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Isolation and Characterization of Extracellular Enzyme Producing Bacilli in the Digestive Tracts of Rohu, *Labeo rohita* (Hamilton) and Murrel, *Channa punctatus* (Bloch)

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Abstract

Isolation of some extracellular enzyme producing aerobic bacteria in the digestive tracts of rohu (*Labeo rohita*, Cyprinidae) and murrel (*Channa punctatus*, Channidae) was carried out. Gut bacteria were isolated on tryptone soya agar (TSA) plates. The isolated strains were qualitatively screened based on their extracellular protease, amylase and cellulase producing ability using selective media. Among the strains isolated, LrB1 and CpB2 isolated from *L. rohita* and *C. punctatus*, respectively, seemed to be the superior strains. The strains were characterized based on morphological, physiological and biochemical characterization and identified as *Bacillus subtilis* (LrB1) and *Bacillus cereus* (CpB2). They tolerated a wide range of temperature (15-55°C), pH (5-11) and NaCl (1-25%). Biochemical characterization suggests that the isolates are capable of hydrolyzing proteins and carbohydrates indicating their importance in fish nutrition. The information generated from the present investigation could contribute towards the utilization of such autochthonous enzyme-producing bacteria for nutrition in fish with better efficiency.

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Introduction

Fish are continuously exposed to microorganisms present in the aquatic environment. Being rich in nutrients, the digestive tract of fish confers a favourable growth environment for microorganisms. The intestinal tract of fish is generally colonized by a good number of heterotrophic bacteria. Ecological studies on the gut microbiota of fish have been presented by several authors (Horsley 1977; Cahill 1990). However, the characteristics and biological significance of the microbiota in the intestine of fish are not well known. It seems logical that the gut bacteria have a role in nutrition, growth and disease susceptibility in fish as it has been established for homeothermic species (Floch et al. 1970). An understanding of the indigenous microbiota in fish may help to improve feeding and other conditions for the intensive rearing of fish.

In all higher vertebrates, digestion of food material occurs through the dual action of the enzyme system of the host itself and also contributed by the intestinal microbiota. There is paucity of information in the field of enzyme producing bacteria in the fish gastrointestinal (GI) tract (Bairagi et al. 2002; Ghosh et al. 2002; Saha et al. 2006). Information on proper characterization of the intestinal bacteria having digestive enzyme producing capacity will help to determine their probable function in fish nutrition. Therefore, an attempt has been made in the present investigation to isolate enzyme-producing bacteria from the GI tracts of two freshwater teleosts having different feeding habits, namely rohu (*Labeo rohita*, Cyprinidae) and murrel (*Channa punctatus*, Channidae). The isolated bacterial strains were assessed for extracellular enzyme production. Finally efforts have been made to characterize two potent enzyme-producing strains through morphological, physiological and biochemical tests to determine their probable role within the fish GI tracts.

Materials and Methods

Test fish

Herbivorous Indian major carp, rohu, *L. rohita* and carnivorous murrel, *C. punctatus* were evaluated for the present study. Both species were collected from local ponds.

Isolation and culture of gut bacterial flora

To isolate autochthonous bacteria, test fish (average weight 68 ± 2.16 g for rohu and 87.6 ± 3.65 g for murrel) were starved for 24 hrs to clear the GI tract before being killed. Five fish of each species were used for the isolation of gut bacteria. Bacteria were isolated from the individual fish separately. Before dissection, the ventral surfaces of each fish were thoroughly scrubbed with 1% iodine solution (Trust & Sparrow 1974). Standard aseptic procedures were used. The GI tracts were removed, cut into pieces and homogenized with sterilized 0.89% NaCl solution (10:1; volume:weight) (Das & Tripathi 1991).

