TÜBİTAK

Turkish Journal of Zoology

http://journals.tubitak.gov.tr/zoology/

Research Article

Turk J Zool
(2014) 38: 79-88
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doi:10.3906/zoo-1205-3

Distribution of extracellular enzyme-producing bacteria in the digestive tracts of 4 brackish water fish species

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Received: 03.05.2012 • **Accepted:** 21.08.2013 • **Published Online:** 01.01.2014 • **Printed:** 15.01.2014

Abstract: Occurrence and distribution of enzyme-producing bacteria in the proximal (PI), middle (MI), and distal (DI) segments of the gastrointestinal tracts of 4 brackish water teleosts (*Scatophagus argus*, *Terapon jarbua*, *Mystus gulio*, and *Etroplus suratensis*) have been investigated. Data were presented as log viable counts g^{-1} intestine (LVC). The heterotrophic bacterial population had the highest occurrence in the DI regions of all fish species studied except *M. gulio*. Proteolytic and amylolytic bacteria had the highest occurrence in the DI of *M. gulio* (LVC = 5.50 and 5.93, respectively), while cellulolytic and lipolytic populations exhibited highest occurrences in the DI regions of *T. jarbua* (LVC = 6.33) and *S. argus* (LVC = 5.78), respectively. Out of the 81 bacterial isolates, the most promising 3 isolates were determined through quantitative enzyme assay and studied through 16S rRNA gene sequence analysis for identification. Both the strains SA2.2 isolated from *S. argus* and TJ2.3 isolated from *T. jarbua* showed high similarity to different strains of *Brevibacillus parabrevis*, while another strain, MG4.2, isolated from *M. gulio*, was similar to *Bacillus licheniformis*. The NCBI GenBank accession numbers of the 16S rRNA gene sequences for isolates SA2.2, TJ2.3, and MG4.2 were KF377322, KF377324, and KF377323, respectively. The present study might offer scope for further research to evaluate prospects for application of the gut-associated extracellular enzyme-producing bacteria in brackish water aquaculture.

Key words: Brackish water fish, gut bacteria, enzyme, *Brevibacillus parabrevis*

1. Introduction

The microflora within the gastrointestinal (GI) tract of marine and freshwater fish species has been widely investigated (Austin, 2002; Ghosh et al., 2010; Ray et al., 2010; Askarian et al., 2011, 2012) The nutrient-rich GI tract of fish is a favorable growth environment for these bacteria (Kar et al., 2008). During the last decade, there has been an improved understanding of the importance of commensal intestinal microflora in fish (Bairagi et al., 2002; Ghosh et al., 2002a, 2010; Ringø et al., 2010; Khan and Ghosh, 2012; Mandal and Ghosh, 2013). The gut microflora may be categorized as either autochthonous (indigenous) or allochthonous (transient) depending upon its ability to colonize and adhere to the mucus layer in the digestive tract (Ringø and Birkbeck, 1999; Ringø et al., 2003). The bacterial flora within the GI tract of fish shows very broad and variable enzymatic potential, and these enzymatic masses may interfere positively in the digestive process of fish (Ray et al., 2010). Fish gut bacterial isolates have been demonstrated to break down chitin (Danulat and Kausch, 1984; MacDonald et al., 1986; Itoi et al., 2006), p-nitrophenyl-b-N-acetylglucosamine and protein (MacDonald et al., 1986; Belchior and Vacca, 2006), cellulose (Saha and Ray, 1998; Bairagi et al., 2002; Ghosh et al., 2002a, 2010; Saha et al., 2006; Mondal et al., 2008), starch (Sugita et al., 1997; Ghosh et al., 2002a, 2010), phytate (Li X et al., 2008, Li XY et al., 2008; Roy et al., 2009; Khan et al., 2011; Khan and Ghosh, 2012, 2013), and tannin (Mandal and Ghosh, 2013). Previous studies conducted in the carps advocated the beneficial aspects of gut-associated microbiota in the host fish with regard to nutrition (Ghosh et al., 2002a, 2002b, 2003; Ray et al., 2010). Meanwhile, information on the enzyme-producing gut bacteria in the brackish water fish species is scanty (Rani et al., 2004; Sivasubramanian et al., 2012). In this context, the search for extracellular enzyme-producing beneficial gut bacteria to be used as probiotics for the culturable brackish water fish species may be of interest.

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Therefore, the primary objective of the present study was to detect the autochthonous extracellular enzyme-producing bacteria from the proximal (PI), middle (MI), and distal (DI) segments of the GI tracts of 4 culturable brackish water teleosts. Furthermore, the study was intended to evaluate the gut bacteria's ability for protease, amylase, cellulase, and lipase production, and to identify the most promising bacterial strains by 16S rRNA gene sequence analysis.

2. Materials and methods

2.1. Fish species examined

Four brackish water fish species, *Scatophagus argus*, *Mystus gulio*, *Terapon jarbua*, and *Etroplus suratensis*, were collected from 3 brackish water culture ponds of the Fish Technological Station, Junput, West Bengal, India (21°43.232′N, 87°48.884′E) and brought to the laboratory in oxygen-packed plastic bags. Physicochemical parameters of the collection ponds ranged between pH 8.1 and 8.4, with water temperature of 29.8–31.4 °C and salinity of 16–18 ppt. Feeding habits, average live weight, average fish length, and average gut weight of the fishes examined are presented in Table 1.

