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Kinetics of Fe^{2+} Oxidation by *Acidithiobacillus ferrooxidans* Using Total Organic Carbon Measurement

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Abstract Kinetic experiments on the biological oxidation of Fe^{2+} by *Acidithiobacillus ferrooxidans* were conducted by measuring the total organic carbon content. The total organic carbon in the solution was determined with different initial concentrations of Fe^{2+} (4, 9, 15, and 20 mg/ml). The growth of *At. ferrooxidans* and substrate utilization were described by the Monod expression. The total organic carbon was found to be an indicator of the biomass concentration and thus may be effectively utilized for estimating cell growth rates in kinetic model development.

Key words: Ferrous ion, kinetic model, *Acidithiobacillus ferrooxidans*, total organic carbon

Since *Acidithiobacillus ferrooxidans*, previously named as *Thiobacillus ferrooxidans*, was discovered in 1947 in acid mine drainage from bituminous coal mines, this iron-oxidizing bacterium has been intensively applied to industrial metal leaching processes. *At. ferrooxidans* is one of the most studied microorganisms in terms of iron- and sulfur-bearing mineral dissolution [4, 9] as well as its effects, in a practical sense, on the bioleaching of various metal ores and coals [6-8]. As such, there have been in-depth studies on the kinetics of the biological oxidation of Fe^{2+} by *At. Ferrooxidans* and several well-designed kinetic models are available in previous literature [14, 15]. Most earlier studies about the kinetics of such systems have relied on the measurement of the Fe^{2+} oxidation rate as an indicator of the culture growth rate. However, despite the close correlation between the rates of Fe^{2+} oxidation and culture growth, it is rarely satisfactory to consider the period of enhanced Fe^{2+} oxidation as the period of the logarithmic phase of cell growth, because specific cellular Fe^{2+} oxidation has been shown to be independent of the stage of culture growth [10]. Instead, the direct measurement of the cell population can give more accurate information on the kinetics of biological Fe^{2+} oxidation.

Since *At. ferrooxidans* can be regarded as $(C_5H_7O_2N)_n$ on the basis of its principal elemental constituents [18], a total organic carbon (TOC) analysis can be used to determine elements or molecules of microbiological origin [2]. When direct cell counting by microscopy is employed, the attachment of the bacterium on colloidal suspensions of iron precipitates and cell agglomeration make it difficult to identify individual cells. In such a case, a TOC measurement, which is easily conducted and accurate, can be used to estimate the biomass in the system. However, relatively few reports exist on the use of TOC to develop a kinetic model.

It is essential to understand the kinetics of bacterial leaching to improve the current ability to utilize this microorganism efficiently in the design and operation of leaching facilities. Accordingly, the current work was undertaken to estimate the kinetics of Fe^{2+} oxidation and cell growth using a TOC analysis as a new approach for modeling the oxidation of Fe^{2+} by *At. ferrooxidans*.

Materials and Methods

Bacterial Culture

A freeze-dried culture of *At. ferrooxidans* (KCTC 2677) was obtained from the Korean Collection of Type Cultures. The cells were cultivated in 150 ml of a 9 K medium in a 300-ml Erlenmeyer flask [17]. The 9 K medium consisted of two solutions as follows; solution A: 3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.0144 g $Ca(NO_3)_2 \cdot 4H_2O$ in 700 ml of distilled water, and solution B: 45 g $FeSO_4 \cdot 7H_2O$ in 300 ml of distilled water. The culture flasks were incubated at 30°C on a shaking incubator at 200 rpm. In order to enhance the cell activity, a serial transfer was performed several times.

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Cell Harvest

To remove any mineral precipitate, the cultures were filtered through a Whatman No. 1 filter (pore size >11 μm), then the cells in the filtrate were harvested by centrifugation at 10,000 rpm for 20 min. The harvested cultures of *At. ferrooxidans* were then inoculated into a semi-continuous reactor.

Semi-Continuous *At. ferrooxidans* Culture Reactor

A 4-l semi-continuous culture reactor was charged with 2 l of the 9 K medium. The top of the reactor was fitted with a cotton plug wrapped in gauze, and aerobic conditions were supplemented by aeration at 315 cm^3/min. The initial pH of the medium was adjusted to 2.0–2.5 using 5 M H_2SO_4, and inoculated with the harvested cells. A homogeneous cell mixture was maintained using a magnetic stirrer. The reactor was incubated at 30°C using a heating-cooling water circulator (PolyScience, U.S.A.). The pH was monitored and the Fe^{2+} concentration measured at regular intervals. When the Fe^{2+} oxidation was complete, 300 mp of a fresh 9 K medium was used to replace the same volume in the culture reactor; this process permitted the continuous growth of the cells.

