Kinetics of Mn(II) oxidation by *Leptothrix discophora* SS1

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Abstract—The kinetics of Mn(II) oxidation by the bacterium *Leptothrix discophora* SS1 was investigated in this research. Cells were grown in a minimal mineral salts medium in which chemical speciation was well defined. Mn(II) oxidation was observed in a bioreactor under controlled conditions with pH, O_2, and temperature regulation. Mn(II) oxidation experiments were performed at cell concentrations between 24 mg/L and 35 mg/L, over a pH range from 6 to 8.5, between temperatures of 10°C and 40°C, over a dissolved oxygen range of 0 to 8.05 mg/L, and with *L. discophora* SS1 cells that were grown in the presence of Cu concentrations ranging from zero to 0.1 μM. Mn(II) oxidation rates were determined when the cultures grew to stationary phase and were found to be directly proportional to O_2 and cell concentrations over the ranges investigated. The optimum pH for Mn(II) oxidation was approximately 7.5, and the optimum temperature was 30°C. A Cu level as low as 0.02 μM was found to inhibit the growth rate and yield of *L. discophora* SS1 observed in shake flasks, while Cu levels between 0.02 and 0.1 μM stimulated the Mn(II) oxidation rate observed in bioreactors. An overall rate law for Mn(II) oxidation by *L. discophora* as a function of pH, temperature, dissolved oxygen concentration (D.O.), and Cu concentration is proposed. At circumneutral pH, the rate of biologically mediated Mn(II) oxidation is likely to exceed homogeneous abiotic Mn(II) oxidation at relatively low ($\approx\mu$g/L) concentrations of Mn oxidizing bacteria.

1. INTRODUCTION

Microbial intervention accounts for much of the Mn(II) oxidation in the environment and therefore the global cycling of Mn is strongly influenced by bacteria. Indeed, biologic catalysis has been well established as the dominant mechanism of Mn oxidation in circumneutral freshwater (Ghiorse, 1984; Nealson et al., 1988) and marine environments (Tebo and Emerson, 1986; Moffett, 1997). The existence of biologic rate controls on Mn(II) oxidation is strongly supported by the recent isolation of Mn-oxidizing enzymes and the identification of the putative genes responsible for Mn(II) oxidation (Tebo et al., 1997). Mn(II) oxidation governs not only the global cycling of Mn, but also the cycling of other trace metals via coupled adsorption processes as described below. Biologic oxidation of Mn(II) may also control Mn removal in water treatment plants (Stumm and Morgan, 1996) and may be an important factor in corrosion of metals (Dickinson et al., 1997).

The cycling of metals in aquatic environments is controlled by an array of biogeochemical process, including adsorption, oxidation/reduction, ligand binding reactions, mineral precipitation/dissolution, and uptake by biologic organisms. Adsorption at the solid/solution interface is thought to control toxic metal residence time and phase distribution in most aquatic systems. Particulate organic material and Fe/Mn oxides have been identified as prominent solid phases controlling transition metal adsorption (Vuceta and Morgan, 1978; Luoma and Bryan, 1981; Tessier et al., 1996; Nelson et al., 1999a; Nelson et al., 1999b; Dong et al., 2000). While trace metal adsorption to Fe oxyhydroxides has been studied extensively (Dzombak and Morel, 1990), adsorption to Mn oxides is less well characterized. Biologically oxidized Mn has recently been shown to bind Pb to a significantly greater extent than either colloidal Fe oxyhydroxide or abiotic Mn oxides (Nelson et al., 1999a). Indeed, the role of Mn oxides in controlling toxic trace metal distribution in the environment may have been underestimated because the abiotic Mn oxides widely employed in adsorption studies typically have lower specific surface areas than biologically oxidized Mn (Nelson et al., 1999a,b; Dong et al., 2000). Integration of the kinetics for biogenic Mn oxide formation into models for toxic metal interactions would likely increase their ability to predict trace metal fate and transport in natural systems. Thus, quantitative mechanistic models that predict either Mn cycling or toxic metal fate could benefit from the capacity to accurately describe bacterially catalyzed Mn(II) oxidation.

A rate law for the autocatalytic abiotic oxidation of Mn(II) was developed by Morgan and Stumm (1964). While thermodynamically favorable, abiotic Mn(II) oxidation is kinetically inhibited at pH values <9.0 and therefore it is likely that, at most ambient pH values, the rate of Mn(II) oxidation is subject to biologic control. Although there has been some prior research on the mechanisms of enzymatic catalysis of Mn(II) oxidation (Larsen et al., 1999; Tebo et al., 1997), no comprehensive rate equation has been proposed for microbially mediated Mn(II) oxidation kinetics.

Recent investigations have suggested that copper-dependent enzymes play a role in Mn(II) oxidation in three different Mn-oxidizing bacteria: *Pseudomonas putida* GB-1, *Bacillus* SG-1, and *Leptothrix discophora* (van Waasbergen et al., 1996; Corstjens et al., 1997; Brouwers et al., 1999; Brouwers et al., 2000a). Addition of Cu to culture media has been reported to increase the Mn(II) oxidizing activity of *P. putida* by a factor

of 5 (Brouwers et al., 1999). Similarly, 1 μM of Cu added to spore suspensions of *Bacillus* SG-1, that had been extracted with ethylenediaminetetraacetic acid (EDTA) (which would have removed Cu ions), optimally enhanced ($\approx 2\times$) their formation of particulate Mn relative to conditions with zero added Cu (van Waasbergen et al., 1996). In addition, Brouwers et al. (2000b) reported that Cu stimulated the Mn(II) oxidation activity of supernatants obtained from stationary-phase suspensions of *L. discophora* SS1 after the cells were grown in the presence of Cu; however Cu did not stimulate Mn(II) oxidation when added directly to the spent medium supernatant after growth of the bacterium without added Cu.

