PN-AAZ-810

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AP. 5045
LIMA – PERU

January 28, 1985.

ACTION COPY

Miss Linda Lou Kelley
Agency for International Development
Av. España 386
Lima.-

Dear Miss Kelley:

Please find enclosed the First Interim Report: Period covered September to December 1984.

Sincerely.

Dr. José Luis Bauer
Professor of Microbiology
Department.

ACTION: HNE
Info: DC
CO
DC

JLB/msp.

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4.6.7

FIRST INTERIM PROGRESS REPORT

Project Nº 364 - 1486

"NATURALLY OCCURING PLASMIDS AND HEAVY RESISTANCE OF Thiobacillus ferrooxidans

This report covers the period between September to December 1984.
In agreement to the established in the project implementation and responsibilities, the following are the advances:
In first place the Laboratory is being implemented and the laboratory equipment and expendable supplies are being adquired through VWR Company.
In relation to the Technical Consultation the second investigator from Universidad Peruana Cayetano Heredia, Jasmin Hurtado is hosted from October by Dr. Anne Summers of the University of Georgia for a period of 4 months, consulting on certain technical and implementation aspects of the research activity (Genetic aspects), and later she will be two months at Ohio State University with Dr. Olli H. Tuovinen, consulting on physiological aspects of the microorganisms involved in the leaching process.
We are now working in the standarization of the techniques for the isolation, purification and characterization of T. ferrooxidans strains.
In this moment, we have five isolates, from mine waters.

1. Cerro Verde - Arequipa
2. Santa Rosa - Arequipa
3. Coricancha - Lima
4. Aurelio - Lima
5. Paracoha - Cerro de Pasco

The microorganisms isolated are authotrophics, Gram negative rods, able to produce oxidation of the ferrous iron, but in order to confirm if the microorganisms are T. ferrooxidans, we should investigated for example, the molecular per cent of Guanine + cytocine

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The problems that we have are the following:

A- The authotrophics microorganisms are susceptible to suffer inhibition of their growth from organic compounds. The agar agar must be purified if we want to use it like a solidifying agent. On the other hand, the metabolic products, like organic acids, that they produce during their growth, cause an inhibition of growth when these acids are accumulated. We are in the process investigating this problem now.

B- The apparition of precipitate that occurs during ferrous iron oxidation, produces interference with the turbidimetric measurement of the cultures, and with the protein dosage.
Using amberlite IR-120 cation exchange resin we are solving this problem.

C-The use of the conventional cultures media for T. ferrooxidans produce poor cultures. It is necessary to improve these formulation in order to obtain better cultures.

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Second Interim Progress Report

Project N° 304-1486

"NATURALLY OCCURRING PLASMIDS AND HEAVY METAL RESISTANCE OF THIOBACILLUS FERROOXIDANS"

This report cover the period between January to June 1985.
The advances are the following:
The laboratory is already implemented, and the laboratory equipment is operating.
In relation to the Technical Consultation, Miss Jasmin Hurtado, M. Sc. carried out during October 6 to March 8 at University of Georgia, Anne O. Summers's Laboratory the following experiments:

1.- Transposition of Tn5 (E. coli pmj 5000: Tn5) to Thiobacillus versatus

2.- Improvement recovery of T. versatus Tn5 mutants.
4.- Characterization of T. versatus Tn5 mutants
5.- Plasmid screen in parentals and T5 mutants strains
6.- Plasmid screen in metal resistant strains. Use of restriction enzymes.
7.- Transformation. Cloning Vectors
8.- Hybridization

Experiments performed during March 8 to May 8 at Ohio State University at Olli H. Tuovinen's Laboratory.

1.- Carboys culture of T. ferrooxidans
2.- Protein determination of T. ferrooxidans
3.- Stimulation/inhibition of sulfur, and sulfur compounds on ferrous oxidation by T. ferrooxidans: clark electrode (oxygen consumption) and ferrous oxidation.

In relation to the techniques to be used in the project, the oxido-reduction and complexemetric techniques in order to determinate Fe^{++}, Fe^{+++} and total Fe are standarizated.

