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Reestablishment of Cellulase-Producing Bacteria in the Intestine of Grass Carp (*Ctenopharyngodon idella*)

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ABSTRACT

Cellulase-producing aeorobic bacterial flora in the intestine of phytophagous Chinese grass carp (*Ctenopharyngodon idella*) were isolated and characterized using selective carboxymethylcellulose agar (CMC-agar) medium. Bacteria were identified and characterized by conventional, biochemical and API kits. Out of 48 bacteria isolated, 26 were cellulase-positive. β-glucosidase activity was measured to discover potential cellulase-producing strains. *Bacillus*, *Erwinia* and *Actinobacillus* species were good β-glucosidase producers (0.186, 0.181 and 0.180 U/ml/h, respectively). Antibiotic sensitivity tests were conducted for all the isolates against 22 antibiotics based on their spectrum in order to find the most sensitive antibiotic and hence making the intestine devoid of cellulase-producing bacteria and again establishing the potent cellulolytic bacteria by a dip treatment. Antibiogram results revealed that almost all isolates were resistant to clindamycin, erythromycin and metronidazole but sensitive to ciprofloxacin and trimethoprim. This information might contribute to the utilization of these extracellular enzyme-producing bacteria in commercial aquaculture.

Keywords: β-glucosidase, cellulolytic, endoglucanase, exoglucanase, hepatopancreas
Abbreviations: CMC, carboxymethylcellulose; **PNPG**, *p*-nitrophenol β-D-glucoside; **MAR**, multiple antibiotic resistance; **NSS**, normal saline solution; **PBS**, phosphate buffer saline

INTRODUCTION

The intensification of aquacultural practices has generated a demand for alternative feed ingredients. In this sense, plant biomass is an inexpensive and highly available nutrient source, mainly composed of cellulose (Peixoto *et al*. 2011). Fermentative digestion typically occurs in animals with a diet composed predominantly of plant material (Bergman 1990) and symbioses with microorganism have been well studied in herbivorous mammals, birds and reptiles (Stevens 1988). Earlier reports suggest that fish do not produce cellulolytic enzymes. Cellulase activity observed in fish is mainly produced by intestinal microbiota (Kar and Ghosh 2008). Only 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). Fish receive bacteria from the aquatic environment as their digestive tract is an open system constantly in contact with the surrounding water. The microflora of water plays an important role in the formation of microflora in the digestive tract of fish (Storm and Olafsen 1990). The fish digestive tract, rich in nutrients, provides a favourable growth environment for these microorganisms. The microbial population grows upon the food absorbed by the host animal, digestive secretions and fragments sloughed off the mucosal epithelium (Lesel 1991). A number of cellulolytic bacteria comprised of diverse physiological groups have been identified in the fish intestine. The predominant bacterial species isolated from most fish digestive tracts have been reported to be aerobes or facultative anaerobes (Trust and Sparrow 1974; Bairagi *et al*. 2002; Saha *et al.* 2006). Cellulolytic bacteria include aerobic species such as *Pseudomonas* and *Actinomycetes*, facultative anaerobes such as *Bacillus* and *Cellulomonas* and strict anaerobes such as *Clostridium* (Heck *et al.* 2002). The first systematic survey of cellulase activity in fish was attempted by Stickney and Shumway (1974), who reported cellulase activity in 17 species out of 62 species of fish examined. They concluded that cellulase activity in fish resulted from the presence of stable microflora maintained within the digestive tract. Lindsay and Harris (1980) assayed cellulolytic activity in the digestive tract of 130 fishes representing 42 species and found 37 fishes with cellulase activity of microbial origin. Cellulase activity has been reported in a variety of fish, including Cyprinids (Prejs and Blaszezyk 1977), roach (*Rutilus rutilus*) and rudd (*Scardinius erythropthalmus*) (Niederholzer and Hofer 1979) rainbow trout (*Oncorhynchus mykiss*), grass carp (*Ctenopharyngodon idella*) and goldfish (*Carassius auratus*) (Lesel *et al*. 1986). Bairagi *et al*. (2004) isolated cellulolytic and amylolytic bacteria from the gastrointestinal tract of several culturable freshwater teleosts *viz*. Indian major carps (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*), Chinese carps (*Hypophthalmichthys molitrix* and *Ctenopharyngodon idella*), common carp (*Cyprinus carpio*), tilapia (*Oreochromis mossambicus*), walking catfish (*Clarias batrachus*) and murrel (*Channa punctatus*) following the enrichment culture technique (Bairagi *et al*. 2002).

Grass carp is basically an aquatic weed feeder (Cross 1969). The present investigation was carried out to isolate the cellulolytic microflora present in grass carp and to re-establish the best cellulase producer so that cellulose digestion in this fish can become more efficient while using aquatic plants, a cheap source of energy compared to formulated feed.

MATERIALS AND METHODS

Collection of experimental fish and processing

Grass carp of different weights were collected from the Central Institute of Freshwater and Aquaculture (CIFA) farm in Bhubanes-

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war, India using a cast net and from a local retail fish market in Bhubaneswar and ventrally dissected to separately extract the hepatopancreas and intestine.