Prior studies of bacterially mediated Mn(II) oxidation kinetics have been performed under a variety of conditions. In most cases, at least one important physical/chemical parameter was not controlled, such as temperature, pH, O_2, the presence of multiple metal species, or the presence of ligands with undefined metal-binding properties in the medium. In this research, the kinetics of Mn(II) oxidation by *L. discophora* SS1 were investigated under well-defined bioreactor conditions. The influence of cell concentration, pH, temperature, dissolved oxygen and Cu concentration on the rate of Mn(II) oxidation was determined using cells grown in a minimum mineral salts (MMS) medium, in which chemical speciation was defined. The bioreactor system permitted control of pH, O_2, and temperature during the Mn(II) oxidation experiments without the use of pH buffers that could potentially affect Mn coordination chemistry.

2. MATERIAL AND METHODS

L. discophora SS1 (American Type Culture Collection [ATCC] 43821) is a heterotrophic, freshwater proteobacterium isolated from a wetland area in Ithaca, New York (Ghiorse and Chapnick, 1983). Pure cultures of *L. discophora* SS1 have been shown to oxidize Mn(II) extracellularly (Adams and Ghiorse, 1985). Cultures were maintained at 4°C on plates of mineral salts–vitamins–pyruvate (MSVP) medium [per liter of deionized water: 0.24 g $(NH_4)_2SO_4$, 0.06 g $MgSO_4 \cdot 7H_2O$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.02 g KH_2PO_4, 0.03 g Na_2HPO_4, 1 mL of 10-mM solution $FeSO_4 \cdot 7H_2O$, 2.5 mL of 4× vitamin solution (Staley, 1968), 5 mL of Na pyruvate stock (20%), 10 mL of Mn stock (0.1 mM), 10 mM HEPES (*N*-2-hydrooxyethylpiperazine-*N*′-2ethanesulfonic acid) buffer, pH 7.2, and agar at 1.5 to 2.0%]. Before the addition of vitamins, pyruvate, and Mn, the medium was autoclaved for 20 min. Vitamins, pyruvate, and Mn (all 0.2 μm filter sterilized) were then added to the solution.

Inocula from plates were first grown in 50 mL of a defined MMS medium (Table 1, from Nelson et al., 1999a) with 0.25 mg/L peptone and 0.5 mg/L yeast extract added in 250-mL flasks on a rotary shaker (New Brunswick Scientific, Innova 200) at 150 rpm. Inocula (2.5 mL) from this initial culture were then transferred to 50 mL MMS prepared without addition of peptone or yeast extract. When cells were in exponential growth, 2.5 mL were again transferred to 50 mL of fresh MMS medium. In some cases, this transfer sequence was repeated again to ensure that a rapidly growing population was obtained in the MMS medium. The resulting cell suspensions were used for inoculum for all subsequent experiments.

It was difficult to achieve reliable growth of *L. discophora* directly in the bioreactor. Therefore, cell suspensions were first grown in shake flasks and then transferred to bioreactors for subsequent Mn oxidation under controlled conditions. Because of the use of this procedure, all Mn oxidation was measured with the culture in stationary growth phase. To prepare the cell suspensions, approximately 30 mL of the rapidly growing culture was then transferred to 600 to 800 mL MMS medium in a 1-L Erlenmeyer flask, and placed on a rotary shaker @ 150 rpm.

Table 1. Composition of MMS medium used for growing *L. discophora* cell suspensions for measurement of Mn oxidation rates.

Component	Concentration in distilled deionized H_2O (μM)
Pyruvate	2900
$CaCl_2 \cdot H_2O$	200
$MgSO_4 \cdot 7H_2O$	140
$(NH_4)_2SO_4$	910
KNO_3	150
$NaHCO_3$	10
KH_2PO_4	5
Vitamin B_{12}	0.0015
$FeSO_4$	0.1

The ionic strength was adjusted to 0.05 M with $NaNO_3$. The medium was not buffered, and the pH increased from 6.0 to 7.8 during cell growth.

After the shake flask culture reached stationary phase (as measured by optical density (O.D.) at 600 nm), the entire cell suspension was transferred to a stirred bioreactor that had pH, temperature, and dissolved oxygen control as described below. The total volume of cell suspension added to each bioreactor was 0.50 L. Dry weights of the cell suspension were determined by analyzing the total suspended solids (American Public Health Association, 1995). Oxidation of added Mn(II) was monitored by removing 20-mL aliquots of broth at approximately 1-h intervals for analysis of dissolved Mn(II) concentration. Samples were filtered through a 0.2-μm membrane to remove cells and oxidized Mn. The Mn(II) in the filtrate was analyzed by atomic absorption spectroscopy (Perkin Elmer, AAnalyst 100) with an air-acetylene flame. Replication of Mn(II) concentrations was within 5% relative standard deviation.

