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DISTRIBUTION OF ENZYME-PRODUCING BACTERIA IN THE DIGESTIVE TRACTS OF SOME FRESHWATER FISH

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Background. The information on gut microflora in fish is scanty and there is a paucity of knowledge regarding microbial enzyme activity in fish gastrointestinal tracts. Although some information is available on the enzyme-producing bacteria in fish digestive tracts, almost nothing is known about their distribution in different regions of the gut. In the present study, an attempt has been made to investigate the distribution of enzyme-producing microflora in the foregut and hindgut regions of seven culturable freshwater teleosts.
Materials and Methods. Isolation and enumeration of aerobic bacterial flora in the foregut and hindgut regions of the gastrointestinal tracts of seven freshwater teleosts of different feeding habits, namely rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*; bata, *Labeo bata*; orange-fin labeo, *Labeo calbasu*; Nile tilapia, *Oreochromis niloticus*; and climbing perch, *Anabas testudineus*, have been carried out. Microbial culture of the gut mucosa on selected nutrient media, following the enrichment culture technique, was done for bacterial isolation. Bacterial isolates were qualitatively screened on the basis of their extracellular enzyme-producing ability. The selected strains were further quantitatively assayed for amylase, cellulase and protease activities.
Results. In general, bacterial population was lower in the foregut region of all the seven species of fish examined. Amylolytic strains were present in higher densities in the foregut region of orange-fin labeo and bata (12.20×10^3 CFU · g^{-1} gut tissue and 11.50×10^3 CFU · g^{-1} gut tissue, respectively) in comparison to the hindgut region. The cellulolytic population exhibited maximum densities in the hindgut region of bata (7.20×10^3 CFU · g^{-1} gut tissue) followed by the foregut region of the same fish (5.50×10^3 CFU · g^{-1} gut tissue). Amylolytic and cellulolytic bacterial flora was not detected in both the fore and hindgut regions of climbing perch. Proteolytic bacterial flora was found in all the species of fish studied and the maximum count was observed in the hindgut region of bata (13.40×10^3 CFU · g^{-1} gut tissue), orange-fin labeo (9.00×10^3 CFU · g^{-1} gut tissue), Nile tilapia (8.30×10^3 CFU · g^{-1} gut tissue) and climbing perch (7.20×10^3 CFU · g^{-1} gut tissue). Minimum count of proteolytic bacterial flora was observed in the foregut region of all the fishes studied. Peak amylase and cellulase activities were exhibited by bacterial strains isolated from the foregut of orange-fin labeo (266.43 ± 0.15 U) and the hindgut of bata (64.01 ± 0.42 U), respectively. Maximum protease activity was exhibited by a strain isolated from the hindgut region of orange-fin labeo (44.33 ± 0.09 U), followed by the strains isolated from the hindgut regions of climbing perch (32.87 ± 0.12 U), bata (29.71 ± 0.11 U), and Nile tilapia (29.46 ± 0.11 U).
Conclusions. The results of the present study indicate that there is a distinct microbial source of digestive enzymes apart from the endogenous sources in fish digestive tracts. The enzyme-producing bacteria isolated from the digestive tracts can be beneficially used as a probiotic while formulating aquafeeds, especially in the larval stages. However, further investigations are required to determine if the addition of such isolates to fish feeds do, in fact, provide some kind of benefit to the fish involved before advocating their use.

Keywords: freshwater fish, gut bacteria, distribution, microbial enzyme activity

INTRODUCTION

Fermentative digestion occurs typically in animals with a diet composed predominantly of plant material (Bergman 1990) and symbioses with microorganisms have been well studied in herbivorous mammals, birds, and reptiles (Stevens 1988). Only recently diverse microbial communities have been reported from the guts of fishes (Clements 1997, Saha and Ray 1998, Bairagi et al. 2002, Saha et al. 2006). These microbial populations grow upon the food absorbed by the host animal, digestive secretions

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and fragments sloughed off the mucosal epithelium (Lésel 1991). In general, the bacterial flora of the gastrointestinal tract represent a very important and diversified enzymatic potential and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a major part of the metabolism of the host animal (Clements 1997). Though considerable information is available regarding the intestinal microflora of homeotherms and their role in digestion, reports on the bacterial population in the gastrointestinal tract of poikilotherms, including fish, and their role in digestion are scanty. Although few reports concerning microbial enzyme production in the gastrointestinal tract of fish are available (Prejs and Blaszczyk 1977, Lindsay and Harris 1980, Lésel et al. 1986, Das and Tripathi 1991, Saha and Ray 1998, Ghosh et al. 2002, Bairagi et al. 2002, Saha et al. 2006), information on the distribution of these enzyme-producing endosymbionts in different regions of the gut are scarce (Trust and Sparrow 1974, Sakata 1990, Mac Donald et al. 1986, Ringø 1993, Ringø and and Strøm 1994).

