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ORIGINAL ARTICLE

Occurrence and Abundance of Phosphatase-Producing Bacteria in Mangrove Ecosystem

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ABSTRACT

The study detailed the isolation and characterization of phosphatase producing bacteria (PPB) from rhizospheric, pneumatophoric and bulk sediments of Avicennia marina along Mumbai coast, India and also estimated alkaline phosphatase activity in sediment in addition to the Phosphatase enzymatic activity of isolates. The inorganic-organic nature of phosphorus was also examined. About, 42 PPB were isolated and identified from the sediment samples. 16S rDNA sequence revealed that all the isolates belong to the genus Bacillus except the one which belong to Geobacillus in the environment and B. anthracis str. Ames showed the phosphatase-producing activity of 84.11±0.01 µg p-NP released per 1×108 cfu in 72 hrs. The isolates, B. cereus strain APT23 and B. thuringiensis strain INRS4 showed the next best phosphatase activity. It was observed that PPB was very much abundant in A. marina mangroves. The pneumatophoric sediment showed significantly higher number of PPB with higher alkaline phosphatase activity, inorganic P and low organic P than those of other sediment types indicating that pneumatophoric region of A. marina harbours favourably the phosphatase-producers than the rhizospheric or bulk sediments and forms the first reference worldwide depicting this relationship. The isolates that showed higher phosphatase-producing potentials can be explored for using as phosphatic bio-fertilizer to enhance the agricultural, aquacultural and mangrove productivity on a larger scale.
***Key words**: Phosphatase-producing bacteria, alkaline phosphatase activity, Avicennia marina*

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INTRODUCTION

Mangrove forests are located in the tropical and subtropical areas between the aquatic and the terrestrial environment [1]. The biodiverse mangrove forests provide a range of benefits as ecosystem services mostly to local communities living in close proximity to the forests [2]. Mangrove productivity is mainly assured by sediment and rhizosphere microorganisms. The Phosphorus (P) in nature gets immobilized rapidly whereas the mobilization is a slow process by the microorganisms which are a major part of the soil P cycle as they help in soil transformation and releasing P from organic pools of soil P through mineralization. In marine sediments, abundance of cation in the interstitial water makes phosphorus gets precipitated and largely unavailable to plants. PPB as potential suppliers of soluble forms of phosphorus would provide a great advantage to mangrove plants which produce extracellular enzymes such as alkaline phosphatase and are able to mineralize organic phosphates into inorganic form that eventually become available to the plants [3]. Alkaline phosphatase is a hydrolase enzyme responsible for removing phosphate groups (dephosphorylation) from many types of molecules including nucleotides, proteins and alkaloids. It is responsible for microbial mineralization of organic phosphate compounds and catalyzes the phosphate-derived molecules to produce inorganic phosphate and a hydrolyzed molecule.

Mangrove sediments act as a sink for phosphorus with high retention capacity [4]. The rhizospheric (true roots) and pneumatophoric (respiratory root) zones may harbour unique bacterial community than that of bulk sediments. Therefore, present study was aimed to isolate and characterize phosphatase-producing bacteria in rhizospheric and also in pneumatophoric sediment which was compared with bulk or non-rhizospheric sediment.

MATERIALS AND METHODS

Sample Collection and Processing:

The sampling was carried at Alibag, Gorai, Mahul and Versova along Maharashtra, India during 2013. The coordinates of the sampling points were recorded using GPS (eTrex Venture® HC, Garmin, USA) and were shown in figure 1. For an elaborate comparison, the sediments were collected by carefully removing the soil adhering in a 2-3 mm thickness around the individual roots of *A. marina* and termed as rhizospheric sediment. Bulk sediment without any roots was collected in the depth of 0-15 cm. The sediment at the base of the pneumatophores was collected and referred as pneumatophoric sediment. All sediment samples were also collected aseptically in sterile Uricol bottles (Hi-Media, India) for the microbiological and phosphatase enzyme study. Organic and inorganic P in sediment was estimated by ignition method [5].

