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Non-Starch Polysaccharide Degrading Gut Bacteria in Indian Major Carps and Exotic Carps

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Abstract

Cellulose and xylan are the most common Non-Starch Polysaccharides (NSPs); they are available in plants and they exhibit anti-nutritional effects. The present study is intended to detect cellulose and xylan degrading autochthonous gut bacteria in Indian major carps (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) and exotic carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio*); it is also meant to identify the most promising strains by molecular methods. The promising strains were also tested for likely antagonism against few pathogenic *Aeromonas* strains. Altogether, 432 microbial strains were isolated on media containing either cellulose or xylan. Seventy strains were primarily selected through qualitative enzyme assay. Finally, the quantitative assay led to the selection of 5 promising NSP-degrading strains (LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X). Amongst these, LRF1X was the best cellulase- and xylanase-producer. Analyses of 16S rRNA partial gene sequence revealed that strains LRF1X and CMF1C were closely related to *Bacillus pumilus* (Accession numbers; KF640221, KF640223, respectively), whereas HMF6X, CtIF1C, and CMH8X were similar to *B. tequilensis* (KF640219), *B. megaterium* (KF640220) and *B. altitudinis* (KF640222), respectively. The culture of the selected microorganisms with autochthonous bacteria and yeasts indicated their co-existence within the fish gut. An appraisal of antagonism against four pathogenic *Aeromonas* species by the cross-streaking method revealed that the selected NSP-degrading strains (except CMH8X) were antagonistic to at least 2 pathogens. In vivo bio-safety assessment through intra-peritoneal injection of the isolates showed no induction of pathological lesions or mortality in healthy laboratory acclimatized rohu, *L. rohita.*

Keywords: : Cellulose, Xylan, Carps, Bacteria, Aeromonas.

1. Introduction

Non-Starch Polysaccharides (NSPs) are complex polysaccharides that are polymers of hexoses and pentoses (e.g., galactose, glucose, arabinose, xylose, mannose, etc.) excluding starch or α-glucans (Van Barneveld, 1999). The NSPs comprise up to 90% of the plant's cell wall (Selvendran and DuPont, 1980), wherein cellulose, hemicelluloses, and pectins are the most abundant (Sinha *et al*., 2011). Cellulose is the basic structural component of plant cell walls and constitutes about 33% of all vegetable materials. Xylan is the most common hemicellulose and represents the major non-cellulosic cell wall polysaccharide in plants. In contrast, pectic polysaccharides (pectins) are only present in modest amounts in plants (Sinha *et al*., 2011). Being an integral part of plant ingredients, NSPs are represented in aquaculture through natural food and supplementary feeds in the form of phytoplankton, algae, aquatic macrophytes, detritus, husks (rice bran or wheat bran) and different oil cakes. The predominant endogenous polysaccharide digesting enzymes, in fish specifically, hydrolyze the α-glycosic linkages of starch to yield glucose. Presence of β-(1→4) glycosidic linkages is the reason why cellulose is indigestible by monogastric animals due to the lack of the enzyme cellulase in their gastrointestinal (GI) tract. Similarly, the other enzymes for NSP digestion, such as β-glucanases and β-xylanases, are also either scarce or not present in fish (Kuz'mina, 1996). Consequently, the dietary NSPs remain mostly indigestible and cannot be used as a nutritional source. In addition, being partially soluble in water, NSPs increase the viscosity of the digesta, leading to changes in the physiology and the ecosystem of the gut, thus, exerting anti-nutritive effects (Sinha *et al*., 2011). Therefore, it appears that unless hydrolyzed or degraded by exogenous enzymes, NSPs would decrease the nutritive value of the plant feedstuffs.

The contribution of endosymbionts in the digestive process requires understanding the relative importance of both, endogenous (produced by self) and exogenous (produced by endosymbionts) enzymes (Clements, 1997).

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During the last few decades, there has been an improved understanding of the enzymes produced by the resident endosymbiotic microbial community in fish (Ringø *et al*., 2010). Several studies have reported the occurrence of cellulase-producing microorganisms within the GI tract of diverse fish species, including carps.[1]

In contrast, reports on xylanase production by fish gut microorganisms are scarce (German and Bittong, 2009; Banerjee and Ghosh, 2014). In the previous studies, association of cellulase (Saha and Ray, 1998; Bairagi *et al*., 2002; Ghosh *et al*., 2002, 2010; Saha *et al*., 2006; Mondal *et al*., 2008), phytase (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012) and tannase (Mandal and Ghosh, 2013) producing microorganisms were demonstrated as an ecological adaptation of the carps towards herbivory. Likewise, the existence of the gut-associated symbiotic microbial community capable of producing NSP-degrading enzymes (in addition to cellulase) could not be excluded in carps.