Rec'd in SCI: AUG 8 1985

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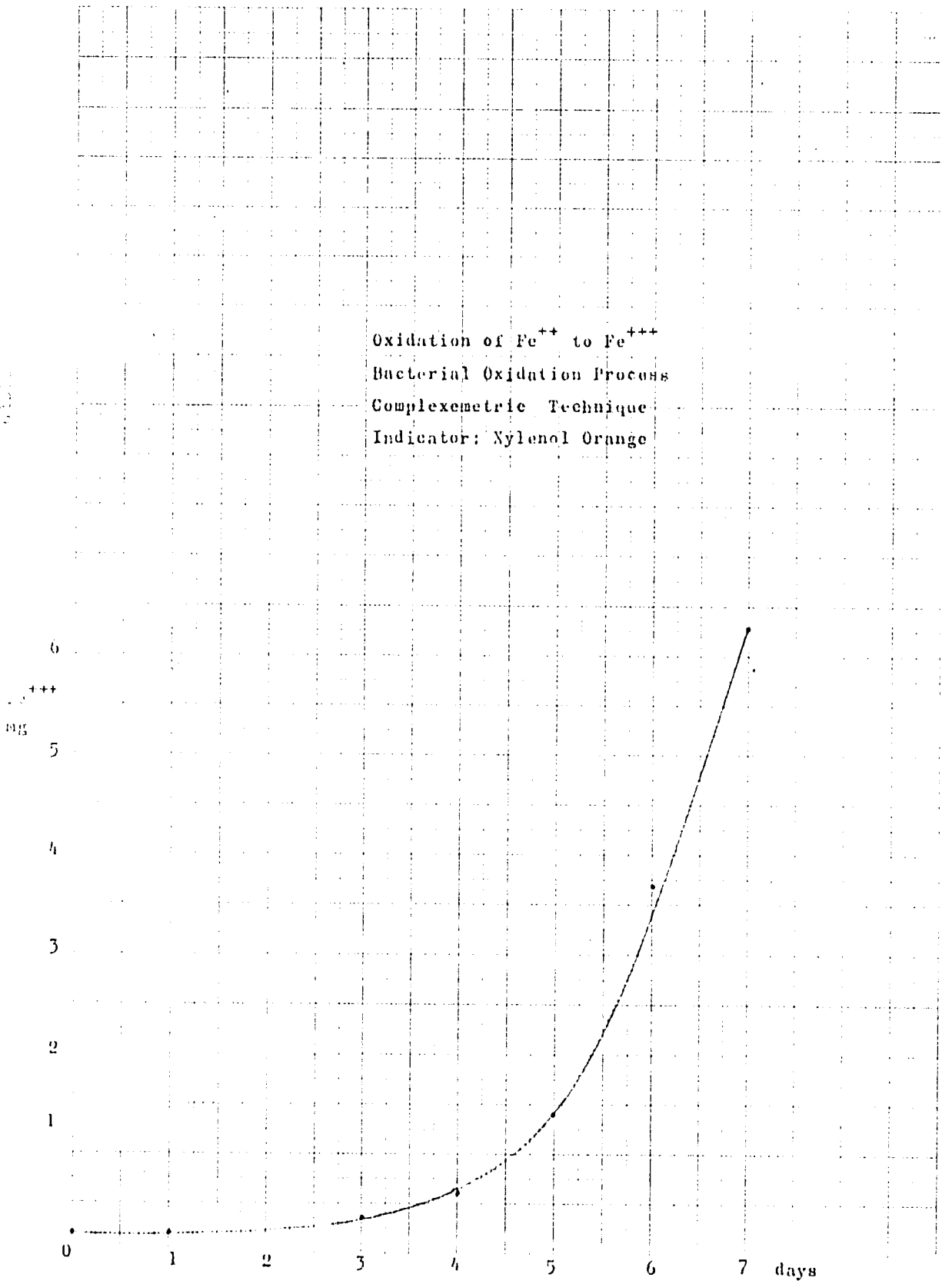
These techniques are used in order to carry out the quantification of iron in the kinetic studies of the chemical and bacterial oxidation of Fe^{++} to Fe^{+++}.

