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Chemosynthetic microorganisms in leaching sulfide minerals

A. Keith Jameson
Brigham Young University - Provo

CHEMOSYNTHETIC MICROORGANISMS IN LEACHING SULFIDE MINERALS

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A. KEITH JAMESON

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Signed,

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Date

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CHAPTER I

INTRODUCTION

The classification of microorganisms is very difficult. Very minute gradations of characteristics are exhibited, from clearly plant like to clearly animal like. Bacteria are often called the "buffer" zone between plant and animal life.[1] The general classification of bacteria is done as in zoology and botany whereby life is subdivided according to kingdom, phylum, class, order, family, tribe, genus, and species.

The class Schizomycetes is a subdivision of the suborder Fungi. Contained in this class are all bacteria. It is characterized by a rather heterogeneous group of organisms which are usually unicellular and quite small (on the order of one micron as the smaller dimension).

Several classifications of true bacteria (Eubacteriales) are possible. They may be classified according to morphological or physiological characteristics. The most widely used classification is that employed by Bergey's Manual.[2] This classification is based upon both morphological characteristics and biochemical properties. Although not satisfactory in many cases, it is a good guide to identification of bacteria.

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[1]C.E. Clifton, "Introduction to the Bacteria," McGraw-Hill Book Company, Inc., New York, N. Y., 1950, p. 78.

[2]"Bergey's Manual of Determinative Bacteriology," 6th ed., Bailliere, Tindall, and Cox, London, 1948.

True bacteria (Eubacteriales) may be defined in several ways, none entirely adequate. They are characterized as simple, undifferentiated, rigid cells. This order contains both spherical and rod-shaped cells and both motile and non-motile species. The cells reproduce by transverse fission.[3]

The sub-order Eubacteriineae contains thirteen classes. These subdivisions are characterized both by morphological and nutritional characteristics. The family Bacillaceae is characterized as containing species which form endospores, while the family Nitrobacteriaceae contain every autotroph in the suborder Eubacteriineae.

The family Nitrobacteriaceae are known as "chemosynthetic autotrophs". They are characterized as having the ability to grow in a completely inorganic medium utilizing carbon dioxide as the only source of carbon. Further breakdown into tribes is dependent upon the substrates which are oxidized. Nitrobacteriae oxidize some form of nitrogen to nitrate and nitrite, Hydrogenomonadeae oxidize hydrogen gas to water, and Thiobacilleae oxidize some inorganic form of sulfur or iron.

In the tribe Thiobacilleae are two species which are particularly noted for having the ability to live in the most acid environment known to man. The pH of solutions containing Thiobacillus thiooxidans and Thiobacillus ferrooxidans is often less than one. T. thiooxidans was characterized in 1922[4] and was found to oxidize sulfur to sulfuric acid. T.

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[3]Clifton, op. cit., p.116.

[4]S.A. Waksman and J.S. Joffe, J. Bact., 7, 239 (1922).

ferrooxidans was characterized in 1951[5] and was found to oxidize ferrous to ferric iron.

Oxidation of sulfidic constituents in waste rock dumps at Bingham Canyon, Utah, and other geogrphical locations has been known for some time. It was found in this laboratory that biological oxidation was responsible for the oxidation and subsequent solubilization of these sulfidic components.[6] The pH of effluent streams from these dumps was in the vicinity of 2.75. This high acidity, autotrophic like metabolism, sulfide oxidation, and morphology suggested that the active form of life in the waste dump effluents be a member of the genus Thiobaccilus.

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[5] K. L. Temple and A. R. Colmer, J. Bact., 62, 605(1951).

[6] L. C. Bryner, et. al., Ind. Eng. Chem., 46, 2587(1954).

CHAPTER II

BASIS OF THE PROBLEM

The oxidation of the sulfide minerals in exposed ore bodies was once thought to be due to direct action of the oxygen from the atmosphere. A literature study revealed that varying results were obtained on the solubilities of iron pyrites and its oxidation rate: In some cases very small amounts of pyrite (less than .2% oxidized) were found to be oxidized while in others relatively large amounts were solubilized. From these findings it seemed likely that biological action was responsible.

Previous studies in this laboratory revealed that iron pyrites, various copper sulfide minerals and molybdenite are oxidized by the bacteria found in the leaching streams in Bingham Canyon, Utah.[8,9,10] The streams that flow from the waste ore dumps and the open pit mining area contain considerable amounts of soluble iron and copper. These waste dumps are obtained by stripping the main ore body of rock debris low in copper bearing minerals. They are very extensive and yield soluble copper and iron when leached with water.

The chief objectives of this study were: (1) to isolate these relatively little known bacteria in pure culture and obtain their description and characterization; (2) to determine the optimum nutrient requirements and the effects of various compounds on their action on sulfide minerals.

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[8] Ibid., p. 18.

[9] D. B. Davis, "Biological Oxidation of Copper Sulfide Minerals" (unpublished Master's thesis, Brigham Young University, 1953), p. 18.

[10] R. Anderson, "Oxidation of Molybdenite with the Aid of Microorganisms" (unpublished Master's thesis, Brigham Young University, 1956), p. 26.

CHAPTER III

EXPERIMENTAL METHODS AND PROCEDURES

In this study on the isolation of the bacterium responsible for metal sulfide oxidation, several methods and varying equipment were employed. Petri plates were used in the isolation on solid medium. Physiological studies on the isolated bacterium were carried out in percolators developed and modified in this laboratory. This apparatii consisted of many members similar to that shown in Figure I. These percolators consist primarily of a large glass tube (40 mm. diameter and 400 mm. long) connected by a sidearm air lift of 5 mm. glass tubing. In the sidearm is a small "tee" which served as the air entrance for the lift. The draining mechanism extends from the base of the percolator.

The percolators were charged in the following manner. Into the bottom of the main tube was placed a small perforated porcelain disc, covered with a layer of pyrex glass wool. The mineral or substrate (one to five grams) was mixed with 100 grams of Ottawa sand (inert silica) and introduced into the percolator by means of an elongated funnel which extended to the pyrex wool layer. One hundred ml. of nutrient solution was added. The side arm air lift was connected to the air source and constant cycling of the leaching solution was provided. The compressed air (5 psig) was washed and filtered through a cotton plug in a calcium chloride tube before it entered the sidearm of the air lift. Washing the air served two purposes, to remove dust and saturate the air with water to minimize evaporation from the solution in the percolators. The air stream leaves through a cotton plug in the top of the percolator. The drain was closed with a pinch clamp. This apparatus was easily sterilized by placing the entire unit in an autoclave, after which aseptic conditions were maintained indefinitely.

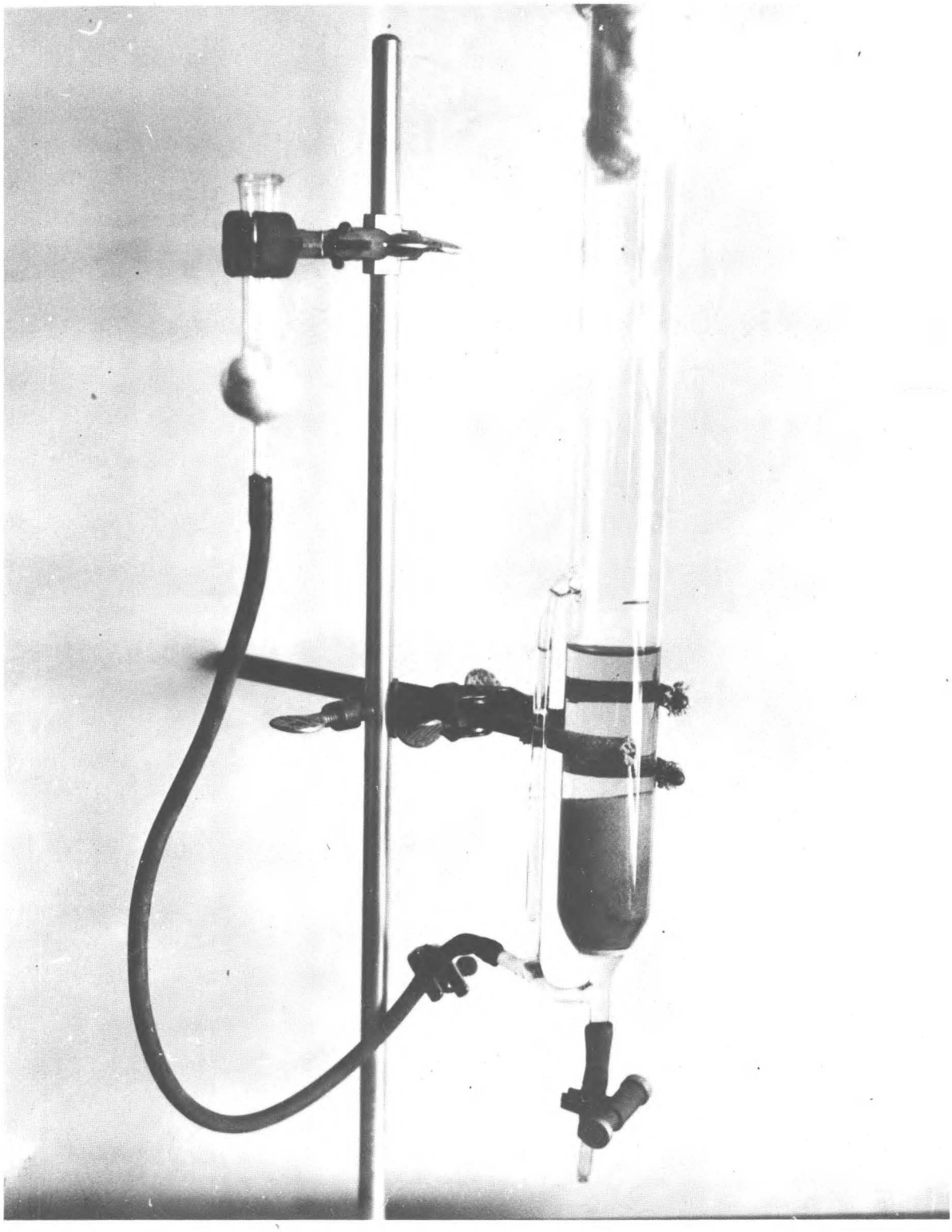

Figure 1 -- A Typical Percolator Used in This Investigation

The sulfide oxidizing organism studied has been shown to be a chemosynthetic autotroph.[11] The percolator system insured a constant supply of oxygen and carbon dioxide, two essentials to autotrophic growth. The air lift also provided a constant flow of solution through the bed containing the substrate. Thus, the bacteria were supplied with a constantly fresh supply of nutrient material and the oxidation products from the bacterial metabolism were carried away. By this means the rate of oxidation of the substrate was kept at a maximum. Mechanical advantages were also inherent in this apparatus since there were no moving parts.

The percolators are particularly useful to conditions of slow growth. Many bacteria complete all phases of their growth on a substrate in a matter of hours or a very few days. The bacterium studied in this investigation showed exceedingly slow growth on most of the substrates employed. With this type of apparatus continuous studies were run for as long as six months and could have been continued indefinitely.

A. The solid media used in isolation studies.

To isolate the bacterium responsible for the oxidation of pyritic materials it was found necessary to utilize modifications of the usual solid medium. Agar-agar was found to be unsuitable because it seemed to inhibit the growth of the bacteria. Since autotrophic bacteria grow better in the absence of organic substances, a silica gel containing the desired nutrients and substrates was developed. The method of preparation of the gel is similar to the one described by Kingsbury and Barghoorn.[12] It was prepared as follows:

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[11] Wilson, op. cit., p. 24.

[12] J.M. Kingsbury and E.S. Barghoorn, Appl. Microbiol., 2, 5(1954).

1. A predetermined amount of Ludox[13] was successively poured through Amberlite resins IR-4B and IR-120 (or equivalent anion and cation exchange resins). This removed foreign ions from the colloidal silica.
2. A predetermined volume was diluted with water (the hardness of the gel depends upon the ratio of Ludox to water). The nutrient material was dissolved in the same concentrations as those in the liquid nutrient described in the following section. The substrate was added to the preparation (ferrous iron, sulfur, or sulfide minerals).
3. Twenty ml. portions of the resulting solution were poured into petri plates and sterilized. Sterilization (autoclaving at 15 psig for 20 minutes) aided the sol-gel transformation. To prevent pocking (boiling) of the gel, compressed air was introduced into the autoclave to maintain a slightly higher pressure than that of the steam after the heat was turned off. At least two hours were allowed for cooling before the pressure was released.
4. After the gel had cooled it was inoculated. This was done by the streak method. A small amount of inoculum was introduced aseptically onto the surface of the gel and spread by a sterile cotton swab. It was found that very gentle streaking as obtained with the swab was necessary to prevent rupturing of the surface of the gel.

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[13] A trade name for a stable commercial colloidal silica preparation.

B. Nutrient Solutions

Several substances must be present for normal growth of bacterial cells. An energy source (relatively large amounts) and the constituents necessary for cell growth (small amounts) must be present. Many trace elements presumed necessary for bacterial growth are present in sufficient quantities as impurities in the sulfide minerals.

Several substances are necessary in noticeable amounts. For the particular bacterium studied in this investigation it was found that oxygen, an oxidizable substrate, a source of nitrogen, a source of carbon, and phosphate had to be supplied to obtain noticeable bacterial growth.[14] Oxygen and carbon dioxide (the source of carbon) were obtained from the air, the oxidizable substrate was mixed with Ottawa Sand and placed in the percolator, and the nitrogen and phosphate sources were supplied in the nutrient solution.