Previous experiments with *L. discophora* indicated that Mn(II) adsorption to cells was negligible under the experimental conditions, as confirmed by the absence of Mn(II) release from the solid phase by rinsing with $CuSO_4$ solution (Nelson et al., 1999a). Also, previous experiments with azide-inhibited *L. discophora* showed no decrease in soluble Mn(II) concentration over the course of several days (Nelson et al., 1999a), further indicating the absence of Mn(II) adsorption to the cells. Therefore, the observed loss of soluble Mn(II) from solution can be reliably attributed to Mn(II) oxidation.

The bioreactor system constructed for the study of Mn(II) oxidation kinetics is illustrated in Figure 1. Water from a constant-temperature circulating bath (Neslab, model 1162) was pumped through the bioreactor jacket to control the temperature (± 0.1°C).

A pH controller (Cole Parmer, Chemcadet) was used to control addition of 0.01 mol/L solutions of NaOH and HNO_3 to

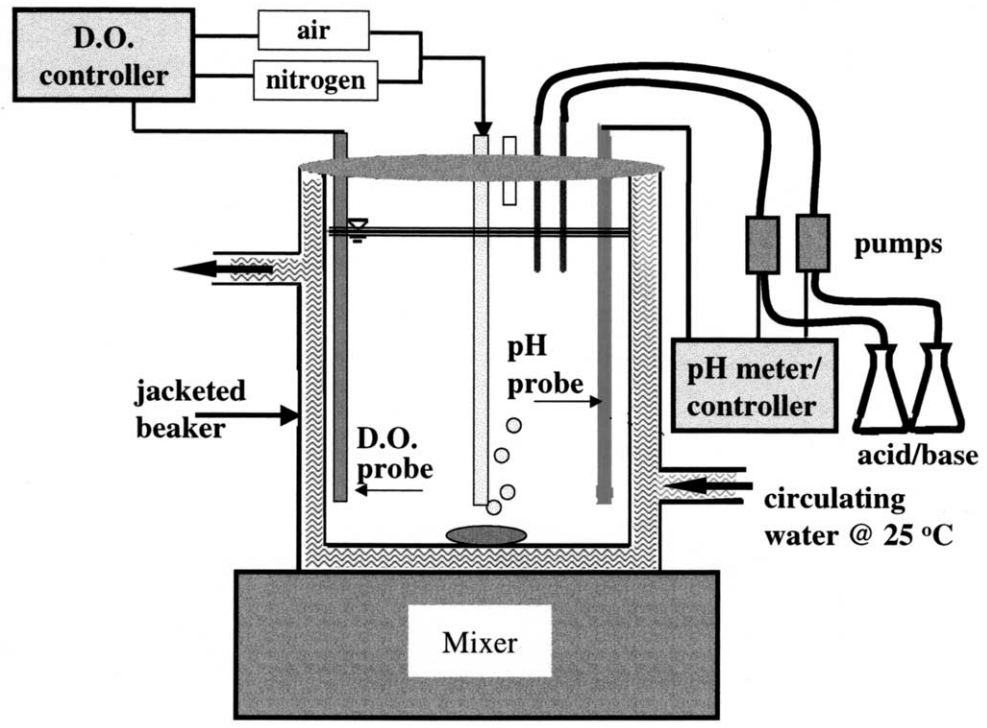

Fig. 1. Schematic drawing of the bioreactor used for study of Mn(II) oxidation kinetics under conditions of controlled temperature, pH, and dissolved oxygen concentration.

maintain the desired pH (±0.1 pH unit). Use of pH control avoided the use of organic acid buffers that would be expected to bind cationic metals via their carboxyl functional groups. The metal binding constants of many commonly used organic buffers have not been determined; therefore, their use precludes knowledge of metal speciation. Alternatively, use of phosphate or carbonate buffers exacerbates the risk of forming metal precipitates at higher pH values. Therefore, use of simple inorganic acid and base additions in conjunction with a pH controller was deemed to be the preferred option for maintaining a known metal speciation.

A dissolved oxygen controller (Cole Parmer, Chemcadet) was used to regulate the flow of nitrogen and air. In experiments with variable dissolved oxygen, the bioreactor solution was purged with pure N_2 and then with air to define conditions of zero dissolved O_2 and 100% saturation.

The influence of copper on Mn(II) oxidation was tested by adding Cu(II) at different concentrations to MMS medium, coincident with the addition of the *L. discophora* SS1 inoculum. The growth of the cells with and without added Cu was monitored by O.D. measurement at 600 nm. After cells grew to stationary phase, they were transferred to the jacketed bioreactor system, and the procedure described above was used to measure the Mn(II) oxidation rate.

3. RESULTS AND DISCUSSION

3.1. Measurement of Mn(II) Oxidation Rates

Typical growth curves of *L. discophora* SS1 in MMS medium in the 1-L flask cultures are shown in Figure 2. The maximum growth rate in exponential phase was 0.11/h (r^2 = 0.98) and the exponential death rate was 6×10^{-5}/h (r^2 = 0.99).

