

Phosphate solubilizing bacteria around Indian peninsula

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Received 29 July 1998, revised 16 August 1999

The occurrence and physiological characteristics of marine phosphate solubilizing bacteria (PSB) around the Indian peninsula were studied. A higher percentage of phosphate solubilizing bacteria was seen in the islands and coastal areas as compared to the offshore and sandy beaches. Most of the PSB belonged to *Bacillus* spp. Additions of nutrients (glucose, yeast extract) enhanced their growth rate and phosphatase activity of the bacteria. Variations in temperature had a negligible effect on enzyme activity. Their ability to survive on carriers up to 60 days indicates that they could be effective seed organisms for field applications. These bacteria were found to be highly adaptive and therefore, can significantly contribute to the phosphate economy of the marine environment.

Phosphorus is a limiting nutrient, inspite of being a major component of seawater as its major forms are either bound to or strongly adsorbed to silt and clay¹. Microorganisms play a direct role by acting as either a sink or a source for phosphates in different niches²⁻⁴. Moreover phosphate uptake has been found to be dominated by bacteria⁵. Orthophosphate appears to be the preferred and apparently universal substrate. A large but variable percentage of the added organic and combined inorganic compounds accumulated outside the cells as soluble reactive phosphorus (SRP), indicating an efficient regeneration of orthophosphate from various phosphorus compounds. The major forms of phosphorus are solubilized enzymatically or by acid hydrolysis. Among the free enzymes, extracellular alkaline phosphatase which is widely distributed in seawater and deep sediment appears to play an important role in the phosphate budget⁷. However, very few reports from Indian waters are available on the study of extracellular phosphatases produced by marine bacteria⁸⁻¹⁰. The present work aims to study the occurrence and taxonomy of phosphate solubilizing bacteria from different niches of Indian coastal waters.

Materials and Methods

Seawater and sediment samples were collected from 8 sites around the Indian peninsula. The

sampling sites were the sandy beaches (Anjuna and Dias beach) and coastal regions of Arabian Sea (Chorao and Vasco Harbour) of Goa, besides the coastal regions of Cuddalore and Nagapattinam (east coast of India), Kalpeni island (Lakshadweep) on the west coast (Fig. 1). Surface water samples were collected using Niskin sampler. Sediment samples were collected using an alcohol sterilized spatula in steriware petridishes from the intertidal region. All

