

Autochthonous Gut Bacteria in Two Indian Air-breathing Fish, Climbing Perch (*Anabas testudineus*) and Walking Catfish (*Clarias batrachus*): Mode of Association, Identification and Enzyme Producing Ability

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Abstract

Scanning electron microscopy (SEM) was used to define the location of epithelium-associated bacteria in the gastrointestinal (GI) tract of two Indian air-breathing fish, the climbing perch (*Anabas testudineus*) and walking catfish (*Clarias batrachus*). The SEM examination revealed substantial numbers of rod shaped bacterial cells associated with the microvillus brush borders of enterocytes in proximal (PI) and distal regions (DI) of the GI tract of both the fish species. Ten (two each from the PI and DI of climbing perch and three each from the PI and DI of walking catfish) isolated bacterial strains were evaluated for extracellular protease, amylase and cellulase production quantitatively. All the bacterial strains exhibited high cellulolytic activity compared to amylolytic and proteolytic activities. Only two strains, CBH6 and CBH7, isolated from the DI of walking catfish exhibited high proteolytic activity. Maximum cellulase activity was exhibited by the strain, CBF2, isolated from the PI of climbing perch. Six most promising enzyme-producing adherent bacterial strains were identified by 16S rDNA gene sequence analysis. The strain ATH1 (isolated from climbing perch) showed high similarity to *Bacillus amyloliquefaciens* whereas, the remaining five strains (isolated from walking catfish) were most closely related to *Bacillus licheniformis*.

Key words: 16S rDNA, air-breathing fish, enzyme production identification, GI tract bacteria identification, SEM

Introduction

The microbial population within the gastrointestinal (GI) tract of fish is rather dense, with number of microorganisms much higher than those in the surrounding water, indicating that the digestive tract provides favourable ecological niches for these organisms (Ringø *et al.*, 1995). Some bacteria in the gastrointestinal tract are considered to be transient (allochthonous), whereas others exist as members of the established microbiota associated with the intestinal mucosa (autochthonous) (Ringø and Birkbeck, 1999). The autochthonous bacteria are responsible for enteric antagonism and colonization resistance, since they are associated closely with the intestinal epithelium, and form a barrier, serving as the first defense to limit direct attachment or interaction of fish pathogenic bacteria to the gut mucosa (Ringø *et al.*, 2007). Bacteria are the main constituents of the gut microbiota in fish (Spanggaard *et al.*, 2000; Pond *et al.*, 2006). The GI tract bacteria produce different types of extracellular enzymes that break down a wide range of substrates such as starch, cellulose,

chitin, protein, phytate and produce vitamin B12 (for review see Ray *et al.*, 2012).

To date, methods for studying the gut microbiota of fish, whether autochthonous or allochthonous, have been undertaken by homogenizing sections of gut and plating the homogenate onto a range of selective media. However, such methods detect only the microorganisms capable of growing on specific, selective media. A proportion of the bacterial population remains undetected. To define the presence of an autochthonous microbiota in fish, electron microscope examination might be a useful tool. This method has been used successfully in studies of the microbial ecology of the digestive tract of chickens, pigs, rodents and humans (Knutton *et al.*, 1987; Tannock, 1987). Although several publications have described the composition of intestinal microbiota in fish (Ringø *et al.*, 1995; Ringø and Birkbeck, 1999; Bairagi *et al.*, 2002; Mondal *et al.*, 2008; Ringø, 2008; Ray *et al.*, 2010; Dan and Ray, 2013), relatively few investigations have attempted to identify the location and mode of association of microorganisms in the GI tract using scanning electron microscopy