Isolation and identification of cellulolytic bacteria

Cellulolytic bacteria were isolated by the enrichment culture technique (Bairagi *et al*. 2004). Intestinal tract weighing 1 g was triturated with 9 ml of sterilized 0.85% normal saline solution. 1 ml of triturated sample was inoculated into 9 ml of cellulose enrichment broth [0.5% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2SO_4, 1% carboxymethylcellulose] and incubated at 37°C for 24 h. Then the enriched samples (100 µl) were cultured using the spread plate method (Kar and Ghosh 2008) on 0.5% carboxymethylcellulose (CMC) agar plates and incubated at 37°C for 48 h. The colonies which appeared on CMC agar plates were pure when cultured and the cellulose hydrolysis by these bacteria was detected by the Congo red binding assay (Teather and Wood 1982). Identification of Congo red-positive bacteria was carried out by Gram staining, colony characteristics and a panel of biochemical tests. Bacterial cultures were preserved in 40% glycerol and stocked at 4°C.

β-Glucosidase estimation from exogenous (bacterial) source

Cellulase-producing strains were cultured in cellulose enrichment broth (pH 8.0) and harvested by centrifuging at 10,000 rpm for 20 min at 4°C. The supernatant was used as crude enzyme extract and was used for estimating β-glucosidase (E.C.3.2.1.21) following the method described by Wood and Bhat (1998) with some modifications. A reaction mixture containing 0.5 ml of enzyme solution was added to 0.5 ml of 0.1% PNPG (*p*-nitrophenol β-D-glucoside) solution prepared in Tris HCl (pH 6.5) and incubated at 37°C in a shaking water bath. After 2 h the reaction was stopped by adding 1 ml of 2% sodium carbonate. The yellow colour of *p*-nitrophenol liberated was determined spectrophotometrically (Jenway 6505 UV/VIS Spectrophotometer, UK) at 400 nm against a standard curve.

Antibiogram of the isolates

An antibiogram of the 48 isolates were carried out by using the Kirby Bauer Disc diffusion method (Bauer *et al*. 1966) against a panel of 22 antibiotics procured from Himedia (Mumbai, India). The multiple antibiotic resistance (MAR%) index was determined using the formula described by Mahapatra *et al.* (2006).

$$MAR\% = \frac{\text{Isolate resistant to no. of antibiotics}}{\text{Number of antibiotics used}} \times 100$$

The most potent cellulase-producing bacterial species was selected from the enzyme activity data. And from the antibiogram results the most sensitive antibiotic was selected against that bacterial species.

Dip treatment

Grass carps were maintained and fed in aerated experimental tanks (30 L capacity). Initially all the fishes were acclimatized for 15 days with control feed (rice bran and ground nut oil cake in a 1: 1 ratio). The experiment was carried out in two steps. In step 1, five fishes, each weighing around 70 g, were placed in tank A and five fishes in triplicates of almost same weight were placed in tank B. Tank A was maintained as the control and tank B as the test tank. The most sensitive antibiotic (50 ppm) was fed to test groups whereas normal feed without antibiotic was fed to the control groups. Sensitivity to antibiotics was determined using antibiotic discs. The most potent antibiotic, which was water-soluble, was used within feed and fed as pellets (prepared in a locally made hand operated extruder) to make the intestine of the fishes devoid of cellulase-producing bacteria so that, when dip treatment was applied, the supplemented bacteria, when tested for cellulase production, might colonize the intestine of experimental fish. Feed was dried at 42°C in an oven and stored in a refrigerator at 4°C until further use.

Fishes in tank B were fed with the pellets (feed) twice daily (morning and evening) for three days continuously at 3% of their body weight. The body weight of fishes was determined during initial stocking in glass tanks/jars. Then, fish were sacrificed up to three days (minimum of one fish daily from both tanks) and intestinal samples were tested for the presence of cellulolytic bacteria (Teather and Wood 1982). In step 2, the test fishes of step 1, treated with antibiotics through feed and now devoid of cellulolytic bacteria, were divided into two groups in tank C and tank D. Each tank contained five fish. Tank C fishes served as control and fishes in tank D as the test group which were given a dip treatment of cellulolytic bacteria for the establishment study. All the fish in tanks C and D in this phase were placed in water filtered through ceramic candle filters (Bajaj Water Filters, India, pore size 1 µm) normally used in Indian households to purify drinking water, free from bacteria. Additionally, as further precaution to maintain the fish devoid of cellulolytic bacteria, fish were treated with antibiotic through feed for 3 days. On the 4th day tank D fishes were given a dip treatment with bacterial species (having highest β-glucosidase activity amongst all the cellulose-positive species isolated) for 1 h with aeration. Bacterial suspensions were prepared as described in the following paragraph. Dip treatment was repeated twice at 7-day intervals, which were selected with an anticipation, probability and experience of working with some lactic acid bacteria, that once colonized, they might remain for a week. After the third treatment, the fishes were sacrificed, at least one per day, up to three days and finally one on seventh day, to isolate cellulolytic bacteria, to determine if they had established.

