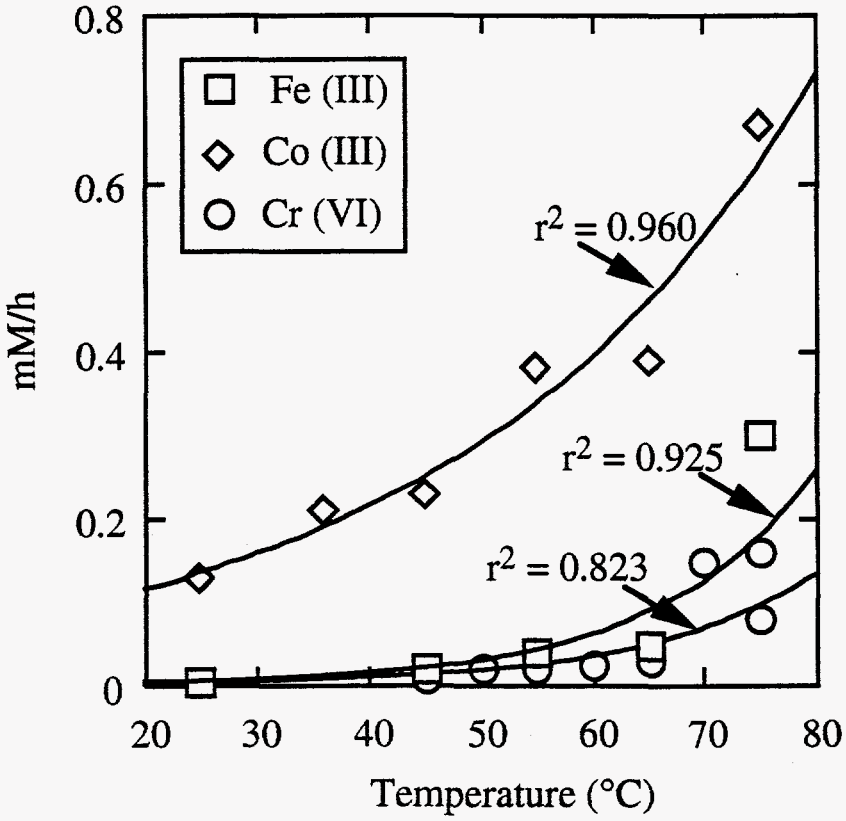

0.8
0.6
0.4
0.2
0
mM/h
Fe (III)
Co (III)
Cr (VI)
r2 = 0.960
r2 = 0.925
r2 = 0.823
20
30
40
50
60
70
80
Temperature (°C)

