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Isolation of Phosphorus Solubilizing Bacteria from Mangrove Rhizospheric Sediment and its Potential Application in Aquaculture

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ABSTRACT

Background: Phosphorus acts as a limiting nutrient in aquatic environments due to its non-availability as it forms insoluble precipitate with Ca, Mg, Fe and Al. Phosphorus solubilising bacteria (PSB) solubilizes the inorganic precipitate into soluble phosphorus.

Methods: The PSB isolates were screened and isolated from rhizospheric sediment of mangrove, *Avicennia marina* of Ennore creek, Tamil Nadu. Their phosphorus transformation potential and the possibilities of their application in aquaculture to solubilize inorganic phosphorus were studied.

Result: In the present study, thirteen nos. of PSB isolates were screened from the rhizospheric sediment samples of *Avicennia marina* collected from Ennore region and identified as *Rhodococcus, Arthrobacter, Bacillus, Enterococcus, Staphylococcus* and *Pseudomonas*. The P-solubilizing activity and Acid phosphatase (ACPase) activity of PSB isolates were found to be in the range of 7.62±0.51- 16.33±0.84 mg/l and 4.40±0.28 - 22.77±0.32 μmol ml^{-1} h^{-1} respectively. The maximum ACPase activity was exhibited by *Arthrobacter* sp (22.77±0.32 μmol ml^{-1} h^{-1}) followed by *Rhodococcus* sp. (18.08±0.78 μmol ml^{-1} h^{-1}). The P-mobilizing potential of the isolates obtained in this study were compared with the commercial product following a microcosm study for a period of 21 days. *Rhodococcus* sp showed higher ACPase activity in sediment (12.00 μmol g^{-1} h^{-1}) than that of the commercial product (11.23 μmol g^{-1} h^{-1}).

Key words: *Arthrobacter* sp, Mangroves, Phosphorus solubilising bacteria, *Rhodococcus* sp.

INTRODUCTION

Phosphorus is one of the essential major nutrients that limits the productivity in the aquaculture ecosystem, even though it is found abundantly in earth's crust. Because of its high reactive nature, it forms insoluble precipitates or forms complexes with soil and organic matter. Phosphorus solubilising bacteria (PSB) dissociate phosphorus (P) from soil complexes through several mechanisms, such as the production of organic acids that dissolve or chelate inorganic phosphate or the production of phosphatase enzymes that dissociate phosphorus from organic sources. Several studies have reported that bacterial species such as *Bacillus*, *Azobacter*, *Pseudomonas* have the potential to solubilise inorganic P (Sundara *et al.*, 2002; Ghaderi *et al.*, 2008; Amutha *et al.*, 2014). Several studies were carried out to screen PSB from a variety of land ecosystems in search of P mineralising and solubilising microbes for their application as biofertilizers in agriculture (Alori *et al.*, 2017; Garcia - Fraile *et al.*, 2017; Kalayu 2019; Aruna and Jain, 2021). Recent researches are focusing on the screening of salt tolerant microbes in warrant of their application in salt affected land to improve soil fertility (Iwaoka *et al.*, 2018; Zhang *et al.*, 2018; Egamberdieva *et al.*, 2019; Aruna *et al.*, 2023). Very few studies have been carried out to screen PSB in aquatic ecosystems, particularly from mangrove areas. Thus, the present study was carried out to isolate potential PSB isolates from the rhizospheric sediments of *Avicennia marina*, grey mangroves. As the mangrove region is prone to salinity fluctuation, the bacterial isolates obtained from this region are able to perform nutrient mobilization in low saline conditions to marine conditions. Hence, PSB can serve as a promising tool in integrated nutrient management in aquaculture. Despite high background phosphorus levels due to its non-availability, extraneous phosphorus fertilizers are still used to increase aquaculture primary productivity. The application of salinity-tolerant PSB as biofertilizer will be an effective, eco-friendly and economical means of improving the P availability or soil fertility.