When we make the ferrous determination using oxido-reduction technique with $KMnO_4$ like valoraizer, we find that this technique doesn't have the sensibility that we need when the iron concentration is low (< 10 mg/ml). On the other hand when we use the complexometric technique with EDTA-Na like valoraizer and salicilic acid or xylenol orange like indicator, we find a very good sensibility, working at low concentration of iron.


Oxidation of Fe++ to Fe+++
Bacterial Oxidation Process
Complexemetric Technique
Indicator: Xylenol Orange
+++
mg
6
5
4
3
2
1
0
1
2
3
4
5
6
7
days

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Fe^{+++} Determination

Complexometric Technique

Valoraizer: EDTA-Na Indicater: Xylenol Orange

A blue complexo of Fe^{+++} - Xylenol Orange is formated and by titulation with EDTA-Na Solution cause a change of the blue color and a yellow color appear.

In order to determinate total Fe, is necessary to oxidize the sampl with 3% H_2O_2 and make the total Fe valuation like Fe^{+++}.

Oxidation of Fe^{++} to Fe^{+++}

Bacterial Oxidation Process

Complexometric Technique

Kelly medium

Time days	EDTA (ml.) Exhausted volume	Fe^{+++} mg.
0	0.3	0.1680696
1	0.35	0.1694701
3	0.6	0.3641508
4	1.0	0.6022494
5	2.5	1.4005800
6	6.6	3.7253248
7	11.2	6.2745984

In relation to the sample colection, we have samples from Arequipa, Lima and Cerro de Pasco, and we are working with the microorganismos isolated from these mine waters.
The growth on solid media is solving with the use of purified agar, and now we can obtain isolated colonies of our strains.

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The apparition of precipitate in our liquid media was solving using the Ferrous Iron Medium (Tuovinen-Kelly, 1973) which has the following formulation.

Mineral Salts Solution:

K_2HPO_4	0.5 g.
$(NH_4)_2SO_4$	0.5 g.
$MgSO_4 \cdot 7H_2O$	0.5 g.
H_2SO_4 0.1 N	1000 ml.

(sterilize by autoclaving)

Ferrous Iron Solution (Per liter)

$FeSO_4 \cdot 7H_2O$	167 g.
H_2SO_4 1 N	50 ml.

(sterilize by filtration).

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Ferrous Iron Medium:

