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CHARACTERIZATION AND IDENTIFICATION OF GUT-ASSOCIATED PHYTASE-PRODUCING BACTERIA IN SOME FRESH WATER FISH CULTURED IN PONDS

Argha KHAN and Koushik GHOSH*

Aquaculture Laboratory, Department of Zoology, The University of Burdwan, Golapbag, Burdwan, West Bengal, India

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Background. Phytase produced by gut bacteria increases the availability of phosphorus and other important nutrients in ruminants by virtue of enzymatic hydrolysis of the phytic acid, an antinutritional factor present in the majority of plant feedstuffs. This topic, however, has been insufficiently investigated in fish. This study was intended: to evaluate the presence of phytase-producing autochthonous bacteria in the gastrointestinal (GI) tracts of 14 freshwater teleost fishes; and to identify the most promising phytase-producing strains by phenotypic characterization and 16S rDNA.

Materials and methods. The GI tracts were removed and divided into proximal (PI) and distal (DI) intestine. Homogenates of intestinal segments were spread onto sterilized tryptone soya agar and modified phytase screening media (MPSM) plates to determine autochthonous culturable heterotrophic and phytase-producing microbiota, respectively. Data were presented as log viable counts (LVC) g-1 intestine. Out of 95 phytase-producing isolates, primarily selected 32 isolates were studied for phytase-assay using MPSM broth. Promising phytase-producing isolates were evaluated for other exo-enzymes (amylase, cellulase, protease, lipase) using 4 selective media. Two most promising phytase-producing isolates were identified by phenotypic characterization and 16S rDNA.

Results. Population of heterotrophic bacteria was highest (LVC = 8.29 g^{-1} intestine) in the DI of *Gudusia chapra* followed by DI of *Hypophthalmichthys molitrix* (LVC = 6.82 g^{-1} intestine). However, more than log 4 reduction of the phytase-producing bacteria was observed compared to heterotrophic bacteria. Phytase-producing microbiota was highest in the PI of *G. chapra* (LVC = 3.95 g^{-1} intestine) followed by PI of *Labeo calbasu* (LVC = 3.78 g^{-1} intestine). The strain LB1.4 isolated from DI of *Labeo bata* showed highest phytase activity (2.33 ± 0.006 U · mL^{-1}) followed by the strain GC1.2 (2.19 ± 0.018 U · mL^{-1}) isolated from PI of *G. chapra.* Both isolates were efficient in producing other exo-enzymes. Phenotypic characterization and nucleotide homology analysis revealed that the isolates LB1.4 and GC1.2 were similar to *Bacillus subtilis* and *Bacillus atrophaeus*, respectively.

Conclusion. Autochthonous phytate degrading bacteria were present in the GI tract of fish that might endow ecological advantages to overcome the anti-nutritional effects of plant phytate.

Keywords: Gut bacteria, phytase, *Bacillus*, freshwater teleost, 16S rDNA

INTRODUCTION

Phytase (E.C.3.1.3.8. myo-inositol hexaphosphate phosphohydrolase) is a hydrolytic enzyme that initiates the release of phosphate from phytic acid (myo-inositol 1, 2,3, 4,5,6-hexakis-dihydrogen phosphate), which is the major phosphorus (P) storage compound in plant feedstuffs (Oatway et al. 2001). To combat the increasing cost and irregular supply of fish meal (FM) on the global scale, there has been an emphasis to replace FM in aquafeeds with alternative ingredients derived from low cost and protein-rich plant sources (Hardy 2010). However, this might increase the risk of exposure of the plant derived antinutritional factors, such as phytate, to cultured fish. Natural feeding on phytoplankton, algae, or aquatic weeds may also increase this problem. Phytate forms compounds with a large number of minerals like K, Mg, Ca (Graf 1983), Zn (Lei et al. 1993), Fe, and Cu (Lee et al. 1988). In addition, it also forms complexes with proteins and amino acids, thereby reducing bioavailability of minerals and decrease digestibility of proteins due to lack of intestinal phytase in monogastric animals including fish (Pointillart et al. 1987). The antinutritional effect of phytate is likely to be reduced by hydrolysing it with phytase (Gatlin et al. 2007). Furthermore, dietary supplementation of microbial phytase, or pre-treatment of plant ingredients through fermentation by microbial phytase was comprehended to increase phytate phosphorus bioavailability, and thereby reduce the use of inorganic phosphorus supplements (Sardar et al. 2007, Cao et al. 2008).

