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The bacterium most frequently used in microbial leaching processes and studies is Thiobacillus ferrooxidans. Applications of this bacterium include the treatment of coal, low-grade ores and industrial solid wastes for the solubilization of pyrite and other trace metals associated with sulfides. The goal of this study was the development of a standard procedure that can be used for screening or comparing various strains of Thiobacillus ferrooxidans.

Measuring the oxidation of ferrous iron is a quick and simple method for predicting the relative effectiveness of a given strain of T. ferrooxidans in microbial leaching operations. When conducting experiments for the relative determination of bacterial activity, 9K medium with a low initial soluble ferrous iron concentration and a pH of approximately 2.0 is recommended. Also it is important to begin with the same

ferric iron concentration in bacterial cultures, while minimizing the amount of precipitated iron. The effect of inoculum size on the maximum oxidation rate of ferrous iron is negligible; however, lag time can be reduced by using larger inocula.

IRON OXIDATION BY *THIOBACILLUS FERROOXIDANS*

by

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1. INTRODUCTION

Several investigators (1-6) have shown that microorganisms are involved in many naturally occurring oxidation processes. The solubilization of metals catalyzed by microorganisms is called microbial leaching, bioleaching or bacterial leaching. At present bacterial leaching is widely used commercially to produce copper from low-grade mine wastes. Although microbial leaching seems to be restricted to metal sulfides, metals such as uranium, silver or gold can be solubilized when they occur in association with sulfides.

The bacteria most frequently used in microbial leaching processes are the species Thiobacillus ferrooxidans and Thiobacillus thiooxidans, which thrive at pH 2-3 and ambient temperatures. T. ferrooxidans is the focus of this project. T. ferrooxidans can utilize both ferrous iron and inorganic sulfur as energy sources. The pyrite oxidation in the presence of T. ferrooxidans has been studied by many researchers (7-11). Reported experimental results give general information on pyrite oxidation in the presence of the different strains of T. ferrooxidans by analyzing for total soluble iron and sulfate concentrations.

However, the role of the microorganism is not fully understood. Bacterial leaching experiments in a batch system have shown that oxidation of metal sulfide is not usually complete and takes a long time to obtain the maximum soluble metal concentration (7). For these reasons, the a simple method for screening new strains of T. ferrooxidans is introduced in this study.

1.1 Background

1.1.1 The Geomicrobiology of Iron

Iron is the fourth most abundant element in the earth's crust, and it is the most abundant element in the earth as a whole. It is found in a number of minerals in rocks, soils, sands and sediments. Table 1 lists mineral types for which Fe is a major or minor structural component (12).

Iron is a very reactive element. It commonly exists in oxidation states of 0, +2 and +3. At pH values greater than 5, its ferrous state is readily oxidized to the ferric state in the presence of air. Under reducing conditions, ferric iron is easily reduced to the ferrous form. In acid solution, metallic iron oxidizes to ferrous iron with the production of hydrogen:

Table 1. Iron Containing Minerals

Primary Minerals	Secondary Minerals	Sedimentary Mineral
Prroxenes	Montmorillonite	Siderite($FeCO_3$)
Amphiboles	Illite	Geothite($Fe_2O_3 \cdot H_2O$)
Olivines		Limonite($Fe_2O_3 \cdot nH_2O$)
Micas		Hematite(Fe_2O_3)
		Pyrite(FeS_2)
		Magnetite(Fe_3O_4)
		Ilmenite($FeO \cdot TiO_2$)

$$Fe^{o} + 2H^{+} \text{ ---> } Fe^{2+} + H_2 \qquad (1\text{-}1)$$

Ferric iron precipitates in alkaline solution and dissolves in acid solution.

Iron is important biologically. Cells use it catalytically in enzymatic electron transfer. Ferrous iron also serves as a major energy source to certain bacteria, especially T. ferrooxidans.

1.1.2 Thiobacillus ferrooxidans, Iron-Oxidizing Bacteria

Many species of bacteria can acquire their energy for growth through the oxidation of inorganic sulfur. These bacteria, especially Thiobacilli, are corrosive due to sulfuric acid generated by microorganisms. However, they play important role in the mining of copper, uranium and other ores. Additionally, they have potential for the desulfurization and demetalization of industrial wastes.

Thiobacillus ferrooxidans was discovered and named by Temple and coworkers (13). Later Leathen et al. (14) isolated an iron-oxidizing bacterium that they claimed was unable to oxidize sulfur, and named it Ferrobacillus ferrooxidans. However, this microorganism can utilize elemental sulfur, thus invalidating the new binomial (3).

T. ferrooxidans is acidophilic, aerobic and rod-shaped bacteria about 0.5 by 1.0 μm in size and can utilize carbon dioxide in air. They also require a nitrogen source which is usually ammonium. Additional requirements are phosphate and some trace elements usually existing in their environments.

The growth rate of T. ferrooxidans is highly dependent on the composition of their medium. Silverman and Lundgren (15) have shown that the 9K medium, which they had developed, is well suited for the growth of T. ferrooxidans. Cell counts ranged from $2x10^8$ to $4x10^8$ cells per 1 ml as opposed to only $2x10^6$ cells per 1 ml in the medium of Leathen (14). Table 2 represents the comparison of media components for growth of T. ferrooxidans (15).

T. ferrooxidans derives energy for growth from the oxidation of ferrous iron;

$$4FeSO_4 + O_2 + 2H_2SO_4 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (1\text{-}2)$$

This reaction occurs in air without T. ferrooxidans present but is very slow. T. ferrooxidans is able to oxidize ferrous iron at a rate about 500,000 times as fast as would occur in its absence (16).

Table 2. The Composition of Media Components

Components	Leathen	9K
Basal salts		
$(NH_4)_2SO_4$	0.15 g	3.0 g
KCl	0.05 g	0.10 g
K_2HPO_4	0.05 g	0.50 g
$MgSO_4 \cdot 7H_2O$	0.50 g	0.50 g
$Ca(NO_3)_2$	0.01 g	0.01 g
Distilled water	1000 ml	700 ml
10 N H_2SO_4	--	1.0 ml
Energy source		
$FeSO_4 \cdot 7H_2O$	10 ml of a 10% solution	300 ml of 14.74% solution

1.2 Application of Microbial Leaching using T. ferrooxidans

1.2.1 Dump, Heap and In Situ Leaching

Dump, Heap and In Situ leaching are the most common methods of solution mining and have been widely applied to the recovery of copper and uranium from low-grade ores and wastes on a large scale. It is estimated that 20% of the worldwide production of copper and a substantial part of the uranium is recovered by one of these simple and inexpensive processes (17).

Dump leaching is used to extract copper from low-grade oxide, metal sulfides and waste materials. Such materials contain less than 0.4% copper and are not normally crushed (1). The leach dumps are usually located in valleys to use natural slopes for stability and recovery of solution.

Heap leaching is primarily used to extract copper and uranium from crushed and uncrushed oxide ores of a higher grade than ores used for dump leaching (4). Most of the problems associated with dump leaching result from poor construction and incomplete information of the internal conditions and reaction occurring in the dump.

In situ leaching of ores is similar in many aspects to heap and dump leaching. When the grade of ores is too low to use conventional winning operations, it may

be feasible to grind the ore and leach the diminished ore in place (6). The method as a process is currently applicable to oxide and mineral sulfides of copper and uranium, but should also be considered for the recovery of other precious metals.

1.2.2 Microbial Desulfurization of Coal

Coal is a major source of energy in the world. Roughly 50% of the total electrical power is generated by the burning of 550 - 600 million tons of coal per year, utilization of domestic coal is expected to accelerate to meet increasing energy requirements within the world (18). An increase in CO_2 and SO_2 emissions associated with conventional coal combustion operation has resulted in a significant environmental problems, such as formation of acid rain, which is detrimentally effecting our lakes, forests and agricultural land.

Microbial desulfurization before precombustion of coal offers a promising conceptual alternative to current chemical or physical methods, because of the characterization of bacteria which catalyze the solubilization of sulfur compounds on coal. T. thiooxidans, T. ferrooxidans, and Sulfolobus acidocaldarius have been considered for use in various processes (19). Most of the

research on microbial desulfurization have dealt with the removal of inorganic sulfur from coal; several studies have presented economic analyses which compared favorably to other physical and chemical process costs (9,18-20). For example, Hoffmann and coworkers (9) presented the results of their study on the ability of different strains of T. ferrooxidans and T. thiooxidans to catalyze the oxidative dissolution of iron pyrite, FeS_2, in coal. This study showed that bacterial desulfurization of coal can yield 90 - 98% removal of pyrite as an inorganic sulfur within 10 days at low pulp densities and small particle sizes. Organic sulfur in coal matrix is not removed from coal by current mechanical methods because of its characteristic structure. Therefore, the microbial method of removing organic sulfur in coal is also of economic interest. Studies on microbial organic sulfur removal and lignin biodegradation need to be done to develop a microbial desulfurization process that will result in sulfur removal to meet EPA standards.

1.2.3 Recovery of Gold and Silver

Frequently, gold and silver are associated with iron sulfide. With gold, microbial leaching takes on a somewhat different role. Gold is often found bound up

with minerals such as arsenpyrite, a mixed sulfide of arsenic and iron. Conventionally, this refractory gold is extracted by attack with cyanide ions in a solvent to form soluble gold cyanide complexes. The association can be broken by roasting but this procedure is technically difficult. Depending on the mineralogy of the ore, up to 60% of the gold may be recovered by this treatment (21). The problem is that much of gold is locked up within the pyrite matrix and is inaccessible to the cyanide. Although gold, unlike copper and iron, is itself biologically inert, Thiobacilli bacteria are able to open up the matrix of the ore, making gold more amenable to attack by the cyanide. With this additional biological step, gold yields may be increased from the original 60% up to as high as 90%, again depending much on the mineralogy (21). Typically this additional biological step is carried out in a pachuca tank, a cone-bottomed column familiar to the mining industry.

1.2.4 Purification of Sand

Sand for glass-making or foundry use is commonly benefited by removal of iron minerals by strong acid leaching. If the metal sulfide is pyrite or marcasite, partial removal of iron can be affected by leaching at

room temperature and pH 2.5 with T. ferrooxidans (22).

1.2.5 Microbial Demetalization

Many solid wastes from various industries contain toxic metals. For example, geothermal power plants, which use hypersaline brine, are considering the use of a biological waste treatment facility for the removal of toxic metals from their precipitated solid waste as an alternative to the costly disposal of the hazardous solid waste (23).

1.3 Objectives and Methods of Approach

The goal of the project was to develop a standard protocol that can be used for screening new strains of T. ferrooxidans and be considered for use in various bioleaching applications.

In order to determine pyrite solubilization rate, in the presence of the microorganism batch kinetic studies have been performed with several different strains of T. ferrooxidans using a sample of pyrite particles supplied by WARD'S Natural Science Establishment, Inc. Experiments were performed to study the effects of pulp

density, particle size and strain of T. ferrooxidans on the solubilization rate of pyrite by analyzing the soluble iron concentration in leachate. Pyrite was chosen because of its significance in coal, low-grade ores, solid waste, and other potential bioleaching applications.

In order to obtain the maximum %conversion rate of ferrous iron to ferric iron, batch kinetic studies have been done in 9K medium by measuring pH and ferrous concentration at various time intervals. Experiments were also conducted to test the effects of different strains of T. ferrooxidans, initial pH, initial ferrous iron concentration, and inoculum size on ferrous iron oxidation.

2. REVIEW OF THE LITERATURE

2.1 Reaction Mechanisms

A general reaction is often used to express the biological oxidation of a mineral sulfide involved in leaching;

$$MS + 2O_2 \xrightarrow{\text{microorganism}} MSO_4 \qquad (2\text{-}1)$$

where M is a bivalent metal. The leaching process is the end result of the bacteria acting upon the metal sulfide, which serves as an energy source in the presence of other nutrients. However, the mechanism of bacteria attack on sulfide minerals is not completely known.

Two different mechanisms are frequently referred to as the indirect and direct contact mechanisms for the oxidation of pyritic sulfur by T. ferrooxidans. No extracellular enzymes are involved in the direct mechanism. This mechanism requires direct contact between bacteria and pyrite surface. Whether or not direct reaction with the surface occurs, there must be an advantage to the organism to be attached to the surface because of the oxidizable products of chemical leaching.

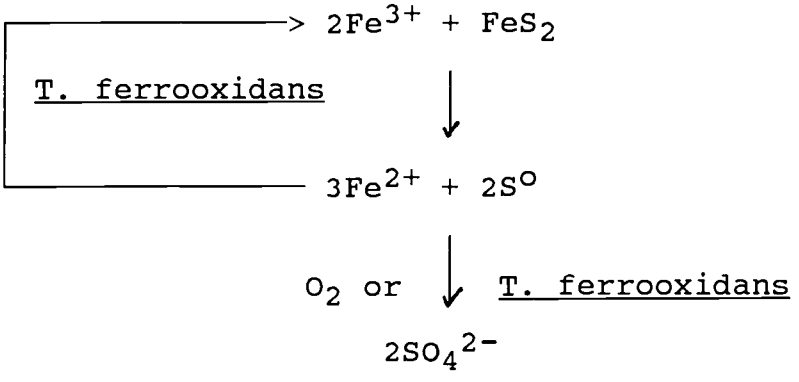
According to the indirect contact mechanism, ferric

iron is the major oxidant, oxidizing metal sulfides while the ferric irons are reduced to the ferrous state. The role of microorganisms in the indirect mechanism is to regenerate the ferric iron for pyrite oxidation. Silverman and Ehrlich (24) have discussed the role of ferric iron and oxygen in the oxidation of mineral sulfides. Reactions to explain the involvement of ferric iron are;

(aerobic) $$MS + 2Fe^{3+} + H_2O + 1.5O_2 \rightarrow M^{2+} + 2Fe^{2+} + SO_4^{2-} + 2H^+ \quad (2\text{-}2)$$

(anaerobic) $$Fe_2(SO_4)_3 + FeS_2 \rightarrow 3FeSO_4 + 2S^o \quad (2\text{-}3)$$

In the presence of iron-oxidizing bacteria, the ferrous iron produced by these reactions can be oxidized ferric ion, thereby establishing a cyclic process. The following reactions is to explain the role of ferric iron on the pyrite oxidation by T. ferrooxidans (20):



Many researchers have concluded that the direct and indirect mechanisms operate together in the solubilization of mineral sulfides (9,15,20).

2.2 Chemistry of Microbial Leaching

The microorganism used in this study is Thiobacillus ferrooxidans. This organism is capable of chemolithotropic growth in acid environments using energy from the oxidation of ferrous iron, various metal sulfides and elemental sulfur.

As a result of its metabolism, T. ferrooxidans oxidizes certain metal sulfides into water-soluble sulfates while catalyzing the production of sulfuric acid from elemental sulfur. That is, T. ferrooxidans can solubilize certain metal ions by the following series of redox reactions;

$$2FeS_2 + 7O_2 + 2H_2O \text{ ---> } 2FeSO_4 + 2H_2SO_4 \qquad (2\text{-}4)$$

$$4FeSO_4 + O_2 + 2H_2SO_4 \text{ ---> } 2Fe_2(SO_4)_3 + 2H_2O \qquad (2\text{-}5)$$

$$MS + Fe_2(SO_4)_3 \text{ ---> } MSO_4 + 2FeSO_4 + S^o \qquad (2\text{-}6)$$

$$2S^o + 3O_2 + 2H_2O \text{ ---> } 2H_2SO_4 \qquad (2\text{-}7)$$

where M is any bivalent metal of interest. Only Reaction (2-6) is independent of bacteria.

In the case of pyrite oxidation in the presence of T. ferrooxidans, the following reaction is believed to take place.

$$FeS_2 + Fe_2(SO_4)_3 \text{ ---> } 3FeSO_4 + 2S^o \qquad (2\text{-}8)$$

The above chemical Reactions (2-4 - 2-8) indicate that pH decreases during pyrite oxidation. However, the microbial oxidation of pure pyrite is proceeded by a lag time in which pH is increased according to an acid-consuming reaction (2);

$$FeS_2 + H_2SO_4 + 1/2O_2 \text{ ---> } FeSO_4 + H_2O + 2S^o \qquad (2\text{-}9)$$

This phenomenon is very similar to that of T. ferrooxidans growth according to Reaction (1-2).

