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### Thiobacilli from Cochin Backwaters & Their Oxidative & Corrosive Activities

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Thiobacilli from mud samples of Cochin backwaters were isolated and cultured using Parker's thiosulphate medium. Oxidative products like sulphates and sulphuric acid were estimated. Sulphuric acid of 0.0033 to 0.0396*N* conc. was produced by the isolates. Corrosive action of Thiobacilli on specially prepared concrete surfaces was reported; net loss in weight ranging from 19.58 to 49.96% was recorded after 92 days of incubation at room temperature.

AMONG the sulphur bacteria, the group Thiobacteriaceae is important in estuarine environment. Representatives of this group are capable of oxidizing sulphur or its reduced inorganic compounds forming them as energy sources in a variety of ways, depending on the species. Even though several studies on Thiobacilli of estuarine and marine origin are available<sup>1-10</sup>, information on Thiobacilli from Indian waters is meagre. Bacterial corrosion of concrete has been reported by Parker and Prisk<sup>11</sup>. Bass Becking and Wood<sup>12</sup> have studied corrosion of various acid sensitive substances including concrete by Thiobacilli. The present paper deals with some aspects of Thiobacilli isolation, culture and assessment of their ability to oxidize thiosulphate to sulphates and to sulphuric acid. Results of experimental corrosion of concrete surfaces by Thiobacilli under laboratory conditions are also reported.

Mud samples from 6 shallow water stations in Cochin backwaters, having proximity to sewage openings, were collected using a grab. Average pH of water at all stations was between 5.6 and 6.7, temperature, 28° ± 1°C and salinity 21‰. Thiosulphate medium and thiosulphate agar having the following composition<sup>11</sup> (g/litre) were employed: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1; K<sub>2</sub>HPO<sub>4</sub>, 4; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; CaCl<sub>2</sub>, 0.1; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.02; MnSO<sub>4</sub>·4H<sub>2</sub>O, 0.02; Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O, 10 and distilled water 1000 ml. The medium was sterilized by steaming for 1 hr

each time for 3 consecutive days. Final pH was adjusted to 6.5. For making solid media, 1.5% agar powder was added to the above composition. One gram of mud (wet basis) from each station was weighed aseptically and incorporated into 150 ml of the medium in 250 ml conical flasks separately. Control flasks were also kept. The 6 flasks, representing 6 stations (I to VI), were incubated at 29° ± 1°C; pH was measured with a pH meter, (Systronics model). Sulphates were estimated gravimetrically by precipitating with barium chloride. Sulphuric acid produced was estimated by titrating with 0.0075*N* sodium hydroxide.

For studying the corrosive action, 3 pieces of specially prepared concrete (gravel:cement, 2:1) of almost similar dimensions were placed in 3 Erlenmeyer flasks (a, b and c) containing 150 ml Parker's thiosulphate medium. The concrete pieces were cured for 10 days before use, sterilized and weighed aseptically before the experiment. The flasks were inoculated (1 ml) with previously grown pure cultures of *Thiobacillus thiooxidans* (a and b) and *T. concretivorus* (c); control flasks were also maintained. Initial pH of the medium in all the 3 flasks was kept at 6.5 and the flasks were incubated at 30° ± 1°C. The concrete pieces were weighed accurately after drying in an oven, after 92 days of incubation. Data presented here are based on one single test block for each of the 3 cultures a, b and c.

**Bacterial growth**—Heavy turbidity was noticed after 3 days in all the 6 flasks representing samples from 6 stations. From this a loopful was removed and streaked on thiosulphate agar and incubated for 3-4 days. White to watery translucent small colonies appeared. All were gram negative small rods (0.5 µm av. diam.) which grew well on thiosulphate agar.

In all the flasks there was no change in pH during the first 4 days. pH changes were significant from 7th to 9th days of incubation. A sudden decrease was observed which continued till 15th day of incubation and then again a further decrease was noticed after 17th day indicating the presence of sulphuric acid. The trend in fall of pH in all 6 flasks was almost similar.