Development of Kinetic Model

The *At. ferrooxidans* used for the kinetic experiments was withdrawn from the semi-continuous reactor when the culture medium in the reactor contained Fe^{2+} concentrations under the detection limit. After filtration through a Whatman No. 1 filter, 10% (v/v) *At. ferrooxidans* was inoculated into 300-ml Erlenmeyer flasks containing 150 ml of the 9 K medium with 4, 9, 15, or 20 mg/ml of Fe^{2+}. The flasks were then incubated in a shaking incubator at 200 rpm and 30°C. Three milliliters of each sample were removed from the flasks at regular intervals and filtered through a Whatman No. 1 filter to determine the TOC and Fe^{2+} concentrations. All experiments were carried out in duplicate.

Analytical Methods

The pH was monitored using a combination electrode connected to an Orion model 250A pH meter (U.S.A.). The Fe^{2+} concentration was determined using a UV-Vis spectrophotometer (model Lambda 12, Perkin Elmer, U.S.A.) at 510 nm after mixing with *o*-phenanthroline [1]. The bacterium was observed through a Nikon Microphot-SA microscope (Japan), and the total organic carbon analysis was performed using a Dohrmann DC-180 TOC Analyzer (U.S.A.).

RESULTS AND DISCUSSION

Estimation of Biomass

The determination of the TOC as a surrogate for the cell concentration was conducted when Fe^{2+} was used as the sole substrate, and TOC increasing curves were obtained for a variety of initial Fe^{2+} concentrations (4, 9, 15, and 20 mg/ml) over a given time period (Fig. 1). The cell biomass in the solution increased with time for the first 50 h of the experiment, and then showed a greater TOC production at higher initial Fe^{2+} concentrations after 50 h. The concentrations of Fe^{2+} in the solution decreased as a function of time due to oxidation by *At. ferrooxidans*. (Fig. 2).

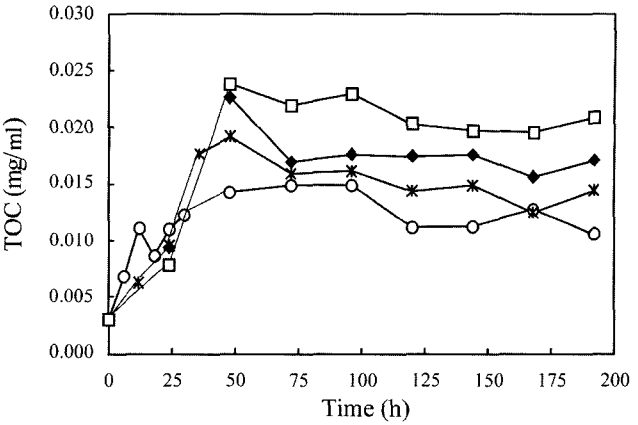

Fig. 1. Experimental biomass profile of *At. ferrooxidans* determined by TOC analysis at various initial Fe^{2+} concentrations. (○)=4 mg/ml, (×)=9 mg/ml, (◆)=15 mg/ml, (□)=20 mg/ml of Fe^{2+}.

Model Development and Determination of Kinetic Parameters

A mathematical model describing the system during exponential cell growth was developed. When Fe^{2+} was assumed to be the sole substrate, the kinetics of the cell growth in the batch reactor were described using the Monod expression [13].

The growth rate, ignoring possible cell decay, is expressed as follows:

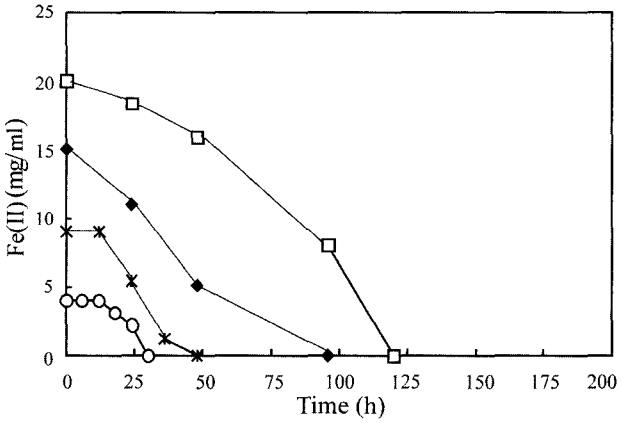

Fig. 2. Experimental oxidation of various initial Fe^{2+} concentrations by *At. ferrooxidans*.
(○)=4 mg/ml, (×)=9 mg/ml, (◆)=15 mg/ml, (□)=20 mg/ml of Fe^{2+}.