The composition of the nutrient solutions used in this investigation are given in Table I. Nutrient 1 was the same as that used by Leathen.[15] This nutrient was used in the initial studies.[16] Nutrient 2 was made up to simulate the composition of the effluents from the waste rock dumps in Bingham Canyon. Nutrient 3 and 4 reflect intermediate conditions when the necessary or optimum concentrations of certain substances had been determined while the optimum concentrations of other components had not. Nutrient 5 is the final nutrient determined. Either urea or ammonium sulfate was found to be adequate as a source of nitrogen. It was found that phosphate in limited amounts was necessary.

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[14] Wilson, op. cit., p. 27.

[15] W. W. Leathen, L. D. McIntyre, and S. A. Braley, Science, 114, 280 (1951).

[16] Wilson, op. cit., p. 10.

TABLE I

VARIOUS NUTRIENTS USED IN THE STUDIES OF THE PHYSIOLOGICAL PROPERTIES OF THE ISOLATED BACTERIUM

Nutrient Component	Nutrient No. (Conc. in g/l) 1	2	3	4	5
$(NH_2)_2CO$	---	---	---	0.1	(0.1)
$(NH_4)_2SO_4$	0.15	1.0	1.0	-----	(1.0)
K_2HPO_4	0.05	0.30	0.10	0.10	0.10
KCl	0.05	0.05	---	---	---
$Al_2(SO_4).18H_2O$	---	8.0	---	---	---
$MgSO_4.7H_2O$	0.50	3.0	3.0	3.0	---
$MnSO_4.H_2O$	---	0.05	---	---	---
$Ca(NO_3)_2.4H_2O$	0.01	0.10	---	---	---
Na_2SO_4	---	0.05	---	---	---
Distilled H_2O	1000 ml.	1000 ml.	1000 ml.	1000 ml.	1000 ml.
pH	2.65	2.65	2.65	2.65	

The pH was adjusted to 2.65 with sulfuric acid.

C. Substrates

A variety of oxidizable substrates was used in the isolation procedures and the physiological studies on the isolated bacterium. Molybdenite concentrate and chalcopyrite samples were obtained from Kennecott Copper Company (Utah Division). Pyrite III and V were museum grade pyrite from the Brigham Young University Geology Department. Sulfur, ferrous sulfate, and copper sulfide were reagent grade materials.

All solid minerals were used in a finely ground state (at least 60% of a -200 mesh, 100% of -100 mesh). The molybdenite and chalcopyrite had been recovered by flotation so they were washed with acetone and water to

remove remaining traces of the reagents used in the flotation process. Analysis of the substrates are given in Table II.

TABLE II

ANALYSIS OF SUBSTRATES USED

Substrate	%Mo	%S	%Cu	%Fe	Insol.
Molybdenite Concentrate	57.58	38.42	0.1	0.0	3.9
Chalcopyrite II	trace	33.45	32.15	18.55	15.85
Pyrite III	0.04	44.45	6.57	46.0	0.08
Pyrite V	trace	42.5	5.36	34.25	17.89
Sulfur	Reagent Grade				
Ferrous Sulfate	Reagent Grade				
Copper Sulfide (CuS)	Reagent Grade				

D. Microorganisms Used

The microorganisms used in this investigation were obtained from the effluent streams from waste dumps in Bingham Canyon, and Cananea, Sonora, Mexico.[17] The leaching stream from Bingham Canyon had previously been used as a culture source. The various cultures were treated by different methods in the isolation procedure. The various cultures which were used are summarized in Table III.

TABLE III

BACTERIAL CULTURES USED IN THE ISOLATION PROCEDURES

Culture	Source
Culture 1	Bingham Canyon Stream Solution

[17] R.C. Weed, Mining Eng., 8, 721(1956).

TABLE III-Continued

BACTERIAL CULTURES USED IN THE ISOLATION PROCEDURES

Culture	Source
Culture 2	Culture 1 after enrichment on iron pyrite
Culture 3	The organism isolated on sulfur from culture 1
Culture 4	The organism isolated on ferrous iron from culture 1
Culture 5	Cananea Stream Solution (Veta)
Culture 6	Cananea Stream Solution (Ronquillo)

E. Methods of Analysis

The physiological properties of the isolated bacterium were determined by analysis of the end products of the oxidation. The metal sulfides, FeS_2, CuS, $CuFeS_2$ have all been shown to be oxidized to sulfates[18,19,20] and soluble metal ions. Ferrous iron is oxidized to ferric iron, sulfur to sulfuric acid, and molybdenite to sulfuric and molybdic acids.

Standard methods with slight modifications were used for making the analyses.

Iron was determined by the standard dichromate method.[21] A sample containing either or both forms of iron (ferrous and ferric) was reduced by stannous chloride so that only ferrous iron remained. Excess stannous

[18]Wilson, op. cit., p. 18.

[19]Davis, op. cit., p. 18.

[20]Anderson, op. cit., p. 26.

[21]I.M. Kolthoff and E.B. Sandell, "Textbook of Quantitative Inorganic Analysis", 3rd ed., The MacMillan Company, New York, N.Y., 1952, p.579.

chloride was removed by oxidation with mercuric chloride. The ferrous iron was then titrated with potassium dichromate in the presence of phosphoric acid with sodium diphenylamine sulfonate as the indicator. To determine the ferrous iron only, no reduction with stannous chloride was made. The ferric iron concentration was determined by taking the difference between the total iron and ferrous iron.

The determination of molybdenum was made by the non-extraction, colorimetric thiocyanate method described and modified by Anderson.[22] This determination depends upon a colored thiocyanate complex formed by pentavalent molybdenum. Certain interfering ions must be absent.

The standard iodometric method was used in the determination of copper.[23] The method consists of reaction of copper with potassium iodide in a properly buffered environment to produce free iodine quantitatively. The liberated iodine was titrated with sodium thiosulfate.

Sulfate was determined gravimetrically as $BaSO_4$ by the standard gravimetric method.[24]

The pH measurements were made with a Beckman Model M pH meter.

Sulfuric acid was determined by titrating an aliquot with standard 0.1 N sodium hydroxide to the pH of the original nutrient solution.

Bacterial cell growth was obtained by determining the dry weight of the cells and correlating it with turbidity measurements. The optical density measurements (625 mμ) were taken of cell suspensions and the dry weight measurements of these same suspensions were made. Centrifugation and washing of the cell suspension eliminated most of the salts from the

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[22]Anderson, op. cit., p. 21.

[23]Kolthoff and Sandell, op. cit., p. 603.

[24]Ibid., p. 322.

solution surrounding the cells. The cells were dried at 110°C.

Staining of the bacteria was accomplished by two methods. Since the bacteria naturally occur in an acid environment an acid dye, carbol-erythrosin was used.[25] The Hucker modification of the Gram stain was used. To utilize the Gram stain the bacterial cells must be in a neutral or slightly basic environment so it was found necessary to centrifuge and wash the cells to obtain this condition.

F. Methods of Isolation

The isolation of the bacteria in pure cultures which are responsible for the oxidation of sulfur, ferrous iron, and sulfide minerals in exposed ore bodies was necessary in order to obtain a more complete picture of the oxidative processes involved. The characteristics of these little known autotrophic bacteria must be determined and compared with the other species that have been isolated and described by other investigators.[26,27]

Two accepted methods of isolation were employed in this study, the enrichment-dilution technique and growth from a single cell on a solid medium.

1. The Enrichment-Dilution Technique

The most generally used technique for enrichment-dilution isolation consists of diluting an enriched culture aseptically to such an extent that only a very few organisms remain. Theoretically, the enriched culture is diluted until only the organisms present in the greatest amount remain. Thus, by dilution, foreign organisms are removed.

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[25]Fred and Waksman, "Laboratory Manual of General Microbiology," McGraw-Hill Book Company, Inc., New York, N.Y., 1928, p. 49.

[26]Waksman and Joffe, op. cit.

[27]Temple and Colmer, op. cit.

A modified enrichment-dilution technique, particularly suited for use with the slow growing organisms studied in this investigation was employed.

This method consisted of an enriching step similar to the usual procedure. In this step a high concentration of organisms was obtained. A single drop of solution was then transferred aseptically to another percolator. When growth was first noticed, a single drop of the resulting solution was transferred to a third percolator. This was continued through several steps. Each step drastically reduced the concentration of slow growing and foreign organisms. At some point the foreign organisms reach a very low concentration. At this concentration, the chances for a single droplet to contain one bacterial species become very good. Several transfers ensure that a pure culture has been obtained.

2. Isolation on Solid Medium

In cases where possible, this method of isolation is much preferred over the enrichment-dilution technique because single, isolated colonies are grown from one organism. These single colonies can be easily seen and are recognized as colonies descending from a single cell.

After several attempts to grow cultures on agar-agar, it was evident that it was not suitable for the isolation of this type of bacteria. It had previously been shown that the bacterium in question was a chemosynthetic autotroph. Since organic materials tend to have a deleterious effect on this type of bacteria, a completely inorganic solid medium was

desired. Thus, the silica gel containing the desired nutrients and substrates was developed for this phase of the investigation. The method of preparation has been previously described.[28] This method of isolation consisted of aseptically spreading a droplet of an enriched solution from the percolators over the surface of the gel. After incubation in a closed container at room temperature for several days or weeks (depending on the substrate used) small, isolated colonies would appear over the streaked areas.

The small colonies were growth from a single or a very few cells. To insure against contaminant organisms a small representative colony was used as the inoculum for another silica gel plate. After several transfers in which the characteristics of the colonies remained the same, the bacterium responsible for the substrate oxidation was assumed to be isolated.

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[28] See page 8.

CHAPTER IV

RESULTS

A. Enrichment Dilution study on Ferrous Iron

The enrichment dilution technique as employed with ferrous iron as the oxidizable substrate consisted first of the enriching step. A percolator containing ferrous iron (2000 ppm.) was inoculated with stream solution (culture 1) to obtain an enriched culture. Second, a series of thirteen percolators were employed to carry forth the enrichment dilution steps. It was assumed that a pure culture was obtained after this procedure had been completed.

At the first indication of oxidation, (by the appearance of the brownish ferric color) one drop of solution was transferred aseptically to the next percolator. Only a few hours were necessary for complete oxidation after the induction period had ended. A typical curve showing the rate of oxidation is shown in Figure 2, culture 2. The induction period for each percolator is shown in Table IV. After this procedure had been completed, it was assumed that all foreign organisms were eliminated.

TABLE IV

THE INDUCTION PERIOD DURING ENRICHMENT DILUTION ISOLATION ON FERROUS IRON

Transfer Number	Induction Period Days
1	5
2	5
3	6
4	-
5	-
6	7

TABLE IV-Continued

Transfer Number	Induction Period Days
7	-
8	5
9	-
10	-
11	4
12	4
13	4

Several tests were run on the culture isolated by this means (culture 4) to make a comparison with culture 1 and others described in the literature.[29,30]

Enrichment dilution studies of this ferrous iron oxidizing culture on sulfur were carried out. This was done to determine if any of the relatively fast growing organisms which grow on sulfur alone had been carried through the isolation procedure as contaminants. The results are given in Table V.

TABLE V

THE EFFECT OF CULTURE 4 (BY ENRICHMENT DILUTION ISOLATION) ON FERROUS IRON AFTER PROLONGED GROWTH ON SULFUR

Transfer Number (From Sulfur Percolator)	Induction Period on Sulfur	Effect on Ferrous Iron
1	14 Days	Oxidized ferrous iron
2	11	Oxidized ferrous iron
3	8	Oxidized ferrous iron
4	8	Oxidized ferrous iron

[29]Waksman and Joffe, op. cit.

[30]Temple and Colmer, op. cit.

It was found that culture 4 can also oxidize sulfur. The data in Table V also show that after several transfers on sulfur this culture retained its ability to oxidize ferrous iron.

The ferrous iron oxidizing organism (culture 4) was used to inoculate sterilized percolators containing 5 grams of pyrite, 100 g. sand, and the required nutrient solution. The results of this study are given in Table VI and shown graphically in Figure 2. These data show a comparison of the rates of oxidation by culture 1 and culture 4 on pyrite. Culture 4 consists of results from both the enrichment dilution technique and from isolation on silica gel. The lowest curve is a sterile control.