In the present study, an attempt has been made to isolate and enumerate the enzyme-producing microflora in the foregut and hindgut regions of a few freshwater culturable teleosts of different feeding habits. The amylolytic, cellulolytic, and proteolytic microbes from selected fish species were isolated in pure culture and comparative assay of extracellular microbial enzyme activity by these isolates was conducted. The strains from the foregut and hindgut regions of the gastrointestinal tracts exhibiting maximum enzyme activity in the case of each enzyme were screened.

MATERIALS AND METHODS

Fish examined. Seven species of adult freshwater teleosts of different feeding habits, namely, the Indian major carps (rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*; orange-fin labeo, *Labeo calbasu*); minor carp (bata), *Labeo bata*; Nile tilapia, *Oreochromis niloticus*; and climbing perch, *Anabas testudineus* were selected for the presently reported study. The fish were sampled from a pond near Santiniketan, West Bengal, India (lat 23°41′30″N, long 87°41′20″E) during December to June, 2005–2006 when temperature varied between 20 and 30°C. The feeding habits, average weight and length of the fishes examined are presented in Table 1. The fish were starved for 36 h prior to sacrifice in order to clear their digestive tracts before being dissected.

Post-mortem examination. Immediately after being pithed, the ventral surface of each fish was scrubbed thoroughly with 1% iodine solution (Trust and Sparrow 1974). The fish were carefully dissected aseptically within laminar airflow on ice slabs. The digestive tract was divided into foregut and hindgut region as described by Ringø and Strøm (1994). The two regions were emptied and thoroughly rinsed five times in sterile 0.9% saline in order to remove non-adherent bacteria. The two regions were separately homogenized with 10 parts of chilled 89% sodium chloride solution with due care (Das and Tripathi 1991).

Microbial culture. Homogenate of the intestinal mucosa of each of the test fish was used for microbial culture after five serial 1 : 10 serial dilutions (Beveridge et al. 1991). Samples (0.1 mL) were taken from each dilution and poured aseptically within a laminar flow on sterilized Tryptone Soya Agar (TSA) plates, in duplicate. These culture plates were incubated at 34°C for 24 h. They were subsequently examined for the developments of bacterial colonies. The well separated colonies with apparently different morphological difference were streaked separately on TSA plates to obtain pure cultures. Single, isolated colonies from the streaked plates were transferred to TSA slants.

To isolate and enumerate amylase-, cellulase-, and protease-producing bacteria, diluted gut homogenate was poured on starch-agar, carboxymethylcellulose (CMC)-agar, and peptone-gelatin-agar media containing plates, respectively. These culture plates were incubated at 37 ± 1ºC for 24 h. It was assumed that the microflora, which had formed colonies on the starch plate, had amylolytic activity. Similarly, microflora grown on CMC plate, and peptone-gelatin plate were assumed to have cellulolytic, and proteolytic activities, respectively. By multiplying the number of colonies formed on each plate by the reciprocal of dilution, colony numbers per unit sample volume of gut homogenate were determined (Rahmatullah and Beveridge 1993).

Media composition. Tryptone Soya agar medium: 40 g of TSA (Hi media Laboratories Pvt. Ltd., Mumbai, India) suspended in 1000 mL of distilled water (pH 7). Starch agar medium ($g \cdot L^{-1}$): starch, 10; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.004; Tryptone, 2; Agar, 15 (pH 7). Carboxymethylcellulose (CMC)-agar medium ($g \cdot L^{-1}$): Carboxymethylcellulose, 10; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.004; Tryptone, 2; Agar, 15 (pH 7). Peptone-gelatin-agar medium ($g \cdot L^{-1}$): Peptone, 5; Gelatin, 4; Beef extract, 3; Agar, 20 (pH 7).

Table 1

Average weight and length of the fishes examined

Fish species	Feeding habits	Average weight*[g]	Average length* [cm]
Labeo rohita	Omnivorous, mostly plant matter	232.8 (1.3)	18.2 (1.7)
Catla catla	Zooplanktophagous	216 (9.9)	13.2 (1.5)
Cirrhinus mrigala	Detritivorous	168.3 (6.5)	15.6 (1.1)
Labeo bata	Herbivorous	117.8 (7.6)	8.2 (0.7)
Labeo calbasu	Detritivorous	124.3 (7.9)	10.7 (1.1)
Anabas testudineus	Carnivorous, mostly insects	22.2 (2.7)	10.5 (1.1)
Oreochromis niloticus	Omnivorous	87.6 (4.8)	13.6 (2.1)

* (in brackets): Standard deviation of mean of 3 determinations.