Preparation of bacterial sample for dip treatment

Nutrient broth (1800 ml) was prepared and sterilized. 100 ml of inoculum was added and incubated at 37°C overnight in a shaker water bath. Bacterial culture was centrifuged at 8000 rpm for 10 min. After washing twice the pellet was dissolved in 18 ml of PBS (Himedia). A dissolved pellet (6 ml) was added to tank D having 5 L of distilled water to make up to a final concentration of 10^7 cells/tank. After 1 h of dip treatment the fish were removed and kept in another tank. Dip treatment was administered twice at 7-day intervals.

RESULTS

Screening and identification of cellulolytic bacteria

In total 48 bacteria were isolated based on colony morphology of which 26 (54.17%) reported to be cellulase-positive on the basis of growth on CMC agar and Congo red reaction. The zone sizes of the isolates are presented in (**Fig. 1**). Out of 26 isolates 19 were found to be Gram-positive whereas, 7 isolates were Gram-negative. Based on the biochemical analyses the Gram-negative species were tentatively identified as *Pseudomonas* (15.38%), *Flavobacterium* (0.04%), *Actinobacillus* (0.04%) and *Erwinia* (0.04%) and the gram-positive species as *Bacillus* (61.54%), *Micrococcus* (0.04%) and *Lactobacillus* (0.08%). Detailed identification of the isolates is presented in **Tables 1** and **2**.

Confirmatory test for *Bacillus* species

A total of 16 *Bacillus* species isolated were further characterized on Hicrome *Bacillus* Agar (Himedia, Mumbai, India) on the basis of chromogenic reaction. Based on colony growth and chromogenic reactions these isolates were identified as *B. cereus* (4, 25%), *B. coagulans* (1, 6.25%), *B. megaterium* (1, 6.25%), *B. subtilis* (4, 25%), *B. circulans* (3, 18.75%), and *B. marinus* (1, 6.25%). However, the rest two species of *Bacillus* isolated could not be identified in chromogenic reaction.

Estimation of bacterial cellulase

β-glucosidase estimation was carried out using the method

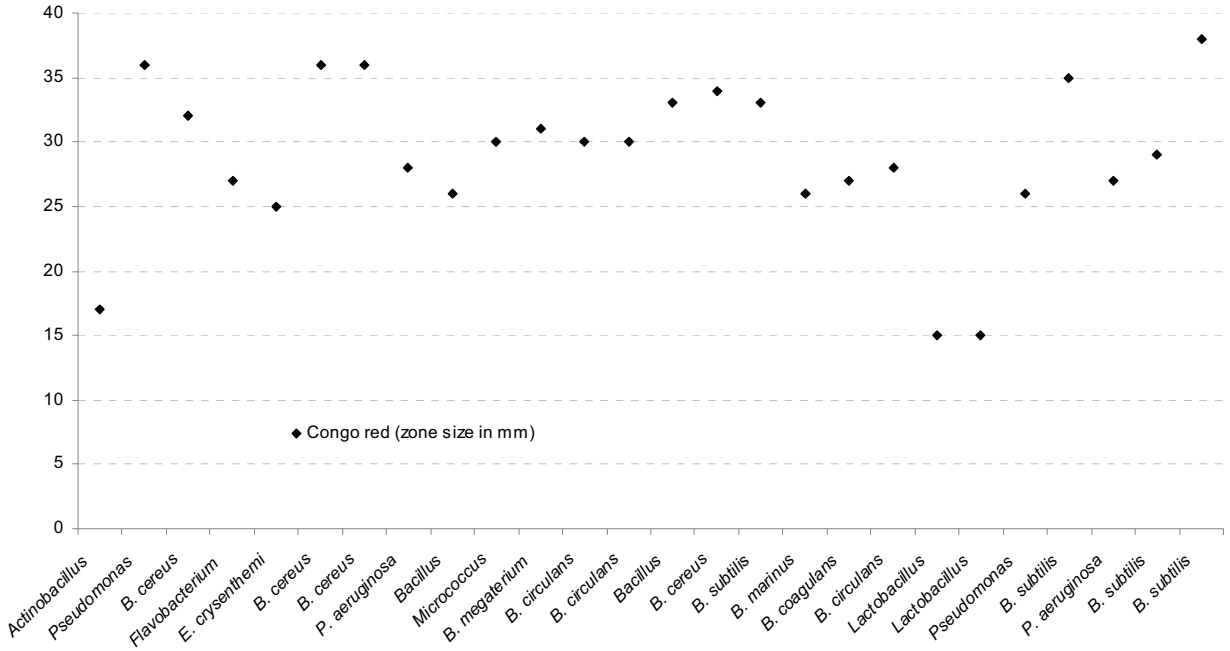

Fig. 1 Cellulose activity of the isolates.

Table 1 Identification of cellulose-producing *Bacillus* species.

