MICROBIAL ECOLOGY OF *THIOBACILLUS FERROOXIDANS*

INSTITUTE OF NORTHERN ENGINEERING

Microbial ecology of thiobacillus ferrooxidans
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FORWARD

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INTRODUCTION

Thiobacillus ferrooxidans is an acidophilic chemoautotrophic bacterium which is capable of aerobically oxidizing reduced iron as a sole energy source for growth and carbon dioxide fixation. *T. ferrooxidans* can also oxidize reduced sulfur compounds and it is this characteristic that is responsible for classification as a thiobacilli (Tuovinen and Kelly, 1972; Apel et al., 1980; Brierley, 1982). It is a particularly interesting bacteria and has been given considerable attention in the literature due to its ecological, economic and biochemical significance.

Ecologically, *T. ferrooxidans* is one of the major organisms responsible for catalyzing the chemical reactions leading to acid mine drainage and to the leaching of toxic heavy metals into groundwaters and streams. Economically, the leaching abilities of these organisms have been harnessed to recover metals from low-grade sulfide ores. By the late 1970s, bioleaching of low-grade copper waste materials in the United States already accounted for greater than 10% of the total annual copper production (Brierley, 1978; Kelly et al., 1979). Similar bioleaching techniques have also been applied to the mining of uranium. Bioleaching processes for recovery of valuable metals are advantageous since they are generally less energy intensive and less polluting than nonbiological methods (Brierley, 1982). The ability of these organisms to oxidize sulfur compounds may also be used to develop an economically feasible method for the commercial desulfurization of coal (Hoffman et al., 1981). Understanding the physiology of the thiobacilli is essential for optimization of these processes.

The physiology of *T. ferrooxidans* is interesting both in relation to its role in leaching of metals and acid production, and, at least equally importantly, in basic studies of the nature of chemoautotrophy. Growth as a result of the oxidation of reduced ferrous iron represents one of the narrowest thermodynamic limits known to occur (Ingledew, 1982). Despite the fact that very little energy is available from the oxidation of ferrous iron, *T. ferrooxidans* is able to fix carbon dioxide. The mechanism of iron oxidation, energy conservation and the nature of the respiratory chain have been extensively studied and are described in several excellent papers and reviews (Ingledew et al., 1977; Cox et al., 1979; Ingledew and Cobley, 1980; Apel et al., 1980; Ingledew, 1982; Cobley, 1982; Cox and Brand, 1982; and Cobley and Cox, 1983). Somewhat less studied is the specific nature and efficiency of CO_2 fixation and

how it relates to the growth and bioenergetics of *T. ferrooxidans* during iron oxidation.

Carbon dioxide (CO_2) fixation in *T. ferrooxidans* occurs predominantly via the Calvin reductive pentose phosphate cycle and secondarily through carboxylation of phosphoenolpyruvate derived from the Calvin cycle (Din, Suzuki and Lees, 1967; Tuovinen and Kelly, 1972). In autotrophic bacteria, in general, about 80% of total ATP available is used in the Calvin cycle, the rest is required for biosynthesis from the hexose level and other metabolic functions (Eccleston and Kelly, 1978). Although energy conservation for CO_2 fixation may not be directly in the form of ATP, it is clear that CO_2 fixation is very energy intensive. Ingledew (1982) calculated a minimum of 22.4 ferrous ions oxidized per CO_2 fixed via a standard Calvin cycle. This value yields a maximum theoretical growth yield of 0.53 grams carbon produced per mole ferrous iron oxidized.

In this study we have investigated the steady-state growth, microbial ecology and biohydrometallurgical potential of *T. ferrooxidans*. The report is divided into two sections which summarize more detailed works which have been published in one thesis, one institute report, and one journal article. A third section summarizes our most recent results which have yet to be published in peer-reviewed literature.

THE EFFECTS OF SURFACE DISTURBANCES ON THE LEACHING OF HEAVY METALS

1. Introduction

Deleterious effects of mine drainage on water quality have been known for years. As early as 1868, a report to the River Pollution Commission in Britain described the injury to livestock and killing of herbage caused by mine drainage of toxic metals from lead, zinc, and arsenic mines (Forstner and Wittman, 1981). Recently, significant deleterious impacts caused by mine drainage from coal fields in the eastern United States have become public, and the difficulties faced with trying to rehabilitate these impacted areas have become known.

Unfortunately, mine drainage does not only come from the mine but also from the mine's refuse (tailings). Tailings often have high concentrations of metal sulfides with the most commonly occurring iron sulfides being in the form of pyrites, pyrrohotites, and marcasites (Forstner and Wittman, 1981). Metals and reduced sulfur in tailings can oxidize causing dissolution of the metals at such a slow rate that tailings are able to leach metals for decades.

This primary mine effluent is commonly known as acid mine drainage. This acidic effluent is indicated by a very rapid pH decrease which can enhance the leaching of metals into the water system. The pH decrease is attributed to the slow oxidation of sulfides and/or sulfo-salts which are associated with ore and coal deposits. The oxidation process can be aided by the acidophilic bacterium *T. ferrooxidans*, which assists in the oxidation of exposed pyrites to produce ferrous iron, sulfate, hydrogen ions, and an indirect leaching of metals that accumulate in the iron sulfides.