The homogenates of the GI tract of each test fish was used after 10 serial 1:10 dilutions (Beveridge et al. 1991). To isolate the heterotrophic bacterial population, diluted samples (0.1 ml) were poured aseptically on sterilized tryptone soya agar (TSA) (Himedia Laboratories Pvt., Mumbai, India) plates in duplicate within a laminar airflow and incubated overnight at 37°C. The well-separated colonies that apparently have different morphological appearance (e.g. colony shape, color, elevation) were isolated and streaked separately on TSA plates to obtain pure cultures. Altogether nine single isolated colonies from the streaked plates were transferred to TSA slants as pure culture and maintained at 4°C in the refrigerator.

Screening of isolates for extra-cellular enzyme production

Screening of isolates was done based on their qualitative extracellular amylase, cellulase and protease production on agar plates with selective media. Spot inoculation of the isolates was given on starch (1%) supplemented nutrient agar plates (SA) and incubated at 37°C for 24 h. The culture plates were then flooded with 1% Lugol's iodine solution for the development of clear zone (halo) to identify amylase activity (Jacob & Gerstein 1960). Similarly, for extra-cellular protease production, the isolates were inoculated on peptone-gelatin enriched nutrient agar (4% gelatin) plates (PG) and incubated at 37°C for 18 h. The appearance of halo around the colony after flooding the plate with 15% $HgCI_2$ indicated the presence of proteolytic activity (Jacob & Gerstein 1960). To determine cellulase production, isolates were grown on carboxymethylcellulose agar plates (CMC) at 37°C for 36 hrs. Appearance of halo after flooding the plates with congo red dye prepared in 0.7% agarose indicated utilization of cellulose as sole carbon source and thereby capacity of cellulase production (Teather & Wood 1982).

Morphological, physiological and biochemical characterization

Colony morphology was studied visually. Gram staining procedure was performed to determine the staining property of the isolates. Endospore forming capacity was determined by staining with 5% aqueous malachite green solution. Culture characteristics were observed in broth and motility was observed under phase contrast microscope.

Growth of the bacterial isolates was studied at different temperatures, starting from 5 to 70°C (at an interval of 5°C) and pH (3-11) (Table 1). Sodium chloride tolerance was determined by supplementing NaCl at different concentrations (1-25%) in nutrient broth medium. Growth of the isolates was studied after 24 hrs of incubation at 37°C.

Table 1. Physiological characteristics of LrB1 (*Bacillus subtilis*) and CpB2 (*Bacillus cereus*).

Parameters	Characteristics	
	LrB1	CpB2
Growth temperatures		
5°C	-	-
10°C	=	-
15°C	+	=
20°-50°C	+	+
55°C	+	=
60°C	+	-
65°C	=	-
70°C	-	-
Growth at pH		
pH 3	-	-
pH 5	+	=
pH 6-10	+	+
pH 11	=	=
NaCl Tolerance (%)		
1 – 4	=	=
5 –15%	+	+
20%	=	=
25%	-	-

+ (Good); = (Moderate); - (Not detected)

Catalase production was tested by pouring aqueous solution of H_2O_2 (10% v/v) over a 24 h old nutrient agar slope culture. Nitrate reduction was determined in Davis & Mingioli (1950) broth medium supplemented with KNO_3 (0.2% w/v) as nitrogen source (instead of $(NH_4)_2SO_4$) after 24 h at 37°C. The H_2S production was detected by inserting lead acetate paper strips into the neck of the culture tube containing nutrient broth medium. Presence of cytochrome oxidase was detected from 18 h old

nutrient broth culture. Appearance of blue color with the mixture of *p*-amino-dimethylaniline oxalate (1%) and α-napthol (1%) indicated a positive test. Indole production was determined in Davis & Mingioli (1950) medium supplemented with 0.1% casein hydrolysate. The appearance of a red color with the introduction of Kovac's reagent indicated the presence of indole. Bacterial growth in synthetic citrate medium indicated the utilization of citrate as sole carbon source. The inoculated Gelatin-agar medium was incubated for 24 hours and the tubes were then chilled to determine liquefaction of the gelatin. Hydrolysis of casein was detected on nutrient agar plates supplemented with casein (1%). Bacteria cultures were streaked on urea-agar plates and incubated for 48 hours for detection of urease activity. Hydrolysis of urea with the liberation of ammonia was detected by the rise of pH value indicated by a color change of the indicator (phenol red) from yellow to red. Carbohydrate utililization tests were performed by using Hicarbonate test kit (Himedia Laboratories Pvt., Mumbai, India, code KB009) comprising of 36 carbohydrate sources.

