Optimum pH for Oxidation of Mn(II) Ions in Model and Actual Manganese Drainages by a Mn-Oxidizing Fungus, *Phoma* sp. Strain KY-1

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A Mn-oxidizing fungus, *Phoma* sp. strain KY-1, showed a Mn-oxidizing activity of more than 1.82 mol m^{-3} of Mn(II) at an optimum pH 6.8. Controlling the pH at between 6.5 and 7.3 was necessary for fungal oxidation of Mn(II). This is probably due to inactivation of the Mn-oxidizing enzyme by decrease in pH during the reaction. Carbon fiber was found to catalyze the oxidation of Mn(II), most significantly under more unfavorable conditions such as high Mn concentrations, coexisting inhibitive components, or lacking nutrients. Examinations of fungus used for actual Mn-rich mine drainage containing more than 1.46 mol m^{-3} of Mn showed that organic nourishments and pH-buffering agents are both essential to obtain acceptable Mn removal rates and that the time required to attain the 10 mg dm^{-3} (0.182 mol m^{-3}), which is the maximum acceptable concentration of Mn in discharged wastewater in Japan, was about 170 h in the presence of carbon fiber.

[doi:10.2320/matertrans.M-MRA2008802]

(Received July 17, 2007; Accepted December 25, 2007; Published March 5, 2008)

Keywords: a manganese-oxidizing fungus, carbon fiber, manganese-rich mine drainage, pH-buffering agent

1. Introduction

Manganese (Mn) is the second most abundant transition metal in the earth's crust behind Fe and, like Fe, its oxidation-reduction reactions are largely mediated by microbiological activity.¹⁾ Microbial catalysis is known to accelerate the kinetics of Mn(II) oxidation and promotes the formation of Mn(III, IV) oxide minerals at neutral pHs in natural waters.²⁻⁴⁾ Manganese oxides are produced biogenetically by numerous species of bacteria, including *Leptothrix discophora* and *Pseudomonas putida* strain GB-1, but few species of fungi were found to be involved in the biogeochemical cycles of Mn.

At one time manganese was mined on a massive scale in Hokkaido, Japan, but at present all Mn-mines are abandoned. Agencies in the Japanese government have taken the responsibility of treating water with high Mn concentrations discharged from many of these mines. The ore at these mines is veins of rhodocrosite (MnCO_3). Insoluble manganese oxides (MnO_2) such as pyrolusite and ramsdellite are in common worldwide. Because rhodocrosite is relatively soluble, drainage with Mn released by weathering spills out throughout the year. To limit the risk for human health, the maximum concentration limit (MCL) for Mn in discharged waters is regulated to be 10 mg dm^{-3} in Japan and US. Treatment of the water to remove Mn(II) is commonly done using a conventional chemical process; a sequence of alkalization, aeration, sedimentation, and neutralization. These chemical treatment costs are high and discharge concentrations of dissolved salts are high enough that additional treatment is often required. Bioremediation is an attractive and alternative approach to remediation of Mn-bearing drainage, because it can be carried out in neutral media, may be less costly, and may be less harmful to environment. Biological treatment is usually slow but it can be applied to constructed wetlands, where the aim is to treat

the water over an extended period of time.

During bioremediation by microbial oxidation of Mn(II), manganese-oxidizing bacteria are usually considered, but we found that they are too delicate for this task.⁵⁾ Previously we reported a new manganese-oxidizing fungus isolated from a hot spring in Hokkaido.⁶⁾ A brief summary of the characteristics of this fungus is as follows:⁶⁻⁸⁾ It is closely related to *Phoma* sp. by morphological and genetic analyses based on the gene sequence of ITS1-5.8SrRNA-ITS2 but is not identical to any in the database, giving the name of *Phoma* sp. strain KY-1; it shows high Mn(II)-oxidizing activity in poorly nourished aqueous media, and with relatively high ambient dissolved Mn concentrations (1.09 mol m^{-3}) at pH 7.3; it entangles with and clings to carbon fibers put in the media and the oxidized Mn species accumulates on the fungus; and its oxidation ability is enhanced in the presence of carbon fiber, while polyethylene terephthalate fiber and unwoven carbon cloth do not enhance the oxidation, nor is adhesion of the fungus observed.

