

## Sulfate Reducing Bacteria from Mangrove Swamps of Goa, Central West Coast of India

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Levels of lactate and acetate oxidising sulfate reducing bacteria in sediment samples of mangrove swamps were in the order of  $10^3 \text{ g}^{-1}$  dry sediment and the counts decreased with depth. Lactate utilisers were generally higher in number than acetate utilisers. Of the 20 strains studied from lactate based medium and 21 from acetate based medium 20 and 47.6% respectively were spore formers. The strains were all mesophilic and euryhaline ( $0-40 \times 10^{-3}$ ). About 60% of the former grew on acetate while 71.4% of the latter grew strictly on formate. The strains were all Gram negative and cytochrome-C positive.

Sulfate reduction in anoxic environments has received considerable attention<sup>1-3</sup>. The microorganisms responsible for the process along with methanogenic bacteria are considered to be situated at the base of the complex anaerobic food web. In the marine and estuarine environment the sulfate reducing bacteria (SRB) play a key role in the anoxic mineralisation of organic matter. Very little is known on the microbial turnover of sulfur in the mangrove swamps in India. The present study aims to estimate the load of SRB in 3 different areas of the estuarine complex of Mandovi and Zuari and their distribution in relation to depth at one of the areas. The SRB estimated on acetate and lactate based media would give an insight into their occurrence. A study of their physiological properties is attempted to find out their affinities to various genera.

### Materials and Methods

Sediment samples were collected from mangrove swamps at Orda, Agacaim and Banastrim (along Cumbarjua canal) (Fig. 1). Initially a single core sample (13 cm of 5 cm dia.) was collected in February 1985 from Banastrim. Surface samples were collected from all the 3 places in March 1985. Bacteriological analyses were carried out within 2-3 h of sampling.

The core was sub-sampled at 1, 5 and 10 cm levels in the laboratory. Only the central portion of the core was used to minimise external contamination. The sub-samples were serially diluted

in sterile 50% seawater blanks to which freshly filter sterilized sodium thioglycolate solution (final concn. 0.03%) was added. Inoculation was carried out with 1 ml of inoculum at  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  dilutions in triplicate.

Sulfate reducing bacteria were enumerated<sup>4</sup> in shake agar in screw capped culture tubes using modified medium of Hatchikian. The techniques followed for enumeration and isolation were essentially those outlined by Pfennig<sup>5</sup>. Enumeration was carried out using lactate (50% Na-lactate, 15  $\text{ml.l}^{-1}$ ) to represent Group I and acetate (Na-acetate, 2  $\text{g.l}^{-1}$ ) to represent Group II. Pure cultures were obtained from the highest positive agar shakes by serial dilutions in new agar shakes containing the same medium as the one used for counting. The isolates were maintained on the same medium without agar. At the time of inoculation, 1 ml.  $(200 \text{ ml})^{-1}$  of  $\text{FeSO}_4 \cdot \text{H}_2\text{O}$  solution at 0.04% conc. acidified with 0.2 ml of 4N  $\text{H}_2\text{SO}_4$  was added and instead of sodium thioglycolate, 0.5 ml.  $(200 \text{ ml})^{-1}$   $\text{Na}_2\text{S}_2\text{O}_8$  at 5% conc. was added to the autoclaved medium.

*Physiological tests*—Tests for NaCl requirement, temperature tolerance, substrate utilisation (ethanol and pyruvate for lactate utilisers, propionate, benzoate and carbonate and formate for acetate utilisers) were carried out with 10-12 d old cultures. The cultures were centrifuged and suspended in 2 ml of the medium. Inocula consisting of 0.1 ml of this suspension were used. For these tests the yeast extract in the medium was reduced to 0.01% and for autotrophic growth, on formate and bicarbonate, addition of yeast extract was totally avoided.