* Correspondence: Dr. Koushik Ghosh, Aquaculture Laboratory, Department of Zoology, The University of Burdwan, Burdwan 713 104, West Bengal, India, phone: (+91) 943 425 1606, fax: (+91 342) 263 4200, e-mail: *kghoshbu@gmail.com* or *kghosh_bu@yahoo.co.in*.

During the last decade there has been an improved understanding on the importance of commensal intestinal microbiota in fish (Ringø et al. 2010). The gut microbiota may be categorized as either autochthonous (indigenous; adherent) or allochthonous (transient) depending upon its ability to adhere and colonize the mucus layer in the digestive tract (Ringø and Birkbeck 1999, Ringø et al. 2003). Previous studies conducted on Indian major carps advocated beneficial aspects of gut-associated microbiota in the host fish with regard to nutrition (Ghosh et al. 2002a, b, Ray et al. 2010). Degradation of phytic acid through the action of phytases produced by microbiota is well known in ruminants (Selinger et al. 1996, Yanke et al. 1998, Lan et al. 2011). Further, phytase-producing bacteria isolated from fish gut have also been reported (Li et al. 2008, Roy et al. 2009, Askarian et al. 2012a, b). Li et al. (2008) documented phytase-producing marine yeast strain *Kodamea ohmeri* BG3 isolated from the gut of a marine fish, *Hexagrammos otakii* Jordan et Starks, 1895. However, the authors did not pay attention whether the marine yeast strain was adherent or transient. To the authors' knowledge, only two reports addressed autochthonous phytase-producing bacteria from fresh water fishes (Roy et al. 2009, Khan et al. 2011), which indicates that this issue merits further investigation.

In view of this context, the major aim of the presently reported study was to evaluate the presence of phytase-producing autochthonous bacteria in the gastrointestinal (GI) tracts of 14 freshwater teleost fishes. In addition, we evaluated other exo-enzymes (protease, amylase, cellulase, and lipase) as these enzymes might contribute in nutrition of fish (Ray et al. 2012). Finally, the two most promising phytase-producing bacteria, which were also capable of producing other exo-enzymes, were identified on the basis of phenotypic characteristics as well as 16S rDNA sequence analysis.

MATERIALS AND METHODS

Fish species examined. Within the frames of this study we examined 14 fish species, namely: rohu, *Labeo rohita* (Hamilton, 1822); catla, *Catla catla* (Hamilton, 1822); mrigal, *Cirrhinus cirrhosus* (Bloch, 1795); orangefin labeo, Labeo *calbasu* (Hamilton, 1822); silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844); common carp, *Cyprinus carpio* Linnaeus, 1758; bata, *Labeo bata* (Hamilton, 1822); pool barb, *Puntius sophore* (Hamilton, 1822); climbing perch, *Anabas testudineus* (Bloch, 1792); Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758); striped dwarf catfish, *Mystus vittatus* (Bloch, 1794); stinging catfish, *Heteropneustes fossilis* (Bloch, 1794); Indian river shad, *Gudusia chapra* (Hamilton, 1822); spotted snakehead, *Channa punctata* (Bloch, 1793). Their food habits, average length and weight, and average weight of the gut are presented in Table 1.

The specimens were sampled by a gill-net from 3 local culture ponds and transported to the laboratory at Golapbag, Burdwan, West Bengal, India inside oxygen-packed plastic bags.

Processing of specimens. Prior to experiment, the fish were reared in glass aquaria (75 L) with tap water for 7 days separately according to their source and species. During this period the fish were fed a sterilized diet (36% protein, 7% lipid, and 29% carbohydrate) consisting of fishmeal and rice bran (1 : 1). The ranges of water quality parameters were: temperature 18–23°C; pH 6.9–7.2; and dissolved oxygen 6.8–7.8 mg · L^{-1}. Nine individuals of each fish species collected from 3 ponds (three from each pond) were used in the presently reported study. The fish were starved for 48 h to empty the gastro-intestinal (GI) tracts (Ray et al. 2010). After starvation, the fish were anaesthetized and sacrificed by applying 0.03% of tricaine methanesulfonate (MS-222). Immediately after being sacrificed, the ventral surface of each fish was thoroughly scrubbed with 1% iodine solution for surface sterilization according to Trust and Sparrow (1974). The fish were dissected aseptically within laminar airflow on ice and their alimentary tracts were removed. Gut samples were processed for isolation of adherent (autochthonous) bacteria as described by Ringø (1993) with minor modification. The GI tracts were divided into PI (proximal part of the intestine) and DI (distal part of the intestine), cut into pieces, and flushed carefully three times with 0.9% sterile saline solution using an injection syringe in order to remove non-adherent (allochthonous) microbiota according to Ghosh et al. (2010). The gut segments were homogenized with 10 parts of sterilized pre-chilled 0.9% NaCl solution as described elsewhere (Das and Tripathi 1991). Pooled samples of 3 fish were used for each replicate to avoid erroneous conclusions due to individual variations in gut microbiota, as described elsewhere (Ringø et al. 1995, Spanggaard et al. 2000, Ringø et al. 2006).