Carps are the freshwater fish of the family Cyprinidae. Various species of carps have been included in aquaculture as food fish across Europe and Asia for centuries. Three Indian major carp species (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) along with three Chinese carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio*) constitute the composite carp culture in India. All these carp species are mostly herbivorous or omnivorous in feeding aptitude (Jhingran, 1997). Intensive carp polyculture demands improvements in feed efficiency, while the replacement of animal protein sources (e.g., fish meal) with the non-conventional plant ingredients is already a concern to trim down the production costs. Enumeration of exo-enzyme producing gut microorganisms capable of degrading complex polysaccharides and their utilization as either feed supplement or *in vitro* processing of plant feedstuffs has been recommended in some of the recent investigations (Khan and Ghosh, 2013; Das and Ghosh, 2015).

Since, cellulose and xylans are the major NSPs in plant feedstuffs, the present study is undertaken to appraise the occurrence of cellulase and xylanase-producing autochthonous microorganisms within the GI tracts of six freshwater carps. The most promising enzyme-producing strains were characterized through 16S rRNA partial gene sequence analysis to corroborate their identity. Bio-safety in fish was evaluated *in vivo* by injecting healthy laboratory acclimatized rohu, *L. rohita*, with fresh cultures of the isolates. In addition, the promising enzyme-producers were evaluated for antagonism against few well-known fish pathogens belonging to the genus *Aeromonas*. Motile aeromonads, being the major bacterial pathogens among tropical freshwater fish, are reported to be the main causative factor behind mass mortalities associated with increased stocking in composite carp culture (Karunasagar *et al*. 1986). *Aeromonas* outbreaks in aquaculture are common in tropical countries and, therefore, the use of pathogen inhibitory microorganisms has been suggested as probiotic bio-control agents to substitute the use of antimicrobial drugs (Balcázar *et al*., 2006; Dutta and Ghosh, 2015). Finally, the selected bacterial isolates were cultured with the previously isolated autochthonous gut bacteria and yeasts to substantiate their co-existence as gut microbiota in fish in view of their likely application in aquaculture.

2. Materials and Methods

2.1. Experimental fishes

Six freshwater carp species consisting of three Indian major carps (rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*) and three exotic carps (silver carp, *Hypophthalmichthys molitrix*; (grass carp, *Ctenopharyngodon idella*; common carp, *Cyprinus carpio*) were selected for the present study. Three specimens of each species were collected by gill net from three different composite fish culture farms at and around Burdwan (23°14′N, 87°39′E), West Bengal, India during June to September, 2012, and kept in 350L Fiber-Reinforced Plastic (FRP) aquaria. Specimens were brought to the laboratory within oxygen-packed bags. Descriptions of the fishes examined along with their feeding habits are depicted in Table1.

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

Fish Species	Food habits*	Average fish live weight (g)	Average fish length (cm)	Average gut weight (g)	Gut length (L_G) ($cm_=$)
Rohu, *L.rohita*	Omnivorous, mostly plant matter	260±13.44	30.5±2.61	11.32±0.62	271.7±8.51
Catla, *C.catla*	Zooplanktophagous	370±10.97	29.4±2.34	12.18±0.59	224.5±7.76
Mrigal, *C. mrigala*	Detrivorous	330±12.33	30.7±2.70	8.29±0.57	431.3±10.27
Silver carp, *H. molitrix*	Phytoplanktophagous	440±14.42	26.6±3.84	8.38±0.68	218.3±8.68
Grass carp,*C. idella*	Herbivorous, mostly macrophytes	450±10.88	28.9±2.21	16.7±0.55	63.2±8.39
Common carp, *C. carpio*	Detrivorous	375±13.44	27.4±2.37	7.81±0.58	47.3±9.81

Data are means ± S.D. of three determinations. * adapted from Jhingran (1997)

[1]For more information, see Ray *et al.* (2012).