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MATERIALS AND METHODS

Sampling sites

The present study was carried out in the mangroves of Ennore creek, Chennai. The sampling stations are given in Table 1 and Fig 1.

Isolation of phosphorus solubilising bacteria

The rhizospheric sediments of *Avicennia marina* from Ennore region were collected aseptically by carefully removing the soil adhering to 2-3 mm thickness around the individual roots. Serially diluted soil samples were plated on NBIRP medium for selective screening of PSB. The bacterial colony that forms hallow zone around the colony were picked up and pure colonies were obtained by sub-cultured by streak plating method. The study was carried out Dr MGR Fisheries College and Research Institute, TNJFU, Ponneri for the period of 2018-2022.

Genomic DNA isolation, 16S rDNA sequencing, RAPD amplification of 16S rDNA

The genomic DNA was extracted from the bacterial isolates (using QIAamp genomic DNA kits (Qiagen, Germany) as per manufacturer's protocol. PCR amplification of DNA using the universal 16S rDNA primer of Weisburg *et al.* (1991). Nucleotide sequencing (forward and reverse) was done by commercial sequencing services (Eurofins, India). The edited gene sequence was compared against the GenBank database of the National Center for Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool) program (http://blast.ncbi.nlm.nih.gov) and submitted to GenBank.

Table 1: Sampling stations.

Stations	Geographical position
Station 1	Lat 13.24948°N; Long 80.3175°E
Station 2	Lat 13.24564°N; Long 80.31255°E
Station 3	Lat 13.25199°N; Long 80.3187°E
Station 4	Lat 13.42191°N; Long 80.3209°E

Measurement of P-solubilizing capability of bacteria (PSB) isolates in solid medium

The isolated pure strains of PSB isolates were incubated at 37°C for 96 h. The P-solubilizing capacity was observed by measuring the width of the hallow zone between the bacterial colony and outer ring of the hallow zone and its size was measured by the P-solubilizing ring is a clarified area around the bacterial colony (Cao *et al.*, 2018).

Measurement of phosphate solubilizing activity of PSB isolates in liquid medium

Inoculating One ml of PSB isolate grown at broth culture at 10^8 CFU/ml in 25 ml of sterilized broth and incubated for 72 h at room temperature at 100 rpm. Then the culture was centrifuged at 10000 rpm for 15 min. And the reactive phosphate in the supernatant was determined spectrophotometrically using ascorbic acid method (Cao *et al.*, 2018).

Acid phosphatase (ACPase) assay

Inoculating 1 ml of PSB culture at 10^8 CFU/ml in 25 ml of sterilized broth in a 100 ml conical flask and incubated at room temperature at 100 rpm for 48 h. The samples were withdrawn by centrifuging at 10000 rpm for 10 min at 4°C and ACPase assay was carried out by using para-nitrophenyl phosphate (p-NPP) as organic P substrate (Tabatabi and Bremner, 1969).