Isolation and 16S r DNA Sequencing of Phosphatase-producing bacteria

Phenolphthalein-phosphate agar was the media used for PPB isolation by selecting the pink coloured colonies on exposure to ammonia vapour. Pour plate method was followed by adopting the standard dilution procedure. The bacterial isolates were sub-cultured continuously and checked for purity using Gram staining technique. The DNA extracted from the isolates was analysed using 1.0% agarose gel pre-stained with ethidium bromide. The 16S rDNA of the bacterial isolates were amplified using primers 27F and 1492R [6]. The amplified PCR products were sequenced at GeNei Pvt. Ltd., Bangalore and the sequence was searched on GenBank Database of National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm and thus the closest similarity was determined.

Estimation of Alkaline Phosphatase Activity of PPB

The phosphatase activity (mineralization) was measured by using para-nitrophenyl phosphate (*p*-NPP) as organic P substrate. When *p*-NPP loses its phosphate group, and becomes para-nitrophenol (*p*-NP), the solution turns yellow. Therefore, the activity of the enzyme is measured by the concentration of *p*-NP released. The bacterial strains were cultured in marine broth for 72 hours.

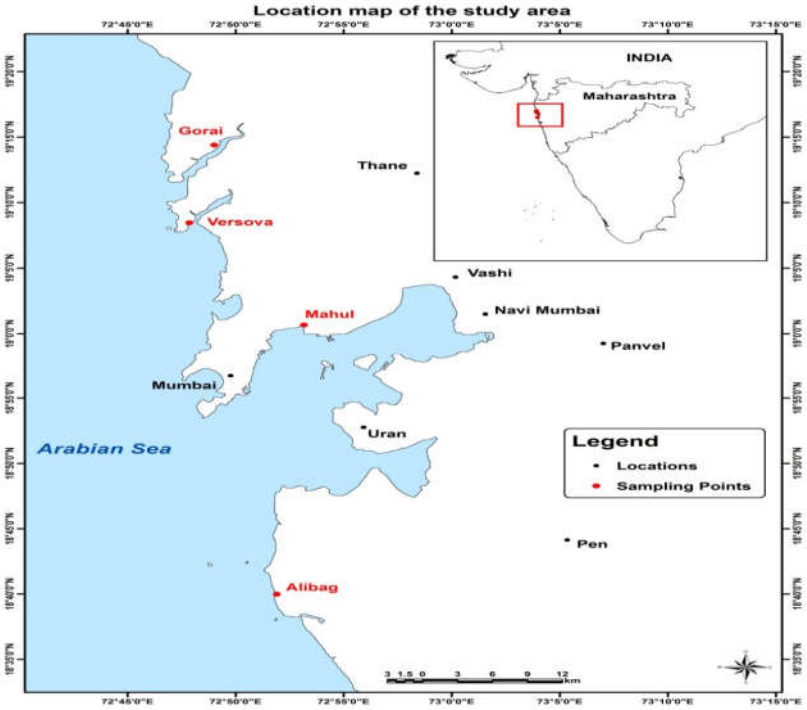

Figure 1: Location of the study area

To this 0.5 ml filter-sterilized solution of the substrate *p*-NPP (0.005 *M*) was added. A corresponding tube was prepared with 0.5 ml substrate, and 1 ml sterilized distilled water was added in place of the bacterial culture and maintained as control. The tubes were incubated for 72 hours at 28 $\pm 1^0$C. After incubation, one ml of 0.2 *N* Sodium carbonate was added; centrifuged at 3000 rpm for 15 minutes and *p*-NP released was measured at 418 nm using spectrophotometer. The absorbance of the control was subtracted from the experimental ones to calculate the amount of *p*-NP released by the isolates. The activity is expressed as µg *p*-NP released per 10^8 cfu in 72 hrs [7].