2.2. Processing of specimens

Nine specimens of each species collected from 3 ponds (3 from each pond) were evaluated for the present study. The fishes were starved for 48 h to empty the GI tracts (Ray et al., 2010). After starvation, fish were anesthetized with 0.03% tricaine methane-sulfonate (MS222), and the ventral surface of each fish was thoroughly scrubbed with 1% iodine solution for surface decontamination (Trust and Sparrow, 1974). The fishes were dissected aseptically within a laminar airflow 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 proximal (PI), middle (MI), and distal (DI) segments, cut into pieces, and flushed carefully 3 times with 0.9% sterile saline solution using an injection syringe in order to remove nonadherent (allochthonous) microflora, according to Ghosh et al. (2010). Gut segments from the 3 specimens of a species collected from the same pond were pooled together region-wise for each replicate, and thus there were 3 replicates for each gut segment from each fish species. The gut segments were homogenized with 10 parts of sterilized, prechilled 0.9% NaCl solution, as described elsewhere (Das and Tripathi, 1991). Pooled samples were used to avoid erroneous conclusions due to individual variations in gut microflora, as described elsewhere (Ghosh et al., 2010).

2.3. Microbial culture

Homogenized samples of each gut segment were used separately after appropriate serial (1:10) dilutions (Beveridge et al., 1991). Diluted samples (0.1 mL) were poured aseptically within a laminar airflow onto sterilized tryptone soy agar (TSA, HiMedia, India) plates to determine the culturable heterotrophic autochthonous aerobic/facultative anaerobic microbial population. For determination of protease-, cellulase-, amylase-, and lipase-producing bacterial populations, diluted samples (0.1 mL) were poured onto peptone gelatin agar (PG), carboxymethylcellulose agar (CMC), starch agar (SA), and tributyrin agar (TA) plates, respectively. The culture plates were incubated at 30 °C for 48 h. The colony-forming units (CFUs) 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), and the data were presented as log viable counts g^{-1} intestine (LVC). Colonies with apparently different morphological appearances (such as color, configuration, surface, margin, and opacity) from a single plate were streaked separately on respective plates to obtain pure cultures.

Table 1. Food habits, average live weight, average fish length (standard length), and average gut weight of the fishes examined.

Fish species	Feeding habit	Average live weight (g)	Average fish length (cm)	Average gut weight (g)
Scatophagus argus	Omnivorous; feeds on fishes, insects, algae, and sand-dwelling invertebrates	53.87 ± 4.92	13.76 ± 0.56	3.02 ± 0.2
Terapon jarbua	mnivorous; feeds on worms, crustaceans, insects, and plant matter	46.5 ± 3.56	14.2 ± 0.81	1.7 ± 0.14
Mystus gulio	Carnivorous	24.2 ± 1.94	11.92 ± 0.45	1.22 ± 0.17
Etroplus suratensis	Omnivorous; feeds on filamentous algae, plant material, and insects	33.46 ± 1.4	11.99 ± 0.28	0.53 ± 0.03

Results are mean ± SD of the 3 observations.

2.4. Screening of isolates by qualitative assay for exoenzyme production

Out of the 81 extracellular enzyme-producing isolates from the fish species examined, 21 isolates were primarily selected (on the basis of growth potential at 30 °C) for qualitative enzyme assay. For extracellular amylase production, isolates were inoculated on SA plates and incubated at 30 °C for 48 h. The culture plates were flooded with 1% Lugol's iodine solution to identify amylase activity by the formation of a transparent zone (halo) surrounding the colony (Jacob and Gerstein, 1960). Similarly, for extracellular protease, the isolates inoculated on PG plates were incubated at 30 °C for 48 h; the appearance of a halo after flooding the plates with 15% $HgCl_2$ indicated the presence of proteolytic activity (Jacob and Gerstein, 1960). For determination of cellulase production, isolates grown on CMC plates at 30 °C for 48 h were flooded with Congo red dye prepared with 0.7% agarose (Teather and Wood, 1982). Congo red selectively binds with unhydrolyzed CMC. Appearance of a halo due to the presence of hydrolyzed CMC surrounding the bacterial colony indicated cellulase production in the medium. Lipase producers showed a halo surrounding their colony in 1% tributyrin plates (Sangiliyandi and Gunasekaran, 1996). There were 3 replicates for each experimental set. Qualitative extracellular enzyme activity was assessed based on the measurement of the halo zone (diameter in mm) around the colony and presented as scores, as follows: 0 (0–5 mm), 1 (low, 6–10 mm), 2 (moderate, 11–15 mm), 3 (good, 16–20 mm), 4 (high, 21–25 mm), and 5 (very high, >25 mm).

2.5. Quantitative enzyme assay

On the basis of the qualitative assay, 10 extracellular enzyme-producing isolates were selected for quantitative assay using broth culture to screen the promising isolates. Quantitative assay for the production of amylase, cellulase, protease, and lipase were performed following the methods described by Bernfeld (1955), Denison and Koehn (1977), Walter (1984), and Bier (1955), respectively. A detailed description for measurement of extracellular enzyme production and quantitative enzyme assay has been mentioned elsewhere (Bairagi et al., 2002). Quantitative enzyme activities were expressed as units (U).

2.6. Identification of isolates by 16S rRNA gene sequence analysis

The most promising 3 of the extracellular enzyme-producing isolates were investigated by means of their quantitative enzyme assays (amylase, cellulase, protease, and lipase) in addition to their 16S rRNA partial gene sequence analysis for identification. The gene encoding 16S rRNA was amplified from the isolates by polymerase chain reaction (PCR) using universal primers 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR reactions were performed using PCR mix containing 200 µM of deoxynucleotides (dNTPs), 0.2 µM of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer, and 0.2 U of Taq DNA polymerase (Invitrogen). The template DNA was obtained by extracting genomic DNA using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) from a fresh colony grown on a nutrient agar slant. The following cycle was used for PCR reaction: initial denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, and a final extension at 72 °C for 3 min (Lane, 1991). PCR products were sent to a commercial house for Sanger sequencing using an automated DNA sequencer (Applied Biosystems Ltd.). Sequenced data were aligned and analyzed for finding the closest homolog of the microbes using a combination of NCBI GenBank and RDP databases. The phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of isolates SA2.2, TJ2.3, and MG4.2 and their phylogenetically closest type strains using MEGA 5.2 software following the minimum evolution method. Partial sequences of 16S rRNA from the 3 selected isolates were deposited in the NCBI GenBank database to obtain accession numbers.

