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Research Article

PHYTATE MINERALIZING BACTERIA AND THEIR PHYTASE ACTIVITY FROM DIFFERENT MATRICES OF TROPICAL FLOODPLAIN WETLANDS

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ABSTRACT: Phytic acid constitutes a significant part of total phosphorus (P) load in aquatic sediments, with potential for P release to water. The present study harnessed phytase producing bacteria from different matrices of two floodplain wetlands of West Bengal, India and examined their phytate mineralization activity. Prominent phytate degrading bacterial isolates were identified from 16S rDNA sequences as *Bacillus megaterium*, *Arthrobacter* sp., *Klebsiella oxytoca*, *Methylobacterium gregans,* and *Fictibacillus* sp. The bacteria from gut of *Cirrhinus mrigala* had much higher phytase activity than those from sediment and water. Requirements of pH for optimum phytase enzyme activity varied widely, in sync with the pH of respective niche, with highest activity at pH 3-4 for gut bacteria and pH 6-7 for sediment isolates, suggesting their P release potential. Although the sediment bacteria had moderate phytase enzyme activity, presence of large amount of organic matter of plant origin, and congenial physio-chemical environments such as temperature and pH might make the wetland sediment an important site of phytate degradation towards phosphorus cycling for primary production.

Key words: Bacteria, Phytase, Water, Sediment, Fish gut.

INTRODUCTION

Phytic acid or the myoinositol 1, 2, 3, 4, 5, 6-hexakis-dihydrogen phosphate (IP6) is one of the major forms of inositol phosphates (IP) and occurs widely in the environment. Phytate, synthesized widely by plants, accounts for 70-80% of the total phosphorus present in seed grains (Lott *et al*. 2000). Human and other monogastric animals including fish consume a large amount of food phytate, which, however, is not digested effectively and is excreted out. The phytate originating from terrestrial runoffs, manures and city sewages containing the undigested nutrient from human and animal excreta have been identified to form a major part of organic phosphorus in aquatic sediments (Suzumura and Kamatani 1995,Turner and Weckström 2009). Plant-based fish feeds, especially those containing oil seed meals or cakes (Bello *et al*. 2013, Yasothai *et al*. 2014) add to this quantity in aquatic environments. Macrophytes, abundantly present in majority of the tropical floodplain wetlands also synthesize and contribute phytate to the ecosystem. For example, raw duckweed (*Lemna polyrrhiza*) leaf has a phytate content of 1.23% (Bairagi *et al.* 2002).

In terrestrial soil IP6 accounts for 5.3-83% of organic P contents (Turner *et al.* 2002) with a potential to provide phosphorus (P) necessary for plant. In aquatic ecosystems IP6 constitutes from negligible to a significant portion of sediment organic P pool (Chessman *et al.* 2014, Paraskova *et al.* 2015). In lakes of tropical China, the level of monoester-P, that includes IP, varied between 21.4 and 82.7 mg kg^{-1}, contributing 1.8-14.3% to the total sediment P (Zhang *et al.* 2009). The concentration of phytic acid in sediments from temporary ponds of the Doijana National Park, Spain, ranged from 9-31 mg kg^{-1} or higher (Serrano *et al.* 2000). In Carolina Bay wetlands the IP6 accounts up to 187 mg kg^{-1} or 46% of total phosphomonoesters and 11% of total soil P (Chessman *et al.* 2014). Phosphorus is a critical and limiting macronutrient for primary producers, including phytoplanktons. However, unlike terrestrial soil where

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IP may meet P requirements of plants, bioavailability of phytate-P in aquatic environments is debated with contradictory observations. Due to higher pH-dependent charge density phytate forms stronger complexes with iron hydroxide, other multivalent cations and humic substances that prevent its enzymatic hydrolysis (He *et al.* 2006). In lake sediments the half-life of clustered monoester-P is about 29 years (Reitzel *et al.* 2007), much higher than other common organic P species, making it a dominant organic P fraction in organic rich wetlands. Due to high degree of recalcitrance and longer environmental persistence, phytate has been argued as a P-specific paleo-indicator in aquatic sediments (Turner and Weckström 2009). Phytate-P is generally considered refractory in nature (Reitzel *et al.* 2007). On the contrary, Zhu *et al.* (2013) reported bulk recovery of IP6 in the labile H_2O and $NaHCO_3$ fractions suggesting its amicable nature for hydrolysis. In Tokyo Bay river and estuarine sediments, Suzumura and Kamatani (1995) estimated IP6 at the level of 0.07-0.66 µmol P g^{-1} which decreased to 0.01-0.1 µmol P g^{-1} in coastal marine zones: this natural mobilization in marine environment matched with hydrolysis of IP in simulated marine sediments. Turner *et al.* (2006) recorded very low levels of IP6 in treatment wetlands and suggested its rapid degradation.