Sample ID	**C**	**G**	**I**	**L**	**N**	**O**	**P**	**Q**	**R**	**T**	**U**	**X**	**H1**	**L1**	**S1**	**U1**
Gram staining	**+Ve bacilli, spore formers**	**+ve bacilli, spore formers**	**+ve bacilli, spore formers**	**+ve rods in chain, spore formers**	**+ve short rod**	**+ve short rods**	**+ve short rods**	**+ve rods in chain, spore formers**	**+ve bacilli, spore formers**	**+short rods**	**+ short rods**	**+ve rods**	**+ve short rods**	**+short rods**	**+short rods**	**+short rods**
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility										+	+			+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease					+	+	+				-	+	+			
MR				-	-	-	-	-			+	-	-			
VP	+	+	+		-	+	+		+	+	+	-	+	+	+	+
Indole	-	-	-	+	-	-	-	+	-			-	-			
Citrate	-	-	-	-	+	+	+	-	-	-	-		+	-	-	-
Starch										+	-			+	+	+
Esculin					+	+	+					+	+			
H_2S						-	-				-	-	-			
Growth in 7% NaCl	+	+	+	+				+	+	+				+	+	+
Sugar utilization																
Glucose	+	+	+		+	+	+		+		+	+	+			
Lactose	-	-	-		-	+	+		-		+	-	+			
Adonitol					-	+	+				+	-	+			
Arabinose	-	-	-	-	-	+	+	-	-		+	+	+			
Sorbitol											+					
Ribose																
Rhamnose					+	+	+					+	+			
Mannitol	-	-	-	-				-	-							
Xylose	-	-	-	-	+	+	+	-	-			+	+			
Dextrose	-	-	-	+				+	-							
Organism	***B. cereus***	***B. cereus***	***B. cereus***	***Bacillus***	***B. megaterium***	***B. circulans***	***B. circulans***	***Bacillus***	***B. cereus***	***B. subtilis***	***B. marinus***	***B. coagulans***	***B. circulans***	***B. subtilis***	***B. subtilis***	***B. subtilis***

of Wood and Bhat (1998) and a standard curve was prepared based on six concentrations of *p*-nitrophenol. From the standard curve, the R^2 value was 0.9997 and y = 0.0632x. Out of 48 isolates 10 (20.83%) were found to be negative for β-glucosidase activity whereas, 9 (18.75%) isolates showed negligible (0.001 U/ml/h) β-glucosidase activity. Thirteen (27.08%) isolates were observed to be good producers of β-glucosidase with ≥ 0.1 U/ml/h enzyme activity. The rest 17 (35.42%) isolates produced ≤ 0.01 U/ml/h enzyme activity. *Actinobacillus*, *Erwinia* and *Bacillus* species were identified as good β-glucosidase producers with enzymatic activity ≥ 0.1 U/ml/h. Thus based on enzyme activity data *Bacillus* species were selected for dip treatment.

Table 2 Identification of cellulose-producing Gram-negative isolates.

Sample ID	A	B	D	E	K	M	I1	J1	K1	R1
Gram staining	–ve cocco bacilli	-ve short rods	-ve long rods in chain	-ve rods	-ve short rods	+ve cocci in tetrad or diplococcus	+ve short rod, non spore formers	+ve short rod, non spore formers	-ve short rods	-ve short rods
Catalase	+	+	+	+	+	+	-	-	+	+
Oxidase	+	+	+	+	+	-	-	-	+	+
Motility	-	+	+	-					+	
Nitrate reduction	+	-	+	+	-	+	+	+	-	-
Urease	+	-	-	-					-	
MR	-	-	+	-					-	
VP	-	-	-	-	-	+	+	+	-	-
Indole	+	+	+	-	-	-	-	-	+	-
Citrate	+	+	-	+	+	-	-	-	+	+
Starch	-	-	+	+					-	
Esculin	+	-	-						-	
H_2S	+	+	-	-					+	
Growth in 7% NaCl					+					+
Sugar utilization										
Glucose	+	+	+	+			+	+	+	
Lactose	+	-	-	-			+	+	-	
Adonitol	+	-	-	+					-	
Arabinose	+	+	-	-	-	-	-	-	+	-
Sorbitol	+	-	-	+					-	
Ribose	-	-	+						-	
Rhamnose	+	-	-	+					-	
Mannitol	-	-	-	+	-	+	-	-	-	-
Xylose	+	+	-	+	+	-	+	+	+	+
Dextrose					-	+	+	+		-
Organism	*Actinobacillus*	*Pseudomonas*	*Flavobacterium*	*E. crysenthemi*	*P. aeruginosa*	*Micrococcus*	*Lactobacillus*	*Lactobacillus*	*Pseudomonas*	*P. aeruginosa*

Antibiogram pattern of bacterial isolates

An antibiogram of the 48 isolates were carried out by using the Kirby Bauer Disc diffusion method to quantify the sensitivity of the isolates with a panel of 22 antibiotics. The MAR% calculated is represented in (**Table 3**). All the isolates showed resistance to clindamycin, erythromycin, metronidazole. Amoxycillin, aztreonam, carbenicillin, chloramphenicol, imipenem, pefloxacin and piperacillin showed maximum sensitivity to 8, 4, 3, 6, 11, 2 and 1 isolates, respectively. It was also found that out of 48 isolates, 14 showed maximum zones of sensitivity against ciprofloxacin and another 14 isolates against trimethoprim. Ciprofloxacin and trimethoprim were sensitive against 44 and 25% of the *Bacillus* species, respectively.