All these and other biochemical tests (e.g. tyrosine clearing, DNase test, lysine decarboxylation, ornithine decarboxylation, phenylalanine deamination, starch hydrolysis) were performed following prescribed methods (Society of American Microbiologists 1957; Skerman 1959; Aaranson 1970).

Table 2 represents the compositions of the media used in the present study.

Statistical analysis

Statistical analysis of data regarding enzyme production by the intestinal isolates (expressed as diameter of halo on culture plates) was made by analysis of variance (ANOVA) followed by Scheffe's F-test for multiple comparison (Das & Das 1993).

Results

Four strains of bacteria from *L. rohita* and five from *C. punctatus* were isolated and maintained as pure culture. The strains were found to produce extracellular enzymes such as protease, amylase and cellulase (Table 3), although significant variation in the intensity of enzyme production was observed. Extracellular protease and cellulase productions were recorded from almost all strains.

Table 2. Compositions of the media used for the study.

Media used*	Composition (g•L^{-1})	
TSA	Pancreatic digest of casein	15
	Papaic digest of soya bean meal	5
	NaCl	5
	Agar	20
CMC	Beef extract	5
	Peptone	5
	NaCl	5
	Carboxymethyl cellulose	2
	Agar	20
SA	Beef extract	5
	Peptone	5
	NaCl	5
	Starch	2
	Agar	20
PG	Beef extract	3
	Peptone	5
	Gelatin	4
	Agar	20
Nutrient Agar	Beef extract	5
	Peptone	5
	NaCl	5
	Agar	20
Davis & Mingioli medium	K_2HPO_4	7
	KH_2PO_4	3
	$(NH_4)_2SO_4$	1
	$MgSO_4.7H_2O$	0.1
	Na-Citrate	0.5
	Glucose	10
	Agar	20
Urea agar medium	NaCl	5
	K_2HPO_4	1
	Peptone	1
	Glucose	1
	Phenol red	0.012
	Agar	20
Citrate medium	NaCl	5
	$NH_4H_2PO_4$	1
	$MgSO_4,7H_2O$	10.2
	K_2HPO_4	1
	Agar	20

* pH for the media used was adjusted to 7 in all cases.

Table 3. Qualitative extracellular enzyme producing ability of the intestinal isolates. Results are mean ± SE of three determinations.

Fish species	Bacterial strains	Enzyme activity* (Diameter of halo in mm)		
		Protease[1]	Amylase[2]	Cellulase[3]
Labeo rohita	LrA1	22.83 ± 0.76^{c}	15.43 ± 0.60^{d}	8.90 ± 0.36^{b}
	LrA2	25.23 ± 0.87^{d}	7.77 ± 0.25^{b}	12.66 ± 0.57^{c}
	LrB1	28.83 ± 1.26^{e}	22.53 ± 0.61^{e}	24.40 ± 0.53^{f}
	LrB2	20.66 ± 0.57^{c}	13.57 ± 0.60^{cd}	22.50 ± 0.50^{ef}
Channa punctatus	CpA1	8.80 ± 0.34^{a}	-	9.07 ± 0.12^{b}
	CpA2	8.77 ± 0.40^{a}	-	6.00 ± 0.20^{a}
	CpB1	15.50 ± 0.70^{b}	-	15.83 ± 0.28^{d}
	CpB2	23.10 ± 0.65^{cd}	5.87 ± 0.23^{a}	33.87 ± 1.03^{g}
	CpB3	21.17 ± 1.04^{c}	-	12.90 ± 0.36^{c}

*With pure culture of the isolates. - Not detected. Values with the same superscript in the same column are not significantly different ($P<0.05$) from each other.
[1]On PG plate flooded with 15% $HgCl_2$ solution; [2]On SA plate flooded with Lugol's iodine solution; [3]On CMC plate flooded with congo red dye.