Estimation of Alkaline phosphatase activity of sediment

Phosphatase activity is measured in terms of quantity of *p*-nitrophenol released from the substrate p-nitrophenyl phosphate. One gram sediment each was taken in two 50 ml conical flasks. Out of these two, one conical flask was used as control and other as substrate flask. 0.2 ml of toluene and 4 ml of MUB (Modified universal buffer) solution of pH 11 was added to both flasks. One ml of p-nitrophenyl phosphate (*p*-NPP) solution was added to substrate conical flasks. After proper swirling stoppered them and placed in incubator at 37˚C for one hour. After incubation, the stopper was removed and one ml of 0.5 *M* $CaCl_2$ and four ml of 0.5 *M* NaOH was added. One ml of *p*-NPP solution was added to the control. All the suspensions were quickly filtered through Whatman No. 2 filter paper and intensity of the yellow colour (*p*-nitrophenol) in the filtrate was measured at 440 nm using UV-visible spectrophotometer and compared with the standard graph prepared with different concentrations of *p*-nitrophenol (*p*-NP). The results are expressed as µg *p*-NP released g^{-1} hr^{-1}. [8]

Statistical Analysis

All statistical analysis was carried out using SPSS 16.0 (SPSS Inc., Chicago, llinois, USA). Duncan homogenous grouping of means was carried out at p =0.05 for the significant effects. One-way ANOVA was carried out for the phosphatase activity of the bacterial isolates.

RESULT

Phosphatase-producing bacteria

Phosphatase-producing bacteria were isolated using phenolphthalein-phosphate agar. The bacteria which are able to produce phosphatase enzyme utilized the phenolphthalein phosphate as a source of phosphorus, and breaks down into phenolphthalein and phosphate. When the colonies were exposed to ammonia vapour, pH of the media increases, pink colouration indicates the formation of phenolphthalein (Fig. 2).

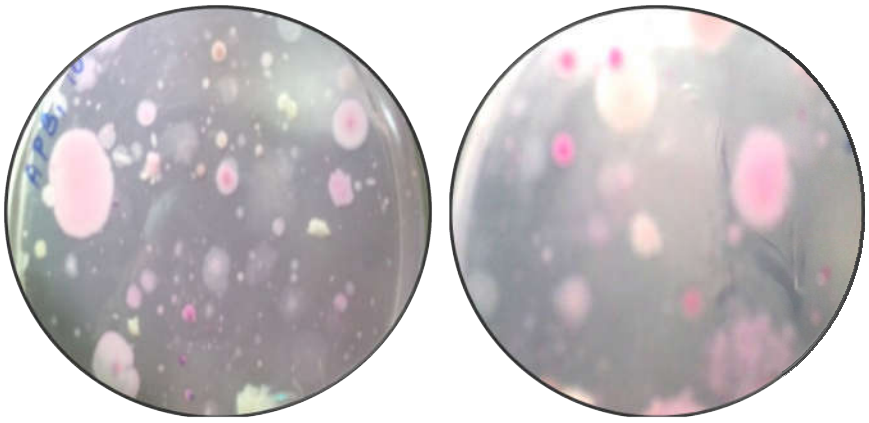

Fig. 2: Phosphatase-producing bacteria on phenolphthalein-phosphate agar (Pink-coloured colonies indicate mineralization of organic phosphate)

Distribution of Phosphatase-producing bacteria (PPB)

The pneumatophoric sediment had higher PPB activity (28.38×10^3 cfu g^{-1}) when compared to bulk and rhizospheric sediments. The sediments of Alibag, Versova and Mahul were found to be having higher PPB load than that of Gorai (Table 1).

Table 1*: Distribution of phosphatase-producing bacteria (cfu × 10^3 g^{-1}) in phenolphthalein-phosphate agar medium

	Sediment type			Mean
Site	Bulk	Pneumatophore	Rhizosphere	
Alibag	1.62	31.73	21.35	18.23^b
Versova	2.98	30.87	19.63	17.83^b
Gorai	0.98	22.65	16.70	13.44^a
Mahul	6.59	28.26	19.39	18.08^b
Mean	3.04^a	28.38^c	19.27^b	16.89

From all the four sampling sites, 64 isolates were selected; purified using repeated streaking and genomic DNA could be extracted only from 42 isolates. 16S rDNA of these 42 isolates were sequenced and all 42

isolates belong to *Bacillus* genus and almost 25 isolates were found to be *B. anthracis* strain mainly from the sediment of Versova mangrove area (Table 2).