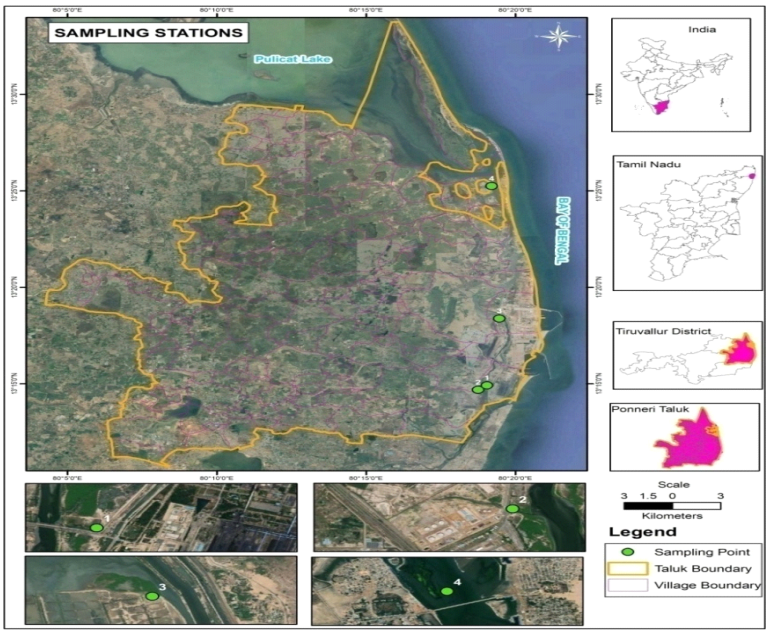

Fig 1: Map showing the study area of Ennore creek.

Production of organic acids

Analysis of organic acids was carried out by inoculating the bacterial culture in 50 ml of broth and incubated at 100 rpm and 37°C for 144 h. One ml of incubated sample was centrifuged at 10,000 rpm (Remi laboratories) for 15 min and filtered through 0.2 μ membrane filter to obtain cell-free culture supernatant and organic acid analysis was carried out using HPLC (Shimadzu - LC-2010C with Autosampler, FLD and UVE Photochemical Derivatisation). The results were compared with organic acid analysis standards following the methods of Yadav *et al.* (2013).

Microcosm experiment with PSB isolates

The study was carried out in a set up of glass tanks (2x1x1 ft). In each tank, the aquaculture pond sediment was placed to 5 cm thickness and 25 L of seawater. The PSB isolates (*B. altitudinis*, *Rhodococcus* sp and *Arthrobacter* sp) isolated from the study and a commercial PSB (FOZBACT, composed of *B. megaterium* from Ashwathy Green Enterprises Pvt. Ltd) was cultured in 25 ml of marine broth for 3 days at a concentration of 10^8 CFU/ml. After centrifugation and washing, the pellets of 5ml of the bacterial cultures were inoculated into the microcosm set up. The available P, total P, organic carbon, pH and soil texture were analysed before the start of the experiment. Both water and soil samples were collected from the microcosm set up on Day 0, 2, 4, 6, 8, 10, 12, 14 and 21 for the analysis of phosphorus concentration in water, available-P content and ACPase activity of sediment samples.

RESULTS AND DISCUSSION

Isolation and identification of PSB

Bacterial colonies which produced clear transparent zones were isolated and purified by subculturing. Thirteen PSB isolates were obtained and maintained during the study period. PCR amplification of 16S rDNA and sequencing analysis showed the identity of the PSB and the details are provided in Table 2. PSB isolated in this study were identified as *Rhodococcus* sp, *Arthrobacter* sp, *B. pumilus*, *B. subtilis*, *B. paramycoides*, *B. aryabhattai*, *B. anthracis, B. altitudinis, Enterococcus* sp, *Staphylococcus* sp (2 nos) and *Pseudomonas* sp (2 nos). Vazquez *et al.*, (2000) reported that PSB species such as *B. amyloliquefaciens, B. licheniformis, B. atrophaeus, Paenibacillus macerans, Vibrio proteolutics, Xanthobacter agilis, Enterobacter* sp., *Kluyvera cryocrescens, Pseudomonas stutzeri* and *Chryseomonas luteola* from the rhizospheric sediment of black and white mangroves in Mexico. Ravikumar *et al.* (2009) screened eight PSB isolates namely, *B. megaterium, Micrococcus roseus, B. subtilis, Proteus mirabilis, Streptococcus aureus, Micrococcus luteus, Athrobacter illicius* and *Enterobacter aerogenes* from mangrove sediments. Armandeh *et al.* (2022) isolated 11 isolates belonging to *Pseudomonas* and *Acinetobacter* from the sediments of aquaculture pond.