However, amylase production by the intestinal isolates from *C. punctatus* remained poorly detected. Protease producing ability of LrB1 isolated from *L. rohita* was found to be greatest and was followed by the other strains from *L. rohita*, LrA2 and LrA1, respectively. The strain LrB1 also recorded for best amylase and moderately good cellulase production. The highest value for cellulase production was obtained with the isolate from *C. punctatus*, CpB2 and was followed by LrB1 and LrB2 respectively. Comparing intensities of protease, amylase and cellulase production by the intestinal isolates of both the fish species, strains LrB1 and CpB2 seemed to be superior bacterial strains. Therefore, these potent strains were selected for further characterization and identification.

On the basis of major morphological, physiological and biochemical characters (Tables 1, 4, 5) the genus of the isolates LrB1 and CpB2 has been identified as *Bacillus* following Bergey's Manual of Systematic Bacteriology (Williams et al. 1986). Organisms were aerobic and capable of endospore formation, one in each cell. The spores were terminal or subterminal in position having elliptical shape and sporangium like vesicles. Both the strains were rod shaped, short or long chain forming gram positive, motile and capable of forming moderate to large round, convex, wavy, opaque colonies (Table 4). Both were capable of utilizing citrate, casein, starch and gelatin, and were positive in ornithine decarboxylation and urease production.

Both the strains effectively utilized carbohydrates such as lactose, maltose, fructose, dextrose, galactose, sucrose, mannose, ribose, cellobiose,

Table 4. Morphological characteristics of LrB1 (*Bacillus subtilis*) and CpB2 (*Bacillus cereus*).

Parameters	Characteristics	
	LrB1	CpB2
Colony morphology		
Shape	Round	Round
Elevation	Convex	Convex
Surface	Rough	Smooth
Pigment	Blackish	Yellowish green
Gram's reaction	+	+
Shape	Rod shaped & chain former	Rod shaped & chain former
Size	Long	Moderate
Spore		
Endospore	+	+
Shape, position	Terminal & elliptical	Subterminal & ellipsoidal

+ (Positive)

Table 5. Biochemical characteristics of LrB1 (*Bacillus subtilis*) and CpB2 (*Bacillus cereus*).

Tests	Characteristics	
	LrB1	CpB2
Catalase test	+	+
Indole production	-	-
Tyrosine clearing	+	-
DNase test	+	+
Citrate utilization	+	+
Lysine decarboxylation	-	-
Ornithine decarboxylation	+	+
Urease	+	+
Phenyl alanine deamination	-	-
Cytochrome oxidase test	+	-
Nitrate reduction	-	-
H_2S production	-	-
Gelatin liquefaction	+	+
Starch hydrolysis	+	+
Casein hydrolysis	+	+
Carbohydrate utilization		
Glucose, Adonitol, Lactose, Maltose, Fructose, Dextrose, Galactose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Inulin, Sodium gluconate, Cellulose, Glucosamine, Sorbitol, Mannitol, ∝-Methyl-D-glucoside, Ribose, Rhamnose, Cellobiose, Melezitol, ∝-Methyl-D-mannoside, Xylitol, D-Arabinose, Citrate, Malonate, Sorbose	+	+
Xylose, Raffinose, Glycerol, Salicin, Esculin	+	-
Dulcitol, Inositol	-	+

+ (Positive); - (Not detected)

etc. The strains were catalase positive. They tolerated a wide range of temperature (15-55°C), pH (5-11) and NaCl concentration (1-20%).