TABLE VI

A COMPARISON OF OXIDATION RATES BEFORE AND AFTER ISOLATION OF CULTURE 4

			After Isolation				
		Before Isolation	Initial Transfer			Transfer 2	
Time Weeks	Sterile Control	%FeS_2 Oxidized	%FeS_2 (1)	Oxidized (2)	Oxidized (3)	%FeS_2 (1)	Oxidized (2)
1	--	0	--	--	--	--	--
2	--	18.3	--	--	--	4.3	8.3
3	--	32.2	1.5	--	--	--	--
4	--	41.1	--	2.1	1.1	--	--
5	--	46.3	--	--	--	--	--
6	--	48.8	2.9	--	--	--	--
7	--	51.5	4.3	3.7	2.1	--	--
8	--	53.6	--	--	--	40.0	53.6
9	--		--	--	--		
10	0.12		7.2	--	--		
15	0.18		--	5.7	--		
18			11.5	--	--		
31			--	30.0	35.8		
34			35.2	--	--		

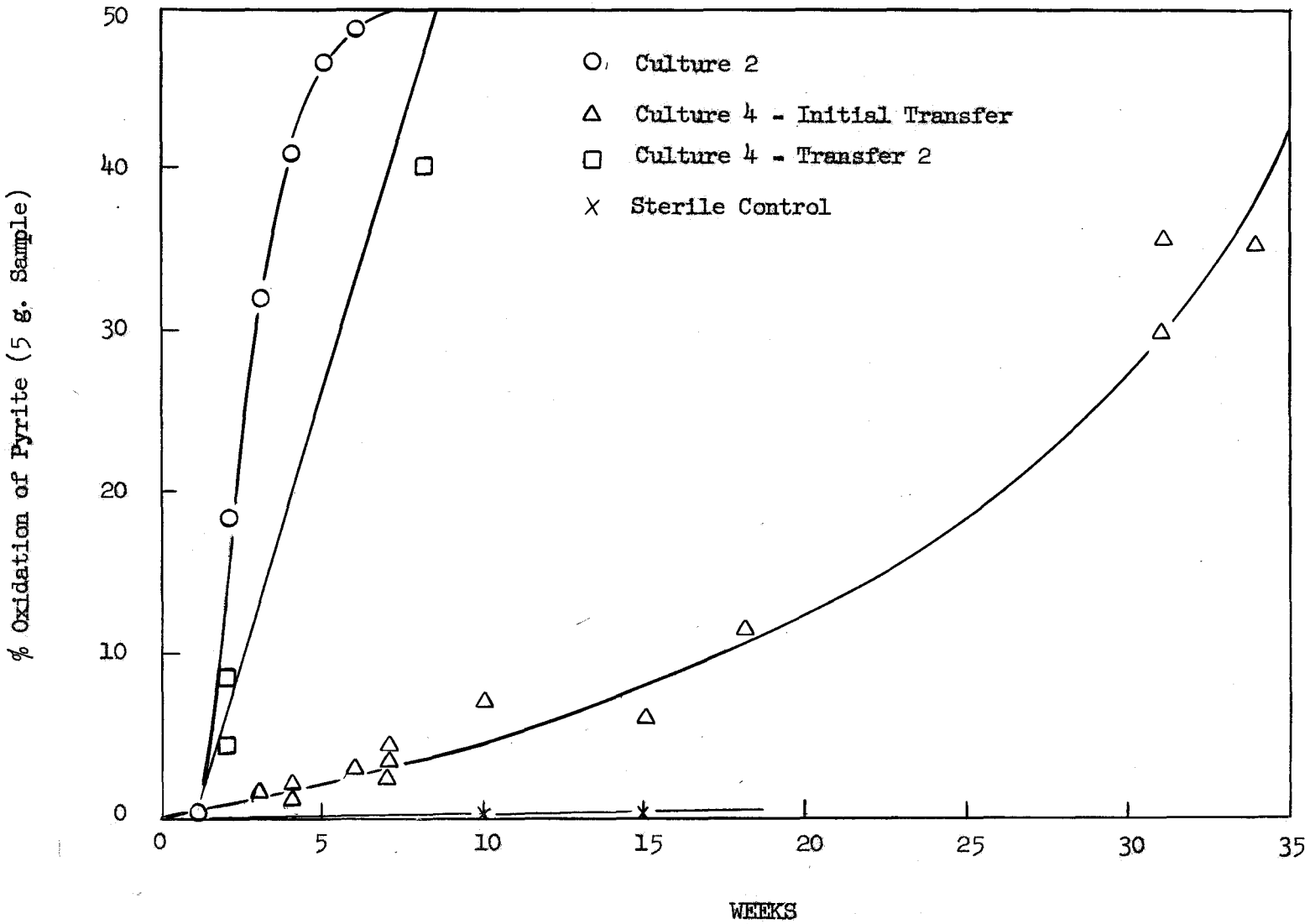

Fig. 2: A comparison of oxidation of pyrite before and after isolation of culture 4.

The graph shows that the rate of oxidation of pyrite was much slower after isolation (about 5% of the original rate) than with the original stream solution. However, the rate increases slowly, as shown by the gradual increase in the slope of the line (culture 4). The second transfer has nearly the same rate of oxidation as the original enriched culture (culture 2). Thus it appears that after prolonged growth and repeated transfers on pyrite the rate of oxidation is increased and eventually reaches the same activity as the initial culture. A similar type of activity on pyrite as a function of cupric ion concentration has previously been shown.[31]

B. Isolation on Silica Gel With Ferrous Iron as the Oxidizable Substrate

The isolation of the iron oxidizing bacterium on silica gel, from the enriched culture (culture 2), was accomplished in the following manner. The petri plates containing silica gel were prepared as previously described.[32] A drop of inoculum from culture 2 was aseptically placed on a sterile cotton swab and a light smear was made on the surface of the gel. Cotton swabs were found to be superior to the conventional wire loop because they did not fracture the surface of the silica gel. The plates were allowed to incubate in desiccators in the dark at room temperature. In three or four days tiny brown colonies began to form along the path of inoculation.

After several days incubation of the plate, a single colony was selected and a transfer made to another petri plate. After the second plate had been allowed to incubate the process was repeated. This was done several times to insure elimination of possible contaminants. The results are shown in Table VII and representative plates were photographed and are shown in Figure 5, a and b.

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[31] Wilson, op. cit., p. 30.

[32] See page 8.

TABLE VII

OBSERVATIONS DURING ISOLATION ON SILICA GEL CONTAINING FERROUS IRON

Inoculum	Observed Result
1. Enriched Culture (culture 2)	Separate and distinct colonies
2. Single Colony from plate number 1 (culture 4)	Separate and distinct colonies
3. Single Colony from plate number 2 (culture 4)	Separate and distinct colonies
4. Single Colony from plate number 3 (culture 4)	Separate and distinct colonies

Single colonies were obtained from all of the transfers. This entire process was repeated several times with identical results.

A transfer from a single colony (culture 4) was made to a prepared sterile percolator containing the desired substrate and the results were observed. This was done either by making quantitative measurements of the oxidation products or by direct observation.

The results of this study are shown in Figure 2. The curve showing the rate of oxidation of pyrite by the isolated organism is identical for both methods of isolation.

C. Enrichment Dilution Study on Sulfur

Essentially the same technique was employed in this study as in the previous enrichment dilution study. A total of ten percolators, charged with 100 g. of Ottawa sand, 5 g. sulfur, and 100 ml. nutrient 2 were used. At the first indication of oxidation (by the appearance of turbidity in the percolators) one drop of solution was transferred aseptically to the next percolator.

After the completion of this series of transfers it was assumed that a pure culture had been isolated.

Tests were made on ferrous iron and pyrite with the isolated organism (culture 3) to determine its physiological properties.

The growth rate in percolators on pyrite and ferrous iron both before and after the isolation study is given in Tables VIII and IX and is shown graphically in Figures 3 and 4. These results show that culture 3 does not oxidize either ferrous iron or pyrite. Thus, it would appear that the pyrite and ferrous iron oxidizing bacterium had been eliminated by this method of isolation.

TABLE VIII

COMPARISON OF THE RATE OF PYRITE OXIDATION BEFORE AND AFTER ISOLATION ON SULFUR

Weeks	(Culture 1) Before Isolation %FeS_2 Oxidized	(Culture 3) After Isolation %FeS_2 Oxidized
1	0.0	-
2	18.3	-
3	32.2	-
4	41.1	0.06
5	46.3	-
6	48.8	-
7	51.5	0.10
8	53.6	-
12	--	0.15
15	--	0.18

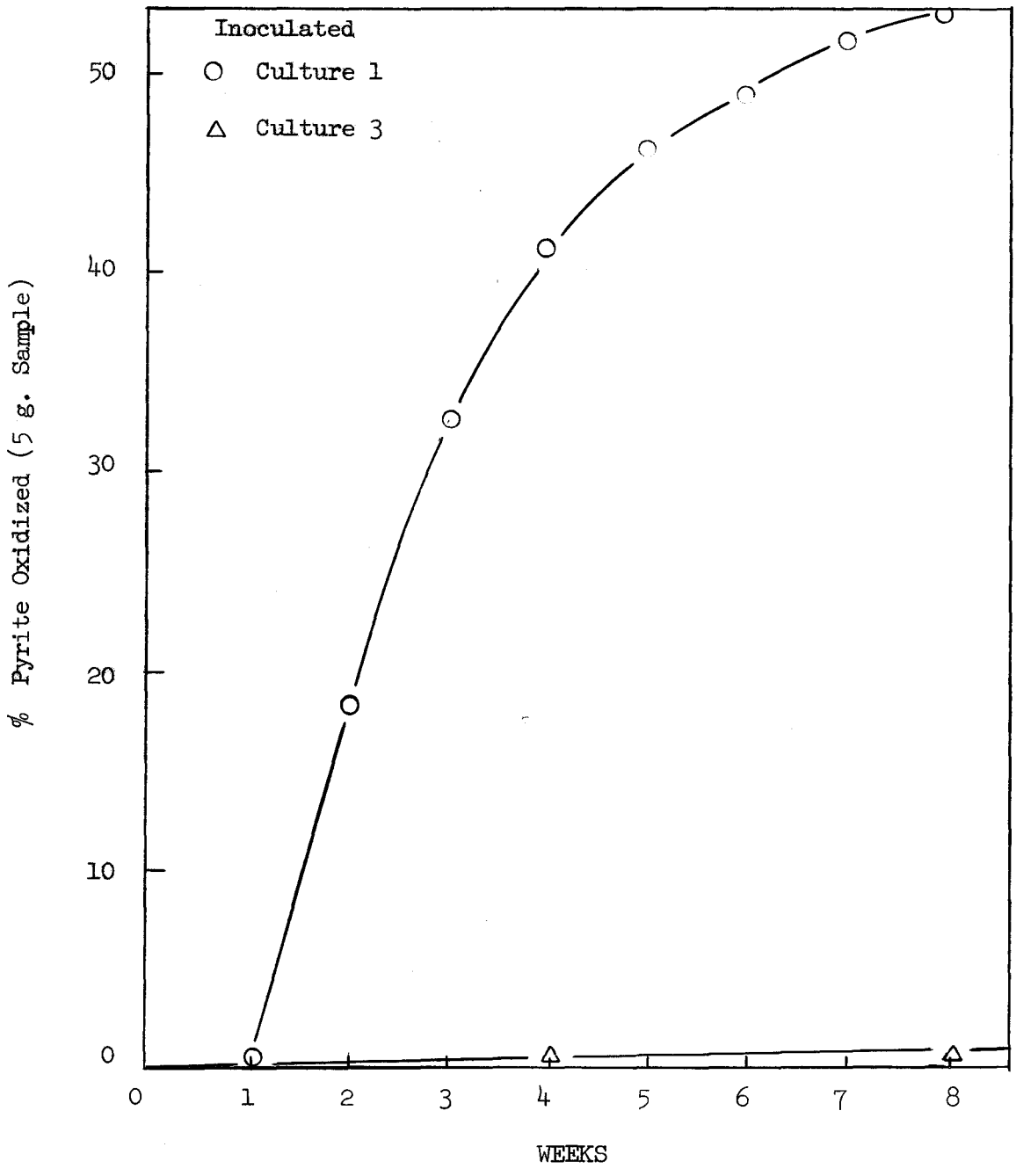


Fig. 3: The oxidation of pyrite before and after isolation of the sulfur oxidizing organism.