Screening of isolates for extra-cellular qualitative enzyme production. For screening of enzyme-producing strains, bacterial isolates were streaked on starch-agar medium, CMC-agar medium and peptone-gelatin-agar medium and incubated for 48 h at 37 ± 1ºC to screen amylase, cellulase, protease producing strains, respectively. For screening of amylase producing strains, isolates were streaked on starch (1%) supplemented nutrient agar plates and incubated at 37 ± 1ºC for 48 h. After appearance of the colonies on the starch-agar medium, the culture plates were flooded with 1% Lugol's iodine solution (Jacob and Gerstein 1960) to identify amylase activity. Similarly, for screening of cellulase producers, isolates were grown on CMC-agar medium containing plates and flooded with 5 mL of Congo red dye prepared in 0.7% agarose (Seakem HGT agarose, Cambrex India Pvt. Ltd., Mumbai, India) according to the method of Teather and Wood (1982). The appearance of a clear zone around the colony after flooding the plates indicated the presence of cellulolytic activity. For extra-cellular protease production, the isolates were streaked on peptone-gelatin enriched nutrient agar (4% gelatin) plates and incubated at 37 ± 1ºC for 48 h. The appearance of a clear zone around the colony after flooding the plate with 15% $HgCl_2$ indicated the presence of proteolytic activity (Jacob and Gerstein 1960).

Quantitative enzyme assay. Respective selective broth media were used as production media for a quantitative assay of amylase, cellulase and protease production. A loopful of selected strain was inoculated into Tryptone soya agar (TSA) broth and incubated for 24 h at 37ºC and was used as the inoculum. The liquid production medium of 25 mL was inoculated with 2% of the inoculum obtained from seed culture. The culture flasks were incubated for 72 h at 37 ± 1ºC. After incubation, the contents were centrifuged at 10 000 g for 10 min, at 4ºC and the cell-free supernatant was used for enzyme assay.

Cellulase assay. The production of reducing sugars due to cellulolytic activity was measured by dinitrosalicylic acid method (Denison and Koehn 1977) using 1% CMC in sodium citrate buffer (0.1 M, pH 5.0) as substrate. The production of reducing sugar (glucose) from CMC substrate because of cellulolytic activity was measured at 540 nm using glucose as the standard. One cellulase unit was defined as the amount of enzyme per mL culture filtrate that released 1 µg of glucose per min.

Amylase assay. Amylase was assayed by the dinitrosalicylic acid method based on the estimation of reducing sugars at 560 nm using maltose as the standard (Bernfeld 1955). One amylase unit was defined as the amount of enzyme per mL culture filtrate that released one microgram reducing sugar per min.

Protease assay. Protease activity was measured by caseinase assay method (Walter 1984). One unit of enzyme activity was expressed as the amount of enzyme required liberating 1 µg of tyrosine per mL culture filtrate per min under standard assay conditions.

Statistical analysis. The data were subjected to analysis of variance (ANOVA) using Origin 6.1 software. Duncan's multiple range test (Duncan 1955) was employed to test differences among means. The significance of differences was tested at the significance level $P = 0.5$.

RESULTS

A considerable population of aerobic bacterial symbionts has been isolated from the fore- and hindgut regions of all the fishes studied (Tables 2 and 3). The bacterial population in tryptone soya agar (TSA) plate was maximal in the hindgut region of bata (1.70×10^6 CFU · g^{-1} gut tissue), followed by mrigal (1.20×10^6 CFU · g^{-1} gut tissue) and minimum in the foregut region of Nile tilapia (0.03×10^6 CFU · g^{-1} gut tissue). In general, bacterial population was lower in the foregut region of all the seven species of fish studied. While enumerating specific enzyme-producing bacterial flora, it was observed that the amylolytic strains were present in higher densities in the foregut region of orange-fin labeo (12.20×10^3 CFU · g^{-1} gut tissue) and bata (11.50×10^3 CFU · g^{-1} gut tissue) in comparison to the hindgut region (2.30×10^3 CFU · g^{-1} gut tissue and 1.30×10^3 CFU · g^{-1} gut tissue in bata and orange-fin labeo, respectively). The cellulolytic population exhibited maximum densities in the hindgut region of bata (7.20×10^3 CFU · g^{-1} gut tissue) followed by the foregut region of the same fish (5.50×10^3 CFU · g^{-1} gut tissue). Amylolytic- and cellulolytic bacterial florae were not detected in both the fore- and hindgut regions of climbing perch. Proteolytic bacterial florae were recorded in all the species of fish studied and the maximum count was observed in the hindgut region of bata (13.40×10^3 CFU · g^{-1} gut tissue), orange-fin labeo (9.00×10^3 CFU · g^{-1}

Table 2

Aerobic bacterial count in foregut of fish digestive tract

Fish species	CFU · g^{-1} gut tissue			
	Bacterial count in TSA plate ($\times 10^6$)	Amylolytic bacteria ($\times 10^3$)	Cellulolytic bacteria ($\times 10^3$)	Proteolytic bacteria ($\times 10^4$)
Labeo rohita	0.06^b	0.70^d	0.10^c	0.02^c
Catla catla	0.10^a	4.00^c	0.09^c	0.08^c
Cirrhinus mrigala	0.08^a	0.90^d	0.03^c	0.40^b
Labeo bata	0.10^a	11.50^a	5.50^a	3.40^a
Labeo calbasu	0.20^a	12.20^a	0.40^b	0.80^b
Anabas testudineus	0.08^a	ND	ND	0.90^b
Oreochromis niloticus	0.03^a	7.30^b	1.50^b	0.90^b

ND = Not detected; Values with same superscripts in the same vertical column are not significantly different ($P < 0.05$).