In contrast to these common characteristics the isolates differed in many others. The isolate LrB1 produced diffusible blackish pigment in nutrient agar medium and formed a short chain of 2-4 cells. The cells were 8.5 - 8.85 µm × 1.5 - 1.9 µm in size. Isolate LrB1 formed a colony with rough texture. It showed positive citrate utilization, negative in phenylalanine deamination, nitrate reduction, and H_2S production. The isolate was positive in utilization of xylose, raffinose, glycerol, salicin and esculin. Hence, it was similar to the species *B. subtilis.*

On the other hand isolate CpB2 showed poor growth at 15 and 55°C and growth was not detected at 10 and 60°C. The cells were 4.5 - 5 µm × 1.5 - 1.9 µm in size. The isolate CpB2 showed yellowish green diffusible pigment and appeared as a chain of variable length (2-16 cells). Smooth textured colonies were formed. In addition the isolate was positive to citrate utilization, casein and starch hydrolysis, gelatin liquefaction and utilized carbohydrates such as dulcitol, inositol. Hence, it was similar to the species *B. cereus*.

Discussion

There is a considerable body of evidence regarding the endogenous digestive enzymes in fish. However, information regarding the enzyme producing intestinal bacteria and their significance in fish is rare. The intestinal microbiota in fish has been classified as indigenous when it is able to colonize the gut ecosystem, or as transient when it only passes through the digestive tract without colonizing (Ringø et al. 2003). In the present study the test fish were starved for 24 hours before preparation of inoculum from their digestive tracts for isolation of autochthonous intestinal bacteria. Thus, it seems that bacterial population detected in the present study forms a natural and persistent population (indigenous) in their digestive tracts (Bairagi et al. 2002).

Bacterial strains isolated in the present study demonstrated their ability for extracellular protease, amylase and cellulase production. Protease and cellulase activities were exhibited by all bacterial isolates, while amylase production remained poorly detected by the strains isolated from the murrel. Microbial amylase activity in the fish gut has been documented

in a few studies (Sugita et al. 1997; Ghosh et al. 2002). Occurrence of amylolytic and proteolytic bacteria in the gut of gold fish was reported by Lesel et al. (1986). Proteolytic, cellulolytic and amylolytic bacteria in the gut of *L. rohita* have been documented by Ghosh et al. (2002) indicating their omnivorous feeding habit. Microbial cellulase production in the digestive tract of carp was reported by Shcherbina & Kazlawlene (1971). Later, abundance of cellulolytic bacteria has been documented in the GI tracts of grass carp (Lesel et al. 1986; Das & Tripathi 1991; Saha et al. 2006), rohu fingerlings (Saha & Ray 1998; Ghosh et al. 2002) and tilapia (Saha et al. 2006). Bairagi et al. (2002) failed to detect cellulolytic bacteria in the GI tract of carnivorous catfish and murrels. However, the result of the present investigation showed the presence of cellulolytic bacteria in carnivorous murrel. Stickney & Shumway (1974) opined that omnivores and carnivores might pick up cellulolytic flora from the invertebrates that harbour the bacteria, which may explain the prevalence of the cellulolytic bacteria within the GI tract of murrel in the present study. Assay of extracellular enzyme production showed the highest value for protease and amylase production in LrB1 and for cellulase production in CpB2. Ghosh et al. (2002) reported very good protease production and moderate cellulase production in the intestinal isolates from rohu. Bairagi et al. (2002), however, reported poor protease and amylase production in the isolates from rohu and murrels, respectively.