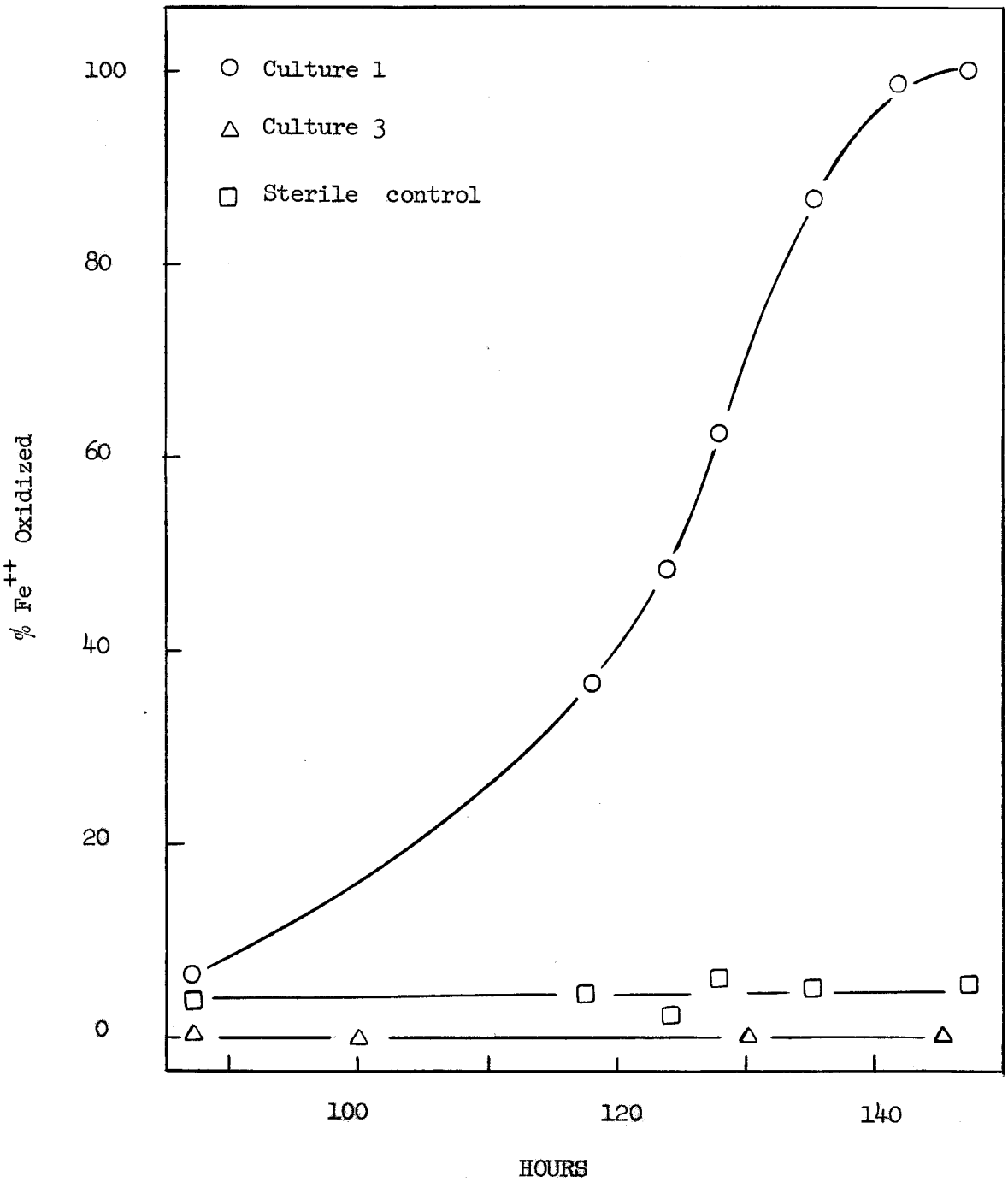


Fig. 4: The oxidation of ferrous iron before and after the isolation of the sulfur oxidizing organism.

TABLE IX

COMPARISON OF THE RATES OF FERROUS IRON OXIDATION BEFORE AND AFTER ISOLATION ON SULFUR

Hours	Before Isolation % Ferrous iron oxidized Sterile	Before Isolation % Ferrous iron oxidized Inoculated	After Isolation % Ferrous iron oxidized (Culture 3)
87	4.0	6.0	-
100	-	-	nil
118	4.0	36.5	-
124	3.0	48.4	-
128	6.0	62.3	-
130	-	-	nil
135	4.0	87	-
141.5	0.0	98.6	-
147	5.0	100.0	-
3 weeks	6.0	-	-
6 weeks	-	-	nil

Attempts were made to isolate the sulfur oxidizing organism on silica gel containing sulfur as a substrate. Significant growth could not be observed using flowers of sulfur. By using colloidal sulfur as the substrate (made from sodium thiosulfate by reaction with HCl and then removing the cations and anions with ion exchange resins) good growth was observed. However, since the colonies and the gel were both the same color, it was difficult to observe single colonies. Only the difference in the luster of the gel and the colonies made observation possible. It was impossible to select good single colonies, since only the largest could be readily seen.

D. Attempted Isolation on Agar-agar

Agar-agar is the semi-solid medium usually used in the isolation of bacteria. However, it is usually used at a pH of approximately 7. In this

study an attempt was made to isolate the ferrous iron oxidizing organism on agar. Washed agar was used as the semi-solid medium to eliminate most of the soluble organic compounds. The salts to be included in the nutrient were sterilized separately. The composition of the sterilized solutions is shown in Table X

TABLE X

COMPOSITION OF THE SOLUTIONS USED IN MAKING WASHED AGAR PLATES

I		II	
Agar	30.0 g/l	$MgSO_4.7H_2O$	3.0 g/l
		$FeSO_4$	20.0
		K_2HPO_4	0.6
		$(NH_4)_2SO_4$	2.0
		pH adjusted to 2.65	

After sterilization and cooling to nearly the solidification point of the agar, the above solutions were mixed and poured into previously sterilized petri plates. This procedure gave very nice plates which streaked easily. Both the pour plate and streak plate methods were used; however, no growth was observed after one month's time and so this method of isolation was abandoned.

It was thought possible that a slight hydrolysis of the agar, even though mixed with the nutrient and the substrate just before solidification, may have liberated organic compounds which inhibited growth.

E. Characteristics of the Organism Isolated on Ferrous Iron (Culture 4)

This organism (culture 4) was found to be a gram negative rod. The conventional gram stain was used. However, excess acid had to be removed by washing and centrifuging. The organism differed slightly in size and shape

depending on the substrate. It was about .5 - .8 microns wide by 1 - 1.3 microns long. It was motile. On ferrous iron it was almost round while on pyrite it had a definite rod shape. Photomicrographs of stains of the organism as it grows on ferrous iron are shown in Figure 5, a and b.

On silica gel, with ferrous iron as the substrate (Figure 5, c and d) the colonies gave the following characteristics:

Color-- Orange to red (iron precipitate)

Size-- 1 to 2 mm. in diameter

Luster-- dull

Margin-- circular

Surface-- smooth, hard crust

Elevation-- flat

On silica gel, with pyrite and chalcopyrite as the substrate the following characteristics were exhibited:

Color-- Yellow-orange

Size-- 1 mm. (maximum)

Luster-- dull

Margin-- circular

Surface-- smooth

Elevation-- flat

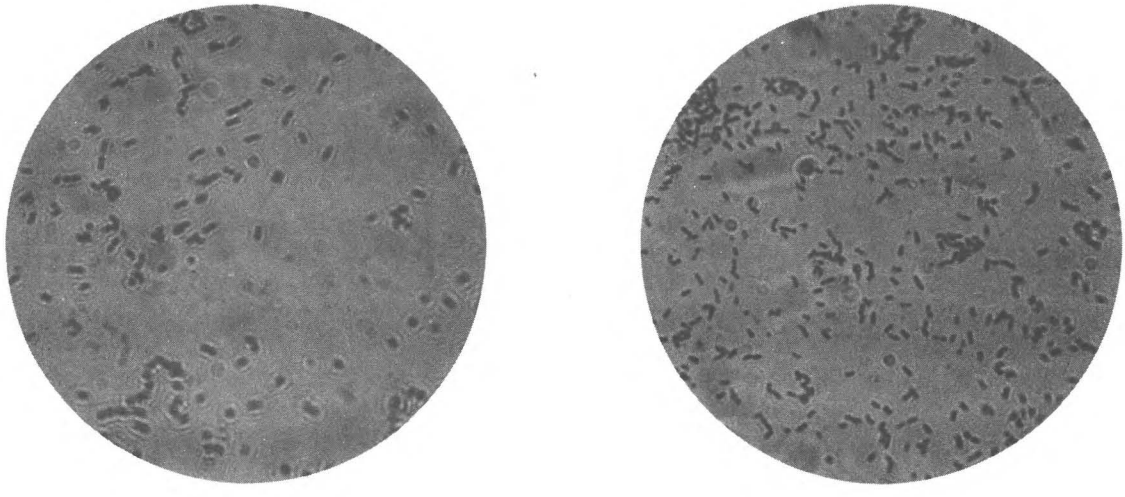
F. Characteristics of the Organism Isolated on Sulfur (Culture 3)

Culture 3 was found to consist of a bacterium which was gram-negative. It was rod shaped, about .5 - .8 micron wide by 1 - 1.3 micron long. It was motile.

On silica gel, with colloidal sulfur as the substrate, the colonies exhibited the following characteristics:

a b

Photographs of Petri plates showing colonies of Ferrous Iron Bacteria



c d

Photomicrographs of Ferrous Iron Bacteria

Fig. 5: Photographs of the microorganisms isolated on ferrous iron: Microscopic and colonies

Color-- White

Size-- about 1 mm.

Luster-- dull

Margin-- circular irregular

Surface-- rough

Surface elevation-- flat

The colonies were very hard to see, being of the same color as the colloidal sulfur silica gel.

G. Physiological Characteristics of the Isolated Organisms

The proper characterization of each bacterium isolated from Bingham Canyon streams requires a knowledge of its ability to oxidize various substrates. It has been shown that the bacterium isolated on sulfur (culture 3) by the enrichment dilution technique would not grow on the sulfides (FeS_2, $CuFeS_2$), but would grow readily on sulfur. Culture 4 has the ability to oxidize iron pyrites, chalcopyrite, molybdenite, free sulfur and iron.

1. Growth on Pyrite and Ferrous Iron

It has been shown that the bacterium isolated on ferrous iron (culture 4) could also oxidize iron pyrites, while the bacterium isolated on sulfur could not oxidize either pyrite or ferrous iron. Curves comparing growth on pyrite and ferrous iron with growth before isolation are shown in Figures 2, 3, and 4.

2. Growth on Sulfur

As stated in the preceding section, both culture 3 and culture 4 were found to oxidize sulfur to sulfuric acid. Isolation of the sulfur oxidizer (culture 3) by the enrichment dilution technique requires that it be faster growing than all other organisms present in the impure culture.

The purpose of this study was to determine the rate of oxidation of each isolated organism and compare it with the rate of those

from the stream solution. The results of this study are given in Table XI and Figure 6. Two separate runs at widely differing times were made with culture 2. These are labeled A and B. Curve A shows growth on a 5 g. sample of sulfur while 2 grams were used in runs shown by the rest of the curves.

TABLE XI

THE RATE OF OXIDATION BY VARIOUS CULTURES OF SULFUR OXIDIZING BACTERIA

Time		% Solubilized					
		Culture 2		Culture 3		Culture 4	
Weeks	Sterile	A	B	%	pH	%	pH
1	0.04	2.9	---	---		---	
2	0.5	8.6	---	---		---	
3	1.6	14.1	---	---		---	
4	2.1	26.7	19.8	19.8		10.2	
5	---	38.4	---	---		---	
6	---	48.1	---	---		---	
7	---	61.0	---	---		---	
7.5	6.4	---	78.6	83.5	0.42	33.6	0.85
8		70.6					
9		78.6					
10		83.6					
11		87.8					
12		93.6					
13		98.6					
14		101.2					

The results of this study show that the enriched culture from the stream solution and the organism isolated on sulfur have approximately the same oxidation rate. However, the sulfur oxidation rate by the organism isolated on ferrous iron is considerably slower (approximately 30-50% that of culture 2).

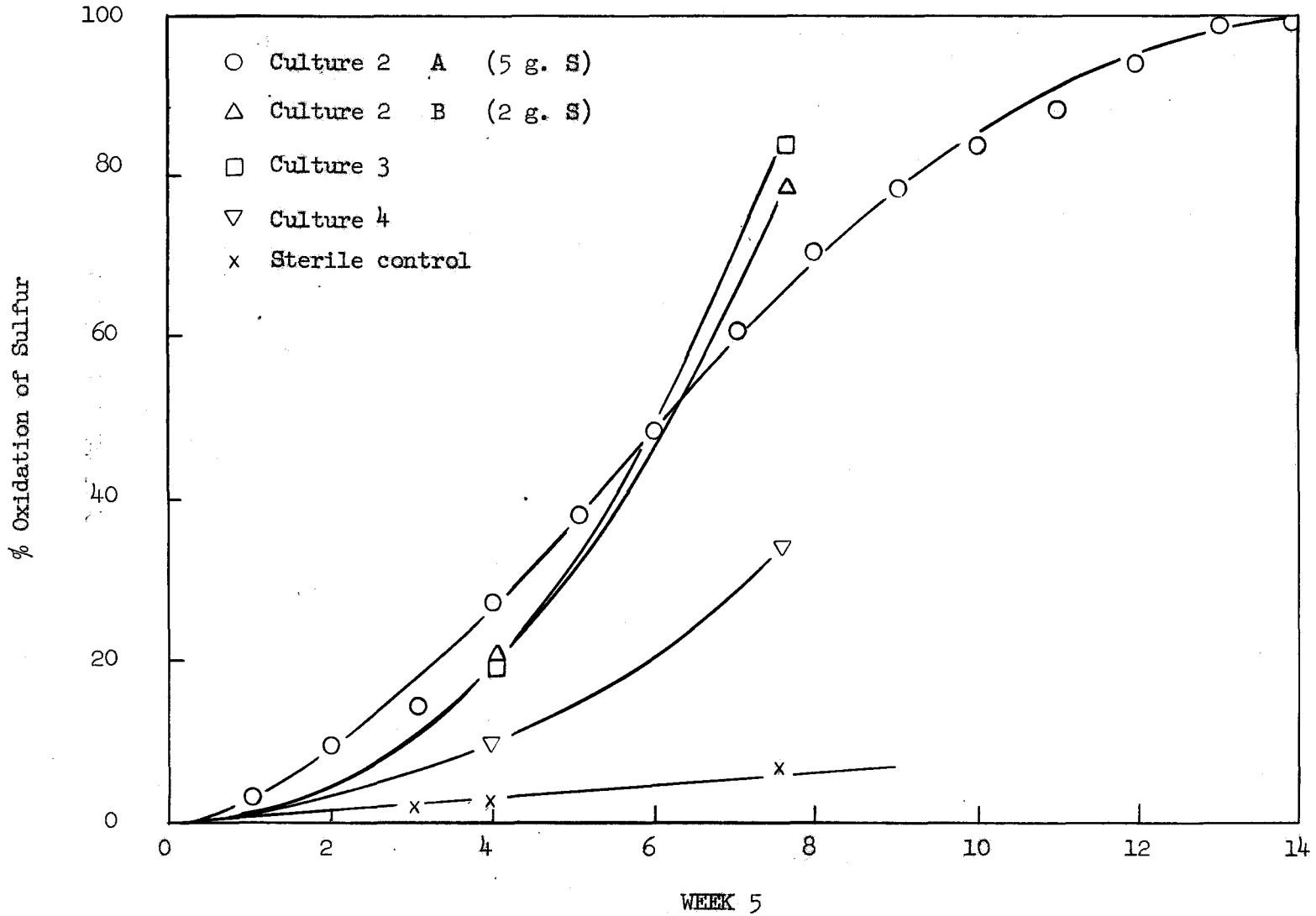


Fig. 6: The oxidation of sulfur by various cultures.

