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Idaho National Laboratory
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Nicolas Hoffman

Office of Science, Science Undergraduate Laboratory Internship (SULI) Program

Brigham Young University-Idaho, Rexburg, ID

Idaho National Laboratory, Idaho Falls, ID

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Prepared under the direction of Yoshiko Fujita and David Reed in the Biological Science Department at Idaho National Laboratory.

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I. ABSTRACT

The use of *Acidithiobacillus ferrooxidans, Thiobacillus thiooxidans,* and other chemoautotrophic microbes in bioleaching have been implemented in a variety of processes.[1] In industry, bioleaching has been studied for its potential to extract valuable metals from low grade ores that would otherwise be cost prohibitive to recover.[2] Other applications of bioleaching include the ability of certain microbes to detoxify waste products and even heavy metal contaminated soils.[3] Another potential application for such bio-oxidative microbial activity is the extraction of tellurium (Te) from mine tailings, a low-cost abundant resource. Tellurium is one of the least common elements on Earth; it is found in the planetary crust at about 1 μg/kg, a rarity most comparable to that of platinum.[4] A major use of Te in the U.S. is in cadmium-telluride (CdTe) solar panels[5]. Te is primarily imported into the U.S. from Canada, and it is usually recovered as a byproduct of copper refining.[6] Since CdTe photovoltaic (PV) cells are the most efficient, cost-effective, and environmentally friendly PV chemistry, the renewables market has seen an increased demand for CdTe PV cells causing some concerns about sustainability and the limited global availability of Te. *Acidithiobacillus ferroxidans* is a microorganism that can oxidize iron and sulfur to produce ferric iron and sulfuric acid, and it is possible that it could also solubilize Te from sulfidic mine tailings. In this project, different media for growth of *A. ferroxidans* were evaluated, and a plan for testing the ability of *A. ferroxidans* to leach Te from mine tailings was developed. Initial characterization of *A. ferroxidans* cultures grown in the presence of copper tailings suggests that conditions suitable for Te bioleaching can be established.

II. INTRODUCTION

This project aims to conduct initial studies of whether the well-known bacterium *Acidithiobacillus ferrooxidans* can promote Te solubilization from sulfidic mine tailings.

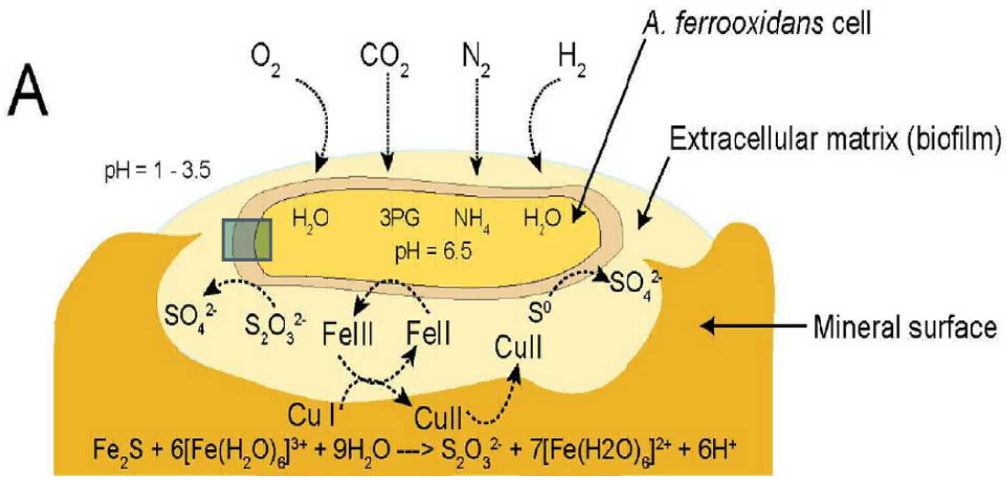

Figure 1: A. ferrooxidans proposed bioleaching mechanism of copper sulfide ores.

A. ferrooxidans oxidizes Fe^{2+} to Fe^{3+} (Fig 1). The Fe^{3+} can then abiotically oxidize copper within the copper sulfides, allowing for solubilization of the valuable ore. This is the process known as "bioleaching," and the main objective in future experiments is to determine if tellurium is also recovered in this same manner. This paper outlines suitable conditions for such a tellurium bioleaching experiment. The first step was to determine the best microbiological media for growing *A. ferrooxidans* and for conducting the leaching studies. Three different media were prepared: *A. ferrooxidans* was grown in three standard media types: DSMZ 882, DSMZ 35, and 9K. To monitor growth and activity, cell numbers were determined by acridine orange direct counts (AODC) and soluble iron was measured using the Ferrozine assay (Stookey, 1970). The Ferrozine

assay measures soluble Fe^{2+}; Fe^{3+} and total soluble iron can also be determined by reduction of Fe^{3+} to Fe^{2+}. Our hypothesis is that if A. ferrooxidans is able to oxidize high concentrations of Fe^{2+} to Fe^{3+}, then the oxidative conditions necessary for indirect Te bioleaching will be microbially created.