Establishment of cellulase-producing *Bacillus* sp. in grass carp intestine

Bacillus species Q was identified as the most potent cellulase producer as a very high β-glucosidase activity of 0.186 U/ml/h was detected in this sample. Hence this bacterium was selected for establishment in the grass carp intestine. In the first step, five control and five antibiotic-treated fishes were sacrificed to detect the presence of cellulolytic bacteria. From the control samples *Bacillus*, *Lactobacillus* and *Erwinia*, *Micrococcus* were isolated and from the antibiotic treated samples *Erwinia*, *Micrococcus* and *Flavobacterium* were isolated and identified. In the second step, five antibiotic treated fish were kept as control and five test fishes were given dip treatment with *Bacillus* sp. three times. After sacrificing the control fishes and processing the intestinal samples, cellulolytic bacteria such as *Erwinia*, *Micrococcus* were identified and from the test samples *Micrococcus*, *Flavobacterium* and *Bacillus* were identified, thus suggesting the establishment of *Bacillus* sp. in the grass carp intestine.

DISCUSSION

Grass carp efficiently degrades the vegetable biomass (rich in cellulose) present in an aquatic environment and grows to a marketable size within a short period (Wijeyratne and Perera 2000). According to Masser (2002), the large consumption of these carbohydrates present in water bodies by grass carp is due to a very short gut compared to other herbivores. This decreases the retention time (less than 8 h) in the gut and reduces the digestive efficiency to only 60 to 70%. Because of this reduced efficiency, they eat more vegetation. During a preliminary investigation high blood glucose level of grass carp (~32 mg/dl) was detected as compared to other fish species like *Catla catla* (catla) (~12 mg/dl), *Cirrhinus mrigala* (mrigal) (~13 mg/dl) and *Labeo rohita* (rohu) (~25 mg/dl). The high blood glucose level in grass carp may be due to the accumulation of more end product (glucose) after cellulose digestion. Previous studies on the digestive enzyme of grass carp indicated the source to be of microbial origin (Das and Tripathi 1991). Similar findings were also marked in *L. rohita* (Saha and Ray 1998). Akolkar *et al.* (2006) identified cellulose producing *E. crysenthemi* in intestine of *L. rohita* and *C. idella*. According to Mondal *et al*. (2008), two explanations have been proposed to account for the presence of cellulase in the digestive tract of fish. The first one suggests that intestinal tract associated cellulase is produced by an endosymbiotic microbial flora resident in the intestinal tract. The second explanation was that cellulase may be derived from ingestion of plant detritus. Jiang *et al*. (2011) investigated the bacterial diversity and population abundance in the gut of starved grass carp by sequencing 16S r RNA gene libraries and the results

Table 3 Antibiogram pattern of the isolates.

