Mahasagar-Bulletin of the National Institute of Oceanography, 11 (1 & 2), 1978, 21-28.

STUDIES ON PHOTOSYNTHETIC BACTERIA: I. ECOLOGY OF PHOTOSYNTHETIC BACTERIA IN AQUATIC ENVIRONMENT IN GOA

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ABSTRACT

Photosynthetic bacteria were isolated from mud samples under strictly anaerobic conditions. Marine and fresh water mud samples were collected from different places in Goa to study the ecology of the bacteria present. The isolated bacteria were studied microscopically for their morphology and other characteristics. Identification of these bactreria was done with the help of Bergey's Manual of Determinative Bacteriology. Three types of bacteria were identified: (1) green sulphur bacteria, (2) purple sulphur bacteria and (3) non-sulphur purple bacteria.

INTRODUCTION

The green and purple bacteria have for many years attracted the attention of microbiologists and considerable biochemical studies employing these bacteria, their ecological role and photosynthetic properties have been extensively reviewed. These organisms are generally divided into three families, which are distinguished by their pigment composition, nature of electron donor in photosynthesis, vitamin requirements, etc. Chlorobacteriaceae are green or yellowish green and contain types of chlorobium cholorophyll. They are phototrophic anaerobes and require sulphide in their medium, which is oxidised to sulphate during growth, CO_2 is the usual source of cell carbon. The green photosynthetic bacteria are widely distributed in shallow polluted waters and mud, where visible development in species may occur when the environment is rich in sulphide. Marine and estuarine species have been reported. The purple sulphur bacteria (Thiorhodaceae) and the non-sulphur purple bacteria (Athiorhodaceae) both contain bacteriochlorophyll. Their reddish brown colour is due to carotenoids. The Thiorhodaceae may utilize sulphide as an electron donor in photosynthesis and CO_2, or various organic acids, serve as carbon sources. The non-sulphur bacteria, however, require reduced carbon compounds, such as malate or succinate, as both electron donors and carbon sources, and with one exception are capable of aerobic growth in the dark as well as anaerobic growth in the light. Species of purple bacteria are generally found in stagnant water, especially if it contains decomposing organic matter. Van Niel (1931) and Pfennig (1962) have done extensive studies on the isolation of photosynthetic bacteria, the culture media and conditions required for their growth. Later on, Larsen (1953) used a modified medium of Van Niel (1944) for the development and isolation of green photosynthetic bacteria.

MATERIALS AND METHOD

Mud samples were collected from different places for the study of photosynthetic bacteria. The place of sample collection have been shown in Fig. 1. These are (1) Chapora river, (2) Estuary at St. Inez, (3) Dona Paula bay, (4) Estuary at Panaji, (5) Chinchinim pond and (6) Sal river.