Measurement of P- solubilizing efficiency of PSB isolates in solid medium

Maximum clear zone of 9 mm thickness observed in *B. altitudinis*. *Rhodococcus* sp and *Arthrobacter* sp showed 4 mm thickness of clear zone. Though the clear zone formation of other isolates such as *B. aryabhattai* and *B. paramycoides* were lower than that of *B. altitudinis*, the solubilizing activity in liquid media was comparatively high.

The PSB isolates that produced very mild clear zones on solid medium, could also solubilize various types of insoluble inorganic phosphate in liquid medium (Das, 1963). This may be because of varying diffusion rates of different organic acids secreted by an organism (Johnson, 1959). Many researchers (El-Tarabily *et al.*, 2008; Park *et al.*, 2012) reported that the P-solubilization by bacterial inoculation results in drop in pH of culture media, which facilitate transformation of insoluble-P into soluble-P (Selvi *et al.*, 2017; Behera *et al.,* 2017a). In the present study on P - solubilizing efficiency, the liquid medium showed a reduction in pH (7.0 to 4.2) within 3 days of incubation. The reduction

Table 2: Details of the identified PSB isolates from sediment samples.

Isolate code	Name of the bacterial isolate	Thickness of hallow zone (mm)	P-Solubilizing rate ($mg.l^{-1}. h^{-1}$)
E/S1/19/06	*Staphylococcus* sp.	2	0.14
E/S1/19/07	*Pseudomonas* sp.	4	0.13
E/S1/20/13	*Rhodococcus* sp.	4	0.17
E/S1/20/14	*Bacillus subtlis*	2	0.11
E/S2/19/01	*Bacillus anthracis*	2	0.12
E/S2/19/03	*Bacillus aryabhattai*	3	0.20
E/S2/19/05	*Bacillus paramycoides*	2	0.23
E/S2/20/06	*Bacillus altitudinis*	9	0.21
E/S3/19/08	*Bacillus pumilus*	2.5	0.21
E/S3/20/04	*Enterococcus* sp.	2	0.15
E/S4/19/02	*Pseudomonas* sp.	2	0.14
E/S4/20/05	*Staphylococcus* sp.	4	0.13
E/S4/20/11	*Arthrobacter* sp.	4	0.16

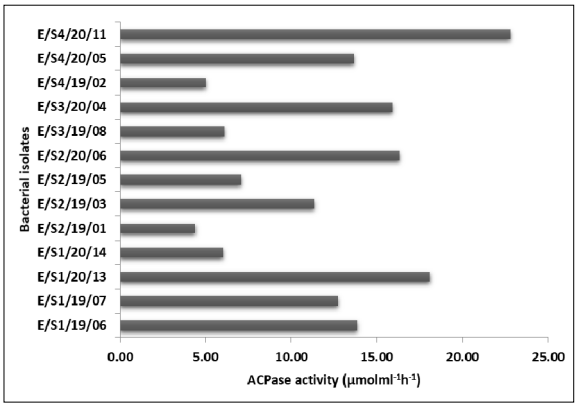

Fig 2: ACPase activity of PSB isolates.

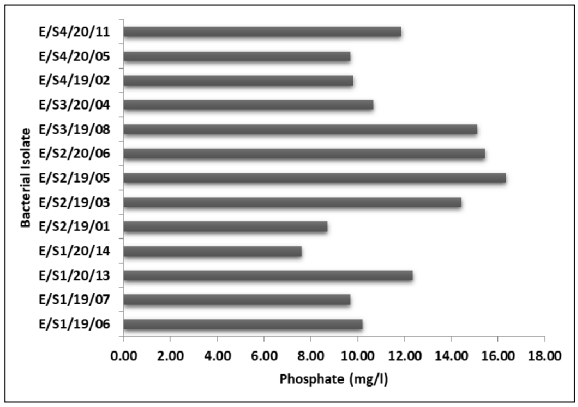

Fig 3: P- solubilizing activity of PSB isolates *Arthrobacter* sp.

in pH of culture media is associated with the production of different organic acids.