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(SEM) and/or transmission electron microscopy (TEM) (Hellberg and Bjerkaas, 2000; Lødemel *et al.*, 2001; Ringø *et al.*, 2001; Ringø *et al.*, 2002; Ringø *et al.*, 2003; Ghosh *et al.*, 2010). An in depth electron microscopical study on gut-associated bacteria is highly relevant as the digestive tract is a potential port of entry of pathogens. One remarkable feature of the indigenous (autochthonous) microbiota of fish gut is that they are affected by certain situations including stress, antibiotic administration and even small dietary changes. The stability of the gut flora is therefore, an extremely important factor in the natural resistance of fish to infections produced by bacterial pathogens in the GI tract. To the authors' knowledge, no information is available on the mode of association of GI tract bacteria in Indian air-breathing fish. Therefore, the aim of the present study was to detect autochthonous gut microbiota in the GI tract of two Indian air-breathing fish, the climbing perch, *Anabas testudineus* and walking catfish, *Clarias batrachus* by SEM. Furthermore, the presently reported study also investigated the protease, amylase and cellulase-producing capacity of selected bacterial strains in the proximal (PI) and distal intestine (DI) of the two air-breathing fish species through quantitative enzyme assay and identification of the most promising isolated gut-associated bacteria by 16S rDNA gene sequencing.

Experimental

Materials and Methods

Fish examined. Two species of adult Indian air-breathing fish, the climbing perch, *A. testudineus* and walking catfish, *C. batrachus* were selected for the present study. The fish were obtained from a local fish farm near Santiniketan, West Bengal, India (23°41'30" N latitude and 87°41'20" E longitude). The feeding habits (Jhingran, 1997), average live weight, relative intestinal length and average intestine weight of the fish species examined are presented in Table I. Five adult specimens

of each species were stocked separately in glass aquaria and starved for 48 hours in order to empty their alimentary tracts before dissection.

Tissue preparation for scanning electron microscopy. The selected portions of the GI tract were cut into two pieces, proximal and distal. The two segments were incised longitudinally. Each tissue segment was prepared step by step as follows: the tissue was fixed in cacodylate buffer (0.1 M) containing 2.5% glutaraldehyde for 1 h at 4°C, mucous was partially removed by washing the sample in heparinized saline (2 g heparin mixed in 20 ml of 0.67% NaCl), again washed with cacodylate buffer for four times (15 min each at 4°C) and kept in fresh buffer overnight at 4°C. Next day, the tissues were dehydrated through graded ethanol as follows: 50% (40 min), 70% (40 min), 90% (1 h) and absolute alcohol for 1 h. Thereafter, three consecutive changes were made in ethanol and amyl acetate in three different ratios 3:1, 2:2 and 1:3 (each for 30 min) and finally, tissues were kept in pure amyl acetate for 12 h. Critical point drying (CPD) was done in liquid nitrogen. The nitrogen was finally removed while in a supercritical state so that no gas-liquid interface is present within the sample during drying. The dry specimens were usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape followed by coating with gold particle in IB ion coater. Coated tissues were placed and observed with a Hitachi S530 Scanning Electron Microscope at an accelerating voltage of 20 kV. Images were taken and recorded digitally in Windows XP.

Isolation of gut associated bacterial flora. The GI tract of fish were dissected out aseptically inside the laminar air flow on an ice plate. It was cut into two parts, proximal (PI) and distal intestine (DI) and thoroughly rinsed three times with PBS (phosphate buffer saline, pH 7.2) to remove undigested food and allochthonous bacterial flora following the method described by Ringø (1993) before homogenization. After homogenization of the two parts separately, one ml of sample from each part were mixed in 0.9% of NaCl solution separately

Table I
Food habits, average live weight, average fish length, relative intestinal length and average intestine weight of the air-breathing fish species examined

Fish species	Feeding habit*	Average live weight (g) (SD)	Average fish length (cm) (SD)	Relative intestinal length	Average intestine weight (g) (SD)
<i>Anabas testudineus</i>	Insects, insect larvae, water fleas, smaller fish, vegetable debris etc.	98.4 (3.2)	16.4 (0.9)	0.43	7.2 (0.61)
<i>Clarias batrachus</i>	Insect larvae, shrimps, worms, small fish, organic debris etc. Carnivorous	138.2 (4.23)	18.3 (1.1)	0.50	9.2 (0.58)

* Jhingran (1997)

SD, Standard deviation ($n=5$)

Relative intestinal length = length of intestine (cm) / total length of fish (cm)

(Das and Tripathi, 1991) and serial dilution was done (Beveridge *et al.*, 1991). One hundred μ l of sample from each test tube was spread on tryptone-soya agar plate separately and incubated for 36 h at 34°C. The well separated colonies with distinct morphology were selected for pure culture. After pure culture bacteria were stored in TSA (tryptone soya agar) slant at 4°C.

