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EXTRACELLULAR TANNASE-PRODUCING BACTERIA DETECTED IN THE DIGESTIVE TRACTS OF FRESHWATER FISHES (ACTINOPTERYGII: CYPRINIDAE AND CICHLIDAE)

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Background. Although, presence of tannase-producing and/or tannin tolerant gut bacteria has been documented in ruminants and non-ruminant herbivores, the topic is rarely addressed in fish. The present study aimed at enumeration of autochthonous tannase-producing bacteria in the gut of freshwater teleosts.

Materials and methods. This study covered seven freshwater teleost fish species: rohu, *Labeo rohita* (Hamilton, 1822); catla, *Catla catla* (Hamilton, 1822); mrigal, *Cirrhinus mrigala* (Hamilton, 1822); grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844); common carp, *Cyprinus carpio* Linnaeus, 1758; silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844); and Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758). Gastrointestinal (GI) tracts of the fish studied were divided into proximal (PI) and distal (DI) parts, homogenized and plated onto Tryptone Soya Agar (TSA) plates. The pure colonies were spotted on selective tannic acid (TA) agar plates to determine the tannase-producing bacteria. Extracellular tannase-producing capacity of the isolates was determined through qualitative and quantitative assay using TA media plates or broth, respectively at three different pH levels (5.5, 7.0, and 8.5). Further, 16S rRNA gene fragments of the promising tannase-producing bacteria were sequenced, aligned, analysed, identified, and deposited to the GenBank.

Results. Totally 685 strains were isolated on TSA plates, of which 116 strains (37 from PI and 79 from DI) grow on TA media and were defined as tannase-producers. The highest percentage of tannase-producing strains was noticed in the DI regions of grass carp, *Ctenopharyngodon idella* (38.98%), and tilapia, *Oreochromis niloticus* (37.74%). The lowest frequency of tannase-producing bacteria was revealed in PI region of catla, *Catla catla* (2.12%). The intestine of Indian major carps was relatively poorly colonized by tannase-producing bacteria compared to that of exotic carps. Evaluation of tannase-producing capacity revealed that the majority of the isolates exhibited maximum extracellular tannase production at pH 7.0. Quantitative evaluation, showed highest tannase activity by strain HMT1 (0.28 ± 0.001 U) isolated from silver carp, *Hypophthalmichthys molitrix*, followed by strains ONH2Ph (0.19 ± 0.005) and ONH13B (0.17 ± 0.009 U) isolated from tilapia. Analyses of the 16S rRNA partial gene sequences revealed that strains ONH2Ph and ONH13B showed high similarity to *Bacillus subtilis* (KP765736) and *Brevibacillus agri* (KP765734), respectively. Whereas, strain HMT1 was most closely related to *Klebsiella variicola* (KP765735).

Conclusions. The study revealed existence of tannase-producing bacterial symbionts within fish GI tracts. Tannin-degrading bacteria detected in the presently reported study might aid in overcoming the anti-nutritional effects of dietary tannins within fish gut.

Keywords: freshwater teleosts, gut bacteria, autochthonous bacteria, tannase

INTRODUCTION

Tannin-acyl-hydrolase (EC.3.1.1.20), commonly known as tannase, is an industrially important microbial enzyme that catalyses hydrolysis of the ester and depside bonds in hydrolysable tannins (e.g., tannic acid) to glucose and gallic acid (Lekha and Lonsane 1997). Tannins are generally considered as plant secondary metabolites that are present in varying amounts depending on species, specific biotic or abiotic environmental stress and phenology (Alonso-Amelot 2011). Apart from the structural polysaccharides (viz., cellulose, hemicelluloses, and lignin), tannins are the most abundant compound formed by vascular plants and probably most common among the plant derived antinutritional factors (Francis et al. 2001).

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Therefore, supplementation of raw plant ingredients in fish feed has been restricted as dietary tannins are reported to hinder protein and total digestibility by interfering with protease or forming indigestible complexes with dietary protein (Krogdahl 1989, Ghosh and Mandal 2015). Indian major carps (IMC), exotic carps and tilapia (*Oreochromis niloticus*) are mostly herbivorous or omnivorous, generally depending on plankton, algae, aquatic macrophytes, and detritus as the food items (Jhingran 1997). Phytoplankton or aquatic vegetation occurring as natural food, together with ingredients commonly used as feed supplements; e.g., oil cakes, leaf meal, or seed meal, contain significant amount of hydrolysable tannin (Mandal and Ghosh 2010a). Exposure to the tannins (200g or less) extracted from terrestrial (*Accacia* sp.) or aquatic macrophytes (*Pistia* sp.) has been found to inhibit or lower the activities of the major digestive enzymes; protease, amylase, and lipase, in the IMCs (Maitra and Ray 2003, Mandal and Ghosh 2010b).