**Production of sulphate**—Result of oxidation by bacteria on 20th and 36th days of incubation are given in Table 1. Oxidation is rapid after 20th day and is almost 3-fold by 36th day in flasks I to III and VI while it is almost 2-fold in flasks IV and V.

**Production of sulphuric acid**—The amount of sulphuric acid produced does not vary much from 20th to 36th day of incubation, yet it is a proof of complete aerobic oxidation of thiosulphate.

**Corrosion of concrete pieces**—pH in flasks containing concrete pieces a, b and c registered an increase during the first 3-4 days, thereafter it steadily decreased. The initial increase is obviously due to the dissolution of the calcium present in the concrete mixture. In all the 3 flasks concrete pieces were corroded in varying degrees. Percentage loss in weight of 3 concrete pieces is given in Table 2. Corrosion was at its maximum in flask a (Table 2). The whole surface presented a gross appearance like that of a honey comb (Fig. 1). The rigidity

SHORT COMMUNICATIONS

TABLE 1 — SULPHATE AND SULPHURIC ACID PRODUCED BY THIOBACILLI AFTER 20 AND 36 DAYS OF INCUBATION

Station (flask)	Depth m	Sulphate g/150 ml medium				Sulphuric acid N		pH after 36 days
		20 days		36 days		20 days	36 days	
			Per cent		Per cent			
I	1.5	0.3202	21.35	1.0282	68.55	0.0213	0.0229	1.5
II	1.5	0.39	26	1.0113	67.42	0.024	0.0382	1.75
III	2.1	0.333	22.2	0.9236	61.57	0.0285	0.0396	2.12
IV	2	0.339	22.6	0.8031	53.54	0.0247	0.0387	2.5
V	2	0.3075	20.5	0.8055	53.7	0.0096	0.0098	2.5
VI	2	0.2662	17.7	0.9682	64.55	0.0084	0.0033	1.8

TABLE 2 — PERCENTAGE LOSS IN WEIGHT OF 3 CONCRETE PIECES

Flask	Concrete piece wt, G		Per cent loss	Final pH after 92 days
	Before expt	After expt		
a	11.3456	5.6771	49.96	1.8
b	7.7477	4.3389	43.97	2
c	10.9468	8.8033	19.58	2.5

of the concrete was significantly lost. In flask b also corrosion was rather similar as in a and the corrosive action was mostly at the surface. In flask c the action was moderate since the rigidity of the surface was not lost.

pH tolerance of the individual species of *Thiobacillus* is specific showing that different species tolerate different acid conditions. It has been proved statistically, based on large numbers of results from marine samples and deposits that it is possible to distinguish between autotrophic species of *Thiobacillus* solely on the basis of the pH values in the culture medium after completion of the sulphur cycle. Fjerdingstad has proposed a scheme to distinguish the *Thiobacillus* spp based on the pH values.

Basing on Fjerdingstad's scheme, the following species may be recognized in the 6 samples representing the 6 stations:

Sample	Causing pH drop up to	Species
I	1.5 ± 0.05	<i>Thiobacillus thiooxidans</i>
II	1.75 ± 0.5	do
III	2.0 ± 0.12	do
IV	2.5 ± 0.5	<i>T. concretivorus</i>
V	2.5 ± 0.5	do
VI	1.8 ± 0.2	<i>T. thiooxidans</i>

Fjerdingstad<sup>1,2</sup> reported in the experiment on concrete corrosion by Thiobacilli, a loss in weight ranging from 11.8 to 6.1% after 100 days of incubation at 30°C by strains of *T. thiooxidans*. Compared to his observations the present experiments show that the corrosive action is much more faster and of high magnitude in the Cochin estuarine environment. This may be due to the temperature factor as reported by Fjerdingstad<sup>1,2</sup>. Microbial corrosion of concrete has also been reported by Parker and Prisk<sup>11</sup>. Bass Becking and Wood<sup>12</sup> also reported corrosion of various acid sensitive substances by Thiobacilli.