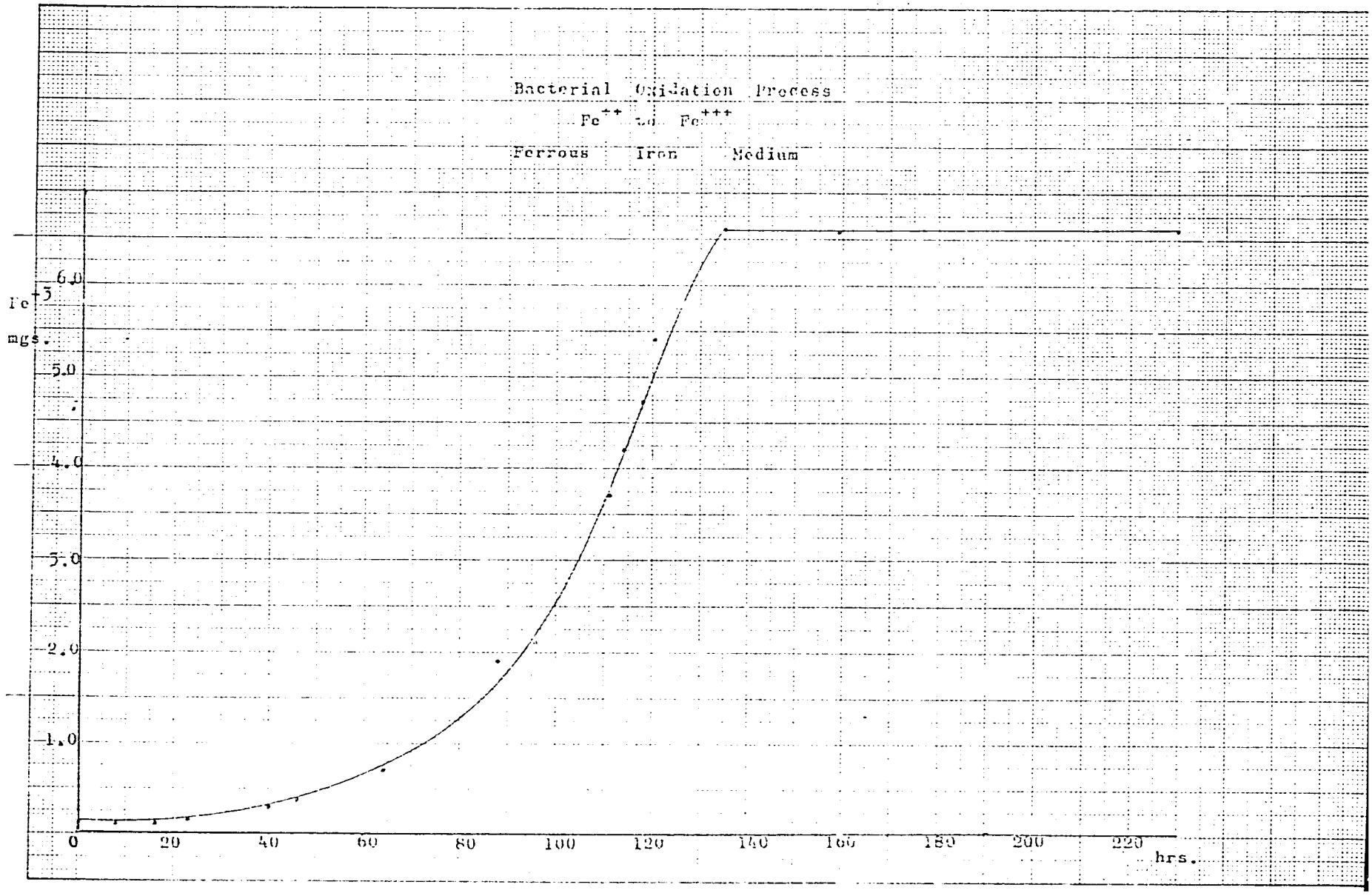
Mix 1 part ferrous iron solution to 4 parts mineral salts solution. The pH of the medium is 1.6.

Bacterial Oxidation of Fe^{++}
Complexometric Technique
Ferrous Iron Medium

Time Hours	EDTA (ml) Exhausted Volume		Fe^{+++} mg
0	0.1,	0.2	0.1052722
8	0.2,	0.2	0.1052722
16	0.2,	0.2	0.1052722
23	0.3,	0.3	0.1579084
40	0.5,	0.5	0.263157
46	0.6,	0.7	0.3421349
64	1.25,	1.25	0.6579517
88	3.5,	3.7	1.894901
96	4.0,	4.0	2.1054456
111	7.0,	7.1	3.7108478
114	8.0,	8.0	4.2108912
117.5	9.1,	9.1	4.7898887
120.5	10.2,	10.2	5.3688862
135.0	12.65		6.6584717
159.0	12.65		6.6584717
231.0	12.65		6.6584717

Total Ferrous Determination
Complexometric Technic

Medium	EDTA (ml) Exhausted Volume	Fe^{+++} mg
Ferrous Iron Medium	12.65	6.67161630


Bacterial Oxidation Process
Fe^{++} to Fe^{+++}
Ferrous Iron Medium
Fe^{+3}
mgs.
6.0
5.0
4.0
3.0
2.0
1.0
0
20
40
60
80
100
120
140
160
180
200
220
hrs.