Phytase enzyme (myo-inositol hexaphosphate phosphohydrolase, E.C.3.1.3.8), responsible for hydrolysis of phytate to lower substituted inositol phosphates and orthophosphate, is synthesized by plants, microorganisms and some animals (Gatlin *et al.* 2007). Several researchers have worked on soil microbial phytases (Powar and Jagannathan 1982, Greiner *et al.* 1993, Greiner *et al*. 1997), and their role in soil productivity improvement by realizing phytate P is well established (Singh *et al.* 2014). However, meagre studies have been conducted on phytate degradation in aquatic ecosystems and phytate degradation capability of aquatic microbes, especially from water and sediment. In a different perspective, phytase producing bacteria and yeast such as *Rhodococcus* sp., *Bacillus* spp., *Candida tropicalis* have been isolated from freshwater fish gut with major objective of their potential use in fish feed development (Khan *et al.* 2011, Khan and Ghosh 2012, Das and Ghosh 2014). The objective of our study was to examine diversity and phytate degradation ability of bacteria from different matrices of freshwater ecosystems with long term goal of enhancing P availability through microbial phytate degradation.

MATERIALS AND METHODS

Study sites

Water, sediment and fish samples were collected from two freshwater floodplain wetlands, namely, Akaipur (23°04'N, 88°43'E) and Bhomra (22°59'N, 88°38'E) wetlands located in rural West Bengal, India. These floodplain wetlands are closed ox-bow lakes with large detritus load that have greatly enriched the sediment with 5470-6040 mg total P kg^{-1} sediment. Sediment total

Table 1. Identification of phytate mineralizing bacterial isolates from 16s rDNA sequence information.

Isolate	Isolation Source	Identification	Closest type strain	Identity	Accession number
APh1	Akaipur sediment	*Bacillus megaterium*	*Bacillus megaterium* ATCC 14581T	100%	ANNS00000000
APh2	Akaipur sediment	*Bacillus megaterium*	*Bacillus megaterium* ATCC 14581T	99%	KM035406
BPh4a	Bhomra sediment	*Bacillus megaterium*	*Bacillus megaterium* ATCC 14581T	99%	JQ965768
PhIn1	Fish intestine	*Arthrobacter* sp.	*Arthrobacter mysorens* DSM 12798T	99%	JX134618
PhIn2	Fish intestine	*Klebsiella oxytoca*	Klebsiella oxytoca ATCC 13182T	99%	JX000001
PhIn3	Fish intestine	*Arthrobacter* sp.	*Arthrobacter mysorens* DSM 12798T	99%	KM035405
PhIn4	Fish intestine	*Methylobacterium gregans*	*Methylobacterium gregans* DSM 19564T	99%	JX134616
PhIn5	Fish intestine	*Fictibacillus* sp.	Bacillus sp. Ca7	100%	KM035407

organic carbon (TOC) contents were high, 2.95 ± 0.09 % and 4.1 ± 0.2 % in Akaipur and Bhomra wetlands respectively, and macrophyte derived detritus forms an important autochthonus food source to the fish population (Samanta *et al*. 2015). Heavy macrophyte production and washings from surrounding agricultural fields are the major sources of nutrients and organic matter inputs into the wetlands; the wetlands receive meager amounts of domestic wastes and no city sewage. Fish species *viz.*, *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala*, and *Hypophthalmichthys molitrix* are stocked in moderate density in these wetlands without any supplementary feeding. Top layers (0-14 cm depth) of bottom sediments from different sampling points in wetlands were collected using Ekman dredge; water samples were taken from 0.5m depth. From each sampling point one litre of water and 1.5-2 kg of sediment samples were collected in sterile plastic containers and brought to the laboratory within 2 hours. *Cirrhinus mrigala*, a detritivore species, was chosen to study the phytase activity of gut bacteria. Live *C. mrigala*, weighing between 750 and 1200 g, were netted from the wetlands, individually packed in plastic bags and transported to the laboratory under ice cover.