Organism ID	Amoxycillin (Am)-30	Ampicillin (A)-10	Azlocillin (Az)-75	Aztreonam (Ao)-30	Carbenicillin (Cb)-100	Ceftazidime (Ca)-30	Cefoperazone (Cs)-75	Chloramphenicol (C)-30	Ciprofloxacin (Cf)-10	Clindamycin (Cd)-2	Erythromycin (E)-10	Imipenem (I)-10	Kanamycin (K)-30	Metronidazole (Mt)-5	Neomycin (N)-30	Pefloxacin (Pf)-5	Penicillin-G (P)-10	Piperacillin (Pc)-100	Polymyxin-B (Pb)-50	Tetracycline (T)-30	Tricarcillin (Ti)-75	Trimethoprim (Tr)-30	MAR %
	Zone size in (mm)																						
A	17	R	14	31	22	28	32	29	32	R	R	33	23	R	21	30	10	24	13	19	R	35	22.75
B	R	R	14	19	30	23	27	R	40	R	R	21	R	R	15	25	R	29	15	11	19	R	36.4
C	R	R	10	19	28	22	23	R	33	R	R	21	R	R	15	21	R	25	11	R	16	R	45.5
D	30	26	14	R	18	R	21	28	28	20	23	35	33	R	24	28	R	21	R	28	10	R	27.3
E	17	11	10	27	20	27	29	32	29	R	R	34	20	R	19	27	11	21	12	23	R	33	18.2
F	15	17	21	25	25	28	31	24	34	R	R	30	21	R	16	24	16	24	14	23	28	37	13.65
G	R	R	15	35	27	29	21	30	30	19	20	30	20	R	22	28	15	27	16	23	10	38	13.65
H	16	12	13	22	16	24	20	23	25	R	R	23	16	R	16	21	10	16	10	18	R	17	18.2
I	R	R	R	16	18	24	19	17	32	R	R	21	12	R	16	24	R	10	R	18	R	R	45.5
J	10	R	13	15	23	20	19	R	35	R	R	20	15	R	18	21	R	25	12	12	13	15	27.3
K	R	R	12	18	23	20	R	19	24	R	R	R	R	R	15	20	R	24	R	13	15	R	50.05
L	R	14	13	17	21	22	19	15	20	R	R	17	12	R	16	24	R	21	12	17	R	21	27.3
M	15	12	11	23	17	20	16	18	14	R	R	R	13	R	14	11	R	15	R	17	R	25	27.3
N	19	14	12	25	R	24	19	28	28	R	R	29	17	R	18	R	14	19	12	22	12	24	22.75
O	15	14	12	13	16	22	22	26	29	R	R	28	17	R	18	R	10	17	12	19	R	28	22.75
P	R	R	13	14	18	22	R	10	24	R	R	21	17	R	15	20	10	16	12	17	10	25	27.3
Q	16	11	11	25	17	23	23	21	27	R	R	27	17	R	18	25	11	15	13	18	R	13	18.2
R	15	12	11	24	17	22	16	17	23	R	R	24	12	R	15	22	14	16	12	18	R	R	22.75
S	26	15	R	12	R	22	10	20	27	R	R	30	15	R	12	25	16	25	11	22	22	34	22.75
T	21	13	17	27	22	27	27	27	27	R	R	26	17	R	17	28	11	19	13	21	R	30	18.2
U	16	12	12	30	18	27	27	15	29	R	R	31	19	R	17	26	R	12	12	23	R	31	22.75
V	29	19	18	28	26	24	26	29	31	R	R	32	15	R	15	25	14	27	11	21	23	30	13.65
W	12	R	20	23	29	23	22	R	40	R	R	R	12	R	10	24	R	R	R	13	R	R	40.95
X	17	12	10	28	18	24	25	26	27	R	R	27	16	R	15	23	11	15	13	16	R	27	18.2
Y	29	16	20	26	26	23	26	29	27	R	R	29	16	R	17	27	15	24	14	15	23	R	18.2
Z	R	R	20	18	27	21	16	R	32	R	R	26	12	R	15	19	R	27	13	16	17	15	31.85
A1	29	15	19	20	27	27	29	31	24	R	R	29	15	R	17	19	12	25	12	25	23	R	18.2
B1	34	20	20	32	30	17	13	20	18	R	R	20	R	R	R	18	14	29	14	14	18	R	22.75
C1	29	19	21	29	26	18	R	24	R	R	R	R	18	R	18	27	13	27	12	20	22	R	31.85
D1	R	R	15	17	25	17	17	15	24	R	R	24	11	R	15	22	R	24	11	14	14	R	31.85
E1	26	14	17	24	23	23	28	27	29	R	R	27	14	R	15	28	13	29	11	20	22	32	13.65
F1	19	17	20	R	23	23	17	30	24	R	R	30	16	R	14	17	20	22	10	15	25	14	18.2
G1	26	16	19	18	28	26	24	24	27	R	R	26	16	R	16	28	13	25	11	16	16	15	13.65
H1	17	12	13	25	18	22	R	25	27	R	R	26	16	R	17	23	R	16	10	20	R	R	31.85
I1	29	15	24	30	30	21	21	25	25	R	R	29	20	R	17	26	13	25	12	20	23	30	13.65
J1	14	18	19	23	24	23	24	18	26	R	R	32	13	R	18	23	13	22	13	18	12	19	13.65
K1	29	15	23	29	27	26	25	31	28	R	R	29	20	R	20	31	11	24	13	23	25	33	13.65
L1	40	29	24	R	35	17	23	33	28	R	22	35	11	R	12	24	31	33	R	14	24	R	22.75
M1	28	18	19	29	28	27	15	29	27	R	11	28	18	R	17	20	13	26	12	21	25	32	9.1
N1	37	29	26	R	33	13	19	30	23	R	18	33	12	R	13	21	32	35	R	33	21	R	22.75
O1	12	R	15	20	28	20	21	18	40	R	R	27	15	R	18	25	R	27	11	18	15	16	22.75
P1	37	24	22	R	31	11	20	23	21	R	15	29	12	R	12	18	29	29	R	17	24	R	22.75
Q1	12	R	17	20	24	23	18	18	24	R	R	27	12	R	20	25	R	19	12	12	22	15	22.75
R1	28	17	18	28	28	26	19	30	22	R	R	26	18	R	16	22	17	28	12	24	17	30	13.65
S1	36	27	30	R	33	14	22	28	22	R	R	30	28	R	13	20	15	30	18	18	21	R	22.75
T1	24	17	20	R	13	12	22	35	29	25	23	38	22	R	20	28	11	19	R	24	R	R	22.75
U1	19	15	14	17	19	23	19	30	28	R	R	21	21	R	16	27	R	19	10	22	11	20	18.2
V1	15	14	14	17	17	25	22	28	25	R	R	22	R	R	15	23	13	16	10	24	R	28	22.75

MAR%: Multiple Antibiotic Resistance Index

revealed that 28 parasitic bacteria from the gut were affiliated to seven genera of *Vibrio*, *Acinetobacter*, *Providencia*, *Yersinia*, *Pseudomonas* and *Aeromonas*. 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 (Strom and Olafsen 1990; Hansen *et al*. 1992). This is probably the reason why a large number of probiotics developed in aquaculture are bacteria directly originating from the aquatic environment (Durand and Durand 2010). 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; Ringo and Strom 1994; Clements and Choat 1995), which are 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). Definitely, not all bacteria in food which gain entry in the digestive tract of the fishes establish themselves there (Wang *et al*. 2008). Part of them adapts themselves in the digestive tract, where as the others are digested by the enzymes produced by the host organism. In this study, 48 cellulolytic bacteria from the intestine and intestinal content samples were identified. The