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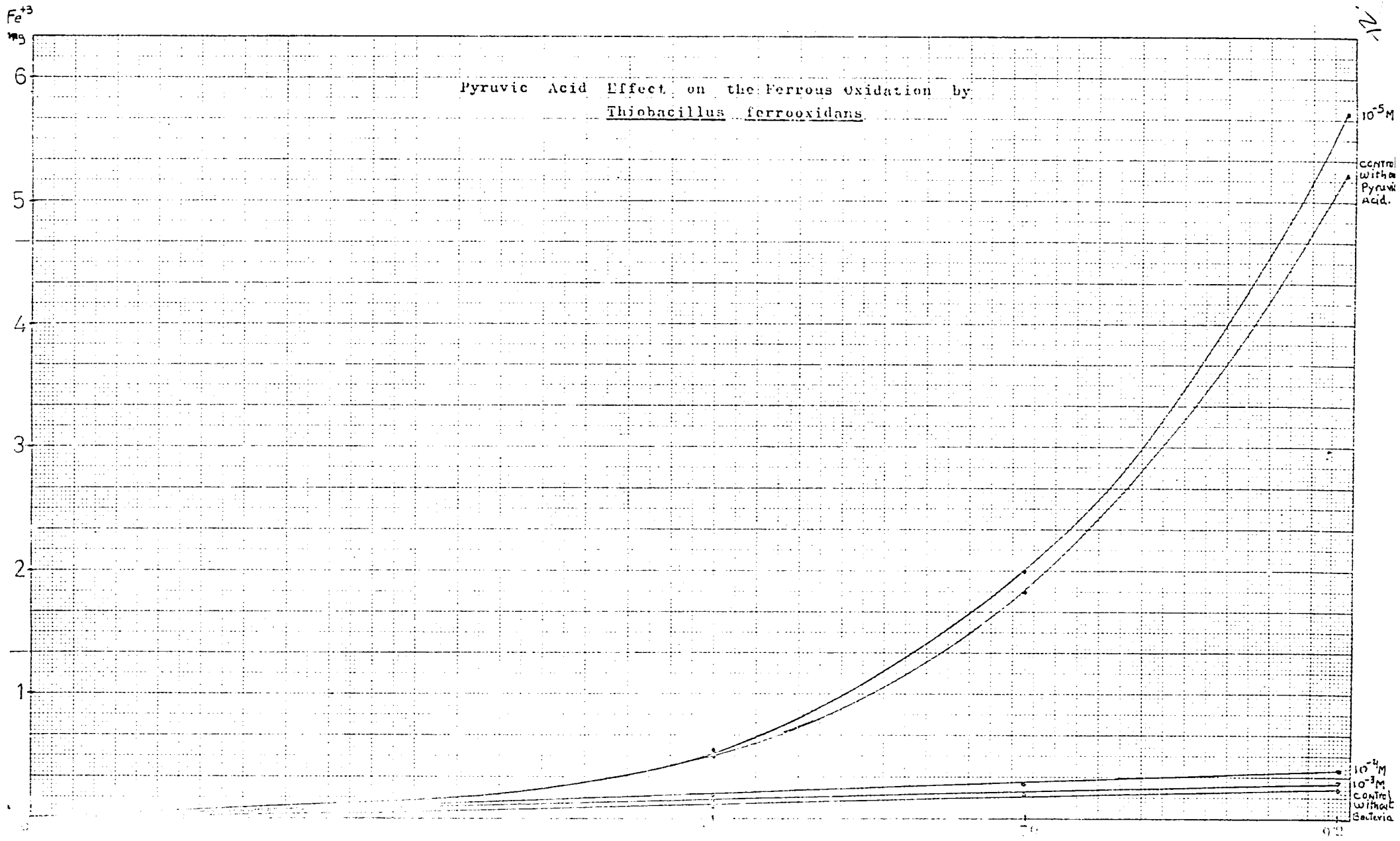
In order to investigate the inhibitory effect of the bacterial growth, from organic compounds, we are investigating the effect of the pyruvic acid on the Ferrous Oxidation by Thiobacillus ferrooxidans.
The sodium pyruvate concentration used were the following 10^{-3}M, 10^{-4}M, 10^{-5}M. We find that sodium pyruvate 10^{-3}M and 10^{-4}M, produce inhibition of the bacterial ferrous oxidation, but when we use sodium pyruvate 10^{-5}M the effect to seen stimulating.