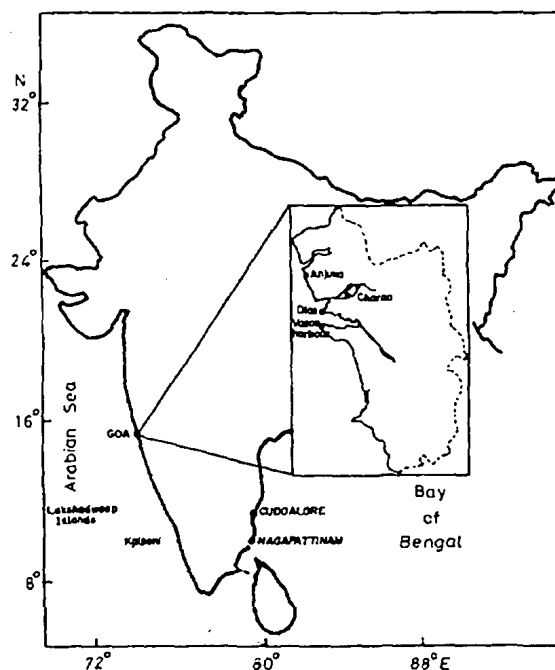


Fig.1—Sampling sites

the samples were transferred to the laboratory for analysis. Water samples were serially diluted and standard plating technique was followed using nutrient agar and Zo Bell 2216e media to isolate the total heterotrophic bacteria. The plates were incubated at $28 \pm 2^\circ\text{C}$. The bacterial isolates were then screened for phosphate solubilization by streaking them on to hydroxyapatite medium¹¹ and incubated for three days at room temperature. The phosphate solubilization was expressed as positive and negative depending on the halo formation. The cultures which showed halo formation around their colonies were considered to be the phosphate solubilizers. These solubilizers of inorganic phosphate were further screened for phosphatase production (mineralisation)¹² also. About 1 ml of culture suspension (1 cm cell $\text{O.D}_{600} = 0.1$) was inoculated into 10 ml of 0.2 μm filtered and sterilized seawater. To this, 1 ml of filter sterilized N.P.P. (p-nitrophenyl phosphate) was added. The tubes were vortexed for 30 sec and incubated at room temperature for 24 hr. The phosphatase activity was measured by the release of nitrophenol from nitrophenylphosphate by the culture. One mole of nitrophenol is equivalent to 1 atom of phosphorus. The absorbance was read at 418 nm using DU-6 Beckmann spectrophotometer. The activity was expressed as $\mu\text{mol.P.ml}^{-1}\text{d}^{-1}$. The cultures which showed good phosphate solubilization and phosphatase production were chosen and identified using Hewlett Packard Microbial Identification System (MIDI Inc. USA). Two potential phosphate solubilizing and mineralizing bacterial cultures of *Klebsiella pneumoniae* (P6) and *Bacillus pumilus* (P43) were selected for further studies. The strain P6 was isolated from the marine sediments of the Goa beach while strain P43 was from the coastal waters of Cuddalore.

Effect of NaCl concentrations, 0, 1, 3 and 4.5% prepared in freshwater, were tested on the bacterial phosphatase production of P6 and P43 at 15° , $27^\circ \pm 2$ and 37°C temperatures after 1, 7, 10 and 24 h of incubation. The influence of seawater on the phosphatase production by P6 and P43 was also tested maintaining the same parameters such as temperature and period of incubation. Relevant controls for substrate and enzyme were also maintained. The phosphatase activity was measured as mentioned earlier.

To measure the effect of NaCl, temperature and period of incubation on growth and activity the above

experiment was repeated in nutrient amended water (0.001% yeast extract and 0.01% glucose) at 1, 4, 22, 26, 30 and 46 h of incubation with 1.0 ml of exponentially growing cells as inocula. Experiments were carried out using suitable controls. Growth was measured at 600 nm using DU-6 Beckmann spectrophotometer and phosphatase activity was measured as described earlier.

For testing the rate of survival of PSB on various carriers 2 g of finely powdered sediment, rice-bran and sugar cane bagasse were autoclaved and cooled in test tubes to which 2 ml of culture suspension ($\text{O.D}_{600\text{nm}} = 0.1$) was added. The uninoculated test tubes were also kept as controls for each of the carriers. The material in the tubes was left to dry at room temperature. After one and two months, the viability was tested by their colony forming ability.

Results and Discussion

Totally, 603 isolates obtained from various niches were tested for their phosphate solubilizing property. Eighty-eight of these (14%) were found to be PSB. Venkateswaran & Natarajan¹³ reported 10-15% of the total viable counts to be capable of solubilizing inorganic phosphate. The number of heterotrophic bacteria solubilizing phosphorus varied at different regions. The variation in numbers depends on the bacterial abundance and the nature of the samples. Around the islands and coastal areas the number of PSB were higher as compared to those in the sandy beaches and offshore areas (Table 1). It is probable that the offshore organisms are poor solubilizers of inorganic phosphate because these organisms are generally low in the uptake of carbon and therefore, do not change the *pH* of the medium drastically. The ubiquitous nature of these groups of bacteria has already been reported, in the intertidal waters of open sandy beaches, estuarine and neritic waters and sediments of Bay of Bengal and in the Veli lake regions^{9,10}. Higher populations were found in clay sediments as they are rich in organic matter^{8,14}. Low numbers recorded in sediments in the present study may possibly be attributed to the sandy nature of the sediments. Five of the 88 phosphate solubilizing isolates showed higher phosphatase activity ($8.68 - 24.78 \mu\text{mol.P.ml}^{-1}\text{d}^{-1}$) with culture P63 showing the highest activity. Two offshore isolates showed very high activity compared to the island and beach isolate (Table 2). Although fewer isolates were obtained from the offshore regions the mean activity was high,

Table 1—Phosphate solubilizing bacteria in various niches.