Quantitative enzyme assay of the selected bacterial strains. For assessing the extracellular enzyme producing capacity of the bacterial strains, they were cultured in different broth media such as peptone-gelatin (g/l): beef extract 3 g, peptone 5 g and gelatin 4 g, starch (g/l): tryptone 2 g, KH_2PO_4 4 g, Na_2HPO_4 4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaCl_2 0.001 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004 g, starch 10 g and carboxymethylcellulose (CMC) broth (g/l): tryptone 2 g, KH_2PO_4 4 g, Na_2HPO_4 4 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, CaCl_2 0.001 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.004 g, CMC 10 for protease, amylase and cellulase, respectively. All the chemicals were supplied by HiMedia Laboratories Private Limited, Mumbai, India. For protease, amylase and cellulase enzyme production, the bacterial isolates were cultured in peptone-gelatin, starch and CMC medium, respectively in a shaker incubator. After incubation, the broth was centrifuged at $8,000 \times g$ and the supernatant was collected for enzyme assay. The assay of protease, amylase and cellulase were done according to Walter (1984), Bernfeld (1955), and Denison and Kohen (1977), respectively. Protein content of the supernatant was estimated by the method of Lowry *et al.* (1951). The specific enzyme activity was expressed as unit (U).

Identification of bacterial strains by 16S rDNA gene sequence analysis. Six most promising bacterial strains (one from *A. testudineus* and five from *C. batrachus*) were identified by 16S rRNA gene sequence analysis as described by Ringø *et al.* (2006). All sequences were aligned and analyzed using bioinformatics tool (Codon-code and Mega 4.0) for finding the closest homolog of the microbes using a combination of NCBI (National Centre for Biotechnology Information) GenBank and RDP (Ribosomal Database Project) database.

Results and Discussion

Mode of association. One of the criteria for testing autochthony of microorganisms in the GI tracts of fish is their association with the epithelial mucosa of the stomach, proximal or distal intestine (Ringø and Birkbeck, 1999). It has been suggested by several authors that electron microscopic (EM) examinations of the GI tract should be included as an important tool for investigating the microbial ecology of the gut ecosystem and determining the presence of autochthonous or allochthonous microbiota (for review, see Ray *et al.*, 2012). In the present study, scanning electron microscopy (SEM)

revealed sporadic colonization of autochthonous bacterial cells in the GI tract of both the fish species examined. Substantial association of rod-shaped bacterial cells on the tips and within the enterocyte microvilli in both PI and DI are demonstrated by SEM (Figs. 1–7). Figure 1 shows the association of rod-shaped bacteria in the apical aspects of the enterocytes in the PI of *C. batrachus*. Previous SEM and/or TEM elevations also demonstrated rod-shaped bacteria associated with the microvilli in the GI tract of rainbow trout, *Oncorhynchus mykiss* (Lesel and Pointel, 1979), Atlantic wolfish, *Anarhichas lupus* (Hellberg and Bjerkås, 2000), Arctic charr, *Salvelinus alpinus* (Ringø *et al.*, 2001) and rohu, *Labeo rohita* (Ghosh *et al.*, 2010). SEM investigation also revealed that the bacterial colonies attached in the intestinal fold were associated with mucous and the attachment of bacteria was clearly visible in the mucous removed intestinal folds (Figs. 2, 3 and 4). Similarly, attachment of autochthonous bacteria in GI tract of *A. testudineus* was also very clear (Figs. 5, 7). In the PI of *A. testudineus*, the luminal end of one bacterium is found protruding above the level of the microvilli (Fig. 6). In the DI of *A. testudineus*, numerous small pores are discernable (Fig. 8). These pores might be due to emptying mucous-producing goblet cells. Bacterial adhesion is a cell-surface interaction phenomenon which makes it ideal for examination by SEM (Knutton, 1995). Several factors are reported to influence adhesion and colonization of the microbiota within the GI tract. These are: (a) gastric acidity, (b) bile salts, (c) peristalsis, (d) digestive enzymes, (e) immune response and (f) indigenous bacteria and the antibacterial compounds produced by them (Ringø *et al.*, 2003). Ringø *et al.* (2007) reported that both bacterial cells and epithelial cells are negatively charged that prevent closer association of bacteria on the epithelial surface. SEM and classical microbiology have been used to investigate bacteria in different regions of the GI tract of rainbow trout, *O. mykiss* (Lesel and Pointel, 1979). In the presently reported study, in some cases, bacterial cells are seen to remain associated with the folds of the intestinal villi wrapped by mucous. This finding corroborates the observation made by Ghosh *et al.* (2010) in the GI tract of *L. rohita*. On the other hand, in the PI of *A. testudineus*, the bacteria are found to protrude above the level of microvilli as reported in Arctic charr, *S. alpinus* by Ringø *et al.* (2001). The mechanisms involved in adhesion of adherent bacteria in the GI tract seem to include adhesive factor(s), such as adhesion (Krovacek *et al.*, 1987), salinity and pH (Balebona *et al.*, 1995) and cell surface hydrophobicity (Parker and Munn, 1984; Bruno, 1988). In addition, receptor-specific interactions, such as pili-like structures, specific receptors on enterocytes such as sugar residues have been reported to be involved in mammalian models (Knutton, 1995).