III. METHODS

a. DSMZ 882, DSMZ 35,and 9K medium preparation:

To prepare a solution of DSMZ 882 medium, all the reagents in Table 1 were combined while stirring to a final volume of 2 L with npH_2O and sterile technique. The DSMZ 882 was prepared in three separate parts, as per the recipe, and not mixed until solutions A, B, and C were completed and sterilized/filtered. These solutions were aseptically added into a 2L final container. In preparing the DSMZ 35 medium, two variations of DSMZ 35 were tested. Each DSMZ 35 medium contained a different sulfur source. The sulfur sources were: tyndallized elemental sulfur, and sodium thiosulfate. Mixing instructions were the same as indicated in Table 2, apart from the indicated sulfur source. For the 9K medium, Solutions A and B (Table 3) were prepared separately, allowing Solution B to be autoclaved while Solution A was filter-sterilized using a 0.20 μm PES bottle-top filter unit (Millipore). See Table 3 for the steps used in the 9K media preparation. During preparation of all three media types, certain ingredients were not available in the lab, and there was a need to slightly adjust which reagent was used. For example, the DSMZ 882 media in Table 1 shows that the recipe called for 147.0 mg of

calcium chloride dihydrate. However, only the anhydrous form was available in the lab, so 110 mg of anhydrous calcium chloride was used in order to provide an equimolar concentration of the necessary reagent.

Table 1: DSMZ 882 Media Preparation

DSMZ Medium 882 as follows:		
Solution A:		
(NH4)2SO4		132.0 mg
MgCl2*6H2O		53.0 mg
KH2PO4		27.0 mg
CaCl2*2H2O	***Replace the dihydrous with 110mg of anhydrous***	147.0 mg
Distilled Water		950.0 ml
Adjust to pH 1.8 with 10 N **(5M)** H2SO4		
Solution B:		
FeSO4*7H2O		20.0 g
0.25 N H2SO4 (**0.125 M**)		50.0 ml
The pH of this solution should be 1.2		
Solution C:		
Trace elements solution (see below)		1.00 ml

Mixing Instructions:		
Sterilize Solutions A and C by autoclaving at 112C for 30 min. Prior to use, mix Solutions A, B, and C. The final pH of the medium should be 1.8. Incubate statically without shaking.		

Trace Elements Solution:		
MnCl2*4H2O		76.0 mg
ZnCl2 ***Replace the ZnCl2 with 143.5 mg of ZnSO4*7H2O due to availability***		68.0 mg
CoCl2*6H2O		64.0 mg
H3BO3		31.0 mg
Na2MoO4	***found as dihydrate, so 11.9 mg of the dihydrate***	10.0 mg
CuCl2*2H2O		67.0 mg
Distilled Water		1000.0 ml

Table 2: DSMZ 35 Media Preparation

DSMZ Medium 35 as follows:		
NH4Cl		0.10 g
KH2PO4	*See Solution A ingredients*	3.00 g
MgCl2*6H2O	*See Solution A ingredients*	0.10 g

CaCl2*2H2O *See Solution A ingredients. Replace w/ 0.105g anhydrous* 0.14 g Sulfur, powdered *Replace w/ 77.4g sodium thiosulfate pentahydrate* 10.00 g Distilled Water 1000.00 ml	
Mixing Instructions: Dissolve all ingredients, except the sulfur source, and adjust to pH 4.2 with .125M H2SO4, then autoclave. For sterilization, place the sulfur in screw-capped tubes or bottles, wet with a few drops of water, and then heat for 3 hours to 90-100C in a water bath on each of the three successive days. Before use, aseptically layer the sulfur onto the surface of autoclaved liquid basal medium. Incubate statically without shaking.	