Isolation of phytate mineralizing bacteria (PMB)

Five grams of each sediment sample and aseptically dissected intestinal contents of individual fish were homogenized in sterile saline (0.85%) solution following Maitra *et al*. (2015). One hundred microlitres of the 10% (w/v) suspension, sample water and their dilutions (10-fold dilutions in 0.85% saline) were plated by spread plate method on to phytate screening medium (also called PMB medium here) containing phytate as the sole source of phosphorus [10 g l^{-1} D-glucose, 4 g l^{-1} Na- phytate, 5 g l^{-1} NH_4NO_3, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4.7H_2O$, 0.01 g l^{-1} $FeSO_4.7H_2O$, 0.01 g l^{-1} $MnSO_4.H_2O$ and 15 g l^{-1} agar, pH 7.0] (Kerovuo *et al.* 1998). Following incubation at 30°C for 7 days, representative phytate degrading colonies with clear zone of halo around them were picked up, restreaked on PMB agar for presumptive confirmation of phytate degradation, and finally given passages on to Reasoner's 2A (R_2A) agar for pure culture isolation.

Quantitative phytate mineralization by bacterial strains

The phytase activity of the bacterial isolates was measured following Yanke *et al.* (1998). Briefly, bacteria were grown in phytate screening broth for 72 h at 30°C, centrifuged at 10,000 × g for 10 minutes at 4°C and filtered through 0.22 µm membrane filters. Cell free culture broth was used for mineralization assay using 0.2% w/v sodium phytate as substrate in 0.1 M Na-acetate buffer (pH 5.0) at 39°C. After 30 minutes of incubation, reaction was terminated with 5% (w/v) trichloroacetic acid, and orthophosphate content in the supernatant was measured by the molybdate-blue method (Murphy and