Since the presence of *T. ferrooxidans* and the leaching of heavy metals by acidic water from coal mines has been described by numerous scientists, it is possible that the same environmental conditions that had arisen in coal mines could also arise in gold mines that had sulfide formations associated with them (Brown et al., 1982). In fact, recent studies in Alaska and Canada have identified acidic subarctic streams and the presence of *T. ferrooxidans* in areas affected by gold mine drainage. Hence, areas of Alaska provide unique opportunities to study problems of acid mine drainage from gold mines because of the long history and wide dispersal of gold mining in Alaska.

The objective of this portion of the project was to determine if the presence of *T. ferrooxidans* in the waters affected by gold mining in Alaska can be used as a biological indicator for heavy metal contamination.

The objective was approached by first reviewing the literature describing the history, geography, and geology of a historic mining area in Alaska. We chose the Wade Creek area of the Fortymile District. Next, the nature of metal pollution in aquatic environments in general, and the environmental affects of metals on the growth of *T. ferrooxidans* was reviewed. Finally, a field study was undertaken to determine the presence of *T. ferrooxidans* and heavy metals at several sites along Wade Creek.

2. Materials and Methods

Growth medium: The growth medium used for the most probable number (MPN) test for *T. ferrooxidans* was the same as that used by Braddock et al. (1984) and contained: $MgSO_4$ - $7H_2O$ (0.4 g/l), $NH_4)_2SO_4$ (0.4 g/l), KH_2PO_4 (0.1 g/l), and 10N H_2SO_4 to bring the medium to a pH between 1.8 and 2.0. Reduced iron as the sole energy source was supplied as $FeSO_4$ - $7H_2O$ at a concentration of 15.0 g/l.

To eliminate iron precipitation, the components of the medium were autoclaved separately in two different solutions and then were mixed after the solutions had cooled to room temperature. Solution 1 contained the $FeSO_4$. Solution 2 contained the remaining salts in deionized water and 1-2 ml of 10N H_2SO_4.

After mixing the two solutions, the final solution was placed aseptically into test tubes which were then sealed to reduce the possibility of contamination.

pH: The pH of streams was measured using a Hach visual color comparison with color discs field testing kit, and VWR digital mini pH meter model 49 with a 0.1 pH resolution and + or - 0.05 pH reproducibility.

Temperature: A gas-filled mercury glass thermometer incremented to 0.1°C was used to measure temperature of streams.

Total Suspended Solids: Total suspended solids were measured using a 500 ml Erlenmeyer flask, filter cup, membrane filter paper (0.45 μm), and an oral vacuum.

Water Samples: 200 ml polypropylene plastic bottles containing 1.0 to 3.0 ml of concentrated nitric or hydrochloric acid were used to store water samples for later analysis of heavy metals.

Field Methods: At each sampling site, the temperature, pH, total suspended solids, and water samples were taken. The direct count MPN test was taken sporadically for each trip into the field and the number of samples taken for *T. ferrooxidans* varied from two on May 31, 1986 to four on July 3-4, 1986.

Five field trips were made throughout the summer: May 31, June 21, July 3-4, August 2, and September 2, 1986.

The temperature was taken by placing the thermometer directly in the creek for a minimum of 10 minutes before recording. The pH was measured from grab samples from Wade Creek and was determined by color comparison using the Hach test kit. On one occasion, the VWR pH probe was used to determine the pH of the water samples by placing it directly into the stream. Water samples for total suspended solids (TSS) and heavy metals were taken.

For TSS and metal analysis, water samples were collected by pouring the water through a filter cup and allowing it to drain through a 0.45 μm membrane filter into a 500 ml flask. Half the water sample was then fixed with nitric acid and the other half was fixed with hydrochloric acid for later laboratory analysis.

The TSS was determined by differences in pre- and post-weighings of the filter paper per volume of water passed through the filter.

Microbial Biomass: *Thiobacillus ferrooxidans* numbers were measured by aseptically removing 1 ml aliquots directly from Wade Creek with a 1 ml pipette. The sample was then diluted serially in sterile medium. Finally, each dilution was placed in five growth tubes. The growth tubes contained 10 ml of *T. ferrooxidans* medium. The growth tubes were incubated for four weeks at room temperature before recording observations.

Total Dissolved Arsenic: Arsenic was analyzed by graphite furnace atomic absorption using an Electrodeless Discharge Lamp (EDL) at 193.7 nm., with a sensitivity of 0.01 ppb, as described for the Perkin and Elmer Model 4000 atomic absorption spectrophotometer (Perkin-Elmer, 1982).

Total Dissolved Copper: Copper was analyzed by flame atomic absorption method using a Hollow Cathode Lamp (CRT) at 324.8 nm., with a sensitivity of 0.001 ppm, as described for the Perkin and Elmer Model 4000 atomic absorption spectrophotometer (Perkin-Elmer, 1982).

Total Dissolved Iron: Iron was analyzed by flame atomic absorption method using a CRT at 248.3 nm., with a sensitivity of 0.001 ppm, as described for the Perkin and Elmer Model 4000 atomic absorption spectrophotometer (Perkin-Elmer, 1982).

Total Dissolved Zinc: Zinc was analyzed by flame atomic absorption method using a CRT at 213.9 nm., with a sensitivity of 0.001 ppm, as described for the Perkin and Elmer Model 4000 atomic absorption spectrophotometer (Perkin-Elmer, 1982).

