

## **Phosphate solubilizing bacteria: Comparison between coastal and deep sea sediments**

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### **Abstract**

Phosphate solubilizing bacteria (PSB) play a key role in the phosphate cycle by enzymatically hydrolyzing a wide variety of organic phosphorus compounds and solubilizing various major insoluble forms of bound phosphorus. PSB are ubiquitous and are found in intertidal waters, estuaries, coastal beaches and even in offshore regions like deep sea. This study compares the abundance, diversity and enzymatic activity of PSB in the coastal sediments where TOC ranged from 0.6 – 1.3% with that of the deep sea where TOC ranged from 0.1- 0.4%. PSB numbers averaged  $1.3 \times 10^4$  cfu g<sup>-1</sup> in the coastal sediments while they were  $5.0 \times 10^3$  cfu g<sup>-1</sup> in the deep sea sediments. PSB were dominated by  $\gamma$ -Proteobacteria *Pseudomonas* in the coast and *Xanthomonas* and  $\beta$ -Proteobacteria *Alcaligenes* in the deep sea sediments. The average enzyme activity of the PSB in coastal region was twice ( $0.46 \mu\text{g P ml}^{-1} \text{h}^{-1}$ ) that of their deep sea counterparts ( $0.24 \mu\text{g P ml}^{-1} \text{h}^{-1}$ ). It related positively to TOC with its variation being more influenced in the CIB sediments ( $r=0.59$ ) than in the coastal sediments ( $r= 0.22$ ). It is apparent that the enzyme activity in the coastal sediments could be more for P mobilization and in the oligotrophic deep sea it could be both for P and C mobilization.

## **Introduction**

Phosphorus (P) is an essential nutrient for sustaining marine life (Karl, 2000). It is an important macromolecule and it plays vital role in the primary production in marine environment. In some organisms it can also be present as intracellular polyphosphate storage granules (Paytan & McLaughlin, 2007). This P is transported to the ocean primarily in the dissolved and particulate phases via riverine influx. P is made available to marine bacteria and phytoplankton in the form of sinking particulate organic matter (POM). The majority of P is remineralized within the water column and very little that reaches the deep ocean floor gets locked up as insoluble inorganic and organic phosphorus compounds specifically P associated with metal oxides and hydroxides (Broecker & Peng, 1982).

Oceanic P exists broadly in two forms, dissolved and particulate. The dissolved fraction includes inorganic (generally in the soluble orthophosphate form), organic compounds, and macromolecular colloidal phosphorus. Particulate P includes living and dead plankton, precipitates of phosphorus minerals, phosphorus adsorbed to particulates, and amorphous phosphorus phases (Paytan & McLaughlin, 2007). Organically bound P is for the most part not directly available to living organisms because it cannot be taken into the cell. To be taken up, organic P must first be converted (hydrolyzed) to orthophosphate. This is done with the help of enzymes secreted by PSB.

PSB play a major role in regulating the inorganic phosphate concentration by secreting extracellular enzymes such as alkaline phosphatases. These enzymes are present in all organisms but only bacteria, fungi, and some algae are able to secrete them outside their cells. As exoenzymes, they participate in the dissolution and mineralization of organic phosphate compounds in the environment (Jones, 2002).

In the marine environment, PSB have been found in the water column (Promod and Dhevendaran, 1987) and in sediments (Ayyakkannu and Chandramohan, 1971, Devendran et al. 1974). Phosphobacteria have been found to produce some organic acids such as monocarboxylic acid (acetic, formic), monocarboxylic hydroxy (lactic, glucenic, glycolic), monocarboxylic, ketogluconic, decarboxylic (oxalic, succinic), dicarboxylic hydroxy (malic, maleic) and tricarboxylic hydroxy (citric) acids in order to solubilize inorganic phosphate compounds. Phosphate solubilization seems to be a complex biochemical phenomenon and it is important to understand the bacterial populations capable of P solubilization, realizing the multiple roles the native bacteria perform (Shri Ramkumar and Kannapiran, 2011). This study aims to compare the abundance, diversity and activity of the PSB in the coastal sediments with that of the deep sea.

## **Materials and Methods**

### **Sample collection**

Coastal sediment samples were collected from beaches of Goa namely, Anjuna, Dias Choroa, Vasco Harbour. Deep sea sediment samples were collected during the 26<sup>th</sup> & 38<sup>th</sup> expedition of R.V. *Akademic Boris Petrov* (December 2006-January 2007 & September -October 2009), of PMN-EIA (Polymetallic Nodules-Environmental Impact Assessment) programme, in the Central Indian Basin, with the help of United States Naval Electronics Laboratory (USNEL) –type box core of 50 cm x 50 cm x 50 cm dimension. Sub-cores were collected using acrylic cores of 6.3 cm inner diameter. The sediment cores were sectioned at 2 cm intervals up to 10 cm and at 5 cm interval

thereafter and collected in sterile plastic bags for further processing. Microbial samples were processed at 4°C and 1 atm pressure onboard immediately after sediment collection.