P-solubilizing activity and ACPase activity of PSB isolates in liquid medium

The ACPase activity and P-Solubilizing activity of PSB isolates in broth after 48 h of incubation is shown in Fig 2 and 3. The ACPase activity of isolates ranged from 4.40±0.28 -22.77±0.32 µmol ml^{-1} h^{-1}. *Arthrobacter* sp exhibited maximum ACPase activity (22.77±0.32 µmol/ml/h) followed by *Rhodococcus* sp (18.08±0.71µmol ml^{-1} h^{-1}), *B. altitudinis* (16.31±0.71 µmol ml^{-1} h^{-1}). The P solubilizing activity and P solubilizing rate in liquid medium observed in the isolates ranged from 7.62±0.51- 16.33±0.84 mg/l and 0.11-0.22 mgl^{-1} h^{-1} in 72 h of incubation respectively.

Kathiresan and Selvam (2006) reported that phosphate solubilizing efficiency of 24 isolates from Vellar estuary at Parangipettai, Tamil Nadu was in the range of 0.012-0.141 mg/l. *Vibrio* sp and *Pseudomonas* sp from the marine sediment reported to solubilize 0.5-0.55 mg/l (Promod and Dhevendaran, 1987). Bacterial species such as *B. subtilis, Pseudomonas* sp and *Azotobacter* sp isolated from mangrove sediment of Chollangi, East Godavari, exhibited a P-solubilising ability in the range of 80-100 mg/l (Audipudi *et al.*, 2012). Much higher phosphate solubilising activity (400 mg/l) was also reported in the bacterial population from the arid mangrove ecosystem in Mexico (Vaquez *et al.,* 2000). Behera *et al.* (2017a) documented that *Serratia* sp. isolated from mangrove soil of Mahanadi River delta showed P-solubilizing activity of 44.84 µg/ml, accompanied by a decrease in pH of the growth medium from 7.0 to 3.15.

Production of organic acids by PSB isolates from sediment samples

The organic acids produced by the selected PSB isolates (*B. altitudinis*, *Arthrobacter* sp and *Rhodococcus* sp) were shown in Fig 4. HPLC analysis of organic acids revealed that *B. altitudinis* produced 66.53 mg/kg of lactic acid, 37.8 mg/kg of acetic acid, 20.21 mg/kg of citric acid, 18.89 mg/kg of succinic acid and 15.1 mg/kg of oxalic acid; *Arthrobacter* sp produced 320.62 mg/kg of acetic acid, 35.36mg/kg of oxalic acid and 14.07 mg/kg of citric acid; *Rhodococcus* sp produced relatively very low quantity of citric acid (3.94 mg/kg), lactic acid (4.03 mg/kg) and oxalic acid (3.95 mg/kg).

The organic acids solubilize P by chelating metals ions that would immobilize it or forming soluble complexes with P via metal ions or both (Neumann *et al.*, 2000). The P-solubilization rate depends not only on the pH reduction of culture media, but also on the structure and type of the organic molecule produced and not the concentration of organic acid (Johnston, 1952). It is reported that oxalic, 2-ketogluconic and succinic acids produced by *Bacillus* sp have the capability to solubilize P (Banik and Dey, 1983). Park *et al.* (2009) documented that *Pseudomonas fluorescens* produced oxalic, trans-aconitic, citric, tartaric, malic, gluconic, succinic and fumaric acids; *Pantoea* sp and *Enterobacter* sp produced lactic, acetic, propionic, pyruvic, malonic, maleatic, tartaratic, oxalic, succinic, fumaric, citric and trans-aconitic acids to solubilize the inorganic P respectively. The P solubilisation activity of *Alcaligenes faecalis* decreased in pH levels 7.0 to 3.2 of the growth medium with the production of organic acids such as oxalic acid, citric acid, malic acid, succinic acid and acetic acid (Behera *et al.*, 2017b).