Table 3

Aerobic bacterial count in hindgut of fish digestive tract

Fish species	CFU g^{-1} intestinal tissue			
	Bacterial count in TSA plate ($\times 10^6$)	Amylolytic bacteria ($\times 10^3$)	Cellulolytic bacteria ($\times 10^3$)	Proteolytic bacteria ($\times 10^4$)
Labeo rohita	0.20^b	0.02^b	0.80^c	4.00^c
Catla catla	1.00^a	0.20^b	0.60^c	4.20^c
Cirrhinus mrigala	1.20^a	0.07^b	0.80^c	3.70^c
Labeo bata	1.70^a	2.30^a	7.20^a	13.40^a
Labeo calbasu	1.10^a	1.30^a	0.95^c	9.00^b
Anabas testudineus	0.20^b	Nil	Nil	7.20^b
Oreochromis niloticus	0.10^b	0.60^b	4.00^b	8.30^b

Values with the same superscripts in the same vertical column are not significantly different ($P < 0.05$).

gut tissue), Nile tilapia (8.30×10^3 CFU · g^{-1} gut tissue) and climbing perch (7.20×10^3 CFU · g^{-1} gut tissue). Minimum count of proteolytic bacterial flora was observed in the foregut region of all the fishes studied (0.02 to 0.90×10^3 CFU · g^{-1} gut tissue).

The intensity of extracellular enzyme production by the bacterial strains isolated from the gut of the selected species of fish was assayed qualitatively (Table 4). Among these isolates, six amylase, six cellulase, and ten protease producers were selected (from both the foregut and hindgut regions) for quantitative enzyme assay (Figs. 1–3). Peak amylase- and cellulase activities were exhibited by the bacterial strains CF5 and BH4 isolated from the foregut of orange-fin labeo and the hindgut of bata, respectively. Maximum protease activity was observed in CH22, the strain isolated from the hindgut region of orange-fin labeo, followed by the strains TH1, BH4, and NH5, isolated from the hindgut regions of climbing perch, bata, and Nile tilapia, respectively.

DISCUSSION

Generally, bacteria are abundant in the environment in which fish live and it is therefore, rather impossible to avoid them being a component of their diet (Strøm and Olafsen 1990, Hansen et al. 1992). The bacteria entering along with the diet of fish during ingestion may adapt themselves in the gastrointestinal tract and form a symbiotic association. Within the digestive tract of fish large numbers of microbes are present (Trust et al. 1979, Rimmer and Wiebe 1987, Clements 1991, Luczkovich and Stellwag 1993, Ringø and Strom 1994, Clements and Choat 1995), which is much higher than in the surrounding water indicating that the digestive tracts of fish provide favourable ecological niches for these organisms (Trust and Sparrow 1974, Horsley 1977, Austin and Al-Zahrani 1988, Sakata 1990). However, the gastrointestinal microflora of fish appears to be simpler than those of endotherms. While the digestive tracts of endotherms are colonized mainly by obligate anaerobes (Finegold et al. 1983), the predominant bacterial genera/species 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. 2002). In the present study, attention has been focused on the aerobic gastrointestinal bacteria of seven Indian freshwater teleosts. But only isolation and identification of bacterial flora do not give a representative picture of the gut flora in the different regions of the digestive tract (Savage 1977). Therefore, more information is required on the adherent bacterial genera in the different regions of the digestive tract (Trust and Sparrow 1974, Trust et al. 1979, MacDonald et al. 1986, Austin and Al-Zaharani 1988, Strøm and Olafsen 1990, Westerdahl et al. 1991, Ringø 1993, Ringø and Strøm 1994). In the present investigation, the presence of considerable population of bacterial flora has been found in the foregut and hindgut regions of the fish species and some of the strains exhibit amylolytic-, cellulolytic-, and proteolytic activities.

