ORE UTILIZATION BY THIOBACILLUS FERROOXIDANS

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by: Barbara Thorsen, MDH, April, 1978

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Method of Analysis and Results.

Three samples of crushed rock were received, designated Erie Unmineralized, AX 9002, and DP 9002. They were assigned chemistry numbers 70551, 70552, and 70559. These samples were tested to determine the effect of the mineral bacteria Thiobacillus ferooxidans upon them. The T. ferrooxidans was obtained from Dr. H.M. Tsuchiya at the University of Minnesota. The growth medium used was called 9-K Medium composed of the following ingredients:

BASAL SALTS		ENERGY SOURCE	
$(NH_4)_2SO_4$	3 gms	$FeSO_4 \cdot 7H_2O$	44.2 gms
$K_2H\ PO_4$	.5 gms	Tap Water	300 mls
$MgSO_4 \cdot 7H_2O$	.5 gms		
K Cl	.1 gm		
$Ca(NO_3)_2$	.01 gm		
Tap Water	700 ml		

This media was autoclaved in two solutions and combined prior to use using 70 ml basal salts to 30 ml energy source. Erlenmeyer flasks, 250 mls, were used as containers, and placed on a shaker at 20°C in a constant temperature room. The culture of T. ferrooxidans was transferred twice before the test started. The pH of these cultures was initially 2.80 and over ten days dropped to 2.2. Slides were able to demonstrate the presence of viable bacteria after about 10 days of incubation. The test consisted of placing 30 gms of the crushed rock (ground to -200 m or finer) and 5 ml of the active bacterial suspension in 100 ml of the basal salts part of the 9-K Medium. The Thirty grams of ore ~~then was~~ had been substituted for the 30 ml of the "energy source" part of the 9-K Medium. For each ore type tested, a corresponding control was set up with 100 ml of the basal salts, 30 gms ore, and 5 ml of sterile 9-K Media added. The control was identical, except for bacteria, to the test. A growth control was included, with sterile 9-K Medium inoculated with 5 ml of the T. ferrooxidans suspension. An additional control, to act as a chemical blank for the metals determination was included. This consisted of 100 ml of the basal salts with 5 ml of the sterile 9-K Medium added. This would demonstrate the effect of media exchange in sampling upon the metals tested. After all the various test components were added together, aliquots for an initial pH reading and metals determination were taken. These readings indicated that the ore tempered the pH. Two drops of conc. H_2SO_4 were found to reduce the pH to 2.53, well within the requested 2.5 to 2.8 pH range. As 10 ml aliquots of the sample were used for the metals determination, and 10 ml for the pH, 20 ml of the basal salts solution were added per sample. The samples were then incubated for three days. In taking these aliquots it was found that it was necessary to let the ore solutions settle for 3 to 4 hrs. before sampling. It took that long for the fine particles to completely settle out. At three days incubation, 20 ml were taken for pH and metals determinations, and 20 ml of sterile basal salts solution were added. The pH had risen to approximately 4.2 to 4.5 so 1 drop of H_2SO_4 (conc.) was added to give a pH of 2.76 to the solutions wi

ore in them. The growth control showed 2.3 and the reagent blank 3.7. The pH was taken at day 8, day 10, and day 15. Additional metals determinations were done on days 3 and 15. Hanging drop slides at day 8 indicated bacterial growth in the growth control and none in the other flasks. The pH of the growth control had dropped to 2.3. From day 8 to day 10 to day 15, no real change in pH or media appearance was seen. Following the outline of the Canadian protocol supplied (A Study of Water Pollution in the Vicinity of Flin Flon, Manitoba, Part A Water Pollution Sources. Canada Environmental Protection Service. Surveillance Report EPS-5-NW-75-7.) an additional 15 gms of ore was added. Twenty four hours later, the pH was not below 3.5 so the test was terminated, and a final metals determination was taken. A table of results follows. (Table 1.)

4/4/78

Table 1. Results of pH and Metals Determinations.

	**	day 0 METALS*				**	day 3 METALS				day 8	day 10		day 15 METALS					day 16 METALS			
	pH	Cd	Cu	Fe	Ni	pH	Cd	Cu	Fe	Ni	pH	pH	pH	Cd	Cu	Fe	Ni	pH	Cd	Cu	Fe	Ni
Erie																						
mineralized	3.9	.83	190	390	180	4.5	3.0	55	240	12000	4.2	4.2	4.1	13	5200	82	24000	4.6	2.4	120	36	16000
ntrol	4.4	.67	92	560	150	4.2	4.1	48	140	13000	4.3	4.2	4.2	14	4400	37	22000	4.7	4.2	22	3.7	16000
9002	3.8	.78	240	420	130	4.5	.96	73	250	8200	4.3	4.3	4.1	10	9700	49	4100	4.7	6.1	300	2.5	4200
ntrol	5.2	.29	63	370	61	4.5	1.3	51	150	3900	4.3	4.2	4.2	8.6	7400	42	5300	4.6	8.0	310	10	7100
P 9002	5.1	1.6	270	450	460	4.5	4.9	70	390	8300	4.0	4.0	4.0	91	14000	180	19000	4.4	6.1	110	127	13000
ntrol	4.9	.67	67	440	240	4.5	7.3	83	370	10000	4.1	4.0	4.0	98	9600	160	16000	4.4	15	130	83	12000
em Blank	4.6	.19	60	460	44	3.7	.12	34	310	16	2.6	2.6	2.6	.18	17	240	35	2.5	.16	14	180	22
rowth Cont.	2.6	.84	850	11200	850	2.3	.67	820	8700	550	2.3	2.3	2.3	.55	300	5200	590	2.3	.63	230	3700	420
															Basal Salts			2.5	.27	4.9	.31	4

*Cd in µg/l, Cu in µg/l, Fe in mg/l, Ni in µg/l

**pH adjusted after reading with H_2SO_4 (conc.)

Discussion of Results.