2.7. Media composition

TSA medium contained (g L^{-1}): pancreatic digest of casein, 15; papaic digest of soybean meal, 5; NaCl, 5; agar, 15; pH of 7.5. PG medium contained (g L^{-1}): beef extract, 3; peptone, 5; gelatin, 4; agar, 20; pH of 7.5. CMC medium contained (g L^{-1}): beef extract, 5; peptone, 5; NaCl, 5; carboxymethylcellulose, 2; agar, 20; pH of 7.5. SA medium contained (g L^{-1}): beef extract, 5; peptone, 5; NaCl, 5; starch (soluble), 2; agar, 20; pH of 7. TA medium contained (g L^{-1}): tributyrin-agar, 10; peptone, 5; agar, 15; pH of 7.5.

2.8. Statistical analysis

Data pertaining to specific extracellular enzyme production by the selected isolates were subjected to analysis of variance (ANOVA) followed by Tukey's test following Zar (1974) using SPSS 10 (Kinear and Gray, 2000).

3. Results

Heterotrophic as well as protease-, cellulase-, amylase-, and lipase-producing bacterial populations present in the PI, MI, and DI segments of the GI tracts of all the fish species examined are presented in Table 2. Analysis of the bacterial populations in the GI tracts of 4 brackish water fish revealed that the heterotrophic population on TSA plates differed among different fish species as well as among different regions of the gut. The bacterial population on TSA plate was highest in the DI region of *M. gulio* (LVC = 7.49 g^{-1} intestine), followed by *T. jarbua* (LVC = 7.48 g^{-1} intestine), while it was lowest in the PI

Table 2. Log viable counts (LVC) of autochthonous adherent bacteria isolated from the proximal (PI), middle (MI), and distal (DI) segments of the GI tracts of the fish species examined.

LVC g^{-1} intestine					
Fish species	Bacterial count in TSA plate	Amylolytic bacteria	Cellulolytic bacteria	Proteolytic bacteria	Lipolytic bacteria
Scatophagus argus					
PI	6.93	4.92	5.06	4.69	5.58
MI	7.11	5.61	5.84	4.99	5.76
DI	7.29	5.73	6.06	5.12	5.78
Terapon jarbua					
PI	6.98	5.33	5.18	4.85	5.09
MI	7.28	5.50	5.92	5.20	5.43
DI	7.48	5.62	6.33	5.41	5.55
Mystus gulio					
PI	6.38	5.61	5.69	5.05	5.31
MI	7.09	5.66	5.29	5.27	5.35
DI	7.49	5.93	4.93	5.50	5.50
Etroplus suratensis					
PI	6.78	4.87	4.83	4.74	4.96
MI	6.92	4.93	4.85	4.89	5.08
DI	7.25	4.94	4.99	5.08	5.23

region of *M. gulio* (LVC = 6.38 g^{-1} intestine). The highest amylolytic bacterial population was detected in the DI region of *M. gulio* (LVC = 5.93 g^{-1} intestine), followed by *S. argus* (LVC = 5.73 g^{-1} intestine); it was lowest in the PI region of *E. suratensis* (LVC = 4.87 g^{-1} intestine). Cellulase-producing bacteria showed the highest concentration in the DI region of *T. jarbua* (LVC = 6.33 g^{-1} intestine), followed by *S. argus* (LVC = 6.06 g^{-1} intestine), while it was lowest in the PI region of *E. suratensis* (LVC = 4.83 g^{-1} intestine). Proteolytic bacterial population showed the highest concentration in the DI region of *M. gulio* (LVC = 5.50 g^{-1} intestine), followed by *T. jarbua* (LVC = 5.41 g^{-1} intestine); the lowest concentration was in the PI region of *S. argus* (LVC = 4.69 g^{-1} intestine). Lipolytic bacteria rates were highest in the DI region of *S. argus* (LVC = 5.78 g^{-1} intestine), followed by the MI region of the same species (LVC = 5.76 g^{-1} intestine). Altogether, 21 enzyme-producing bacterial isolates were primarily selected from different fish species, and extracellular enzyme production by the bacterial isolates was assayed qualitatively. Qualitative extracellular enzyme activities were presented as scores (Table 3), maximum and minimum scores being 18 and 2, respectively. Based on the qualitative assay, 10 bacterial isolates were selected for the quantitative enzyme assay. Results of the quantitative enzyme assay revealed significant differences in the enzyme activities among different bacterial isolates (Table 4). Maximum amylase and cellulase activities were recorded in SA2.2 isolated from the DI of *S. argus* (44.03 ± 0.43 U and 13.12 ± 0.23 U, respectively). Protease activity was highest in TJ2.3 isolated from the DI of *T. jarbua* (26.89 ± 0.28 U), while the best lipase activity was noticed in MG4.2 isolated from the DI of *M. gulio* (11.1 ± 0.31 U). Considering all 4 enzymatic activities, isolates SA2.2, TJ2.3, and MG4.2 were found to have the most potential among the 10 selected isolates. Based on the nucleotide homology and phylogenetic analysis of the 16S rRNA gene sequences, isolates SA2.2 and TJ2.3 were both identified as *Brevibacillus parabrevis* (GenBank Accession Nos. KF377322 and KF377324). The isolate SA2.2 showed 100% similarity with *B. parabrevis* HDYM-18 (EF428244), while isolate TJ2.3 showed 99% similarity with *B. parabrevis* M3 (AB215101). Thus, SA2.2 and TJ2.3 were 2 different strains of *B. parabrevis*. The isolate MG4.2 was identified as *Bacillus licheniformis*

Table 3. Qualitative extracellular enzyme activity of some bacterial strains isolated from the GI tracts of the fish species examined. Enzyme activities were presented as scores as described in the text.