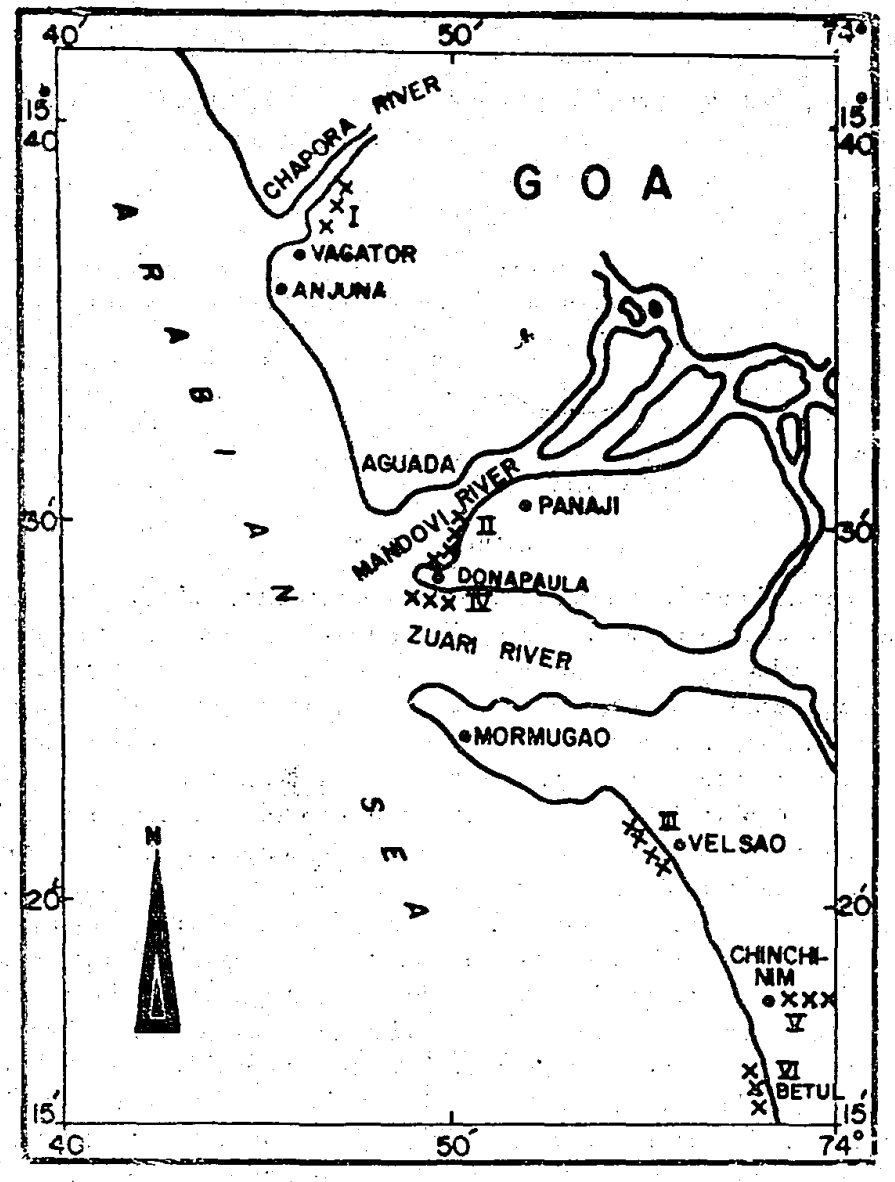

Fig. 1. Map of Goa coastline showing the sites of sample collection.

A little mud with some water was taken from a depth of about 30 cm below the surface of water into a previously sterilized MacCartney bottle or a closed beaker, with the help of clean sterile spatula. The container was immediately closed, to avoid the entrance of any aerial microorganism. The sample was brought to the laboratory within 18 hours collection, to avoid any changes in the microflora, as the result of the change of the environmental conditions.

To 0·2 g to 0·5 g of the above mud sample, 2·0 ml of sterile distilled water was added, made into a homogenous suspension on a 'Vortex" mixer and inoculated into strerile MacCartney bottles (15 ml) with the help of a sterile pasteur pipette. The bottles were then filled upto the brim with the respective media and were covered tightly, to ensure the absence of any air bubbles. The samples were inoculated into the following types of media.

(1) Green sulphur bacteria medium of Larsen (1953), with the following composition: NH_4Cl, 1·00 g, KH_2PO_4, 1·00 g, $Na_2S.9H_2O$, 1·00 g, $MgCl_2$, 0·50 g, $NaHCO_3$, 2·00 g, NaCl, 10·00 g, distilled water, 1000 ml, pH, 7·3.

The $NaHCO_3$ is prepared as a 5·00% solution and sterilised by filtration through seitz filter, the $Na_2S.9H_2O$ is prepared as a 10·00% solution and autoclaved separately, the remainder of the ingredients being autoclaved in bulk. After sterilisation 10·00 ml per litre of $Na_2S.9H_2O$ are added to the bulk along with 20·00 ml per litre of $NaHCO_3$ solution to complete the medium.