Table XI also shows that cultures 2 and 3 were able to grow in solutions of pH= 0.42. On titration this corresponded to an acidity greater than 0.8 N sulfuric acid.

3. Growth on Chalcopyrite by Cultures 3 and 4

The purpose of this study was to determine the rates of oxidation of chalcopyrite by cultures 3 and 4 and to compare them with that of culture 2. The results of this study are shown in Table XII. The period of oxidation was 13 days.

TABLE XII

A COMPARISON OF THE AMOUNT OF SOLUBILIZED CHALCOPYRITE BY CULTURES 2, 3, AND 4 AFTER 13 DAYS

Culture	Solubilized Copper (mg)	Solubilized Iron (mg)
2	7.9	454
3	0.9	12
4	7.9	491
Sterile Control	0.7	8

From the preceding results it may be seen that almost exactly the same characteristics were exhibited on chalcopyrite by both culture 2 and culture 4 and that both cultures were capable of the oxidation of chalcopyrite. The bacterium isolated on sulfur (culture 3) did not show appreciable oxidation of chalcopyrite.

4. Growth on Molybdenite by Culture 4

The purpose of this study was to determine the rates of oxidation of molybdenite by cultures 3 and 4 and to compare them with that of culture 2. The nutrient solution contained 4000 ppm ferric iron. The results of this study are shown in Table XIII. The period of oxidation was 13 days.

TABLE XIII

A COMPARISON OF THE OXIDIZED MOLYBDENITE BY CULTURE 2 AND BY CULTURES 3 AND 4 AFTER 13 DAYS

Culture	% Oxidation of MoS_2
2	0.61
3	0.09
4	0.53
Sterile Control	0.06

The preceding results show that almost the same amount of molybdenite was solubilized by both culture 2 and culture 4. Table XIII also shows that both were capable of molybdenite oxidation and that the organism isolated on sulfur (culture 3) was not capable of molybdenite oxidation.

H. A Comparison of the Isolated Organisms with Similar Organisms described In the Literature

The organism found at Bingham Canyon (culture 1) oxidizes ferrous iron, sulfur, and various sulfide minerals. It has been found to be autotrophic[33] so it belongs in the family of bacteria known as Nitrobacteraceae. It oxidizes sulfur and ferrous iron, therefore it belongs to the tribe Thiobacillae.

It may be seen from Table XIV that only two organisms, T. thiooxidans and T. ferrooxidans, are able to exist in the same high acid concentrations as the organisms in question. From Table XIVb it may be seen that T. thiooxidans can oxidize sulfur but not ferrous iron or pyrite while T. ferrooxidans is able to oxidize ferrous iron but is unable to oxidize

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[33] Wilson, op. cit., p. 24.

sulfur.[34,35] One organism (culture 4) found in Bingham Canyon is able to oxidize ferrous iron, sulfur, and pyrite as well as several other sulfide minerals. Thus, it would appear as though this organism is quite closely related to T. thiooxidans and T. ferrooxidans, but possesses somewhat distinguishing characteristics.

The second organism isolated from the Bingham Canyon stream solution (culture 3) possesses almost exactly the same characteristics as T. thiooxidans. The only substrate in question is thiosulfate. As has recently been pointed out, this is a very poor substance to check bacterial oxidation on in acid solution.[36] Thiosulfate decomposes in acid solution into free sulfur and sulfur dioxide; therefore the oxidation could be attributed to the free sulfur present.

Thus all reliable tests yield similar results for culture 3 and for T. thiooxidans. It appears as though they may be two strains of the same organism.

TABLE XIV

pH RANGE FOR MEMBERS OF THIOBACILLUS

Organism	Min.	Max.	Opt.
T. thioparus			7
T. thiooxidans	0.5	6.0	2-3.5
T. novellus			5-9
T. coprolitícus			7-7.5
T. denitrificans			7
T. ferrooxidans			2-3.5
Culture 3	0.4		ca. 2-3

[34] "Bergey's Manual of Determinative Bacteriology," op.cit.

[35] K. L. Temple and E. W. Delchamps, Appl. Microbiol. 1, 255(1953).

[36] W. W. Leathen and S. A. Braley, J. Bact., 69, 481(1955).

TABLE XIV--Continued

pH RANGE FOR MEMBERS OF THIOBACILLUS

Organism	Min.	Max.	Opt.
Culture 4	0.7	ca. 2-3	

TABLE XIVb

SUBSTRATES OXIDIZED BY VARIOUS BACTERIA

Substrates	T. thiooxo.	T. ferro	T. thiop.	Culture 4	Culture 3
Sulfur	+	-	+	+	+
Thiosulfate	+	+	+	?	?
Ferrous Iron	-	+	-	+	-
Copper Sulfide				+	
Iron Pyrite	-	+		+	-
Chalcopyrite				+	
Molybdenite				+	
Chalcocite				+	
Bornite				+	
Tetrahedrite				+	

I. Biological Oxidation of Sulfide Minerals by Bacteria From Locations Other than Bingham Canyon

It has been known for several years that biological action was responsible for the oxidation of various sulfides in the waste dump at Bingham Canyon.[37] The purpose of this phase of the investigation was to show that

[37] Wilson, op. cit., p. 18.

biological oxidation of sulfide minerals occurs under similar conditions at other geographical locations.

At Cananea, Sonora, Mexico, a similar operation to that at Bingham Canyon is being successfully carried out.[38] This oxidation of sulfide minerals under favorable conditions seems to be a general phenomenon.

1. Oxidation of Pyrite by Various Cultures

Preliminary runs under aseptic conditions showed that the oxidation of pyrite by stream solutions from both the Cananea waste dumps (Ronquillo, culture 6) and the underground operation (Veta, culture 5) were biological. A series of sterile percolators were set up to compare the rate of pyrite oxidation by the organisms from Bingham Canyon with those from Mexico. The results are shown in Table XV and in Figure 7. The initial inoculum in each case was from an enriched culture on pyrite.

TABLE XV

OXIDATION OF PYRITE BY ORGANISMS FROM VARIOUS GEOGRAPHICAL LOCATIONS

Time Days	Cumulative Soluble Iron (mg.)			
	Sterile	Culture 2	Culture 6	Culture 5
12	20	187	152	193
26	25	404	313	482
51	30	985	1098	1127

The results given in Table XV and Figure 7 show that all cultures have very nearly the same action on pyrite.

2. Oxidation of Chalcopyrite by Various Cultures

The purpose of this portion of the study was to determine the effect of cultures 2, 5, and 6 on chalcopyrite. The results of this study are shown in Table XVI.

[38] Weed, op. cit.

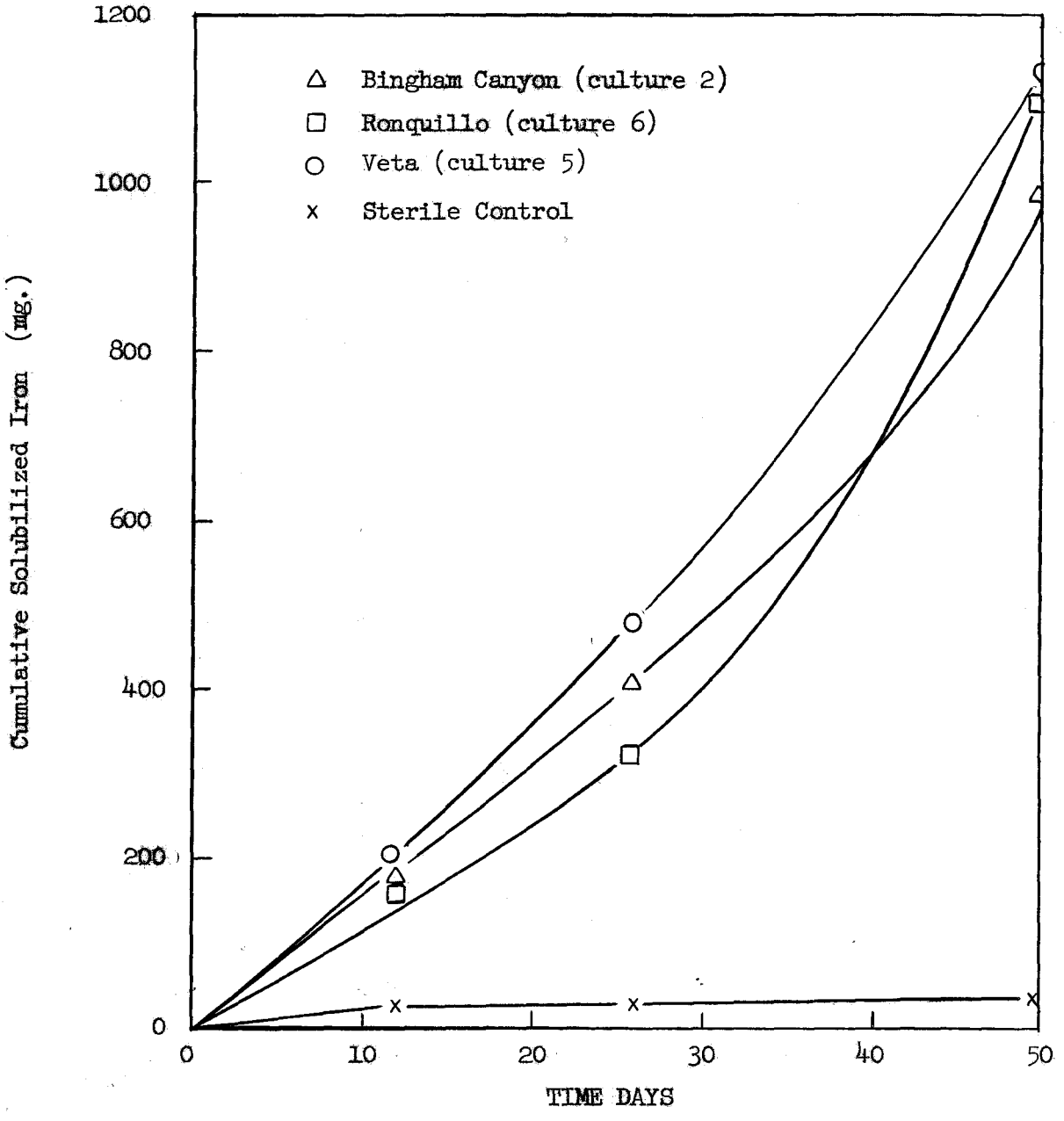

Fig. 7: The oxidation of pyrite by organisms from Utah and Mexico.

TABLE XVI

OXIDATION OF CHALCOPYRITE BY ORGANISMS FROM VARIOUS GEOGRAPHICAL LOCATIONS AFTER 13 DAYS

Culture	Solubilized Iron (mg)	Solubilized Copper (mg)
2	539	7.9
5	428	11.5
6	494	8.5
Sterile Control	8	0.7

The preceding results show that oxidation of chalcopyrite was effected by all three inoculated percolators in substantially the same amounts.

3. Oxidation of Molybdenite by Various Cultures

The purpose of this portion of the study was to determine the effect of cultures 2, 5, and 6 on molybdenite. Ferric iron of a concentration of 4000 ppm. was included in the nutrient. The results of this study are shown in Table XVII.

TABLE XVII

OXIDATION OF MOLYBDENITE BY ORGANISMS FROM VARIOUS GEOGRAPHICAL LOCATIONS AFTER 13 DAYS

Culture	% Oxidation of MoS_2
2	0.70
5	1.00
6	0.61
Sterile Control	0.06

The preceding results show that oxidation of molybdenite occurred in all three inoculated percolators in very nearly the same amounts.

J. Preservation of the Bacteria over Various Periods of Time

Many bacterial species have very fast growth rates. For such species the best way of preserving them has been to maintain them on petri plates by successive transfers. Preservation of a single culture has been accomplished by keeping the culture in an ice bath. By this method cultures of fast growing organisms have been kept alive for over a month.[39]

Various preservation methods have been attempted to maintain virile cultures of bacteria from Bingham Canyon stream solution.

It was observed that the bacteria could be maintained in solution for several months at room temperature but a gradual lessening in the ability of the culture to grow was apparent. Stock cultures were also maintained by continuous growth on pyrite.[40]

A second method of preservation of cultures was carried out as follows: An active culture was grown in a percolator on a moist Ottawa sand suspension of the mineral. Portions of this suspension of the actively growing culture were placed in small glass tubes and either sealed or left unsealed. The moisture was allowed to evaporate to differing degrees of dryness before sealing the tubes. After varying lengths of time the cultures were run in sterile percolators to determine if any live bacteria remained. The results are summarized in Table XVIII.