$$\frac{dX}{dt}=\frac{\mu_m S}{K_S+S}X \quad (1)$$

where μ_m=maximum specific growth rate, 1/h,
S=concentration of substrate, mg/ml,
K_S=half-saturation constant, mg/ml,
X=concentration of microorganism, mg/ml.

The rate of substrate utilization is expressed as follows:

$$\frac{dS}{dt}=\frac{1}{Y}\frac{\mu_m S}{K_S+S}X \quad (2)$$

where Y=yield coefficient.

In Eq. (2), the term μ_m/Y is replaced by the term k:

$$k=\frac{\mu_m}{Y} \quad (3)$$

where k=maximum specific rate of substrate utilization, 1/h.

The Lineweaver-Burk method was used to evaluate the kinetic parameters. As such, it is more convenient to transform Eq. (1) into a linear form. Taking the reciprocal of both sides of Eq. (1) gives

$$\frac{1}{\left(\frac{1}{X}\frac{dX}{dt}\right)}=\frac{K_S}{\mu_m}\frac{1}{S}+\frac{1}{\mu_m} \quad (4)$$

A plot of $((1/X)(dX/dt))^{-1}$ versus 1/S yields a straight line with an intercept of $1/\mu_m$ and slope of K_S/μ_m. In the current study, the biomass concentration (X) and substrate concentration (S) in the exponential phase were determined by polynomial interpolation from measured values to determine the kinetic parameters. A least-squares analysis was used to find the best straight line through the experimental points in the Lineweaver-Burk plot; Fig. 3 shows the plot from Eq. (4).

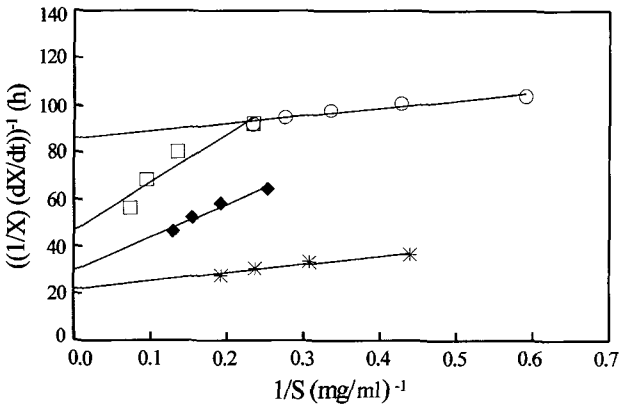

Fig. 3. Lineweaver-Burk plot for determination of maximum specific growth rate (μ_m) and half-saturation coefficient (K_S) at various initial Fe^{2+} concentrations.
(○)=4 mg/ml, (✳)=9 mg/ml, (◆)=15 mg/ml, (□)=20 mg/ml of Fe^{2+}.

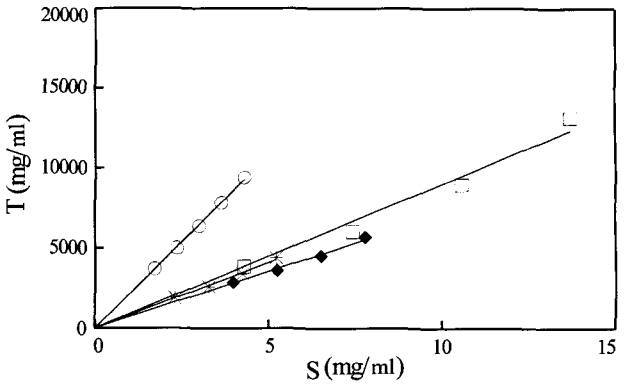

Fig. 4. Estimation of growth yield coefficient (Y) of *At. ferrooxidans* at various initial Fe^{2+} concentrations.
(○)=4 mg/ml, (✳)=9 mg/ml, (◆)=15 mg/ml, (□)=20 mg/ml of Fe^{2+}.