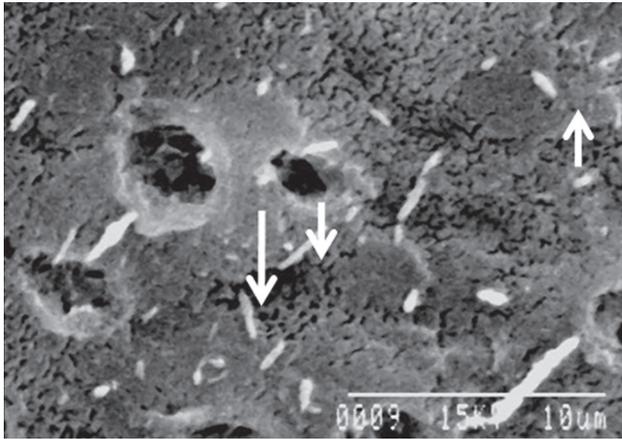


Fig. 1. Apical aspect of enterocytes in the PI showing association of rod-shaped bacteria (arrows) ($\times 5000$).

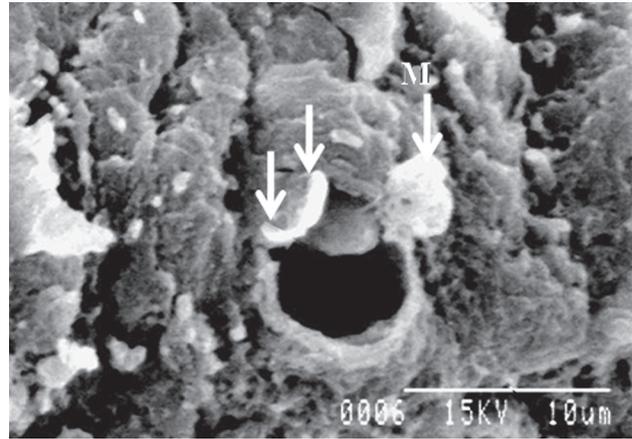


Fig. 2. Bacterial cells (arrows) are seen adjacent to the pore of goblet cell ($\times 4000$). M- indicate mucus.

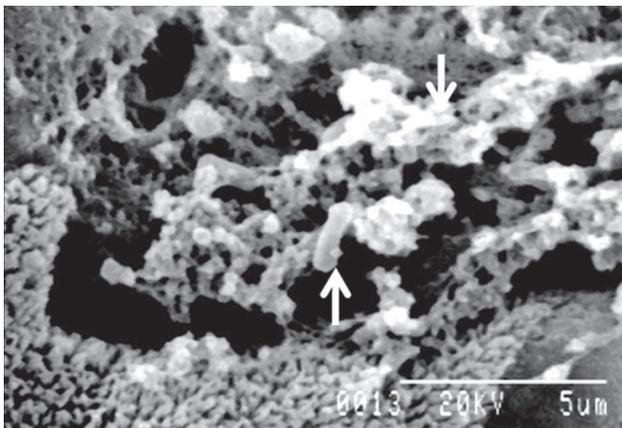


Fig. 3. Bacterial cells (arrows) are visible in close association with mucus ($\times 8000$).