Microcosm experiment with PSB isolates

The P-mobilizing potential of the PSB isolates obtained in this study were compared with the commercial P biofertilizer following a microcosm study. The initial water quality parameters in the microcosm study were pH-8.2; alkalinity-160 mg/l; salinity-35ppt; ammonia-0.017mg/l; phosphorus-0.31 mg/l. And the initial sediment characteristics such as pH, EC, Available-P, Total -P are given in Table 3. Fig 5 shows the phosphate in water and ALP activity and available-P in the sediment during microcosm experiment for PSB isolates. The control tank showed stable available-P (48.10-52.31 ppm) and ACPase activity (17.86-20.80 µmol/g/h) in sediment and orthophosphate (3.07-3.17 mg/l) in water from 0-21 days. The tanks inoculated with *B. altitudinis*, *Rhodococcus* sp, *Arthrobacter* sp and commercial product showed significant increase in ACPase activity and available-P

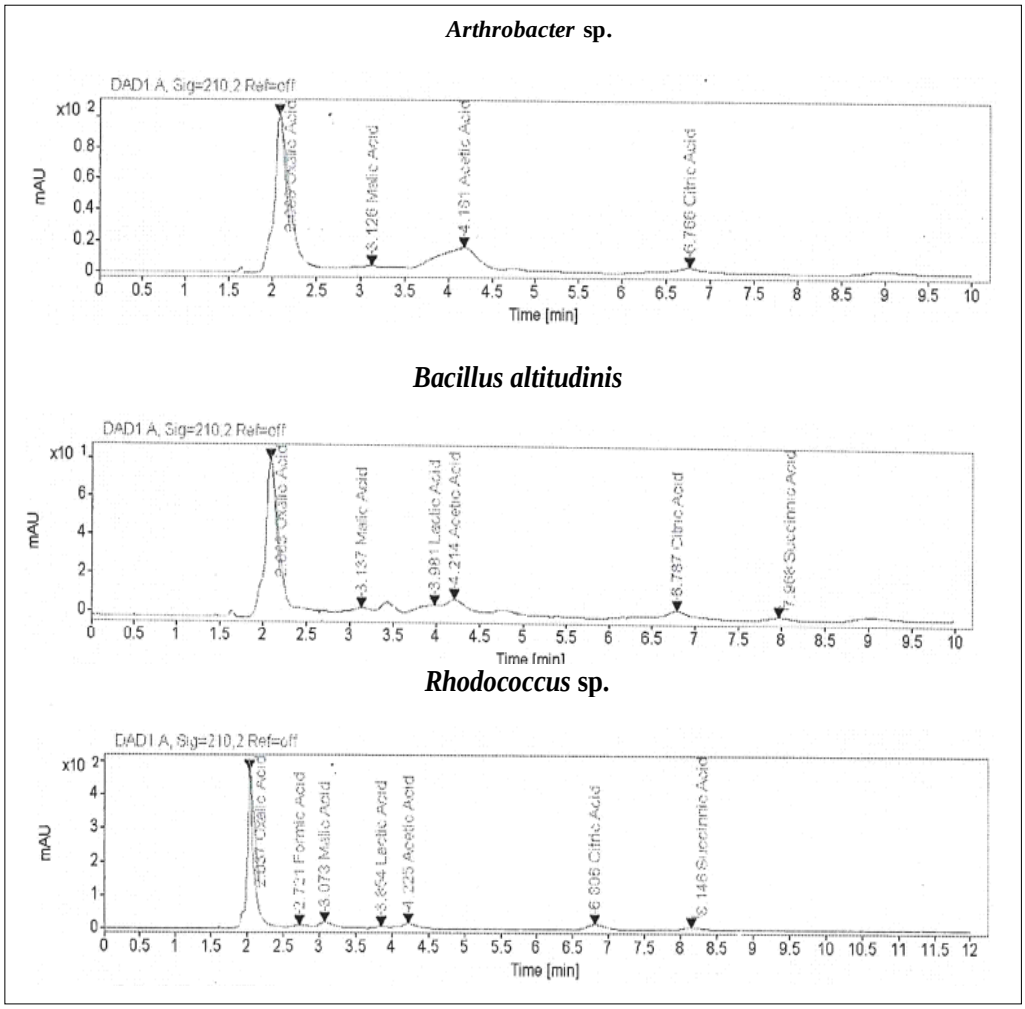

Fig 4: HPLC analysis of organic acids.