(2) The purple sulphur bacteria medium of Van Niel (1944) with the following composition: NH_4Cl, 1·00 g, K_2HPO_4, 0·50 g, $MgCl_2$, 0·20 g, $NaHCO_3$, 1·00 g, $Na_2S.9H_2O$, 1·00 g, distilled water, 1000 ml, pH, 8·0–8·5. The $Na_2S.9H_2O$ is autoclaved separately and $NaHCO_3$ seitz filtered as described above.

(3) Non-sulphur bacteria medium of Van Niel (1944) with the following composition: $(NH_4)_2SO_4$ or NH_4Cl, 1·00 g, K_2HPO_4, 0·50 g. $MgSO_4.7H_2O$, 0·50 g, NaCl, 2·00 g, $NaHCO_3$, 5·0 g, proteose peptone or organic substrate, 1·50 to 20·0 g, distilled water, 1000 ml, pH, 7·1–7·3, trace metal solution, 10·0 ml. The composition of the trace metal solution is as under: $CaCl_2$, 1·00 mg, $FeCl_3$, 5·00 mg, H_3PO_3, 1·0 mg, $ZnSO_4$, 1·00 mg, $CO(NO_3)_2$, 0·5 mg, $CuSO_4$, 0·05 mg, $MnCl_2$, 0·05 mg, distilled water, 1000 ml. The solution is sterilised by autoclaving. The mud samples were also used in Winogradsky's cylinder technique described by Carr (1969) for isolating photosynthetic bacteria. The MacCartney bottles and the Winograsdsky column were placed around 40 watt bulb at the distance of about 60 cm.

The photosynthetic bacteria appeared after 3 weeks and were enriched by transferring repeatedly every week into fresh medium with the help of sterile pasteur pipettes. They were then purified by the roll tube method as described by Hungate (1969). A little of the bacterial suspension was taken and introduced into a tube containing agar medium. The tube was then rolled to help in dispersing the bacteria to obtain isolated colonies immersed in the surface of agar, which provided the anaerobic conditions. These tubes on incubation gave isolated colonies, which were then inoculated carefully into fresh sterile medium to give a pure culture. The isolated organisms were then examined microscopically for morphology, size, motility and gram staining characteristics.

The organisms were identified and classified with the help of Bergey's Manual of Determinative Bacteriology (1974). In identifying the green bacteria (*Chlorobium* species), the thiosulphate medium described by Larsen (1953) was used to grow *Chlorobium thiosulfatophilum*. Media containing various organic substrates were used to grow the non-sulphur purple bacteria (Carr, 1969), which helped in the identification of the isolates.

The organic media used for the identification of *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* was as under: KH_2PO_4, 0·50 g, K_2HPO_4, 0·50 g, $MgSO_4 \cdot 7H_2O$, 0·20 g, $MnSO_4 \cdot 4H_2O$, 2·23 mg, $CaCl_2 \cdot 2H_2O$, 0·040 g, Ferric citrate, 0·01 g, Sodium glutamate, 1·90 g, DL–malic acid, 2·70 g, Biotin, 10·0 µg, Nicotinic acid, 1·0 mg, Thiamine hydrochloride, 500 mg, distilled water, 1000·0 ml, pH, 6·8.

The medium used to identify and grow *Rhodopseudomonas palustris* is as under: $(NH_4)_2SO_4$, 0·40 g, $MgSO_4 \cdot 7H_2O$, 0·60 g, $CaCl_2 \cdot 2H_2O$, 0·15 g, Sodium glycerophosphate, 2·00 g, Potassium citrate, 0·50 g, Sodium acetate, 0·20 g, L–glutamic acid, 3·00 g, Tyrosine, 0·10 g, *p*–amino benzoic acid, 200·0 µg, distilled water 1000·0 ml, pH, 6·5. After sterilisation, 10·00 ml of the trace metal used earlier for growing non–sulphur purple bacteria were added to complete the medium.

RESULTS

The following characteristics were observed on physical examination of the mud samples that were collected from the different places (Table I).