Table 3: 9K Media Preparation

Reagents:	Solution 1	Ammonium Sulfate	3.0 g	Lot# 030M0139
		K2HPO4 (anhydrous)	0.5 g	Lot# 974351
		Magnesium Sulfate Heptahydrate	0.5 g	Lot# 025626
		Potassium Chloride	0.1 g	Lot# 072772
		Ca(NO3)2	0.01 g	Batch# 18620CB
		NanoPure Water	700mL	
	Solution 2	Iron (II) Sulfate Heptahydrate	44.22 g	Lot# MKCJ9113
		NanoPure Water	300mL	

Mixing instructions:

1. Beginning with Solution 1, add the ingredients together and then adjust the pH to 5.5 with drops of 5M H2SO4.
2. Then, autoclave Solution 1 at 120C on setting 3 (LIQ40)
3. Solution 2, add the iron (II) sulfate to the npH2O
4. Adjust pH to 1.4 with 5M H2SO4.
5. Then, filter sterilize Solution 2.
6. After Solution 1 cools from autoclaving, combine both solutions into a 1L screw-cap container.
7. Adjust final pH (between 1.5-2.0.) using the pH electrode.
8. Because the pH electrode is not clean, a sterile filtration of the entire medium into a final container is the last step.
9. Final medium should be a clear blue color (no precipitate).

b. Growth studies:

Initially, *A. ferrooxidans* was inoculated as a 5% (v/v) inoculum with a final volume of 20mL in all 3 media types. *A. ferrooxidans* used in the following analyses was obtained from Michael Guzman at LLNL. This strain was viable and grew in 9K and 882. No growth of *A. ferrooxidans* was observed in either of the DSMZ 35 media. Initial inoculation of the cultures used an inoculum ratio of 1:20 (1mL of inoculum into 19mL of media) was used, then a ratio of 1:50 (1mL of inoculum and 49mL of media) was used for growth studies. Static incubation was attempted initially with the nonviable strains, but not with the viable strain acquired from LNLL. Growth conditions were controlled with the temperature at 30C, shaking at 150rpm, and growth media pH 1.8-2.0.

c. Acridine Orange Direct Counts (AODC):

Samples for AODC cell enumerations were prepared by mixing 900uL of culture with 100uL of acridine orange solution containing 54uL 37% formaldehyde. Acridine orange stains the DNA of the bacteria for observation under a fluorescence microscope. The stained cells were filtered onto a nylon membrane placed atop a cellulose filter under vacuum suction. Counting was performed on a 0.01mm grid (100µm x 100µm). The diameter of the stained area was 16.5mm with a stained area of 213.82mm^2, at 21.382 grids/filter. The fluorescence microscope was a A15 Nikon 1000X; the excitation and emission wavelengths were: blue light/green fluorescence = Excitation (Ex) 480nm +/- 30nm and the Emission (Em) 535nm +/- 40nm with the dichronic selectivity filter (Tm)

at 505; green light/orange fluorescence = Ex 540/25 and Em 605/55, and Tm at 565, respectively. The number of cells/field was calculated as an average across various fields until at least 300 cells had been counted.

d. Measurement of soluble iron:

Soluble iron was measured via the Ferrozine assay (Stookey, 1970). The reagents used were HCl 1M, 0.1% (w/v) Ferrozine reagent in 50% (w/v) ammonium acetate (stored in the dark at 4C), and hydroxylamine hydrochloride (HAHC) 10% (w/v) in 1M HCl. The dissolved Fe^{2+} reacts with the Ferrozine reagent and turns purple; the higher the ferrous concentration the darker the purple color, which is measured using a spectrophotometer at 562nm. A calibration curve was generated using ammonium ferrous sulfate hexahydrate in 1M HCl. HCl and Ferrozine reagent was used as the blank. A 100uL sample was diluted into 900uL of 1M HCl and vortexed. Then, 100uL of this solution was added to a cuvette, along with 900uL of 1M HCl and 1000uL of Ferrozine reagent. After 10mins, the absorbance was measured at 562nm and recorded.

IV. RESULTS AND DISCUSSION

Growth from laboratory stocks was not observed by wet mount. It was determined that the laboratory strain could not be revived by any of the above media types. *A. ferrooxidans* used in the following analyses was obtained from Michael Guzman at LLNL. Growth curves comparing *A. ferrooxidans* growth in DSMZ 882 and 9K media show higher cell densities are achieved by growth in the 9K medium as compared to growth in the DSMZ 882 medium over

the same period of time (196 hours). (Fig. 2) Growth was slow and linear in the DSMZ 882 medium, and growth appeared to be much greater in the 9K medium. In a separate growth experiment, *A. ferrooxidans* was inoculated in 9K medium to determine what cell densities could be reached. Growth was observed to be logarithmic and inflection points were seen after 48 hours and significantly after 96 hours of incubation time. (Fig. 3) This indicated that after 96 hours of growth, the bacteria had entered their stationary phase of growth. The standard curve generated for the Ferrozine assay was used to calculate the concentrations of Fe^{2+} and had an R value of 0.9999, indicating a high accuracy of measurement. (Fig. 4) The Ferrozine assay applied to the 9K culture medium showed that after 96 hours, the amount of detectable ferrous iron was dramatically decreased as compared to the uninoculated 9K medium as a positive control. (Fig. 5)