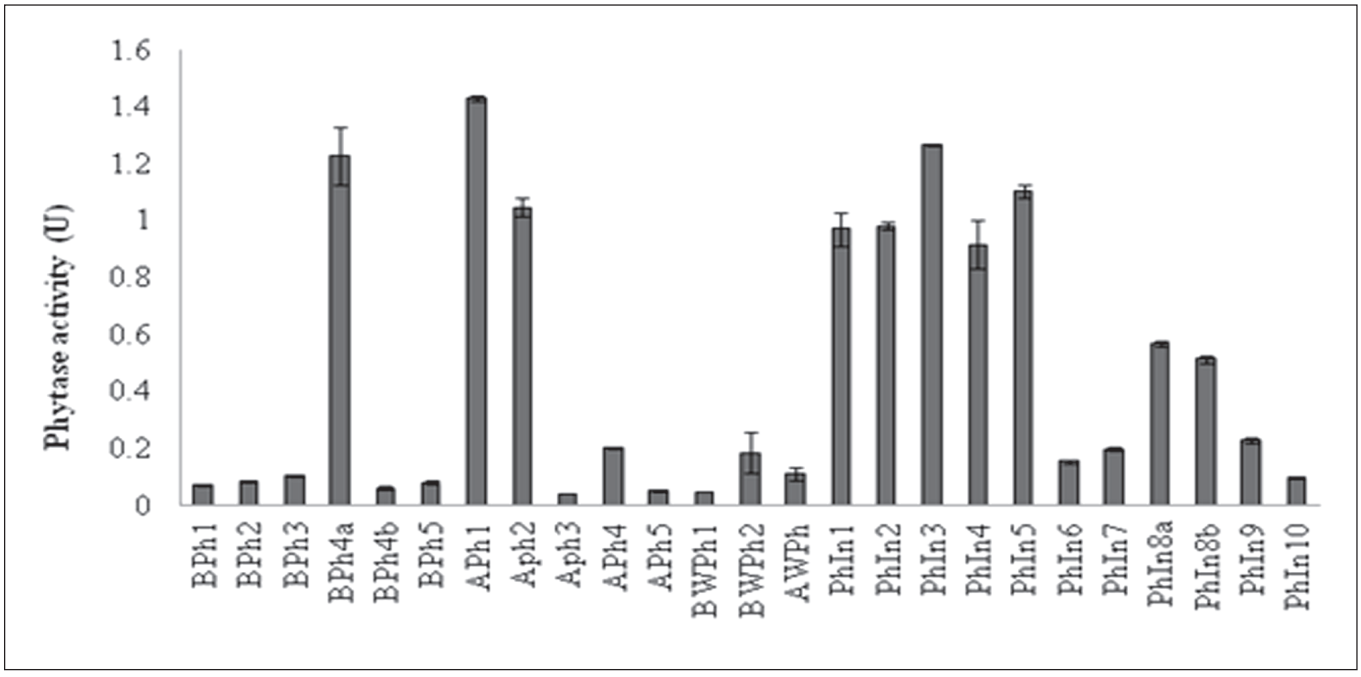

Fig. 1. Phytase activity of the bacterial isolates from wetland ecosystems.
(Strains isolated from Akaipur wetland sediment are named with 'APh', those from Bhomra wetland sediment are named with 'BPh', those from water are named as either "AWPh' or 'BWPh', and those from fish intestine are named with 'PhIn').

Riley 1962). Uninoculated medium served as negative control. One phytase unit (U) was defined as 1 µg of inorganic phosphorus released per 1 ml of culture filtrate per 1 min.

pH optima of phytase activity

Since bacteria from different matrices were tested where pH ranged from around 4 in fish gut to 5.79-7.61 in water and sediment, the optimum pH for phytase activity of prominent phytate mineralizing strains was determined at pH 3-7. Briefly, cells were grown for 5 days and cell-free culture broth, prepared by centrifugation and filtration as mentioned above, were tested for phytase activity following Yanke *et al.* (1998) at pH 3-7 in 0.1 (M) citrate buffer (pH 3-5), 0.1 (M) acetate buffer (pH 5, 6) and 0.1 (M) Tris HCl buffer (pH 6, 7) using 0.2% w/v Na-phytate as a substrate. The time and temperature conditions for obtaining pH optima were kept similar to enzyme assay stated earlier.

Identification of bacteria

PMB with prominent phytase activity (activity >0.9 U) were identified by PCR amplification and sequencing of 16S rDNA as per Maitra *et al.* (2015). Bacterial Genomic DNA was extracted using GenElute™ Bacterial genomic DNA Kit (SIGMA- Aldrich) from a fresh pure culture colony grown on nutrient agar, and the gene for 16S rRNA was amplified by polymerase chain reaction (PCR) using bacterial universal primers 8F (5´-AGAGTTTGATCCTGGCTCAG-3´) and U1492R (5´-GGTTACCTTGTTACGACTT-3´) following cycle condition of 95° C for 1 minute, followed by 30 cycles of 95° C for 30 seconds, 57° C for 30 seconds and 72° C for 2 minutes, and a final extension at 72° C for 5 minutes. The PCR reaction mix consisted of 0.2 µM of each primer, 10 µM of each deoxynucleotides, 1.5 mM of MgCl2, 10X PCR buffer and 0.5 U of Taq DNA polymerase (Invitrogen). The PCR amplicons were separated and visualized in 1% agarose gel, purified using QIAquick® gel extraction kit (Qiagen) and sequenced by Sanger method. The nucleotide sequences were verified for quality by Sequence Scanner v1.0 software (Applied Biosystems, Inc., US), checked for chimera using Bellerophon (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl) and forward and reverses sequences were aligned with codon code aligner (Codon Code Corporation, US) to form contigs which were compared with synonymous sequences using nucleotide BLAST program (Altschul *et al.* 1997) in GenBank, Ribosomal Database Project (RDP), and Greengenes (http://greengenes.lbl.gov). The closest match of phylogenetic affiliation was used to assign the sequenced strains to specific taxonomic groups. A phylogenetic tree of the identified bacteria and their type strains, SSU sequences of which were obtained from Taxon passports, was prepared using the Maximum Likelihood method in