Table 3: Physico-chemical characteristics of sediment in microcosm set up of PSB isolates.

Samples	pH	EC	Available - P (ppm)	Total - P (ppm)
Experimental control	7.8	2.3	48.10±1.03	531.32±2.35
Rhodococcus sp	8	2.26	49.32±0.09	540.36±1.24
Arthrobacter sp	7.9	2.23	49.46±1.19	538.29±2.73
Commercial product	7.9	2.24	49.35±1.07	539.87±1.86

in sediment and orthophosphate in water from 2nd day onwards. The ACPase activity of the sediment was found to be higher in the tank inoculated with *Rhodococcus* sp (12.00 μmolg^{-1}h^{-1}) followed by commercial product (11.23 μmol g^{-1} h^{-1}), *B. altitudinis* (10.03 μmol g^{-1} h^{-1}) and *Arthrobacter* sp (8.10 μmol g^{-1} h^{-1}). The available P of the sediment on 21st day was in the following order: Commercial product (128.25 ppm) > *Rhodococcus* sp (110.2 ppm) > *B. altitudinis* (85.77 ppm) > *Arthrobacter* sp (82.65 ppm). The phosphate level in the water of microcosm study on 21st day was observed in the following order: *Rhodococcus* sp and commercial product (0.49 mg/l) > *B. altitudinis* (0.46 mg/l) > *Arthrobacter* sp (0.417 mg/l). With regard to phosphate level in the water, there is no significant difference observed between *Rhodococcus* sp and commercial product. The initial and final PSB count in water samples of treatment tanks during the study period were in the range of 2-3 CFU/ml and 26-42 CFU/ml. The initial and final PSB count in soil samples of treatment tanks during the study period were in the range of 5-7 CFU/g and 1-3×10^3 CFU/g. In the present study, water-P, ACPase activity and available-P of sediment were increased up to 0.32-0.53 times, 0.56-1.16 times and 0.58-1.45 times respectively. This study gives evidence that apart from *Bacillus* sp, *Rhodococcus* sp and *Arthrobacter* sp could be used as biofertilizer in aquaculture.