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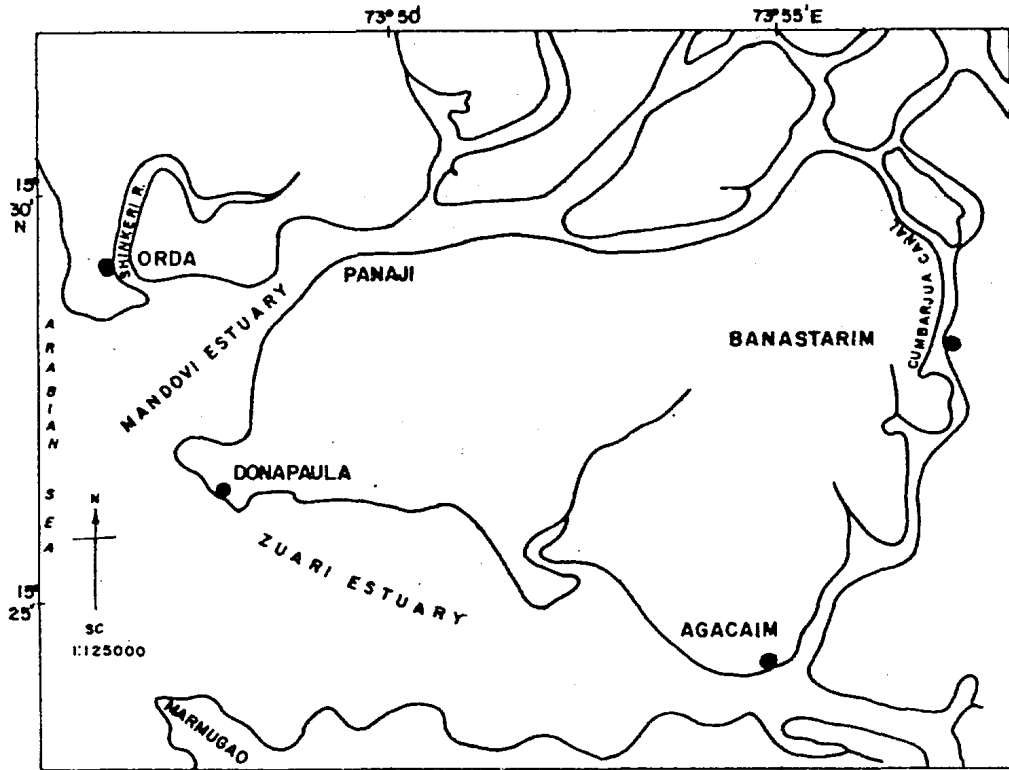


Fig. 1 - Sampling area

**Pigment identification**—Desulfovirdin test: Fluorescence of NaOH added cultures were detected with spectrofluorometer (Perkin-Elmer Model LS-3). Excitation wave length was fixed at 365 nm and emission was scanned from 400 to 700 nm. Cultures were centrifuged and the pellet resuspended in 2 ml of the medium. One or two drops of 2 M NaOH were added into the cell suspension just before scanning.

**Cytochrome identification:** Absorbance spectra of air oxidized and sulfide reduced suspensions of whole cells were determined using Beckman Scanning Spectrophotometer Model-DU6. Scanning was carried out from 500 to 650 nm. The culture supernatant was used as blank.

**Cytological characteristics**—Cell dimension and morphology were determined in Gram stained preparation and live cell suspensions of 10-12 d old cultures. The arrangement of flagella was determined by Rhodes staining technique.

**Results**

The salinity of water at sampling sites was  $35.6 \times 10^{-3}$ ,  $35.2 \times 10^{-3}$  and  $28.5 \times 10^{-3}$  at Orda, Agacaim and Banastarim respectively and pH 6.97, 7.03 and 6.07. The temperature was 28.2°C at Agacaim and Banastarim and 29°C at Orda.

Maximum number of acetate utilizers were present at the surface of the sediment and their number decreased with depth (Table 1). The number of lactate utilizers also decreased with depth but the decrease in number was not as marked as in the case of acetate utilizers. More number of colonies were recovered from lactate based medium than from acetate based one from the higher depths. SRB capable of oxidizing lactate were more in all the 3 regions as compared to the acetate utilizers. The difference between lactate and acetate utilizers in Banastarim was much less as seen from the surface as well as core samples.