Preliminary experiments showed that if Mn(II) was added 24 h after the cells reached stationary phase, there was incomplete oxidation of added Mn(II). However, when Mn(II) was added while cells were in the early stationary phase, the Mn(II) oxidation rate was similar in bioreactors that received Mn(II) additions within 5 h of the onset of stationary phase. Thus, Mn(II) oxidation kinetics were reasonably constant in the first 5 h of the stationary phase. This result is consistent with the findings of Adams and Ghiorse (1985) who reported that the highest Mn(II) oxidizing activity of *L. discophora* SS1 was obtained in early stationary-phase cultures. In all subsequent experiments Mn(II) was added within the first 5 h of the stationary phase of growth.

3.2. Effect of Cell Concentration

To determine the effect of cell concentration on the rate of Mn(II) oxidation, a series of Mn(II) oxidation experiments was performed at four different cell concentrations ranging from 24 to 35 mg/L (at room temperature (≈22°C), pH = 7.5, and 100% air saturation). The results showed that the Mn oxidation rate was directly proportional to cell concentration, with a maximum specific oxidation rate of 0.0052 ± 0.0005 μmol Mn(II)/(min · mg cell) (r^2 = 0.99) under these conditions.

3.3. Effect of Mn(II) Concentration

Biologic Mn oxidation was expected to follow Michaelis-Menten enzyme kinetics, with the corresponding rate law:

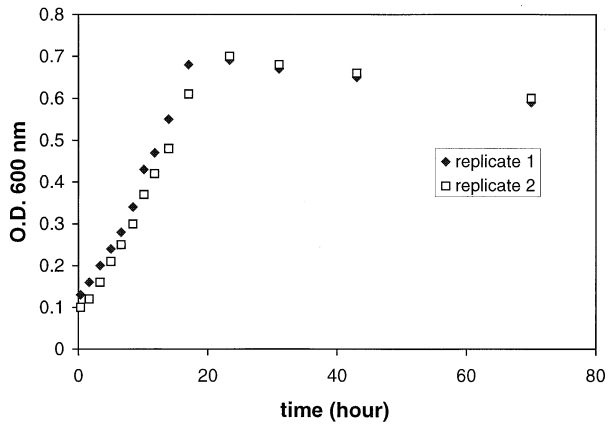

Fig. 2. Growth curve of *L. discophora* SS1. Batch cultures were grown at room temperature with no added Cu and without pH control in shake flasks with MMS medium.

$$\frac{dMn(II)}{[X]dt} = -\frac{k[Mn(II)]}{K_S + [Mn(II)]} \quad (1)$$

where K_S is the half velocity constant, (the concentration of Mn(II) when the oxidation rate is half of the maximum), k is the maximum Mn(II) oxidation rate [μmol Mn(II)/(mg cell · min)], and [X] is the cell concentration (mg/L).

In this model, the value of k is expected to vary with pH, temperature, dissolved oxygen concentration (D.O.), and copper concentration. When these parameters were kept constant (D.O. = 8.05 mg/L, T = 25°C, pH = 7.5, and zero added Cu), the Mn(II) oxidation rate increased with increasing Mn(II) concentration as described by the Michaelis-Menten model (Fig. 3). A nonlinear least squares analysis of the data gave K_S = 5.7 μmol Mn(II)/L and k = 0.0059 μmol Mn(II)/(min · mg cell). This value of k was used for a reference datum, k_{ref}, in our subsequent analysis of pH, O_2, T, and Cu effects on the Mn(II) oxidation rate. It should be noted that the K_S we observed in these experiments is close to the value of 6 μM reported by Adams and Ghiorse (1987) for Mn(II) oxidation by

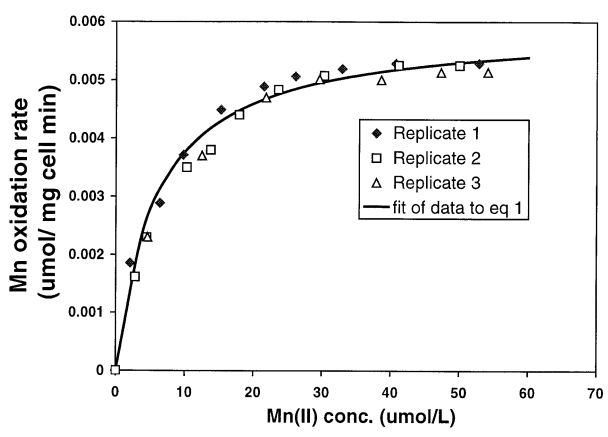

Fig. 3. Mn(II) oxidation rates as a function of Mn(II) concentration, showing Michaelis-Menten oxidation kinetics for Mn(II) at T = 25°C, pH = 7.5, and O_2 = 8.05 mg/L, and zero added Cu.

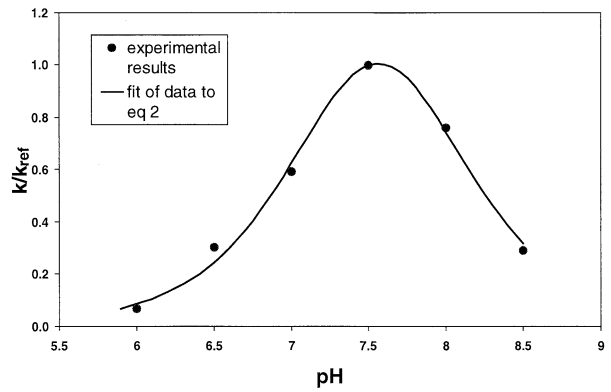

Fig. 4. pH influence on Mn(II) oxidation rate in the bioreactor at T = 25°C, O_2 = 8.05 mg/L, and zero added Cu.