Numerous studies (26-31) have been carried out on other sulfide minerals such as chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S), covellite (CuS) and sphaierite (ZnS). The ferric irons resulting from reaction (2-5) can chemically solubilize metal sulfide such as pyrite by the following reaction;

$$FeS_2 + Fe_2(SO_4)_3 + 3O_2 + 2H_2O \text{ ---> } 3FeSO_4 + 2H_2SO_4 \qquad (2\text{-}10)$$

This reaction proceeds by two steps: First, Reaction(2-8)

which is independent of bacteria occurs, and then Reaction(2-7) is catalyzed by bacteria.

2.3 Factors Affecting Bacterial Oxidation

2.3.1 Effect of Temperature

T. ferrooxidans is mesophilic bacteria which grows optimally at moderate temperatures, i.e., between 20 and 35°C. Optimal bioleaching of metal sulfide ores occurs between 25 and 45°C (3); whereas, the optimal temperature range for the coal desulfurization in the presence of this organism is 28 - 35°C (19).

2.3.2 Effect of pH

T. ferrooxidans is active in the pH range of 1.5 - 5.0. Optimal pH is between 2.0 and 2.5 for the oxidation of metal sulfide and ferrous iron (8,16). T. ferrooxidans has been adapted to grow at lower pH values at which the formation of jarosite is decreased (7). Optimal pH for microbial desulfurization of coal by T. ferrooxidans is around 2.0 (32).

2.3.3 Effect of Particle Size

Particle size is a major parameter affecting the rate of pyrite solubilization and metal extraction. Silverman and coworkers (20) showed that the bacteria oxidation of pyrite in coal was effective when the smallest particle sizes were used. Greater yields and extraction rates of metals from mineral sulfides occur when the specific surface area of the ore is increased by reducing particle sizes.

2.3.4 Effect of Pulp Density

The concentration of solid substrate is expressed as the pulp density or as the solid/liquid ratio. Atkins (7) showed that the rate of solubilization from pyrite is proportional to pulp density up to approximately 4 wt% with an optimum pulp density at about 10 wt% using *T. ferrooxidans* adapted to high concentrations of iron, copper, zinc and arsenic. Kargi (19) has also shown that the optimal ratio of cell number-to-surface area of coal was nearly 10^8 cells per cm^2 coal surface area.

2.3.5 Effect of Other Factors

T. ferrooxidans has a high tolerance to toxic metals as compared to most other microorganisms (33,34). However, the presence of toxic metals in coal may in some cases have an effect on pyrite leaching rates. Oxygen and CO_2 transport are also important factors to be considered in pyrite leaching operation. Another factor is the cell number of an inoculum. Atkins (7) investigated the importance of inoculum size on the bacterial oxidation of pyrite. He showed that varying the inoculum size from 10^5 cells to 10^{10} cells per ml for a 1% pulp density (weight/volume) gave similar leaching rates except a difference in the lag phase of the typical logarithmic leaching curves.

3. EXPERIMENTAL MATERIALS AND METHODS

3.1 Bacterial Culturing

Strains of bacteria, ATCC 13598, ATCC 13661, ATCC 19859 and ATCC 33020 used in this study were obtained from the Environmental Biotechnology Group at Brookhaven National Laboratory. All T. ferrooxidans cultures were performed in a stationary state. However, T. ferrooxidans, which was used as an inoculum, had been grown for a week in a shaker (Lab Line Environ-Shaker Model 3527) at 28^{o}C and 120 rpm to obtain a maximum cell number. The cultures of T. ferrooxidans were transferred into a new 9K medium every week to keep a continuous supply of the bacteria. At the end of the exponential phase of growth and after the removal of ferric iron which is an inhibitor of cell growth, all of the strains of T. ferrooxidans were stored in a refrigerator until they were needed.

3.2 Pyrite Preparation

The main pyrite sample was obtained from WARD's Natural Science Establishment,INC. The pyrite samples were prepared by crushing and grinding at the U.S. Bureau

of Mines in Albany. The pyrite was separated in three different sizes; +80 mesh, 80-150 mesh and -150 mesh. To minimize soluble aqueous-phase iron before beginning of each experiments, the pyrite samples were washed for 2 hr in a 0.1 N HCl solution, rinsed thoroughly with distilled water, and subsequently dried overnight in a oven.

3.3 Oxidation Experiments

3.3.1 Ferrous Iron Oxidation

General Experimental Procedure. The experiments were conducted at an ambient temperature of 28°C and under sterile conditions. For sterile runs, 9K medium with basal salts was poured into each 250-ml erlenmeyer flask. While this was autoclaved at 18 psia for 30 min, 14.74% $FeSO_4 \cdot 7H_2O$ solution (weight/volume) as an energy source was filtered through 0.45 micron filter for sterilization. Next, 30 ml of ferrous iron solution was mixed with the rest of 9K solution when it cools. Duplicate runs were performed to ensure reproducibility of all experiments. 9K medium was inoculated with bacteria culture in measured aliquots using an aseptic transfer technique. A sterile micro pipette was used to transfer the inoculum from the bacteria culture to the experimental

flask. The mouth of flask was then covered with aluminum foil. Following inoculation, the flask was placed on a horizontal shaker set at 120 rpm. First sampling was performed immediately after inoculation by inserting a sterile pipette into a flask to remove 1-ml liquid sample. Each liquid sample was filtered through a 0.45 micron filter to separate bacteria from liquid sample. Beckmann model 21 pH meter was used to determine the pH of samples by inserting a thin electrode into each flask. Sampling has been done almost every 6 hr to analyze ferrous iron concentration by a volumetric titration method since a pH started to increase.

Effect of Different Strains of T. ferrooxidans. The 9K medium including basal salts was adjusted to the two appropriate pH's with concentrated sulfuric acid before autoclaving; this was necessary because later addition of 30 ml of 14.74% $FeSO_4 \cdot 7H_2O$ solution will result in increasing pH of approximately 0.2. Each 100 ml of 9K medium was inoculated with bacteria cultures of T. ferrooxidans strains 13598, 13661, 19859, and 33020 in 1-ml aliquots. The experiment was followed by the general procedure.

Effect of Initial pH. The effect of initial pH on ferrous iron oxidation by T. ferrooxidans strain 13661 was investigated. The pH of each 9K medium was adjusted to the appropriate values by addition of concentration

sulfuric acid. The experiments were performed as for a general experimental procedure section.

Effect of Initial Ferrous Iron Concentration. To examine the effect of initial ferrous iron concentration on ferrous iron oxidation by T. ferrooxidans strain 13661, 5, 10, 20, and 30 ml of 14.74% $FeSO_4 \cdot 7H_2O$ solution as energy sources were mixed with cooled 70 ml of 9K medium. Next, distilled water was added to bring the volume up to 100 ml in each flask.

Effect of Inoculum Size. The experiments were performed to determine the effect of inoculum size on ferrous iron oxidation because the number of cells in aliquots was not counted. The above general procedure was followed with one variation: bacteria cultures of T. ferrooxidans strain 13661 in 1, 2, and 4-ml aliquots were inoculated to each 9K medium.

3.3.2 Pyrite Oxidation

For sterile experiments the media and the pretreated pyrite samples were autoclaved separately at 18 psia for 30 min. The weighed pyrite samples were put into erlenmeyer flasks and the pH of 9K medium was adjusted to pH 2.0 with concentrated sulfuric acid before autoclaving. Next, an inoculum of 10% (volume/volume) was transferred

(i.e., 5 ml of inoculum per 50 ml of total volume) into each flask. The rate of evaporation was found to be nearly constant over the duration of the experiment at 0.641 ml per week. The correction for evaporation was performed by adding distilled water once per week. In the control experiment, the pyrite sample was mixed with 50 ml of medium without inoculum. All flasks were placed on a horizontal incubator shaker set at 200 rpm. Sampling was done weekly by inserting a sterile pipette to remove a 1.0 ml liquid sample. Samples were centrifuged to separate suspended solids. The supernatant from each sample was stored in sampling bottles after the addition of 0.1 ml of 3 M HNO_3 to keep ions stable until it was to ready for analyzing the soluble iron by Atomic Absorption Spectrophotometry (AAS). After the 1.0-ml sample was withdrawn for analysis, the 1.0 ml of 9K medium which had been adjusted to pH 2.0, was added to bring the original volume in the flask back up to 50 ml. The pH of the sample was measured by inserting the thin electrode of a Beckmann model 21 pH meter into the flask after immediately the sample was withdrawn. The care was taken to maintain sterile conditions when sampling and measuring the pH.

3.4 Analytical Methods

3.4.1 Permanganate Analysis of Fe^{2+} Samples

Volumetric titration techniques are used to analyze Fe^{2+} chemical species. First, 50 ml of distilled water were added to a 250-ml flask and followed by addition of 10 ml 3 M H_2SO_4 and 5 ml 85% phosphoric acid. Finally, the appropriate sample amounts was put into a Erlenmeyer flask. A clean 50-ml buret was filled with the 0.001 M $KMnO_4$ solution. The flask was swirled constantly and waited until the solution became colorless before adding more 0.001 M $KMnO_4$ solution. Titration was done slowly at first. The equivalence point corresponds to the first permanent tinge of pink-purple color.

3.4.2 Atomic Absorption Spectrophotometry

The samples were analyzed for the total soluble iron concentration with a Perkin-Elmer 560 Atomic Absorbance Spectrophotometer. The machine setting for iron is as followed;

a) Metal: Iron

b) Linear range (mg/L): 0 - 5

c) Slit width (nm): 0.2

d) Wave length (nm): 248.3

e) Flame: air-acetylene

The machine was zeroed with distilled water and calibrated using the standard solution, which was the highest concentration measured throughout the analysis in the linear working range. Standards of 1, 3, and 5 ppm were used to establish a linear absorbance range. If a sample exceeded the maximum value of the linear working range, it was diluted with a measured amount of distilled water until an absorbance reading was obtained that fell within the linear range.

4. RESULTS AND DISCUSSION

4.1 Ferrous Iron Oxidation

4.1.1 Sigmoidal Model for the Oxidation of Ferrous Iron to Ferric Iron

A general rate equation, known as the Michaelis-Menten equation, describes the reaction in which a single substrate is enzymatically converted to a single product. The Michaelis-Menten (M-M) rate equation for the irreversible conversion of substrate S catalyzed by a typical enzyme is:

$$V = \frac{V_{max}\,[S]}{K_M + [S]} \qquad (4\text{-}1)$$

where V is the velocity or rate of reaction of S, K_M is the M-M constant, V_{max} is the maximum velocity, and [S] is the substrate concentration. For such a system, a double reciprocal plot of 1/V versus 1/[S] gives a straight line which facilitates the estimation of V_{max} and K_M.

Frequently, allosteric enzymes have more complex kinetics. They are often represented by sigmoidal curves in plots of V as a function of [S]. As a result, their double reciprocal plots are nonlinear. However, their

kinetic behavior can be described by an equation similar to Equation (4-1);

$$V = \frac{V_{MAX}\,[S]^{n_H}}{K_M + [S]^{n_H}} \qquad (4\text{-}2)$$

where n_H is the Hill coefficient (35).

The observed conversion of ferrous iron to ferric iron as a function of time exhibits a sigmoidal behavior. Therefore, a sigmoidal model equivalent to Equation (4-2) can be used to model the oxidation of ferrous iron to ferric iron:

$$X = \frac{X_{max}\,t^m}{K + t^m} \qquad (4\text{-}3)$$

where X is the %conversion of ferrous iron to ferric iron, K and m are constants, X_{max} is the maximum %conversion, and t is the time.

In case of ferrous iron oxidation, all ferrous iron is assumed to be oxidized to ferric iron. Therefore, the maximum conversion should be 100%.

Equation (4-3) can be rearranged to give:

$$\log [X/(X_{max}-X)] = m \log t - \log K \qquad (4\text{-}4)$$

Thus, a log-log plot of $X/(x_{max}-X)$ vs. t yields K and m from the y-intercept and slope. For example, the values of K and m for ferrous iron oxidation by T. ferrooxidans 13661 at 28°C with 9K medium, and an inoculum concentration of 2% (i.e, 2 ml of inoculum/100 ml of solution) were obtained by the plot of Eq. (4-4) illustrated in Fig. 1.

The rate of %conversion of ferrous iron to ferric iron is the derivative of Equation (4-3):

$$\frac{dX}{dt} = \frac{X_{max}\ K\ m\ t^{m-1}}{(K + t^{m})^{2}} \qquad (4\text{-}5)$$

A maximum value of Equation (4-5) is the maximum rate of %conversion of ferrous iron to ferric iron. A lag time is defined as the time needed to reach the maximum conversion rate.

Parameter estimates for four different strains of T. ferrooxidans at 28°C with 9K medium, an inoculum concentration of 1% and a shaker speed of 120 rpm are given in Table 3. The values of constants for ferrous iron oxidation by T. ferrooxidans at various initial pH's, ferrous iron concentrations and inoculum sizes are listed in Table 4.

As reported in Tables 3 and 4, values of maximum rate of %conversion for T. ferrooxidans strain vary

Figure 1

A Graphical Method to Determine the Constants in the Sigmoidal Model for Ferrous Iron Oxidation in the Presence of T. ferrooxidans Strain 13661 with 9K Medium.

Ferrous Iron Concentration of 0.16 M, Temperature 28°C, Initial pH 1.90, Inoculum Concentration of 2% (2 ml Inoculum/100 ml Medium Solution).

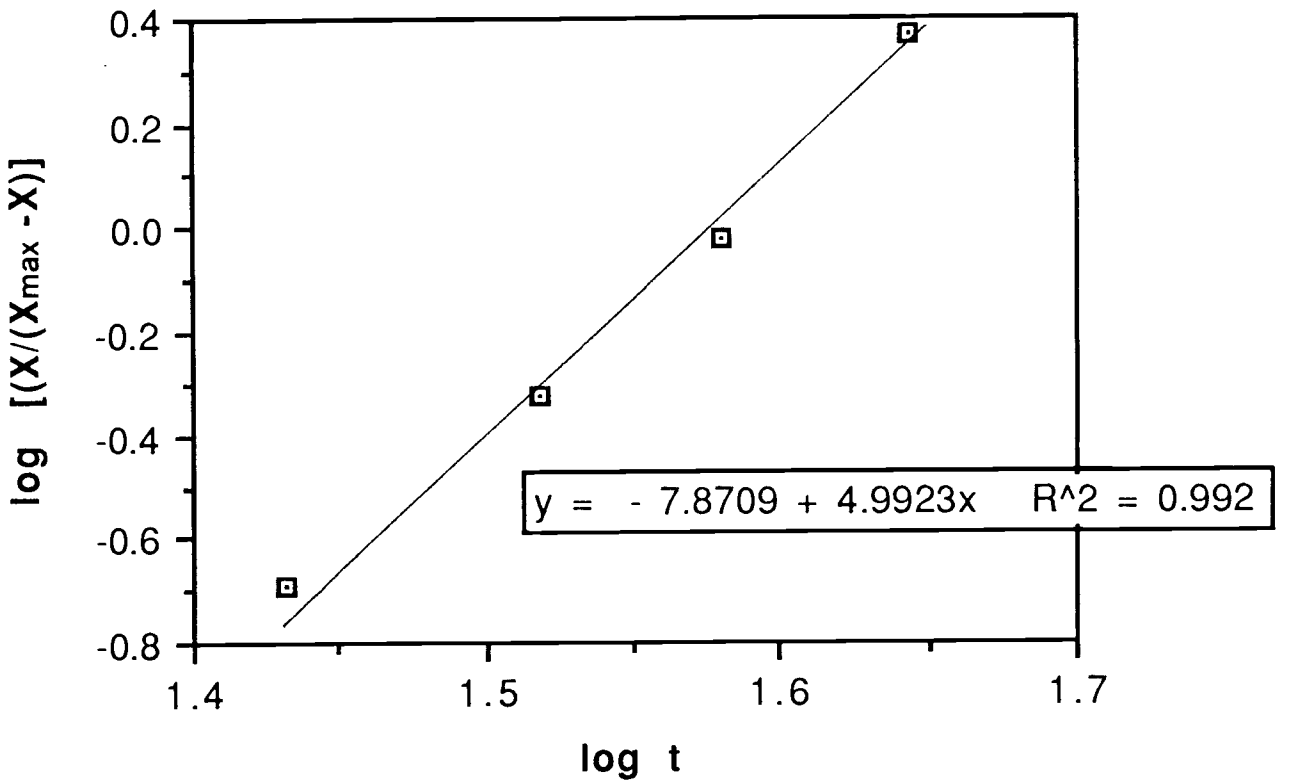

0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
log [(X/(Xmax -X)]
1.4
1.5
1.6
1.7
log t
y = - 7.8709 + 4.9923x R^2 = 0.992