2.2. Processing of Specimens

Prior to sacrifice, experimental fishes were starved for 48 h to clear their gastrointestinal (GI) tracts and to remove traces of any undigested food or fecal matter therein (Mondal *et al.*, 2010). Specimens were anesthetized using 0.03% tricainemethanesulfonate (MS-222). Ventral surfaces were sterilized using 70% ethanol and fishes were dissected aseptically to remove the GI tracts (Ghosh *et al.*, 2010). GI tracts were divided into proximal (PI) and distal (DI) regions, cut into pieces, and flushed carefully three times with Sterile Saline Solution (SSS) using an injection syringe in order to remove non-adherent (allochthonous) microbiota (Ghosh *et al.*, 2010; Khan and Ghosh, 2012). Gut segments from 3 specimens of each species, collected from the same pond, were pooled together region-wise for each replicate, therefore providing 3 replicates for each fish species. Pooled samples were utilized to avoid erroneous conclusions due to individual variation in gut microorganisms as described elsewhere (Ringø *et al.*, 1995; Spanggaard *et al.*, 2000; Ringø *et al.*, 2006).

2.3. Microbial Culture

Pooled segments for each replicate were homogenized separately with pre-chilled SSS (1:10; weight: volume), serially diluted (1:10) up to 10^{-7}, and used as inoculums for isolation of gut microorganisms (Beveridge *et al.*, 1991). Diluted samples (100 µL) were spread aseptically onto sterilized tryptone soya agar (TSA; HiMedia Laboratories, Mumbai, India) plates to obtain the culturable heterotrophic aerobic/facultative anaerobic autochthonous microbial population. In order to isolate cellulase and xylanase-producing microorganisms, diluted samples were plated onto carboxy-methyl-cellulose (CMC, gL^{-1}: carboxy-methyl-cellulose 5, Yeast extract 5, peptone 5, NaCl 5, agar 20) and xylan (XY, gL^{-1}: peptone 5, yeast extract 2, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $CaCl_2$ 0.15, Birchwood xylan 20, agar 20) supplemented selective media plates as described in Dutta and Ghosh (2015). The culture plates were incubated at 30°C for 24 h. Colony counts were determined utilizing the dilution plate count technique. The average values of the replicates were expressed as log viable count g^{-1} GI tract (LVC). The well-separated colonies were randomly collected with inoculation loop and streaked individually onto respective media plates repeatedly to acquire pure cultures. Pure cultures were maintained on slants in a refrigerator (4°C) for further study.

2.4. Screening of Potent Cellulase and Xylanase-Producing Isolates by Qualitative and Quantitative Enzyme Assay

Gut isolates were primarily evaluated for a qualitative determination of extracellular cellulase and xylanase-producing capacities following growth (30°C, 48 h) on the selective media plates containing respective substrates. The cellulase-producing capacity was determined on CMC plates flooded with Congo red prepared with 0.7% agarose (Teather and Wood, 1982). Congo red selectively binds with unhydrolyzed carbohydrate polymers. The appearance of a clear zone (halo), due to the presence of hydrolyzed CMC, indicated a cellulase production in the medium. Positive xylanolytic isolates were distinguished after flooding the XY plates with Congo red solution [0.5% Congo red (w/v) and 5% ethanol (v/v)] 5 min., followed by repeated decolorizaion with 1 M NaCl (Ninawe *et al.*, 2006). The appearance of halo, owing to the hydrolyzed XY surrounding the bacterial colony, indicated xylanase production in the medium. Isolates producing a halo of ≥10 mm in excess of colony growth in each case were selected for quantitative enzyme activity.

Proficient NSP-degrading strains were identified through the quantitative assay of the extracellular cellulase and xylanase production. Respective selective broth media were used to obtain the enzymes. The culture flasks were incubated (37°C, 72 h) with vigorous shaking (150–170 rpm), centrifuged at 10,000*g* (4°C, 10 min), and the cell-free supernatant containing the enzymes was collected (Bairagi *et al.*, 2002). The quantitative assay for cellulase production was performed following the method described by Denison and Koehn (1977) using 1% CMC in sodium citrate buffer (0.1 M, pH 5.0) as substrate. Xylanase activity was assayed using 1% birch-wood xylan as the substrate as described by Bailey *et al.* (1992) and using D-xylose as the standard. Production of reducing sugar (glucose) from the substrate due to cellulolytic or xylanolytic activity was measured at 540 nm by the dinitrosalicylic acid method (Miller, 1959) using glucose or D-Xylose as the standard. Unit activity (U) of cellulase was defined as the µg of glucose liberated mL^{-1} of enzyme extract min^{-1}. Xylanase activity (U) was defined as the mg of D-xylose liberated mL^{-1} of enzyme extract min^{-1}.