Pyruvic Acid Effect on the Ferrous Oxidation by Thiobacillus ferrooxidans.-

Culture with Sodium Pyruvate	Fe^{+3} mg/ml 22 hrs.	Fe^{+3} mg/ml 48 hrs.	Fe^{+3} mg/ml 70 hrs.	Fe^{+3} mg/ml 92 hrs.
10^{-3}	0.092	0.131	0.189	0.294
10^{-4}	0.105	0.184	0.255	0.381
10^{-5}	0.118	0.523	1.946	5.76
Control without Pyruvate	0.118	0.504	1.87	5.22
Control without bacteria	0.071	0.105	0.226	0.273

Bacterial Growth Control.-

Culture	Bacterial Number Initial	Bacterial Number Final (92hrs.)
Without Sodium Pyruvate	1×10^5 bact./ml.	9×10^7 bact./ml.
With Sodium Pyruvate 10^{-5}M	1×10^5 bact./ml.	1.08×10^8 bact./ml.



Pyruvic Acid Effect on the Ferrous Oxidation by
Thiobacillus ferrooxidans

XN - AAZ-810-B

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THIRD INTERIM PROGRESS REPORT

Project Nº 364-1486

"NATURALLY OCCURRING PLASMIDS AND HEAVY METAL RESISTANCE OF THIOBACILLUS FERROOXIDANS

Principal Investigator :

DR. JOSE L. BAUER

Rec'd in SCI: FEB 12 1986

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This report cover the period between July 1985 to December 1985. The advances are the following:

1) Studies of the physiology and genetics of the isolated strains of Thiobacillus ferrooxidans.

To confirm the purity of T. ferrooxidans strains it was neccesary to grow these strains in solid media, and then inoculate one colony in liquid medium. This procedure needed to be repeated at least three times. After third passage in liquid media, these strains were completely purified through resistance to silver. Theyware grown in Tuovinen-Kelley Medium with increasing concentrations of silver nitrate until they can not oxidize ferrous iron. After we search for acidophiles presence in cultures of T. ferrooxidans: T. acidophilus and Acidophilum cryptum (the pattern strains were a generous gift of Dr. Harrison, University of Missouri). The media used for the isolation of A. cryptum contains the following (% w/v): 0.2 $(NH4)_2SO_4$, 0.01 KCl, 0.025 K_2HPO_4, 0.025 $MgSO_4$ $7H_2O$, 0. 0.1 Ca $(NO_3)_2$, 0.1 % glucose and 0.01% yeast extract. T. acidophilus was looked for using Basal 9 K medium of silver man and Lundgren with 1% glucose and 10 p.p.m. of ferrous sulfate.

With the techniques used we have demostrated that our cultures are purified (strains CH5, CH6 and CH18).

We used these strains for the following experiments.

A) Utilization of Sulfur and Thiosulfate as energy source

T. ferrooxidans strains CH5, CH6, CH18 were inoculated in sulfur and thiosulfate media.

Sulfur medium were prepared by adding 0.5% powdered - sulfur (W/v) to basal media adjusted to pH 4.2. 001% thymol blue was used as pH indicator.

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Growth of the bacteria in sulfur and thiosulfate media were detected by microscopic assay and verified by pH measurement. The sulfuric acid formed from the oxidation of sulfur and thiosulfate caused lowering of pH concomitent with growth.
The results of growth in thiosulfate and sulfur are in the Table 1.

Energy Source	pH	STRAIN CH5	CH6	CH18
Sulfur	initial	4.2	4.2	4.2
	final	2.1	2.2	2.1
Thiosulfate	initial	4.3	4.3	4.3
	final	5.0	2.1	2.1

TABLE 1. Growth of *T. ferrooxidans* in Thiosulfate and Sulfur.

B) Organic Compounds Inhibition

Organic compounds inhibition were assesed by examining growth and oxidation of ferrous sulfate in Tuovinen-Kelley (T-K) medium with different concentrations of sodium piruvate, sodium citrate, lactose and glucose.
The profile of inhibition in *T. ferrooxidans* strains CH5, CH6 and CH18 are in the table 2.