Microbial culture. Homogenate of the pooled intestinal segments of each of the 3 replicates for each fish species and each region of gut was used separately after appropriate serial (1 : 10) dilutions (Beveridge et al. 1991). Diluted samples (0.1 mL) were poured aseptically (each in triplicate) within a laminar airflow on sterilized tryptone soy agar (TSA; Himedia, India) and incubated at 37°C for 48 h to determine culturable heterotrophic autochthonous bacteria. Phytase-producing bacteria was determined by using a modified phytase-screening media (MPSM) (Howson and Davis 1983) with some modifications. Briefely, the composition of MPSM medium was (g L^{-1}): glucose, 10; $(NH_4)_2SO_4$, 1; urea, 10; citric acid, 3.0; sodium citrate, 2; $MgSO_4$ $7H_2O$, 1; sodium phytate, 3; $FeSO_4$ $7H_2O$, 0.01; and agar, 20. For the preparation of MPSM, 0.3 g of sodium phytate was dissolved in 10 mL of deionized H_2O, sterilized separately and thereafter added 90 mL sterilized sodium phytate-free MPSM. pH of phytate-free MPSM was adjusted to 7.0 before sterilization according to Roy et al. (2009). Diluted samples (0.1 mL) were poured aseptically within a laminar airflow on MPSM plates and incubated at 37°C for 72 h as phytase-producing strains required prolonged incubation to grow (authors' personal observation). The colony forming units (CFU) per unit sample volume of gut homogenate were determined by multiplying the number of colonies formed on each plate by the reciprocal dilution

(Rahmatullah and Beveridge 1993). Data is presented as log viable counts (LVC) g^{-1} intestine. Colonies with apparently different morphological appearances (such as colour, configuration, surface, margin, and opacity) from a single plate were streaked separately on MPSM plates to obtain pure cultures.

Screening of isolates by quantitative assay for extra-cellular phytase production. Out of the 95 phytase-producing bacterial strains isolated from the fish species examined; 32 randomly selected isolates (33% of total isolates) were further evaluated for quantitative phytase assay. Quantitative phytase assay was done with MPSM broths and one phytase unit (U) was defined as 1 µg of inorganic phosphorus released per 1 mL of culture filtrate per 1 min (Yanke et al. 1999). Potent phytase-producers were further studied to quantify extracellular protease, amylase, cellulase, and lipase production capacities.

Quantitative assay for production of protease, amylase, cellulase and lipase by the gut isolates. Quantitative assay for the production of amylase, cellulase, protease and lipase by the most promising phytase-producing gut bacteria were performed using the methods described by Bernfeld (1955), Denison and Kohen (1977), Walter (1984), and Bier (1955), respectively. Quantitative measurement of enzymes; cellulase, amylase, protease, and lipase activity were determined as described elsewhere (Bairagi et al. 2002).

Morphological, physiological, and biochemical characterization. The two most promising phytase-producing isolates (GC1.2 and LB1.4) were subjected to morphological, physiological, and various biochemical tests following standard methods. Identification of the strains was primarily based on the phenotypic characters described in the Bergey's Manual of Systematic Bacteriology (Holt et al. 2000).

Identification of isolates by 16S rDNA sequence analysis. Results of the identification based on the phenotypic characters were further confirmed by the analysis of partial 16S rDNA sequences as described by Roy et al. (2009). Sequenced data were aligned and analyzed for finding the closest homolog of the microbes using a combination of NCBI GenBank and RDP database. Phylogenetic trees were constructed in MEGA 4.1 software using the neighbor joining method with bootstrap analysis to obtain information on their molecular phylogeny. Partial sequences of 16Sr DNA from the 2 selected isolates were deposited in the NCBI GenBank database to obtain accession numbers (HM 246635, HM 352551).

Statistical analysis. Statistical analysis of the observed data were performed according to Zar (1999) using SPSS Ver10 (Kinnear and Gray 2000) software, if applicable. Data pertaining to extracellular phytase production were subjected to analysis of variance (ANOVA) followed by Tukey's test.