The nutrient-rich micro-environment of fish gut provides favourable ecological niches for the microorganisms (Kar and Ghosh 2008, Mondal et al. 2008, Denev et al. 2009). Although, the variability of the intestinal environment strongly depends on the particular herbivore diet and supposed to create a unique gut microbial community. Some information is available on the presence of tannase-producing and tannin tolerant microbiota in the gastrointestinal (GI) tracts of ruminants and non-ruminant herbivores feeding on tannin rich plant matters (Nelson et al. 1995, Sasaki et al. 2005, Goel et al. 2005). Occurrence of tannase-producing microorganisms has been suggested as an ecological adaptation of herbivores to overcome the antinutritional effects of tannins present in plant feed ingredients (Mandal and Ghosh 2010b). Although tannins are toxic as well as bacteriostatic (Scalbert 1991), tannase is produced by a group of tannin tolerant microorganisms; fungi, yeast, and bacteria (Lekha and Lonsane 1997). Tannin tolerant or tannin degrading microorganisms have been isolated from the gut of several animals; koalas (*Phascolarctos cinereus*) (see Osawa 1992), goats (*Capra hircus*) (see Brooker et al. 1994, Nelson et al. 1995), and horses (*Equus caballus*) (see Nemoto et al. 1995) feed on tannin-rich forages. Previous studies documented that fungi and bacteria were the major tannase-producing microorganisms within the gut of diverse animals (for review see Goel et al. 2005). While, Mandal and Ghosh (2013) demonstrated that autochthonous tannase-producing microorganisms within the gut of freshwater teleosts were represented predominantly by diverse yeast species (e.g., *Pichia* sp., *Candida* sp.) and to a lesser extent by bacteria (*Enterobacter* sp.). Although, numerous studies have indicated that bacteria colonizing the GI tract of fish may have beneficial effect by being enzyme-producers and contribute to nutrition and the digestive process in fish (for review see: Ringø et al. 1995, Pond et al. 2006, Ray et al. 2012), less information is available on the tannase-producing gut bacteria in fish (Mandal and Ghosh 2013). Thus, the aim of the presently reported study was to:

- Isolate autochthonous tannase-producing bacteria within the GI tracts of seven freshwater teleosts;
- Screen the tannase-producing bacteria through in vitro qualitative and quantitative tannase assay; and
- Identify the most promising strains by 16S rRNA partial gene sequence analysis in view of their possible use in aquaculture.

MATERIALS AND METHODS

Fish species examined. Within the frames of the study we examined three species of Indian major carps: rohu, *Labeo rohita* (Hamilton, 1822); catla, *Catla catla* (Hamilton, 1822); and mrigal, *Cirrhinus mrigala* (Hamilton, 1822); three species of exotic carps: grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844); common carp, *Cyprinus carpio* Linnaeus, 1758; and silver carp, *Hypophthalmichthys molitrix* (Valenciennes, 1844); and Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758). Along with natural feeding, the fishes received a mixture of fishmeal, de-oiled rice bran, and different oil cakes as supplementary feed (approximately, 30% crude protein, 6% lipid, and 40% total carbohydrate) at 2%–3% of their body weight. Three specimens of each species were collected from each of three polyculture ponds located at and near Burdwan (23°14′N, 87°39′E), West Bengal, India. Altogether nine specimens of each species were evaluated in the presently reported study. During rearing in culture ponds, the fishes were offered mixture of fishmeal, rice bran, and different oil cakes as supplementary feed together with natural feeding. The collected specimens were transported to the laboratory inside oxygen packed plastic bags and kept separately in 350 L *fibre-reinforced plastic* (FRP) aquaria according to their source and species. Water quality parameters during the collection period were: dissolved oxygen 6.5–7.8 mg $\cdot$ L^{-1}, temperature 25.3–28.3°C, and pH 6.7–7.3. Feeding habits, mean weight and length of the fish studied, and mean length and weight of the gut are presented in Table 1.