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Organic Compound	Concentration (M)	STRAIN CH5	CH6	CH18
Sodium	1×10^{-4}	ND	ND	15 d
	1.5×10^{-4}	15d	15d	NG
	1×10^{-3}	NG	NG	NG
Sodium Citrate	1×10^{-3}	0d	0d	0d
	1×10^{-2}	2 d	0d	0d
	1×10^{-1}	NG	NG	NG
Lactose	1×10^{-5}	0d	ND	0d
	1×10^{-4}	2d	ND	0d
	1×10^{-3}	NG	ND	NG
Glucose	1×10^{-3}	0d	0d	0d
	1×10^{-2}	0d	0d	0d
	5×10^{-2}	0d	0d	0d

TABLE 2: Effect of organic compounds on growth and oxidation of ferrous sulfate. ND: No data, ; NG : no growth ; Nº d: Number of days of lag period.

C) Metal Resistance

Metal resistance were assesed by examining growth and oxidation of ferrous sulfate in TK medium with a range of metal concentrations. The metals used were Antimony (Sb) as -- $C_4H_4KO_7Sb$, Arsenic (As) as Na_3AsO_4, Cadmium (Cd) as $CdCl_2$. H_2O, Cobalt (Co) as $Co\ Cl_2$. H_2O, Copper (Cu) as SO_4Cu. $5H_2O$, Chromium (Cr) as $(NO_3)_3\ Cr$. $9H_2O$, Tin (Sn) as Cl_2Sn. $2H_2O$ Mercury (Hg) as $HgCl_2$, Nickel (ni) as SO_4Ni. $6H_2O$, Selenium (Se) as Se, Tellurium (Te) as K_2teO_3, zinc (Zn) as $ZnSO_4$.

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$7H_2O$ and Molybdenum (Mo) as $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$.

The profiles of metal resistance in T. ferrooxidans strains CH5, CH6 and CH18 are in Table 3.

D) Nitrogen compounds utilization

It was assesed utilization of urea, aminoacids and no nitrogen source in TK medium by examining growth and ferrous iron oxidation.

The results of these experiments in T. ferrooxidans strains CH5, CH6 and CH18 are in Table 4, 5 and 6.

metalic ion \ Strain	CH5	CH6	CH18
Ni	5×10^{-1}M	5×10^{-1}M	5×10^{-1}M
Cu	1×10^{-1}M	1×10^{-1}M	1×10^{-1}M
Zn	5×10^{-1}M	5×10^{-1}M	5×10^{-1}M
Cd	3×10^{-2}M	1×10^{-2}M	3×10^{-2}M
As	7×10^{-2}M	7×10^{-2}M	1×10^{-1}M
Hg	7×10^{-7}M	7×10^{-7}M	7×10^{-7}M
Mo	1×10^{-5}M	1×10^{-5}M	1×10^{-5}M
Cr	1×10^{-3}M	1×10^{-3}M	1×10^{-3}M
Sn	1×10^{-3}M	1×10^{-3}M	1×10^{-3}M
Co	1×10^{-2}M	1×10^{-2}M	1×10^{-2}M

Table 3: Metal resistance of T. ferrooxidans strains CH5, CH6 and CH18.

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Concentration (bact/ml) \ Strain	CH18			CH6			CH5		
	TK	U	-N	TK	U	-N	TK	U	-N
Initial	1×10^6	1×10^6	1×10^6	2×10^6	2×10^6	2×10^6	3×10^6	2×10^6	2×10^6
Final	2×10^8	2×10^8	2×10^7	2×10^8	2×10^8	2×10^7	2×10^8	2×10^8	2×10^7

Table 4: Effect of growth and oxidation of ferrous sulfate using Amonium Sulfate (TK medium), Urea (U) and no nitrogen source (-N).