Location	No. of cultures		No. of positive cultures						*Total (%)
	Seawater	Sediment	Seawater			Sediment			
			Range of halo						
			+	++	+++	+	++	+++	
Beaches	12	50	.	-	-	1	3	1	8.1
Islands	36	-	-	6	-	-	-	-	16.7
Coasts	460	22	8	30	32	2	1	2	15.6
Offshore	23	-	-	2	-	-	-	-	8.7

* 100% = 603 bacterial isolates

Halo formation: + Poor (0.5-1.4 cm in diameter)

++ Moderate (1.5-2.5 cm in diameter)

+++ Good (> 2.5 cm in diameter)

- Nil (0 cm in diameter)

Table 2—Phosphatase activity of the selected isolates.

Location	Number of cultures	Range ($\mu\text{g.at.P.ml}^{-1}\text{d}^{-1}$)	Mean + S.D
Beaches	5	0.102-14.694	8.065 + 5.852
Islands	6	1.287-3.450	2.584 + 0.754
Coasts	75	0.230 - 33.687	10.53 + 8.5
Offshore	2	10.902-11.241	11.173

as compared to other locations. Kobori & Taga⁷ have shown that there is an increase in activity from the coastal to the offshore region, but a decrease in activity with depth, which may be due to biotope difference¹¹. In general, phosphatase activity at offshore regions and coastal areas showed almost similar values as compared to other locations. It was seen that isolate P6 showed higher activity compared to that of P43, which may be due to strain difference and location¹⁵.

Phosphate solubilizing bacteria were not restricted to a few genera as it mainly depends on the regions. In this study the strains were mainly *Bacillus*, in addition few strains of *Vibrio* and *Klebsiella* were also encountered, whereas in oceanic regions *Pseudomonas* was the dominant group and in the Tokyo Bay it was *Flavobacterium* and *Acinetobacter*⁵. Pauluch & Szulicaka¹⁶ and Niewolak¹⁷ reported that *Pseudomonas* spp., *Bacillus* spp., *Micrococcus* spp. and *Aeromonas* spp. decomposed tribase calcium phosphate. In the Porto Novo area the dominant groups were reported¹⁸ to be *Pseudomonas* spp. and *Bacillus* spp.

The optimum temperature for enzyme activity for the bacterial isolates was found to be $27\pm 2^\circ\text{C}$. Negligible activity was found at lower temperatures. The cultures showed good activity in seawater (Fig. 2) and were not adversely affected by the change in

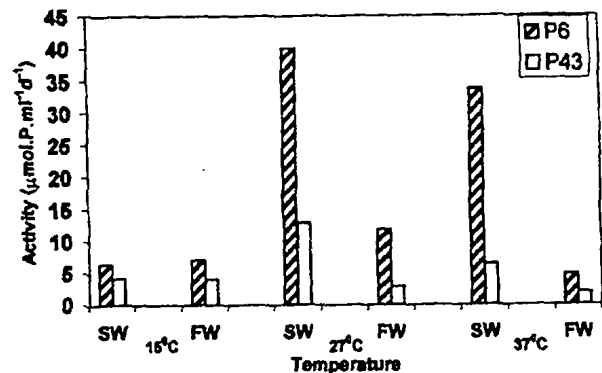


Fig.2—Effect of temperature and seawater/freshwater on phosphatase activity.

concentration of NaCl (data not shown). Strain P6 showed higher activity than strain P43 at all three temperatures. Lower and higher temperatures caused a delay in the expression of phosphatase activity in both the isolates, although 37°C was found to be much more stable of the two.

Addition of nutrients (0.001% yeast extract, 0.01% glucose) to seawater increased the growth rate and the activity showed a corresponding increase. Thus, phosphatase is growth related in seawater medium. In some cases the extracellular activity increased considerably as compared to growth which may possibly be due to cell lysis. In the case of varying concentrations of NaCl and addition of nutrients, the cultures were found to be poor growers and thus gave low levels of activity. This problem was not encountered in seawater amended with nutrients, proving seawater to be the medium for these cultures, hence strengthening the fact that the isolates are of marine origin. Maximum activity of $5 \mu\text{mol.P.ml}^{-1}\text{d}^{-1}$ was observed for strain P6 in 4.5% NaCl

Table 3—Survival of bacteria on carriers

Carriers	Population (C F U. x 10 ⁶ g ⁻¹)			
	30 days		60 days	
	P6	P43	P6	P43
Sediment	142	0.46	1.04	0.16
Ricebran	0.03	0.02	0.02	0.02
Bagasse	1.04	0.63	0.88	0.07

0day = 0.1 x 10⁸ CFUg⁻¹

concentration at 37°C whereas, it was only 0.7 µmol.P.ml⁻¹d⁻¹ for strain P43.