Fish species	Bacterial strains	Enzyme activity (scores)*					Total score
		Isolated from	Amylase[1]	Cellulase[2]	Protease[3]	Lipase[4]	
Terapon jarbua	TJ3.1	PI	0	2	0	0	02
	TJ1.2	MI	2	2	3	2	09
	TJ1.1	DI	4	3	3	2	12
	TJ2.3	DI	5	4	5	4	18
	TJ4.1	DI	4	3	4	4	15
Scatophagus argus	SA3.1	PI	0	4	3	2	09
	SA1.1	MI	4	3	3	4	14
	SA3.2	MI	0	2	3	3	08
	SA2.2	DI	5	4	5	3	17
	SA1.2	DI	5	3	3	2	13
	SA2.1	DI	3	2	3	2	10
	SA4.3	DI	0	4	3	2	09
Mystus gulio	MG3.1	PI	0	2	0	4	06
	MG4.1	MI	4	2	4	3	13
	MG1.1	MI	0	3	4	2	09
	MG4.2	DI	5	3	5	5	18
	MG1.2	DI	4	2	3	2	11
Etroplus suratensis	ES3.1	PI	0	2	4	2	08
	ES1.2	MI	0	2	4	0	06
	ES2.1	DI	3	3	4	0	10
	ES4.3	DI	2	2	4	0	08

*: With pure culture of bacterial isolates.
[1]On starch (SA) plate; [2]on carboxymethylcellulose (CMC) plate; [3]on gelatin-peptone (GP) plate; [4]on tributyrin-agar (TA) plate.

(GenBank Accession No. KF377323). It showed 100% similarity with *B. licheniformis* GLU 113 (FN678352). It appeared from the phylogenetic tree that strains SA2.2 and TJ2.3 were closest to the *B. parabrevis* type strain (AB112714) and were grouped together, whereas MG4.2 was distantly placed with *B. licheniformis* (Figure).

4. Discussion

Diverse microbial communities in the GI tracts of freshwater or marine carnivorous, herbivorous, and omnivorous fish species have been reported abundantly (for review, see Ray et al., 2012). However, the endosymbiotic community among the brackish water fish species has remained poorly investigated (Esakkiraj et al., 2009). Digestive tracts of endotherms are colonized mainly by obligate anaerobes (Finegold et al., 1983), while the predominant bacterial genera isolated from most fish guts have been aerobes or facultative anaerobes (Trust and Sparrow, 1974; Horsley, 1977; Sakata, 1990; Bairagi et al., 2002; Ghosh et al., 2002a). In the present investigation, aerobic/facultative anaerobic extracellular enzyme-producing bacterial symbionts were detected in the GI tracts of 4 brackish water fish species. As the fish were starved for 48 h and their GI tracts were thoroughly

Table 4. Profile of specific enzyme activities (mean ± SE) in the selected isolates from the GI tracts of the fish species examined.

Bacterial strain	Enzyme activity (U)			
	Amylase*	Cellulase$	Protease#	Lipase@
MG4.1	26.82 ± 0.34^{c}	7.42 ± 0.24^{a}	20.31 ± 0.28^{e}	8.20 ± 0.27^{c}
SA2.2	44.03 ± 0.43^{e}	13.12 ± 0.23^{d}	20.95 ± 0.20^{e}	8.45 ± 0.51^{c}
TJ2.3	42.25 ± 0.70^{e}	11.76 ± 0.16^{c}	26.89 ± 0.28^{g}	8.79 ± 0.38^{d}
TJ4.1	34.19 ± 0.51^{d}	12.59 ± 0.16^{d}	22.87 ± 0.20^{f}	7.56 ± 0.52^{b}
MG4.2	36.98 ± 0.72^{d}	10.03 ± 0.13^{b}	11.78 ± 0.12^{a}	11.1 ± 0.31^{e}
TJ1.1	30.22 ± 0.69^{c}	10.80 ± 0.38^{c}	12.31 ± 0.32^{b}	5.66 ± 0.60^{a}
SA1.1	22.13 ± 0.08^{b}	9.95 ± 0.32^{b}	12.43 ± 0.27^{b}	7.78 ± 0.25^{b}
SA1.2	31.98 ± 0.16^{c}	9.1 ± 0.13^{a}	13.43 ± 0.38^{c}	5.82 ± 0.27^{a}
MG1.2	22.43 ± 0.12^{b}	9.61 ± 0.62^{b}	18.17 ± 0.43^{d}	8.92 ± 0.22^{d}
ES2.1	17.43 ± 0.27^{a}	8.41± 0.54^{a}	19.91 ± 0.33^{d}	Nil

Data are means ± SE of 3 determinations. Values with the same superscripts in the same vertical column are not significantly different ($P < 0.05$).
*: 1 unit (U) = 1 µg maltose liberated mL^{-1} of enzyme-extract min^{-1}
#: 1 unit (U) = 1 µg tyrosine liberated mL^{-1} of enzyme-extract min^{-1}
$: 1 unit (U) = 1 µg glucose liberated mL^{-1} of enzyme-extract min^{-1}
@: 1 unit (U) = 1 µmol fatty acid liberated mL^{-1} of enzyme-extract min^{-1}