Enzymatic Characterization of Bacteria

Twenty-four isolates were selected (isolates with > 40 µg *p*-NP released per 10^8 cfu in 72 hrs) for the Duncan grouping based on phosphatase activity. The enzyme activity of isolate *B. anthracis* str. Ames strain Ames *(*84.11±0.01 µg P released per 1×10^8 cfu in 72 hrs) was higher than other 23 isolates. It was observed that greater numbers of isolates were found from pneumatophoric sediment. Rhizospheric sediment also had higher number of PPB when compared to bulk (Table 2).

Table 2*: Distribution and phosphatase activity of PPB (Mean±SD; µg *p*-NP released per 10^8 cfu in 72 hrs)

Isolate	Site	Sediment	Activity
Bacillus sp. NII-73	Alibag	B	42.10±0.01^{abc}
B. cereus strain Chi	Alibag	P	45.54±0.02^{abcde}
B. cereus strain JKR62	Gorai	P	42.56±0.01^{abc}
B. cereus strain BQAR-01d	Mahul	P	51.06±0.03^{efgh}
B. cereus isolate BRL02-31	Mahul	P	47.65±0.02^{cdef}
B. anthracis str. Ames strain Ames	Versova	P	43.93±0.01^{abcd}
B. anthracis str. Ames strain Ames	Versova	B	51.98±0.02^{efgh}
B. thuringiensis strain IAM 12077	Versova	R	53.76±0.01^{efghi}
B. thuringiensis strain	\Versova	R	60.47±0.01^{defgh}
B. anthracis str.	Versova	P	41.92±0.01^{abc}
B. anthracis str. Ames strain	Versova	P	74.18±0.01^{l}
B. anthracis str. Ames strain	Versova	P	44.74±0.02^{abcd}
B. thuringiensis strain IAM 12077	Versova	P	40.06±0.02^{a}
B. anthracis str. Ames strain Ames	Versova	P	49.52±0.01^{defgh}
B. anthracis str. Ames strain Ames	Versova	P	84.11±0.01^{m}
B. anthracis str. Ames strain Ames	Versova	P	49.40±0.01^{defgh}
B. cereus strain APT23	Versova	P	71.99±0.00^{l}
B. cereus strain Chi	Versova	P	47.72±0.01^{defg}
Bacillus sp. DU98(2010)	Versova	P	46.81±0.01^{bcde}
B. thuringiensis strain INRS4	Versova	P	65.06±0.01^{l}
B. cereus strain TD2	Versova	R	43.53±0.01^{abcd}
Bacillus sp. NII-12	Versova	R	59.24±2.52^{opqr}
B. anthracis str. Ames strain	Versova	R	42.27±0.01^{abc}
B. anthracis str. Ames strain	Versova	R	46.92±0.02^{bcdef}

B-Bulk sediment, P-Pneumatophoric sediment, R-Rhizospheric sediment

Alkaline Phosphatase Activity

Alkaline phosphatase activity in Gorai sediments (10.04 ± 4.04 µg *p*-NP released g^{-1} hr^{-1}) was significantly higher than that of other sites. Alibag sediment showed significantly lower activity (7.02 ± 2.65 µg *p*-NP released g^{-1} hr^{-1}) than that of Mahul and Versova. The alkaline phosphatase activity of pneumatophoric sediment (14.53 ± 3.22 µg *p*-NP released g^{-1} hr^{-1}) was higher than that of other sediment types (Table 3).