Figure 1

Table 3. Parameter Estimates for Four Different Strains of T. ferrooxidans at 28°C with 9K Medium (0.16 M Ferrous Iron), an Inoculum Concentration of 1% and a Shaker Speed of 120 RPM

Bacteria	initial pH=1.94			
T. ferrooxidans strain	log K	m	max. rate (%/hr)	lag time (hr)
13598	9.73	5.33	2.06	62.3
13661	9.43	5.04	1.76	68.7
19859	10.73	6.15	2.85	52.6
33020	9.57	5.34	2.24	57.6

Bacteria	initial pH=2.26			
T. ferrooxidans strain	log K	m	max. rate (%/hr)	lag time (hr)
13598	11.03	6.32	2.91	52.9
13661	10.12	5.72	2.51	55.3
19859	13.13	7.95	4.51	43.4
33020	10.16	5.90	2.88	49.7

Table 4. Parameter Estimates for T. ferrooxidans 13661 at 28°C with 9K Medium and a Shaker Speed of 120 RPM

(a) Initial Ferrous Iron of 9000 ppm (= 0.16 M)
1 ml Inoculum/100 ml Solution

initial pH	log K	m	max. rate (%/hr)	lag time (hr)
1.93	13.05	7.24	2.91	61.1
2.15	13.33	7.44	3.06	59.7
2.39	13.79	7.72	3.21	59.1
2.70	14.90	8.35	3.48	59.1

(b) Initial pH=2.19
1 ml Inoculum/100 ml Solution

initial ferrous conc.	log K	m	max. rate (%/hr)	lag time (hr)
9000 ppm	14.48	8.15	3.46	58.0
6000 ppm	13.67	7.80	3.50	54.7
3000 ppm	13.44	7.97	4.17	47.1
1500 ppm	11.52	7.08	4.26	40.7

(c) Initial Ferrous Iron of 9000 ppm
Initial pH=1.90

inoculum conc. (ml inoculum/100 ml total volume)	log K	m	max. rate (%/hr)	lag time (hr)
1	10.16	6.07	3.30	44.7
2	7.87	4.99	3.44	34.8
4	5.66	3.82	3.38	26.3

significantly at the same composition as 9K medium, a 1 ml aliquots of bacteria culture, and approximately the same pH. See Table 5 for a comparison of values.

The difference in values of maximum %conversion rate may have resulted from pH meter error or from various number of cells in the 1 ml aliquots. However, an error analysis for the pH meter shows that the average standard deviation is only 0.085 (Table 6). Whereas, the effect of cell number in aliquots, which was not measured but shown to be approximately 10^8 cells/ml by previous researchers (15), appears to be negligible according to the maximum %conversion rate given in Table 4-(c).

The maximum %conversion rate of 1.76%/hr for case 1 of Table 5 is much lower than that of other cases. This may have occurred because only three data were used to determine the values of K and m for the sigmoidal function; whereas, nine and seven data points respectively used for the other two cases. Furthermore, the three data points were collected in case 1 before the maximum %conversion rate occurred. Hence, the extrapolated value of maximum %conversion rate for case 1 is expected to be less accurate than the interpolated values for the other two cases.

While the three explanations discussed above are not plausible sources of error, they are not sufficient enough to explain the large variation in maximum %conversion rate

Table 5. Comparison of Maximum %Conversion Rate and Lag Time for T. ferrooxidans strain 13661 at 28°C with 0.16 M Ferrous Iron and 1 ml Aliquots of Bacteria Cultures

case	initial pH	max. rate (%/hr)	lag time (hr)	number of data collected
1	1.94	1.76	68.7	3
2	1.93	2.90	61.1	9
3	1.90	3.30	44.7	7

Table 6. pH Meter Error Analysis

	Standard Solution		
Run	pH1	pH2	pH3
1	2.01	2.26	2.71
2	2.08	2.34	2.79
3	2.13	2.39	2.84
4	2.09	2.34	2.77
5	2.14	2.38	2.80
6	2.18	2.41	2.80
7	2.04	2.29	2.71
8	1.85	2.12	2.58
9	2.08	2.33	2.75
10	2.14	2.39	2.80
Average	2.07	2.33	2.76
standrad deviation	0.094	0.086	0.074

from 1.76 to 3.3%/hr observed at similar sets of conditions. The more likely source of error among the various data sets can be attributed to the variability in the growth of T. ferrooxidans prior to the beginning of the experiments. All bacteria cultures were grown at 28°C in medium adjusted to appropriate pH with concentrated sulfuric acid until near the end of the growth phase. Therefore, measured aliquots used as an inoculum for each ferrous iron oxidation experiments have different pH, cultured age, and ferric iron concentration.

4.1.2 Factors Affecting Ferrous Iron Oxidation

Silverman and Lundgren (15) have suggested that T. ferrooxidans readily oxidizes ferrous iron to ferric iron according to the following stoichiometric equations;

$$4FeSO_4 + O_2 + 2H_2SO_4 \rightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (4\text{-}6)$$

$$3Fe^{3+} + 2SO_4^{2-} + K^+ + 6H_2O \rightarrow \underbrace{Fe_3(SO_4)_2(OH)_6}_{\text{jarosite}} + 6H^+ \qquad (4\text{-}7)$$

where Reaction (4-6) is catalyzed by the microorganism.

They showed that Reaction (4-6) is catalyzed by T. ferrooxidans under acidic conditions by determining the

amount of O_2 consumption. Data shown in Figure 2 support this claim that these two reaction occur in the 9K medium during cell growth. During cell growth, pH increases as a result of the acid-consuming reaction of (4-6). The subsequent generation of hydrogen ions by Reaction (4-7) leads to the development of acidic conditions and precipitation in the growing medium. That is, precipitation of jarosite occurs for pH's in the range of 2 to 4. This precipitate can also exist in the H^+, NH_4^+ or Na^+ forms. The pH changes of each strains of T. ferrooxidans during cell growth were shown on Fig. 2-A and B. Fig. 2-A indicates similar behavior for all four strains of T. ferrooxidans. The same points is also illustrated, to a lesser extent, in Fig. 2-B. However, the shapes of pH changes curve were a little bit different from each strains.

Effect of Bacteria Strain. The effect of different strains of T. ferrooxidans on ferrous iron oxidation was studied to compare with the maximum %conversion rate among ATCC strain 13598, 13661, 19859, and 33020; experiments were conducted with the same initial ferrous iron concentration and inoculum size at two different initial pH's (see Table 3). Although all strains have slightly different activities to oxidize ferrous iron to ferric iron in 9K medium, %conversion of ferrous iron to ferric iron as a function of time in the presence of each strains

Figure 2

The Change in pH during Cell Growth of Four Different Strains of T. ferrooxidans in 9K medium.

Temperature 28°C, Inoculum Concentration of 1%.

(A) Initial pH of 1.94

(B) Initial pH of 2.26

Figure 2-A

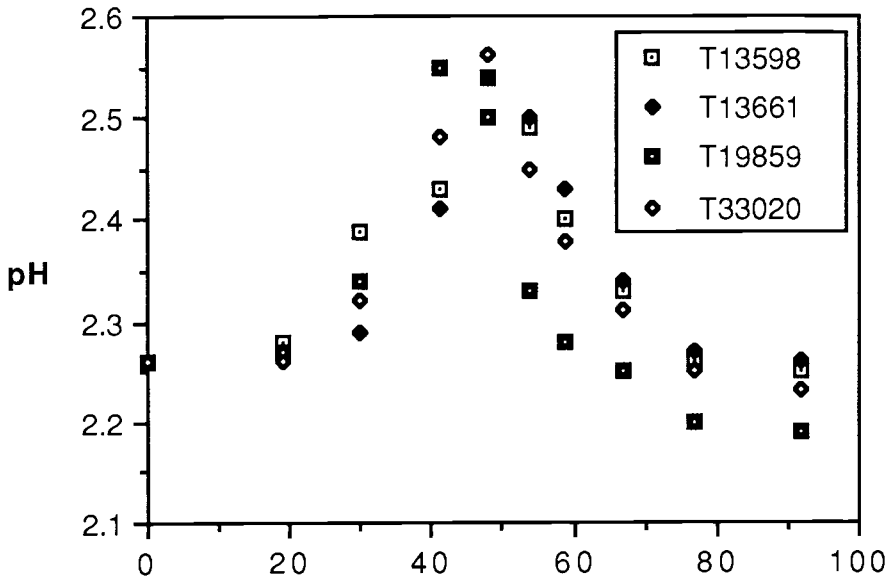


Figure 2-B

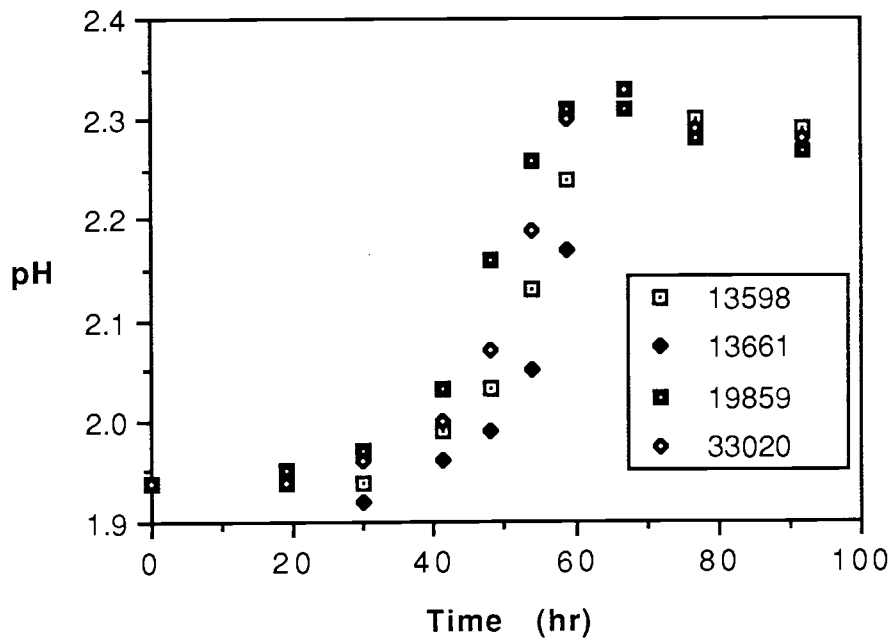


Figure 2

of T. ferrooxidans exhibits a sigmoidal curve (Fig. 3). The T. ferrooxidans strain 19859 is the most effective strain in terms of both the maximum %conversion rate and lag time (Fig. 4).

Effect of Initial pH. The effect of initial pH on ferrous iron oxidation has been measured for T. ferrooxidans strain 13661. Comparisons between maximum %conversion rate and lag time are illustrated as a function of initial pH in Fig. 5. In the initial pH range of 1.9 to 2.7, the maximum %conversion rate is directly proportional to the initial pH; while lag time tends to decrease as the initial pH is increase up to 2.4, at which point it levels off. When the bacteria in its iron-containing media was mixed with fresh 9K medium, precipitation occurred at an initial pH above 2.0. Furthermore, participation occurred at pH's above 2.4 when ferrous sulfate was mixed with the rest of the 9K medium. The precipitate is believed to be iron (both ferrous and ferric) phosphate. As the bacteria thrive, they consume ferrous iron; hence, ferrous phosphate redissolves. The change in pH during ferrous iron oxidation were also monitored as a function of time for various initial pH's (see Figure 6). The medium with a higher initial pH had a lower final pH. This suggests that precipitation, which occurs more readily at higher pH's, results in more acidic conditions via Reaction (4-7).

Figure 3

Conversion of Ferrous Iron to Ferric Iron in the Presence of Various Strains of T. ferrooxidans.

Temperature 28°C, Inoculum Concentration of 1%, Shaker Speed of 120 RPM.

Δ : Initial pH of 2.26

+ : Initial pH of 1.94

——: Sigmoidal Model

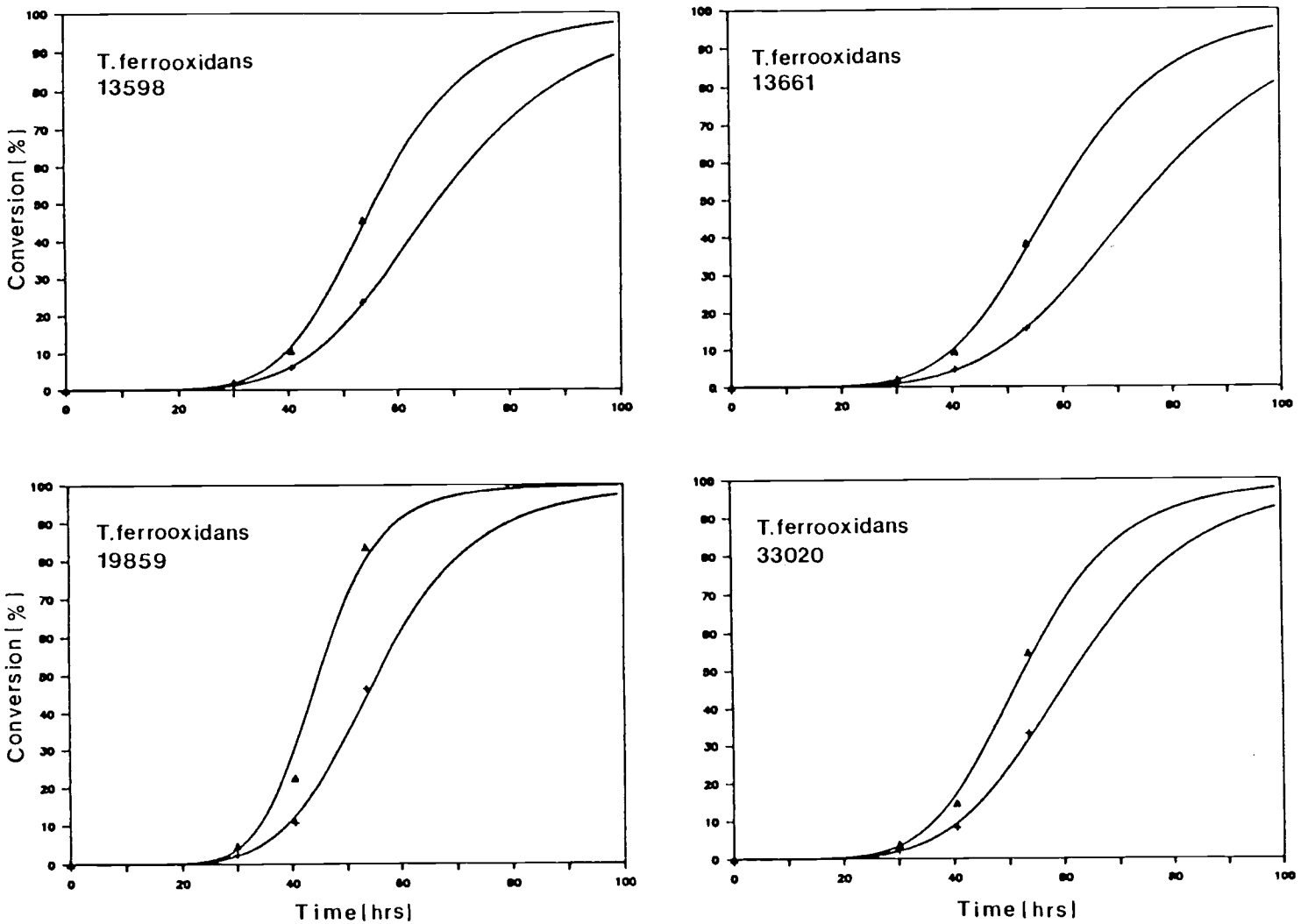
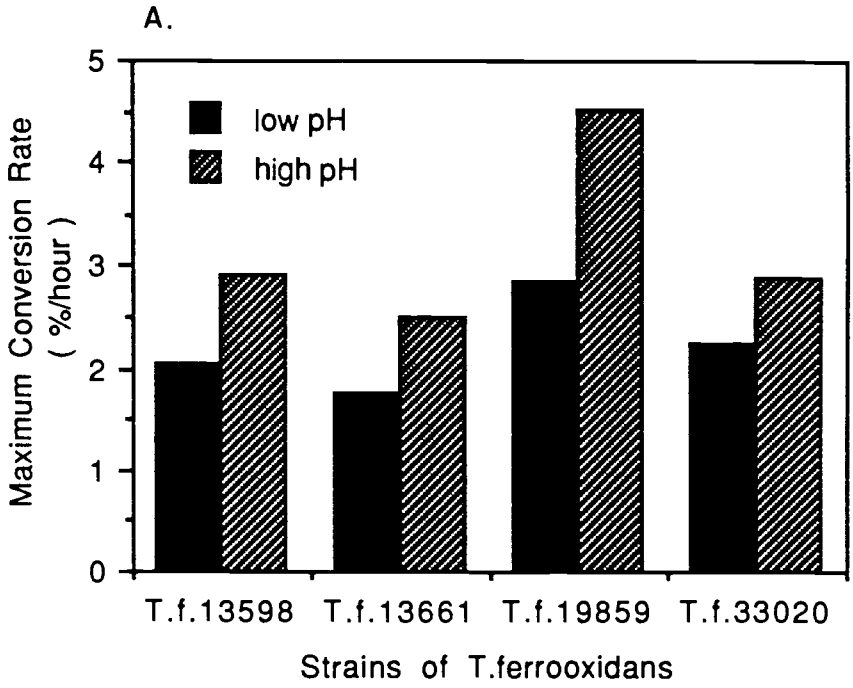

T.ferrooxidans
13598
T.ferrooxidans
13661
T.ferrooxidans
19859
T.ferrooxidans
33020
Conversion [%]
Time [hrs]

Figure 3

Figure 4

Comparison of Maximum %Conversion Rate (A) and Lag Time (B) during Ferrous Iron Oxidation in the Presence of T. ferrooxidans.