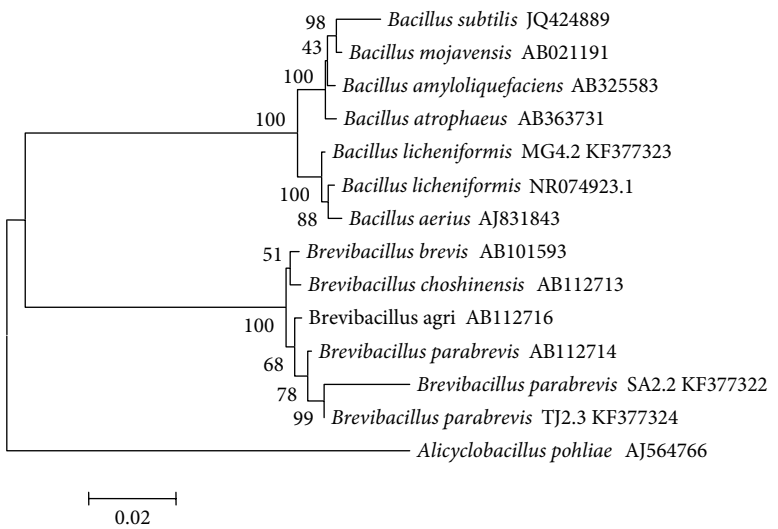

Figure. The phylogenetic tree showing the relationship among *Brevibacillus parabrevis* strains SA2.2 and TJ2.3, *Bacillus licheniformis* MG4.2, and their phylogenetically closest type strains. The GenBank accession numbers of the type strains and studied strains are shown following species names. Distance matrix was calculated by Kimura's 2-parameter model. The scale bar indicates 0.02 substitutions per nucleotide position. *Alicyclobacillus pohliae* AJ564766 served as an out-group.

washed with sterile, chilled 0.9% saline prior to isolation of microflora, it may be suggested that the microorganisms isolated in the present study belong to the autochthonous adherent microflora, as suggested elsewhere (Ghosh et al., 2010). The rate of microflora present within the GI tract of fish is much higher than that of the surrounding water, indicating that the GI tract of fish provides favorable ecological niches for these microorganisms (Mondal et al., 2008). However, isolation and identification alone might not give a realistic depiction of the gut microflora in different regions of the GI tract with an appraisal of their likely function (Khan and Ghosh, 2012). Therefore, it was considered legitimate in the present study to quantify heterotrophic bacteria along with specific extracellular enzyme-producing bacteria in different regions of the GI tracts in the fish species studied, as the major endeavor in the present study was to gather information on extracellular enzyme-producing gut bacteria in some brackish water fishes.

In the present study, gut bacteria were isolated by conventional culture-based methods. It is generally argued that culture-dependent techniques are time-consuming, lack accuracy (Asfie et al., 2003), and do not represent a correct picture of the bacterial diversity in the fish gut, even if several different media are used (Ray et al., 2010). However, the use of a culture-based technique employing a specific substrate containing selective media is justifiable, as the major aim of the present study was to detect different extracellular enzyme-producing gut bacteria. Besides, in the present study, conventional methods in combination with 16S rRNA analysis have been employed to identify the potent enzyme-producing gut isolates, as suggested elsewhere (Ghosh et al., 2010; Mondal et al., 2010; Ray et al., 2010).

Proper information regarding the relative importance of exogenous enzymes produced by the endosymbionts of the GI tract and digestive enzymes produced by the host is essential for understanding the contribution of endosymbionts in digestion (Clements, 1997). In the present investigation, all the fish species examined exhibited considerable amylolytic, proteolytic, cellulolytic, and lipolytic bacterial populations (Table 2). This can be correlated with their feeding habits. Being omnivore fish species, the occurrence of protease-, amylase-, cellulase-, and lipase-producing bacterial populations in the digestive tracts of *S. argus*, *T. jarbua*, and *E. suratensis* is justified. The occurrence of proteolytic, cellulolytic, and amylolytic bacteria in the gut has been suggested as an omnivorous feeding aptitude of the fish by Creach (1963) and Ghosh et al. (2002a, 2010). Previously, Bairagi et al. (2002) failed to detect cellulolytic bacteria in the GI tracts of carnivorous catfish and murrels; however, the results of the present investigation showed the presence of cellulolytic bacteria in carnivorous *M. gulio*. Stickney and Shumway (1974) opined that omnivores and carnivores might pick up cellulolytic flora from invertebrates that harbor the bacteria, which might explain the presence of the cellulolytic bacteria within the GI tract of *M. gulio* in the present study. The present study indicated that cellulolytic bacteria exist in the GI tracts of all the brackish water fish species studied, which supports the hypothesis that bacteria might contribute to the degradation of cellulose in fish (Ray et al., 2010). The presence of a huge population of cellulolytic bacteria and their vital role in extracellular cellulase production in fish has been documented in several investigations (Das and Tripathi, 1991; Saha and Ray, 1998; Bairagi et al., 2002; Saha et al., 2006; Mondal et al., 2008; Mondal et al., 2010). In their previous study with carp, Shcherbina and Kazlawlene (1971) suggested that cellulose absorption takes place in the DI, which may indicate the presence of microbial cellulase in this region. Our observation is in accordance with this hypothesis as most cellulase-producing bacteria were recorded in the DI of all fish species studied except in *M. gulio*, which was supposed to be a carnivorous fish. Furthermore, it may be mentioned that except for cellulolytic bacteria in *M. gulio*, the heterotrophic microbial population was observed to be highest in the DI regions of all the fish species studied when compared to the PI and MI regions, which is in harmony with previous reports (Mondal et al., 2008; Ghosh et al., 2010; Ray et al., 2010).

The assay of extracellular enzyme production showed the highest values for amylase and cellulase production in SA2.2 isolated from the DI of *S. argus*. However, protease and lipase productions were highest in TJ2.3 isolated from the DI of *T. jarbua* and MG4.2 isolated from DI of *M. gulio*, respectively. Qualitative and quantitative determination of extracellular enzyme production exhibited poor performance by the isolates from *E. suratensis* when compared with the isolates from the other fishes studied. In the present study, the 2 efficient enzyme-producing strains (SA2.2 and TJ2.3) were established through quantitative enzyme assay and identified as *Brevibacillus parabrevis* based on 16S rRNA sequence analysis. Although both promising isolates belonged to *B. parabrevis*, the strain SA2.2 isolated from DI of *S. argus* showed the most similarity to *B. parabrevis* HDYM-18 (EF428244) (Ping and Ge, 2007; unpublished data), while strain TJ2.3 isolated from DI of *T. jarbua* showed closeness to *B. parabrevis* M3 (AB215101) (Suzuki et al., 2005; unpublished data). Another strain, MG4.2, isolated from the DI of *M. gulio*, showed 16S rRNA sequence similarity to *B. licheniformis* GLU 113 (FN678352) (Shariati, 2010; unpublished data). Diverse strains of extracellular enzyme-producing bacteria have been identified from the GI tracts of freshwater and marine fishes (for review, see Ray et

al., 2012). The occurrence of *B. licheniformis* within the gut of freshwater fishes has been reported previously by several authors (Mondal et al., 2010; Dan and Ray, 2013). However, to the authors' knowledge, extracellular enzyme-producing *Brevibacillus* sp. has not been reported from fish gut previously. In addition, reports on gut-inhabiting extracellular enzyme-producing bacteria from brackish water fish species are scanty (De et al., 2012).