### **Total counts of bacteria**

Total counts of bacteria were estimated according to Hobbie *et al.*, 1977. About 0.5 g of sediment was diluted with 5 ml of sterile seawater and were divided into 2 sets. The first set of this slurry was fixed with buffered formalin at an end concentration of 2% and stored at 4°C until analysis.

### **Direct Viable Counts (DVC)**

Direct viable cell numbers (DVC) were determined according to Kogure *et al.*, (1984). The second set was amended with 0.001% final concentration of yeast extract and 0.0016% final concentration of an antibiotic cocktail solution (Joux and Lebaron, 1997) containing piromedic, pipemic and nalidixic acid in the ratio 1:1:1 and incubated statically in the dark at 4°C for 30h for aerobic viable count. At the end of the incubation the aliquots were fixed with 2% buffered formalin and stored at 4°C until analysis. At the on-shore laboratory, the 2.5 ml aliquot of all the sets was sonicated at 15 hertz for 15 seconds. The supernatant (1ml) was stained with 75 µl of 0.01% acridine orange (3mins, in dark) and filtered onto 0.22µm a black polycarbonate filter paper (Millipore, USA). This procedure minimized masking by sedimentary particles. About 10-15 microscopic fields per sample were counted on a Nikon 80i epifluorescence microscope. The cell counts were normalized to one gram of dry sediment.

### **Phosphate solubilizing activity (PSA)**

The PSA was measured by the release of Nitrophenol from p-nitrophenyl phosphate (substrate) by the culture (Taga and Kobori 1978). The phosphatase activity was calibrated by using Nitrophenol as standard. 1 mole of Nitrophenol is equivalent to 1 atom of phosphorus. A uniform culture suspension was prepared in filtered and sterilized seawater. 1ml of culture suspension was inoculated into 10 ml of 0.2µm filtered and sterilized seawater in screw capped tubes. To this, 1ml of filter sterilized p-NPP p-Nitrophenyl phosphate was added. The tubes were vortexed and incubated at ambient temperatures for 24 hrs. The absorbance was read at 410nm using Beckman DU-6 spectrophotometer. The activity was expressed as µg P ml<sup>-1</sup>h<sup>-1</sup>.

### **Total Organic Carbon**

Total inorganic carbon (TIC) was analysed using a UIC CM 5014 Coulometer with CaCO<sub>3</sub> as a standard and total carbon with an NCS 2500 elemental analyser (Patience, et al 1990), cross-checked with a UIC CM 5014 Coulometer. Total organic carbon (TOC) was determined indirectly by subtracting the values of TIC from total carbon (Gupta and Jauhari, 1994).

### **Pore water phosphate**

Sediment pore water phosphate was extracted and measured spectrophotometrically as described earlier (Nath and Mudholkar, 1989) using technique described in Grasshoff, 1983. Pore water was squeezed from sediment sections immediately using temperature-controlled centrifuge at 6000 rpm. The pore water obtained was filtered through 0.45µm Millipore filters and stored at 4°C. The extraction was completed within 3-4 hours of retrieving the sample. Dissolved PO<sub>4</sub><sup>3-</sup> was determined colorimetrically using standard photometric methods for seawater analyses (Grasshoff *et al.*, 1983).

Briefly, to 10 ml of distilled water blank, working standards (1, 2, 3  $\mu\text{M}$ ), diluted pore water (2 times; 5 ml sample + 5 ml distilled water), 0.5 ml ascorbic acid (reducing agent) was added. After mixing, 0.5 ml of ammonium molybdate reagent was added to the blanks, standards and pore waters. The absorbance of complex was measured at 880 nm after 30 minutes. From these absorbance values, concentration of pore water phosphate was calculated. Duplicates were run in order to check the precision of the analyses.

## Result and Discussion

The deep sea and coastal sediments possess different characteristics. The deep sea sediments are organically poor with high pressure, extreme temperature and are usually under darkness (Li and Kato 1999). The coastal sediments are exactly the opposite with high organic load and productivity (Baars, et al 1998, Desai and Bhargava, 1998), normal temperature and pressure and plenty of light. These two systems are compared to study the PSB with respect to their abundance, diversity and activity. Phosphate solubilizing bacteria are ubiquitous in the nature, but may vary in their numbers and function in various ecosystems as here in deep sea and the coastal sediments. Generally, in the marine environment, P input flux, largely consists of P bound in the organic matter (Ruttenberg, 2003). This bound P has to be liberated from the organic matter prior to release from the sediment, which is done by extracellular enzymes such as phosphatases (Steenbergh et. al., 2011).