A.
Maximum Conversion Rate
(%/hour)
5
4
3
2
1
0
low pH
high pH
T.f.13598
T.f.13661
T.f.19859
T.f.33020
Strains of T.ferrooxidans

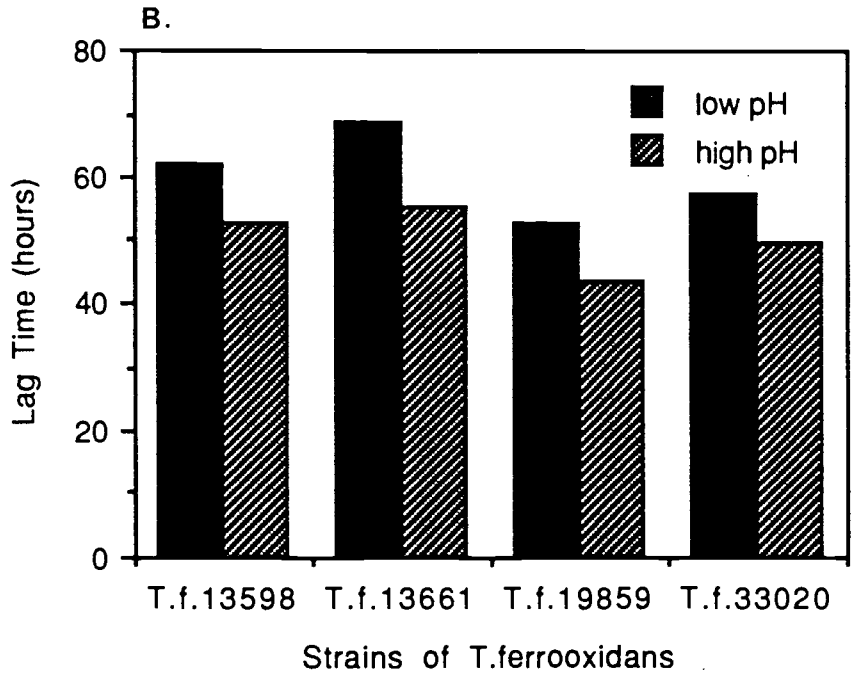

B.
Lag Time (hours)
80
60
40
20
0
low pH
high pH
T.f.13598
T.f.13661
T.f.19859
T.f.33020
Strains of T.ferrooxidans

Figure 4

Figure 5

Comparisons of Maximum %Conversion Rate and Lag Time during Ferrous Iron Oxidation by T. ferrooxidans Strain 13661 at Various Initial pH's.

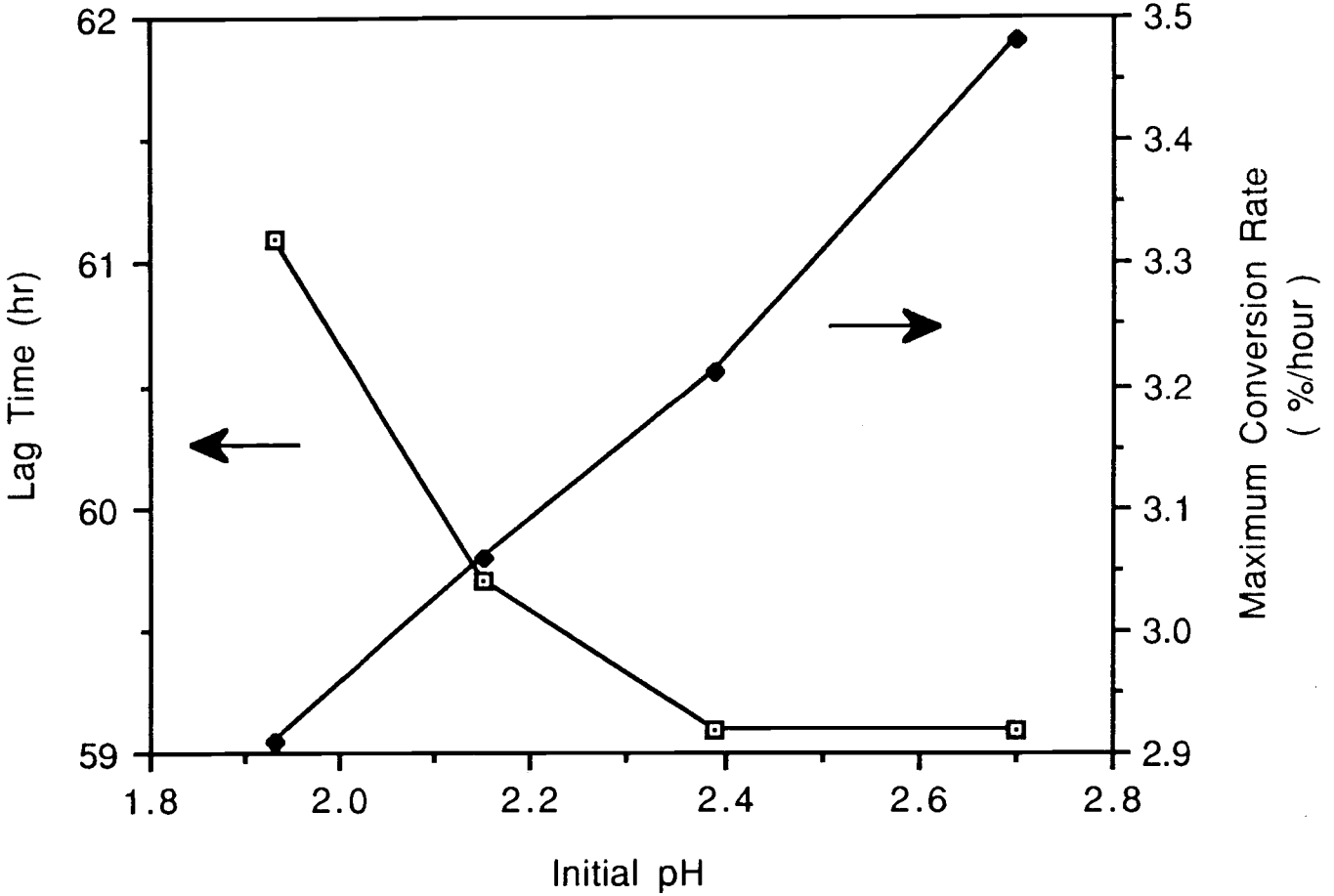

62
61
60
59
Lag Time (hr)
3.5
3.4
3.3
3.2
3.1
3.0
2.9
Maximum Conversion Rate
(%/hour)
1.8
2.0
2.2
2.4
2.6
2.8
Initial pH

Figure 5

Figure 6

The Change in pH during Ferrous Iron Oxidation in the Presence of T. ferrooxidans Strain 13661 at Different Initial pH's.

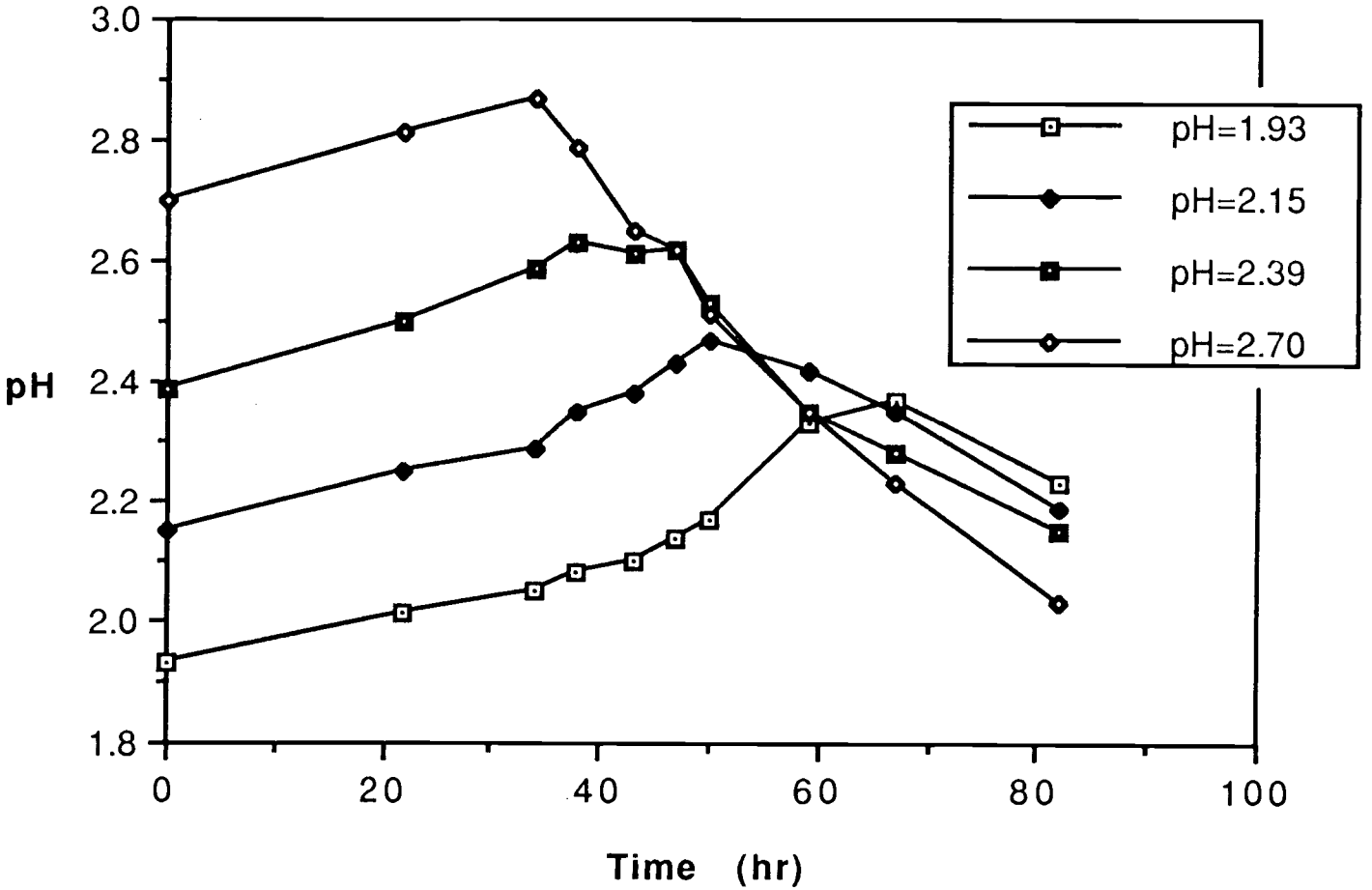

3.0
2.8
2.6
2.4
2.2
2.0
1.8
pH
0
20
40
60
80
100
Time (hr)
pH=1.93
pH=2.15
pH=2.39
pH=2.70

Figure 6

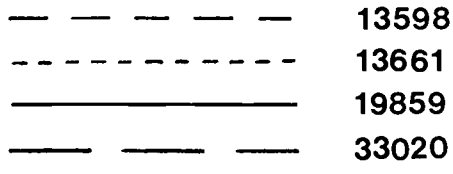
The effect of initial pH on ferrous iron oxidation in the presence of each four strains of T. ferrooxidans is illustrated in Figure 7. All four strains have a higher maximum %conversion rate and a smaller lag time at the higher initial pH.

The change in pH during ferrous iron oxidation were monitored at two different initial pH's (see Figure 8). They all exhibit a similar behavior in that run with the higher pH ends up having the lower final pH. Because precipitation occurs above pH 2.0, an initial pH below than 2.0 should be used to screen or compare new strains of T. ferrooxidans.

Effect of Initial Ferrous Iron Concentration. The impact of the initial ferrous iron concentration on ferrous iron oxidation was investigated. Initial ferrous iron concentrations of 1500, 3000, 6000, and 9000 ppm were prepared and oxidized in the presence of T. ferrooxidans strain 13661. The tests were initiated at a pH of about 2.15 and with bacteria cultures of 1 ml aliquots per 100 ml medium solution. Comparisons of maximum %conversion rate and lag time during ferrous iron oxidation by T. ferrooxidans strain 13661 at different initial ferrous iron concentrations is illustrated on Figure 9. The medium with less initial ferrous iron concentration resulted in a higher maximum %conversion rate and a smaller lag time. The pH changes during ferrous iron

Figure 7

Comparison of %Conversion of Ferrous Iron to Ferric Iron as a Function of Time in the Presence of Various Strains of T. ferrooxidans at Two Different Initial pH's.