In the present work, the optimum pH range for Mn(II) oxidation by this fungus and its tolerance to Mn(II) ions were investigated using simulated drainages. In addition, oxidation experiments using actual drainage at an abandoned manganese mine in Hokkaido were carried out.

2. Experimental Conditions

Details about the isolation and culture of the fungus are described elsewhere.⁶⁾ Commercial PAN-based carbon fiber supplied as a 2–3 mm wide yarn tape with 7–10 μm diameter individual fibers was used as the carrier for the fungus. The fiber was cut into 30 cm lengths and then tied into a loop to avoid loose threads during experiments. Sizing agents on the fiber surface were removed by heating at 500°C for 2 h in air. With this treatment, the surface became hydrophilic. As already reported this carbon fiber neither adsorbs Mn(II) ions nor oxidizes Mn(II) ions catalytically.^{7,8)}

The composition of the simulated drainage is similar to the

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Fig. 1 Batch experiment Mn(II) removal rates at different pHs (a) with and (b) without carbon fiber. The standard deviations were omitted for simplicity.

growth medium for the Mn-oxidizing bacteria, *L. discophora*, but organic nourishments were at low concentrations (per 1 dm³ of distilled water: MgSO₄·7H₂O 0.6 g, CaCl₂·2H₂O 0.07 g, peptone 0.05 g, yeast extract 0.05 g, pH-buffering agent 0.15 mol). In this solution, peptone and yeast extract are organic nourishments for the fungus. This drainage was sterilized by autoclaving at 120°C for 15 min. Up to 2.73 mol m⁻³ of filter-sterilized MnSO₄·5H₂O solution was then added as a Mn(II) ion source and the solution pH was adjusted to a desired value. An appropriate pH-buffering agent was selected depending on the pH; for pH 6.3, 2-(N-morpholino)ethanesulfonic acid, monohydrate (MES, $pK_a = 6.15$); for pH 6.5, 6.8 and 7.0, piperazine-N,N'-bis(ethanesulfonic acid) (PIPES, $pK_a = 6.80$); and for pH 7.3 and 7.5, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES, $pK_a = 7.55$). The pHs of the buffer solutions were adjusted with a dilute NaOH solution. A 500 cm³ Erlenmeyer flask was filled with 150 cm³ of the model drainage and either with or without the loops of sterilized carbon fiber, then 0.75 g (wet weight) of fungus, which was cultured in the standard medium (pH 7.3, MgSO₄·7H₂O 0.6 g, CaCl₂·2H₂O 0.07 g, peptone 0.5 g, yeast extract 0.5 g, glucose 0.5 g, HEPES 3.57 g (0.15 mol) per 1 dm³ of distilled water), was inoculated into each flask. Carryover of the organic nourishments by the above inoculation was less than a few percent of those contained in the model drainage.

All oxidation experiments were carried out in a rotary-shaking culture-apparatus operated at 75 rpm at an incubation temperature of 25°C and for a maximum 250 h under light shielding. At timed intervals, the supernatants were sampled, membrane filtered to 0.22 µm, and appropriately diluted with 1 mol dm⁻³ hydrochloric acid. Dissolved Mn species were determined by atomic absorption spectrometry (Hitachi, Z-6100).