Total Dissolved Cadmium: Cadmium was analyzed by flame atomic absorption method using a CRT at 228.8 nm., with a sensitivity of 0.001 ppm, as described for the Perkin and Elmer Model 4000 atomic absorption spectrophotometer (Perkin-Elmer, 1982).

3. Summary and Conclusions

The history of gold mining in Alaska is long and rich with adventure. Yet, in the last few years, the means of regulating gold mining by government agencies has been based upon the effectiveness of the mines to meet total settleable solids discharge limits, dampening a lot of the mining activity. Presently, miners feel that the discharge limit for turbidity is often impossible to meet, and the regulating agencies feel that it is often impossible to enforce. Therefore, it seems probable that if new economically reasonable techniques are not developed in the future, the governing agencies may change their means of regulation and enforcement.

Here, an examination has been conducted describing the discharge of dissolved zinc, iron, copper, arsenic, and total suspended solids caused by gold mining, relandscaping of gold mine tailings, and natural

weathering processes in Wade Creek. It was determined that *T. ferrooxidans* did not occur in high concentrations in this mining area. Therefore, the variations in dissolved metal concentrations cannot be attributed to the presence of large numbers of this bacterium but rather to the site disturbances and natural weathering processes.

The importance of this study illustrates the fact that in the Wade Creek drainage, discharge of dissolved metals associated with mining or surface disturbance is a short term and site specific problem, and the long range impact of the dissolved metals caused by these activities will be negligible. The long-term effects will result from stockpiled tailings, and settling ponds filled with settleable solids which have heavy metals sorbed onto them. These two types of mining refuse may lead to long term impacts on the environment over time if the heavy metals are able to leach from the tailings or the settled solids.

The detailed results and discussion of this project are found in Dixson and Brown (1987) which is Appendix A of this report.

ARSENIC REMOVAL BY USE OF BIOLEACHING AFTER FLOTATION CONCENTRATION OF AN ALASKAN SILVER ORE

1. Introduction

Historically, most of the precious metal mining in Alaska has been dominated by the placer mining method. However, many deposits in Alaska can be operated as lode mines. Lode mining is defined as "hard rock" mining using either open pit or underground methods of mining minerals that are in place as originally deposited in the earth's crust or that have been reconsolidated into a composited mass with waste rock.

Arsenic often occurs in metallic sulfide ores with the usual forms as orpiment (As_2S_3) and realgar (AsS), or as the sulfarsenides or arsenides of heavy metals, especially arsenopyrite (FeAsS). The latter mineral is most common. Arsenic sulfide ores often occur wherever metallic sulfides are found, and small amounts regularly occur in conjunction with silver, lead, copper, antimony and iron.

Arsenic-rich groundwater samples have been located in all the mineralized belts which extended from Pedro Dome-Cleary Summit to Ester Dome. Many groundwater samples with high concentrations of arsenic occur near known arsenopyrite-bearing veins, some of which are currently being mined for their gold content. It may be solubilized in mining and milling by oxidation of the ore and appears in the effluent stream (Hawkins et. al, 1982).

Inorganic arsenic is released into surrounding areas from anthropogenic sources which include copper, zinc and lead smelters. The atmosphere is a main conduit of arsenic emitted from anthropogenic sources (dry and wet) which fall onto soil and may be followed by movement through soil in ground or surface water. Transportation of arsenic in the surface waters is often followed by sedimentation. Pentavalent arsenic in an aqueous medium which is acidic is an oxidant, and in the presence of oxidizable material will react to form trivalent arsenic.

Concentrations of arsenic from 100 mg/l can be severely poisonous to humans, and 130 mg/l has proved fatal. Arsenic can accumulate in the body much faster than it is excreted and can build to a toxic level in the human body (U.S. EPA, 1983).

The Federal Water Pollution Control Act Amendments of 1972 (Public Law 92-500) are some of the primary sources of regulations involving

environmental issues critical to the mining industry. The goal of this act is "to restore and maintain the chemical, physical and biological integrity of the nations' water" (U.S. Congress, 1972). As a result of this Act, both federal and state agencies were responsible for achieving elimination of pollutant discharge into navigable water by 1985. Under this Act, the following requirements were established:

(1) The application of the best practicable control technology currently available (BPT) shall be achieved not later than July 1, 1977.

(2) The application of the best available technology economically achievable (BAT) shall be achieved not later than July 1, 1983.

Best available technology (BAT) permits were issued by the Environmental Protection Agency (EPA) to 445 placer mines on June 14, 1984. The basic technology for BAT is settling ponds. The maximum settleable solids discharge limit is 1.5 ml/l and the monthly average is 0.7 ml/l. Monitoring is required twice per day when sluicing.

Permits must include end-of-pipe effluent limitations necessary to achieve Alaska water quality standards. In 1984, EPA regulations included effluent limitations for turbidity not to exceed 5 Nephelometric Turbidity Units (NTU) and 0.05 mg/l for arsenic. EPA issued both the modified permits to miners holding permits in 1984 and the new permits issued to 1985 applicants as of May 10, 1985.