RESULTS

Enumeration of microbial community in the GI tracts of the 14 fish species studied revealed that autochthonous culturable heterotrophic and phytase-producing microbiota are present in both PI and DI regions in all the fish species studied (Table 2). Population levels of culturable autochthonous heterotrophic aerobic/facultative anaerobic bacteria were highest in the DI region of all the fish species studied. Highest number of culturable heterotrophic microbiota was noticed in the DI region of *Gudusia chapra* followed by DI region of *Hypophthalmichthys molitrix* (LVC = 8.29 and 6.82 g^{-1} intestinal tissue respectively); whereas phytase-producing microbiota on MPSM plate was highest in the PI region of *G. chapra* followed by the PI region of *Labeo calbasu* (LVC = 3.95 and 3.78 g^{-1} intestinal tissue, respectively). Compared to heterotrophic bacteria, the phytase-producing bacteria level was generally lower in the DI region of most of the fish species examined, except *Cyprinus carpio*.

Table 1

Food habits, live weight, fish length, and gut weight of the fishes examined

Fish species	Food habits	Live weight [g]	Fish length (TL) [cm]	Gut weight [g]
Labeo rohita	Omnivorous/phytophagous	89.71 ± 2.53	20.05 ± 1.69	4.24 ± 0.05
Catla catla	(Zoo) planktophagous	81.07 ± 2.66	20.62 ± 0.59	1.71 ± 0.04
Cirrhinus cirrhosus	Detritivorous	82.67 ± 1.17	20.66 ± 2.22	3.72 ± 0.02
Labeo calbasu	Detritivorous	91.71 ± 2.10	26.32 ± 0.90	4.2 ± 0.01
Hypophthalmichthys molitrix	(Phyto) planktophagous	104.80 ± 7.50	26.43 ± 1.16	2.16 ± 0.02
Cyprinus carpio	Detritivorous	157.95 ± 5.14	21.30 ± 1.03	4.12 ± 0.02
Labeo bata	Herbivorous	46.81 ± 3.38	16.69 ± 1.42	1.21 ± 0.03
Puntius sophore	Nymphs, algae (diatoms)	23.76 ± 3.96	13.63 ± 1.23	0.33 ± 0.03
Anabas testudineus	Carnivorous	22.83 ± 2.36	10.45 ± 1.20	0.63 ± 0.03
Oreochromis niloticus	Omnivorous	22.44 ± 2.98	9.34 ± 0.91	0.95 ± 0.02
Mystus vittatus	Carnivorous	12.11 ± 0.92	9.41 ± 1.70	0.63 ± 0.02
Heteropneustes fossilis	Carnivorous	47.70 ± 4.96	24.98 ± 1.36	0.95 ± 0.01
Gudusia chapra	Planktophagous	11.66 ± 1.53	8.82 ± 1.30	0.281 ± 0.01
Channa punctata	Carnivorous	134.33 ± 3.32	15.39 ± 1.20	1.94 ± 0.05

Values are mean ± standard deviation of nine specimens of each species; TL = total length.

Thirty-two selected isolates (17 isolates from the PI region and 15 isolates from the DI region) were evaluated for quantitative phytase assay. It was observed that strains LB1.4 isolated from the DI of *Labeo bata* and GC1.2 isolated from the PI of *G. chapra* were the two most promising phytase-producers; 2.33 ± 0.006 and 2.19 ± 0.018 U · L^{-1}, respectively (Table 3). Data pertaining to extracellular protease, amylase, cellulase, and lipase production by some potent phytase producers were presented in Table 4. Both the promising phytase producers (LB1.4 and GC1.2) were efficient in the production of the other exo-enzymes as well. Therefore, the isolates LB1.4 and GC1.2 were finally selected for identification in view of probable future use.

Table 2

Log values of culturable autochthonous aerobic heterotrophic (grown on TSA plates) and phytase-producing (grown on MPSM plates) bacteria isolated from the GI tracts of 14 different fish species

Fish species	Log viable counts (g^{-1} intestinal tissue)			
	Proximal intestine		Distal intestine	
	TSA	MPSM	TSA	MPSM
Channa punctata	5.23	2.89	6.68	2.48
Labeo rohita	4.93	3.08	6.36	3.48
Labeo bata	5.30	3.30	6.45	2.28
Catla catla	5.27	3.28	6.28	2.95
Gudusia chapra	6.88	3.95	8.29	3.24
Cirrhinus cirrhosus	5.71	3.41	6.38	2.71
Labeo calbasu	5.34	3.78	6.32	3.48
Hypophthalmichthys molitrix	4.78	2.49	6.82	2.48
Cyprinus carpio	4.90	3.13	6.34	3.30
Puntius sophore	4.51	2.22	6.63	3
Anabas testudineus	4.67	3.48	5.48	2.60
Oreochromis niloticus	4.52	3.48	5.32	3.1
Mystus vittatus	4.60	2.30	5.14	2.72
Heteropneustes fossilis	5.10	2	6.34	2.79

TSA = tryptone soy agar; MPSM = modified phytase screening medium.