Typical numbers of bacteria in fish intestines are 10^8 aerobic heterotrophic bacteria per 1 g and approximately 10^5 anaerobic bacteria per 1 g (Trust and Sparrow 1974, Trust et al. 1979, Kamei et al. 1985). Austin and Al-Zaharani (1988) noted a progressive decline in numbers (3×10^5 to 2×10^4) of heterotrophic bacteria along the digestive tract (oesophagus, stomach, upper, and lower intestines) of farmed rainbow trout, *Oncorhynchus myskiss*. In contrast, Ringø (1993) recorded a progressive increase in the numbers of bacteria in cultivated Arctic charr from the foregut (2×10^4) to the rectal region (4×10^5) of the intestine. In the present study, total bacterial population was recorded highest in the hindgut region of all the seven species of fish. However, on the basis of their enzyme production ability, amylase producing bacteria were found to be highly colonized in foregut region rather than in the hindgut whereas, cellulase and protease producing strains were highly colonized in the hindgut region rather than in the foregut region in case of all the seven species of fishes studied. Maximum numbers of amylase producing strains (12.2×10^3) were isolated from foregut region of *Labeo calbasu* whereas, maximum number of cellulase (7.2×10^3) and protease (13.4×10^4) strains were detected in the hind gut region of *Labeo bata.*

An understanding of the contribution of endosymbionts to digestion requires information on the relative importance of exogenous (produced by gastrointestinal endosymbionts) and endogenous (produced by the host) digestive enzymes (Clements 1997). In the present study, some selected strains isolated from both foregut and

Table 4

Bacterial strains isolated from fish gut and qualitative extracellular enzyme activity

Fish species	Bacterial strains	Amylase activity	Cellulase activity	Protease activity
Labeo rohita	RH2	+	+	+
	RF3	+	+	+
	RH5	+	+	+
Catla catla	KH2	+	+	+
	KF1	+	+	+
	KH3	+	+	+
	KF2	+	+	+
Cirrhinus mrigala	MH5	+	+	+
	MF2	+	+	+
	MH3	+	+	+
	MF4	+	+	+
Labeo bata	BH4	+++	++++	+++
	BF2	+	++	+
	BH6	++	+++	++
	BF3	+	+	+
Labeo calbasu	CH22	+	++	++++
	CF8	+	+	++
	CH13	+	+	++
	CF6	+	+	+
	CF5	++++	+++	++
	CH7	+	+	+
	CF3	+++	++	+
	CH8	+	+	+
Oreochromis niloticus	NH5	+	+++	++
	NF2	++	++	+
	NH4	+	++	++
	NF3	+	+	+
Anabas testudineus	TH1	—	—	+++
	TF1	—	—	+
	TH2	—	—	++
	TF2	—	—	+

++++, very high; +++, high; ++, moderate; +, low; —, nil.

hindgut regions were quantitatively assayed for cellulase, amylase and protease activities to ascertain their role in exogenous production of digestive enzymes.

The most important group of exogenous enzymes in symbioses between terrestrial vertebrate herbivores and microorganisms are cellulases, which degrade the cell walls of vascular plants (Clements 1997). A number of studies have examined cellulase activity in the alimentary tracts of fishes, with mixed results. Much of the controversy concerning the source of cellulase activity in the intestinal tract of fish has arisen due to the inability to isolate cellulase-producing microorganisms from the intestinal contents and to document diet-related fluctuations in the level of cellulase activity (Luczkovich and Stellwag 1993). Two explanations have been proposed to account for the presence of cellulase in the digestive tracts of fish. The first one suggests that intestinal tract-associated cellulase is produced by an endosymbiotic microbial flora resident in the intestinal tract. This hypothesis is supported by the fact that no vertebrate has been shown to produce endogenous cellulase (Yokoe and Yasumasu 1964, Barnard 1973). The presence of cellulolytic bacteria in the digestive tracts of fish and inhibition of cellulase production after antibiotic treatment (Stickney and Shumway 1974, Saha and Ray 1998, Bairagi et al. 2002, Saha et al. 2006) also confirm the presence of endosymbiotic cellulolytic bacterial flora in fish gut. The results of the presently reported study indicated that carboxymethylcellulolytic bacteria exist in the digestive tracts of fish and support the hypothesis that bacteria contribute to the exogenous production of cellulase in fish. The presence of considerable population of cellulolytic bacteria and their active role in extracellular cellulase production in fish has also been confirmed in a number of investigations (Lésel et al. 1986, Das and Tripathi 1991, Saha and Ray, 1998, Bairagi et al. 2002, Saha et al. 2006). The second explanation regarding the presence

of cellulase activity in the digestive tracts of fish is that cellulase may be derived from ingestion of plant detritus. In six cyprinid and salmonid species, Prejs and Blaszczyk (1977) showed that the activity of cellulase was correlated positively with the amount of dead plant materials, presumably detritus, present in the digestive contents. The