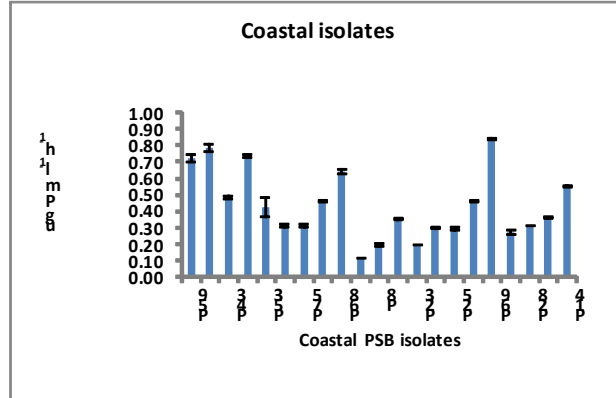
The total bacterial numbers of the coast were comparable with that of the deep sea (Table 1). In the coast it ranged from  $1.15 - 1.79 \times 10^{10}$  cells  $\text{g}^{-1}$  and in the deep sea it ranged from  $0.86 - 1.41 \times 10^9$  cells  $\text{g}^{-1}$ . The viability count of the coastal sediments averaged to  $4.57 \times 10^8$  cells  $\text{g}^{-1}$  and that of the deep sea were  $3.27 \times 10^8$  cells  $\text{g}^{-1}$ . The heterotrophic bacterial numbers were high in the coast and ranged from  $1.10 - 9.30 \times 10^7$  cfu  $\text{g}^{-1}$  as compared to their deep sea counterparts which ranged from  $1.50 - 7.82 \times 10^3$  cfu  $\text{g}^{-1}$ . Phosphate solubilizing bacteria formed 11% of the total heterotrophic counts in the coastal beaches where as, they formed only 1% in the deep sea. Das et. al., 2007 reported 7.57% of total heterotrophic bacteria to be PSB in the Bay of Bengal sediments.

Phosphate solubilizers counts in the coast were one order higher than the deep sea with average values of  $1.3 \times 10^4$  cfu  $\text{g}^{-1}$  and  $5.0 \times 10^3$  cfu  $\text{g}^{-1}$  respectively. De Souza et al, 2000 also reported higher phosphate solubilizing bacterial population in coastal areas than offshore areas.

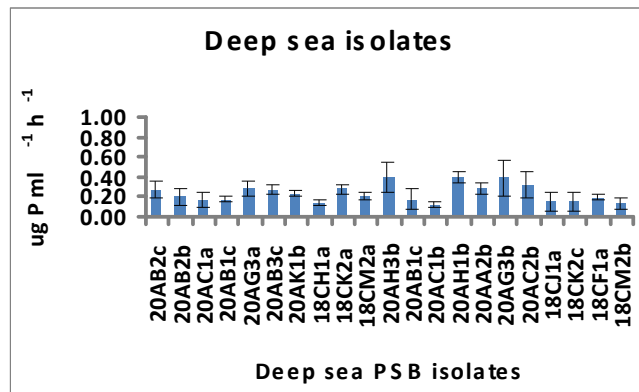
The biochemical identification of the Phosphate solubilizing bacterial isolates showed that most of the coastal cultures belonged to *Bacillus* of division Firmicutes and *Pseudomonas* of  $\alpha$ -Proteobacteria, in addition few strains of *Vibrio* and *Klebsiella* were also encountered. Kannapiran and Ravindran also encountered *Bacillus*, *Pseudomonas* and *Vibrio* spp from coral reefs of Gulf of Mannar. Promod and Dhevendaran (1987) and De Souza et al (2000) reported *Bacillus* as the dominant group in the inshore areas. In the deep sea isolates *Xanthomonas* and *Alcaligenes* were dominant. The other genera participating in this activity were *Flavobacterium* and *Micrococcus*.

Phosphate solubilizing activity (PSA) of the coastal isolates were twice higher than those of the deep sea isolates. It ranged from  $0.102 - 0.865$   $\mu\text{g P ml}^{-1}\text{h}^{-1}$  in the coast and  $0.119 - 0.397$   $\mu\text{g P ml}^{-1}\text{h}^{-1}$  in the deep sea (fig:1a & 1b). The higher phosphate solubilization was observed in the bacterial cultures isolated from coastal regions than in the deeper regions by Das et al. 2007.