According to federal and state water quality regulations, the removal of arsenic for maintaining clean water is required of the mining industry. Many known gold deposits, including several in Alaska, are associated with arsenopyrite. Arsenopyrite is a particularly strong inhibitor of gold extraction by cyanide leaching. Removal of arsenopyrite from gold bearing material by microorganisms increases gold or silver recovery in some ores from 60% before removal to 94% after removal (Bruynesteyn et al., 1983). According to United States environmental regulations there is a prohibition against construction of new pyrometallurgical processing facilities. Thus biohydrometallurgical processing is extremely important in Alaska. However, detailed evaluations of ore types, microorganisms and biohydrometallurgical processes have not been studied for application in Alaska. In this section of the project, the following were analyzed:

1. The effectiveness of bioleaching a well described ore.

2. The effectiveness of mining bioleaching on water quality.

3. Practical design of an industrial scale bioleaching system.

2. Materials and Methods

T. ferrooxidans strain AKL was used in all bioleaching experiments. This organism was first isolated from Eva Creek in Fairbanks, Alaska which was heavily impacted by placer mining operations (Luong et al., 1985). Pure culture of *T. ferrooxidans* was maintained in an iron media solution and kept at 25°C. The composition of the iron media is listed below.

Composition of Iron Media

Material	Quantity
Ferrous Sulfate ($FeSO_4$ $7H_2O$)	30 g/l
Ammonium Sulfate ($(NH_4)_2SO_4$)	0.4 g/l
Magnesium Sulfate ($MgSO_4$ $7H_2O$)	0.4 g/l
Potassium Phosphate (KH_2PO_4)	0.15 g/l
10 N or Sulfuric Acid (H_2SO_4)	2.5 ml/l

Sulfuric acid (10N) was used to maintain the pH level below 1.5 to 2. To eliminate iron-precipitate, acid-ferrous sulfate ($FeSO_4$ $7H_2O$) was autoclaved separately from the salt solution and mixed with salt solution after being cooled to room temperature. The media was clear to light green and free from iron-precipitate. Even though isolate, AK1 showed a moderate resistance to arsenic (Luong et al., 1985); it did not rigorously attack the Wackwitz ore. To adapt the organism to the Wackwitz ore material, six flasks containing different amounts of ore material were prepared (0.5, 2, 5, 10, 15, and 20 g). A mixture of 1 ml of bacteria culture and 20 ml of fresh iron media was then added to each flask and incubated at 25°C on a shaker. The resultant culture was then

checked for bacterial growth after 10 days of incubation. Only the 0.5 g flask showed bacterial growth. As a result, bacteria from this flask were used to inoculate the 10 g flask containing 30 ml of fresh salt solution (same as iron media except no ferrous sulfate). The bacteria from the 10 g sample, with 300 ml of salt solution, were used in all the batch leaching experiments.

Batch leaching experiments were conducted on the 5.7% of sulfide silver mine materials provided by the Wackwitz Mine near Fairbanks, Alaska. The materials were ground to -65 mesh before bioleaching in a disc pulverizer. The precise protocol followed is described in Appendix B.

All flotation tests were conducted using Z-11 (Sodium isopropyl xanthate) and Aeroxanthate-350 (potassium amyl xanthate) as the collector and Dowfroth-65 (Polypropylene glycol methyl ether) as the frother. Before bioleaching, the flotation concentrate was washed eight times with distilled water and dried 24 hours at room temperature. Dissolved metals, except for arsenic, were determined on a Perkin-Elmer model 603 Atomic Absorption Spectrometer (AAS). The arsenic was determined on the Perkin-Elmer model 4000 Atomic Absorption Spectrometer using the graphite furnace method.

Particulate procedures for analyzing the metal contents in the ore or the leaching residue were as follows: (1) 2 g of dried sample were digested in 100 ml of aqua regia; (2) the solution was boiled for one and a half hours and then cooled at room temperature; (3) the cooled solution was filtered through a fiberglass filter and the filtered residue was rinsed with distilled water; and (4) the filtrate was made up to 100 ml and used for analysis of various metal ions.

3. Summary and Conclusions

This experimental work showed that bioleaching is much better than sulfuric acid for leaching of arsenic and iron from the Wackwitz mine ore. Both fresh and after flotation samples showed some instability, such as decreasing arsenic and iron, from day 0 to day 9. The instability may be caused by nonfixed iron. After 9 days, arsenic was removed from the crystal lattice by bacterial oxidation or indirect acid ferric sulfate leaching with fixed iron. Luong et al. (1985) reported the same trend from similar experiments.

Bioleaching of chalcopyrite, the main source of copper, has been studied intensively by Sakaguchi et al. (1976) using *T. ferrooxidans*. In these experiments, bioleaching of chalcopyrite was nearly complete because copper is relatively soluble in the leaching solution.

One problem with the substrate used in this experiment is that it contained alkaline gangue, which resulted in an increase in pH during the initial leaching phase. Alkaline gangue affected the pH of the control solution which started with pH 3 and went up to 6. In the after flotation sample, the leaching solution which started with pH 3 went down to 1.2. The acid formation is due to hydrolysis of ferric sulfate.

The mineral composition of Wackwitz ore is mainly arsenopyrite, galena, boulangerite and small amounts of covellite and chalcopyrite associated with gangue minerals. The chemical composition of the fresh hand-picked sample shows 0.1% silver, 0.1% copper, 5.7% iron and 5.8% arsenic. Also, the chemical composition of the flotation concentrate sample shows 0.44% silver, 0.192% copper, 4.5% iron, and 2.7% arsenic.