Figure 3A

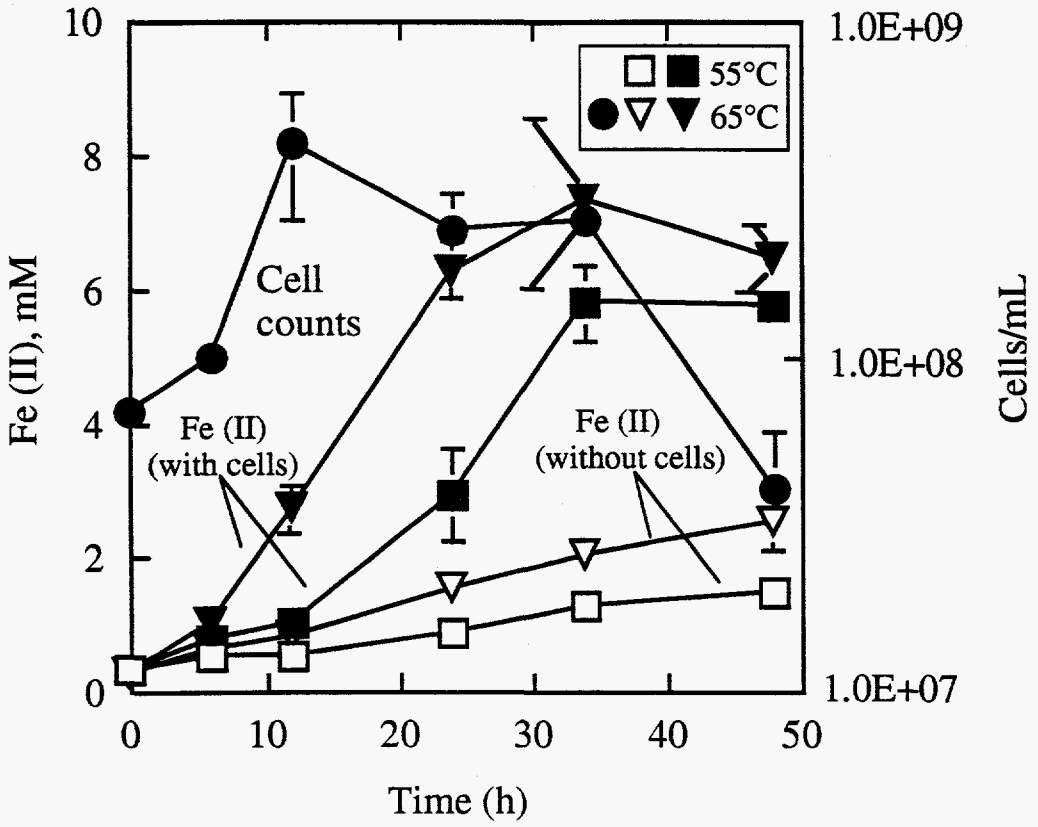

55°C
65°C
Cell counts
Fe (II) (with cells)
Fe (II) (without cells)
Fe (II), mM
Cells/mL
Time (h)
0
2
4
6
8
10
0
10
20
30
40
50
1.0E+07
1.0E+08
1.0E+09

Figure 3B

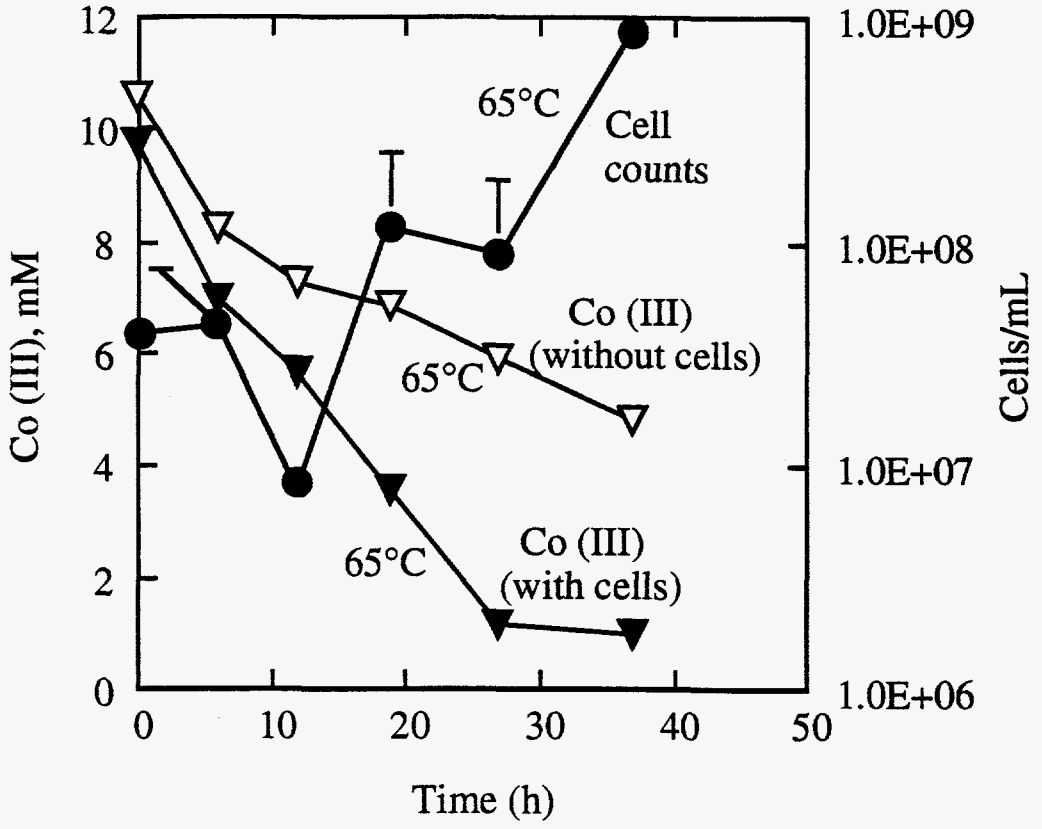

12
10
8
6
4
2
0
Co (III), mM
0
10
20
30
40
50
Time (h)
1.0E+09
1.0E+08
1.0E+07
1.0E+06
Cells/mL
65°C
Cell
counts
Co (III)
(without cells)
65°C
65°C
Co (III)
(with cells)