Table 3

Primarily selected bacterial isolates from fish gut with their quantitative extra-cellular phytase activity

Fish species	Proximal intestine		Distal intestine	
	Isolate	Phytase activity (U)*	Isolate	Phytase activity (U)*
Labeo rohita	LR1.2	1.51 ± 0.035^{b}	LR1.1	1.15 ± 0.013^{d}
	—	—	LR3.1	1.02 ± 0.017^{e}
Catla catla	CC1.1	1.95 ± 0.008^{a}	CC2.1	1.03 ± 0.012^{e}
Cirrhinus cirrhosus	CM1.1	1.23 ± 0.012^{c}	CM1.2	1.30 ± 0.003^{b}
Labeo calbasu	LC1.1	1.22 ± 0.036^{c}	LC2.1	1.06 ± 0.015^{e}
Hypophthalmichthys molitrix	SC1.1	0.86 ± 0.012^{f}	SC1.2	1.34 ± 0.000^{b}
	SC3.2	0.99 ± 0.020^{f}	—	—
Cyprinus carpio	CP1.2	1.17 ± 0.039^{d}	CP3.1	1.20 ± 0.032^{c}
Labeo bata	LB1.1	1.15 ± 0.018^{d}	LB1.4	2.33 ± 0.006^{a}
	LB2.1	1.05 ± 0.015^{e}	—	—
Puntius sophore	PS1.3	0.97 ± 0.010^{f}	PS3.2	0.91 ± 0.006^{f}
Anabas testudineus	AT1.1	0.84 ± 0.015^{f}	AT2.3	0.88 ± 0.003^{f}
Oreochromis niloticus	ON1.1	1.05 ± 0.003^{e}	ON3.2	1.02 ± 0.006^{e}
Mystus vittatus	MV1.1	1.32 ± 0.010^{b}	MV2.3	1.19 ± 0.007^{d}
Heteropneustes fossilis	HF1.2	1.12 ± 0.019^{d}	HF2.2	1.06 ± 0.015^{e}
Gudusia chapra	GC1.2	2.19 ± 0.018^{a}	GC3.2	0.84 ± 0.015^{f}
	GC2.3	1.23 ± 0.049^{c}	—	—
Channa punctata	CP1.1	1.00 ± 0.023^{f}	CP1.2	1.22 ± 0.009^{c}

Data are means ± SE of three determinations; Means with same superscript do not vary significantly ($P < 0.05$).
*1phytase unit (U) = 1 mg of inorganic phosphorus liberated per mL of enzyme extract per min.

Phenotypic characterization, nucleotide homology and phylogenetic analysis of the 16S rDNA partial sequences by nucleotide blast in the NCBI GenBank and RDP database revealed that LB 1.4 and GC 1.2 belonged to *Bacillus subtilis* cluster. The isolates, LB1.4 and GC1.2, shared some common phenotypic characteristics, e.g., Gram positive, rod shaped, motile, and capable of endospore formation. Physiological characterization revealed that both could grow within a wide range of temperatures (10–42°C), at moderate pH (6–8), and exhibited NaCl tolerance up to 7%. The majority of the biochemical characters expressed by both the isolates were similar except Voges Proskauer and oxidase tests. In addition, the isolate GC1.2 produced black pigment in the culture medium. Differences between the two strains as evident from the conventional studies were presented in Table 5. Based on the descriptions given in Bergey's Manual of Systematic Bacteriology (Holt et al. 2000) it was revealed that isolate LB1.4 isolated from the DI of *L. bata* was similar to *Bacillus subtilis*, whereas, the isolate GC1.2 isolated from PI of *G. chapra* was similar to *B. atrophaeus*. Results based on the traditional characterization were confirmed by the 16S rDNA sequence analysis. The isolate LB1.4 showed 100% similarity with *B. subtilis* strain BL 4 (accession No. GU 826160) and isolate GC1.2 showed 100% similarity with *B. atrophaeus* C34 (accession No. DQ153971). The NCBI GenBank accession numbers of the sequences for LB1.4 and GC1.2 are HM 352551 and HM 246635, respectively. The phylogenetic relations of the bacterial isolates with other closely related bacteria are presented in the dendrogram (Fig. 1).