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[39] Bryner, et. al., op. cit.

[40] Davis, op. cit., p. 12.

TABLE XVIII

BACTERIAL GROWTH AFTER VARYING METHODS OF PRESERVATION ON SUSPENSIONS OF OTTAWA SAND

Method of Storing	Growth after--- 2 Months	6 Months	14 Months
Room Temperature			
Dry	-	-	
Partially Dry	-	-	
Moist	+	-	
40°F			
Dry	-	-	
Partially Dry	+	-	
Moist	+	+	+
Deep Freeze			
Moist			-

From the results of Table XVIII it is apparent that storage of cultures at temperatures slightly above freezing maintained them for well over a year in sealed tubes.

K. The Effect of Urea as a Source of Nitrogen

The nutrient solution previously described (page 10) contains ammonium sulfate as a source of nitrogen. In this study the effect of urea as a source of nitrogen was determined. Ammonium sulfate was replaced by urea in varying concentrations and the rate of oxidation of pyrite was then determined. A control was run containing the optimum concentration for ammonium sulfate. The results are shown in Table XIX and in Figures 8 and 9.

As may be seen from the first graph, the optimum concentration of urea is in the vicinity of .05 g/l. The second graph shows that the optimum urea concentration provides nearly the same rate of oxidation of pyrite as the optimum concentration of ammonium sulfate in the nutrient solution.

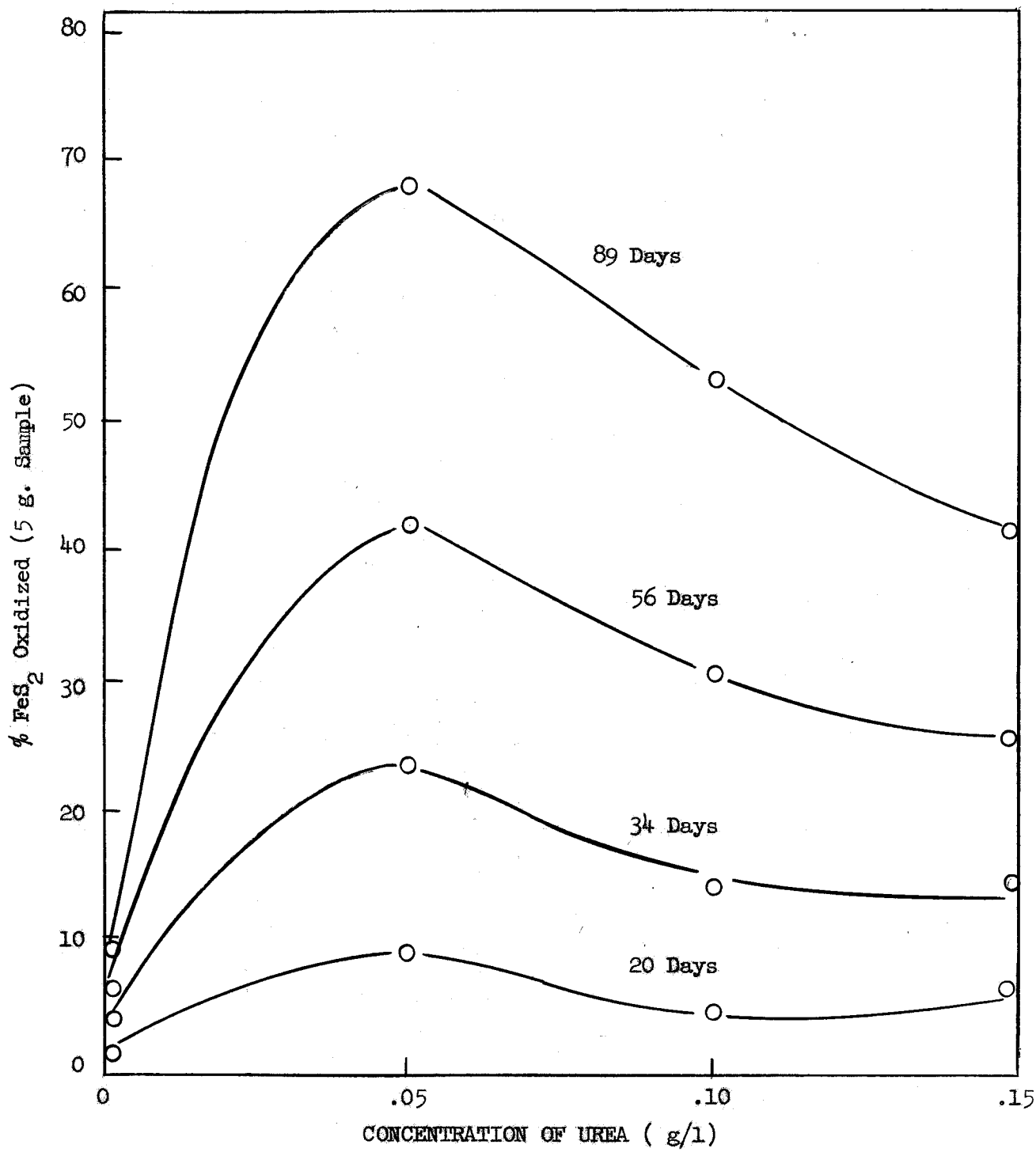

Fig. 8: The effect of urea as the nitrogen source in the biological oxidation of pyrite.

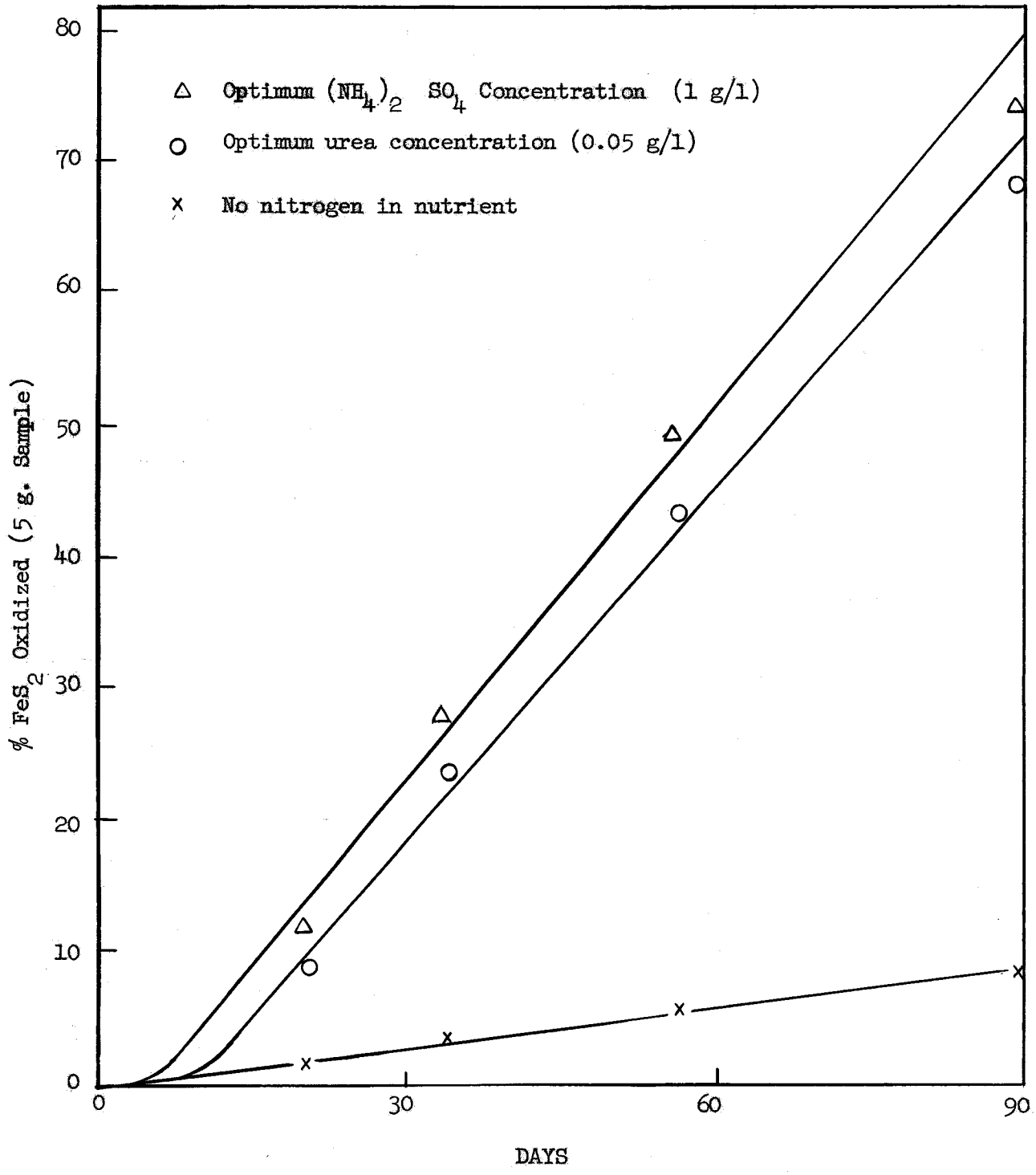

Fig. 9: The oxidation of pyrite using the optimum concentrations of urea and ammonium sulfate.

TABLE XIX

THE EFFECT OF UREA AS THE NITROGEN SOURCE IN THE BIOLOGICAL OXIDATION OF PYRITE

Time Days	Control Reg. Nutrient	% Pyrite Oxidation 0g/l Urea	.05g/l Urea	.10g/l Urea	.15g/l Urea
20	12.0	1.9	9.0	4.7	5.6
34	27.9	3.7	24.0	14.1	14.2
56	50.0	6.0	42.5	30.5	26.2
89	74.0	8.9	68.4	53.2	41.6

L. Determination of the Optimum Nutrient Solution

The concentrations of the media which were used in this study are shown in Table XI. Davis[41] determined the optimum concentration of ammonium sulfate and Anderson[42] determined the optimum phosphate concentration. The effect of the aluminum ion on the oxidation of pyrite was also determined.[43] Since its optimum was not significant it was not included in the final nutrient. Thus, previous workers have determined the optimum concentrations of many substances on the oxidative reactions.

The purpose of this study was to determine the effect of the concentrations of the remaining ions and thus to obtain the optimum nutrient solution for the bacterial oxidation of pyrite.

1. The Effect of Potassium on the Biological Oxidation of Pyrite

In this investigation nutrient 2 was used with the potassium ion concentration as the only variable. The effect of the potassium ion concentration is shown in Table XX. The inoculum was culture 2. Five grams of pyrite 5 were used.

[41] Ibid., p. 34.

[42] Anderson, op. cit., p. 60.

[43] Ibid., p. 66.

TABLE XX

THE EFFECT OF POTASSIUM ION CONCENTRATION ON THE BACTERIAL OXIDATION OF PYRITE

Time Weeks	Cumulative % Oxidation of Pyrite					
	0 ppm Potassium	10 ppm	20 ppm	40 ppm	100 ppm	200 ppm
2	6.2	7.7	5.3	5.7	4.8	7.7
3	14.6	19.3	15.5	17.4	10.9	17.8
4	21.3	31.2	25.1	29.8	16.2	27.5
5	29.3	41.8	34.0	41.8	25.4	37.0
6	38.6	51.1	43.2	51.8	34.8	45.8
7	44.5	57.0	50.1	58.3	41.0	50.8
8	50.7	62.5	57.0	62.5	44.3	54.0

These data show that the concentration of added potassium ion had no significant effect on the oxidation of pyrite, however, there may have been sufficient potassium in trace amounts in the mineral to satisfy the potassium requirements.

2. The Effect of Magnesium on the Biological Oxidation of Pyrite

In this investigation, nutrient 5 (containing ammonium sulfate as the nitrogen source) was used with concentration of magnesium sulfate as the only variable. The effect of the magnesium ion concentration is shown in Table XXI. Culture 2 was used as the inoculum. Five grams of pyrite 5 were used.

TABLE XXI

THE EFFECT OF MAGNESIUM SULFATE CONCENTRATION ON THE BACTERIAL OXIDATION OF PYRITE

Time Days	% Oxidation of Pyrite					
	0 g/l $MgSO_4 \cdot 7H_2O$	.1 g/l	.5 g/l	1 g/l	3 g/l	5 g/l
13	4.2	2.4	3.9	2.1	3.3	2.1

The results shown in Table XXI indicate that the concentration of magnesium ion in the nutrient solution has no significant effect on the oxidation of pyrite. Trace amounts which may be necessary for growth are evidently present as impurities in the minerals.

M. Alternate Nitrogen Sources in the Nutrient Solution

It was shown in the preceding section that urea could serve as the nitrogen source in the nutrient. The purpose of this study was to find out the effect of some other nitrogen containing materials on the biological oxidation of pyrite.

1. The Effect of Sodium Thiocyanate and Thiourea

Two compounds, sodium thiocyanate and thiourea, containing both nitrogen and sulfur were tried in this phase of the investigation. Small amounts were introduced into nutrient 2 (page 10) and the results observed. These results are shown in Table XXII. Both compounds acted as bactericides.