**Figure1a: Coastal isolates showing phosphate solubilizing activity.**

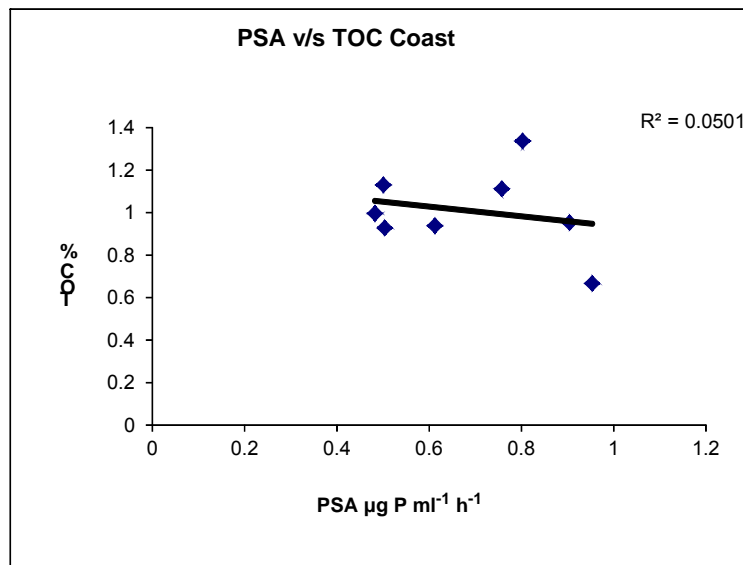


**Figure1b: Deep sea isolates showing phosphate solubilizing activity.**

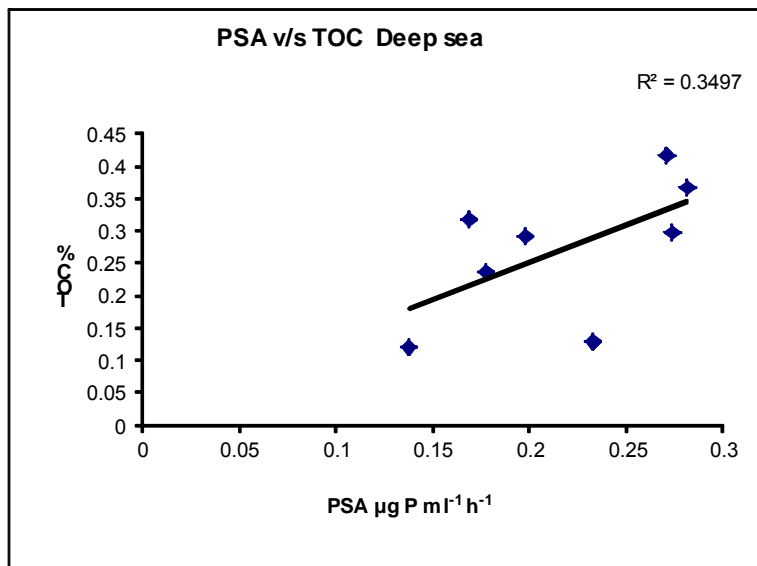


Total organic carbon content of the two regions was different with the values ranging from 0.6 – 1.3% in the coastal sediments (Gonsalves et al, 2011) and 0.1 – 0.4% in the deep sea (Das et al, 2011). In the deep sea, about 34 % of the variation in PSA was due to variation in TOC where as in the coastal region it was only about 5% (fig: 2a &2b). While all the bacterial parameters like heterotrophic, total and viability counts were more in the coastal sediments, the relationship of PSA with TOC ( $r= 0.59$ ) was higher in the deep sea.

**Fig:2a-Scatter plot of phosphate solubilizing activity v/s Total organic carbon in the coast**



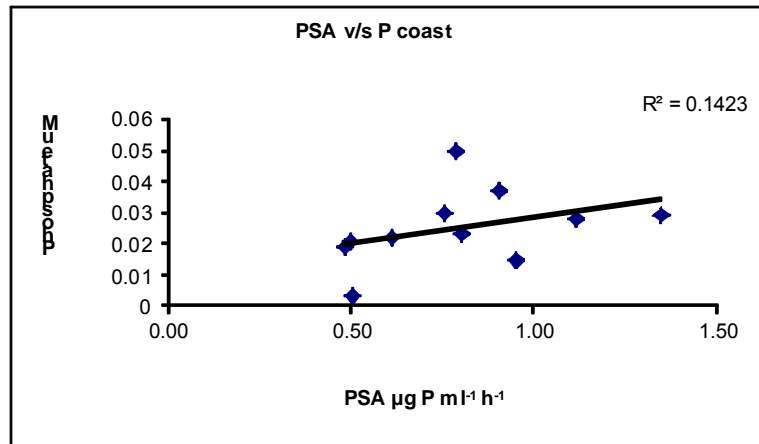
**Fig:2b-Scatter plot of phosphate solubilizing activity v/s Total organic carbon in the deep sea**