DISCUSSION

Reports on phytase activity in fish (Ellestad et al. 2003, LaVorgna unpublished*) were contradictory and confusing. At present, it is generally accepted that the fish, like other monogastric and ruminant animals, lack phytase enzyme (Cho and Bureau 2001, Cao et al. 2008). However, as in ruminants, possibility of phytate degradation through the action of phytase produced by the fish gut microbiota has been suggested in some of the recent investigations (Li et al. 2008, Roy et al. 2009, Khan et al. 2011). In the presently reported study, phytase-producing bacterial symbionts were detected in the GI tracts of 14 freshwater fish species studied. As the fish were starved for 48 hours and their GI tracts were thoroughly washed with sterile chilled 0.9% saline prior to isolation of microbiota, it may be affirmed that the microorganisms isolated in the present study belonged to the autochthonous microbiota as suggested elsewhere (Ghosh et al. 2010). In the presently reported study, gut isolates were isolated by culture dependant methods. It is generally argued that conventional culture-based techniques are time consuming, lack accuracy (Asfie et al. 2003), and do not represent a correct picture of the bacterial diversity in fish gut, even if numerous different media are used (Ray et al. 2010). Besides, one has to admit that presence of any microorganism within the GI tract does not necessarily signify its functional role that it could play (Ray et al. 2012). As the major aim of the present study was to detect a specific enzyme (phytase) producing gut bacteria in some freshwater fish, the use of a culture-dependent technique is reasonable.

It may be apprehended that only isolation and identification would not give a representative picture of the gut microbiota in different regions of the GI tract (Mondal et al. 2008) with relation to their functional significance. Therefore, it was considered legitimate in the presently reported study to quantify heterotrophic bacteria along with phytase-producing bacteria at different regions of the GI tracts in the fish species studied, since this study was intended to gather information on phytase-producing gut bacteria in fish. Heterotrophic and phytase-producing populations were recorded highest in the DI and PI regions, respectively in planktophagous *G. chapra.* It may be mentioned that heterotrophic microbial population was observed highest in DI regions of all the fish species studied when compared to the PI regions, which is in agreement with the previous reports (Mondal et al. 2008, Ghosh et al. 2010, Ray et al. 2010). Out of the 14 fish species examined in this study, 10 species (8 carps: *L. rohita*, *C. catla*, *C. cirrhosus*, *L. calbasu*, *H. molitrix*, *C. carpio*, *L. bata*, and *P. sophore*; as well as *O. niloticus* and *G. chapra*) were either herbivore or omnivore, or feeding on detritus arising out from the plant feedstuffs (Jhingran 1997). Presence of appreciable quantity of both culturable heterotrophic and phytase-producing microbiota in both PI and DI regions of the GI tracts of the fish species studied might signify their probable role in degradation of phytate in the plant feedstuffs. Although, it may be noted

Table 4

Quantitative extracellular cellulase, protease, amylase and lipase activities of some selected isolates from fish GI tracts

Fish species	Isolate	Extracellular enzyme activity (U)			
		Cellulase[a]	Protease[b]	Amylase[c]	Lipase[d]
Catla catla	CC1.1	42.56 ± 1.12	9.23 ± 1.21	158.56 ± 1.95	7.89 ± 0.58
Hypophthalmichthys molitrix	SC1.2	36.21 ± 1.07	8.32 ± 1.07	182.67 ± 3.08	6.84 ± 0.81
Labeo bata	LB1.4	48.33 ± 1.14	12.14 ± 1.23	212.35 ± 3.98	8.88 ± 0.99
Mystus vittatus	MV2.3	22.01 ± 0.97	8.07 ± 1.01	147.08 ± 2.01	6.97 ± 0.83
Gudusia chapra	GC1.2	46.54 ± 0.88	12.32 ± 0.37	208.65 ± 3.02	9.32 ± 0.86
	GC2.3	31.02 ± 0.98	11.07 ± 0.21	101.32 ± 1.84	5.08 ± 0.47

Data are means ± SE of three determinations; [a] µg glucose liberated mL^{-1} of enzyme extract min^{-1}; [b]µg tyrosine liberated mL^{-1} of enzyme extract min^{-1}; [c]µg maltose liberated mL^{-1} of enzyme extract min^{-1}; [d] µmole fatty acid liberated mL^{-1} of enzyme-extract min^{-1}.