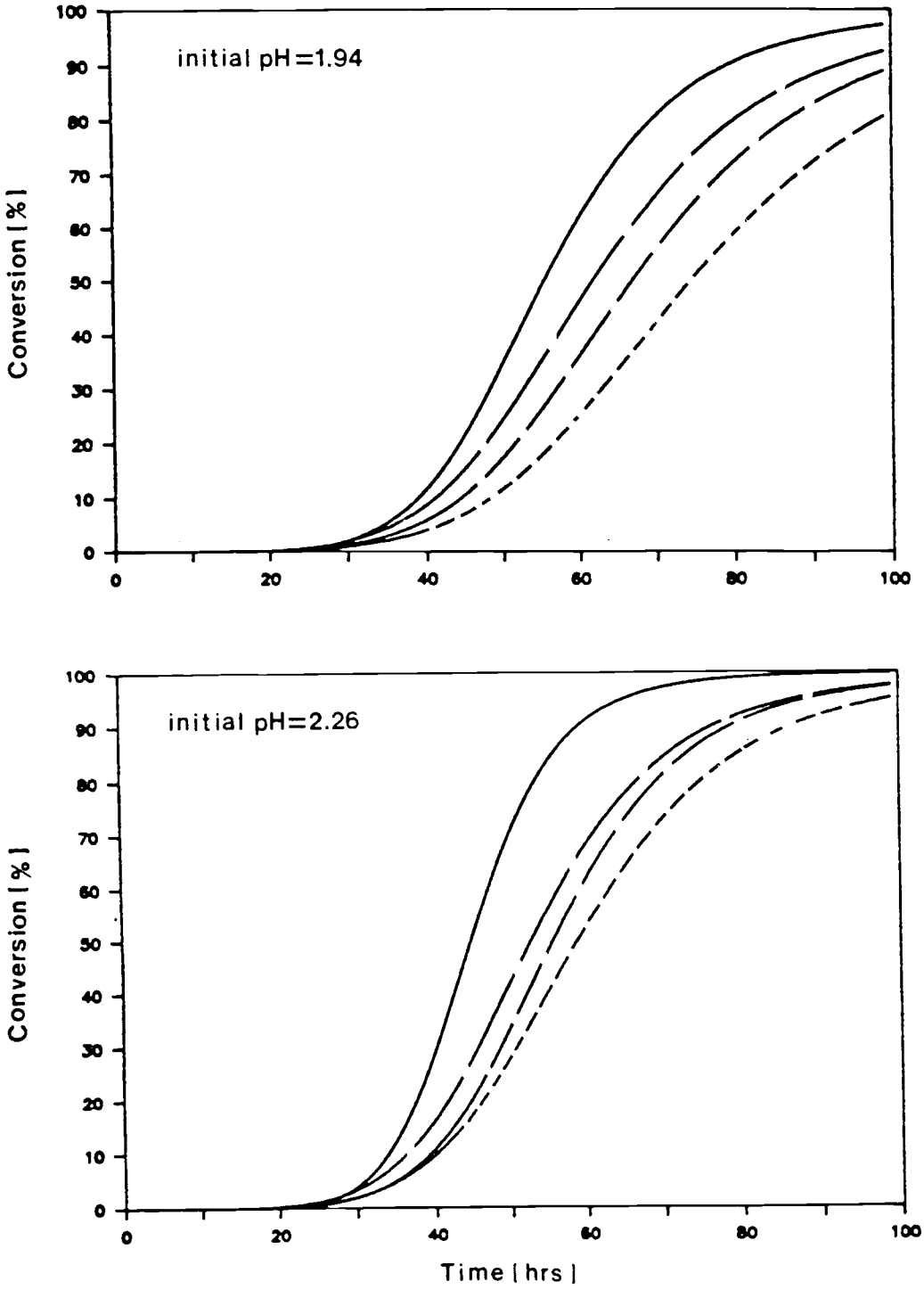
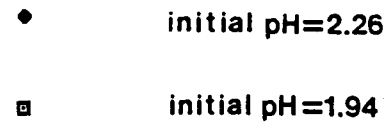

initial pH=1.94
Conversion [%]
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
initial pH=2.26
Conversion [%]
Time [hrs]

Figure 7

Figure 8

The Effect of Initial pH on the Change in pH during Ferrous Iron Oxidation in the Presence of Four Different Strains of T. ferrooxidans.



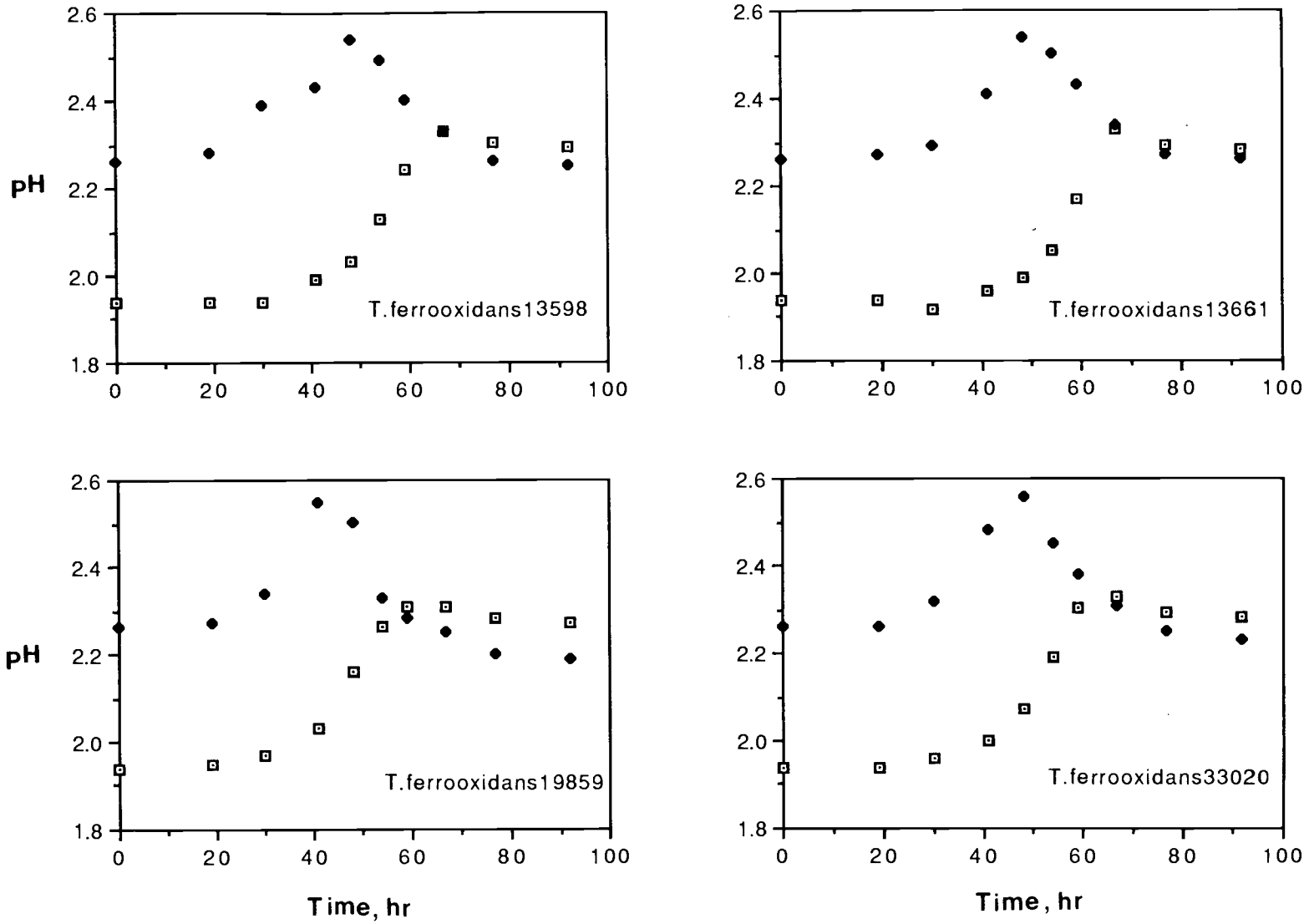

pH
2.6
2.4
2.2
2.0
1.8
0
20
40
60
80
100
T.ferrooxidans13598
T.ferrooxidans13661
T.ferrooxidans19859
T.ferrooxidans33020
Time, hr

Figure 8

Figure 9

Comparisons of Maximum %Conversion Rate and Lag Time during Ferrous Iron Oxidation by T. ferrooxidans Strain 13661 at Different Initial Ferrous Iron Concentrations.

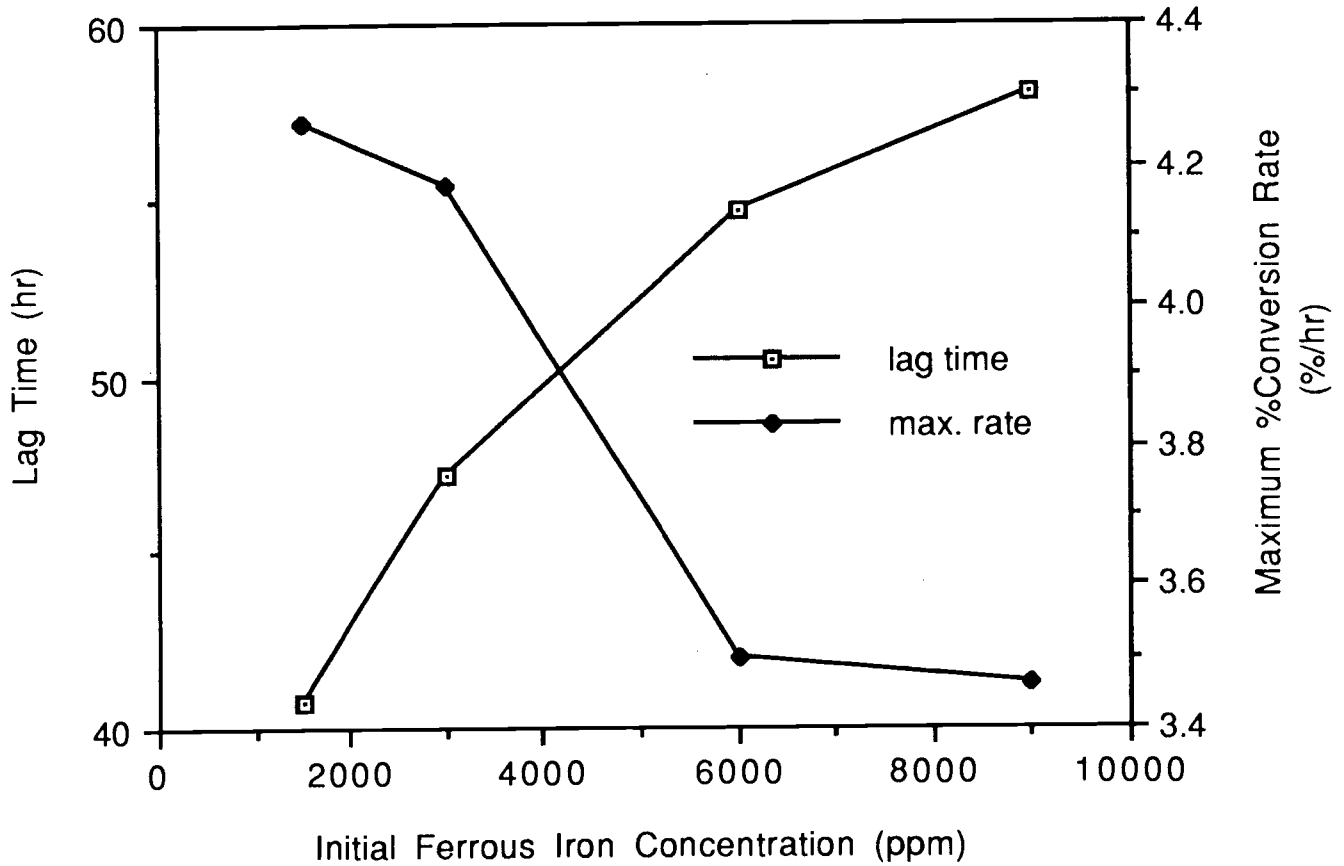

60
50
40
4.4
4.2
4.0
3.8
3.6
3.4
0
2000
4000
6000
8000
10000
Lag Time (hr)
Maximum %Conversion Rate (%/hr)
lag time
max. rate
Initial Ferrous Iron Concentration (ppm)

Figure 9

oxidation in the presence of different ferrous iron concentration have the same general behavior for the same initial pH (Figure 10). Because of the different ferrous iron amounts to be oxidized to ferric iron, each medium has a significantly different final pH due to precipitation. However, the media containing ferrous iron concentrations of 6000 and 9000 ppm seem to have the same final pH. This may mean that there is a critical ferric iron amount to produce precipitation. To screen new strains of T. ferrooxidans, 9K medium with smaller ferrous iron concentration as an energy source should be used.

Effect of Inoculum Size. To establish the effect of inoculum size on ferrous iron oxidation, a series of experiments using 9K medium was conducted by measuring ferrous iron concentration and pH over time. The %conversion of ferrous iron to ferric iron at various inoculum sizes as a function of time is shown on Figure 11. Comparison of maximum %conversion rate and lag time at each inoculum size is given in Figure 12. A larger inoculum size gives a smaller lag time, while values of maximum %conversion rate are not consistent as those of lag time. However, the effect of inoculum size on maximum %conversion rate can be neglected due to small variations from 3.30 to 3.44%/hr. The change in pH was also monitored at different inoculum sizes (Figure 13). They all represent a similar behavior in that the run with the

Figure 10

The Change in pH during Ferrous Iron Oxidation by *T. ferrooxidans* Strain 13661 at Various Initial Ferrous Iron Concentrations.

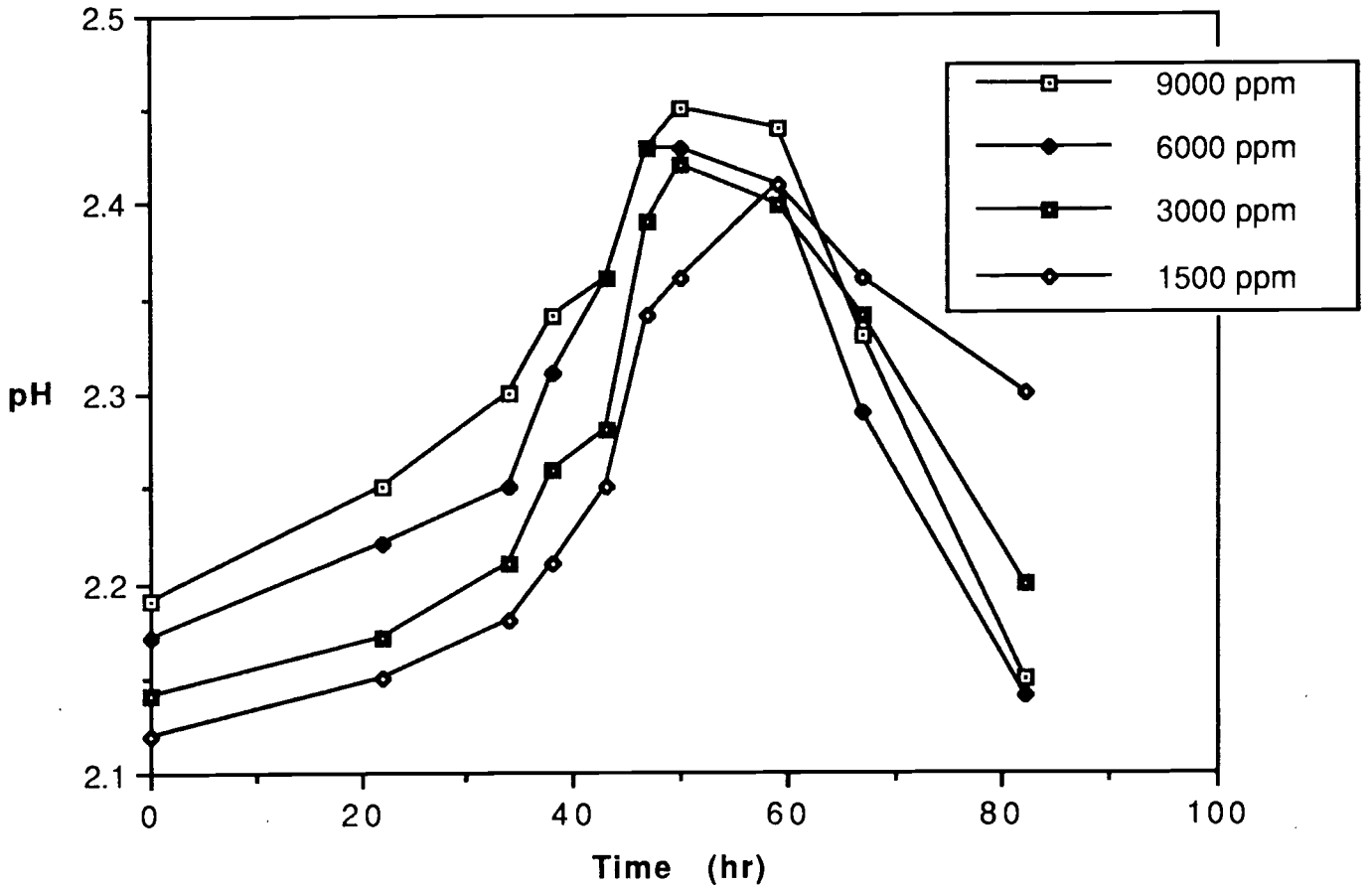

2.5
2.4
2.3
2.2
2.1
pH
0
20
40
60
80
100
Time (hr)
9000 ppm
6000 ppm
3000 ppm
1500 ppm

Figure 10

Figure 11

Conversion of Ferrous Iron to Ferric Iron in the Presence of T. ferrooxidans Strain 13661 at Various Inoculum Sizes.