Fig. 1 — Experimental concrete piece (A) showing corrosion along with the normal control (B)

In the light of these informations and the present studies it could be said that in Cochin backwaters the acidic conditions that prevail during the major period of the year, can give rise to a suitable environment for the growth of *T. thiooxidans* and *T. concretivorus*.

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**Distribution & Activity of L-Asparaginase Producing Fungi in the Marine Environment of Porto Novo**

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Of the total (97) fungal isolates screened 40.2% exhibits L-asparaginase activity. Though the frequency of asparaginase producers is more among the fungi isolated from molluscs, estuarine water and rhizospheres, the sediment samples harbour more potent strains. Generally the fungi exhibit low asparaginase activity and their activity is mainly restricted to the members of the genus *Aspergillus*.

THE enzyme L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) received much attention since Broome<sup>1</sup> showed that it was responsible for the anti-tumour activity of guinea-pig serum<sup>2</sup>. This observation prompted the search for potent sources for therapeutic use<sup>3-10</sup>. Information on the occurrence and activity of this enzyme in fungi is limited<sup>7,8,11-16</sup>. Occurrence of L-asparaginase in the marine sediments of Porto Novo was reported by Selvakumar *et al.*<sup>17</sup>. In the present investigation, fungi isolated from the marine environment at Porto Novo (11°29'N; 79°49'E) were screened for their L-asparaginase producing potential.

*Isolation of fungi* — Samples were collected from the marine environment of Porto Novo once in a month for 7 months (1st August, 1976 to 28th February, 1977). Shell surface scrapings, mantle and gut macerates of molluscs (*Crassostrea madrasensis*, *Meretrix meretrix* and *Anadara rhombia*), marine sediments, estuarine water and rhizosphere samples (of *Ipomoea pes-capra*, *Spinifex littorus* and *Sesuvium portulacastrum*) (Table 1) were serially diluted with 50% sterile sea water and plated using

Rose Bengal medium. The plates were incubated at 25° ± 2°C for 10-14 days. The fungal colonies were selected and then streaked for obtaining pure cultures. The fungi were grouped on the basis of their morphological characters in lactophenol cotton blue preparations.

Mycelial mat (10 mm<sup>2</sup>) of the fresh culture was inoculated into 30 ml of M-medium<sup>8</sup> in 250 ml Erlenmeyer flask and incubated on a rotary shaker for 5 days.

*Enzyme studies* — After growth, the cultures were harvested by centrifugation at 5000 rpm for 20 min in a refrigerated centrifuge and the supernatant was used as the enzyme preparation.

To the reaction mixture containing 0.5 ml of 0.04M L-asparagine and 1 ml of 0.1M Tris-HCl buffer (pH 7.2), 0.5 ml of the enzyme preparation was added and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 0.5 ml of trichloroacetic acid. Controls were included in all the assays in which the substrate was added after the addition of TCA. The ammonia liberated was estimated using Conway microdiffusion method as described by Selvakumar *et al.*<sup>17</sup>. Ammonium sulphate (AR) was used as the standard.

TABLE 1 — DISTRIBUTION AND L-ASPARAGINASE ACTIVITY OF FUNGI ISOLATED FROM DIFFERENT SOURCES

No. of isolates	Source	No. of isolates showing activity	No. of isolates showing 0.15-1 unit/ml
<i>Anadara rhombia</i>			
7	Shell surface	2	2
6	Mantle	4	4
3	Gut	1	1
<i>Meretrix meretrix</i>			
7	Shell surface	4	4
—	Mantle	—	—
—	Gut	—	—
<i>Crassostrea madrasensis</i>			
4	Shell surface	2	2
7	Mantle	3	3
10	Gut	5*	4
SEDIMENTS			
4	Vellar estuary	0	0
30	Marine Beach	9*	8
WATER SAMPLE			
7	Vellar estuary	3	3
RHIZOSPHERE			
4	<i>Ipomoea pes-capra</i>	2	2
6	<i>Spinifex littorus</i>	3	3
2	<i>Sesuvium portulacastrum</i>	1	1
97		39	37

\*One isolate shows 1.01-2.45 units/ml activity.