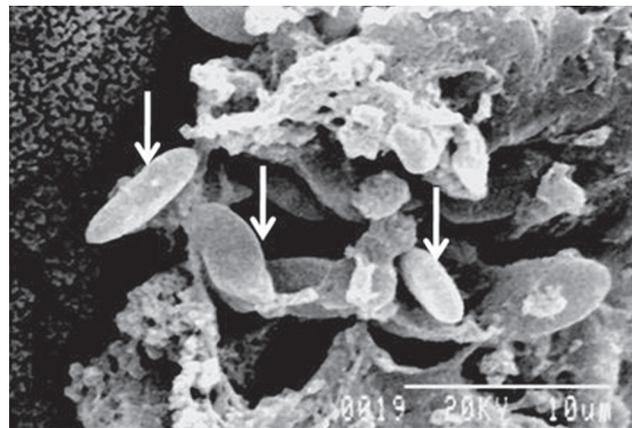


Fig. 4. Arrows indicate the presence of rod shaped bacteria which are comparatively larger in size ($\times 4000$).

Figs. 1–4. Scanning electron micrographs of PI and DI of *C. batrachus* showing bacterial cells associated with enterocytes.

Enzyme producing ability. Out of 21 strains isolated from the GI tract of the two fish species, 10 stains (2 each from the PI and DI of *A. testudineus* and 3 each from the PI and DI of *C. batrachus*) were the most promising with regard to enzyme producing ability. The results of quantitative enzyme activity exhibited by the bacterial isolates are presented in the Table II. All the bacterial strains exhibited high cellulase activity compared to amylase and protease activities. The strain CBF2 isolated from the PI of *C. batrachus* exhibited the highest cellulolytic activity (15.34 ± 0.11 U) followed by CBF4 and CBH5 (10.66 ± 0.24 U and 10.42 ± 0.073 U, respectively) isolated from the PI and DI, respectively of the same fish. The bacterial strain ATH1 isolated from DI of *A. testudineus* was also a good cellulase producer (1.92 ± 0.018 U). The presence of huge population of cellulolytic bacteria and their active role in extracellular cellulase production in fish has been confirmed in several investigations (Das and Tripathi, 1991; Saha and Ray, 1998; Bairagi *et al.*, 2002; Saha *et al.* 2006; Kar and Ghosh, 2008; Mondal *et al.*, 2008; 2010; Ray

et al., 2010). Considerable cellulolytic bacterial population has also been reported in the digestive tract in carnivorous murrel, *Channa punctatus* and stinging catfish, *Heteropneustes fossilis* (Kar and Ghosh, 2008; Banerjee *et al.*, 2013). Niederholzer and Hofer (1979) detected highest levels of cellulase activity in roach, *Rutilus rutilus*, and rudd, *Scardinius erythrophthalmus*, feeding on zooplankton and arthropods. Lindsay and Harris (1980) recorded moderate or high cellulase activity in gut contents of most “invertivores” feeding almost exclusively on invertebrates compared to omnivorous and piscivorous fish and concluded that cellulase activity in fishes is the direct result of ingestion of invertebrates containing cellulase or a cellulolytic microflora. Luczkovich and Stellwag (1993) also supported the hypothesis put forward by Lindsay and Harris (1980) and opined that cellulolytic bacteria present in the invertebrates consumed by pinfish, *Lagodon rhomboides* might have served as a source for the establishment and maintenance of the microbial flora. Our results corroborate these hypotheses and may explain