After substituting the values of K_s and μ_m obtained from Fig. 3, the cell yield coefficient (Y) was determined using Eq. (5) which is a rewritten form of Eq. (2).

$$-\frac{1}{X}\frac{dS}{dt}\frac{1}{\mu_m}(K_S+S)=\frac{1}{Y}S \quad (5)$$

Letting the left side of Eq. (5) be T, T was plotted versus S, and Y was accordingly determined as the slope (Fig. 4).

The values of the various kinetic parameters, μ_m, K_S, k and Y, in the presence of different Fe^{2+} concentrations are summarized in Table 1. In contrast with these results, McDonald and Clack [12] reported μ_m=0.15/h and K_S= 0.40 mg/ml, Braddock *et al.* [5] found μ_m=0.07/h and K_S= 0.04 mg/ml, and Shrihari *et al.* [16] reported μ_m=0.18/d and K_S=5.37 mg/ml. These discrepancies between the results reported by many authors are due to the different conditions and procedures employed for estimating these kinetic parameters [10]. According to the kinetic constants presented in Table 1, enhancement of the substrate concentration increased the apparent values of K_S, and this is consistent with the results of Smith [18] who mentioned that the value of K_S is dependent on the substrate concentration.

In the current study, the kinetic parameters were attributed to both the substrate activation related to the activity of *At. ferrooxidans* and the inhibitory effects of a high Fe^{2+} concentration [3, 15]. While an increase in the initial Fe^{2+} concentration of up to 9 mg/ml enhanced the

Table 1. Kinetic parameters of *At. ferrooxidans* growth with various initial Fe^{2+} concentrations.

Ferrous ion concentrations (mg/ml)	μ_m (1/h)	K_S (mg/ml)	k (1/h)	Y
4	0.01	0.38	24	0.0005
9	0.05	1.60	38	0.0012
15	0.03	4.73	24	0.0014
20	0.02	4.40	19	0.0011

rate of Fe^{2+} oxidation, Fe^{2+} concentrations higher than 9 mg/ml appeared to inhibit the reaction (Table 1). In Table 1, the values of the specific growth rate (μ_m) and maximum specific rate of substrate utilization (k) decreased at higher concentrations (15 and 20 mg/ml of Fe^{2+}), which were attributed to the limited presence of an enzyme capable of oxidizing Fe^{2+} to Fe^{3+}, in terms of the amount of available substrate. At higher substrate concentrations, the amount of biocatalyst present (enzyme) becomes less than the amount of the substrate (Fe^{2+}), plus a second substrate molecule binds to the enzyme-substrate complex to form an unreactive intermediate [15]. With regards to the cause of the inhibition effect, Shrihari *et al.* [16] proposed a mechanism of cell poisoning by Fe^{3+}, and Kelly and Jones [11] mentioned that the pH and potassium level in the solution might influence substrate inhibition.

As shown in Table 1, *At. ferrooxidans* exhibited low yield coefficients (Y), which is typical for autotrophic organisms. Hence, such low yield coefficients apparently resulted in relatively low TOC values in the current experiments. In spite of variation between the determined yield coefficients, as shown in Table 1, the Y values for the individual runs seemed similar. The yield coefficient determined by Smith [18] was around 0.0016 and a value of 0.0013 was reported by Tomizuka and Yagisawa [19]. Since the results of the present study were relatively similar, this indicates that a TOC measurement can be used effectively in estimating cell concentrations during the kinetic model development of biological Fe^{2+} oxidation.

CONCLUSIONS

The determination of carbon in cells ($(C_5H_7O_2N)_n$) as the total organic carbon (TOC) seemed to be a sensitive and reliable method for monitoring cell growth and a useful technique in the study of the growth kinetics of *At. ferrooxidans*. The kinetics of Fe^{2+} oxidation by *At. ferrooxidans* were studied with various initial Fe^{2+} concentrations, and the reaction rate appeared to be influenced by the Fe^{2+} concentration. For 4, 9, 15, and 20 mg/ml initial Fe^{2+} concentrations, the maximum specific substrate utilization rate coefficients (k) were 24, 38, 24, and 19/h, the values for the half-saturation coefficient (K_S) were 0.38, 1.60, 4.73, and 4.40 mg/ml, the yield values (Y) were 0.0005, 0.0012, 0.0014, and 0.0011, and the maximum specific growth rates (μ_m) were 0.01, 0.05, 0.03, and 0.02/h, respectively. Ferrous ion concentrations above 9 mg/ml decreased the value of the maximum specific substrate utilization rate coefficient (k), thereby suggesting that excess amounts of substrate resulted in an inhibition of the *At. ferrooxidans* activity.

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