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Occurrence of thiobacilli in Tuticorin harbour waters

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The number of thiobacilli obtained as colony forming units on thiosulphate agar was the highest ever reported from marine sources. Seven isolates, out of 15 from enrichment cultures, were *Thiobacillus* spp., *T. thiooxidans* and *T. ferrooxidans* produced the most acidic conditions in liquid cultures. Laboratory experiments with decaying alga *Ulva lactuca* in seawater showed that thiobacilli were involved in the oxidation of protein sulphur. The study not only indicates the status of pollution in habour waters but also shows that thiobacilli may have wide distribution in coastal waters.

Sulphur cycle plays an important role in the ocean[1]. Representatives of the genus *Thiobacillus* are responsible for the oxidation of sulphur and reduced sulphur compounds to sulphate and/or sulphuric acid. Information on the distribution of thiobacilli in Indian waters[2-4] and elsewhere[5-10] is limited. Elemental sulphur which is being shipped at Tuticorin harbour finds its way into the coastal waters during handling and storage and, from this point of view, studies on marine thiobacilli are of special interest. The present study deals with the occurrence and characterisation of thiobacilli present in the harbour waters. In an earlier study[11] acid producing thiobacilli have been isolated from decaying flesh of barnacles and hence, in the present investigation, the role of sulphur-oxidising bacteria in decomposing organic matter is also studied.

Materials and Methods

Seawater was collected by a conventional, all-glass bacteriological sampler (250 ml). Samples from 4 locations were considered for enumeration of thiobacilli, 2 each from open sea and sheltered harbour waters (Fig. 1). Of the seawater samples, one was collected from near-shore area and another from a distance approximately 1 km away from shore line.

The 2 media employed for enumeration of thiobacilli were essentially those used by Tilton *et al.*[5] and Ormerod[10]. Seawater and distilled water mixtures were used to adjust salinity[10] in the media to a value between 20 and 22×10^{-3}. For both media, *p*H 6 and 4 were used. Seawater sample (5 ml) was used for inoculation and colony forming units (CFU) on petridishes were counted after 15 d of incubation at room temperature (av.28°C). Enumeration was carried out during the first week of October 1988 and results on CFU are the average of 15 replicate samples.

Enrichment cultures were prepared in 3 different media by adding 250 ml of freshly drawn seawater to 500 ml of pre-sterilized broth in stoppered flasks. The thiosulphate mineral medium (TMM) contained the ingredients of Ormerod's formulation[10] except agar. Elemental sulphur medium[2] (SM1) contained (g) K_2HPO_4, 0.5; NH_4Cl, 0.5; $MgCl_2.6H_2O$, 0.2; $CaCO_3$, 20; and sulphur, 10 per litre of 70% seawater. In the third medium, SM2, sulphur was the only source of energy at 10 $g.l^{-1}$ of seawater. These cultures were prepared thrice between October and December 1988 employing seawater from the sheltered harbour area. *p*H in flasks was measured using a Phillips digital *p*H

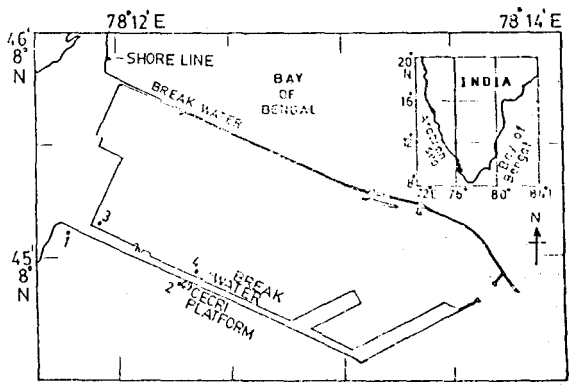

Fig. 1—Station locations in Tuticorin harbour area

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meter at intervals of 15 d for 76 d. Cultures from the flasks were streaked on Ormerod's thiosulphate agar at random intervals of time after incubation. Morphologically diverse colonies were picked and repeatedly streaked on the same medium to obtain purity. Characterisation and identification of the isolates were made[12].

For experiments on putrefaction, fronds of *Ulva lactuca* were placed in stoppered flasks containing 750 ml of fresh seawater. The amount of algal material introduced was roughly 10% (wt/v) of seawater. Density of thiobacilli on Ormerod's agar and gravimetric determination of sulphate concentration[13] were followed for 26 d.

Results

Enumeration on thiosulphate agar—CFU on Ormerod's medium outnumbered those on Tilton's ($t = 17.75, 20.59, 54.07$ and 51.65 respectively for sts 1 to 4, and $P < 0.05$ in all cases). Also, thiobacilli were present in higher numbers in sheltered harbour waters than in open sea ($t = 29.55$ and 33.32 respectively for st 1 *vs* st 2 and st 2 *vs* st 4 on Ormerod's medium with $P < 0.05$ in both cases). The highest numbers obtained was 89 CFU.ml^{-1} at st 3 (Table 1). The variations in CFU as a function of *p*H was too small although the numbers were slightly higher at *p*H 4.

Characteristics of isolates—In all, 15 isolates were obtained from enrichment cultures of which 7 were members of *Thiobacillus*. *T. thiooxidans* and *T. ferrooxidans* were most frequently recorded in plate cultures during enumeration. They grew at both *p*H 6 and 4, but preferred the acidic condition. Considerable acid production was observed in cultures of both organisms. In liquid cultures of *T. thiooxidans* (SM2), *p*H dropped to 1.5 after 25 d. On thiosulphate agar, colonies were minute, circular and whitish-yellow.