Post mortem examination. The fish were starved for 48 h in order to clear their GI tracts prior to sacrifice as recommended by Ray et al. (2010). After starvation, fish were anaesthetized and sacrificed by applying 0.03% tricaine methanesulfonate (MS-222) following guidelines of the Institutional Ethical Committee. The ventral surface of each fish was thoroughly scrubbed with 1% iodine solution (Trust and Sparrow 1974) and dissected aseptically within laminar airflow to remove the GI tract. Gut samples were processed for isolation of adherent (autochthonous) bacteria as described by Ringø (1993), with minor modification. Briefly, the GI tracts were divided into proximal (PI) and distal (DI) parts, cut into pieces, and flushed carefully three times with 0.9% sterile saline solution using an injection syringe to remove non-adherent (allochthonous) bacteria (Ghosh et al. 2010). Gut segments from three individuals of a species collected from the same pond were pooled together region-wise for each replicate, with three replicates for the study. The tissues were homogenized with sterilized pre-chilled 0.9% sodium chloride solution (1 : 10, w/v) (Beveridge et al. 1991). Pooled samples were used to avoid erroneous conclusions due to individual variations in the gut microbiota, as described elsewhere (Ringø et al. 1995, Spanggaard et al. 2000, Ghosh et al. 2010).

Table 1

Food habits and principal biometric parameters of the fishes examined

Fish species	Food habits	Live weight [g]	Total length [cm]	Gut length [cm]	Gut weight [g]
Labeo rohita	Omnivorous, mostly plant matter	188.32 ± 8.35	24.2 ± 1.85	191.87 ± 7.35	7.32 ± 0.48
Catla catla	Zooplanktophagous	278.78 ± 9.32	22.7 ± 1.97	181.44 ± 8.65	7.98 ± 0.59
Cirrhinus mrigala	Detrivorous	209.79 ± 8.65	26.1 ± 1.77	215.73 ± 9.74	7.13 ± 0.56
Ctenopharyngodon idella	Herbivorous, mostly macrophytes	380.75 ± 10.35	28.3 ± 2.16	68.75 ± 8.89	8.24 ± 0.69
Cyprinus carpio	Detrivorous	386.33 ± 12.36	26.7 ± 2.45	41.32 ± 9.45	7.12 ± 0.65
Hypophthalmichthys molitrix	Phyto-planktophagous	313.35 ± 9.35	23.8 ± 2.06	198.75 ± 1.38	7.98 ± 0.42
Oreochromis niloticus	Omnivorous, mostly plankton, weeds	105.33 ± 7.35	16.43 ± 1.42	24.34 ± 3.36	2.45 ± 0.39

Values are mean ± standard deviation (SD) of nine specimens.

Microbial culture. Homogenate of the pooled intestinal tissue of each of the three replicates for each fish species and each gut segments were used separately after appropriate serial (1 : 10) dilutions (Beveridge et al. 1991). For microbial culture, diluted samples (0.1 mL) were poured aseptically within a laminar airflow on sterilized Tryptone Soya Agar (TSA; HiMedia, Mumbai, India) plates and incubated at 30°C for 48 h to determine the culturable, heterotrophic, autochthonous, aerobic/facultative anaerobic microbial population. Incubation temperature of 30°C was used as fish were reared at this temperature. Colony forming units (CFU) per unit sample volume of gut homogenates were determined according to Rahmatullah and Beveridge (1993) and the data were transformed as log viable counts (LVC). The well-separated colonies were randomly selected and streaked separately on TSA plates until purity. Tannase-producing bacteria were determined by spotting the pure colonies on selective tannic acid (TA) agar media at 30°C for 72 h (Bradoo et al. 1996, Mondal et al. 2001a). Briefly, the composition of TA media was ($g \cdot L^{-1}$): tannic acid 5; K_2HPO_4 0.5; KH_2PO_4 0.5; MgSO4, $7H_2O$ 0.5; NH_4Cl 1, $CaCl_2$ 0.01; Agar 20; pH 6.5. It was assumed that bacteria forming colonies on the selective TA plates, had tannase activity as depicted elsewhere (Mandal and Ghosh 2013).