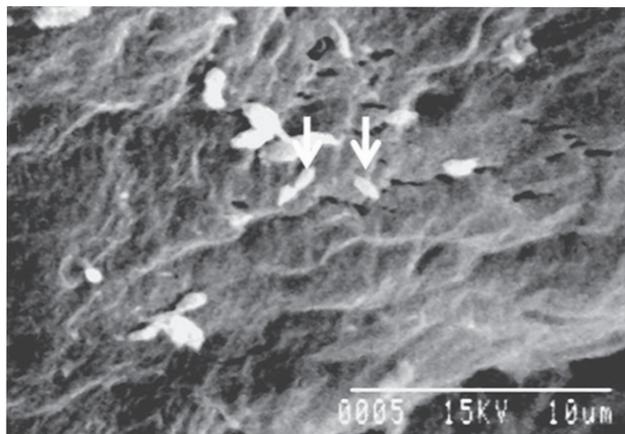


Fig. 5. Associated bacteria (arrows) are located at the apical brush border ($\times 5000$).

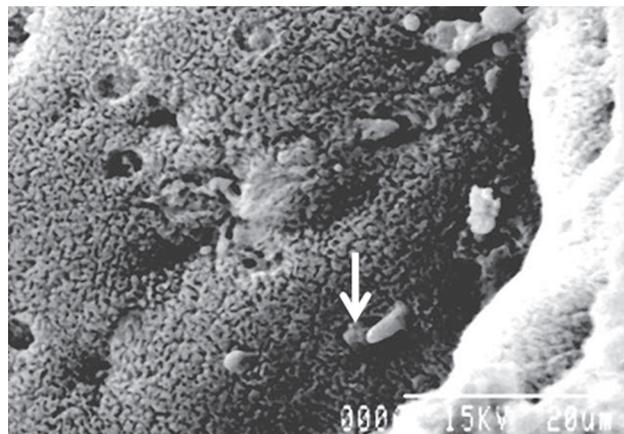


Fig. 6. The luminal end of one bacterium is seen protruding above the level of the microvilli (arrow) in the PI region ($\times 2000$).

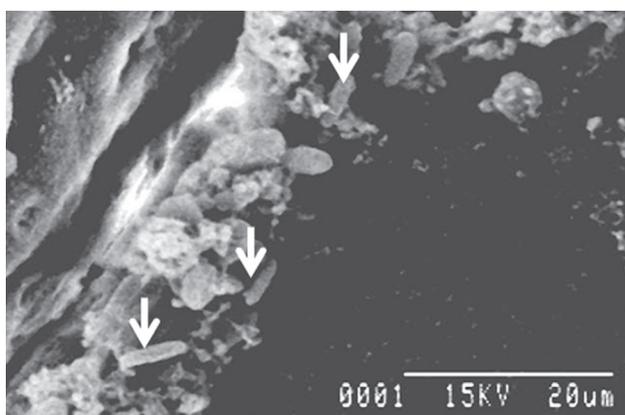


Fig. 7. Long rod-shaped bacteria (arrows) present on the epithelial surface of DI ($\times 2000$).

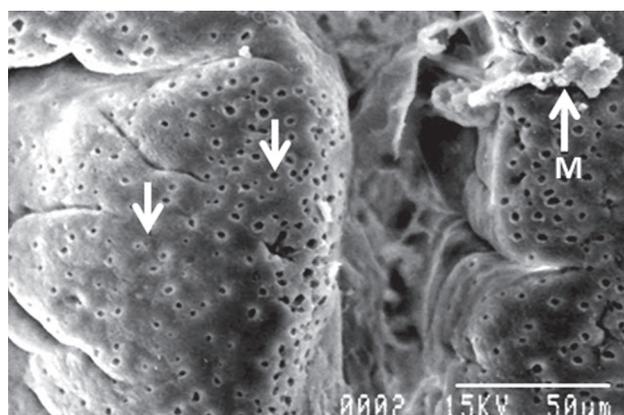


Fig. 8. SEM of DI showing numerous small pores (arrows) on the apical aspects of enterocytes ($\times 600$). *M. mucous*

Figs. 5–8. Scanning electron micrographs of the PI and DI of *A. testudineus* showing association of bacteria on the cell apices.

high cellulase activity in all the bacterial strains isolated from the GI tract of both the fish species studied which feed exclusively on invertebrates. In experimen-

tal conditions, cellulose was found to be poorly utilized by fish (Leary and Lovell, 1975; Anderson *et al.*, 1984; Shiau *et al.*, 1989; Hilton *et al.*, 1989). The results