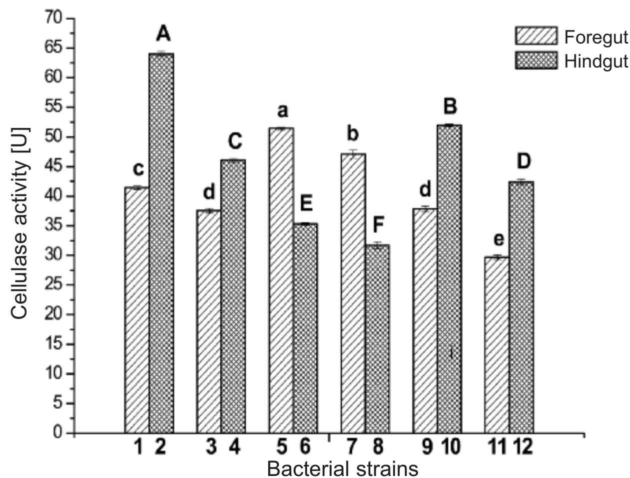

Fig. 1. Cellulase activity (U = μg glucose liberated · mL^{-1} of enzyme extract per min) in different strains (1, BF2; 2, BH4; 3, BF3; 4, BH6; 5, CF5; 6, CH7; 7, CF9; 8, CH8; 9, NF2; 10, NH5; 11, NF3; 12, NH7) isolated from the foregut and hindgut of bata, orange-fin labeo, and Nile tilapia; error bar showing standard deviation among three replicates; means with different letters are significantly different ($P < 0.05$)

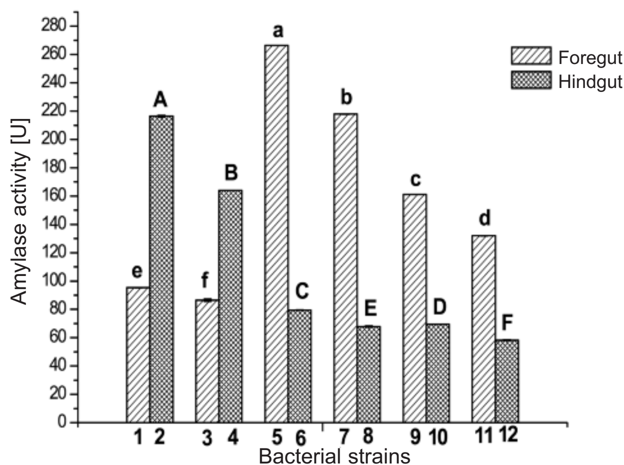

Fig. 2. Amylase activity (U = μg maltose liberated · mL^{-1} of enzyme extract per min) in different strains (1, BF2; 2, BH4; 3, BF3; 4, BH6; 5, CF5; 6, CH7; 7, CF9; 8, CH8; 9, NF2; 10, NH5; 11, NF3; 12, NH7) isolated from the foregut and hindgut of bata, orange-fin labeo, and Nile tilapia; error bar showing standard deviation among three replicates; means with different letters are significantly different ($P < 0.05$)

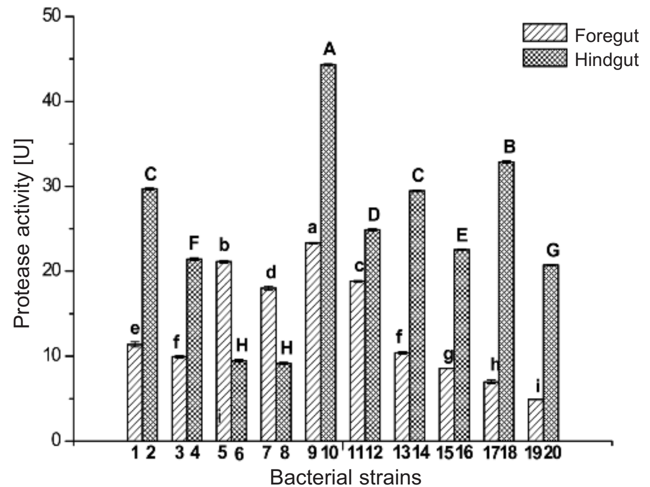

Fig. 3. Protease activity (U = μg tyrosine liberated · mL^{-1}l of enzyme extract per min) in the different strains (1, BF2; 2, BH4; 3, BF3; 4, BH6; 5, CF5; 6, CH7; 7, CF9; 8, CH8; 9, CF2; 10, CH22; 11, CF6; 12, CH23; 13, NF2; 14, NH5; 15, NF3; 16, NH7; 17, TF1; 18, TH1; 19, TF2; 20, TH2) isolated from the foregut and hindgut of bata, orange-fin labeo, Nile tilapia, and climbing perch; error bars showing standard deviation among three replicates; means with different letters are significantly different ($P < 0.05$)

authors suggested that the detritus was colonized intensively by bacteria before ingestion, which implied that the detritus-colonizing bacteria were responsible for the cellulase activity detected in the gut contents. In the present study, cellulolytic bacterial flora was not detected in the digestive tracts of the carnivorous climbing perch, *Anabas testudineus*. Bairagi et al. (2002) also did not detect any cellulolytic bacteria in the gut of a carnivorous catfish, *Clarias batrachus* and murrel, *Channa punctatus*. But the fish with herbivorous and omnivorous feeding habits exhibited significant cellulolytic bacterial flora. Therefore, plant- and detritus-associated cellulase source in fish digestive tracts also cannot be ruled out. Shcherbina and Kazlauskiene (1971) suggested that an endogenous cellulase is secreted in the anterior portion of the digestive tract of carp, while the remaining cellulose absorption takes place in the posterior portion of the digestive tract, indicating the presence of microbial cellulase in this region. Our observation is in agreement with this probable microbial cellulolytic action since more pronounced microbial cellulase activity was recorded in the bacterial strains isolated from the hindgut regions of *Labeo bata* (strains BH4 and BH6) and *Oreochromis niloticus* (strains NH5 and NH7). On the contrary, the strains isolated from the foregut region of *Labeo calbasu* (strains CH7 and CH8) exhibited higher cellulolytic activity.