Qualitative tannase-producing capacity of selected isolates. Extracellular tannase production by the isolated bacterial strains was analysed on TA plates at three different pH levels; moderately acidic (pH 5.5), neutral (7.0), and moderately alkaline (8.5). After incubation of 96 h, a greenish brown zone appeared around the colonies. The TA plates were flooded with $FeCl_3$ solution (0.01 M $FeCl_3$ in 0.01 N HCl) and kept for 5–10 min at 30°C. $FeCl_3$ reacts with tannic acid and forms a brown colour. The clear zone formed on a dark-brown background indicated tannase activity (Kumar et al. 2010). There were three replicates for each experimental set. Qualitative extracellular enzyme activity was assessed through measurement of halo zones (diameter in excess of colony growth) around the colony (Ray et al. 2010) and presented as follows: low (4–6mm); moderate (7–9 mm); high ((≥ 10 mm).

Screening of isolates by quantitative assay for extracellular tannase production. Based on the result of the qualitative assay, primarily selected isolates were further evaluated for quantitative tannase assay following the procedure described by Mandal and Ghosh (2013). Gut isolates were grown in selective Tannic Acid Broth (TAB) for tannase production. The broth media were inoculated with the seed culture (0.5 mL, 1.5×10^7 cells $\cdot$ mL^{-1}) prepared in the same media and incubated (30°C, 48 h) in a rotary shaker (130 rpm). After incubation the broth was centrifuged (5000 × *g*, 20 min, 4°C) and the supernatant was used as the source of crude enzyme. Tannase activity was determined spectrophotometrically based on the measurement of residual tannic acid content following enzymatic reaction (Mondal et al. 2001b). The reaction mixture containing tannic acid [0.3 mL, 0.5% (w/v) in phosphate buffer, pH 6.5] and crude enzyme (0.2 mL) was incubated at 30°C for 1 h. The reaction was terminated by adding bovine serum albumin (3 mL, 1 mg $\cdot$ mL^{-1}) to precipitate the residual tannic acid. Heat-denatured enzyme was used in the control. Afterward, the tubes were centrifuged (5000 × *g*, 20 min) and the precipitate was dissolved in a mixture of sodium dodecyl sulphate (SDS) and triethanolamine [1% (w/v) of SDS in 5% (v/v) triethanolamine, 3 mL] solution. The absorbance (530 nm) was measured with $FeCl_3$ (1 mL) using a spectrophotometer (Shimadzu UV-1800). One unit (U) of tannase activity was defined as µmol of tannic acid hydrolysed per mL of enzyme extract per minute under assay conditions.

Identification of the gut isolates by 16S rRNA partial gene sequence analysis. The most promising tannase-producing bacteria were examined and identified through 16S rRNA partial gene sequence analysis following the methods described by Das et al. (2014). Briefly, the genes encoding 16S rRNA was amplified by polymerase chain reaction (PCR) using universal primers, 27f (50-AGAGTTTGATCCTGGCTCAG-30) and 1492r (50 GGTTACCTTGTTACGACTT-30). Sanger sequencing was employed to sequence the PCR products by a commercial house using automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA).

Sequenced data were edited using BioEdit Sequence Alignment Editor (Version 7.2.0), aligned and analysed for finding the closest homologue using National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP). Sequences were deposited to the NCBI GenBank, obtained accession numbers and a phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using MEGA 5.2.2 software following the Minimum Evolution Method.

Statistical analysis. Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by Tukey's test according to Zar (1999) using SPSS Ver10 (Kinnear and Gray 2000) software.

RESULTS

Enumeration of the autochthonous heterotrophic bacteria on TSA plates revealed highest bacterial population in the DI regions (Table 2), and highest numbers were noticed in the DI region of grass carp, followed by the DI region of tilapia; LVC = 6.07 and 5.89 g^{-1} intestinal tissue, respectively. The lowest bacterial population was revealed in the PI region of rohu; LVC = 4.25 g^{-1} intestinal tissue.