+ : 1 ml, △ : 2 ml, ▽ : 4 ml

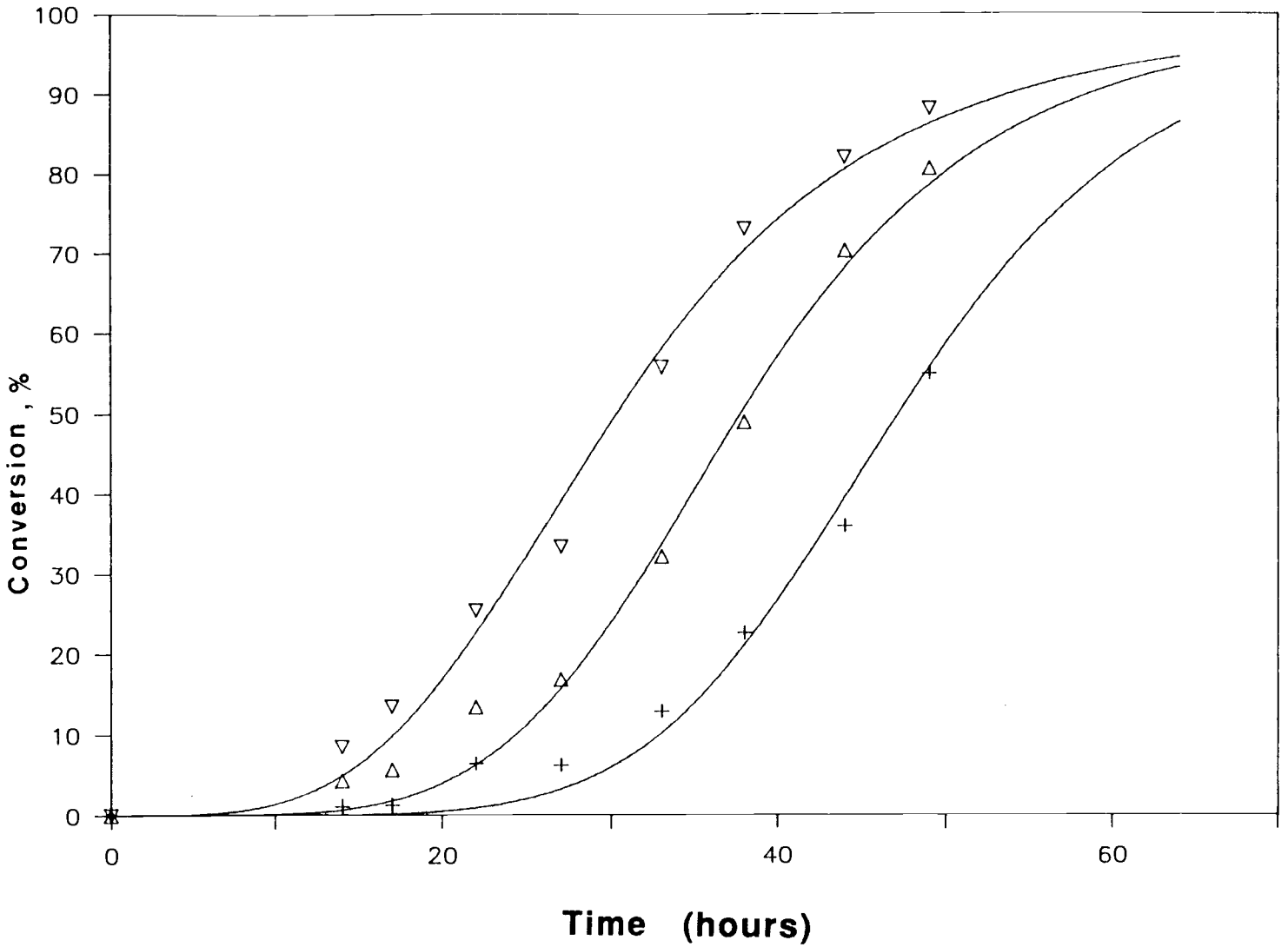


Figure 11

Figure 12

Comparisons of Maximum %Conversion Rate and Lag Time during Ferrous Iron Oxidation by T. ferrooxidans Strain 13661 at Various Initial Inoculum Sizes.

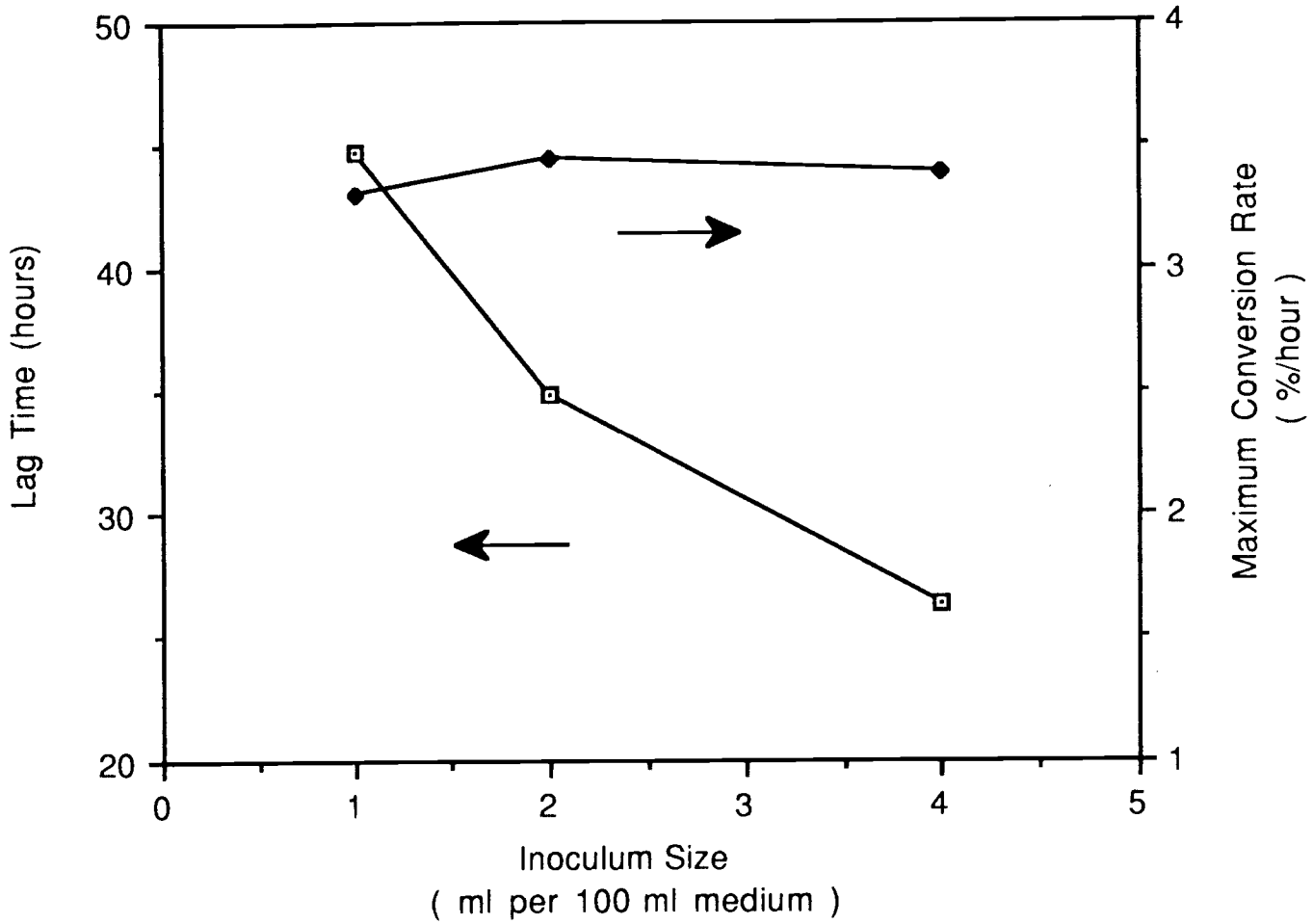

50
40
30
20
0
1
2
3
4
5
4
3
2
1
Lag Time (hours)
Maximum Conversion Rate
(%/hour)
Inoculum Size
(ml per 100 ml medium)

Figure 12

Figure 13

The Change in pH during Ferrous Iron Oxidation in the Presence of T. ferrooxidans Strain 13661 at Different Initial Inoculum Sizes.

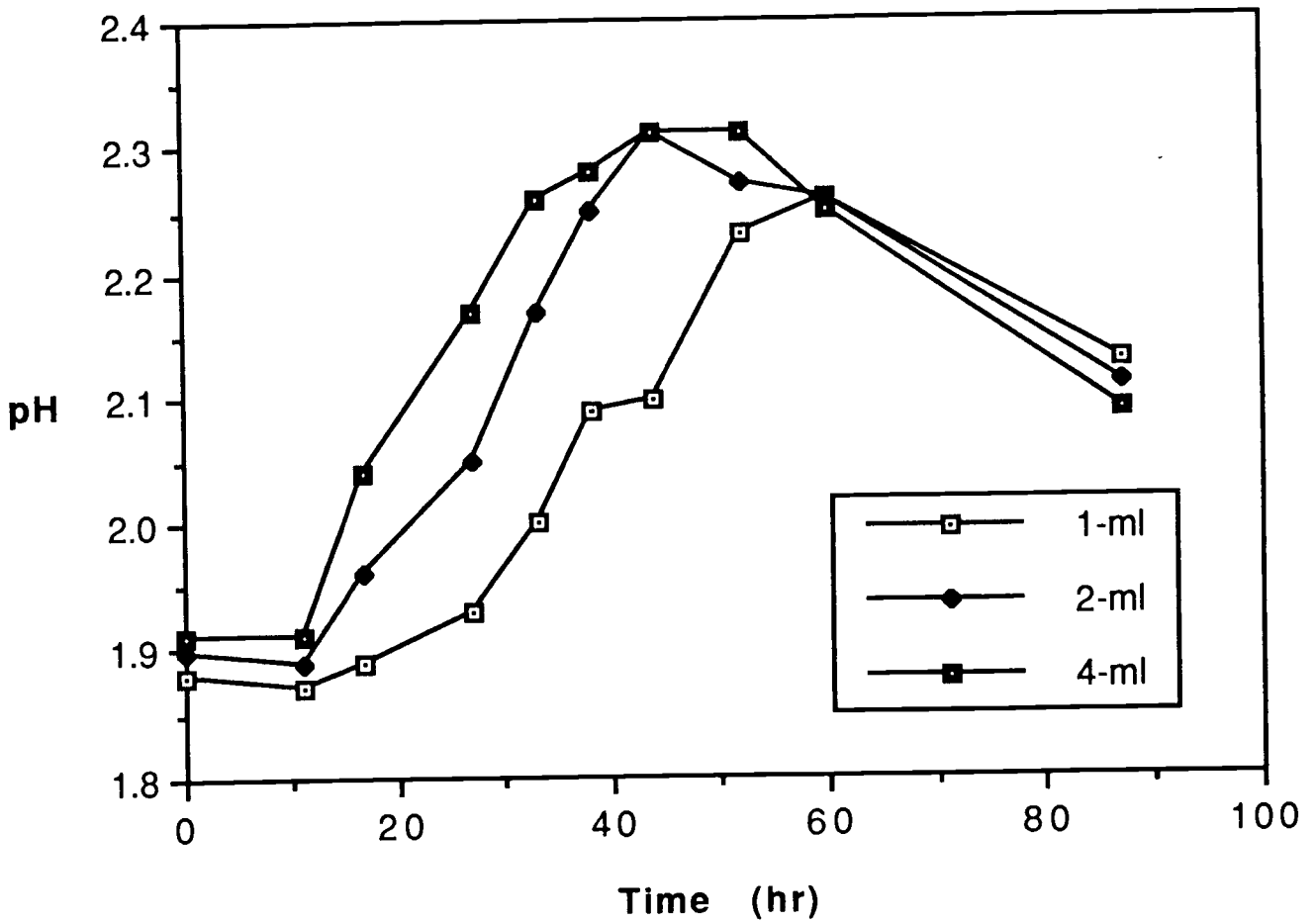


Figure 13

higher inoculum size gives a much higher pH during the early part of run, but ends up with a lower final pH. The main conclusion to be drawn from this study is that a larger inoculum size should be used to reduce a lag time for screening new strains of T. ferrooxidans.

4.2 Pyrite Iron Solubilization

4.2.1 Sigmoidal Model for Pyrite Solubilization

The observed solubilization of pyrite as a function of time exhibits a sigmoidal curve. Therefore, a sigmoidal model equivalent to Equation (4-3) for ferrous iron oxidation can be used to model the solubilization of pyrite:

$$C = \frac{C_{max}\, t^m}{K + t^m} \qquad (4\text{-}8)$$

where C is the total soluble iron concentration, C_{max} is the maximum total soluble iron concentration measured, K and m are constants, and t is the time. The maximum total soluble iron concentration is different from each pulp density, particle sizes, and strains of T. ferrooxidans.

Therefore, C_{max} can be obtained at the end of each experiment. A log-log plot of $C/(C_{max}-C)$ vs. t gives K and m values from the y-intercept and slope. The pyrite solubilization rate can be calculated by taking the first derivative of Equation (4-8) with respect to time:

$$\frac{dC}{dt} = \frac{C_{max}\ K\ m\ t^{m-1}}{(K + t^{m})^{2}} \qquad (4\text{-}9)$$

The maximum pyrite solubilization rate occurs at the lag time where $d^2C/dt^2 = 0$.

Parameter estimates for different particle sizes and different pulp densities at 28°C with 9K medium excluding an energy source, an inoculum concentration of 10% and a shaker speed of 200 rpm are listed in Table 7. C_{max} corresponds to be the concentration of total iron at the end each experiment. Parameters were also estimated for pyrite solubilization in the presence of three different strains of T. ferrooxidans, i.e., 13598, 13661, and 19859 (see Table 8). Results of the maximum solubilization rate of pyrite with T. ferrooxidans strain 13661 at 28°C, 50 ml 9K salts in 250-ml erlenmeyer flask, a shaker speed of 200 rpm, and 2 wt% pulp density are compared with Olson's results using 100-200 mesh South Carolina pyrite in Table 9.

Table 7. Parameter Estmates for Pyrite Solubilization

(a) T. ferrooxidans strain 13661
(b) 5 ml Inoculum/50 ml Total Volume
(c) Temperature=28°C
(d) Shaker Speed=200 RPM
(e) Initial Ferric Iron Concentration=70.4 ppm

particle size (mesh)	80-150			-150
pulp density (wt%)	0.5	1	2	2
max. soluble conc. (ppm)	210	390	730	1950
max. solubilization rate (ppm/hr)	0.06	0.09	0.91	5.17

Table 8. Parameter Estmates for Pyrite Solubilization in the Presence of Three Different Strains of T. ferrooxidans

(a) 5 ml Inoculum/50 ml Total Volume
(b) Temperature=28°C
(c) Shaker Speed=200 RPM

T. ferrooxidans strain	13598	13661	19859
initial ferric iron concentration (ppm)	57.6	70.4	115.3
maximum soluble concentration (ppm)	650	670	570
max. solubilization rate (ppm/hr)	0.71	0.91	1.05

Table 9. Comparison of Maximum Solubilization Rate of Pyrite in the Presence of T. ferrooxidans Strain 13661 at 2 wt%, 28°C and a Shaker Speed of 200 RPM

	max. solubilization rate (ppm/hr)	particle size (mesh)
Thesis	0.91	80-150
	5.17	-150
Olson	2.15 ± 0.2	100-200

4.2.2 Factors Affecting Pyrite Solubilization

Effect of Particle Size. The effects of decreasing particle (i.e., increased surface area) on pyrite solubilization in the presence of T. ferrooxidans strain 13661 have been examined. Figure 14 illustrates that the pyrite solubilization rate greatly increases with a decrease in particle size for a fixed mass of pyrite. From the data presented Table 7, the maximum solubilization are 0.95 and 5.17 ppm/hr, respectively, for a particle size fractions of 80-150 mesh and -150 mesh.

Several differences in experimental conditions for the comparison of our maximum solubilization rates with Olson's are particle size, pyrite source, and energy source for bacteria cultures. Dr. Olson used pyrite as an energy source for bacteria culturing, while ferrous sulfate solution was used in this study. The most distinct sources of difference in maximum pyrite solubilization rate among known experimental conditions may have resulted from initial ferrous iron concentration and particle size (see Table 9).