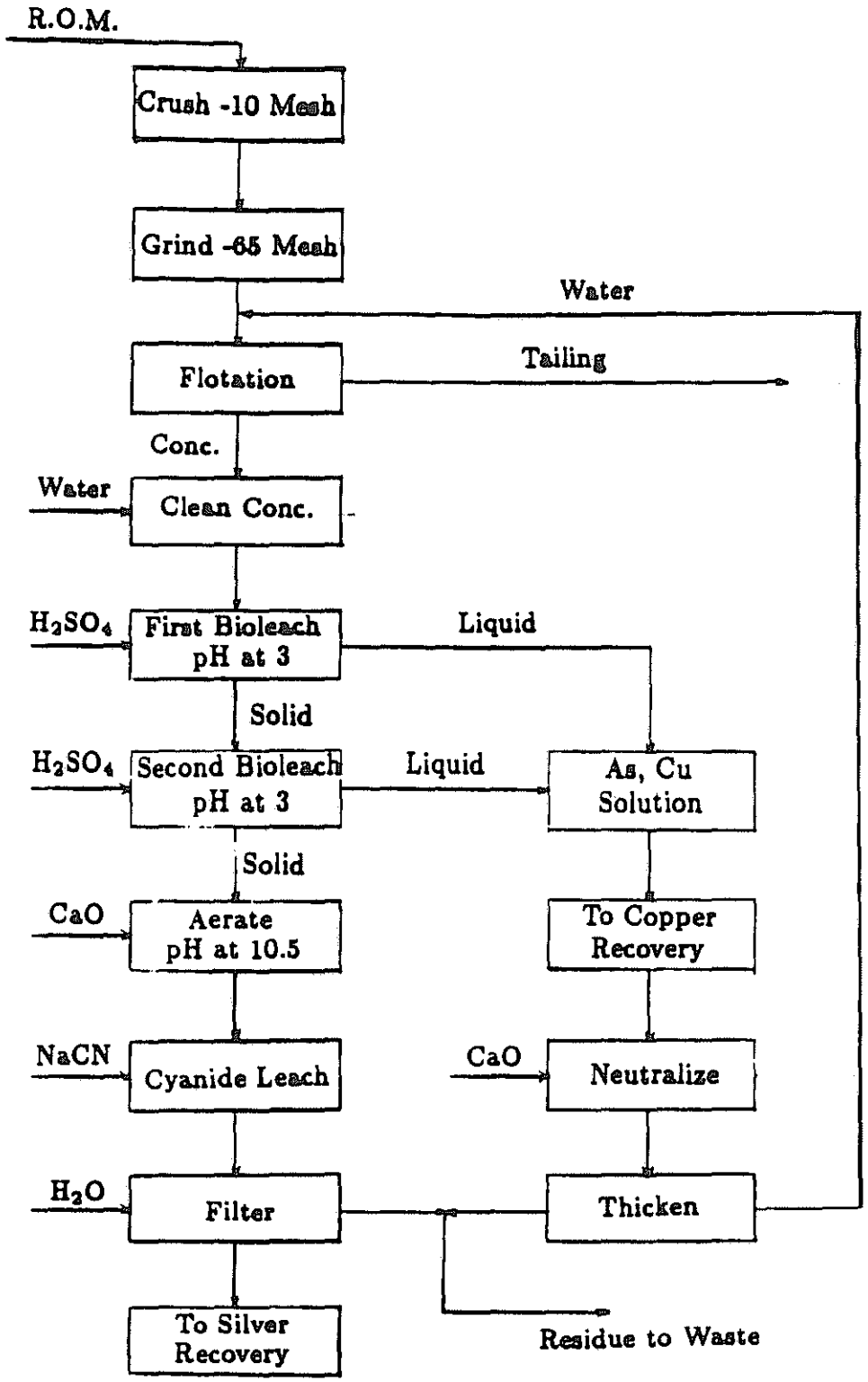
Froth flotation tests compared collectors such as Z-11 and Aeroxanthate-350 for the Wackwitz ores and showed that Aeroxanthate-350 is much better than Z-11 in any pH condition. Optimum flotation conditions yielded 28.52% recovery with a concentration ratio of about 3.5. Conditions used were: 10 minutes grinding time, 0.22 lb/ton of Aeroxanthate-350, 0.1 lb/ton of Dowfrother-65, pH = 6.90, and a collection time of 5 minutes.

Sixty-five ppm of arsenic, 183 ppm of iron and 18 ppm of copper were dissolved in the leaching solution from the fresh sample. Also, 56.8 ppm of arsenic, 68.5 ppm of iron and 23.1 ppm of copper were dissolved in the leaching solution of the flotation concentrate sample. The peak cell number count of the after flotation was three days earlier than for the fresh sample.