TABLE XXII

THE EFFECT OF ADDITION OF THIOUREA AND SODIUM THIOCYANATE TO NUTRIENT 2

	Cumulative % Oxidation of Pyrite			
Time Days	Sterile Control	Nutrient 4	Nutrient 2 plus .1 g/l thiourea	Nutrient 2 plus .1 g/l thiocyanate
18	---	---	---	0.40
25	---	---	---	0.65
32	---	---	---	0.90
34	0.2	24.0	0.6	---
90	0.4	68.0	0.8	---

2. The Effect of β-Alanine and L-Cystine as Sources of Nitrogen

The ammino acids, β-alanine and L-cystine, were tried as nitrogen sources in nutrient 4. Urea was eliminated. The results of this study are shown in Table XXIII and in Figure 10.

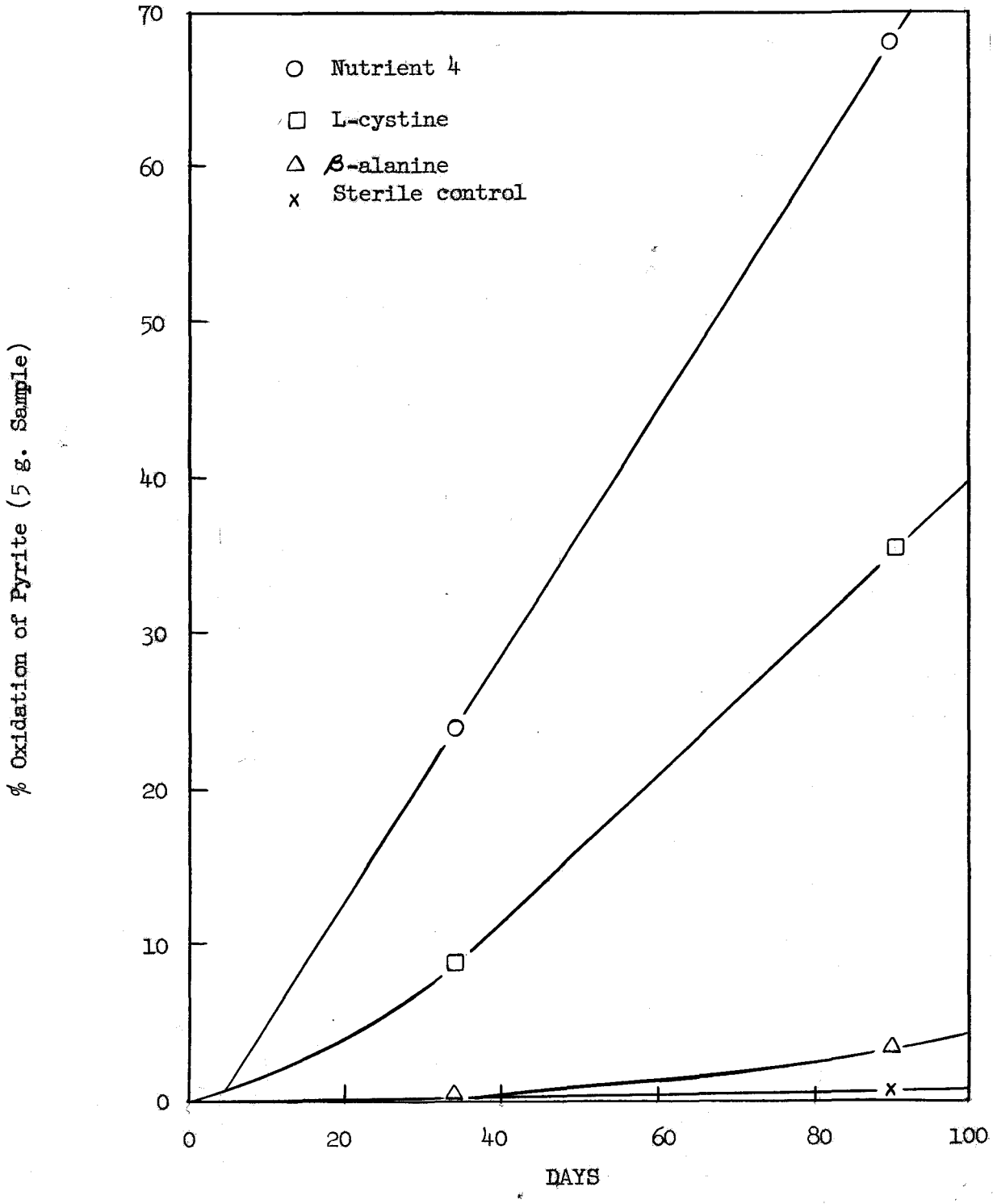

Fig. 10: The effect of substituting various nitrogen containing compounds into nutrient 4.

TABLE XXIII

THE EFFECT OF SUBSTITUTION OF VARIOUS NITROGEN CONTAINING COMPOUNDS INTO NUTRIENT 4

Time Days	Sterile Control	% Oxidation of Pyrite Nutrient 4	.1 g/l β- alanine	.06 g/l L-cystine
34	0.2	24.0	0.1	8.6
90	0.4	68.0	2.7	35.3

3. The Effect of the Nitrate Ion

The purpose of this study was to determine if the nitrate ion could be used as a source of nitrogen by culture 2 in the oxidation of pyrite. Varying quantities of sodium nitrate were introduced into nutrient 4. Urea was eliminated. The results of this study are shown in Table XXIV.

TABLE XXIV

THE EFFECT OF SUBSTITUTION OF $NaNO_3$ INTO NUTRIENT 4

Time Days	Sterile Control	Nutrient 4	% Oxidation of Pyrite Concentration of nitrate ion (ppm) 0	20	50	100	200	500
13	0.35	3.3	0.26	0.29	0.18	0.29	0.55	0.29

From the preceding investigations, several results may be observed. Ammonium sulfate, urea, and L-cystine contain nitrogen which supplies the requirements of culture 2; sodium nitrate and β-alanine do not serve as sources of nitrogen; while sodium thiosulfate and thiourea act as bactericides.

N. The Effect of Organic Materials on the Biological Oxidation of Pyrite

It has been shown previously that the chemosynthetic bacteria in the Bingham stream solution were capable of utilizing certain inorganic substances as their only requirements for growth.

The purpose of this study was to determine the effects of various organic compounds upon the oxidation of pyrite by culture 2.

Several representative organic compounds were utilized in this study. Glucose, sucrose, acetone, kerosene, and benzene were used as representative monosaccharide, disaccharide, ketone, and hydrocarbons respectively.

The results of this study are shown graphically in Figure 11. It may be observed from these data that kerosene, benzene, and acetone have a definitely inhibitory action while little or no change was observed in the oxidation rate in the presence of glucose or sucrose. The effect of benzene was particularly pronounced.

Glucose and sucrose were introduced into percolators in solution. Acetone was introduced in 1 ml. portions twice weekly (due to volatilization of the acetone, this study was only qualitative). Kerosene and benzene were introduced as a layer on top of the solution in the percolator to maintain a saturated solution.

A second study was undertaken to determine the effect of kerosene and benzene on the oxidation of pyrite. This was done using a modified apparatus. The apparatus used in this portion of the investigation consisted of a percolator in which a glass tube (20 mm. diam.), open on both ends, was supported and held in position by an "S" shaped glass tube (6 mm. diam.). This 6 mm tube was hooked inside of the 20 mm tube and over the top of the percolator. After the percolator was set up the immiscible liquid was put inside the 20 mm diameter tube. In this way the surface of the nutrient solution was only partially covered by the kerosene or benzene. The liquid rising in the air lift column of the percolator did not have to pass through the immiscible layer as previously. Air was bubbled through the 6 mm tube. Thus, mixing of the benzene or kerosene with the nutrient solution was enhanced.

The effect of kerosene on the oxidation was particularly desired

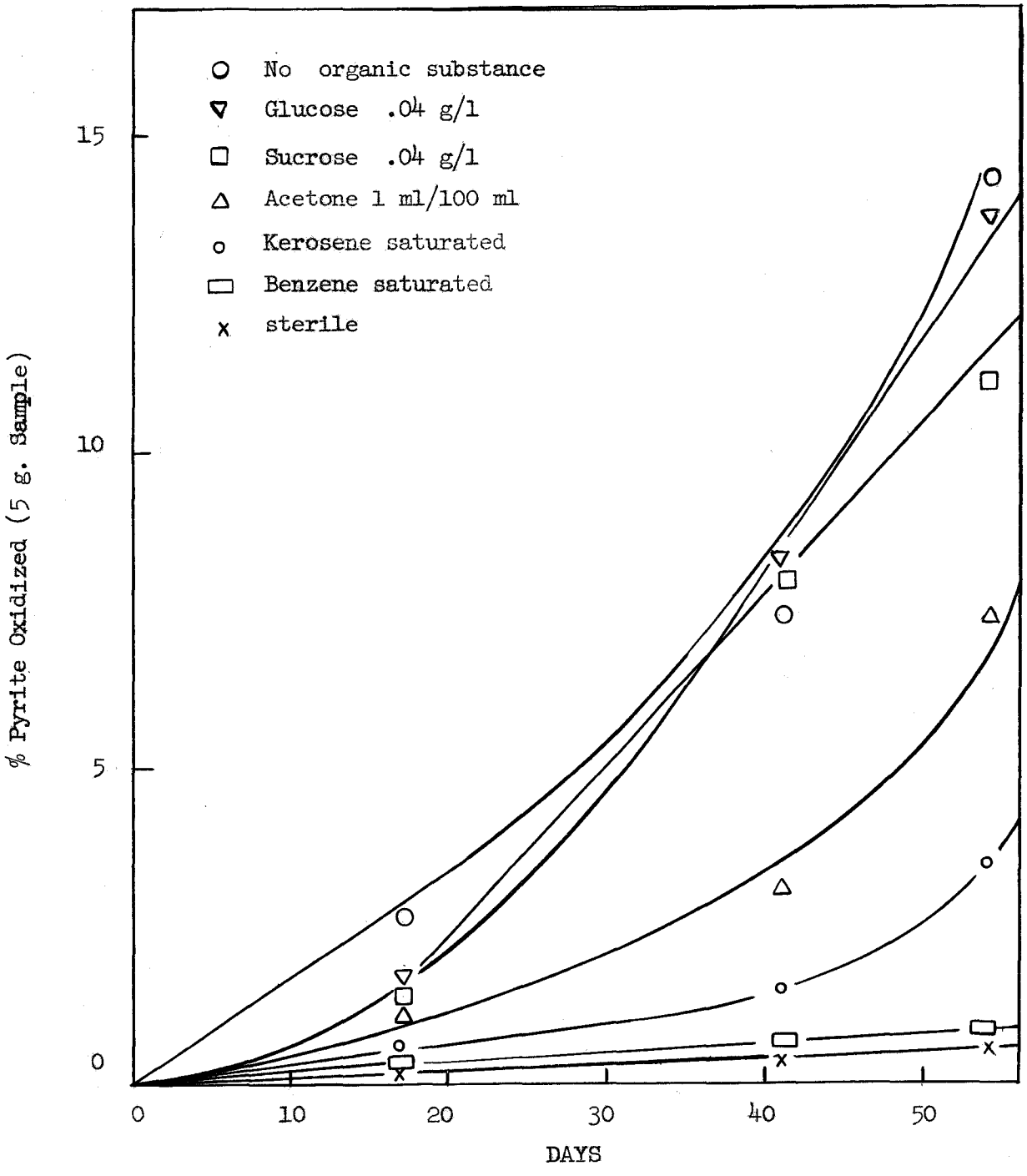

Fig. 11: The oxidation of pyrite in the presence of various organic compounds.

because many procedures for recovery of ions from solution depend upon liquid-liquid extraction using kerosene as the organic phase. At Bingham Canyon, large amounts of water are recycled to the dumps. It was desired to determine what effect small concentrations of kerosene (as would be present if liquid-liquid extraction measures were used) would have on the oxidation of dump materials.

The results obtained in this portion of the study are shown in Table XXV. They show that benzene definitely inhibits bacterial action on pyrite oxidation while kerosene has little effect when the surface of the liquid is only partially covered.

TABLE XXV

THE EFFECT OF KEROSENE AND BENZENE ON THE OXIDATION OF PYRITE UNDER CONTROLLED CONDITIONS

Time Days	% Oxidation of Pyrite			
	Sterile Control	No Organic Substance	Benzene Added	Kerosene Added
14	0.35	6.8	0.7	9.5

O. The Effect of Iron on the Inorganic Oxidation of Sulfide Minerals

Often in the past it was thought that ferric iron in an acid solution was responsible for the oxidation of various sulfide minerals in nature. It has been shown that biological oxidation is the primary cause of solubilization of pyrite.[44] The purpose of this study was to determine the effect of ferric iron on the inorganic oxidation of various sulfide minerals.

In order to obtain these data ferric sulfate in varying concentrations was used as the lixiviant. A normal percolator assembly was used with one exception; to eliminate the effect of oxygen, compressed nitrogen gas was used in the "air lifts".

[44] Wilson, op. cit., p. 18.

1. The Effect of Ferric Iron on the Inorganic Oxidation of Molybdenite

Varying concentrations of ferric iron at a pH of 1.8 to 2.0 were used in this study. The results are shown in Tables XXVI and XXVII.