Table I. Characteristics of the various sediments

Sample	Colour	Characteristics
Chapora river	brown	coarse granules, H_2S present
Estuary at St. Inez	black	fine, less granular, H_2S present
Dona Paula bay	brown	coarse granules, H_2S present
Estuary at Panaji	black	fine, H_2S present
Chinchinim pond	light brown	sandy, H_2S absent
Sal river	brownish black	fine, H_2S present

Though the green bacteria took about two weeks to make their initial appearance in the MacCartney bottles and in another week they started growing fast. The purple bacteria took over a month to appear and grew more slowly, they adhered to the sides of the bottles and had to be carefully scraped off.

In the agar roll tube method, used in purification, the green photosynthetic bacteria appeared as diffused colonies in the agar surface but the purple sulphur and the purple non–sulphur bacteria appeared in 15 days and were seen reddish bias–convex discs in the agar surface. The characteristics and identification is described in Table II.

The Winogradsky's column gave interesting results as all forms of photosynthetic bacteria appeared as coloured spots on the mud–glass interphase after 30 days of incubation. The coloured spots (colonies) which were carefully scooped and purified by agar roll tube method showed the presence of *Chlorobium limicola*, *C. thiosulfatophilum*, *Rhodopseudomonas spheroides*, *Rhodospirillum rubrum* and *Thiospirillum jenese*.

The various organic media used for the growth of non–sulphur photosynthetic microorganisms showed that *Rhodopseudomonas palustris* grew best when tyrosine and para–amino benzoic acid were supplemented into the medium but *Rhodo-*

Table II. Morphological characteristics and identification of the bacterial isolates.

Type No.	Colour of colonies	Shape	Size in microns	Motility (by Hanging Drop Technique)	Gram staining	Growth in $Na_2S_2O_3$	Identity
	Green sulphur bacteria						
1.	green	rods (single and in chains)	1.5×0.8	non-motile	Gram negative	growth	*Chlorobium thiosulfatophilum*
2.	green	rods in chains	1·5×0.7	-do-	-do-	no growth	*Chlorobium limicola*
3.	chocolate brown	rods slightly bent	1.5×0.7	-do-	-do-	no growth	*Chlorobium phaeobacteriorides*
	Purple sulphur bacteria						
4.	reddish	diplococci	1.0	non-motile	Gram negative	not tested	*Thiocapsa pfennigii*
5.	brown	spirals	30×2.5	motile	-do-	,,	*Thiospirillum jenense*
6.	rose-red	diplococci, surrounded by slime layer	1.2	non-motile	-do-	,,	*Thiocapsa roseopersicina*
	Non-sulphur bacteria						
7.	brown-red	clusters, rods curved	1.2×0.6	motile	Gram negative	not tested	*Rhodopseudomonas palustris*
8.	greenish brown	single cocci	1·5	-do-	-do-	,,	*Rhodopseudomonas spheroides*
9.	pink-red	spiral	10×1.5	-do-	-do-	,,	*Rhodospirillum rubrum*

pseudomonas spheroides and *Rhodospirillum rubrum* grew best due to the presence of biotin and nicotinic acid as growth factors. The organisms grew poorly in media without these growth factors.

DISCUSSION

The chief physiological property of the photosynthetic bacteria is its ability to grow rapidly under strict anaerobic conditions using light as the ultimate energy source. This property makes the photosynthetic bacteria virtually unique in the biological world.

This fact has been found true during the growth and isolation of the photosynthetic bacteria from the collected mud samples. These organisms grew best under strict anaerobic conditions in the presence of light with the exception of few non-sulphur bacteria which grew anaerobically in the dark. These organisms are found in mud and stagnant waters containing H_2S, as they require such reducible substrates as electron donors for photosynthesis. Hence the superiority of Winogradsky's column over other isolation procedures was due to the fact that it not only provides large amount mud as inoculum but the mud also serves as a generator of H_2S, through the proteolytic activity therein, which stimulated the growth of the various photosynthetic forms described earlier.