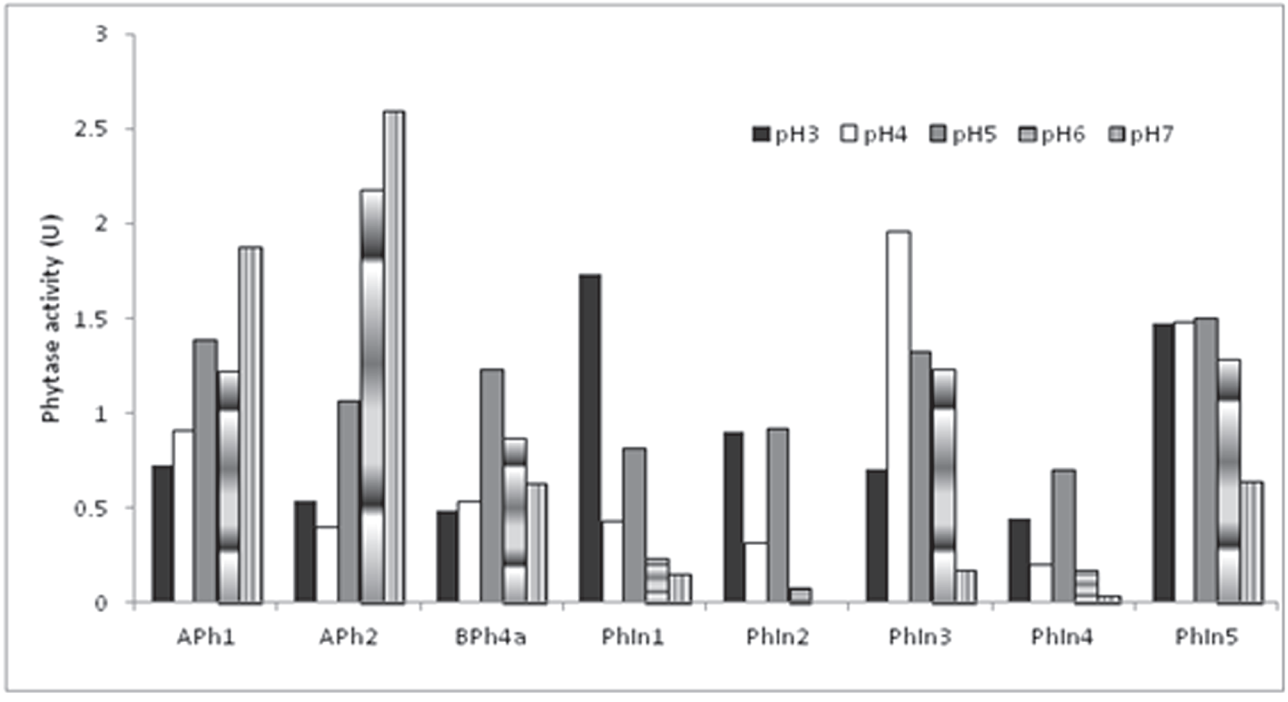

Fig. 2. pH optima of phytase activity of the prominent phytate mineralizing strains.