Smaller particles yield higher ferrous iron concentrations in solution during the lag phase according to the acid-consuming reaction (Reaction 2-9), which is provided as an energy source for cell growth. Therefore, standard methods for measuring solubilization rates need

Figure 14

The Effect of Particle Size on Pyrite Solubilization in the Presence of T. ferrooxidans Strain 13661.

Temperature 28°C, Inoculum Concentration of 10%, Shaker Speed 200 RPM.

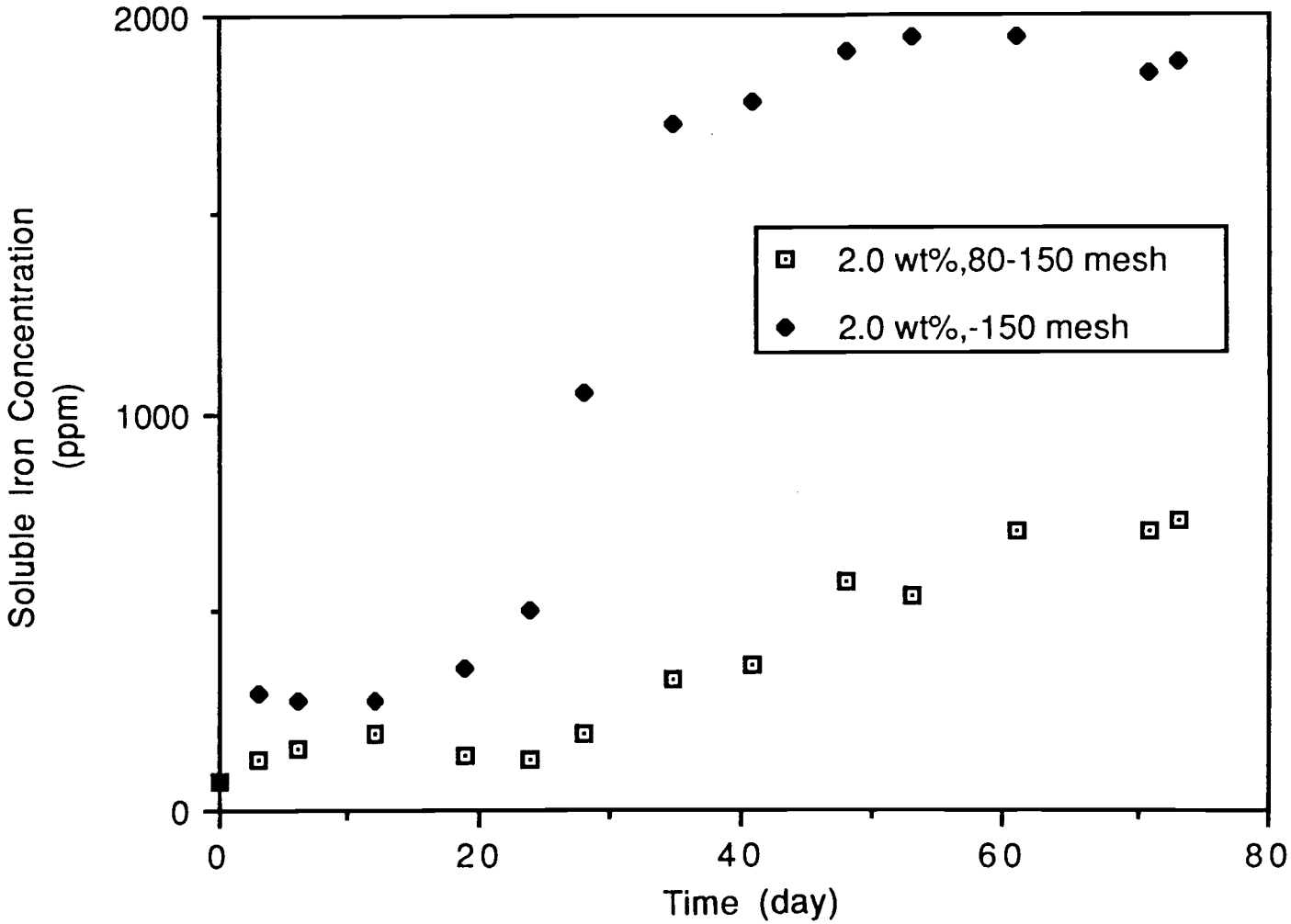

2000
1000
0
Soluble Iron Concentration (ppm)
0
20
40
60
80
Time (day)
2.0 wt%,80-150 mesh
2.0 wt%,-150 mesh

Figure 14

to be developed.

Effect of Pulp Density. The effect of increasing pulp density, which is defined as weight of solids/ volume of liquid, on the rate of pyrite solubilization when the particle size fraction is held constant (i.e., 80 < D_p < 150 mesh) is illustrated in Figure 15 in the presence of T. ferrooxidans strain 13661. The maximum solubilization rate at 2 wt% is much higher than that at 0.5 and 1 wt% (see Table 7). This is probably primarily due to large errors involved in the calculation of maximum solubilization rates for the lower pulp densities, which resulted from the fact that the experiments did not reach the maximum soluble iron concentration. Hence, we suspect that the predicted values of maximum soluble iron concentrations for the 0.5 and 1 wt% pulp densities are not very accurate due to extrapolation methods.

The higher initial solid loading yields higher ferrous iron according to the acid-consuming reaction (Reaction 2-9) during the initial period of experimental run, which in turn is oxidized to ferric iron. Higher ferric iron concentration leads to higher solubilization rate and shorter lag time.

Effect of Strains of Bacteria. With a constant pulp density and particle size, the soluble iron concentration was monitored as a function of time in the presence of different strains (see Figure 16). Due to the high

Figure 15

The Effect of Pulp Density on Pyrite Solubilization in the Presence of T. ferrooxidans Strain 13661.

Particle Size of 80-150 Mesh, Temperature 28°C, Inoculum Concentration of 10%, Shaker Speed 200 RPM.

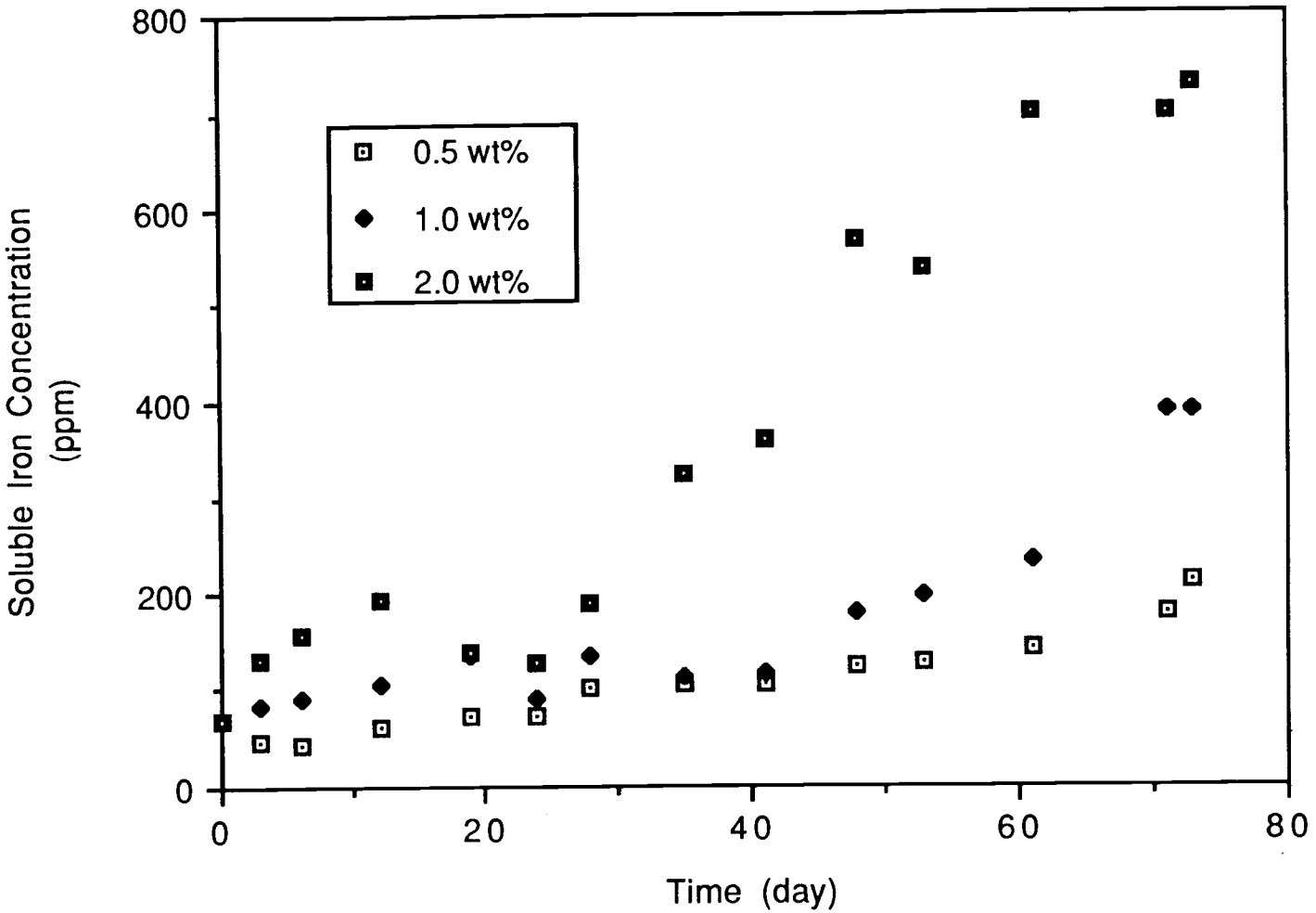

800
600
400
200
0
0
20
40
60
80
Soluble Iron Concentration (ppm)
Time (day)
0.5 wt%
1.0 wt%
2.0 wt%

Figure 15

Figure 16

The Effect of Strains of T. ferrooxidans on Pyrite Solubilization.

Pulp Density of 2 wt%, Particle Size of 80-150 Mesh, Temperature 28°C, Inoculum Concentration of 10%, Shaker Speed 200 RPM.

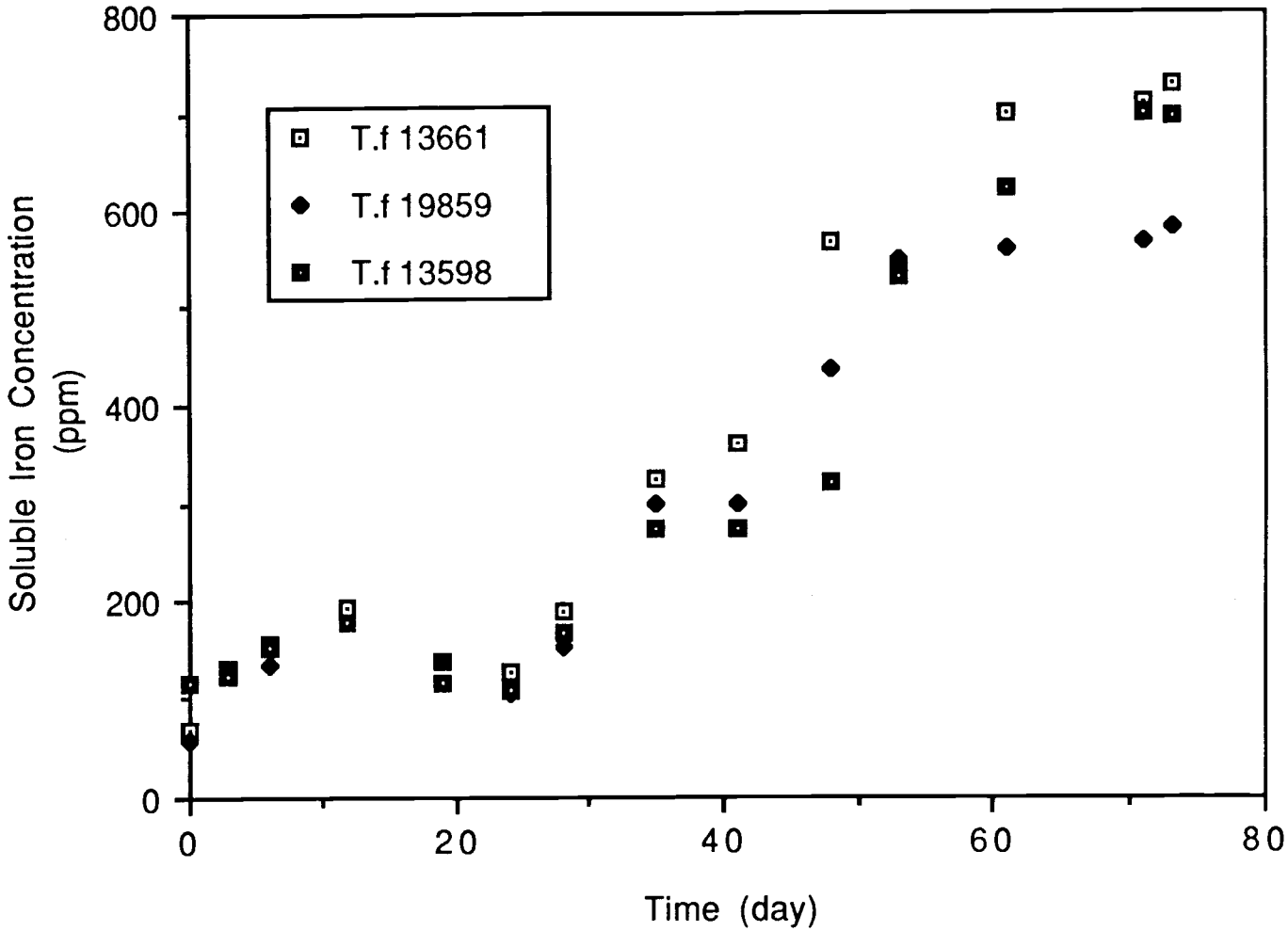

800
600
400
200
0
0
20
40
60
80
Soluble Iron Concentration (ppm)
Time (day)
T.f 13661
T.f 19859
T.f 13598

Figure 16

initial ferric iron concentration and the insufficient experimental run with T. ferrooxidans strain 19859, we can not conclude that this strain is the most effective one in terms of the maximum solubilization rate. The higher initial ferric iron concentration results in the higher maximum solubilization rate (see Table 8). It is also impossible to conclude that the variations in maximum solubilization rate from 0.71 to 1.05 ppm/hr in the presence of each different strain result from the different activities of strains or the different initial ferric iron concentration due to the lack of information. Strain 19859 appears to be better, but we can not be sure because of higher initial ferric iron concentration. However, strain 19859 also gave a higher rate for ferrous iron oxidation. Hence, if we had to select one strain for further study, we would suggest the use of strain 19859.

5. CONCLUSIONS

1. Ferrous iron oxidation can be considered as a simple and fast method to test for effectiveness of new strains of T. ferrooxidans on metal solubilization. When experiments for ferrous iron oxidation are performed to screen new strains of T. ferrooxidans on metal solubilization, 9K medium with low initial ferrous iron concentration should be used at lower pH than 2.0 and high inoculum size and it is important to maintain the same ferric iron concentration in bacteria cultures used as an inoculum through a whole ferrous iron oxidation experiments. Hence, to secure cell suspensions containing a minimum of precipitated iron and constant cell number, the bacteria should be harvested using a supercentrifuger.

2. The most important factor affecting pyrite solubilization is a particle size. Smaller particle size gives a much higher pyrite solubilization rate.

3. The effect of inoculum size on the maximum rate of %conversion of ferrous iron to ferric iron appears to be negligible, while the lag time is reduced by using a high inoculum size.

4. Lower initial ferrous iron concentrations give a higher maximum %conversion rate and smaller lag time.

5. Results of the parametric study of factors affecting ferrous iron oxidation show that the maximum %conversion rate was a function of T. ferrooxidans strains, initial pH, initial ferrous iron concentration, and inoculum size. The lag time in batch reactor using a shaker is found to be function of same parameters as oxidation of ferrous iron.

6. RECOMMENDED PROCEDURES FOR COMPARING VARIOUS STRAINS OF THIOBACILLUS FERROOXIDANS

In this work two different methods of comparing iron oxidation by T. ferrooxidans were studied namely: (1) the oxidation of solubilized ferrous iron and (2) the oxidation of solid particle of pyrite. Of these two methods, the oxidation of ferrous irons in solution is preferred because it is a much quicker method for comparing various strains of T. ferrooxidans, and it is simpler to use. However, before settling on the Ferrous Iron Oxidation Method (FIOM), we need to compare the two methods for consistency. That is, a series of studies with various strains of T. ferrooxidans needs to be conducted to determine if the relative rate of iron oxidation in both the ferrous iron and pyrite is the same.