The activity of carbohydrases in general, and of amylase in particular, differs from species to speies, and appears to be related to their feeding habits (De Silva and Anderson 1995). Amylase is secreted by the entire intestine in Indian major carps, *Catla catla*, *Labeo rohita*, and

Cirrhinus mrigala, and its activity is high towards the proximal end (Dhage 1968). Although reports on microbial amylase activity in fish gut are scanty, endogenous amylase activity in fish is evident. 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. In the present investigation, a considerable population of amylolytic bacteria was detected in the fish species with herbivorous and omnivorous feeding habits. Amylolytic bacteria could not be detected in the carnivorous climbing perch. Similar observation was also made by Bairagi et al. (2002), who also could not detect any amylolytic bacteria in the gut of carnivorous catfish and murrel. In contrast to the cellulase activity, exogenous amylase production was intense in the foregut region of the fish species studied except in *Labeo bata*, where the hindgut exhibited higher exogenous amylase activity. Das and Tripathi (1991) are of opinion that there is a possibility of introduction of these enzyme-producing microflora in fish digestive tracts along with the food ingested, but, whether they form a persisent population in the gut is doubtful. Since, the amylolytic bacteria have been detected in fish guts after 36 h of starvation in our study, it seems that some of the flora forms a persistent population.

Although fish have an endogenous source of protease in their digestive tracts, not much attention has been paid to the microbial source of protease in fish. Ghosh et al. (2002) suggested from their in vitro studies on enzyme-producing bacterial flora that *Bacillus circulans*, *B. pumilus*, and *B. cereus*, isolated from the alimentary canal of *Labeo rohita* fingerlings were good producers of proteolytic enzymes, though they did not quantify the enzyme activity. 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*. In the present investigation, proteolytic bacteria were detected in the gut of all the fish examined and maximum density of proteolytic bacterial population was observed in the hindgut region of *Labeo bata* (13.40×10^4 CFU · g^{-1} intestinal tissue). However, assay of extracellular protease activity of the bacterial isolates showed highest value in CH22, a strain isolated from the hindgut region of *Labeo calbasu*. In general, the strains isolated from the hindgut region of the fish exhibited higher proteolytic activity.

CONCLUSION

The results of the present study indicate that a diverse bacterial flora exists in the gastrointestinal tracts of freshwater teleosts, which are highly colonized in the hindgut rather than in the foregut region. There is also a distinct microbial source of digestive enzymes (cellulase, amylase, and protease) apart from the endogenous sources in fish gastrointestinal tracts. The presence of a diet-dependent microbial population is also evident in the present investigation. The results also present a scope for fish nutritionists to utilize the enzyme-producing bacterial isolates as a probiotic in formulating cost-effective aquafeeds, especially for the larval stages when the enzyme system is not efficient. However, further investigations are required to know about the metabolic pathways used by these microorganisms in the alimentary tracts of fish.

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REFERENCES

Austin B., Al-Zahrani A.M. 1988. The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Biology **33**: 1–14.

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

Barnard E.A. 1973. Comparative biochemistry and physiology of digestion. Pp. 133–164. *In*: Prosser C.L. (ed.) Comparative Animal Physiology. Academic Press, New York.

Bergman E.N. 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Review **70**: 567–590.

Bernfeld P. 1955. Amylase [alpha] and [beta]. Pp. 149–150. *In*: Kolowick S.P., Kaplan N.O. (eds.) Methods in enzymology. Vol. 1. Academic Press, New York.

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

Clements K.D. 1991. Endosymbiotic communities of two herbivorous labroid fishes, *Odax cyanomelas* and *O. pullus*. Marine Biology **109**: 223–229.

Clements K.D. 1997. Fermentation and gastrointestinal microorganisms in fishes. Pp. 156–198. *In*: Mackie R.I., White B.A. (eds.) Gastrointestinal microbiology. Vol. 1. Gastrointestinal Ecosystems and fermentations. Chapman & Hall, New York.

Clements K.D., Choat J.H. 1995. Fermentation in tropical marine herbivorous fishes. Physiology and Zoology **68**: 355–378.

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

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

De Silva S.S., Anderson T.A. 1995. Fish Nutrition in Aquaculture. Chapman & Hall, London.