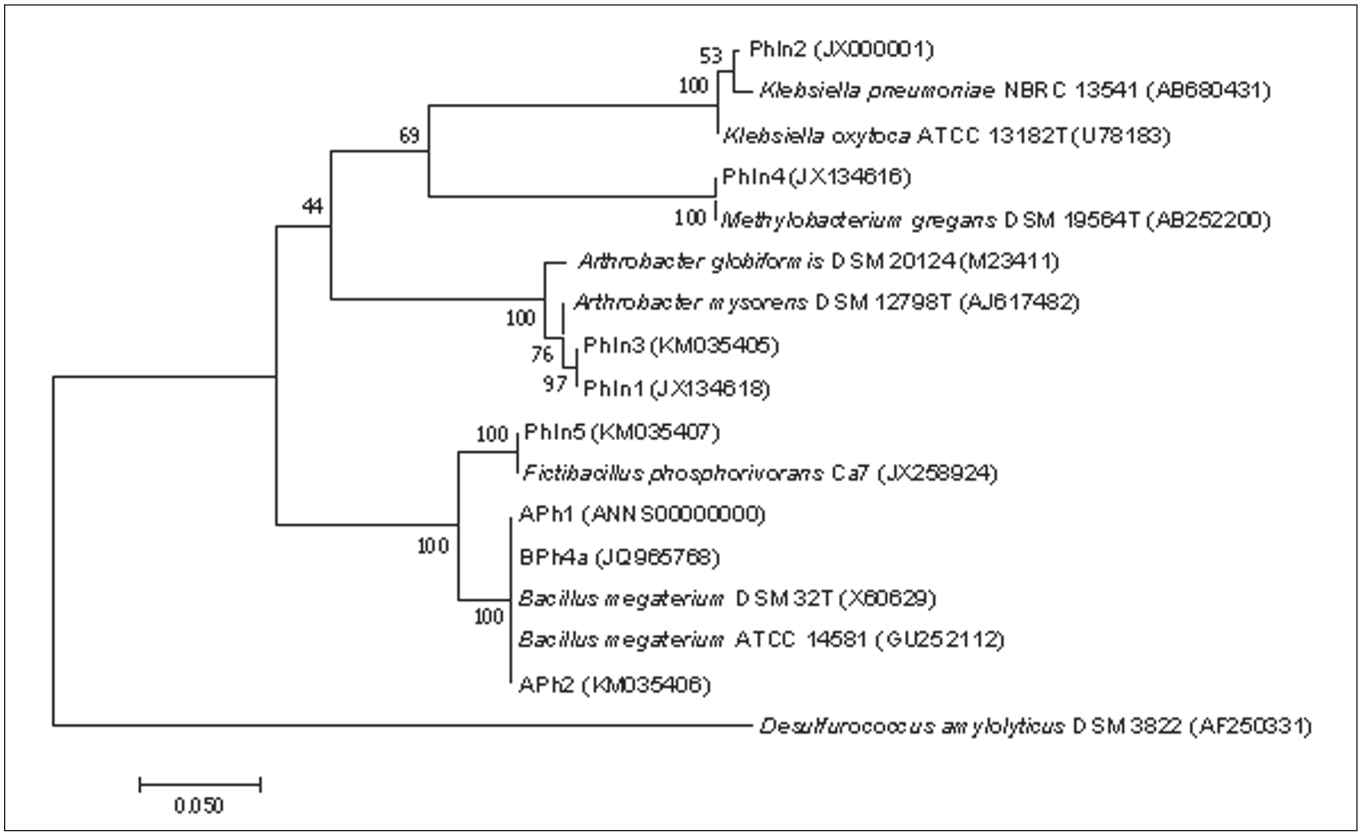

Fig. 3. Phylogenetic tree showing the relationship among PMB and their closest type strains.
[The tree with the highest log likelihood (-2435.15) is shown. Distance matrix was calculated by Tamura-Nei model and Bootstrap analysis was calculated with 1000 replicates bootstrap].

MEGA7 (Kumar *et al*. 2016), following Tamura-Nei model (Tamura and Nei 1993). The distance matrix was calculated by γ distribution method and bootstrap analysis with 1000 replicates was used to test the phylogeny (Felsenstein 1985).

Statistical analysis

Phytase activities of the bacterial isolates are presented as mean ± S.E. Difference in phytase activity among the isolates were examined by one sample t-test using SAS Enterprise Guide (4.2).

RESULTS AND DISCUSSION

Phytate mineralizing bacteria from different freshwater matrices

Following primary culture, a large number of colonies developed on plates for all the sediment and fish intestinal contents, but a few colonies developed for water samples. Representative colonies with prominent zone of clearing around them were primarily selected for further study. Thus, a total of 25 strains of PMB (11 strains from fish gut, 11 from sediments and 3 from water samples) were isolated from the wetlands.

Quantitative phytate mineralization by bacterial strains

Phytate mineralization study conducted in acetate buffer, pH 5.0, using Na-phytate as the P source showed that the strains from water, sediment and fish had low to moderate activity, ranging between 0.046 U and 1.427 U (Fig. 1). The enzyme activities of the strains isolated from fish gut (0.635±0.129 U) were significantly higher than those from sediment (0.398±0.164 U) and water sources (0.112±0.039 U). The bacteria isolated from Akaipur wetland sediment had insignificantly higher activity (0.552±0.287 U) than those from Bhomra sediment (0.269±0.191 U). *Bacillus megaterium* from sediments had the highest activity among all the strains.

pH optima of phytate degradation

Phytase activity of selected strains were tested at different pH ranging from pH 3 to pH 7 to determine the pH optima. Results showed that pH optima of different strains varied. While most of the fish gut isolates were effective in phytate degradation at pH 3-4, majority of the sediment isolates did so at pH 7 (Fig. 2). The enzyme activity of the fish gut bacteria was the lowest at pH 7.