The most apparent comment that can be made is that the bacterium used had no apparent effect upon the ore. That the Thiobacillus was viable at the start of the experiment was demonstrated by its growth in the 9-K Medium. Literature sources indicate that metal ion concentration changes of upwards of 50-60% should be seen over the control values. The chemical blank included demonstrated relatively tight control over the physical sampling procedures as seen by the following table:

Table 2. Chemical Blank Control Statistical Values.

METALS	MEAN	STANDARD DEVIATION	STANDARD ERROR	UNITS
Cd	.1625	.031	.014	ug/l
Fe	297.5	120.7	53.96	mg/l
Cu	31.25	21.09	9.41	ug/l
Ni	29.25	12.63	5.65	ug/l

Any differences of 50-60% from the test to controls should have been clearly demonstrated. However, large disparities in control and test samples were found. Due to test limitations, duplicate analyses were not run. Clearly they should have been. Causal inspection of Table 1 demonstrates that nickel and cadmium concentrations were not markedly affected. The figures for copper and iron are more erratic. Replicate testing possibly could have determined if there was a difference in reduction of the iron between the test and controls. The iron determination appeared to have less fluctuation than the copper. Possibly, a repeat of the experiment with 3 replicates, concentrating on only one ion (Fe) would be of more value. In addition, a mixture of mineral bacteria could be used. It should be noted that the pH range of the experiment was well within the 2-6 pH range for viability for this bacterium. Thus the pH seen in the test and control flasks, although higher than the growth control, would not have acted as an inhibitory or lethal factor. If proof that the ore is or is not a toxic substance to the bacteria is required addition of the ore to a freshly inoculated culture in a growth medium should be done. At least one transfer from this medium should be made after 10 days to new growth medium to test for viability.

Additional Notes.

In working with this medium, doubt arose that it was the best medium suited for the experiment. Additional bacterial strains were ordered from the American Type Culture Collection (ATCC). Upon subsequent culture, the medium suggested by the ATCC clearly demonstrated its superiority, allowing faster growth, as evidenced by a heavy red precipitate occurring days sooner than the 9-K Medium. It is suggested that this medium be used in any subsequent experiments. The formulation is:

BASAL SALTS		ENERGY SOURCE	
$(NH_4)_2SO_4$	.8 gms	$FeSO_4 \cdot 7H_2O$	20 gms
KH_2PO_4	.4 gms	1N H_2SO_4	2 ml
$MgSO_4 \cdot 7H_2O$	.16 gms	distilled H_2O	200 ml

Autoclave and combine in 80 ml basal salts to 20 ml energy source aliquots in sterile 250 ml Erlenmeyer flasks.

REVISED ORE TEST PROTOCOL

Barbara Thorsen, MDH, April, 1978

3 Test Flasks: Ore - 10 gms (used by Dr. Tsuchiya, not the 30 gms used by the Canadian study)
Basal Salts - 100 mls
Inoculum - 1 ml

3 Control Flasks: Ore - 10 gms
Basal Salts - 100 mls
Sterile Growth Medium - 1 ml

Toxic Growth Control: Basal Salts - 80 ml
Inoculum - 1 ml
Energy Source - 20 ml
Ore - 10 gms

Growth Control: Basal Salts - 80 ml
Energy Source - 20 ml
Inoculum - 1 ml

Chemical Blank: Basal Salts - 100 mls
Sterile Growth Medium - 1 ml

All flasks set 3 hrs. with no shaking before aliquots are drawn.

1. Initial pH* and Fe aliquots are taken on day one.
2. Incubation on shaker at 20°C.
3. At day 4 take pH and Fe aliquots.
4. Observe growth in growth control as color change.
5. At day 6, 8, 10, and 12 take pH's.
6. When pH in growth control drops to a stabilized reading concurrent with heavy red precipitate, take additional Fe aliquot. At this point, proceed with one of the following.
   a. If pH in test flasks remains constant, add 5 more grams of ore, shake for 4 more days, take pH and Fe aliquot. If pH has remained constant terminate experiment. If pH has fallen and is below 3.5, add an additional 5 gms of ore, shake 4 more days, take pH and Fe aliquot and terminate experiment.
   b. If pH in test flasks fluctuates or starts falling, continue taking pH every 2 days until the pH stabilizes. After 2 readings that are within $\pm$ 0.1 pH unit, proceed as in A above.
7. Subcultures to additional growth controls should be made from the toxic growth control at day 10, and at the experiments' termination. If the test extends beyond 25 days, an additional transfer should be made at day 25.

*The pH is used as a useful indicator of microbial activity as H_2SO_4 is formed as a byproduct of metal ion utilization. Specific pathways for metal utilization by various bacteria for different ores can be found in Zajic, James E., 1969, Microbial Bio geo chemistry, Academic Press, New York, p. 345.