L. discophora SS1 in a complex medium in which the speciation of other metals, Mn(II), and metal-binding ligands was not defined. These results suggest that K_S was not affected by the presence of undefined metals or by metal complexation with organic ligands in the previous investigations.

3.4. Effect of pH

The effect of pH on Mn oxidation was explored by varying pH in 0.5 pH unit intervals from 6 to 8.5, while keeping temperature at 25°C, D.O. = 8.05 mg/L, added Cu = 0, and other conditions constant. In these experiments, a maximum in Mn oxidation rate was observed at pH 7.5 (Fig. 4).

The observed pH optimum of 7.5 matches the value reported by Boogerd and de Vrind (1987) and is close to the pH 7.3 optimum reported by Adams and Ghiorse (1987); both prior investigations used a medium in which Mn(II) speciation was not defined and that included buffers with overlapping pH ranges. All studies have found that the rate of biologic Mn(II) oxidation is strongly influenced by pH and is very slow at pH values less than 6 or greater than 8.5.

Theoretically, pH can affect the oxidation rate of Mn(II) via both the maximum reaction rate k, as well as the half velocity coefficient K_S. However, Bailey and Ollis (1986) suggest that pH effects on K_S are usually relatively insignificant and indicate that the effect of pH on the maximum rate of an enzymatic reaction can be modeled by an equation of the following form:

$$\left[\frac{k}{k_{ref}}\right]_{pH} = \frac{k_{pH}}{1 + [H^+]/K_1 + K_2/[H^+]} \quad (2)$$

where k is the Mn(II) oxidation rate per cell, $k_{ref} = k$ @ pH = 7.5, D.O. = 8.05 mg/L, T = 25°C, added Cu = 0, K_1 and K_2 are constants, and k_{pH} equals the value of $(1 + [H^+]/K_1 + K_2/[H^+])$ determined at pH = 7.5.

Least square data analysis for the experimental data in Figure 4 gave $K_1 = 3.05 \times 10^{-8}$, and $K_2 = 2.46 \times 10^{-8}$ ($r^2 = 0.99$), with a resulting value of k_{pH} = 2.82. The ability of Eqn. 2 to fit the experimental data was excellent (see Fig. 4).

Because Mn(II) speciation varies as a function of pH, Mn(II) speciation could have affected the above results. Mn speciation was calculated by the chemical equilibrium program MINEQL (Westall et al., 1976) for the defined MMS medium, and is

Table 2. Metal speciation in MMS medium as determined using the chemical speciation program MINEQL ([Mn(II)] = 50 μM, [Cu(II)] = 0.1 μM, $P_{CO_2} = 10^{-3.5}$ atm., T = 25°C).[a]

	pH					
Species (%)	6.0	6.5	7.0	7.5	8.0	8.5
Mn^{2+}	78.5	78.5	78.4	78.2	35.6	3.6
$MnSO_4$	10.9	10.9	10.9	10.0	5	0
$MnHPO_4$	9.9	10	10	10	10	0
Rhodochrosite[$MnCO_3$(s)]	0	0	0	0	48.8	95.8
Cu^{2+}	83.6	69.9	28.4	4.2	N/A	N/A
$Cu(OH)^+$	0	2.2	2.8	1.3	N/A	N/A
$Cu(OH)_2$(aq)	1.7	14.6	59.4	88.4	N/A	N/A
$CuSO_4$	13.1	10.9	3.3	0	N/A	N/A
$CuCO_3$(aq)	0	0	4.4	5	N/A	N/A

N/A = not applicable.
[a] Note that below pH 7.5, over 78% of the total Mn(II) is in the aquo metal ion form, while above pH 8.0 the speciation program predicts precipitation of $MnCO_3$(s).

dominated by the presence of Mn^{+2} below pH 7.5 (Table 2). Thus, the observed increase in Mn(II) oxidation rate between pH 6.0 and 7.5 (Fig. 4) is most likely the result of pH-dependent enzymatic activity rather than an influence of Mn(II) speciation. Above pH 7.5, the calculated free Mn^{2+} ion concentration decreases with increasing pH because of the thermodynamically favorable precipitation of rhodochrosite ($MnCO_3$) (Table 2). Although a decrease in Mn^{2+} availability could conceivably explain the decreasing Mn(II) oxidation rate at high pH, this explanation is thought to be unlikely because formation of a precipitate was not observed. Lack of a visible precipitate suggests that either a disequilibrium with atmospheric CO_2 (g) existed or that $MnCO_3$ (s) was supersaturated and consequently Mn(II) remained as a dissolved solute over the time frame of the experiments. Additionally, if a $MnCO_3$ (s) precipitate had formed, it would have been removed by filtration along with the Mn(III/IV) oxide. This removal of Mn(II) would have been attributed to Mn(II) oxidation and would have increased the "apparent" Mn(II) oxidation rate. Thus, the observed decrease in rate of Mn(II) oxidation above pH 7.5 is most likely a result of pH-dependent enzyme deactivation.