Figure 3C

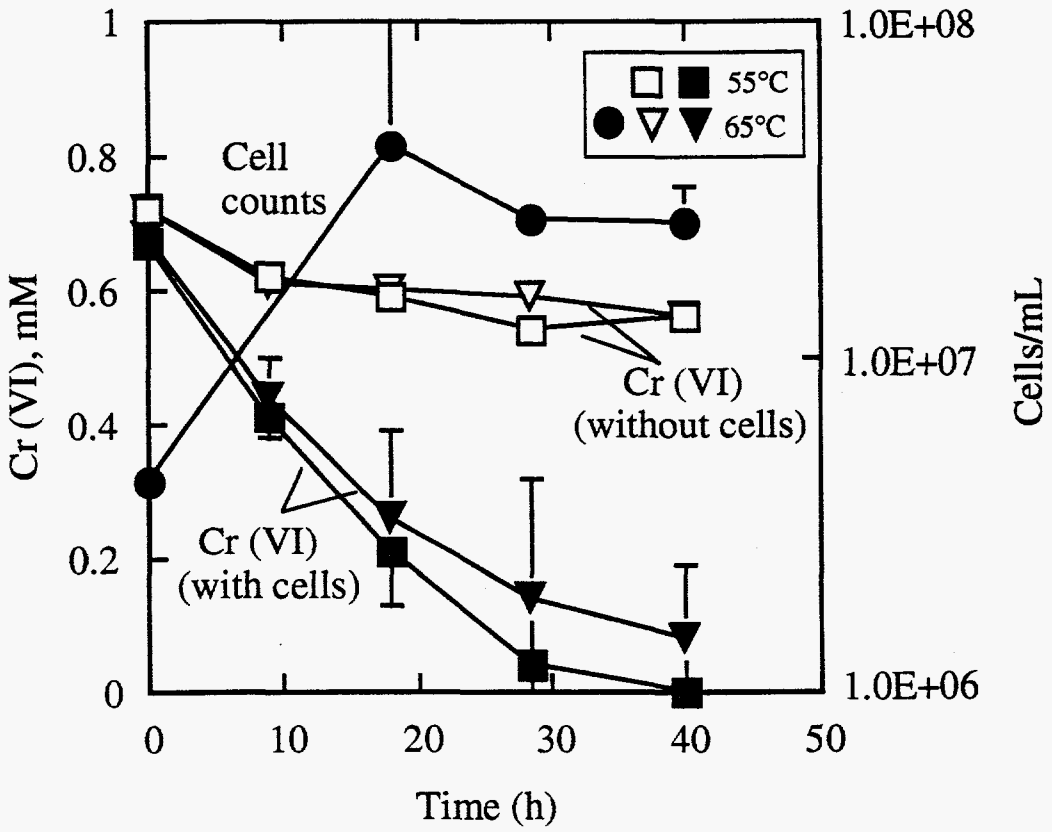
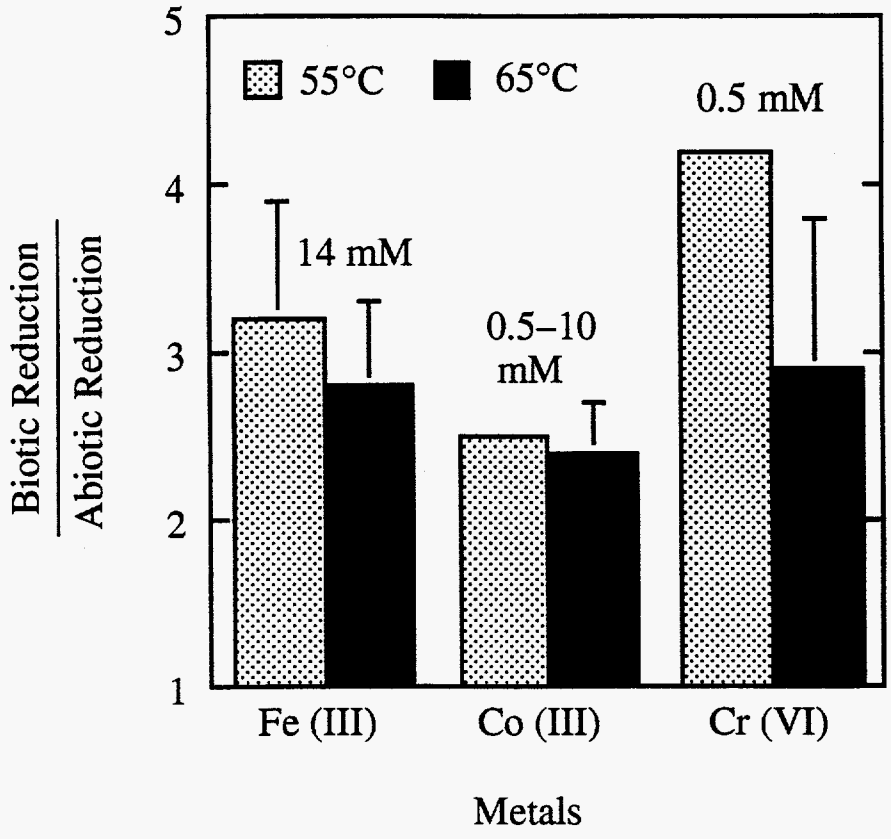

1
0.8
0.6
0.4
0.2
0
Cr (VI), mM
1.0E+08
1.0E+07
1.0E+06
Cells/mL
0
10
20
30
40
50
Time (h)
55°C
65°C
Cell counts
Cr (VI) (without cells)
Cr (VI) (with cells)

Figure 4


5
4
3
2
1
55°C
65°C
0.5 mM
14 mM
0.5–10 mM
Biotic Reduction
Abiotic Reduction
Fe (III)
Co (III)
Cr (VI)
Metals