Actual manganese-bearing drainages were collected from the Jokoku Mine located in the southern Hokkaido, Japan. The pHs of these drainages which are nearly saturated with CO₂ are 6 to 7 depending on the season. The drainage used in the present work was collected in August 2004, and has a dissolved manganese concentration [Mn(II)] of 1.86

mol m⁻³. The water was aerated to remove dissolved CO₂ until a steady pH of 7.8 was attained. Suspended matters formed due to aeration were filtered using paper filters No. 5A, resulting in a final Mn(II) concentration of 1.48 (1σ = 0.029) mol m⁻³. Major components were determined by atomic absorption spectrometry and ion chromatography (DIONEX DX-AQ) include Fe (0.0578 mol m⁻³), Na (8.26 mol m⁻³), K (1.73 mol m⁻³), Mg (2.45 mol m⁻³), Ca (11.1 mol m⁻³), F (0.016 mol m⁻³), Cl (27.6 mol m⁻³), PO₄ (0.032 mol m⁻³), SO₄ (12.9 mol m⁻³). The total organic carbon content of the water measured using a TOC meter (Shimadzu, TOC-5000A) was 0.924 mol m⁻³. Oxidation experiments with this drainage were first carried out after sterilizing it as described above with or without a pH-buffering agent and/or nourishing components. The sterilization in an autoclave precipitated a part of Mn(II) ions due to the formation not of manganese oxides but of MnCO₃ and/or Mn(OH)₂, therefore, the dissolved Mn(II) ion concentration was decreased to 1.01 (1σ = 0.042) mol m⁻³. After these experiments, the unsterilized drainage was used. Detailed conditions are described below.

Unless otherwise noted, the oxidation experiments were done simultaneously in four replicates of flask for each experimental condition, and the results were averaged, with or without one standard deviation as indicated.

3. Results and Discussion

3.1 Oxidation experiment with simulated drainages

As the fungus used in the present work is known to oxidize 60 mg dm⁻³ (1.09 mol m⁻³) of Mn(II) ions at pH 7.3,⁶ the effect of solution pH was examined at this initial Mn(II) concentration. As shown in Figure 1, Mn(II) oxidation activity by this fungus was observed in the pH range of 6.5 to 7.3 with and without adding the carbon fiber into the drainages. During the Mn(II) oxidation, pH decreased due to the formation of organic acids depending on the amounts of organic compounds and Mn(II) added in the medium. Under the optimal conditions at the initial pH 6.8 in Fig. 1, the final pH was 6.3. In Fig. 1 the curves for pH 6.3 appear to be on a positive slope, however, this is only due to averaging of

Fig. 2 Batch experiment Mn(II) removal rates for different initial Mn(II) ion concentrations (a) with and (b) without carbon fiber at pH 6.8. Error bars are $\pm 1\sigma$.

scattered data. It is expected that the minimum pH for the Mn(II)-oxidizing enzyme is a little lower than that for the fungal metabolism to release the enzyme. The maximum oxidation rate was attained at the initial pH 6.8 to 7.0, and with the carbon fiber added the Mn(II) ion concentration decreased to less than 10 mg dm^{-3} (0.182 mol m^{-3}), which is the MCL for Mn in wastewater discharged in Japan, within 50 h. The catalyzing effect of carbon fiber was evident at all pH values tested, but it was more prominent outside of the optimum pH range. In the experiments shown in Fig. 1, appropriate pH buffering agents were used for each pH as described in the experimental section. Additional experiments were carried out at pH 6.8 and 7.0 using a HEPES ($pK_a = 7.55$) pH buffering agent instead of PIPES ($pK_a = 6.80$) to evaluate the sensitivity of the experiment to the type of buffering agent used. When the carbon fiber was present in the solution, Mn(II) removal rate curves were very similar, indicating that the oxidation rate was not affected by the kind of pH buffering agents. In the absence of carbon fiber, however, the time required to attain the MCL was somewhat longer with HEPES than with PIPES. This decreased rate was accompanied by a decrease in pH during the fungal oxidation of Mn(II) ions, indicating that the pH buffering ability of HEPES is not sufficient at these pH values. At both starting pH values, pH decreased to 6.5 or lower after about 40 h with or without the carbon fiber with this decrease in pH in the test done without carbon fiber, while the solutions with the carbon fiber were not affected. We have many data which indicate that the more unfavorable conditions become, the more marked the catalyzing effect of the carbon fiber. Rational explanations for the catalyzing behaviour of the carbon fiber have not been developed. Similar experiments using HEPES at pH 6.5 resulted in the termination of oxidation at $[\text{Mn(II)}] \approx 0.273 \text{ mol m}^{-3}$ after 40 h, even in the presence of the carbon fiber. It is a natural consequence, since pH values became lower than 6 after about 30 h.