Identification of selected bacteria and phytase genes

The bacterial strains belonged to diverse groups, from Gram positive *Bacillus* to Gram negative *Klebsiella*, distributed among *Alphaproteobacteria*, *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes*. The identified PMB were *Bacillus megaterium* from wetland sediments and *Arthrobacter* sp., *Klebsiella oxytoca*, *Methylobacterium gregans*, and *Fictibacillus* sp. from fish gut (Table 1, Fig. 3).

Phytate often forms complexes with other soil components that reduce its accessibility to enzyme hydrolysis (Lung and Lim 2006). However, phytate in wetlands shows relatively higher solubility and degradability (Lim *et al.* 2007). Suzumura and Kamatani (1995) observed complete hydrolysis of IP in 40 days period under anaerobic condition and partial hydrolysis in aerobic conditions in simulated marine sediments with potential to fortify the overlying water with P for primary producers. Although the exact mechanism of higher oxidation of phytate in marine environments remains unclear, reducing environments typical of wetland soils favour iron dissolution and may cause enhanced solubility of metal-bound-phytate for microbial hydrolysis (Turner *et al.* 2002). In a study of 28 freshwater wetlands of varied climatic, hydrogeomorphic and vegetation types Chessman *et al*. (2014) concluded that IP6 level in sediment is a result of biogeochemical conditions including Fe or Al oxides and redox state of the ecosystem that govern its stabilization and turnover. Thus, organic P present in sediment is amenable to hydrolysis by microbial processes. The present study showed presence of phytate mineralizing bacteria suggesting that many microbes were capable of utilizing phytate as a nutrient source in aquatic environments. More number of bacteria, which also showed higher phytate mineralization activity, was isolated from fish gut and sediment probably due to higher substrate availability in these niches, as compared to those from water.

Out of 137 phytate degrading colonies primarily selected, 25 were studied further based on their detectable phytate degradation on culture plates. Although 25 strains theoretically represent only a small proportion of huge microbiota in wetlands ecosystems, identification revealed that the strains belonged to various distantly related genera (Fig. 3) and might represent major taxonomic classes of phytate degrading bacteria present in the studied wetland ecosystems.

Among the isolates, sediment bacteria had an average phytase activity of 0.398±0.164 U, with insignificantly higher activity of the isolates from Akaipur than those from Bhomra for undefined reasons. *Bacillus megaterium* was identified as an important sediment bacterium with phytase activity 1.04-1.43U, which was higher than those of intestinal isolates. Aquatic bacteria have been found to produce phytase earlier, however, since its pH optima were pH 2.5 and 5.0 it was doubted whether the enzyme would be significant in phytate turnover in natural waters having slightly acidic or alkaline pH (Klotz 1991). Unlike the fish gut bacteria having optimum phytase activity at low pH, pH optima of sediment bacterium *Bacillus megaterium* in present study, were pH5 and pH7. Yoon *et al.* (1994) and Demirkan *et al.* (2014) had also observed neutral to alkaline pH optima for soil *Bacillus* strains. The sediment pH 5.89±0.03 in Akaipur and 7.43±0.04 in Bhomra wetlands were within the favorable ranges for phytase activity of *Bacillus*, implying that the bacterium might play significant roles in P cycling in floodplain wetlands with near neutral pH, high total organic carbon and total P contents. P is an essential element determining trophic state of aquatic ecosystems. Microbial degradation of sediment InP might thus enhance available P levels for uptake by algae.

Bacillus as a promising phytase producer in aquatic system was also evident in the work of Das and Ghosh (2013), who reported an activity of 2.31 U in *Bacillus subtilis* isolated from *C. mrigala* gut. In our study, unlike water and sediment isolates which showed wide range of phytase activity from 0.046-1.428 U, the average activity of fish gut isolates were higher, probably due to bottom feeding nature of the fish species studied that exposed it to higher levels of detritus phytate leading to selection of high activity microbiome in gut. Among the phytate degraders from gut, *Arthrobacter* sp. had the highest activity. However, the phytate mineralization activities of all our isolates were low (0.07 - 1.27 U), in comparison to 1.03-2.33 U activity of the phytate degrading bacteria described earlier from gut of cultured carps (Roy *et al*. 2009, Khan *et al*. 2011, Khan and Ghosh 2012). Presence of a diverse bacterial species with moderate phytase activity might liberate a significant amount of available P for fish.