An extensive range of enzymes produced by GI bacteria could be a contributing source of digestive enzymes in fish (Ray et al., 2012). Characterization of the microbial populations in the intestinal microenvironment of fish, and understanding of the physiological interactions between the indigenous microflora and the host, might have important implications (Silva et al., 2005). Enzymes produced by intestinal fish microflora might have a significant role in digestion, mainly for substrates such as cellulose, which few animals can digest, and also for other substrates (Smith, 1989). Luczkovich and Stellwag (1993) opined that the GI microflora of pinfish (*Lagodon rhomboides*) might contribute to the breakdown of plant material. Kar et al. (2008) indicated that the enzyme-producing gut bacteria are able to utilize carbohydrates such as mannose, xylose, raffinose, cellobiose, and cellulose. These substances are mainly found in plant foodstuffs. Therefore, cellulase and amylase activities by the gut bacteria might indicate their ability to aid in the digestion of plant foodstuffs. The use of beneficial bacteria as probiotics has a long tradition in animal husbandry (Stavric and Kornegay, 1995). Beneficial bacteria could be introduced in commercial aquaculture by incorporating them into formulated fish diets, or in the form of bacteria biofilm to achieve colonization in the GI tract to a higher degree (Bairagi et al., 2002, 2004; Ghosh et al., 2002b, 2003, 2004a, 2004b; Ramachandran et al., 2005; Ramachandran and Ray, 2007; Askarian et al., 2011; Saha and Ray, 2011). It has been suggested that beneficial gut bacteria are continuously competing with pathogens through competitive exclusion (Ray et al., 2012). These topics could be addressed in upcoming studies. Whether the gut bacteria can contribute to the host's nutrition has not been elucidated in the present study. Assessment of the role of the enzyme-producing gut bacteria in brackish water fish culture should therefore be given high priority in future studies.

Acknowledgments

The authors are grateful to the Head of the Department of Zoology, The University of Burdwan, West Bengal, India; the Department of Science and Technology (FIST Programme), New Delhi, India; and the University Grants Commission (Special Assistance Programme), New Delhi, India, for providing research facilities and financial support. The authors are obliged to Nilanjan Maitra for rendering help in the analyses of sequenced data.

References

Asfie, M., Yoshijima, T. and Sugita, H. 2003. Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. Fisheries Sci. 69: 21–26.

Askarian, F., Kousha, A., Salma, W. and Ringø, E. 2011. The effect of lactic acid bacteria administration on growth, digestive enzymes activity and gut microbiota in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fry. Aquacult. Nutr. 17(5): 488–497.

Askarian, F., Zhou, Z., Olsen, R.E., Sperstad, S. and Ringø, E. 2012. Culturable autochthonous gut bacteria in Atlantic salmon (*Salmo salar* L.) fed diets with or without chitin. Characterization by 16S rRNA gene sequencing, ability to produce enzymes, and *in vitro* growth inhibition of four fish pathogens. Aquaculture 326–329: 1–8.

Austin, B. 2002. The bacterial microflora of fish. Sci. World J. 2: 558–572.

Bairagi, A., Ghosh, K.S., Sen, S.K. and Ray, A.K. 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult. Int. 10: 109–121.

Bairagi, A., Sarkar Ghosh, K., Sen, S.K. and Ray, A.K. 2004. Evaluation of nutritive value of *Leucaena leucocephala* leaf meal inoculated with fish intestinal bacteria *Bacillus subtilis* and *Bacillus circulans* in formulated diets for rohu, *Labeo rohita* (Hamilton) fingerlings. Aquacult. Res. 35: 436–446.

Belchior, S.G. and Vacca, G. 2006. Fish protein hydrolysis by a psychrotrophic marine bacterium isolated from the gut of hake (*Merluccius hubbsi*). Can. J. Microbiol. 52: 1266–1271.

Bernfeld, P. 1955. Amylase. In: Methods in Enzymology (Eds. S.P. Colowick and N.O. Kaplan). Academic Press, New York, NY, USA, pp. 149–158.

Beveridge, M.C.M., Sikdar, P.K., Frerichs, G.N. and Millar, S. 1991. The ingestion of bacteria in suspension by the common carp *Cyprinus carpio* L. J. Fish Biol. 39: 825–831.

Bier, M. 1955. Methods in Enzymology. Vol. I. Academic Press, New York, NY, USA, pp. 627–642.

Clements, K.D. 1997. Fermentation and gastrointestinal microorganisms in fishes. In: Gastrointestinal Microbiology. Vol I. Gastrointestinal Ecosystems and Fermentations (Eds. R.I. Mackie and B.A. White). Chapman and Hall, New York, NY, USA, pp. 156–198.

Creach, P.V. 1963. The proteolytic enzymes of poisons. In: Nutrition in Poecilothermes (Ed. CNRS). CNRS, Paris, France, pp. 375–471.

Dan, S.K. and Ray, A.K. 2013. Characterization and identification of phytase-producing bacteria isolated from the gastrointestinal tract of four freshwater teleosts. Ann. Microbiol. DOI: 10.1007/s13213-013-0664-3.

Danulat, E. and Kausch, H. 1984. Chitinase activity in the digestive tract of cod, *Gadus morhua* (L.). J. Fish Biol. 24: 125–133.