3.5. Effect of O_2 Concentration

The effect of O_2 on Mn oxidation was explored by measuring Mn(II) oxidation rate at different dissolved oxygen levels at pH = 7.5, T = 25°C, and added Cu = 0 (Fig. 5). The observed Mn(II) oxidation rate was directly proportional to O_2 concentration above 0.4 mg/L, suggesting that all O_2 concentrations considered were below the levels at which enzyme activity would be saturated with respect to oxygen. There was experimental difficulty with accurate O_2 control for O_2 levels below 0.4 mg/L. Because the deviation from a linear response was not great (Fig. 5), for purposes of rate estimation Mn(II) oxidation rate was considered to be directly proportional to dissolved oxygen concentration across the entire range of O_2 concentrations employed. The following relationship was used to account for the influence of O_2 on the rate of Mn(II) oxidation:

$$\left(\frac{k}{k_{ref}}\right)_{O_2} = k_{O_2}[O_2] \tag{3}$$

where $[O_2]$ is the oxygen concentration in mg/L and k_{O_2} = $(8.05\ \text{mg/L})^{-1}$ [note: 8.05 mg/L = O_2 saturation for air at 25°C and 0.05 mol/L ionic strength (Tchobanoglous and Schroeder, 1985)].

3.6. Effect of Temperature

Mn(II) oxidation rates were determined at different temperatures, ranging from 10°C to 40°C at pH = 7.5, with added Cu = 0 and 100% air saturation at each temperature. Because dissolved oxygen was shown to affect the Mn(II) oxidation rate, the experimental results were all normalized to O_2 = 8.05 mg/L (100% saturation at T = 25°C and I = 0.05 mol/L). The optimal temperature for Mn(II) oxidation was ~30°C (Fig. 6). In comparison, an optimal temperature of 28°C for *L. discophora* SS1 was previously reported by Adams and Ghiorse (1987) in an undefined medium.

The increase in Mn(II) oxidation rate between 10 and 30°C is attributed to temperature activation of the reaction. The rate is expected to vary according to the Arrhenius equation in this region:

$$\left(\frac{k}{k_{ref}}\right)_T = Ae^{-E_a/RT} \tag{4}$$

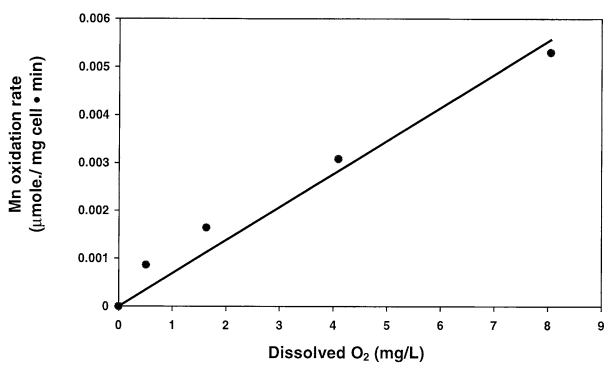

Fig. 5. Effect of dissolved oxygen concentration on Mn oxidation rate in the bioreactor at T = 25°C, pH = 7.5, and zero added Cu.

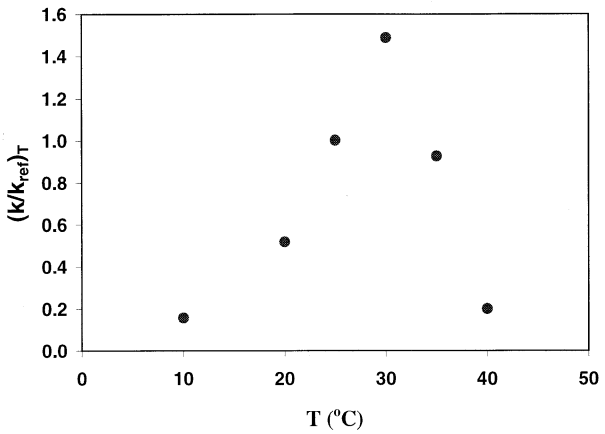

Fig. 6. Effect of temperature on Mn oxidation rate in the bioreactor at pH = 7.5, O_2 = 8.05 mg/L, and zero added Cu.

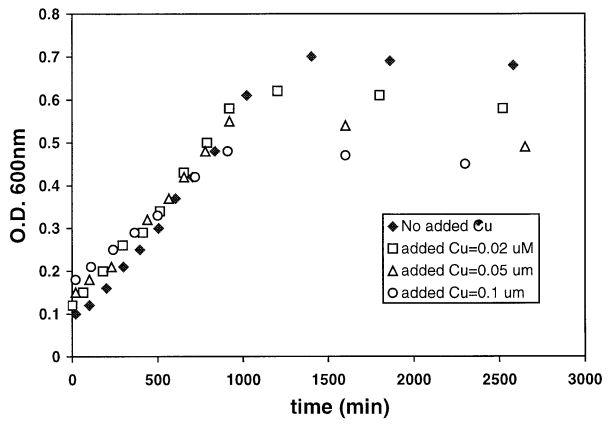

Fig. 7. Effect of added Cu on *L. discophora* SS1 growth curves in shake flask experiments at pH = 7.5, T = 25°C, and O_2 = 8.05 mg/L.

where k = maximum reaction rate per cell unit, E_a = activation energy, A is a constant (equal to $e^{E_a/RT}$ when T = 298 K), and T = absolute temperature.