present study was restricted to the aerobic bacterial population in the digestive tract of grass carp, as preliminary study showed lack of anaerobic flora. Moreover, the absence of microorganisms in CMC culture plates, incubated with hepatopancreatic extracts, in our experiment corroborated with the observations of Trust and Sparrow (1974), who also failed to demonstrate the presence of viable bacteria in the liver of freshwater salmonids. More than 60% of the isolates identified in this study, were *Bacillus* species (*B. subtilis*, *B. circulans*, *B. marinus*, *B. coagulans*, *B. cereus* and *B. megaterium*) based on their growth and pigmentation on Hicrome *Bacillus* agar. In resemblance to our study, Hicrome *Bacillus* agar based on the formulation of MYP (Mannitol Yolk Polymyxin B) agar by (Mossel *et al*. 1967) was efficiently used for the rapid identification of *Bacillus* species based on chromogenic property. In this study the cellulase producing strains produced various degrees of β-glucosidase and exoglucanase activities. Some isolates such as *Actinobacillus* (0.180 U/ml/h), *Erwinia* (0.181 U/ml/h) and *Bacillus* (0.186 U/ml/h) were good producers of β-glucosidase and strains such as *Micrococcus* (0.190 U/ml/h) and *Bacillus* (0.107 U/ml/h) were good producers of exoglucanase whereas some strains produced negligible activity. It was previously reported that *Bacillus* sp. being most potent producer of cellulase showed an activity of 0.112 U/ml/h (Dey *et al*. 2002). Ghosh *et al*. (2002) isolated *Bacillus* species (*B. circulans*, *B. pumilus* and *B. cereus*) from the gut of rohu and reported to be potent producer of enzyme cellulase. Saha *et al*. (2006) also reported that strains isolated from the gut of *C. idella* with maximum cellulolytic property were aerobic, Gram-positive Bacilli. The aim of screening and selecting most sensitive antibiotics was to make the intestine devoid of native bacteria, particularly the cellulose producing ones, as far as possible. The assessment of cellulase production by individual isolated cellulase producing bacteria would be possible when presence and effect of other native intestinal bacteria are negated. Hence, the total cellulase production would be attributed to the individual bacterium in a controlled environment. The treatment of the grass carp with suitable antibiotics and bath treatments with *Bacillus* species initially proved its contribution to enzyme activity in the intestine although the quantity of enzyme produced by bacteria alone could not be ascertained. Earlier, Ninawe and Selvin (2009) also suggested that *Bacillus* has been used successfully as probiotic specie in fishes. In our study establishment of a potent cellulase producer in the intestine of grass carp suggested that such potent cellulase producers could be used for formulating better aquafeed and incorporating them as probiotic in other carp species. This will in turn improve the digestion process and hence enhance the ability of stress resistance and health of the fish.

CONCLUSIONS

The results of the present study indicated that the strains isolated from the gut of grass carp are capable of producing cellulolytic activity. Hence, the enzyme producing microorganisms can be beneficially used as a probiotic while formulating the diet for fish, especially in the larval stages when the enzyme system is not efficient.

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REFERENCES

Akolkar D, Samanta M, Mohanty S, Mukhopadhyay PK, Maiti NK (2006) Molecular characterization of cellulytic bacteria from two freshwater cyprinids by immunoblotting and RAPD-PCR. *Journal of aquaculture in Tropics* **21 (3-4)**, 133-147

Austin B, Al-Zahrani AM (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 KS, Sen SK, Ray AK (2002) Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquaculture International* **10**, 109-121

Bairagi A, Ghosh KS, Sen SK, Ray AK (2004) Evaluation of the 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. *Aquaculture Research* **35 (5)**, 436-446

Bauer AW, Kirby WMM, Sherris JC, Turk M (1966) Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology* **45**, 493-496

Bergman EN (1990) Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiological Review* **70**, 567-590

Clements KD (1991) Endosymbiotic communities of two herbivorous labroid fishes, *Odax cyanomelas* and *O. pullus*. *Marine Biology* **109**, 223-229

Clements KD (1997) Fermentation and gastrointestinal microorganisms in fishes. In: Mackie RI, White BA (Eds) *Gastrointestinal Microbiology*, *Gastrointestinal Ecosystems and Fermentations* (Vol 1), Chapman and Hall, New York, pp 156-198

Clements KD, Choat JH (1995) Fermentation in tropical marine herbivorous fishes. *Physiology and Zoology* **68**, 355-378

Cross DG (1969) Aquatic weed control using grass carp. *Journal of Fish Biology* **1**, 27-30

Das KM, Tripathi SD (1991) Studies on digestive enzyme of grasscarp, *Ctenopharyngodon idella* (Val.). *Aquaculture* **92**, 21-32

Dey R, Pal KK, Chauhan SM, Bhatt DM, Misra J B (2002) Groundnut shell decomposition potential of some cellulolytic microorganisms. *Indian Journal of Microbiology* **42**, 165-167

Durand FC, Durand H (2010) Probiotics in animal nutrition and health. *Beneficial Microbes* **1 (1)**, 3-9

Ghosh K, Sen SK, Ray AK (2002) Characterization of *Bacilli* isolated from the gut of Rohu, *Labeo rohita* fingerlings and its significance in digestion. *Journal of Applied Aquaculture* **12 (3)**, 33-42

Hansen GH, Strom E, Olafsen JA (1992) Effect of different holding regimes on the intestinal microflora of herring (*Clupea harengus*) larvae. *Applied Environmental Microbiology* **58**, 461-470