Das, K.M. and Tripathi, S.D. 1991. Studies on the digestive enzymes of grass carp, *Ctenopharyngodon idella* (V). Aquaculture 92: 21–32.

De, D., Ghoshal, T.K. and Ananda Raja, R. 2012. Characterization of enzyme-producing bacteria isolated from the gut of Asian seabass, *Lates calcarifer* and milkfish, *Chanos chanos* and their application for nutrient enrichment of feed ingredients. Aquacult. Res. DOI: 10.1111/are.12099.

Denison, D.A. and Koehn, R.D. 1977. Cellulase activity of *Poronia oedipus*. Mycologia 69: 592–601.

Esakkiraj, P., Immanuel, G., Sowmya, S.M., Iyapparaj, P. and Palavesam, A. 2009. Evaluation of protease-producing ability of fish gut isolate *Bacillus cereus* for aqua feed. Food Bioprocess Technol. 2(4): 383–390.

Finegold, S.M., Sutter, V.L. and Mathisen, G.E. 1983. Normal indigenous intestinal flora. In: Human Intestinal Microflora in Health and Disease (Ed. D.J. Hentgens). Academic Press, New York, NY, USA, pp. 3–31.

Ghosh, K., Roy, M., Kar, N. and Ringø, E. 2010. Gastrointestinal bacteria in rohu, *Labeo rohita* (Actinopterygii: Cypriniformes: Cyprinidae): scanning electron microscopy and bacteriological study. Acta Ichthyol. Piscatoria 40(2): 129–135.

Ghosh, K., Sen, S.K. and Ray, A.K. 2002a. Characterization of bacilli isolated from the gut of rohu, *Labeo rohita*, fingerlings and its significance in digestion. J. Appl. Aquacult. 12(3): 33–42.

Ghosh K., Sen, S.K. and Ray, A.K. 2002b. Growth and survival of rohu, *Labeo rohita* (Hamilton) spawn fed diets supplemented with fish intestinal microflora. Acta Ichthyol. Piscatoria 32(1): 83–92.

Ghosh, K., Sen, S.K. and Ray, A.K. 2003. Supplementation of an isolated fish gut bacterium, *Bacillus circulans*, in formulated diets for rohu, *Labeo rohita*, fingerlings. Israeli J. Aquacult. 55: 13–21.

Ghosh, K., Sen, S.K. and Ray, A.K. 2004a. Growth and survival of rohu, *Labeo rohita* (Hamilton, 1822) spawn fed diets fermented with intestinal bacterium, *Bacillus circulans*. Acta Ichthyol. Piscatoria 34(2): 155–165.

Ghosh, K., Sen, S.K. and Ray, A.K. 2004b. Supplementation of *Lactobacillus acidophilus* in compound diets for *Labeo rohita* fingerlings. Indian J. Fish. 51(4): 521–526.

Horsley, R.W. 1977. A review of the bacterial flora of teleosts and elasmobranches, including methods for its analysis. J. Fish Biol. 10: 529–553.

Itoi, S., Okamura, T., Koyama, Y. and Sugita, H. 2006. Chitinolytic bacteria in the intestinal tract of Japanese coastal fishes. Can. J. Microbiol. 52: 1158–1163.

Jacob, M.B. and Gerstein, M.J. 1960. Handbook of Microbiology. D. Van Nostrand Co. Inc., Princeton, NJ, USA.

Kar, N., Roy, R.N., Sen, S.K. and Ghosh, K. 2008. Isolation and characterization of extracellular enzyme-producing bacilli in the digestive tracts of rohu, *Labeo rohita* (Hamilton) and murrel, *Channa punctatus* (Bloch). Asian Fish. Sci. 21(4): 421–434.

Khan, A. and Ghosh, K. 2012. Characterization and identification of gut-associated phytase-producing bacteria in some freshwater fish cultured in ponds. Acta Ichthyol. Piscatoria 42(1): 37–45.

Khan, A. and Ghosh, K. 2013. Evaluation of phytase production by fish gut bacterium, *Bacillus subtilis* for processing of *Ipomea aquatica* leaves as probable aquafeed ingredient. J. Aquat. Food Prod. Technol. 22(5): 508–519.

Khan, A., Mandal, S., Samanta, D., Chatterjee, S. and Ghosh, K. 2011. Phytase-producing *Rhodococcus* sp. (MTCC 9508) from fish gut: a preliminary study. Proc. Zool. Soc. 64: 29–34.

Kinnear P.R. and Gray, C.D. 2000. SPSS for Windows Made Simple. Release 10. Psychology Press, Sussex, UK.

Lane, D.J. 1991. 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Eds. E. Stackebrandt and M. Goodfellow). Wiley, Chichester, UK, pp. 130–141.

Li, X., Chi, Z., Liu, Z., Yan, K. and Li, H. 2008. Phytase production by a marine yeast *Kodamea ohmeri* BG3. Applied Biochem. Biotechnol. 149: 183–193.

Li, X.Y., Liu, Z.Q. and Chi, Z.M. 2008. Production of phytase by a marine yeast *Kodamaea ohmeri* BG3 in an oats medium: optimization by response surface methodology. Bioresour. Technol. 99: 6386–6390.

Luczkovich, J.J. and Stellwag, E.J. 1993. Isolation of cellulolytic microbes from the intestinal tract of pinfish, *Lagodon rhomboids*: size-related changes in diet and microbial abundance. Mar. Biol. 116: 381–388.

MacDonald, N.L., Stark, J.R. and Austin, B. 1986. Bacterial microflora in the gastrointestinal tract of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. FEMS Microbiol. Lett. 35: 107–111.

Mandal, S. and Ghosh, K. 2013. Isolation of tannase-producing microbiota from the gastrointestinal tracts of some freshwater fish. J. Appl. Ichthyol. 29: 145–153.