Dhage K.P. 1968. Studies on the digestive enzymes in the three species of the major carps of India. Journal of Biological Sciences **11**: 63–74.

Duncan D.B. 1955. Multiple range and multiple *F*-tests. Biometrics **11**: 1–42.

Finegold S.M., Sutter V.L., Mathisen G.E. 1983. Normal indigenous intestinal flora. Pp. 3–31. *In*: Hentgens D.J (ed.) Human intestinal microflora in health and disease. Academic Press, New York.

Ghosh K., Sen S.K., Ray A.K. 2002. Characterization of bacilli isolated from gut of rohu, *Labeo rohita*, fingerlings and its significance in digestion. Journal of Applied Aquaculture **12**: 33–42.

Hansen G.H., Strøm E., Olafsen J.A. 1992. Effect of different holding regimes on the intestinal microflora of herring (*Clupea harengus*) larvae. Applied Environmental Microbiology **58**: 461–470.

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

Jacob M.B., Gerstein M.J. 1960. Handbook of Microbiology. D. Van Nostrand Co. Inc., Princeton, New Jersey.

Kamei Y., Sakata T., Kakimoto D. 1985. Microflora in the alimentary tract of Tilapia: characterization and distribution of anaerobic bacteria. Journal of General and Applied Microbiology **31**: 115–124.

Lésel R. 1991. Does a digestive active bacterial flora exist in fish? Pp. 655–664. *In*: Kaushik S.J., Luquet P. (eds.) Fish Nutrition in Practice. IVth International Symposium on Fish Nutrition and Feeding, Biarritz (France), 1993. INRA, Paris.

Lésel R., Fromageot C., Lésel M. 1986. Cellulose digestibility in grass carp, *Ctenopharyngodon idella* and in goldfish, *Carassius auratus*. Aquaculture **54**: 11–17.

Lindsay G.J.H., Harris J.E. 1980. Carboxymethylcellulase activity in the digestive tracts of fish. Journal of Fish Biology **16**: 219–233.

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

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

Prejs A., Blaszczyk M. 1977. Relationships between food and cellulase activity in freshwater fishes. Journal of Fish Biology **11**: 447–452.

Rahmatullah S.M., 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.

Rimmer D.W., Wiebe W.J. 1987. Fermentative microbial digestion in herbivorous fishes. Journal of fish Biology **31**: 229–236.

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.). Canadian Journal of Microbiology **39**: 1169–1173.

Ringø E., Strøm E. 1994. Microflora of Arctic charr, *Salvelinus alpinus* (L.). I. The gastrointestinal microflora of free-living fish and the effect of diet and salinity on intestinal microflora. Aquaculture and Fisheries Management **25**: 623–629.

Saha A., Ray A.K. 1998. Cellulase activity in rohu fingerlings. Aquaculture International **6**: 281–291.

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

Sakata T. 1990. Microflora in the digestive tract of fish and shell-fish. Pp. 171–176. *In*: Lésel R. (ed.) Microbiology in poecilotherms [sic]. Elsevier Science, Amsterdam.

Savage D.C. 1977. Interactions between the hosts and its microbes. Pp. 277–310. *In*: Clarke R.T.J., Bauchop T. (eds.) Microbial ecology of the gut. Academic Press, London.

Shcherbina M.A., Kazlauskiene O.P. 1971. The reaction of the medium and the rate of absorption of nutrients in the intestine of carp. Journal of Ichthyology **11**: 81–85.

Stevens C.E. 1988. Comparative physiology of the vertebrate digestive system. Cambridge University Press, New York.

Stickney R.R., Shumway S.E. 1974. Occurrence of cellulase activity in the stomachs of fish. Journal of Fish Biology **6**: 779–790.

Strøm E., Olafsen J.A. 1990. The indigenous microflora of wild-captured juvenile cod in net-pen rearing. Pp. 181–185. *In*: Lésel R. (ed.) Microbiology in poecilotherms [sic]. Elsevier Science, Amsterdam.

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

Trust T.J., Bull L. M., Currie B. R., Buckley J.T. 1979. Obligate anaerobic bacteria in the gastrointestinal microflora of the grass carp (*Ctenopharyngodon idella*), goldfish (*Carassius auratus*), and rainbow trout (*Salmo gairdneri*). Journal of Fisheries Research Board of Canada **36**: 1174–1179.

Trust T.J., Sparrow R.A.H. 1974. The bacterial flora in the alimentary tract of freshwater salmonid fishes. Canadian Journal of Microbiology **20**: 1219–1228.

Walter H.E. 1984. Methods of enzymatic analysis. Verlag Chemie, Weinheim.

Westerdahl A., Olsson J.C., Kjelleberg S., Conway P.L. 1991. Isolation and characterization of turbot (*Scophthalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology **57**: 2223–2228.

Yokoe Y., Yasumasu I. 1964. The distribution of cellulase in invertebrates. Comparative Biochemistry and Physiology **13**: 223–238.

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