TABLE XXVI

THE INORGANIC OXIDATION OF MOLYBDENITE BY FERRIC IRON

Time Days	Cumulative % Oxidation of Molybdenite					
	0 ppm ferric	1000 ppm	2000 ppm	3000 ppm	4000 ppm	5000 ppm
10	0.27	0.49	0.54	0.53	0.55	0.52
23	0.42	0.62	0.76	0.73	0.75	0.70
53	0.45	0.66	0.81	0.77	0.79	0.74

TABLE XXVII

THE REDUCTION OF FERRIC IRON BY MOLYBDENITE

Time Days	Cumulative ferrous iron formed (ppm)					
	0 ppm ferric	1000 ppm	2000ppm	3000ppm	4000 ppm	5000 ppm
10	0	28	33	40	38	39
23	0	54	62	72	68	66
53	0	89	103	108	100	97

The preceding results show that only very small amounts of molybdenite were oxidized by ferric iron and that no further oxidation of molybdenite was observed at ferric iron concentration greater than 2000 ppm.

2. The Effect of Ferric Iron on the Inorganic Oxidation of Chalcopyrite

The conditions used in this study were exactly the same as those of the previous study except chalcopyrite was used as the oxidizable substrate. Each sample was washed in the percolator to remove any oxidized materials. No observable copper was solubilized in this study. The results are shown in Table XXVIII.

TABLE XXVIII

CUMULATIVE SOLUBLE FERROUS IRON IN THE INORGANIC OXIDATION OF CHALCOPYRITE BY FERRIC IRON (TOTAL IRON IN PARENTHESIS)

Time Days	Cumulative ferrous iron (mg) solubilized by varying ferric iron concentrations: 0 ppm ferric	300 ppm ferric	450 ppm ferric	900 ppm ferric	1800 ppm ferric	3000 ppm ferric
9	22.3(27.8)	32.8(40.8)	59.8(65.2)	125(133)	208(256)	414(452)
26	26.8	44.4	108	219	421	718
35	31.3	64.4	139	304	640	932
48	43.0	103	212	462	910	---

It may be seen that some iron was solubilized in each percolator. Practically all of the ferric iron was reduced by the chalcopyrite but no soluble copper was found.

P. Biological Oxidation of Molybdenite in the Presence of Ferric Iron

It has previously been shown that both ferrous iron and iron pyrite materially aid the biological oxidation of molybdenite.[45] However, it was noted that ferrous iron was oxidized to ferric.

In this investigation, the concentration of ferric iron was the only variable. The results are shown in Figure 12 and in Table XXIX. Culture 2 was used as the inoculum.

TABLE XXIX

THE EFFECT OF FERRIC IRON CONCENTRATION ON THE BIOLOGICAL OXIDATION OF MOLYBDENITE AFTER 50 DAYS

Ferric Iron Concentration ppm	% Oxidation of Molybdenite
0	1.4
1000	2.6

[45] Anderson, op. cit., p. 28.

TABLE XXIX-Continued

THE EFFECT OF FERRIC IRON CONCENTRATION ON THE BIOLOGICAL OXIDATION OF MOLYBDENITE AFTER 50 DAYS

Ferric Iron Concentration ppm	% Oxidation of Molybdenite
2000	3.4
3000	4.2
4000	5.1
5000	4.6

It may be observed from Figure 12 that a decided optimum in the molybdenite oxidation occurs at a ferric iron concentration of 4000 ppm. This is in agreement with previous results on ferrous iron.[46]

[46] L.C. Bryner and Ralph Anderson, unpublished data.

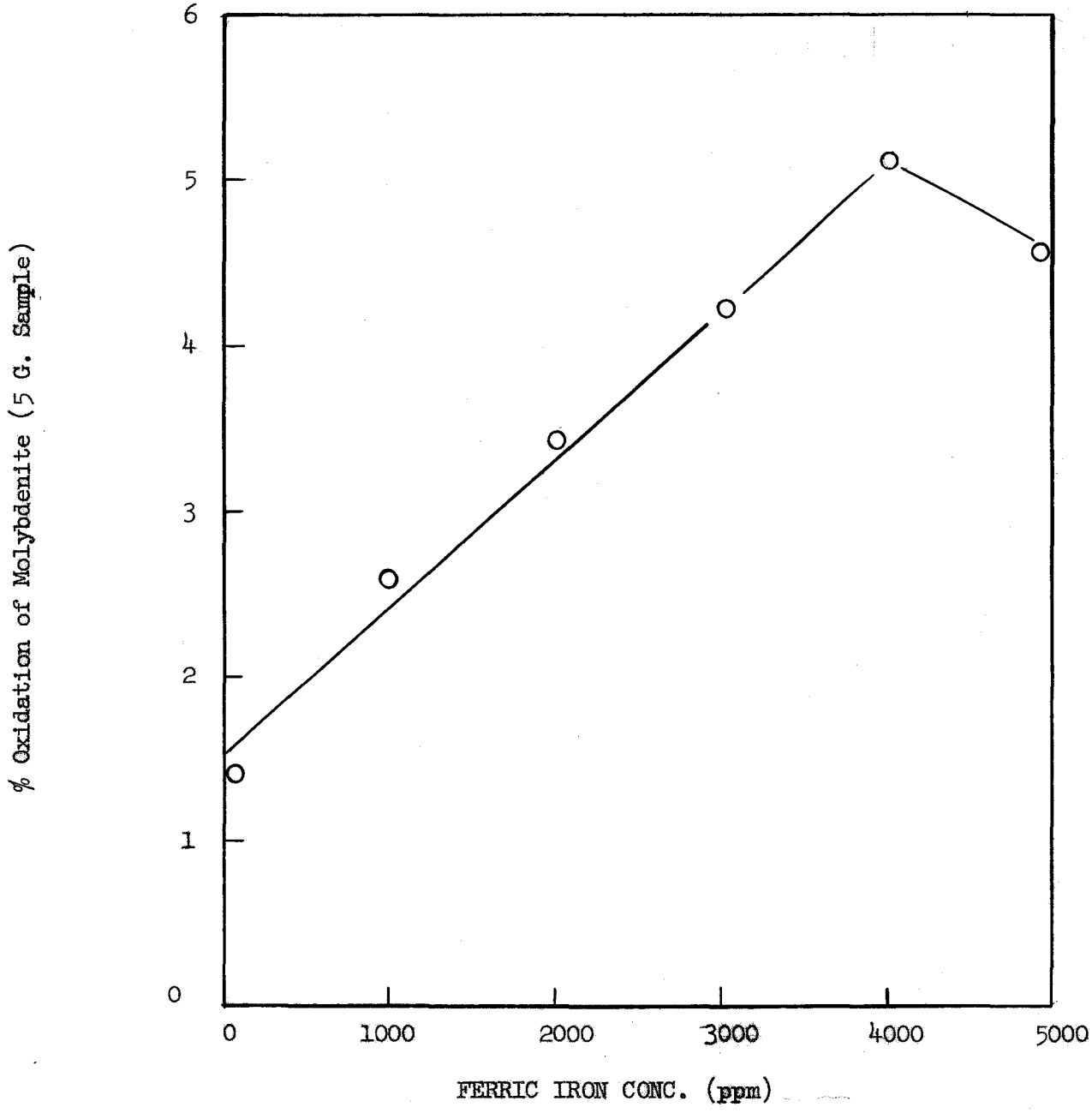

Fig. 12: The effect of ferric iron concentration on the biological oxidation of molybdenite

CHAPTER V

SUMMARY AND CONCLUSIONS

In the leaching streams from exposed ore bodies in Bingham Canyon, large amounts of soluble iron and copper are found. It was previously shown that this oxidation was biological. The primary result from this series of investigations was the isolation of the chemosynthetic bacterium responsible for this oxidation. Two methods were employed in the isolation study. These were the enrichment dilution technique, and silica gel as a solid medium.

The isolated bacteria were compared with similar organisms described in the literature. They were found to correspond most closely to the genus Thiobacillus. Only two organisms from this group tolerate the same high acid concentration (pH = 2.0 - 3.5) exhibited by the bacteria isolated in this investigation. They were T. thiooxidans and T. ferrooxidans. Differences were observed in the physiological properties exhibited by the various organisms. T. thiooxidans oxidizes sulfur but not pyrite or ferrous iron. T. ferrooxidans oxidizes pyrite and ferrous iron in acid solution but not free sulfur. One bacterium isolated in this study oxidized ferrous iron, sulfur, and pyrite as well as other sulfide minerals. Thus, this organism exhibited somewhat different characteristics than either T. thiooxidans or T. ferrooxidans, although nearly the same as T. ferrooxidans.

A second organism was isolated on sulfur and exhibited characteristics very similar to those of T. thiooxidans.

An investigation was made to determine the optimum nutrient material for oxidation of pyrite by culture 4. It was found that a source of nitrogen and phosphate were the only materials which must be included in the nutrient.

Impurities in the sulfide minerals and chemicals used evidently provided sufficient trace elements for growth.

The use of urea as a source of nitrogen was studied. It was found that the optimum concentration of urea was in the vicinity of 0.05 g/l. This is decidedly lower in nitrogen content than the previously used ammonium sulfate concentration of 1.0 g/l, indicating that urea is the better nitrogen source.

The effect of various other nitrogen compounds on the oxidation of pyrite was also determined. It was found that thiocyanate and thiourea are toxic and that nitrate and β-alanine are not utilized as nitrogen sources, but that L-cystine is.

It was found that these bacteria could be stored for long periods of time in sealed tubes on a moist mixture of the mineral and Ottawa sand.

The effect of various organic substances on the oxidation of pyrite was observed. It was found that glucose and sucrose had little effect and that acetone showed a moderately inhibitory action. If the surface of the nutrient solution was not completely covered, the addition of kerosene produced little change in the oxidation of pyrite. Benzene under all conditions inhibited the biological oxidation.

The possibility of the inorganic oxidation of sulfide minerals by ferric iron was studied. Little if any oxidation was observed on molybdenite. Nearly all ferric iron present was reduced to the ferrous state in chalcopyrite. Iron was solubilized from the chalcopyrite but copper was not. An optimum of 4000 ppm ferric iron was exhibited in the biological oxidation of molybdenite.

A process similar to that in the waste dumps at Bingham Canyon has

been observed at Cananea, Sonora, Mexico. The stream solution from this source exhibited the same general characteristics as those from Bingham Canyon. Oxidation of pyrite, chalcopyrite, and molybdenite was observed in very nearly the same amounts. Thus, it may be concluded that the biological oxidation of sulfide minerals is not unique to any one area but occurs wherever conditions are favorable.

LITERATURE CITED

Anderson, Ralph, "Oxidation of Molybdenite with the Aid of Micro-organisms" (unpublished Master's thesis, Brigham Young University, 1956).

"Bergey's Manual of Determinative Bacteriology," 6th ed., Baillière, Tindall, and Cox, London, 1948.

Bryner, L.C., et. al., Ind. Eng. Chem., 46, 2587(1954).

Clifton, C. E., "Introduction to the Bacteria," McGraw-Hill Book Company, Inc., New York, N. Y., 1950.

Davis, D. B., "Biological Oxidation of Copper Sulfide Minerals" (unpublished Master's thesis, Brigham Young University, 1953).

Fred and Waksman, "Laboratory Manual of General Microbiology," McGraw-Hill Book Company, Inc., New York, N. Y., 1928.

Kingsbury, J. M., and Barghoorn, E. S., Appl. Microbiol., 2, 5(1954).

Kolthoff, I.M., and Sandell, E. B., "Textbook of Quantitative Inorganic Analysis," 3rd ed., The MacMillan Company, New York, N. Y., 1952, p. 579.

Leathen, W. W., and Braley, S. A., J. Bact., 69, 481 (1955).

Leathen, W.W., McIntyre, L. D., Braley, S. A., Science, 114, 280 (1951).

Temple, K. L., and Colmer, A. R., J. Bact., 62, 605(1951).

Temple, K. L., and Delchamps, E. W., Appl. Microbiol., 1, 255(1953).

Waksman, S.A., and Joffe, J.S., J. Bact., 7, 239(1922).

Weed, R.C., Mining Eng., 8, 721(1956).

Wilson, D. G., "Studies on the Biological Oxidation of Iron Pyrite" (unpublished Master's thesis, Brigham Young University, 1952).

ABSTRACT OF THESIS

CHEMOSYNTHETIC MICROORGANISMS IN LEACHING SULFIDE MINERALS

This investigation showed that a single bacterium was capable of sulfide oxidation in exposed ore bodies. This single bacterium was isolated and characterized. It exhibited different characteristics than any previously known organism. It was found to be a chemosynthetic autotroph.

An optimum nutrient concentration was determined for pyrite oxidation. The nutrient concentration was determined for pyrite oxidation. The nutrient contains only a source of phosphate and nitrogen. Urea was found to be a better source of nitrogen than ammonium sulfate which had previously been used.

The effect of various organic compounds on the oxidation of pyrite was observed. Glucose and sucrose showed no affect. Acetone slightly inhibited the oxidation. Benzene almost completely stopped the reaction. Kerosene showed no effect if the surface of the nutrient solution was not covered completely.

It was found that a bacterium of the same type as that isolated in this investigation is responsible for sulfide oxidation at a location in Mexico. Thus, the oxidation of sulfides by bacteria is not a unique occurrence but is believed to occur wherever proper conditions are present.