T. ferrooxidans produced a *p*H of 2.1 in TMM. It turned ferrous medium (broth) to brownish red in colour. In plate cultures it exhibited typical variations in colony appearance. Colonies in presence of equal amounts of thiosulphate and ferrous sulphate were reddish yellow and complete while growth on thiosulphate alone produced colourless, irregular ones which became whitish in centre on ageing.

T. novellus, *T. intermedius* and *T. perometabolis* dominated in cultures close to neutral *p*H in TMM. They reduced the *p*H to 6.1, 5.7 and 5.9 respectively from an initial *p*H 7. Colonies of *T. novellus* were 1 mm diam., colourless, and became whitish in old cultures. Growth of *T. intermedius* (flat, yellow-opaque colonies) was considerably increased by the addition of yeast extract. In *T. perometabolis*, supplementation of 2% yeast extract not only enhanced growth but also transformed the barely visible colonies to creamy, circular, yellow ones which were 2-3 mm in diam.

T. thioparus and *T. neopolitanus* were less frequent than the others. Colonies of both species were circular, whitish yellow which became pink in centre. *T. thioparus* showed a further change by becoming dull brown in old cultures. The more precise clue to differentiation between these 2 species, however, was provided by *p*H measurements. *T. neopolitanus*, thus, produced typically lower *p*H (3.6) than *T. thioparus* (4.4) from an initial value of 6 in TMM.

In TMM and SM2, changes became evident within a month. On termination of the experiment, SM2 showed a *p*H of 2.7 while TMM showed 2.9. SM1 did not show appreciable acid production and showed *p*H 5.7.

Thiobacilli in putrefaction—Changes as a function of time in the density of thiobacilli and the concentration of sulphate in 10% decaying *U. lactuca* are shown in Table 2. At the end of 24h, an appreciable increase in the density of thiobacilli (all 7 species) and sulphate concentration was evident. This trend attained a peak after about 3d with thiobacilli population reaching high numbers (3200.ml^{-1}). There was a reduction in number of thiobacilli, which became zero after 7d.

Table 1—Density of thiobacilli and water characteristics at different stations

Location of station and distance from shore (m)	Salinity ($\times 10^{-3}$)	Oxygen (mg.l^{-1})	*p*H	Density of thiobacilli (CFU.ml^{-1})			
				Tilton's medium		Ormerod's medium	
				*p*H 6	*p*H 4	*p*H 6	*p*H 4
Open seawater (10)	31.8	5.4	8.2	9.07±1.98	9.93±1.34	23.4±2.30	20.13±1.67
Open seawater (1000)	31.6	5.3	8.1	8.07±1.73	9.27±1.18	21.0±2.45	25.00±2.03
Harbour waters (10)	32.5	ND	7.6	30.0 ±3.89	33.07±1.73	87.13±3.32	89.07±2.64
Harbour waters (1100)	32.0	4.7	7.7	25.53±1.99	27.13±2.31	75.13±3.52	78.07±2.41

ND = not determined

Table 2—Changes in density of thiobacilli and sulphate concentration in putrefying seawater containing 10% *Ulva lactuca*

Period (d)	Density of thiobacilli ($CFU.ml^{-1}$)	Sulphate (ppm)
0	27	2315
1	512	2881
2	2715	3429
3	3200	ND
4	1410	4446
5	215	6229
6	12	5127
8	0	4183
13	ND	2918
15	ND	2010
17	ND	596
23	ND	586
26	ND	575

ND = not determined

Sulphate concentration, on the other hand, reached its peak after 6d (6229 ppm) and by 15 d a rapid depletion was observed which continued thereafter and fell to a value as low as 575 ppm on termination of the study.

Discussion

The density and number of isolates of thiobacilli obtained in the present study are the highest compared to other marine sources[5,10]. As many as 7 isolates of the genus *Thiobacillus* have been obtained of which 4 species—*T. thiooxidans, T. ferrooxidans, T. neopolitanus* and *T. thioparus*—produce typically acidic conditions. Droop and Jannasch[14] have opined that the presence of *T. thiooxidans* is indicative of local pollution. Isolates from polluted and unpolluted beaches of Kerala[4] show that *T. thioparus, T. concretivorus* and *T. neopolitanus* are always present at places having proximity to sewage and organic waste disposal. Since the present study has revealed the occurrence of 4 acid producers including *T. thiooxidans*, it can be stated that the harbour waters are polluted. Spilling of sulphur during shipment and storage appear to be the reasons. This is evidenced by the fact that st 3 (situated closer to sulphur storage yard) contains greater number of thiobacilli than st 4 (Table 1; $t = 6.57$, $p < 0.05$).

Earlier studies on decomposition of *Codium* sp. and tunicates by Efird and Lee[15] have considered the sequence of changes involving oxygen, nitrogen and sulphur. The reason for initial acidification of seawater prior to oxygen depletion and rapid sulphide production has been attributed to the possible formation of HNO_3 and H_3PO_4. It is well known that sulphur is stored in the protoplasm of marine plants and animals[16]. Evidence for oxidation of sulphur in seaweed by thiobacilli and the consequent formation of an acidic substance such as H_2SO_4 may be envisaged from present results. Although thiobacilli may not be responsible wholly for the decrease in *p*H, it is apparent that they are an additional factor not previously recognised. The peak in sulphate concentration after 6 d (Table 2) by which time thiobacilli density showed a declining trend can be attributed to the role of microaerophilic forms such as *Thiovulum* spp.[17], which seldom grow on agar media. Based on the present results, thiobacilli appear to have wide distribution in the world ocean contrary to doubts raised by Tilton *et al.*[5].

The wide variation in the numbers of CFU, observed with media of Tilton and Ormerod, is surprising. The high amount of acid produced in seawater enriched with sulphur alone (SM2) rather than in other with additional ingredients (SM1) causes considerable discrepancy in data which can arise from the choice of media.

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