Table 3: Distribution of alkaline phosphatase activity (µg *p*-NP released g^{-1} hr^{-1}) of sediments

Site	Sediment type			Mean
	Bulk	Pneumatophore	Rhizosphere	
Alibag	4.68 ± 0.31	10.55 ± 0.92	5.82 ± 0.25	7.02 ± 2.65^{a}
Mahul	3.17 ± 0.61	13.88 ± 1.15	4.79 ± 0.65	7.28 ± 4.87^{a}
Versova	4.44 ± 0.79	19.06 ± 1.27	3.32 ± 0.82	8.94 ± 7.37^{b}
Gorai	10.43 ± 0.48	14.65 ± 0.50	5.04 ± 0.69	10.04 ± 4.04^{c}
Mean	5.68 ± 2.89^{b}	14.53 ± 3.22^{c}	4.74 ± 1.10^{a}	8.32 ± 5.12

Organic Phosphorus

Organic P in the sediment was significantly higher in Mahul (422.76±295.82 mg kg^{-1}) than the other sites. Alibag (125.12±50.28 mg kg^{-1}) had significantly lower organic P. Among the sediment types, rhizospheric sediment (632.86±269.68 mg kg^{-1}) had significantly higher organic P than that of bulk and pneumatophoric sediment. Pneumatophoric sediment had significantly the lowest organic P (Table 4).

Table 4: Distribution of Organic phosphorus (mg kg^{-1}; mean ±S.D)

Site	Sediment type			Mean
	Bulk	Pneumatophore	Rhizosphere	
Alibag	145.51 ± 3.31	57.31±4.28	172.53±4.27	125.12±50.28[a]
Mahul	398.49±3.92	80.03±3.42	789.75±6.99	422.76±295.82[d]
Versova	329.63±3.66	96.09±3.54	774.88±3.49	400.20±286.96[c]
Gorai	253.58±10.06	73.05±1.36	794.27±4.80	373.64±312.33[b]
Mean	281.80±95.4[b]	76.62±14.48[a]	632.86±269.68[c]	330.43±282.98

Inorganic Phosphorus

The Mahul sediments (1078.90±382.37 mg kg^{-1}) showed significantly higher inorganic P than that of others. Alibag sediments (396.69±10.01mg kg^{-1}) had the significantly lower inorganic P. The pneumatophoric sediment showed significantly higher inorganic P than other two sediment types. The rhizospheric sediment had significantly the lowest inorganic P (Table 5).

Table 5: Distribution of inorganic P (mg kg^{-1}; mean ±S.D) in different sediment types of the study sites

Site	Sediment type			Mean
	Bulk	Pneumatophore	Rhizosphere	
Alibag	395.90±3.30	408.57±2.42	385.58±3.28	396.69±10.01[a]
Mahul	1577.30±2.89	987.50±3.28	671.93±4.49	1078.90±382.37[d]
Versova	917.14±2.09	1343.50±8.72	553.57±5.07	938.08±329.05[b]
Gorai	1020.20±4.61	1278.70±4.31	675.99±2.94	991.61±251.61[c]
Mean	977.61±425.31[b]	1004.60±374.58[c]	571.77±119.95[a]	851.32±386.44

DISCUSSIONS

The pneumatophoric sediment had low level of organic P when compared to bulk and rhizospheric sediment. Moreover, high density of phosphatase-producing bacteria was observed in the pneumatophoric sediment, and hence the conversion of organic P to inorganic form occurred by the microbial action. It has been suggested that higher phosphatase in rhizosphere, compared to the bulk soil, can induce significant depletion of organic P in the rhizosphere [10]. It is also reported that microbes involved in mineralizing of organic P into orthophosphates, solubilize the inorganic P compounds converting the inorganic available anion into cell protoplasm and bringing about an oxidation or reduction of inorganic P compounds [11]. Alkaline phosphatase was observed to be higher in pneumatophoric sediment. The presence of significantly higher number of phosphatase-producing bacteria in the pneumatophoric region when compared to other samples justifies the higher alkaline phosphatase activity.