The application of microbiological technology is being considered for the leaching of metals from low grade ores and sulfides ores. Essentially, there is a prohibition against construction of new pyrometallurgical processing facilities according to United States environmental regulations. Thus, biohydrometallurgical processing should be considered to develop the Alaska mineral industries. Moreover, detailed evaluation of ore types, microorganisms and biohydrometallurgical processes have not yet been studied for

application in Alaska. As Bruynesteyn et al. (1982) shows, removal of arsenopyrite from gold bearing material by bioleaching increases gold or silver recovery in some ores from 64% before removal to 94% after removal. This study found that 56.8 ppm of arsenic was removed for the after flotation sample and shows the feasibility of removing arsenic from Wackwitz mine ore. The advantages of bioleaching beneficiation should be considered as an alternative to development of conventional beneficiation processes that generally heavily tax the quality and quantity of water resources.

A recommended practical flowsheet for the application of the bioleaching process to control arsenic concentration, and recovery of silver and copper from run of the mine sample is shown on the following page. The crushing section of this 100 ton per day plant consists of a two stage circuit. Run of the mine ore is fed to a jaw crusher by means of a feeder. Ore is scalped at 6" on a grizzly, and crushed to -2". The crushed ore and undersize from the grizzly are conveyed to a 10 mesh opening vibrating screen. Oversize is crushed in a roll crusher to -10 mesh and combined with the undersized product from the vibrating screen and sent to a fine ore bin. The crushed -10 mesh ore is fed to a ball mill which is operated in closed circuit to produce -65 mesh product. In the flotation section, a 6 cell flotation machine is used. The -65 mesh product is fed to a conditioner where collector and frother are added sequentially. This rougher flotation cell separates sulfide mineral from gangue. After flotation the concentrated sulfide mineral goes to a cleaner to remove organic material (such as excess collector and frother) and then goes to the first bioleaching tank. At the same time sulfuric acid is added to control the acidity at pH 3 and to destroy residual xanthate. After 15 days, solid material and liquid are separated. The solid material goes to the second bioleaching tank. The solution from the filtered bacteria-leached solid is neutralized with lime to remove soluble arsenic, to recover copper, and to recover water for re-use. The bioleached concentrate is slurried with water to a 1:1 ratio, lime slurry is added to obtain a pH of 10.5, and aerated for 24 to 48 hours. Cyanide is added and after leaching the slurry is filtered. The insoluble matter is washed fresh of cyanide and discarded. The cyanide solution is then treated to remove the precious metals either by the Merrill-Crowe (zinc power precipitation process) or by the newer electrolytic deposition process.



Recommended flowsheet for Wackwitz mine

CHEMOAUTOTROPIC GROWTH OF *THIOBACILLUS FERROOXIDANS*

1. Introduction

Studies of the iron-limited growth and iron oxidation kinetics of an arsenic-tolerant subarctic isolate (AK1) using batch and dilute (low biomass) continuous culture have been reported (Forshaug, 1983; Forshaug and Brown, 1983; Braddock et al., 1984).

For the growth and iron oxidation kinetic studies, investigators in this laboratory developed a two-phase continuous culture apparatus which provides optimum conditions for substrate oxidation by *T. ferrooxidans* and eliminates previously reported problems of reactor flask wall build-up of iron bacteria (MacDonald and Clark, 1970).

The steady-state cell yield of *T. ferrooxidans* decreased with increasing dilution rate. This observed inverse relationship between yield and growth rate is perhaps one of the most interesting results of previous studies and indicated that the growth kinetics of *T. ferrooxidans* (and perhaps other chemautotrophs) are not well described by traditional kinetic models.

The steady-state carbon/cell ratio also decreases with increasing dilution rate for isolate AK1 (Braddock et al., 1984). Other investigators have reported similar observations (Tempest and Neijssel, 1983). These results indicated that at low growth rates, strain AK1 is most efficient at utilizing reduced iron. The yield at low growth rate is close to the theoretical maximum growth rate yield of 0.53 mg of carbon per mmol of iron oxidized for *T. ferrooxidans* growth at pH 2 calculated by Ingledew (1982).

In the current study, we attempted to investigate the relationships among cell yield, growth rate, iron concentration and excretion of organic products (glycolate). Difficulty was encountered in kinetic interpretations when chemical analysis of glycolate in spent culture filtrates containing large amounts of iron failed to reveal significant differences in glycolate concentrations under various conditions. This problem led to an investigation of alternate energy sources for growth of *T. ferrooxidans* which would not compound glycolate analysis. We report here the first evidence that *T. ferrooxidans* will grow chemoautotrophically with hydrogen gas as an energy source.

2. Materials and Methods

Glycolate Determination: Colormetric

There are several methods available for estimating glycolate concentration in various types of solution. We have tried many of these techniques with limited success due to the high concentration of iron in *T. ferooxidans* culture filtrates. The following are the methods we have used:

1. 2,7-Dihydroxynaphthalene
   (Calkins, 1943; Fiegl and Anger, 1966)

2. Chromotropic acid (1,8-Dihydroxynaphthalene-3,
   6-disulfonic acid)
   (Fiegl and Anger, 1966)

3. Gas Chromatography
   Using FID detector and packed column
   (Tsukioka et al., 1986)

4. Gas chromatography
   Using ECD and capillary column
   (Tsukioka et al., 1986)

5. High Performance Liquid Chromatography
   (Miwa, 1985)

Several attempts to remove iron from the sample before analysis ended in failure because either a minute concentration of iron remained, which interfered with color development in some methods, or the removed iron (iron oxides, hydroxides) carried with it significant amounts of organic acids.