Concentration (bact/ml)	9K Medium	A	B
Initial	5×10^5	5×10^5	5×10^5
Final	3×10^7	5×10^7	9×10^5

Table 5: Effect of growth and oxidation of ferrous sulfate using two aminoacids pools A: Leucine, tirosine, aspartate and serine and B: Cisteyne, Isoleucine, triptophane and glutanic acid in CH18.

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Concentration (bact/ml)	CH5		CH6		CH18	
	TK	A	TK	A	TK	A
Initial	2×10^6	2×10^6	2×10^6	3×10^6	1×10^6	2×10^6
Final	2×10^8	3×10^8	2×10^8	3×10^8	2×10^8	3×10^8

Table 6: Effect of growth and oxidation of ferrous sulfate using pool A in strains CH18, CH5 and CH6.

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E) Plasmid Profile

For plasmid isolations, batch cultures were grown in 1 liter flask. The bacteria were harvested and the pellet was washed in acidic water. The resulting pellet were resuspended in TES buffer (50mM Tris, 2mM EDTA, 25% sucrose, pH 8.0) to induce Sphecoplast formation. Lysis of the cells was accomplished by adding 15% SDS and Proteinase K (2 mg ml^{-1}).
Sodium chloride 5M was added to a final concentration of 1 M and was held at 4ºC overnight to precipitate chromosomal DNA and SDS protein complexes. Centrifugation at 35,000 xg for 40 min. at 4ºC precipitated the cellular debris. The supernatant containing various plasmid formswere precipitated with 2.5 volumes of 95% ethanol and glucogen (1 mg ml^{-1}). This mixture was held at -20ºC overnight to aid in the precipitation of the nucleic acids followed by centrifugation. The pellet was resuspended in TE buffer and stop mix.
The covalentely closed circular forms of plasmid were resolved by 0.7% agarose gel electrophoresis (4h 30mA) in TEA buffer (40mM Tris, 2 mM EDTA, 20mM Sodium acetate, pH 8.05). Plasmids of known molecular weights were used as standards. The sizes of plasmids of CH5, CH6 and CH18 are indicated in Table 7.

Strain	Plasmid designation	Plasmid Size(x 10^{-6})
CH5	pCH - 51	22
CH6	pCH - 52	6.5
	pCH - 61	22
CH18	pCH - 181	22
	pCH - 182	6.5

Table 7: Plasmid profile in T. ferrooxidans strains CH5, CH6 and CH18.

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F) Guanine plus citosine content of the DNA and electrophoretic whole -cell protein patterns.

There were some problems using these two techniques that we are trying to solve.
In G-C content we were utilizing some reagents with impurities and low-cell concentration. In Sodium Dodecyl Sulfate-Polyacrilamide Gel Electrophoresis (SDS-Page) we used a low cell concentration also.
We have recently received T. ferrooxidans strains we can use as standards of G-C content and SDS-Page from Dr. A. Harrison, University of Missouri.

CONCLUSIONS

Our results indicate that there is only one difference between T. ferrooxidans strains CH5, CH6, CH18 and is thiosulfate utilization. We are trying differents pH and thiosulfate concentrations to demonstrate it. If that is true thiosulfate utilization would be the first genetic marker.
We will study growth and ferrous oxidation with anotheraminoacids and the posible utilization of organic compounds.
Restriction endonucleases will be used in order to characterize the plasmids isolated.

2) Collection of mine water samples. Isolation and purification of T. ferrooxidans strains.

We began to visit mining zones. Three mine water samples were collected and they were proceed to isolate T. ferrooxidans by culture in TK medium.

The results are in Table 8.
We are purifying two isolations of Koricancha mine. Kor strain is from mine water and Kor -S strain is from sediments of mine. Kor-S has better growth in solid and liquid media.

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Mine	Zone	Mine water pH	Mine water T°	Mine water O_2 (mg/ml)	Mine water Metals	*T. ferrooxidans* isolation
Koricancha	Junín	4	25° C	4.5	Sb Zn Co Fe S	Yes
El Baron	Lima	7	25° C	4.4	Fe Sb S	No
Perú Bar	Lima	9	25° C	5.1	No	No

Table 8 : Characterization of mine water sample.