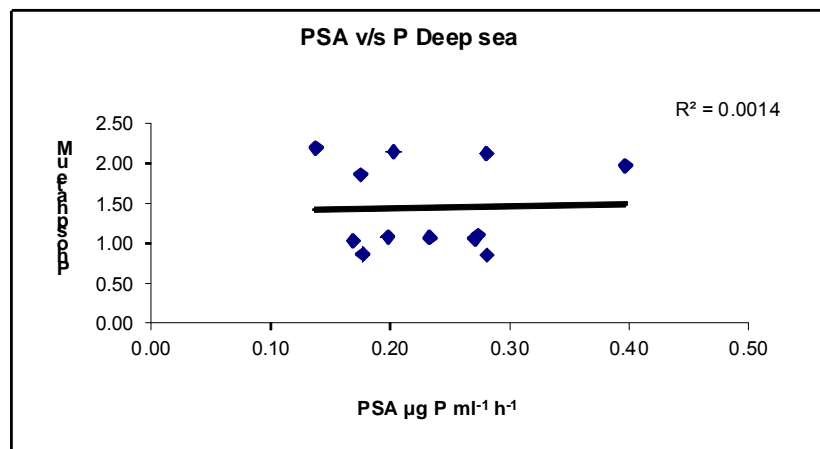
Phosphate in the coastal sediments ranged from 0.01-0.05 and 1-2 $\mu\text{M}$  in the deep sea sediments. It is a major component of the seawater and mostly is a limiting nutrient in the coastal waters, as inorganic phosphates in association with metal cations become insoluble and therefore unavailable to organisms (Overbeck and Chrost, 1994). In the coast, P and PSA showed positive relationship where 14% of the variation in PSA was responsible for variation in the phosphate (fig 3a). PSA in the deep sea showed no relationship with P (fig 3b) indicating that bacteria here were not P-limited. Thus, it is probable that PSB in the coastal region are mostly engaged in P regeneration while in the oligotrophic deep sea they are responsible for C mobilization. Also, the deep sea bacteria are poor

solubilizers of inorganic phosphate, as these organisms are held back by an insufficient supply of easily degradable organic compounds (Hoppe and Ullrich, 1999).

**Fig:3a Scatter plot of phosphate solubilizing activity v/s Pore water phosphate in the coast**



**Fig:3b Scatter plot of phosphate solubilizing activity v/s Pore water phosphate in the deep sea**



From this study it is evident that PSB are active in the coastal sediments with twice the PSA of CIB sediments. Also, it is probable that PSA in the coastal sediments could be more for P mobilization whereas in oligotrophic CIB sediments, it could be for both P and C mobilization. Hoppe H.G. 2003 also avers that phosphatases play a dual function of supplying inorganic phosphorus as well as organic carbon to the deep sea sediments, as these bacteria are C limited.

Further studies on how these PSB interact with organic polymers like nucleic acids, phospholipids and phosphoproteins will throw more lights on P dynamics in these contrasting systems. These bacteria can therefore have wide ranging implications/applications in the ecosystems that are P limited.

**Table:1 Average values of measured parameters at coast and deep sea.**

Parameter	Coast	Deepsea
TC	$1.49 \times 10^{10}$ cells $g^{-1}$	$1.41 \times 10^9$ cells $g^{-1}$
TVC	$4.57 \times 10^8$ cells $g^{-1}$	$3.27 \times 10^8$ cells $g^{-1}$
THC	$3.56 \times 10^7$ cfu $g^{-1}$	$6.11 \times 10^3$ cfu $g^{-1}$
PSB	$1.30 \times 10^4$ cfu $g^{-1}$	$4.99 \times 10^3$ cfu $g^{-1}$
Activity	0.46 $\mu g$ P $ml^{-1} h^{-1}$	0.24 $\mu g$ P $ml^{-1} h^{-1}$
PO <sub>4</sub>	0.03 $\mu M$	1.45 $\mu M$
TOC	1% 10076.25 $\mu g$ $g^{-1}$ 839.69 $\mu g$ atoms	0.2% 2720 $\mu g$ $g^{-1}$ 226.67 $\mu g$ atoms

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