Table 2 gives the percentage isolates positive for various physiological tests. Most of them were

Table 1 - Number of SRB in Sediment Samples

Sampling site	Core (cm)	No. of SRB ( $\times 10^3 \cdot g^{-1}$ )	
		Acetate	Lactate
Orda	0	6.83	27.65
Agacaim	0	8.03	18.15
Banastarim	0	8.61	9.63
	1	16.04	17.62
	5	6.32	20.98
	10	2.52	11.34

Table 2 – Percentage of Isolates Positive for the Physiological Tests

Core (cm)	Media	No. of isolates	Spore former	Motility	Growth														
					NaCl conc. (%)					Temp. (°C)				Ethanol	Acetate	Pyruvate*	Formate	Propionate	Benzoate
					0	1	2	3	4	15°	30° ± 2°	40°	45°						
1	L	5	0	100	60	60	60	100	100	60	100	80	60	20	10	0	—	—	—
	A	3	33.3	100	100	100	100	100	66	60	100	66	33	—	—	—	66	60	0
5	L	8	25.0	100	100	100	100	100	71	71	100	75	50	50	62	0	—	—	—
	A	9	77.8	89	78	100	100	100	100	55	100	78	33	—	—	—	78	78	100
10	L	7	28.6	85	100	100	100	100	100	71	100	71	57	57	85	0	—	—	—
	A	9	22.2	100	66	100	100	100	89	55	100	89	11	—	—	—	66	89	78
1 + 5 + 10	L	20	20.0	94	90	90	90	100	90	100	100	75	55	50	60	0	—	—	—
	A	21	47.6	95	76	100	100	100	90	57	100	81	24	—	—	—	71	90	76

L = lactate; A = acetate; \*SO<sub>4</sub><sup>2-</sup> not added; Desulfovibrin absent in all the isolates; Cytochrome present in all the isolates.

able to grow at 0% salt (NaCl) concentration; 90% of the isolates from lactate based medium were able to grow at 0, 1, 2 and 4% salt conc. Of the strains from the surface samples 40% did not grow in the absence of NaCl. SRB from acetate based medium showed 100% growth at 1, 2 and 3% salt conc.

Growth of SRB decreased with increase in temperature. Only 23.8% of acetate utilizers grew at 45°C as compared to 55% of lactate utilizers.

Of the lactate utilizers 50% used ethanol as a substrate and 60% of the same grew on acetate also. Among acetate utilizers 90.5% showed growth in the medium containing propionate while 76.4% of these strains grew in media containing formate and bicarbonate. The sub-surface isolates were able to breakdown benzoate.

All strains were Gram negative; 40% of the lactate oxidisers and 47.6% of the acetate oxidisers were spore formers. There was not much variation in the cell size and it ranged from 1-4 µm in length and 0.7-2 µm in breadth. Most of the bacteria were motile, possessing a single flagellum. All strains were desulfovibrin negative and contained cytochrome-C as evidenced by absorption peaks of reduced live suspensions of cells at 555/556 nm. Reduced minus oxidized spectra suspensions of whole cells showed a single peak at 556 nm. Scanning of whole cells under air oxidized and Na<sub>2</sub>S.9H<sub>2</sub>O reduced condition did not show peaks at 545, 580 or 630 nm. Hence absence of desulfovibrin and desulforubidin was confirmed.

Some of the isolated strains shared many features in common with *Desulfovibrio salexigenes*, *D. baculatus*, *D. vulgaris*, *Desulfotomaculum ru-*

*minis*, *Desulfobacter postgatei* and *Desulfosarcina variabilis* (Table 3).

## Discussion

Distribution of SRB with depth showed (Table 1) that these anaerobic forms are present in higher number at the surface. However, the lactate utilizers were slightly higher at 5 cm than at 1 cm depth. The decrease in acetate utilizers was less by 3 times at 5 cm and 7-8 times at 10 cm.

A gradual decrease in SRB (on lactate medium) was recorded by Jorgensen<sup>6</sup> in the coastal marine sediments of Limfjorden. However at 3-5 cm depth the numbers were much higher than at 0 or 7 cm depth. In Wilkinsons basin Lyon *et al.*<sup>7</sup> reported an increase in number with increase in depth i.e. 660 at 5 cm depth and 2100 at 15 cm depth, highest number being at 15 cm depth. Nedwell and Abram<sup>2</sup> observed a general decrease in activity and in counts of SRB with depth. Hines and Buck<sup>8</sup> reported that lactate oxidising SRB were restricted to the upper parts of the sediment at all the locations studied in the Long Island sound.