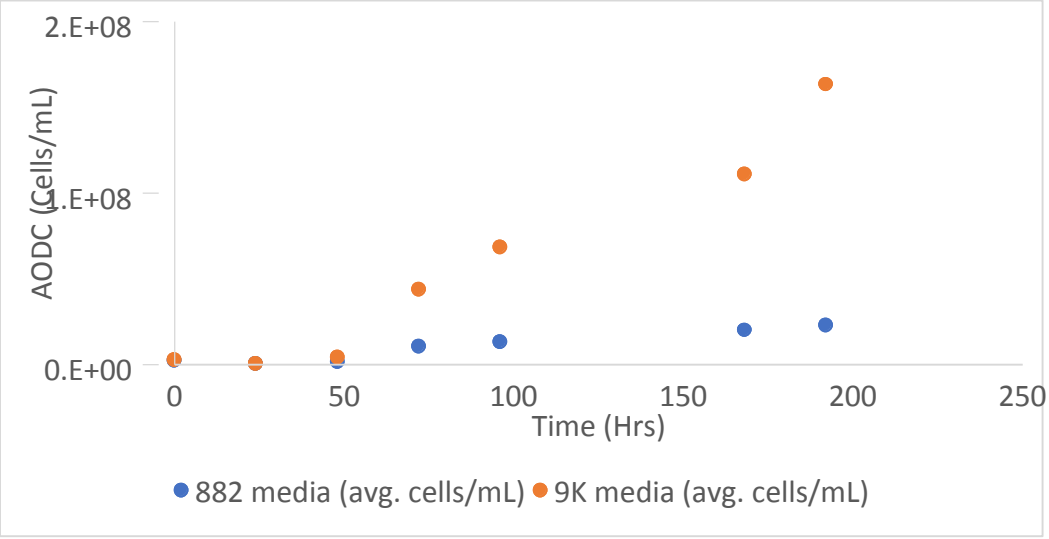

Figure 2: Cell densities of A. ferrooxidans in DSMZ 882 and 9K media

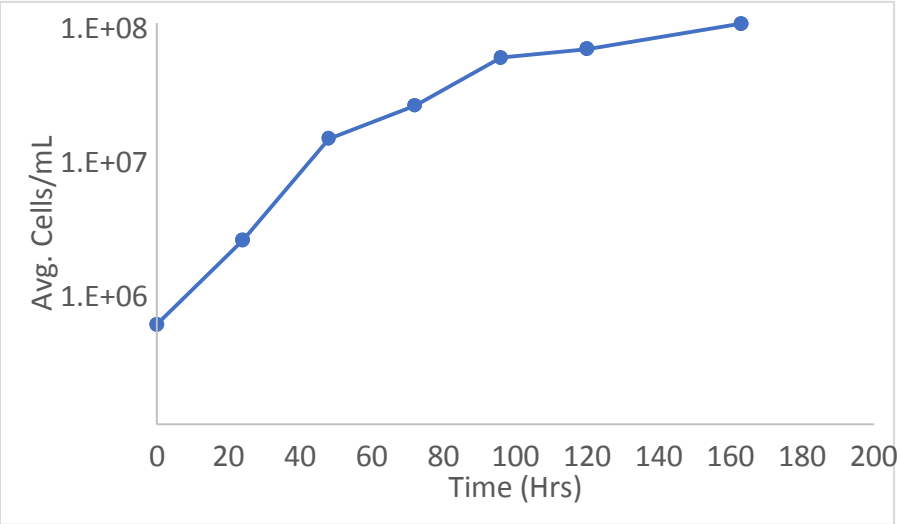

Figure 3: Cell density of A. ferrooxidans in 9K medium

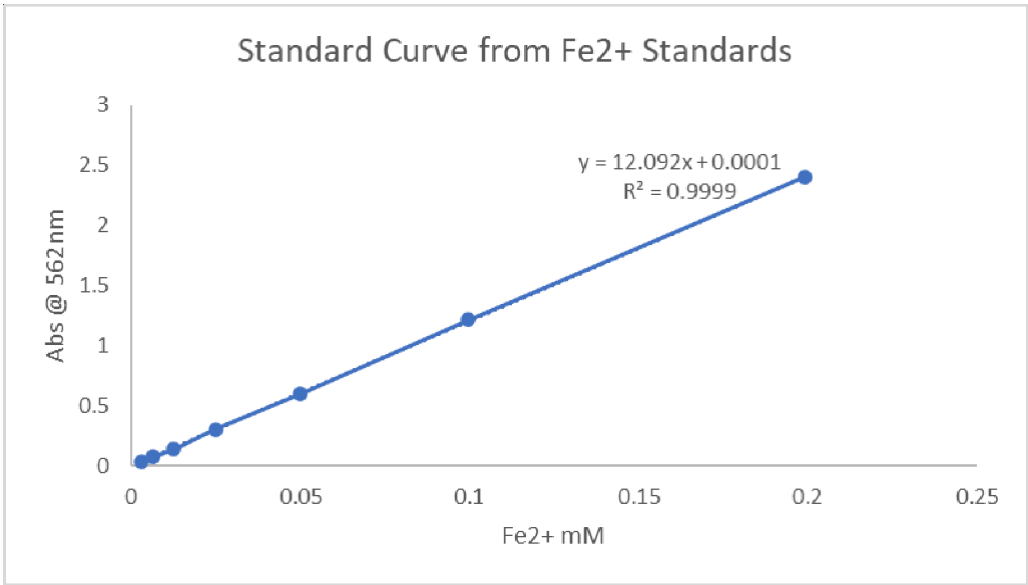

Figure 4: Standard curve generated from Fe^{2+} standards

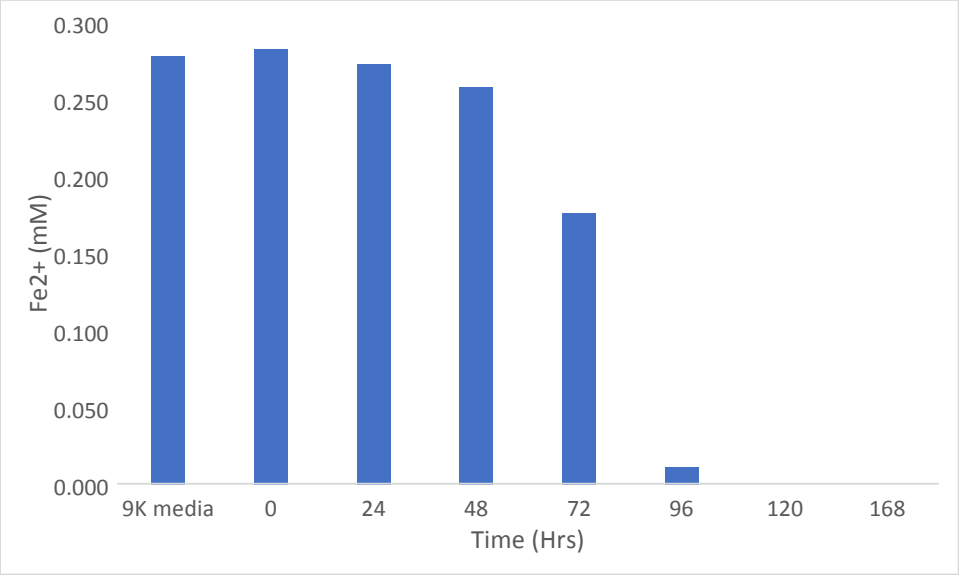

Figure 5: Ferrozine measurements of 9K cultured with A. ferrooxidans incubated for 168 hours

V. CONCLUSIONS

As evidenced by the growth analysis of *Acidithiobacillus ferrooxidans* in 9K media, optimal cell density was achieved under the following incubation conditions: temperature at 30C, shaking at 150rpm, pH 1.8-2.0, and after a period of at least 96 hours incubation. The incubation conditions were not varied in this experiment, however future optimization experiments may involve adjusting these parameters. The time point of 96 hours was identified to be a sufficient starting point for preliminary bioleaching studies based on the results in Figures 3 and 5. Although there is an inflection point in Figure 3 after the first 48 hours, it is not until after 96 hours that the doubling time of the bacteria significantly increases. At these conditions, cell densities of 1.64×10^8 were achieved in the lab with the 9K medium. These greater cell densities are beneficial for bioleaching studies, corresponding to higher levels of potential bio-oxidative activity. Likewise, after 96 hours of culture growth in the 9K medium, the amount of detectable ferrous iron was dramatically decreased, indicating that *A. ferrooxidans* was in fact

oxidizing virtually all the ferrous iron into ferric iron (Fig 5). This oxidation activity is exactly what we would expect to see during a bioleaching experiment as we seek to leach Te from sulfidic minerals found in mine tailings. Actual Te biorecovery will be tested in future experiments, but the results reported here will serve to inform the selection of the bioleaching conditions to be applied in future experiments at INL.

VI. ACKNOWLEDGMENTS

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