Study shows that three species of phosphobacteria belonging to the same genus (*Bacillus*) was isolated from Pichavaram mangrove, India [12] supports our results. The species diversity was found maximum in the roots and rhizospheric sediment of mangrove plant species. This is due to the secretions from root exudates of plants enhancing the growth and multiplication of bacterial species [13, 14]. Tarafdar and Jungk, (1988) reported that almost half of the microorganisms in soil and on plant roots were able to mineralize the organic P through the production of phosphatase. The phosphatase-producing bacteria in phenolphthalein-phosphate agar ranged from 0.98×10^3 to 21.35×10^3cfu g^{-1}. Phosphatase-producers showed higher activity in pneumatophoric sediment. The higher bacterial load, alkaline phosphatase activity, inorganic P and low organic P in the pneumatophoric sediment indicates the possibility of harbouring favourably the phosphatase-producers in pneumatophore than rhizosphere or bulk sediments. Phosphatase-producing bacteria have capability to produce extracellular enzymes such as phosphatase [3]. This enzyme is able to mineralize organic phosphates into inorganic phosphates that provide high P for plant. Soil phosphatases play a major role in the mineralization processes(dephosphorylation) of organic P substrates. The colonization of pneumatophores by

endophytic bacteria enhances growth of the entire plant, increasing productivity and the yield of reproductive organs [15]. It was noted that all 42 phosphatase-producing bacterial isolates identified belonged to the genus *Bacillus*. One isolate was *Geobacillus* sp and almost 25 isolates were found to be *B. anthracis* strain observed mainly from sediment samples of Versova mangrove area. The all stages of the anthrax life cycle occur in soil, including germination of spores, bacterial reproduction and formation of new spores. And the research demonstrates that anthrax can complete its full life cycle without a mammalian host [16]. Hence, Versova receives discharges from agricultural and animal husbandry apart from domestic sewage may harbour *B. anthracis* strains. *Bacillus* is the most abundant genus in the rhizosphere and the PGPR activity of some of these strains has been known for many years, resulting in a broad knowledge of the mechanisms involved [17]. About 95% of Gram-positive soil bacilli belong to the genus *Bacillus*. The strain with significantly higher activity was found as *B. anthracis* str. Ames (84.11±0.01 µg P released per 1×108 cfu in 72 hrs), followed by *B. cereus* strain APT23 and *B. thuringiensis* strain INRS4. It was observed that higher numbers of isolates could be isolated from pneumatophoric sediment. Rhizospheric sediment also had higher PPB isolates when compared to bulk sediment. It is reported that strains produced extracellular alkaline phosphatase ranged between 2.96±0.28 and 12.65±0.13 mU ml-1 [18]. *Bacillus* cereus has been reported as the dominant form in mangrove swamps, and also with higher phosphatase activity [19]. It is reported that phosphatase activity of the PSB strain KPB6 had higher activity (28.78±1.18 µmole g-1 h-1) followed by the strain KPB5 with 26.13±1.10 µmole g-1 h-1 as phosphatase activity [20]. As *B. anthracis* is an opportunistic animal and human pathogen, in spite of it showing the maximum phosphatase activity, other two isolates (*B. cereus* strain APT23 and *B. thuringiensis* strain INRS4) with second best activity can be selected for further study to explore them as potential phosphatase-producing bacteria to enhance the P availability in the salt-affected areas.

CONCLUSIONS

All phosphatase-producing bacteria isolated and identified in the mangrove sediment belong to the genus *Bacillus* except the one (*Geobacillus* sp.) and *Bacillus anthracis* was the predominant bacteria found in Versova mangrove sediments. Higher abundance of phosphatase-producing bacteria was found in the pneumatophoric sediment with clear indication of low organic P in the pneumatophoric sediment than that of rhizospheric and bulk sediment. Alkaline phosphatase activity was higher in the pneumatophoric sediment. Lower organic P in pneumatophoric sediment implies the utilization of organic P by the bacterial load and conversion to inorganic forms. As *B. anthracis* is a pathogenic strain, other two isolates (*B. cereus* strain APT23 and *B. thuringiensis* strain INRS4) with second best activity can be selected for further study to explore them as potential phosphatase-producing bacteria to enhance the P availability in the salt-affected areas.

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