Distribution of lactate (Group I) and acetate utilizers (Group II) at Banastarim did not show much difference. This was particularly true with the surface samples. In Orda and Agacaim the lactate utilizing bacteria were more than acetate utilizers. The trend in the distribution of both these bacteria with depth was same as reported earlier<sup>9</sup>.

In estuarine beach sediments<sup>4</sup> where the substrate is more sandy than clayey, the SRB counted at 5 cm level was generally higher than that at 3

Table 3 – Characteristics of SRB Isolated from Lactate and Acetate based Media and their Relatedness to Existing Species

Culture No.	Cell shape: length × width (av. μm)	Spore	Motility; Flagellation	Cytochrome-C (peak in nm)	Growth										Related to				
					NaCl conc. (%)				Temp. (°C)				Ethanol	Lactate		Pyruvate*	Acetate		
					0	1	2	3	4	15°	30°	40°						45°	
Lactate media																			
27	Vibrioid 2.7 × 1.3	-	+	556	-	-	-	+	+	+	+	+	-	-	+	-	-	<i>Desulfovibrio salexigenes</i>	
28	Rods 3.0 × 2.0	-	+	556	-	-	-	+	+	+	+	+	+	+	+	+	-	-	<i>D. salexigenes</i>
51	Rods 2.8 × 1.8	-	+	556	+	+	+	+	+	+	+	+	+	+	+	+	-	-	<i>D. baculatus</i>
79	Rods 3.1 × 1.4	-	+	555	+	+	+	+	+	-	+	+	+	+	+	+	-	-	<i>D. baculatus</i>
			Lophotrichous																
44	Vibrioid 3.3 × 1.5	+	+	555	+	+	+	+	+	+	+	+	+	-	+	-	-	-	<i>Desulfotomaculum ruminis</i>
78	Rods 3.3 × 1.5	+	+	556	+	+	+	+	+	-	+	+	+	-	+	-	-	-	<i>De. ruminis</i>
83	Spirilloid 4.4 × 0.8	-	+	555	+	+	+	+	+	+	+	+	-	+	+	-	-	-	<i>D. vulgaris</i>
			Polar																
Acetate media																			
21	Rods 4.5 × 1.4	-	+	556	-	+	+	+	+	-	+	+	+	-	+	-	-	-	<i>Desulfobacter postgatei</i>
17	Rods 3.2 × 1.3	-	+	555.5	+	+	+	+	+	-	+	+	-	+	+	+	+	+	<i>Desulfosarcina variabilis</i>
20	Rods 4.3 × 0.7	-	+	556	-	+	+	+	+	-	+	+	-	+	+	+	+	+	<i>Des. variabilis</i>
24	Rods 3.0 × 1.6	-	+	556	+	+	+	+	+	+	+	-	-	+	+	+	+	+	<i>Des. variabilis</i>
22	Rods 2.9 × 1.6	-	+	555.5	+	+	+	+	+	+	+	+	-	+	+	+	+	+	<i>Des. variabilis</i>
			Polar																

\*SO<sub>4</sub><sup>2-</sup> not added; + Spore/Growth present; - Spore/Growth absent; Desulfovibrin not present in all the isolates; all isolates are Gram negative

cm and 1 cm levels and the numbers were one order of magnitude less than the numbers recorded in the present study.

The occurrence of higher numbers of SRB in the surface sediments compared to the subsurface sediments could be due to the reason given by Jorgensen<sup>1</sup> that these organisms must be inhabiting the anaerobic microhabitats in the aerobic layer. It could also be due to the freely available fatty acids<sup>10</sup> and mineralization of organic nitrogen<sup>11</sup> detectable in the top 5 cm of the sediment. Nedwell<sup>12</sup> also emphasised that labile organic carbon, easily available for microbial utilization was confined to the surface layers of the sediment.