Table III gives the different types of bacteria that were isolated from different places. It is observed that the green bacteria *C. limicola* and *C. thiosulfatophilum* were found in all mud samples tested hence were the most predominant flora. There was one place-Chinchinim where only *C. thiosulfatophilum* was isolated. The lack of H_2S in the Chinchinim sample could have resulted for the absence of *C. limicola*.

Mud samples from Chapora and Dona Paula have similar characteristics and the microorganisms isolated from these two places are mostly green bacteria and a few purple sulphur and non-sulphur bacteria. Samples from the estuaries

Table III. Types of organisms isolated from the different places.

Places	Types of organisms*
Chapora river	1, 2, 4, 5, 8, 9.
Estuary at St. Inez	1, 2, 3, 4, 5, 6, 7, 8, 9.
Dona Paula beach	1, 2, 4, 5, 8, 9.
Estuary at Panaji	1, 2, 3, 4, 5, 6, 7, 8, 9.
Chinchinim pond	1.
Sal river	1, 2, 3, 5, 9.

*1. *Chlorobium thiosulfatophilum*
2. *Chlorobium limicola*
3. *Chlorobium phaeobacteriorides*
4. *Thiocapsa pfennigii*
5. *Thiospirillum jenense*
6. *Thiocapsa roseopersicina*
7. *Rhodopseudomonas palustris*
8. *Rhodopseudomonas spheroides*
9. *Rhodospirillum rubrum*

at St. Inez and Panjim are also similar in their appearance and characteristics. These have been found to contain plenty of H_2S gas which may be due to sewage which is being discharged into these areas after treatment. It could also be due to domestic refuse which thrown into these estuaries, which may cause these waters and muds to contain a lot of organic matter. Mandovi and Zuari are the two important rivers on the central west coast of India which form the navigational network for transportation of iron and manganese ores to be shipped from the Mormugoa Harbour. These barges discharge oil which floats on the surface of water thus causing some anaerobic conditions. Iron and manganese waste may be used by these bacteria as trace metals for growth and for their chlorophyll synthesis. Once there are some organic matter and proper conditions, the photosynthetic bacteria flourish in the presence of light. These mud samples have been found to contain almost all types of photosynthetic bacteria, *i. e.*, green sulphur and non-sulphur bacteria. This shows that these muds contain the necessary ingredients of the growth of these bacteria, which are thus predominant here. The mud sample from the Sal river is somewhat similar to the above samples from Panjim and Dona Paula and has been found to contain only certain types of photosynthetic bacteria. The above studies reveal that the best isolates of photosynthetic bacteria were obtained from the two estuaries where these bacteria are abundant, as compared to the sample from Chinchinim where there are hardly any such bacteria, probably due to the absence of H_2S and other organic matter (Table III).

One mud sample was taken from a freshwater pond in Chinchinim, from which only *C. thiosulfatophilum* was isolated. The purple bacteria require a high content of NaCl for good growth and pH of the medium has to be about 8–8·5, which makes them less predominant in the freshwater samples. The green bacterium *C. thiosulfatophilum* is more predominant here due to the absence of H_2S gas in the mud.

The above studies reveal that the photosynthetic bacteria grew in an environment containing H_2S gas and CO_2 or any organic carbon compound which are necessary for bacterial photosynthesis. Most of the reported values of CO_2 assimilation in Goa estuarine waters relate the uptake of $NaH^{14}CO_3$ to algal photosynthesis (Aditi and Devassy, 1976). The data presented here suggests that the assimilation in the environment may also be due to bacterial photosynthesis, since photosynthetic bacteria are abundant in such places.

ACKNOWLEDGEMENT

The authors express their sincere thanks to Dr. S. Z. Qasim, Director, National Institute of Oceanography, Goa, for his keen interest, valuable suggestions and for going through the manuscript. They are also grateful to Dr. M. A. D. Affonso, Principal, Goa College of Pharmacy, Panaji for the laboratory facilities provided.

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