* LaVorgna M. 1998. Utilization of phytate phosphorus by tilapia. Ph.D. dissertation, University of Maryland, Eastern Shore, Princess Anne, MD, USA.

that phytase-producing microbial count was far less than the heterotrophic microbial count in all the fish species studied, which is in accordance with the previous report (Roy et al. 2009). In addition, phytase-producing bacteria were detected in the carnivorous fish species also. Carnivores might pick up the phytase-producing bacteria with their food organisms, as suggested by Stickney and Shumway (1974) regarding the presence of cellulase producing bacteria in carnivore fish species.

To the authors' knowledge, previously only two reports have considered phytase activity by gut bacteria from freshwater teleosts. Roy et al. (2009) reported phytase-producing microbiota in 10 freshwater teleosts and the strains LF1 and LH1 isolated from *L. rohita* were identified as *B. licheniformis*. Khan et al. (2011) isolated an efficient phytase-producing strain CC 1.1 from *Catla catla* and identified it as *Rhodococcus* sp. MTCC 9508. Apart from these limited information in freshwater fish, Li et al. (2008) documented several marine yeast strains from the gut of sea cucumber (*Holothuria scabra*) and marine fish: *Hexagrammos otakii* and "*Synechogobius hasta*" = *Acanthogobius hasta* (Temminck et Schlegel, 1845); having ability to produce large amount of extra-cellular phytase, and opined that such marine yeasts might play important role in degradation of phytate within the guts of marine animals. Askarian et al. (2012a) demonstrated phytase activity by *B. subtilis*, *Acinetobacter* sp., *B. thuringiensis*, *B. cereus*, and *Bacillus* sp. isolated from the GI tract of Atlantic salmon, *Salmo salar*, fed with or without chitin supplemented diet. Further, *Brochothrix* sp. and *Brochothrix thermosphacta* isolated from the GI tract of Atlantic cod were also described as phytase producers (Askarian et al. 2012b). However, in both the studies the authors did not quantify phytase activity of the bacterial strains. In this study, phytase-producing strains were noticed through quantitative phytase assay and 2 promising strains (GC1.2 and LB1.4) were identified as *B. atrophaeus* and *B. subtilis*, respectively based on phenotypic characters as well as 16S rDNA sequence analysis as suggested elsewhere (Roy et al. 2009, Ghosh et al. 2010, Mondal et al. 2010, Ray et al. 2010). Although both the isolates belonged to *B. subtilis* cluster (Xu and Côté 2003), biochemical characterization revealed that they differed in the capacity of pigment production, Voges Proskauer and oxidase tests (Table 5). In addition, both the potential phytase-producing isolates exhibited their capacities for extracellular protease, amylase, cellulase and lipase production. It has been opined by several authors that enzymes produced by such intestinal microbiota might have a significant role in

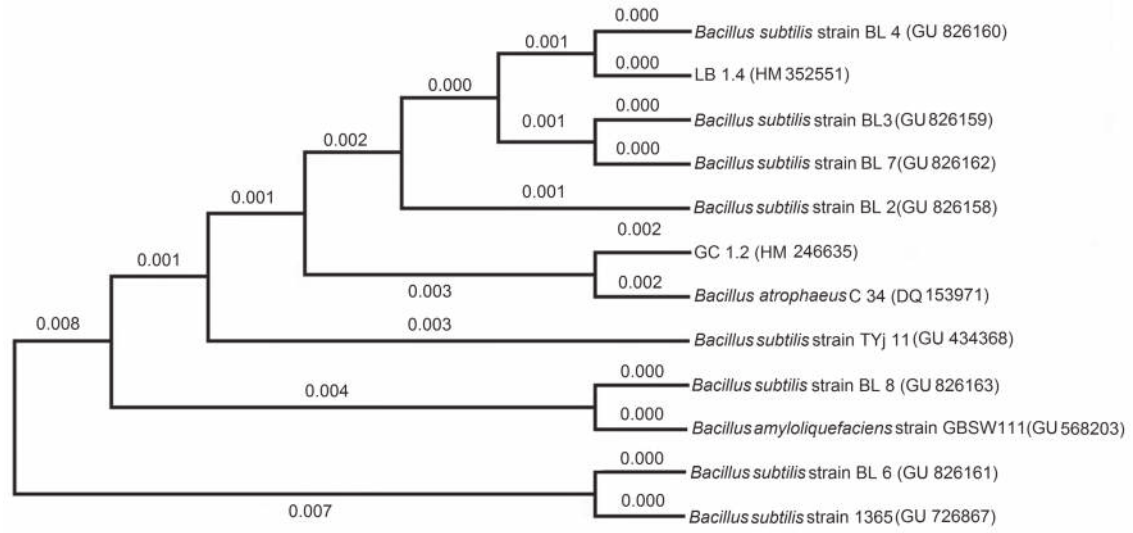

Fig. 1. Dendrogram showing phylogenetic relations of the bacterial strains LB1.4 (*Bacillus subtilis*) and GC1.2 (*Bacillus atrophaeus*) with other closely related bacterial strains; Horizontal bars in the dendrogram represent the branch length; Similarity and homology of the neighbouring sequences have been shown by the bootstrap values