The PSB were then checked for their culturability and survival on three carrier materials (sediment, rice-bran and bagasse) by physical adsorption techniques. Survival rates of *Klebsiella pneumoniae* (P6) and *Bacillus pumilus* (P43) on various substrates (Table 3) showed that sediment was a better carrier, as they survive up to 60 days in air dried marine sediments. Shimizu *et al.*¹⁹ observed that conditions for optimal growth rate for immobilization were often different from free cells. It is also known that the attachment of microbial cells on carriers alter the permeability of cell membrane²⁰. However, Brodelius *et al.*²¹, opined that the understanding of the relationship between the catalytic behaviour of the fixed cell is inadequate inspite of many observations. Primarily the change in the behaviour was attributed to the availability of the nutrients.

In conclusion, the phosphate solubilizing bacteria are widely distributed in different niches with the coastal area having a high density. Alkaline phosphatase production and ability to solubilize inorganic phosphate are linked. Sediment was found to be the most efficient carrier. Hence these isolates

could serve continuously to fertilize a niche by solubilizing insoluble phosphorus compounds especially in environments where low concentrations of phosphorus causes various limitations.

References

- 1 Pomeroy L R, Smith E & Giant C M, *Limnol Oceanogr*, 10 (1965) 167
- 2 Gachter R, Meyer } S & Mares A, *Limnol Oceanogr*, 33 (1988) 1542.
- 3 Yamamoto Y, Satoh N & Hawyashi H, *Jap. J Limnol. Rikusuizatsu*, 52 (1991)205
- 4 Sinke A J C, Cottaar F H M & Keizer P, *Limnol Oceanogr*, 38(1993)1081
- 5 Lebo M E, *Limnol Oceanogr*, 35 (1990) 1279.
- 6 Bjoerkman K & Karl D M, *Mar Ecol Prog Ser*, 111 (1994) 265
- 7 Kobori H & Taga N, *J Exp Mar Biol Ecol*, 36 (1978) 23.
- 8 Ayyakkannu K & Chandramohan D, *Curr Sci*, 39 (1970) 398.
- 9 Eapen A, Maya K, Dhevendaran K & Natarajan P, *Fish Technol Soc Fish. Technol Kochi*. 30 (1993) 62.
- 10 Dhevendaran K, Sundararaj V, Krishnamurthy K & Chandramohan D, *Indian J Mar Sci*, 3 (1974) 139
- 11 Ayyakkannu K & Chandramohan D, *Mar Biol*, 11 (1971) 201
- 12 Taga N & Kobo H, *Mar Biol*, 49 (1978) 223.
- 13 Venkateswaran K & Natarajan R, *Indian J Mar Sci*, 13 (1984) 85
- 14 Lmgaraja J, Chandramohan D & Natarajan R, *Indian J Mar Sci*, 5 (1976) 218
- 15 Kobon H, Taga N & Simidu S, *Bull Jap Soc Sci Fish*, 45 (1979) 1429.
- 16 Pauluch J & Szulicka J, *XVI Ziazd Pol Tow Mikrobiol*, (1967) 352
- 17 Niewolak S. *Acta Hydrobiol*, 13 (1971) 131.
- 18 Rajendran M T, *Chemistry and microbiology of regeneration of nutrients in Vellar estuary*, Ph.D thesis, Annamalai University, India, 1974
- 19 Shimizu S, Tani Y & Yamada, *American Chem Soc Sym Ser*, 106(1979)87
- 20 Atkinson B & Fowler H W, *Adv Biochem Eng*, 3 (1974) 221.
- 21 Brodehus P, Deus B, Mosbach K & Senk M H, *FEBS Lett*, 103 (1979) 93