Thus, we developed a new strategy based on two colormetric methods which rely on both chemical and physical means to separate the color forming reagents from the interfering agents such as iron and other inorganic materials. By heating culture filtrate in concentrated sulfuric acid and purging formaldehyde (formed by the reaction of sulfuric acid and glycolate) into the colormetric reagent, a clean and simple system has been developed. With this procedure, we have developed a new method to analyze glycolate in the presence of high concentrations of any

inorganic oxidizing or reducing agents which can cause interference in the standard colormetric processes for glycolate.

A simple purge and trap apparatus was constructed from a glass wash bottle ("degas" bottle). The bottle consists of an inlet glass tube for cleaned air to bubble into the reactor and an outlet glass tube for formaldehyde flow into the colormetric reaction test tube. The reactor bottle was placed on a hot plate set at 90 - 95°C while the test tube was placed in a block heater set at 80 - 85°C. Since formaldehyde tends to adsorb onto synthetic materials, the apparatus was made entirely of glass.

The reactor is filled with 45 ml of concentrated sulfuric acid. Four ml 0.03% 2,7-Dihydroxynaphthalene in sulfuric acid (Calkins, 1943) are placed into the test tube. The sample (up to 10 ml) is placed in the reactor on the hot plate. Cleaned air is bubbled through the reactor and the test tube for two hours. The test tube is then removed from the heating block and allowed to cool before analyzing on a spectrophotometer at 545 nm.

This method has a detection limit of 55 µg/l glycolate (95% confidence). A typical standard curve for concentrations between 0 and 300 µg/l is linear with r values greater than 0.99.

<u>Glycolate Determination: High Performance Liquid Chromatography</u>

The analysis for glycolate was carried out by using a slightly modified version of a method developed by Miwa (1985). The modifications to the method consisted primarily of a change in solvent composition (90% H_2O/10% MeOH vs. 40% H_2O/60% MeOH) and the use of a C_{18} instead of a C_8 column. The glycolate and all other fatty acids are converted into their 2-nitrophenylhydrazide derivatives which can be detected at visible wavelengths after separation by high performance liquid chromatography.

The advantages of this analysis method include specificity, relatively little interference from the iron media and the ability to derivatize the compound without complicated extraction/drying procedures. The detection limit for glycolate using this method is 5 ppm, (Fig. 1) although lower concentrations could be detected by concentrating the samples using freeze-drying techniques. The disadvantage of this method is that the detection limit is 5 to 10 times greater than the estimated concentration of the analyte in the cultures. Further investigation of