Physiological and biochemical characterization of the intestinal isolates are important in elucidating their functions in the GI tract. In the present investigation, two potent strains LrB1 and CpB2, isolated from the gut *L. rohita* and *C. punctatus* respectively, have been characterized and identified. Although the digestive tract of the endotherm is colonized mainly by obligate anaerobes (Finegold et al. 1983), the predominant bacterial species observed in most of the fish digestive tracts have been reported to be aerobes or facultative anaerobes (Trust & Sparrow 1974; Sakata 1990; Bairagi et al. 2002; Saha et al. 2006). This may be due to the fact that a comprehensive number of studies evaluated aerobes and facultative anaerobes in the GI tracts of fish. In the present study, the strains isolated from the gut of both fish were identified as aerobic gram positive Bacilli. The selected strains LrB1 and CpB2 were identified as *B. subtilis* and *B. cereus* respectively on the basis of morphological, physiological and biochemical characteristics described in the Bergey's Manual of Systematic Bacteriology (Williams et al. 1986). Ghosh et al. (2002) isolated three strains of Bacilli, Lr1.1, Lr1.2 and Lr2.2 from the gut of Indian major carp rohu, *Labeo rohita* and identified them as *B. circulans*, *B. pumilus* and *B.*

cereus respectively, on the basis of morphological, physiological and biochemical characteristics. Saha et al. (2006) also isolated Bacilli from the alimentary tracts of Chinese grass carp, *Ctenopharyngodon idella* and tilapia, *Oreochromis mossambica* and identified them as *B. megaterium* (CI3) and *B. circulans* (TM1), respectively, in the same way.

The major biochemical activity of the heterotrophic bacteria is the dissimilation of organic matter. In the present investigation biochemical characterization indicated that both the isolated organisms were capable of hydrolyzing proteins such as casein and gelatin. In addition, the strains were able to utilize a wide variety of carbohydrates including cellulose. Enzymes produced by intestinal fish microbiota might have a significant role in digestion, especially for substrates such as cellulose, which few animals can digest, and also for other substrates (Smith 1989). Luczkovich & Stellwag (1993) opined that the gastrointestinal microbiota of pinfish (*Lagodon rhomboides*) might contribute to the breakdown of plant materials. Recent observations have documented that fish harbour proteolytic, amylolytic and cellulolytic bacteria in their digestive tracts (Bairagi et al. 2002; Ghosh et al. 2002; Saha et al. 2006), which is in agreement with the present study.

In addition to utilizing various proteins and carbohydrates, both the strains were ornithine decaroboxylation and urease positive. The enzyme ornithine decarboxylase has been reported to catalyse for synthesis of polyamines such as spermine and spermidine, which are used in DNA packaging and required in large amounts in rapidly dividing cells (Lehninger et al. 1993). The ability to decarboxylate ornithine for the intestinal isolates may be indicative of their growth and colonization potential in the GI tract. In general, urease catalyzes hydrolysis of urea into carbon dioxide and ammonia, thereby raising the pH of the media. The urease positive nature of the isolated strains could be helpful in maintaining a neutral or alkaline environment in the GI tract to facilitate microbial growth.

The bacteria ingested by fish in its diet may become adapted to the GI tract to form a mutual association. In the present investigation characterization of isolated bacteria revealed that they could grow within a wide range of temperature (10–65°C) and pH (5-11). This capacity probably enabled these isolates to adapt themselves within the gastrointestinal micro-environment of fish, which are poikilotherms. The range of temperature is relatively narrow (15C-55°C) for the strain CpB2 isolated from the gut of *C. punctatus*.

Conclusion

Earlier investigations have suggested that microorganisms have a beneficial effect in the digestive processes of fish (Ringø et al. 1995). Characterization of the microbial populations in the intestinal microenvironment of fish and understanding the physiological interactions between the indigenous microbiota and the host may have important implications (Silva et al. 2005). The present study 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 feedstuffs. Therefore, cellulase and amylase activities by the gut bacteria may indicate their ability to aid in the digestion of plant feedstuffs. The use of such beneficial bacteria as probiotics has a long tradition in animal husbandry (Stavric & Kornegay 1995). These 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 fish GI tract at a higher degree. The enzyme-producing gut bacteria characterized in the present study may be used beneficially for fish especially in the larval stages. However, further research involving such beneficial bacteria should be conducted for evaluating their efficacy under actual farm conditions to explore their full potential in the field of aquaculture.

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