Mondal, S., Roy, T. and Ray, A.K. 2010. Characterization and identification of enzyme-producing bacteria isolated from the digestive tract of bata, *Labeo bata*. J. World Aquacult. Soc. 41: 369–377.

Mondal, S., Roy, T., Sen, S.K. and Ray, A.K. 2008. Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. Acta Ichthyol. Piscatoria 38: 1–8.

Rahmatullah, S.M. and Beveridge, M.C.M. 1993. Ingestion of bacteria in suspension by Indian major carps (*Catla catla, Labeo rohita*) and Chinese carps (*Hypophthalmichthys molitrix, Aristichthys nobilis*). Hydrobiologia 264: 79–84.

Ramachandran, S. and Ray, A.K. 2007. Nutritional evaluation of fermented black gram (*Phaseolus mungo*) seed meal in compound diets for rohu, *Labeo rohita* (Hamilton), fingerlings. J. Appl. Ichthyol. 23: 74–79.

Ramachandran, S., Bairagi, A. and Ray, A.K. 2005. Improvement of nutritive value of grass pea (*Lathyrus sativus*) seed meal in the formulated diets for rohu, *Labeo rohita* (Hamilton) fingerlings after fermentation with a fish gut bacterium. Bioresour. Technol. 96: 1465–1472.

Rani, S., Garg, S.K., Sabhlok, V.P. and Bhatnagar, A. 2004. Intestinal enzyme activity and enzyme-producing microbial flora in relation to feeding behaviour in some brackish water teleosts. J. Aquacult. 12: 55–68.

Ray, A.K., Ghosh, K. and Ringø, E. 2012. Enzyme-producing bacteria isolated from fish gut: a review. Aquacult. Nutr. 18: 465–492.

Ray, A.K., Roy, T., Mondal, S. and Ringø, E. 2010. Identification of gut-associated amylase, cellulase, and protease-producing bacteria in three species of Indian major carps. Aquacult. Res. 41:1462–1469.

Ringø, E. 1993. The effect of chromic oxide (Cr_2O_3) on aerobic bacterial populations associated with the epithelial mucosa of Arctic charr (*Salvelinus alpinus* L.). Can. J. Microbiol. 39: 1169–1173.

Ringø, E. and Birkbeck, T.H. 1999. Intestinal microflora of fish larvae and fry. Aquacult. Res. 30: 73–93.

Ringø, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H., Hemre, G.I. and Bakke, A.M. 2010. Prebiotics in aquaculture: a review. Aquacult. Nutr. 16: 117–136.

Ringø, E., Olsen, R.E., Mayhew, T.M. and Myklebust, R. 2003. Electron microscopy of the intestinal microflora of fish. Aquaculture 227: 395–415.

Roy, T., Mondal, S. and Ray, A.K. 2009. Phytase-producing bacteria in the digestive tracts of some freshwater fish. Aquacult. Res. 40: 344–353.

Saha, A. and Ray, A.K. 1998. Cellulase activity in rohu fingerlings. Aquacult. Int. 6: 281–291.

Saha, S. and Ray, A.K. 2011. Evaluation of nutritive value of water hyacinth (*Eichhornia crassipes*) leaf meal in compound diets for rohu, *Labeo rohita* (Hamilton, 1822) fingerlings after fermentation with two bacterial strains isolated from fish gut. Turkish J. Fish. Aqua. Sci. 11: 199–209.

Saha, S., Roy, R.N., Sen, S.K. and Ray, A.K. 2006. Characterization of cellulase-producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (P) and grass carp, *Ctenopharyngodon idella* (V). Aquacult. Res. 37: 380–388.

Sakata, T. 1990. Microflora in the digestive tract of fish and shellfish. In: Microbiology in Poecilotherms (Ed. R. Lesel). Elsevier, Amsterdam, the Netherlands, pp. 171–176.

Sangiliyandi, G. and Gunasekaran, P. 1996. Extracellular lipase-producing *Bacillus licheniformis* from an oil mill refinery effluent. Indian J. Microbiol. 36: 109–110.

Shcherbina, M.A. and Kazlawlene, O.P. 1971. The reaction of the medium and the rate of absorption of nutrients in the intestine of carp. J. Ichthyol. 11: 81–85.

Silva, F.C.P., Brito, M.F.G., Brito, L.M. and Nicoli, J.R. 2005. Composition and antagonistic activity of the indigenous intestinal microbiota of *Prochilodus argenteus* Agassiz. J. Fish Biol. 67: 1686–1698.

Sivasubramanian, K., Ravichandran, S. and Kavitha, R. 2012. Isolation and characterization of gut microbiota from some estuarine fishes. Marine Sci. 2(2): 1–6.

Smith, L.S. 1989. Digestive functions in teleost fishes. In: Fish Nutrition (Ed. J.E. Halver). Academic Press, San Diego, CA, USA, pp. 331–42.

Stavric, S. and Kornegay, E.T. 1995. Microbial probiotics for pigs and poultry. In: Biotechnology in Animal Feeds and Animal Feeding (Eds. R.J. Wallace and A. Chuson). Wiley, New York, NY, USA, pp. 205–231.

Stickney, R.R. and Shumway, S.E. 1974. Occurrence of cellulase activity in the stomachs of fishes. J. Fish Biol. 6: 779–790.

Sugita, H., Shibuya, K., Harada, H. and Deguchi, Y. 1997. Antibacterial abilities of intestinal microflora of the river fish. Fisheries Sci. 63: 378–383.

Teather, R.M. and Wood, P.J. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. 43: 777–780.

Trust, T.J. and Sparrow, R.A. 1974. The bacterial flora in the alimentary tract of fresh water salmonid fishes. Can. J. Microbiol. 20: 1219–1228.

Walter, H.E. 1984. Methods of Enzymatic Analysis. Verlag Chemie, Weinheim, Germany.

Zar, J.E. 1974. Biostatistical Analysis. Prentice Hall, NJ, USA.