Altogether 685 strains were isolated from the seven fish species; 92 from rohu, 104 from catla, 96 from mrigal, 100 from grass carp, 97 from common carp, 101 from silver carp, and 95 from tilapia. Amongst these, 116 isolates (37 from PI and 79 from DI) were detected as tannase producers as they grew well on TA plates and revealed greenish-brown halo surrounding their colonies (Table 3). Percentage of tannase-producing strains, calculated on the basis of total isolates on TSA plates, were considerably higher in the DI regions than in the PI regions of all the studied fish species (Table 3). The maximum abundance of tannase-producing strains was noticed in the DI region of grass carp; 23 tannase-producing strains out of 59 isolates and in the DI region of tilapia; 20 tannase-producing strains out of 53 isolates. Among the PI regions investigated, the highest percentage of tannase-producing strains was revealed in tilapia (28.57%), followed by the grass carp (26.82%). Conversely, minimum percentage of tannase-producing isolates was revealed in the PI region of catla; only 1 tannase-producing strains out of 47 isolates. In terms of percentage occurrence, Indian major carps were relatively poorly colonized by the tannase-producing bacteria compared to exotic carps. Qualitative extracellular tannase activities of 30 selected isolates are presented in Table 4. Nine of the selected strains were isolated from tilapia, followed by seven strains isolated from grass carp, while only two strains were selected from rohu and catla each. Qualitative analysis of tannase-producing capacity at different pH levels (5.5, 7.0 and 8.5) showed that most tannase-producing fish gut isolates generally revealed highest extracellular tannase production at pH 7.0 (Table 4).

Among the tannase-producing isolates, only those with high and moderate activity scores at pH 7 were further evaluated through quantitative tannase assay (Table 5). The highest tannase activity was exhibited by isolate HMT1 (0.28 ± 0.001 U · mL^{-1} · min^{-1}) from silver carp, followed by strains ONH2Ph (0.19 ± 0.005 U·mL^{-1}·min^{-1}) and ONH13B (0.17 ± 0.009 U·mL^{-1}·min^{-1}) isolated from tilapia.

These three isolates (HMT1, ONH2Ph, and ONH13B) were suggested as promising extracellular tannase-producers and further studied for characterization and identification. Light microscopy evaluation after Gram-staining revealed that isolate HMT1 was a Gram-negative, small, round shaped bacterium, whereas, the other two isolates (ONH2Ph and ONH13B) were Gram-positive. Based on the nucleotide homology and phylogenetic analyses of the 16S rRNA partial gene sequences by nucleotide blast in the NCBI GenBank and RDP databases, the tannase-producing strain ONH2Ph showed high similarity to *Bacillus subtilis* (AJ276351), while isolate, ONH13B was identified as *Brevibacillus agri* being closest to *B. agri* (AB112716). The strain showing highest qualitative tannase-activity, isolate HMT1 was similar to *Klebsiella variicola* (AJ783916). The phylogenetic relation of the identified bacterial isolates with other closely related type strains retrieved from the RDP database are presented in a dendrogram (Fig. 1). The dendrogram clearly depicted that the isolate HMT1 (KP765735) is an outgroup of type strain *K. varicola* (AJ783916).

Table 2

Log viable counts of culturable heterotrophic autochthonous aerobic/facultative anaerobic microbial population in the digestive tracts of the fish species examined

Fish species	Log viable counts·g^{-1} intestinal tissue (on TSA plate)			
	Proximal intestine		Distal intestine	
	Mean	Range	Mean	Range
Labeo rohita	4.25	4.21–4.29	4.98	4.90–5.07
Catla catla	5.38	5.32–5.43	5.74	5.71–5.78
Cirrhinus mrigala	4.56	4.46–4.65	5.27	5.24–5.29
Ctenopharyngodon idella	5.32	5.3–5.34	6.07	5.98–6.15
Cyprinus carpio	4.26	4.21–4.31	5.62	5.60–5.64
Hypophthalmichthys molitrix	5.12	5.1–5.14	5.82	5.78–5.87
Oreochromis niloticus	4.84	4.81–4.86	5.89	5.84–5.95

Data represents mean values of triplicate observations.

Table 3

Percentage occurrence of tannase-producing strains in the proximal and distal parts of the gut of Indian major carps, exotic carps, and tilapia

Fish species	Proximal intestine			Distal intestine		
	Isolates on TSA	Tannase-producing strains	% of tannase producers	Isolates on TSA	Tannase-producing strains	% of tannase producers
Labeo rohita	42	2	4.76	50	4	8.00
Catla catla	47	1	2.12	57	3	5.26
Cirrhinus mrigala	40	2	5.00	56	5	8.92
Ctenopharyngodon idella	41	11	26.82	59	23	38.98
Cyprinus carpio	43	6	13.95	54	12	22.22
Hypophthalmichthys molitrix	44	3	6.81	57	12	21.05
Oreochromis niloticus	42	12	28.57	53	20	37.74

Percentage of tannase-producing strains calculated on the basis of total isolates on TSA plates.