2.5. Identification of Isolates by 16S rRNAGene Sequence Analysis

The most promising NSP-degrading strains (bacteria) were identified through 16S rRNA partial gene sequence analysis after isolation and PCR (polymerase chain reaction) amplification following the methods described in Das *et al.* (2014). To prepare template DNA, pure colonies were suspended in sterilized saline, centrifuged (12,000*g*, 10 min), supernatants removed and the pellets suspended in InstaGene Matrix (Bio-Rad, USA). DNA isolation was carried out following the manufacturer recommendations. PCR amplification of the 16S rRNA gene was performed using universal primers, 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). PCR was executed using a PCR mix containing 200 µM of deoxynucleotides (dNTPs), 0.2 µM of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 U of Taq DNA polymerase (Invitrogen) and 1 µL of template DNA. The cycle used for PCR was: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 3 minutes (Lane, 1991). *E. coli* genomic DNA was included as positive control. PCR products were purified using Montage PCR Clean up kit (Millipore, USA). The sequencing of the purified PCR products was performed using the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an automated DNA sequencing system (Applied BioSystems 3730XL, USA). Sequence data were edited using BioEdit Sequence

Alignment Editor (Version 7.2.0); then they were aligned and analyzed to find the closest homolog using Basic Local Alignment Search Tool (BLAST) in National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases. Sequences were deposited in the NCBI GenBank and accession numbers were obtained. A phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using the MEGA 5.1Beta4 software following the Minimum Evolution Method.

2.6. Fish Pathogens and Culture Maintenance

Four fish pathogenic strains: *Aeromonas salmonicida* MTCC-1945 (AS), *Aeromonas sobria* MTCC-3613(AB), *Aeromonas hydrophila* MTCC-1739 (AH), and *Aeromonas veronii* (KT737240) (AV) were used to evaluate pathogen inhibitory activity of the promising cellulase and xylanase-producing strains. The pathogenic strains were maintained in the laboratory on T SA (HiMedia, Mumbai, India) slants at 4°C. Stock cultures in Tryptone Soya Broth (TSB) were stored at -20°C in 0.85% NaCl with 20% glycerol to provide stable inoculums throughout the study (Sugita *et al.*, 1998).

2.7. In Vitro Antagonistic Activity and Test of Compatibility

Pathogen inhibitory activity of the selected strains was studied against the four *Aeromonas* spp. utilizing the 'cross-streaking' method (Madigan *et al.*, 1997). The NSP-degrading bacterial isolates were cultured with previously isolated eight autochthonous fish gut bacteria and two yeasts: *Bacillus subtilis subtilis* (JX292128), *Bacillus atrophaeus* (HM246635), *Bacillus subtilis* (HM352551), *Bacillus pumilus* (KF454036), *Bacillus flexus* (KF454035), *Bacillus methylotrophicus* (KF559344), *Bacillus subtilis* subsp. *spizizenii* (KF559346), *Enterobacter hormaechei (KF559347)*, *Pichia kudriavzevii* (KF479403), and *Candida rugosa* (KF479404). Source and description of these autochthonous gut microorganisms were presented in previous studies (Das and Ghosh, 2013; Khan and Ghosh, 2012; Banerjee *et al*. 2015; Mukherjee and Ghosh 2014; Banerjee and Ghosh, 2014). Selected strains were inoculated as a line onto TSA media plates and incubated at 30°C for 24 h. Subsequently, pure cultures of the autochthonous fish gut microorganisms (8 bacteria, 2 yeasts) were inoculated as a p erpendicular line to the NSP-degrading bacterial strains keeping a gap of about 1 mm. Following incubation (30°C, 24 h) , the growth of microorganisms was examined and the disappearance of the gap indicated compatibility (co-existence) of the autochthonous yeast and bacteria strains with the NSP-degrading bacterial strains.