The tolerance of the fungus to high initial Mn(II) concentration, $[\text{Mn(II)}]_0$, was tested at an initial pH of 6.8 (with PIPES) and the results were shown in Fig. 2. At high

initial concentrations (e.g. $> 2 \text{ mol m}^{-3}$), the effect of the carbon fiber is significant. With the carbon fiber, 80 h is required to attain $[\text{Mn(II)}] < 10 \text{ mg dm}^{-3}$ from $[\text{Mn(II)}]_0$ of 1.82 mol m^{-3} and 210 h is required to attain $[\text{Mn(II)}]_0 < 10 \text{ mg dm}^{-3}$ from $[\text{Mn(II)}]_0$ of 2.18 mol m^{-3} . Without the carbon fiber, the MCL is attained after 145 h for $[\text{Mn(II)}]_0 = 1.82 \text{ mol m}^{-3}$ but is not achieved until $> 240 \text{ h}$ when the $[\text{Mn(II)}]_0$ was 2.18 mol m^{-3} and 2.73 mol m^{-3} . With or without the carbon fiber, the oxidation rate after 100 h becomes slow. This slowdown is not due to the decrease of pH below the lower limit. As described in the next section, in the case of Jokoku mine drainage this fungus did not oxidize Mn(II) when organic nourishments were not added. It is possible that the inactivity may be due to a lack of organic nourishments for the fungus. A difficult aspect of the behavior of this fungus is that when large amounts of organic nourishment are included in the initial solution, it does not oxidize Mn(II).⁷⁾ It was found that the initial Mn amounts should be $0.91\text{--}1.82 \text{ mol m}^{-3}$. Therefore, for further experiments, a slow continuous supply of organic nourishments should be considered.

3.2 Treatment of a Mn-rich mine drainage

Figure 3(a) shows the rates of Mn(II) decrease for the mine drainage containing the added organic nourishments (0.05 g dm^{-3} each of peptone and yeast extract) and PIPES pH buffer under the same concentration as simulated drainages at pH 6.8. These solutions were sterilized by autoclaving prior to inoculation of the fungus. It was confirmed in preliminary experiments that the fungal oxidation of larger amounts of Mn(II) requires larger amounts of organic nourishments, but also that too much organic nourishment inhibits fungal oxidation of Mn(II). Figure 3(a) indicates that the fungus can oxidize 1.00 mol m^{-3} Mn(II) ions in the Jokoku mine drainage under these conditions. The PIPES pH-buffering reagent buffered the pH of the experiment at 6.7 with and without carbon fiber. This pH is in a range of 6.5 to 7.3 where the Mn-oxidation by the fungus is active (Fig. 1(a)). In the Jokoku drainage $[\text{Mn(II)}]$ decreased to less than 0.182 mol m^{-3} over about 60 h with carbon fiber and

Fig. 3 Batch experiment Mn(II) removal rates for actual mine drainage sterilized at 120°C (a) with nutritious compounds and PIPES and (b) with nutritious compounds but no PIPES. The initial pH was 6.8 for both. Filled symbols are with carbon fiber and open symbols without carbon fiber. Error bars of concentration are $\pm 1\sigma$ and no error bar for pH.