6.1 Recommended Procedure for Cell Growth and Harvest of T. ferrooxidans for Iron Oxidation Methods

Each culture is to be grown at 28^{o}C in 9K medium that has a pH of 3.0 to 3.6 and contains ferrous iron concentration of 9000 ppm. First, 70 ml of basal salts are poured into a 250-ml erlenmeyer flask and then autoclaved for about 30 min at 18 psia, while the ferrous

iron solution is filtered through a 0.45-micron filter to remove any microorganism that may be present. The sterilized 30 ml of iron solution is mixed with 70 ml of cooled basal salts to give 100 ml of 9K medium in each 250-ml erlenmeyer flask. The mouth of each flask is then plugged with sterile cotton. Next, 1-ml aliquots of the bacteria cultures are inoculated into each flask containing 100 ml of 9K medium. A sterile micropipette is used to transfer the inoculum from the bacteria to the 9K medium. Care should be taken not to touch the sides or the mouth of flasks, which are flame sterilized (see Aseptic Technique Procedure). Following inoculation, each flask is placed on a temperature controlled shaker set at 120 rpm. Batch cultures are grown for 4 or 5 days (i.e., until the end of the growth phase). Note that the opalescent and green color of 9K medium should turn to a brown color with a precipitate.

The contents of each flask is centrifuged in a supercentrifuger at the rate of 2 to 3 liter per hr. The cell paste is suspended in approximately 300 ml of cold distilled water (adjusted to pH 3.0 with H_2SO_4), shaken vigorously in a 1-L erlenmeyer flask for 1 min, and allowed to stand overnight at 4^oC to precipitate residual iron. The turbid supernatant solution is carefully removed from the underlying layer of precipitated iron. This procedure is repeated twice. The cells, contained in

the combined supernatant solution, are separated by centrifugation, washed several times in distilled water (pH 2.6), and brought to a volume of 50 ml with distilled water adjusted to a pH of 3.5. Cells are stored in a refrigerator until needed.

6.1.1 Aseptic Technique

In order to obtain a pure culture, which is defined as a culture containing only one species of a microorganism, the aseptic technique should be used in transferring bacteria. The procedure is as follows:

(a) Shake the erlenmeyer flask from side to side to put the microorganism into suspension. Do not moisten the plug with culture.
(b) Remove the cotton plug and flame the mouth of the flask. Do not contaminate the cotton plug by placing on the table.
(c) Remove the measured volume of bacteria cultures by inserting a sterile micropipette as quickly as possible. Do not touch the sides and the mouth of the flask.
(d) Flame the mouth of flask, again.
(e) Return the cotton plug to the flask in its original

position.

(f) Remove the cotton plug of the reactor while holding the micropipette taken the microorganism.

(g) Briefly heat the mouth of the reactor in a burner flame before putting the measured bacteria cultures.

(h) Inject the bacteria cultures taken into the reactor.

(i) Flame the mouth of the reactor immediately after inoculation.

(j) Return flask to the shaker immediately after replacing the cotton plug.

6.1.2 Method to Calculate the Necessary Volume of Bacterial Cultures as an Inoculum

Cells are stored in a 50 ml of distilled water adjusted to a pH of 3.5 in a refrigerator until needed. There are three steps accounting for various cell numbers in solution. First, bacteria cultures grown for several days reach approximately 10^8 cells/ml. Secondly, after harvesting and washing the cells, the 50 ml of distilled water with a cell suspension of approximately $2x10^9$ cells/ml should be obtained. Cell counting should be done to obtain the exact cell number in the solution. Lastly, the reactor solution gives a 10^8 cells/ml by inoculating the calculated volume of bacteria cultures in second step.

6.2 Recommended Procedure for the Oxidation of Ferrous Iron

The recommended procedure for ferrous iron oxidation will require less than two days to complete the screening of T. ferrooxidans.

6.2.1 Experimental Procedure

The following experimental procedure is to be conducted at 28°C under sterile conditions for each strain T. ferrooxidans to be compared. First, the appropriate volume of 9K medium including basal salts, which is needed to complete all runs, is adjusted to pH 1.9 with the addition of concentrated sulfuric acid. Next, 70 ml of 9K medium is poured into each 250-ml erlenmeyer flask to be used in the study (three flasks per strain plus controls). While this flask with 70 ml of 9K medium is autoclaving for 30 min at 18 psia, a necessary volume of 1500 ppm ferrous iron solution is passed through a 0.45-micron filter to remove any microorganisms that may have been present in the solution. After autoclaving, 30 ml of ferrous iron solution sterilized is put into a 250-ml flask which contains 70 ml of 9K medium. The addition of ferrous iron solution will result in increasing pH up to

approximately 2.0. Finally, 100 ml of fresh 9K medium is prepared to conduct the experiments.

The bacteria cultures in measured aliquots, which have been stored in refrigerator after harvesting and washing, are inoculated to fresh 9K medium (see Section 6.1 for the further information). A sterile micropipette is used to transfer the inoculum, following the aseptic technique (see Section 6.1.1). Following inoculation, each flask is placed on a horizontal shaker set at 28^{o}C and 120 rpm. Triplicate runs for each strain of T. ferrooxidans are performed to ensure reproducibility of all experiments. Two controls, which exclude the bacteria inoculum, are also prepared to check the ferrous iron oxidation in the absence of bacteria.

Sampling is begun immediately after inoculation by inserting a sterile pipette into a flask to remove 2-ml of liquid sample. Each sample is filtered through a 0.45-micron filter to remove bacteria from the liquid sample. The pH of a sample is measured by inserting the thin electrode of a pH meter into the flask after the sample is withdrawn. Care should be taken to maintain sterile conditions when sampling and measuring pH. Sampling will be done continuously every 3 to 4 hr for 2 days.

6.2.2 Data Analysis

At least, ten samples should be collected to accurately determine the maximum conversion rate during ferrous iron oxidation. Each sample is analyzed to measure the ferrous iron concentration in solution by a volumetric titration method (see Section 3.4.1). The maximum conversion rate is obtained by applying the sigmoidal model for the oxidation of ferrous iron to ferric iron (see Section 4.1.1).

6.3 Recommended Procedure for Pyrite Solubilization

The recommended procedure for the pyrite oxidation method of screening various strains of T. ferrooxidans will need one month to complete. This method should be continued until no more pyrite iron is solubilized (i.e., until the soluble iron concentration levels off).

6.3.1 Experimental Procedure

The selected pyrite sample (the same pyrite should be used for any given comparison) is pulverized to pass through a 200-mesh screen; with particles being collected

in the range of 200 to 250 mesh. To minimize soluble aqueous-phase iron before the beginning of each experiment, the pyrite samples are shaken in a 2 N HCl solution for 30 min, rinsed thoroughly with distilled water, and subsequently dried overnight in a oven. Two-gram samples of dried pyrite are added to 250-ml erlenmeyer flasks, which are then covered with sterile cotton.

The appropriate volume of 9K medium, excluding an energy source, is adjusted to pH 2.0 by the addition of concentrated H_2SO_4. For sterile runs, the 9K medium without ferrous sulfate and the flask that contains the pyrite samples are autoclaved separately at 18 psia for 30 min. Next, 100 ml of 9K medium is put into a flask to be mixed with pyrite sample when cooled. The inoculation should be performed using the aseptic technique procedure described above within 30 min after 9K medium and pyrite are mixed together. All flasks are placed on a horizontal incubator shaker set at 200 rpm.

The triplicate runs for each strain are conducted per standard microbiology experimental procedure. In the duplicate control runs, the pyrite sample is mixed with 100 ml of 9K medium without inoculating bacteria cultures. The first samples are taken immediately after inoculation by using a sterile pipette to remove 1-ml liquid samples after suspended solids have settled. The liquid sample is

filtered through a 0.45-micron filter, and then is stored in a 15-ml sampling bottle after the addition of 0.1 ml of 3 N HNO_3 in the sample to ensure that no iron precipitates before analyzing the soluble iron by Atomic Absorption Spectrophotometry. The 1.0 ml of 9K medium adjusted to pH 2.0 is added after every sampling to bring the volume in the flask back to its original volume. The pH of the sample is measured immediately after the sample is withdrawn. Care should be taken to maintain sterile conditions. Samplings are performed to remove 1-ml liquid samples every 2 or 3 days.

6.3.2 Data Analysis

The pyrite oxidation experiment should be conducted until no more pyrite iron solubilized. The final iron concentration is assumed to be the maximum soluble iron concentration which can be obtained in a batch reactor. Each sample is analyzed for soluble iron concentration by AAS (see Section 3.4.2). The maximum pyrite solubilization rate can be obtained from the sigmoidal model described in Section 4.2.1.

7. RECOMMENDATIONS FOR FUTURE WORK

1. The experiments for comparing various strains of *T. ferrooxidans* should be performed by following the two recommended procedures.

2. If the relative rate of iron oxidation in both the ferrous iron and pyrite iron is the same, the simple and quick Ferrous Iron Oxidation Method should be used to compare all strains of *T. ferrooxidans* and to screen new strains of *T. ferrooxidans* coming in terms of the maximum conversion rate.

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APPENDICES

A.1 Soluble Iron Concentration in Pyrite Oxidation by *T. ferrooxidans* 13661

Time (day)	80-150 mesh			-150 mesh
	0.5 wt%	1 wt%	2 wt%	2 wt%
0	70.4	70.4	70.4	70.4
3	47.8	84.7	130.0	293.0
6	43.2	90.3	156.0	273.8
12	63.5	104.7	193.2	272.3
19	71.6	134.5	139.3	349.0
24	74.5	90.0	127.0	500.7
28	100.2	136.1	190.2	1047.2
35	104.8	111.7	322.7	1732.5
41	104.9	114.7	361.0	1785.5
48	122.5	177.0	567.8	1910.7
53	125.8	194.6	536.8	1946.5
61	142.1	233.7	697.6	1950.0
71	177.6	387.3	700.0	1851.4
73	210.6	390.0	726.9	1883.0

A.2 Soluble Iron Concentration in Pyrite Oxidation by Different Strains of T. ferrooxidans

Time (day)	T. ferrooxidans strain, 80-150 mesh, 2 wt%		
	13661	19859	13598
0	70.4	57.6	115.3
3	130.0	126.1	123.7
6	156.0	136.0	151.9
12	193.2	179.5	178.2
19	139.2	136.8	114.6
24	127.0	104.7	108.0
28	190.2	151.2	167.7
35	322.7	297.5	272.5
41	361.0	297.4	272.5
48	567.8	435.2	318.7
53	536.8	550.1	531.1
61	697.6	560.7	623.3
71	700.0	568.4	700.0
73	726.9	582.8	694.2

A.3 The pH and %Conversion of Ferrous Iron to Ferric by Different Strains of T. ferrooxidans for an Initial pH of 1.94

Time (hour)	T. ferrooxidans strain, initial pH 1.94							
	13598		13661		19859		33020	
	pH	conv.	pH	conv.	pH	conv.	pH	conv.
0	1.94		1.94		1.94		1.94	
19	1.94		1.94		1.95		1.94	
30	1.94	1.4%	1.92	1.0%	1.97	2.4%	1.96	2.2%
41	1.99	6.2%	1.96	4.6%	2.03	10.8%	2.00	8.3%
48	2.03		1.99		2.16		2.07	
54	2.13	23.7%	2.05	15.7%	2.26	46.5%	2.19	33.2%
59	2.24		2.17		2.31		2.30	
67	2.33		2.35		2.32		2.33	
77	2.30		2.29		2.28		2.29	
92	2.29		2.28		2.27		2.23	

A.4 The pH and %Conversion of Ferrous Iron to Ferric by Different Strains of T. ferrooxidans for an Initial pH of 2.26

Time (hour)	T. ferrooxidans strain, initial pH 2.26							
	13598		13661		19859		33020	
	pH	conv.	pH	conv.	pH	conv.	pH	conv.
0	2.26		2.26		2.26		2.26	
19	2.28		2.27		2.27		2.26	
30	2.39	2.1%	2.29	2.2%	2.34	4.8%	2.32	3.8%
41	2.43	10.6%	2.41	9.4%	2.55	22.6%	2.48	14.8%
48	2.54		2.54		2.50		2.56	
54	2.49	45.5%	2.50	38.2%	2.33	83.6%	2.45	54.7%
59	2.40		2.43		2.28		2.38	
67	2.33		2.34		2.25		2.31	
77	2.26		2.27		2.20		2.25	
92	2.25		2.26		2.19		2.23	

A.5 The pH during Ferrous Iron Oxidation by *T. ferrooxidans* 13661 at Different Initial pH's

Time (hours)	initial pH				
	1.93	2.15	2.39	2.61	2.70
0	1.93	2.15	2.39	2.61	2.70
22	2.01	2.25	2.50	2.72	2.81
34	2.05	2.29	2.59	2.80	2.87
38	2.08	2.35	2.63	2.80	2.79
43	2.10	2.38	2.61	2.65	2.65
47	2.14	2.43	2.62	2.61	2.62
50	2.17	2.47	2.53	2.53	2.51
59	2.33	2.42	2.35	2.32	2.35
67	2.37	2.35	2.28	2.24	2.23
82	2.23	2.19	2.20	2.06	2.03

A.6 The %Conversion of Ferrous Iron to Ferric Iron at Different Initial pH's by T. ferrooxidans 13661

Time (hours)	initial pH				
	1.93	2.15	2.39	2.61	2.70
0	0	0	0	0	0
22	0.6	0.8	0	0	0
34	1.5	2.9	2.2	1.9	1.7
38	1.3	6.0	2.8	2.4	2.4
43	5.5	6.3	4.7	4.6	4.6
47	9.9	11.3	10.8	11.3	11.3
50	14.5	16.7	18.9	17.2	16.6
59	37.0	38.0	40.0	38.5	37.9
67	60.1	66.4	68.9	69.0	72.7
82	98.9	99.6	99.7	99.7	99.7

A.7 The pH during Ferrous Iron Oxidation with Different Initial Ferrous Iron Concentrations by *T. ferrooxidans* 13661

Time (hours)	initial ferrous iron conc. (ppm)			
	9000	6000	3000	1500
0	2.19	2.17	2.14	2.12
22	2.25	2.22	2.17	2.15
34	2.30	2.25	2.21	2.18
38	2.34	2.31	2.26	2.21
43	2.36	2.31	2.28	2.25
47	2.43	2.43	2.39	2.34
50	2.45	2.43	2.42	2.36
59	2.44	2.41	2.40	2.41
67	2.33	2.29	2.34	2.36
82	2.15	2.14	2.20	2.30

A.8 The %Conversion of Ferrous Iron Oxidation with Different Initial Ferrous Iron Concentrations by T. ferrooxidans 13661

Time (hours)	initial ferrous iron conc. (ppm)			
	9000	6000	3000	1500
0	0	0	0	0
22	0	0	1.8	2.5
34	0.8	2.3	8.4	17.9
38	3.0	6.8	12.5	27.0
43	6.2	10.3	22.8	45.7
47	10.9	18.5	40.8	69.9
50	18.1	28.3	58.5	90.8
59	41.8	59.8	98.0	99.5
67	75.3	96.3	99.7	99.8
82	99.7	99.9	99.9	99.9

A.9 The pH and %Conversion of Ferrous Iron to Ferric Iron at Different Inoculum Sizes by T. ferrooxidans 13661

Time (hours)	inoculum size					
	1 ml		2 ml		4 ml	
	pH	conv.%	pH	conv.%	pH	conv.%
0	1.87		1.90		1.91	
11	1.87		1.89		1.91	
17	1.89	1.3	1.96	5.6	2.04	13.6
27	1.93	6.3	2.05	16.8	2.17	33.4
33	2.00	12.9	2.17	32.2	2.26	55.7
38	2.09	22.7	2.25	48.9	2.28	73.0
44	2.10	36.0	2.31	70.2	2.31	91.9
52	2.23	66.6	2.27	97.8	2.31	98.4
60	2.26	94.8	2.26	99.9	2.25	99.9
87	2.13		2.11		2.09	