Table 4

Qualitative extracellular tannase activities of selected fish intestinal isolates of Indian major carps, exotic carps, and tilapia

Fish species	Microbial isolate	Qualitative tannase activity		
		pH 5.5	pH 7.0	pH 8.5
Labeo rohita	LRH5C		+	
	LRH5X		+	
Catla catla	CCH2X	+	++	+
	CCF2A		+	
Cirrhinus mrigala	CMHC2	+	+++	
	CMF2A		++	+
	CMFX3	+	++	
Ctenopharyngodon idella	CIH2C		+++	+
	CIF1C		+	
	CIH2PH	+	++	+
	CIH1C		+	
	CIH2A	+	+++	
	CIH5C		+	
	CIH4CH		+	
Cyprinus carpio	CyC7		+	+
	CyC6		++	
	CyCH3		++	+
	CyCF4	+	++	
Hypophthalmichthys molitrix	HMT1	++	+++	++
	HM1F8X		+	
	HM1H4X		+	
Oreochromis niloticus	ONH1Ph		++	
	ONH2A		+	
	ONF3X		+++	+
	ONF1T		+	
	ONFIX		+	
	ONH13B		+++	+
	ONH2Ph	+	+++	+
	ONH3L		+	
	ONF1A		+	

+++ = high activity (≥ 10 mm); ++ = moderate activity (7–9 mm); + = low activity (4–6 mm).

Table 5
Quantitative tannase activities of the selected fish gut isolates

Fish species	Microbial isolate	Quantitative tannase activity [U]
Labeo rohita	CCH2X	0.09 ± 0.006^{cd}
Cirrhinus mrigala	CMHC2	0.15 ± 0.007^{ef}
	CMF2A	0.06 ± 0.008^{a}
	CMFX3	0.07 ± 0.008^{bc}
Ctenopharyngodon idella	CIH2C	0.08 ± 0.001^{c}
	CIH2Ph	0.09 ± 0.006^{cd}
	CIH2A	0.14 ± 0.005^{e}
Cyprinus carpio	CyC6	0.07 ± 0.011^{bc}
	CyCH3	0.08 ± 0.009^{c}
	CyCF4	0.08 ± 0.001^{c}
Hypophthalmichthys molitrix	HMT1	0.28 ± 0.001^{h}
Oreochromis niloticus	ONH1Ph	0.08 ± 0.04^{c}
	ONF3X	0.11 ± 0.003^{d}
	ONH2Ph	0.19 ± 0.005^{g}
	ONH13B	0.17 ± 0.009^{g}

Unit activity (U) has been expressed as µmol of tannic acid hydrolysed per mL of enzyme extract (culture supernatant) per minute; Data are mean ± standard error ($n = 3$); Values with the same superscript represents no significant variation ($P < 0.05$).

DISCUSSION

Although tannase has been reported in plants and animals, it is mostly produced by yeasts, fungi, and bacteria (Aguilar et al. 2007). Numerous studies have documented tannase-producing microorganisms since the discovery of tannase in 1867 (van Tieghem 1867), even if, tannase-producing bacteria have been less studied (Aguilar-Zárate et al. 2014). To the authors' knowledge, the first study reporting that bacteria, an *Achromobacter* sp., was capable of hydrolysing tannins as sole energy source was reported by Lewis and Starkey (1969). Since then the interest on tannin-degrading bacteria has increased and isolation of new strains from different sources have been reported (Aguilar-Zárate et al. 2014).

In the presently reported study, autochthonous tannase-producing bacteria were detected in the PI and DI of 7 freshwater fish species. However, as the fish were starved for 48 h and their GI tracts were thoroughly washed with sterile chilled 0.9% saline, population level of tannase-producing bacteria in the GI tracts is probably underestimated as allochthonous bacteria was not investigated. Gut microorganisms were isolated on a general growth medium (TSA) by culture dependant methods, amongst which, tannase-producing bacteria were determined in selective TA media following enrichment culture technique. The heterotrophic bacterial population