Table 5

Differences of the phenotypic characteristics of isolates LB1.4 and GC1.2

Parameter	LB1.4 (*Bacillus subtilis*)	GC1.2 (*Bacillus atrophaeus*)
Pigment	Off-white	Black
Cell shape and size	Rod shaped, 1–2 µm	short rods, 0.5–1.5 µm
Arrangement	Chains	Very small chain
Growth at pH 9.0	+	–
Voges Proskauer test	+	–
Oxidase test	+	–

+ positive; – negative.

digestion (Ghosh et al. 2002 a, b, Ray et al. 2010, 2012). Diverse strains of exo-enzyme producing *Bacillus* spp. have been identified from the GI tract of freshwater teleosts (for review see Ray et al. 2012). However, to the authors' knowledge, *B. atrophaeus* has not previously been reported from fish gut. Besides, phytase-producing ability by a gut inhabiting *B. subtilis* from freshwater fish species has not been documented so far.

Fish lack the phytase enzyme, so supplemental inorganic phosphate is added to their feed to meet the phosphate requirement and to ensure good growth (Cao et al. 2008). However, supplemental inorganic phosphate does not diminish the antinutritive effect of phytate (Roy et al. 2009). Addition of microbial phytase has been reported to improve phytate phosphorus bioavailability and thereby reduce the use of inorganic phosphorus supplements in poultry (Lei and Stahl 2000), pig (Han et al. 1997) and fish (Van Weerd et al. 1999, Robinson et al. 2002, Sardar et al. 2007). During the last decade, phytase has been widely used by aqua-feed industries to enhance the growth performance, nutrient utilization and bioavailability of macro and micro minerals in fish and also to reduce the fecal phosphorus pollution into the aquatic environment (Kumar et al. 2011). Soil fungus, *Aspergillus* sp. is the chief source of commercial phytase used in the animal feeds (Maenz 2001). However, bacterial phytase might be alternative to the fungal enzymes due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency (Konietzny and Greiner 2004). Results of the present study might suggest considerable opportunities for using phytase-producing bacterial symbionts from fish gut as aquaculture probiotics that may reduce or inhibit the toxicity of phytate within the gut microenvironment. Both the strains grew well within a pH range of 6–8 that correspond with the pH within fish gut and exhibited NaCl tolerance almost up to 8%. The strains could grow within a wide range of temperature (10–42°C). This capacity probably enabled these two isolates to adapt themselves within the gastrointestinal micro-environment of fish, which are poikilotherms. Similar ranges of temperature and pH tolerance have been reported in other strains of Bacilli isolated from fish gut (Ghosh et al. 2002a, Saha et al. 2006, Kar et al. 2008). Because of neutral pH condition in the small intestine of fish, phytic acid might change from the protonated form into phytates, predominantly Ca-phytate, which is true substrate of *Bacillus* phytase (Oh et al. 2001). Thus, *Bacillus* phytase appears to be most promising for phytase activity within the small intestine of fish. Apart from the phytase-producing ability, biochemical characterization and an appraisal of different extracellular enzyme production indicated that the selected isolates were able to utilize various substrates. Capacity for phytase production as well as probable supplementation of other exo-enzymes like amylase, protease, cellulase and lipase might expand the scope for their use in aquaculture nutrition.

CONCLUSIONS AND FUTURE PERSPECTIVES

This preliminary investigation confirms the existence of phytase-producing bacterial symbionts within fish GI tracts. Whether the gut microbiota isolated in the present study can contribute to the host's nutrition has not been addressed and an appraisal of their role should therefore be given high priority in future studies. Phytate degrading microbiota detected in the present study may endow the host with some ecological advantages by enabling them to overcome the antinutritional effects of plant phytate. Exploitation of such gut microbiota for degradation of phytate in feedstuffs of plant origin appears to be promising to improve the nutritive value of phytate-rich feeds. Investigations are enduring to optimize fermentation conditions to enhance phytase production by the gut isolates. However, the potential beneficial effects of those bacteria isolated in the present study are worth to further investigations to determine their role in fish nutrition and health before advocating their application in commercial aquaculture.

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