2.8. Safety of the Selected Isolate

The safety of the selected isolates (LRF1X, CMF1C, HMF6X, CtIF1C, and CMH8X) was evaluated through *in vivo* studies conducted in 350 L FRP tanks using 90 healthy *L. rohita* (average body weight: 15±1.7 g) divided into six groups (five experimental and one control), each with three replicates. The fish were acclimatized for 2 weeks in FRP aquaria under laboratory conditions. Group 1 fishes were kept as control, and each fish received Intra-Peritoneal (IP) injection of 1.0 mL SSS (Mesalhy *et al.*, 2008). The five selected NSP-degrading bacterial isolates were grown in Tryptone Soya Broth (TSB) at 30°C for 24 h, centrifuged (2800*g* for 15 min, 4°C), and cell pellets were suspended in SSS. Each fish in the experimental groups (groups 2, 3, 4, 5, and 6) was given IP injection of 1.0 mL saline containing 10^9 cells ml^{-1} of each test bacterium. All groups were kept under observation for 21 days for likely development of any disease symptom.

2.9. Statistical Analysis

Statistical analysis of quantitative enzyme activity data was performed using the analysis of variance (ANOVA) followed by Tukey's test according to Zar (1999) using Statistical Package for the Social Sciences (SPSS) Version 10 (International Business Machines Corporation, Armonk, New York) (Kinnear and Gray, 2000).

3. Results

Enumeration of gut microbial communities in the GI tracts revealed that considerable amounts of autochthonous aerobic or facultative anaerobic culturable heterotrophic, as well as cellulose and xylan-degrading microorganisms, were present in both the PI and DI regions of all the fish species studied (Table 2). The highest counts of cellulase-producing bacteria were noted in the DI region of mrigal, C. mrigala (LVC=6.14 g^{-1} intestinal tissue), followed by the DI region of catla, C. catla (LVC=5.97 g^{-1} intestinal tissue). Similarly, the highest counts of xylanase-producing bacteria were noted in the DI region of silver carp, H. molitrix (LVC=5.34 g^{-1} intestinal tissue), followed by the DI region of rohu, L. rohita (LVC=5.04 g^{-1} intestinal tissue).

Table 2. Log viable counts (LVC) of cellulase and xylanase producing autochthonous (adherent) bacteria isolated from theproximal (PI) and distal (DI) parts of intestine of the fish species examined.

Fish species and intestinal site of isolation		LVC g^{-1} intestinal tissue		
		Heterotrophic count on TSA plate	Bacterial count on cellulase plate	Bacterial count on xylanase plate
L. rohita	PI	6.98	5.12	4.94
	DI	7.11	5.45	5.04
C. catla	PI	6.32	5.45	4.19
	DI	6.65	5.97	4.54
C. mrigala	PI	6.95	5.07	4.26
	DI	7.54	6.14	4.82
H. molitrix	PI	7.25	4.95	4.93
	DI	7.85	5.26	5.34
C. idella	PI	5.54	4.26	4.14
	DI	5.96	4.69	4.28
C. carpio	PI	6.48	4.65	3.81
	DI	6.89	4.86	4.56

Altogether, 432 microbial strains were isolated on the media containing either cellulose or xylan. Seventy strains were primarily selected through qualitative enzyme assay, from which 30 microbial strains were further studied by quantitative assay to select the most promising isolates.

Data pertaining to quantitative assay of cellulase and xylanase activities for the 30 promising strains (12 from PI and 18 from DI), 5 strains from each of the 6 fish species, are presented in Table 3. This led to the selection of 5 promising NSP-degrading strains (LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X)

Table 3. Quantitative assay of the enzyme activity (unit activity, U) by the selected bacterial isolates. Numbers within the parenthesis denote total numbers of isolates from the respective fish species examined.