The PMB having prominent activity were identified to a number of Gram positive and Gram-negative genera and species, with *Bacillus megaterium* as the prevalent bacterium in freshwater sediment. Earlier studies identified *Bacillus licheniformis*, *B. atrophaeus* and *B. subtilis* as major phytase producers in gut of freshwater fish species like *Labeo rohita*, *Gudusia chapra* and *L. bata* (Roy *et al.* 2009, Khan and Ghosh 2012). Askarian *et al*. (2012) isolated *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus cereus* and other *Bacillus* sp. from gastro-intestinal tract of Atlantic salmon. However, in

this study, PMB from gut of detrivorous fish *C. mrigala* were identified as *Arthrobacter* sp., *Klebsiella oxytoca*, *Methylobacterium gregans* and *Fictibacillus* sp. Several other workers also reported such niche specific bacterial distribution. *Rhodococcus* sp. was isolated from *Labeo catla* by Khan *et al.* (2011). Phytase producing *Bacillus subtilis*, *Acinetobacter* sp., *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus* sp. and *Brochothrix* sp. have been isolated from Atlantic salmon, *Salmo salar* and Atlantic cod (Askarian *et al.* 2012) and we hypothesize this as fish species-specific and niche-specific differences in distribution of different phytate degrading microbiota in the aquatic environment. Occurrence and higher activity of *Bacillus megaterium* in sediment in this study and other *Bacillus* species in fish gut in earlier studies suggest that members of the genus *Bacillus* might have potential applications in phytate degradation in aquaculture. *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus megaterium* are widely used in aquaculture in the form of water probiotics, and this study suggests that they might contribute significantly to sediment phytate degradation and P release. *Klebsiella oxytoca* (Jareonkitmongkol *et al.* 1997) and *Arthrobacter bergei* have been shown to have phytate mineralization property earlier (Hill *et al.* 2007). The UniProt database (http://www.uniprot.org) also revealed presence of phytase gene in *Arthrobacter* sp. SJCon (Vikram *et al.* 2013). The phytate degrading strain PhIn4 closely matched with *Methylobacterium gregans*, a well-known P solubilizer (Jayashree *et al.* 2011, Agafonova *et al.* 2013); UniPort database of complete genome sequence of two *Methylobacterium* spp. [*Methylobacterium* sp. (strain 4-46), GenBank accession no. NC010511; *Methylobacterium radiotolerans* strain JCM 2831, GenBank accession no. NC010505] (Marx *et al.* 2012) revealed presence of phytase genes in these bacteria. Strain PhIn5 displayed 100% similarity with *Fictibacillus nanhaiensis* strain JSM 082006 and *Fictibacillus phosphorivorans* strain Ca7, and low similarities (98%) with both *Bacillus arsenicas* (Shivaji *et al.* 2005) and *Bacillus barbaricus*. However, Greengenes (http://greengenes.lbl.gov) revealed a 100% similarity only with *Bacillus* sp. str. JSM 082006, and both Greengenes and RDP did not show the presence of any genus named *Fictibacillus*. *Bacillus* sp. str. JSM 082006 which was later identified as *Bacillus nanhaiensis* (Chen *et al.* 2011), has recently been reclassified into a new genus *Fictibacillus* (Glaeser *et al.* 2013). Thus, we identified the strain as *Fictibacillus* sp. The genus *Fictibacillus* have been detected with phytate mineralizing as well as P solubilizing activity (Maitra *et al*. 2015) possibly for the first time. Overall, *Bacillus* and *Arthrobacter* were found to be the major P solubilizers in freshwater ecosystems.

CONCLUSION

The enzyme phytase has found wide applications to enhance feed digestibility and nutrient availability in animal husbandry. However, pre-treatment of plant-based feed ingredients with phytase or its dietary supplementation in fisheries sector is limited. Use of phytate degrading bacteria for nutrient release in aquaculture systems is also very meager, largely due to scanty information on sediment phytate contents and bacteria that can degrade it. The present work has identified a substantial microbial diversity with higher phytate degrading ability of *Arthrobacter* sp. in fish gut and *Bacillus megaterium* in floodplain wetlands sediments. Close relationships between optimum pH of phytase enzyme activity and pH of the ecosystem of the bacterial isolates suggest potential phytate degradation by the bacteria in their respective niches. Besides enhancing P availability in fish gut, microbial phytate mineralization might play an important role in P cycling and availability in these nutrient rich floodplain wetlands.

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