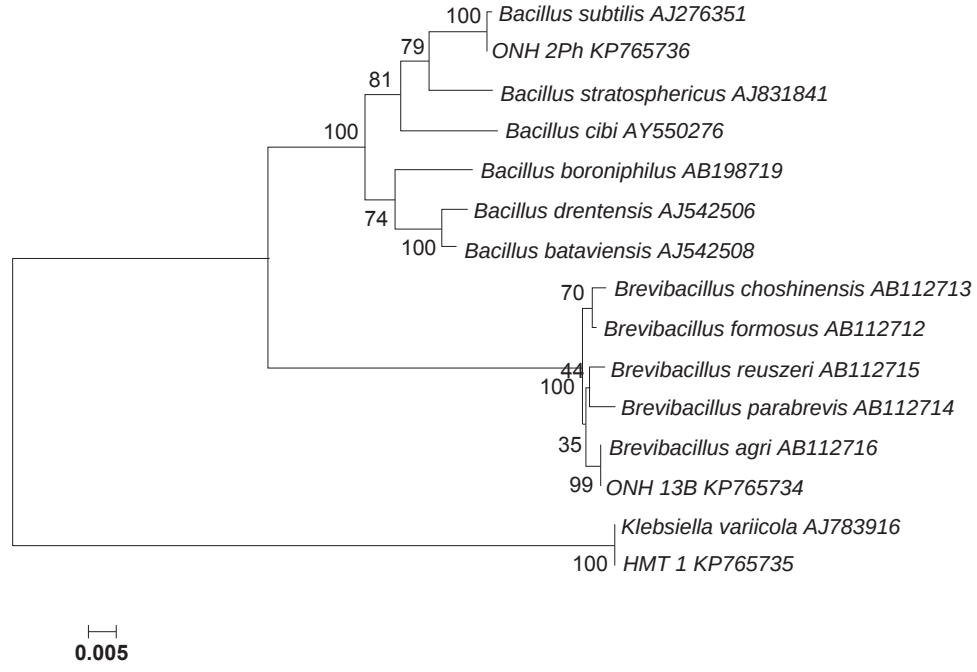

Fig. 1. Dendrogram showing phylogenetic relations of the two potential tannase-producing bacterial strains, *Bacillus subtilis,* ONH2Ph (KP765736) and *Brevibacillus agri*, ONH13B (KP765734), with other closely related strains retrieved from NCBI GenBank and RDP; The GenBank accession numbers of the reference strains are shown besides the names; Horizontal bars in the dendrogram represent the branch length; Similarity and homology of the neighbouring sequences have been shown by bootstrap values; Distance matrix was calculated by P-distance model; The scale bar indicates 0.005 substitutions per nucleotide position; Another isolate, *Klebsiella varicola*, HMT1 (KP765735) exists as an out group with the type strain *K. varicola* (AJ783916)

levels were highest in DI regions of all the fish species studied, which is in accordance with the previous reports (Mondal et al. 2008, Ray et al. 2010, Ghosh et al. 2010). As bacteria revealing tannase-producing ability were highest in the DI regions of all the fish species studied, this finding might indicate likely degradation of dietary tannin in the DI regions of the fish as suggested by Mandal and Ghosh (2013) for the fish with long and coiled intestine (e.g., the carps).

Although there are several reports of tannase-producing microbes in the GI tract of ruminants and non-ruminant herbivores (Bhat et al. 1998, Odenyo et al. 1999, Goel et al. 2005), less information is available on tannase-producing bacteria in the GI tract of fresh water teleosts (Mandal and Ghosh 2013). Although varied among the different species, the presently reported study detected tannase-producing bacteria in the GI tracts of the fish species studied. The occurrence of tannase-producing bacteria might have a co-relation with their food and feeding habits, as the PI and DI regions of grass carp and tilapia were highly colonized with tannase-producing bacteria. Both fish species are herbivores feeding on aquatic macro-vegetation (Anonymous 1976); in contrast to catla feeding mostly on zooplanktons had less tannase-producing bacteria in the GI tract. Based on the results of the presently reported study, we suggest that elimination and detoxification of plant derived tannins by the autochthonous gut bacteria towards the evolutionary adaptation and is in accordance with that proposed for ruminants and non-ruminant herbivores (McBee 1971). It is revealed that tannins impede proper growth of herbivores grazing on tannin rich plant feedstuffs and thus tannins play a major role in plant defence against invasion and herbivory (Goel et al. 2005). Consequently, the occurrence of tannase-producing gut microorganisms in herbivores and existence of tannin like secondary metabolites in plant food stuffs might be a co-evolution, a hypothesis previously suggested by Mandal and Ghosh (2013). The herbivores may overcome the adverse effects of tannin by harbouring tannase-producing microbiota within their gut (Bhat et al. 1998).