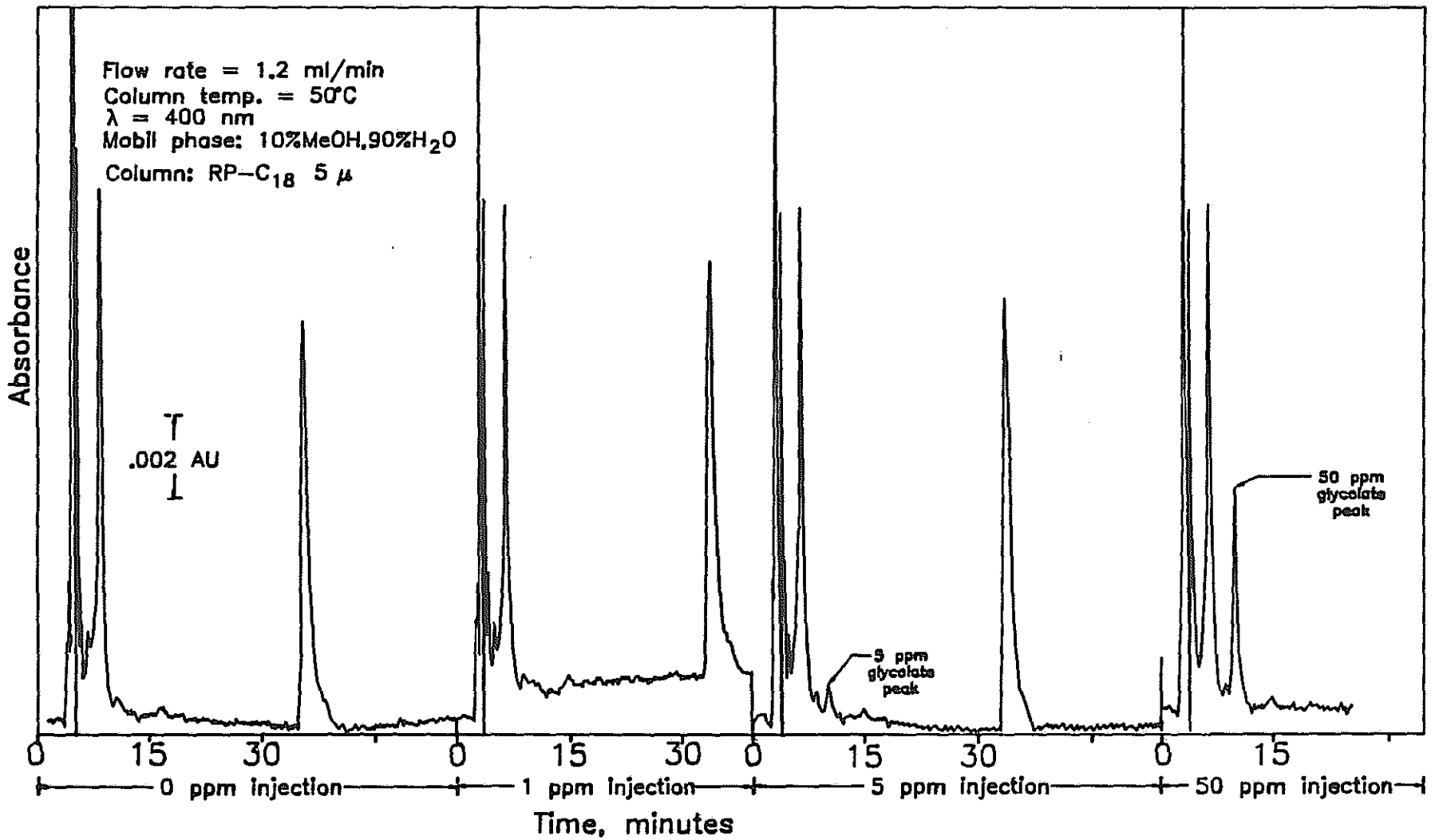


Figure 1. HPLC detection limit determination of the 2–nitrophenylhydrazine derivative of glycolic acid. Analytical conditions as listed above remained constant for all injections. A 5ppm injection was required to obtain a signal to noise ratio of 2:1.

post-derivative concentration techniques is expected to lower the detection limit to levels acceptable for culture assay.

Continuous Cultures

For this study, we used the method previously developed in our laboratory for continuous cultivation of *T. ferrooxidans* continuous cultures (Braddock et al., 1984). A two-phase continuous-culture apparatus was used. Culture medium is stored in 20 liter glass carboy and is pumped by a peristaltic pump directly into a specially designed reactor vessel. The reactors are large boiling flasks modified by the addition of an effluent tube and a sampling port. The effluent port is placed to maintain the volume of liquid in the reactor flask at a specified level. The exact volume of each reactor flask will be measured for that flask. Hydrogen gas and carbon dioxide were individually added to the culture via gas sparging tubes inserted into the reactor vessel stopper (depending on the experiment). A magnetic stirring device is used to continually agitate the contents of the reactor flask. The flasks are supported slightly above the stirrer to avoid any effect on the culture vessel from the heat generated by the motor of the stirrer. Silicone stoppers are used to seal the reactor vessel and feed carboy. Sterile cotton filters inserted in the feed carboy stopper and reactor vessel stopper are used to maintain ambient pressure throughout the system. Glass tubing and a minimal amount of silicone tubing were used to connect the feed carboy to the reactor vessel. The system was periodically monitored for leaks. All continuous cultures were maintained at 20°C.

Enumeration

Numbers of cells were determined by epifluorescent direct-count microscopy by a method adapted from that described by Hobbie et al. (1977). Measured aliquots of reactor vessel contents were stained with acridine orange solution and filtered onto irgalan black-stained Nuclepore filters. Numbers were estimated by counting 20 microscope fields per filter using a Zeiss standard microscope with an epifluorescence light source. Duplicate or triplicate filters were prepared and counted for each sample.

3. Summary and Conclusion

The original hypothesis that glycolate excretion rates of *T. ferrooxidans* are a function of growth rate and ratios of O_2/CO_2 could not be verified in the study. While we have developed a sensitive and accurate method of glycolate analysis for use in chemically complex systems, we have been unable to show a significant difference in glycolate concentrations in culture filtrates of *T. ferrooxidans* grown under a variety of conditions.

However, the difficulty with glycolate analysis in culture filtrates of *T. ferrooxidans* grown on iron led us to investigate alternate energy sources for chemoautotrophic growth of *T. ferrooxidans*. We are now able to report the first evidence of the chemoautotrophic growth of *T. ferrooxidans* on molecular hydrogen and carbon dioxide. All H_2-oxidizing bacteria are thought to be facultative heterotrophs (Schuler and Conrad, 1990). Growth of *T. ferrooxidans* on H_2 provides the first evidence that chemolithotrophic organisms may also be H_2-oxidizers. These results also provide an explanation as to why *T. ferrooxidans* can be routinely isolated from soils and streams where there is no evidence of acidification or iron or sulfur oxidation.

T. ferrooxidans from pH 2 iron medium was inoculated into a continuous culture growth vessel containing normal pH 6 mineral salts (Braddock and Brown, 1984) supplemented with vitamin B_{12}, biotin, thiamine and trace amounts of nickel, tungsten, cobalt and aluminum. The growth rate was held constant at 0.013 hr^{-1} with a saturating flow of H_2 and CO_2. Oxygen was not added but the feed reservoir was fully equilibrated with atmospheric oxygen. Cell numbers have remained constant at about 8 x 10^6 cells/ml for several months. Cells transferred from the continuous culture vessel to pH 2 iron medium grow without apparent inhibition. Cells from either batch (iron) or continuous culture (H_2) were unable to grown on nutrient agar plates.

These results indicate that the physiological ecology of the thiobacilli in general and *T. ferrooxidans* in particular is much more complex than generally believed.

The bioenergetic significance of our findings is yet to be determined, however, they must now be considered when future studies consider the role of these organisms in the biogeochemistry of soil gases, geothermal vents, metal corrosion and leaching of sulfide minerals.

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