A plot of ln k versus $1/T$ (not shown) resulted in a line of slope $-E_a/R$ (r^2 = 0.99), giving an activation energy for Mn(II) oxidation reaction equal to 22.9 kcal/g mol.

The decrease in Mn(II) oxidation rate above 30°C was attributed to thermal deactivation of the Mn-oxidizing enzyme system. Although models have been proposed for thermal deactivation (e.g., Shuler and Kargi, 1992), insufficient data were obtained at high temperatures to permit a meaningful estimation of the model parameters.

3.7. Effect of Cu

To test the effect of Cu on the growth and enzymatic Mn(II) oxidation activity of *L. discophora* SS1, Cu concentrations ranging from 0 to 0.15 μM were added to the medium upon inoculation. Effects on cell growth rate and yield were determined in the shake flasks used for preparing a cell suspension for observation of Mn(II) oxidation kinetics, whereas effects on Mn oxidation were investigated in bioreactors containing cell suspensions grown in the presence of different Cu concentrations. At the level of zero added Cu, which served as the reference condition for the experiments described above, a trace level of Cu may still be present in MMS medium as a reagent contaminant. $FeSO_4$ was the sole reagent-grade chemical employed for which the manufacturer reported a metal impurity. Based on the manufacturer's specifications, the calculated concentration of Cu in 0.1 μmol/L reagent grade $FeSO_4$ salt was less than 0.5 nM, and experimental measurement of copper concentration in MMS medium by specific ion electrode showed that the Cu^{+2} concentration was less than 1 nM. It was also possible that a Cu contaminant was introduced in the inoculum obtained from the initial culture of *L. discophora* in which peptone and yeast amendments were present. A Cu concentration = 0.088 μmol/L was measured in the concentrated peptone plus yeast extract stock used for the initial culture. Dilution of this stock solution and the additional sequential dilution achieved through $\geq 2\times$ transfer of inocula to MMS without added peptone and yeast extract give a maximum Cu concentration = 0.005 ng/L that could be carried over from a Cu impurity in the peptone and yeast extract solution. It is noteworthy that *L. discophora* produced an active Mn(II) oxidizing enzyme under the zero added Cu growth conditions, suggesting that either the cellular Cu requirement for synthesis of the putative copper-containing enzyme is extremely low ($\leq$0.5 nM), or that other metals may substitute for copper. In the latter regard, it is noteworthy that Nelson et al. (1999a) report inclusion of 0.1 μM Fe was needed in the MMS growth medium (that contains no added Cu) to obtain *L. discophora* cultures that actively oxidized Mn(II).

Experimental results for growth of *L. discophora* in the presence of Cu are shown in Figure 7 and the corresponding exponential growth rates are given in Table 3. The exponential growth rate (Table 3) and the cell yield (Table 4) both decreased as added Cu increased. The lag phase of growth also increased as added Cu increased (data not shown). There was no growth observed within 5 d when 0.15 μmol/L Cu was added upon inoculation.

The effect of Cu on cell yield can be modeled by the following empirical equation (@ [Cu] less than 0.1 μM):

$$Y = Y_{max}\left(\frac{K_I}{K_I + [Cu]}\right) \tag{5}$$

where Y is the cell yield, Y_{max} is the observed cell yield with zero added Cu, K_I is an inhibition constant, and $[Cu]$ is the total copper concentration in μmol/L. A nonlinear least square data analysis gives K_I = 0.24 μmol Cu/L (r^2 = 0.94).

Copper speciation may affect its bioavailability and toxicity.

Table 3. The effect of copper on the growth rate of *L. discophora* SS1 as determined in batch experiments in shake flasks.

Added Cu concentration (μmol/L)	Exponential growth rate (h^{-1})
0	0.110
0.02	0.102
0.05	0.096
0.1	0.072
0.15	0

Table 4. Effect of Cu on *L. discophora* SS-1 Mn oxidizing activity and cell yield (the yield and activity of a culture without added Cu(II) are defined as 100%).[a]

[Cu(II)] μM	Relative cell yield, %	Mn(II) oxidizing activity, %	Specific Mn(II) oxidizing activity (per equal cell weight), %
0	100	100	100
0.02	94.7	118.4	125.0
0.05	82.4	129.8	157.5
0.1	70.2	132.6	189.0
0.15	0	N/A	N/A

N/A = not applicable.
[a] Relative cell yields were determined in batch shake flask experiments, while Mn oxidizing activity was measured in bioreactors.

Cu speciation in MMS medium was calculated by MINEQL and is shown in Table 2. Because MMS medium contained no organic ligands with undefined metal binding properties (other than 1.5 nM vitamin B_{12}), the speciation of added Cu was entirely in the form of the free ion or as a complex with inorganic ligands. During the growth of *L. discophora* SS1, pH increased from the initial pH value of 6 to a final value of 7.8. Based on the calculations for Cu speciation (Table 2), the free Cu^{2+} concentration is expected to decrease from 84% of total Cu to 4% as the pH is increased from 6.0 to 7.5. This decrease is attributed almost entirely to the predicted formation of $Cu(OH)_2$ (aq). Because the inorganic hydroxyl complexes of Cu are expected to be labile, the total Cu concentration in the defined MMS medium is expected to be approximately equal to the bioavailable Cu concentration. Therefore, the total Cu concentration was used in the model calculations.