Table II

Quantitative extracellular enzyme activities of the selected bacterial strains isolated from the GI tract of two air-breathing fish species

Fish species	Region	Strain designation	Protease (U) ¹	Amylase (U) ²	Cellulase (U) ³
<i>Anabas testudineus</i>	Proximal intestine	ATF4	0.51 (\pm 0.016)	0.02 (\pm 0.006)	0.71 (\pm 0.016)
		ATF5	0.69 (\pm 0.011)	0.46 (\pm 0.012)	1.80 (\pm 0.021)
	Distal intestine	ATH1	0.90 (\pm 0.019)	1.63 (\pm 0.13)	1.92 (\pm 0.018)
		ATH2	0.72 (\pm 0.02)	0.02 (\pm 0.004)	1.14 (\pm 0.011)
<i>Clarias batrachus</i>	Proximal intestine	CBF2	0.83 (\pm 0.026)	0.04 (\pm 0.01)	15.38 (\pm 0.11)
		CBF3	0.10 (\pm 0.015)	0.08 (\pm 0.02)	4.58 (\pm 0.14)
		CBF4	0.39 (\pm 0.032)	0.34 (\pm 0.041)	10.66 (\pm 0.24)
	Distal intestine	CBH5	0.16 (\pm 0.016)	0.14 (\pm 0.025)	10.42 (\pm 0.073)
		CBH6	1.13 (\pm 0.065)	0.16 (\pm 0.015)	4.58 (\pm 0.05)
		CBH7	1.82 (\pm 0.081)	0.06 (\pm 0.01)	6.51 (\pm 0.08)

Data are means \pm SE of three determinations.

¹ μ g of tyrosine liberated/mg protein/min; ² μ g of maltose liberated/mg protein/min; ³ μ g of glucose liberated/mg protein/min.

of those studies might raise questions regarding the role of cellulolytic bacteria in utilization of cellulose in fish. However, the presence of cellulolytic bacteria alone does not provide evidence that fish can utilize plant materials and/or cellulose. Symbiotic microbial enzymes, which hydrolyse cellulose, also generally require relatively long digestion times (Kristensen, 1972). So, in ruminant animals, a large fermentation/digestion chamber is present with a slow transfer rate to allow sufficient time and space for effective digestion. As fish do not possess a specialized chamber for bacterial fermentation, cellulose is sometimes poorly utilized by fish in spite of the presence of symbiotic cellulolytic bacteria. On the other hand, highest proteolytic activity was recorded in the bacterial strain CBH7 and CBH6 isolated from DI of *C. batrachus* (1.82 ± 0.081 U and 1.13 ± 0.065 U, respectively) followed by ATH1 isolated from DI of *A. testudineus* (0.9 ± 0.019 U). There are some published reports on protease production by fish GI tract bacteria (Skrodenytė-Arbačiauskienė, 2007; Ray *et al.*, 2010; Askarian *et al.*, 2012; Banerjee *et al.*, 2013). Bairagi *et al.* (2002), however, quantified the proteolytic activity in the bacterial strains isolated from nine freshwater teleosts. They recorded highest proteolytic activity in the bacterial strain TP3A, isolated from the gut of *Oreochromis mossambica*. While, Mondal *et al.* (2008) and Ray *et al.* (2010) also reported high proteolytic activity in the bacterial strains isolated from the DI of *Labeo calbasu* and three Indian major carps, *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*. Extracellular protease activity in these bacterial isolates indicates that there exists a definite source of microbial protease apart from the endogenous protease in fish GI tract which have contribution in host digestion process. In the present study, most of the isolated bacterial strains were not good amylase producers except ATH1 (1.63 ± 0.13 U), isolated from DI of *A. testudineus*. Kar and Ghosh (2008), however, reported amylase-producing bacteria in the digestive tracts of rohu (*L. rohita*) and murrel (*C. punctatus*). Mondal *et al.* (2008), however, could not detect amylolytic bacteria in the GI tract of carnivorous climbing perch, *A. testudineus*. They opined that amylase production by GI tract bacteria in herbivorous fish is much higher than that in carnivorous fish. Das and Tripathi (1991) reported high amylase activity in the gastrointestinal tract of grass carp (*Ctenopharyngodon idella*) which appeared to be the result of its omnivorous feeding habit. They are of opinion that there is a possibility of introduction of these enzyme producing microflora in fish GI tracts along with the food ingested, but, whether they form a persistent population in the gut is doubtful. Since, the amylolytic bacteria have been detected in fish GI tract after 48 h of starvation in the present study, it seems that some of the flora form a resistant population.