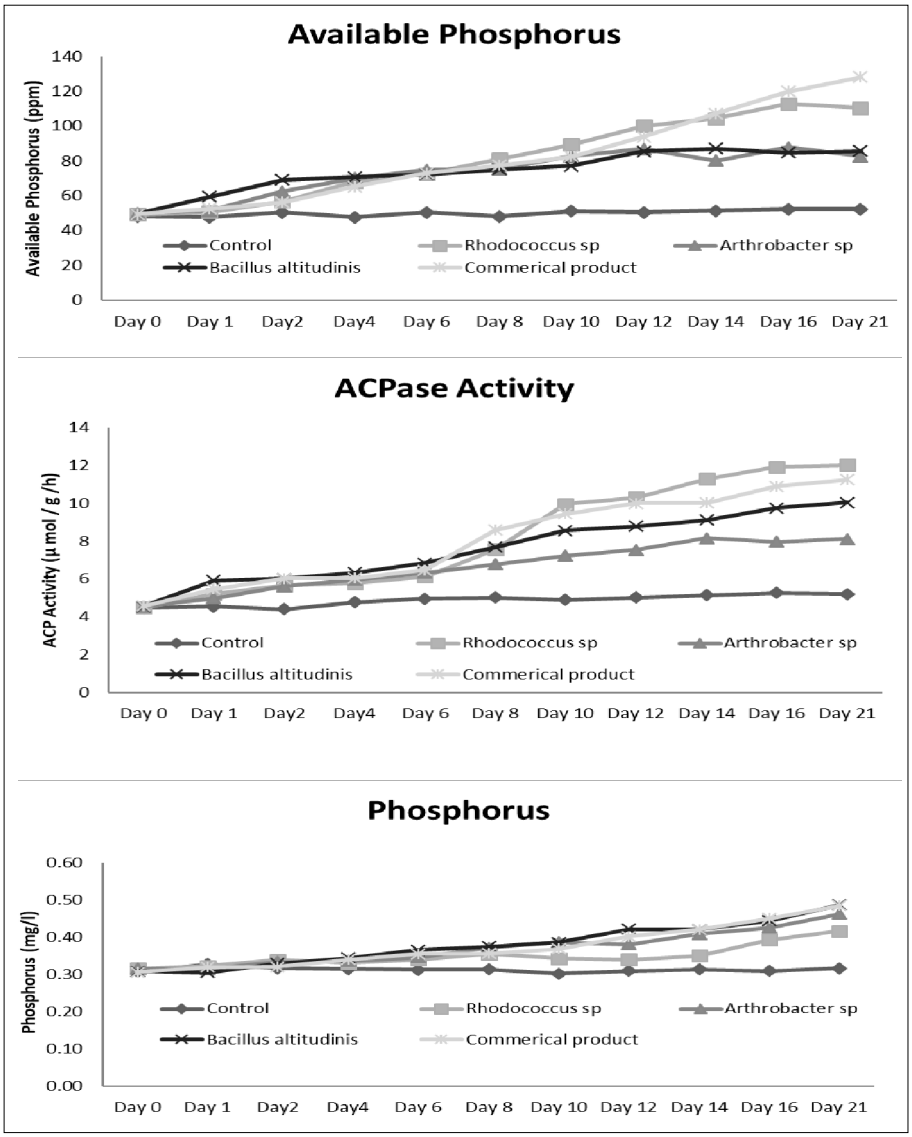

Fig 5: Available-P and ACPase activity in sediment and Phosphate in water of microcosm experiment.

Microcosm study with PSB isolates of Vovk *et al.* (2013), was similar to the present study, where they warranted that application of *Paenibacillus polymyxa* contributes to an increase in mineral phosphorus in pond water by 1.3-3.7 times compared to the control. It was carried out in a 30 L tank consisting of pond water with concentration of PSB, *Paenibacillus polymyxa* KB $(4.50\pm0.50) \times 10^2$ - $(5.63\pm0.46) \times 10^3$ CFU/ml for 35 days. They also inferred that introducing bacterial fertilizer on the floor increased the mineral phosphorus content in the aquatic environment more rapidly (15-17 days) than introducing it on the water surface (25-30 days). Similarly, the study of Zhou *et al.* (2023) revealed that application of *B. cereus* mutant strain S458-M in Crucian carp culture systems improved the concentration of phosphorus in aquaculture water. Further studies are needed to evaluate the rate of application based on soil texture, organic carbon and total phosphorus.

CONCLUSION

This study describes the isolation, characterization and identification of PSB bacteria from rhizospheric sediments of *A. marina* from the Ennore region. As the study region belongs to the brackishwater category, the saline tolerant bacteria were isolated which are able to work at salinity conditions. Further, this study provides evidence for application of *Rhodococcus* sp and *Arthrobacter* sp as biofertilizers in aquaculture systems too. Knowledge on mineralizing potential of the native bacterial isolates of *Bacillus* sp, *Rhodococcus* sp and *Arthrobacter* sp with respect to various environmental parameters will be helpful

in the development of management activities utilizing these isolates in aquaculture application.

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Conflict of interest: None.

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