Heck JX, Hertz PF, Ayub MAZ (2002) Cellulase and Xylanase production by isolated amazon *Bacillus* strains using soybean industrial residue based solid state cultivation. *Brazilian Journal of Microbiology* **33**, 213-218

Horsley RW (1977) A review of the bacterial flora of teleosts and elasmobranches, including methods for its analysis. *Journal of Fish Biology* **10**, 529-553

Jiang Y, Xie C, Yang G, Gong X, Chen X, Xu L, Bao B (2011) Cellulase-producing bacteria of *Aeromonas* are dominant and indigenous in the gut of *Ctenopharyngodon idellus* (Valenciennes). *Aquaculture Research* **42 (4)**, 499-505

Kar N, Ghosh K (2008) Enzyme producing bacteria in the gastrointestinal tracts of *Labeo rohita* (Hamilton) and *Channa punctatus* (Bloch). *Turkish Journal of Fisheries and Aquatic Sciences* **8**, 115-120

Lesel R (1991) Does a digestive active bacterial flora exist in fish? In: Kaushik SJ, Luquet P (Eds) *Fish Nutrition in Practice, IVth International Symposium on Fish Nutrition and Feeding*, Biarritz (France), INRA, Paris, pp 655-664

Lesel R, Fromageot C, Lesel M (1986) Cellulose digestibility in grass carp, *Ctenopharyngodon idella* and in goldfish, *Carassius auratus*. *Aquaculture* **54**, 11-17

Lindsay GHJ, Harris JE (1980) Carboxymethylcellulose activity in the digestive tract of fish. *Journal of Fish Biology* **16**, 219-233

Luczkovich JJ, Stellwag EJ (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

Mahapatra S, Rath CC, Dash SK, Mishra RK (2006) Microbial evaluation of wounds and their susceptibility to antibiotics and essential oil. *Journal of Microbial World* **8**, 101-109

Masser MP (2002) Using grass carp in aquaculture and private impoundments. *SRAC* Publication No, 3600

Mondal S, Roy T, Sen SK, Ray AK (2008) Distribution of enzyme-producing bacteria in the digestive tracts of some freshwater fish. *Acta Ichthyologica et Piscatoria* **38 (1)**, 1-8

Mossel DAA, Koopman MJ, Jongerius E (1967) Enumeration of *Bacillus cereus* in foods. *Applied Microbiology* **15**, 650

Niederholzer R, Hofer R (1979) The adaptation of digestive enzymes to temperature, season and diet in roach *Rutilus rutilus* L. and rudd *Scardinus erythropthalmus* L. Cellulase. *Journal of Fish Biology* **15**, 411-452

Ninawe AS, Selvin J (2009) Probiotics in shrimp aquaculture: avenues and challenges. *Critical Reviews in Microbiology* **35 (1)**, 43-66

Peixoto SB, Cladera-Olivera F, Daroit DJ, Brandelli A (2011) Cellulase-producing *Bacillus* strains isolated from the intestine of Amazon basin fish. *Aquaculture Research* **42**, 887-891

Prejs A, Blaszezyk M (1977) Relationships between food and cellulose activity in freshwater fishes. *Journal of Fish Biology* **11**, 447-452

Rimmer DW, Wiebe WJ (1987) Fermentative microbial digestion in herbivo-

rous fishes. *Journal of Fish Biology* **31**, 229-236

Ringo E, Strom 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 AK, Ray AK (1998) Cellulase activity in rohu fingerlings. *Aquaculture International* **6**, 281-291

Saha S, Roy RN, Sen SK, Ray AK (2006) Characterization of cellulose producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngodon idella* (Valenciennes). *Aquaculture Research* **37 (4)**, 380-388

Sakata T (1990) Microflora in the digestive tract of fish and shell-fish. In: Lesel R (Ed) *Microbiology in Poecilotherms*, Elsevier Science, Amsterdam, pp 171-176

Stevens CE (1988) *Comparative Physiology of the Vertebrate Digestive System*, Cambridge University Press, New York

Stickney RR, Shumway SE (1974) Occurrence of cellulose activity in the stomach of fish. *Journal of Fish Biology* **6**, 779-790

Strom E, Olafsen JA (1990) The indigenous microflora of wild-captured juvenile cod in net-pen rearing. In: Lesel R (Ed) *Microbiology in Poecilotherms*, Elsevier Science, Amsterdam, pp 181-185

Teather RM, Wood PJ (1982) Use of Congo-red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Applied and Environmental Microbiology* **43**, 777-780

Trust TJ, Bull LM, Currie BR, Buckley JT (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 TJ, Sparrow RAH (1974) The bacterial flora in the alimentary tract of freshwater salmonid fishes. *Canadian Journal of Microbiol*ogy **20**, 1219-1228

Wang YB, Li JR, Lin J (2008) Probiotics in aquaculture: Challenges and outlook. *Aquaculture* **281**, 1-4

Wijeyaratne MJS, Perera WMDSK (2000) Studies on the feasibility of using indigenous fishes for controlling aquatic macrophytes in Sri Lanka. *Journal of Aquaculture in Tropics* **15 (3)**, 253-260

Wood TM, Bhat KM (1998) Methods for measuring cellulase activities. *Methods of Enzymology* **160**, 87-112