Based on about 12 test characters certain similarities were drawn between the isolates from the swamps (Table 3) and the tables given by Pfennig *et al.*<sup>5</sup>. Culture No. 27 and 28 shared some of the features of *D. salexigenes* except that both are desulfovibrin negative. But culture No. 27 could not utilize ethanol. Culture No. 51 and 79 resembled *D. baculatus* except for the fact that both the cul-

tures were able to tolerate high temperatures. Culture No. 79 was lophotrichous instead of being single polar. Culture No. 44 and 78 differed from *Desulfotomaculum ruminis* in the cytochrome type. Culture No. 83 resembled *D. vulgaris* in all characters excepting that it was desulfovibrin negative.

Among the acetate utilizers no Gram positive organism was encountered (*Desulfonema limicola* and *D. magnum*), in the medium and the technique employed. Coccoid forms were also not detected. Though many spore formers were isolated the typical spore former *Desulfotomaculum acetoxidans* with spherical spores and gas vacuoles were also strangely absent. The occurrence of these forms seemed to be tied to the gastrointestinal tracts of animals or to manure polluted fresh waters<sup>9</sup>. The spore formers were similar to those isolated from lactate in that the spores were all terminal and these could not be identified with the number of tests performed.

Culture No. 21 resembled *D. postgatei* in that it

was a rod with a single flagellum, with cytochrome-C, desulfovirdin negative, unable to grow in 0% NaCl and unable to make use of propionate, benzoate or grow autotrophically with formate or bicarbonate. Culture No. 17, 20, 22, and 24 are rod shaped with cytochrome-C, able to grow up to 40°C, can utilize both propionate and benzoate and also grow autotrophically at the expense of formate. Hence they resemble *Desulfosarcina variabilis* except that these cultures are motile.

SRB from mangrove swamps were able to tolerate a wide range of salt concentration (Table 2). It was already established that these organisms can tolerate a wide range of salt concentration<sup>13,14</sup>. Moreover, these swamps along the Mandovi and Zuari estuaries are continuously exposed to a wide diurnal seasonal and annual fluctuations in salinity which made them euryhaline.

The number of spore forming isolates were 4 from lactate based medium and 10 from acetate based medium. These included all halotolerant forms except one which showed no growth at 4% salt conc. Spore forming isolates were generally not tolerant to salt concentration above 2.5%. But Skyring *et al.*<sup>15</sup> were able to isolate a number of spore formers from highly saline environments. Similarly, in the present work the spore forming isolates were also halotolerant.

In the present study 71% of acetate utilizers could grow autotrophically at the expense of bicarbonate and sodium formate. Hence, as Laanbroek and Pfennig<sup>9</sup> suggested, lactate and possibly acetate may not be the only primary substrate for sulfate reduction in anaerobic sediments. Hydrogen or sodium formate could be more important electron donors for the reduction of sulfate in the mangrove swamps.

All the isolates (9) from 5 cm and 78% of the isolates from 10 cm depths decomposed benzoic acid. The occurrence of *p*-hydroxy benzoic acid and other phenolic acids in these sediments was reported by Karanth *et al.*<sup>16</sup>

Of the 20 strains that were isolated from the lactate based medium 60% were also able to use acetate. Recovery of such high percentage of isolates from lactate based medium using acetate may be due to (1) lactate may also be used by members of Group-II, (2) acetate utilizers may grow in lactate based medium in the later stages when acetate is released by lactate utilizers and (3) yeast extract in the medium may act as the electron/carbon source. Hence, it might be possible that sulfur turnover in these sediments could be mostly through acetate pool. Banat *et al.*<sup>17</sup> also showed similar acetate oxidizing sulfate reducers

representing a distinct functional group and occupying a metabolic niches separate from that of the majority of sulfate reducers known with a much wider distribution than so far known.

It could therefore be concluded that sulfate reduction in the swamps was brought about by both lactate and acetate utilizers. As majority of the lactate utilizers could also oxidize acetate, it might be possible that the SRB were responsible for the oxidation of acetate in these regions. Two recent studies<sup>17,18</sup> confirm that SRB were indeed responsible for acetate oxidation in anaerobic high sulfate sediments and are therefore, responsible for a functional cycle of complete oxidation of organic carbon under anaerobic conditions.

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