The majority of the tannase-producing bacteria, isolated from the soil, showed optimum tannase production at lower or acidic pH levels. Lekha and Lonsane (1997) documented that tannase is an acidic protein that required pH around 5.5 for optimal activity. Mondal and Pati (2000) isolated *Bacillus licheniformis* KBR6 from lateritic soil and demonstrated maximum tannase production at pH 5.0. Another tannase-producing bacteria, *Bacillus cereus* KBR9 isolated from the lateritic forest soil exhibited highest enzyme activity at pH 4.5 and maximum bacterial growth occurred at pH 5.0 (Mondal et al. 2001a). Similar results were also achieved to demonstrate biodegradation capacity of tannic acid by *Citrobacter freundi* isolated from the tannery effluent (Kumar et al. 1999). In the presently reported study, isolation of the strains was carried out at pH 7 assuming the agastric nature of the carps and long coiled GI tracts in carps and tilapia with neutral or slightly alkaline pH in the gut microenvironment. However, when the tannase activities were evaluated at acidic (pH 5.5), neutral (pH 7.0) and alkaline (pH 8.5) ranges, most of the isolates revealed maximum tannase-producing capacity at neutral pH (pH 7.0), which might be due to the fact that the bacterial symbiont isolated from the fish GI tracts were adapted to the neutral or alkaline pH therein as evidenced previously with the phytase-producing strains isolated from the gut of a minor carp, *Labeo bata* (Hamilton, 1822) (see Khan and Ghosh 2013). Tannase-producing ability of the selected isolates in the presently reported study ranged between 0.17–0.28 $U \cdot mL^{-1}$, which were in accordance with the earlier observations recorded for *Bacillus licheniformis* KBR6 (0.17 $U \cdot mL^{-1}$ at pH 5.0) (Mondal and Pati 2000) and *B. cereus* KBR9 (0.22 $U \cdot mL^{-1}$ at pH 4.5) (Mondal et al. 2001a).

In the presently reported study, the three promising strains (HMT1, ONH2Ph, and ONH13B) were identified as *Klebsiella variicola*, *Bacillus subtilis*, and *Brevibacillus agri*. Previous investigations have revealed that *Klebsiella* spp. is efficient tannase producer (Deschamps et al. 1983, Sivashanmugam and Jayaraman 2011). In the presently reported study, strain *K. variicola* HMT1 isolated from *H. molitrix* was noticed as the most promising tannase-producer. However, we cannot recommend to use the isolate in aquaculture application because *Klebsiella* spp. is reported as pathogenic to fish (Austin and Austin 2007), and human beings (Maatallah et al. 2014). On the other hand, tannase-producing ability of *B. subtilis* has seldom been reported (Jana et al. 2013) and tannin bio-degradation by *B. agri* has not been documented previously. Further studies should therefore be directed to utilize the tannase-producing ability of *B. subtilis* and *B. agri* to catalyse tannin in plant ingredients supplemented to fish diets.

CONCLUSIONS AND FUTURE PERSPECTIVES

Diverse strains of extracellular tannase-producing *Bacillus* spp. have been identified from different environmental sources (for review see Aguilar-Zárate et al. 2014). However, to the authors' knowledge, tannase-producing *Bacillus subtilis* and *B. agri* has either rarely or not at all been reported previously. Except for information on *Enterobacter asburiae* LRB (GU939631) isolated from the gut of rohu (Mandal and Ghosh 2013), tannase-producing ability by fish gut bacteria has not been properly investigated. The presently reported study, revealed the existence of tannase-producing bacterial symbionts within fish GI tracts. Although it should be noted that the analyses was based on culture dependant methods that could reflect only a small proportion of the gut microbiome. Whether the isolated strains contribute to the host's nutrition has not been addressed in the presently reported study and therefore an appraisal on their likely function should be prioritized in future studies. There might be ample opportunities for development of bacterial feed supplements to overcome tannin toxicity within the gut ecosystem. Therefore, the presently reported study might hold promise to manipulate the fish gut microflora to aid in microbial degradation of dietary tannins improving the nutritive value of tannin-rich feedstuffs.

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