over 80 h without the carbon fiber. These are slower rates of Mn removal than for the simulated drainage of similar $[\text{Mn(II)}]_0$ (Fig. 1), but the rates are acceptable. Without the organic nourishments, and with 0.15 mol dm^{-3} of PIPES and 0.924 mol m^{-3} of organic carbon, Mn(II) concentrations did not decrease measurably (Fig. 3(b)). Additional experiments with small amounts of ammonium added as a source of nitrogen also failed. Accordingly, organic nourishments, such as peptone and yeast extract, are essential to this fungus. In separate experiments, it was found that the addition of either peptone or yeast extract is sufficient to initiate oxidation of Mn(II) by the fungus. When PIPES was not added, the pH of the Jokoku drainage dropped steeply over 30 h to 6.1–6.2, as shown in Fig. 3(b), at which point the oxidation of Mn(II) and decreases in Mn concentrations cease. This indicates that some pH control is required.

As previously reported,⁷⁾ this fungus produces H_2O_2 and it is consumed during Mn(II) oxidation. The Mn-peroxidase (MnP) is likely to be involved in the oxidation of Mn(II) by this fungus. Perez and Jefferies⁹⁾ described the role of MnP during the oxidation of Mn(II) in a white rot fungus, *Phanerochaete chrysosporium*, as follows:

Organic acids stabilize Mn(III) and facilitate its diffusion from the active site, stimulating MnP activity. In all fungal Mn(II) oxidation reactions, the primary product is not Mn oxides, but soluble Mn(III)-complex, which is eventually reduced back to Mn(II) upon reaction with organic matter. When suitable complexing reagents are unavailable, the Mn(III) can disproportionate to form Mn(IV) oxides and Mn(II) ions. Reaction (4) causes the pH to decrease and the onset of mineralization by the precipitation of Mn oxides. When the pH decreases to less than the range where MnP is active, the fungal oxidation of Mn(II) becomes stagnant.

Fig. 4 Batch experiment Mn(II) removal rates for unsterilized mine drainage with nutritious compounds and PIPES at pH 6.8. Filled symbols are with carbon fiber and open symbols without carbon fiber. Error bars are $\pm 1\sigma$.

Aiming for practical application, oxidation experiments were carried out in the unsterilized Jokoku mine drainage containing 1.48 mol m^{-3} Mn(II) plus peptone, yeast extract (50 mg dm^{-3} each), and PIPES. The results for the experiment conducted at an initial pH of 6.8 are shown in Fig. 4. The oxidation rate becomes much slower, but with the carbon fiber the MCL for Mn is attained after about 170 h. Without carbon fiber the MCL is not attained within 250 h. This may be because the mine drainage contains components or microorganisms that are hostile to the fungus. High concentrations of chloride are present in the Jokoku drainage (997 mg/L) and might interfere with the fungal Mn(II) oxidation in the unsterilized drainage. As described above, here also the promoting effect of carbon fiber is displayed under unfavorable conditions. The results again illustrate the catalyzing effect of the carbon fiber on the fungal oxidation of Mn(II).

4. Conclusions

A Mn-oxidizing fungus, which was isolated from a constructed wetland and identified to be closely related to *Phoma* sp., showed the Mn-oxidizing activity more than 100 mg dm⁻³ of Mn(II) in the optimized medium at pH 6.8. The pH-buffering is inevitable for the fungal Mn(II) oxidation between 6.5 and 7.3. Addition of carbon fiber enhanced the fungal Mn(II) oxidation under any conditions. The promoting effect of carbon fiber was more significant under more unfavorable conditions like high Mn concentrations, coexisting inhibitive components, and lacking nutrients. According to the application experiments of the fungus to actual Mn-rich mine drainage containing more than 80 mg dm⁻³ (1.45 mol m⁻³) of Mn, it was found that the time required to attain the MCL for Mn was about 170 h in the presence of carbon fiber, although both organic nourishments and pH-buffering agent are essential to obtain the acceptable Mn removal rate.

Acknowledgement

Financial support was provided by the Grant-in-Aid for Scientific Research (B) (No. 16360445) from Japan Society for the Promotion of Science (JSPS).

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