Table III
Identification of selected bacterial strains isolated from the GI tract of *Clarias batrachus* and *Anabas testudineus* with partial sequence of 16S rDNA genes referenced to accession no. in Gen Bank

Isolate	Closest relative (obtained from BLAST search)	Similarity (%)	Accession no.
ATH1	<i>Bacillus amyloliquefaciens</i>	99%	HG328254.1
CBF2	<i>Bacillus licheniformis</i>	97%	HQ005269.1
CBF4	<i>Bacillus licheniformis</i>	99%	HQ005269.1
CBH5	<i>Bacillus licheniformis</i>	98%	HQ005269.1
CBH6	<i>Bacillus licheniformis</i>	98%	HQ005269.1
CBH7	<i>Bacillus licheniformis</i>	100%	HQ005269.1

Identification of bacterial strains. All the strains isolated from the GI tract of both the fish species are Gram-positive, rod shaped aerobic bacteria with irregular configuration. Out of ten bacterial strains, six most promising ones (one from *A. testudineus* and five from *C. batrachus*) were identified by 16S rDNA sequence analysis (Table III). The 16S rDNA is the most conserved (least variable) gene in all cells. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. The bacterial strain ATH1 isolated from the DI of *A. testudineus* showed 99% similarity to *Bacillus amyloliquefaciens* (accession no. HG328254.1). *B. amyloliquefaciens* is a Gram-positive, rod shaped spore forming bacterium. This species of *Bacillus* has been isolated from different sources such as, fish gut (Ghosh *et al.*, 2010), brackishwater sediment (Cao *et al.*, 2011), plant (Chen *et al.*, 2009) and oil contaminated soil (Liu *et al.*, 2012). Due to high level of amylase production, it is considered to be a commercial producer in different industries (Deb *et al.*, 2013). The remaining five bacterial strains isolated from the GI tract of *C. batrachus* were close to *Bacillus licheniformis* (accession no. HQ005269.1). Different strains of *Bacillus* have been identified from the GI tract of fish (Ringø, 2008). In the present study, all the 6 identified strains belong to genus *Bacillus*. One of our strains (ATH1) showed 99% similarity to *B. amyloliquefaciens* (accession no. HG328254.1). *B. licheniformis* is a Gram-positive, mesophilic spore forming bacterium, previously reported in the GI tract of different freshwater fish species (Roy *et al.*, 2009; Mondal *et al.*, 2010; Banerjee *et al.*, 2013; Dan and Ray, 2014). *B. licheniformis* from different sources has been reported to be a valuable source for protease production (Asokan and Jayanthi, 2010; Degering *et al.*, 2012; Sangaralingam *et al.*, 2012; Banerjee *et al.*, 2013). Due to high protease producing capacity, *B. licheniformis* is used in different industries (Degering *et al.*, 2012).

Conclusions. The results of the present study provide evidence that autochthonous bacteria colonize in both the PI and DI of the two air-breathing fish species studied. The investigation further confirms that there is also a distinct microbial source of digestive enzymes (protease, amylase and cellulase) apart from the endogenous sources in fish GI tracts. Characterization and identification by 16S rDNA sequence analysis revealed that all the strains belong to the genus *Bacillus*. However, to present more reliable information on the gut microbiota in fish, several methods, such as random amplified polymorphic DNA, denaturing gradient gel electrophoresis (DGGE), fluorescence *in situ* hybridization (FISH), and confocal microscopy have been used to identify and detect the microbial community in the GI tracts of fish. These techniques should be used in future studies while evaluating the bacterial community in the GI tract of Indian fishes.

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