In the MMS medium, added Cu as low as 0.02 μM inhibited the growth rate and yield of *L. discophora* SS1, but Cu added at levels at and below 0.1 μM stimulated the Mn(II) oxidation activity (Table 4). It is noteworthy that similar results were reported by Browers et al. (2000b) where an optimum Cu concentration of 40 μM was reported for stimulation of Mn oxidation by *L. discophora* SS1. However this observation was obtained using a complex medium containing dissolved organic materials that could bind an undefined portion of the added Cu, reducing the activity of the free cupric ion, and perhaps altering Cu bioavailability and reducing Cu toxicity to cells.

The stimulatory effect of Cu on Mn(II) oxidation can be modeled by the following empirical equation:

$$\left(\frac{k}{k_{ref}}\right)_{Cu} = 1 + k_C[Cu] \tag{6}$$

where k is the Mn(II) oxidation rate with added Cu, and k_C is a stimulation constant. A least square analysis for the data gives $k_C = 8.8/(\mu\text{mol/L})$ ($r^2 = 0.97$).

In a separate experiment, Cu was added to the supernatant of stationary cultures grown in standard MMS medium after removal of cells by centrifuging. Although 76% of the enzymatic activity for oxidation of Mn(II) remained in solution after separation of cells, no stimulatory effect of added Cu on Mn(II) oxidation was observed. This result suggests that actively growing cells are required for the Cu stimulation effect to occur, presumably through cellular incorporation of copper in the active site of the enzyme as postulated by Brouwers et al. (2000b).

3.8. Rate Law for Biologic Mn(II) Oxidation

Based on this research, the following equation may be used to describe biologic catalysis of Mn(II) as a function of pH, cell concentration, O_2, Cu concentration and temperature at T $\leq$ 30°C, $[O_2] \leq 8.05$ mg/L, and added Cu ≤ 0.1 μM.

$$\frac{d[Mn(II])}{dt} = \frac{k[X][Mn(II)]}{K_S + [Mn(II)]}(k_{o_2}[O_2]) \cdot (Ae^{-Ea/RT})\left(\frac{k_{pH}}{1 + [H^+]/K_1 + K_2/[H^+}\right)(1 + k_c[Cu(II)]) \tag{7}$$

where $[X]$ = cell concentration, mg/L; $[O_2]$ = dissolved oxygen concentration, mg/L; $[Cu]$ = total dissolved copper concentration, μmol/L; $k = 0.0059$ μmol Mn(II)/(mg cell · min); $K_S = 5.7$ μmol Mn(II)/L; $k_{o_2} = 1/8.05 = 0.124$ L/mg ($[O_2]$ = 8.05 mg/L at 25°C and I = 0.05 mol/L); $E_a = 22.9$ kcal/(g cell · mol); $A = 2.3 \times 10^{14}$; $K_1 = 3.05 \times 10^{-8}$; $K_2 = 2.46 \times 10^{-8}$; $k_{pH} = 2.82$; $k_C = 8.8$ L/μmol Cu.

At T = 25°C, pH = 7.5, $[O_2]$ = 8.05 mg/L and zero added copper (i.e., Cu < 5nM), the above rate law for Mn(II) oxidation simplifies to the following equation:

$$-\frac{d[Mn(II)]}{dt} = \frac{k[X][Mn(II)]}{K_S + [Mn(II)]} \tag{8}$$

3.9. Comparison of Biotic and Abiotic Mn Oxidation Rates

The rate law for abiotic oxidation of Mn(II) is reported as (Morgan and Stumm, 1964; Morgan, 2001):

$$-\frac{d[Mn(II)]}{dt} = k_1[Mn(II)] + k_2[Mn(II)]_{aq}[Mn(III)] \tag{9}$$

where k_1 is the rate constant for homogeneous oxidation and k_2 is the rate constant for catalysis by the oxide reaction product.

In the absence of an existing Mn oxide solid phase, the term for homogeneous oxidation governs the rate of the abiotic reaction. Because the abiotic Mn(II) oxidation reaction proceeds very slowly at circumneutral pH, until recently values for k_1 had been reported only at pH 8.95 and above (Davies and Morgan, 1989). Recently, Von Langen et al. (1997) reported k_1 values in seawater ranging from 1.1×10^{-6} to 7.0×10^{-6}/min (normalized to $P_{o_2} = 1$ atm at 25°C) over a pH range from 8.03 to 8.67. By way of comparison, the concentration of Mn-oxidizing bacteria required to give a comparable biotic Mn(II)

oxidation rate would be 0.30 μg/L at pH 8.03 and 6.0 μg/L at pH 8.67 (under similar normalized conditions of $P_{O_2} = 1$ atm, at 25°C, and assuming [Mn(II)] $\ll K_S$ and [Cu] = 0). Thus, a relatively small population of bacteria capable of oxidizing Mn(II) is likely to be sufficient to ensure that the initial production of Mn oxide is dominated by biologic catalysis at circumneutral pH values.

This research, and the resulting rate equation (Eqn. 7) set the stage for incorporation of bacterially catalyzed Mn(II) oxidation kinetics into quantitative environmental models where Mn cycling is of direct interest. Additional applications would include modeling the effects of Mn(III/IV) oxide formation on the fate of toxic trace metals and other indirect effects of biologic Mn oxidation such as conversion of humic substances into metabolically available carbon substrates (Sunda and Kieber, 1994).

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