Source and number of isolates*	Strains[#]	Activity of NSP-degrading enzymes	
		Cellulase[†]	Xylanase[+]
L. rohita (PI-38, DI-47)	LRH4X	55.48±2.48^{f}	12.38±1.21^{d}
	LRF1X	64.61±2.39^{g}	24.25±1.29^{g}
	LRH3C	44.55±1.12^{e}	7.07±0.26^{b}
	LRH5C	48.61±1.04^{e}	9.26±0.29^{c}
	LRH5X	49.14±1.19^{e}	8.81±0.31^{c}
C. catla (PI-25, DI-39)	CCF1C	17.34±1.62^{ab}	11.38±0.44^{d}
	CCF1X	20.41±1.71^{b}	7.59±0.65^{b}
	CCF2X	21.51±1.64^{b}	7.67±0.54^{b}
	CCF3X	23.58±1.81^{b}	8.48±0.54^{bc}
	CCH4C	19.36±1.64^{ab}	7.73±0.59^{b}
C. mrigala (PI-30, DI-35)	CMF1C	36.51±1.26^{d}	15.41±1.17^{f}
	CMF3C	13.35±1.08^{a}	15.18±1.06^{f}
	CMH2C	16.73±0.75^{a}	10.58±0.72^{cd}
	CMH3X	20.68±1.01^{b}	11.43±1.06^{f}
	CMH8X	27.62±1.25^{c}	16.34±1.21^{f}
H. molitrix (PI- 34, DI-50)	HMF1C	24.34±1.11^{bc}	10.24±0.27^{c}
	HMF1X	23.44±1.25^{b}	8.13±0.45^{bc}
	HMF6X	48.46±2.15^{d}	23.28±1.37^{g}
	HMH1C	23.53±1.72^{b}	13.25±1.27^{e}
	HMH5X	22.41±1.29^{b}	8.23±0.31^{bc}
C. idella (PI-26, DI-37)	CtIF1C	26.35±1.21^{c}	13.41±1.29^{e}
	CtIF2C	24.46±1.20^{bc}	11.33±1.01^{d}
	CtIH1X	14.41±1.23^{a}	8.95±0.31^{c}
	CtIH2X	21.48±0.94^{b}	8.14±0.29^{bc}
	CtIH3X	29.14±1.18^{c}	4.65±0.28^{a}
C. carpio (PI-26, DI-45)	CyCH3C	22.26±1.72^{b}	7.43±0.31^{b}
	CyCH4C	17.89±1.25^{ab}	6.84±0.29^{a}
	CyCH5C	17.39±1.19^{ab}	7.24±0.27^{b}
	CyCH6C	21.76±1.14^{b}	8.35±0.26^{bc}
	CyFH5X	16.65±1.14^{a}	6.66±0.27^{b}

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

*PI: Proximal intestine; DI: Distal intestine

[#]The alphabets 'F' and 'H' before the numeric value indicate isolates from PI and DI, respectively

[†]µg of glucose liberated mL^{-1} of enzyme extract min^{-1}

[+] mg of D-xylose liberated mL^{-1} of enzyme extract min^{-1}

The strain LRF1X, isolated from the PI of *L. rohita*, exhibited the highest cellulolytic activity (64.61±2.39 U). A considerable cellulase activity was also noted with other strains isolated from *L. rohita*. On the other hand, the highest xylanase activity was recorded in LRF1X (24.25±1.29 U), followed by the strain HMF6X (23.28±1.37 U). Strains CMF1C (15.41±1.17 U), CMH8X (16.34±1.21 U), and CtIF1C (13.41±1.29 U) also exhibited a substantial xylanase activity. Considering the cumulative activities of the two NSP-degrading enzymes, isolates LRF1X, CMF1C, HMF6X, CtIF1C, and CMH8X were finally selected for identification and for possible future evaluation of use.

Based on nucleotide homology and phylogenetic analysis of the 16S rRNA partial gene sequences and using the nucleotide blast in the NCBI GenBank and RDP databases, the strains LRF1X and CMF1C were identified as *Bacillus pumilus* (Accession no. KF640221 and KF640223, respectively), which were closest to the type strain *Bacillus pumilus* (Accession no. AY876289). Another isolate, HMF6X, was identified as *Bacillus tequilensis* (Accession no. KF640219) as it showed a maximum similarity with the type strain *Bacillus tequilensis* (Accession no. HQ223107). Likewise, NCBI GenBank and RDP databases revealed that CtIF1C and CMH8X were similar to *Bacillus megaterium* (Accession no. KF640220) and *Bacillus altitudinis* (Accession no. KF640222), respectively. The isolate CtIF1C showed a similarity with *Bacillus megaterium* (Accession no. D16273), while the isolate CMH8X was closest to *Bacillus altitudinis* (Accession no.AJ831842). The identities of the promising NSP-degrading fish gut bacteria and their homology with the closest type strains are presented in Table 4. Phylogenetic relation of the five identified cellulase and xylanase-producing bacteria with closely related type strains, retrieved from the RDP database, are presented in the dendogram (Figure 1).

To verify the pathogen inhibitory activity, five selected NSP-degrading bacterial isolates were screened against four different strains of fish pathogenic *Aeromonas* spp. Results of the pathogen inhibitory activity, as revealed by the cross streaking method, are depicted in Table 5. Four strains (LRF1X, CMF1C, HMF6X, and CtIF1C) were antagonistic towards two of the tested *Aeromonas* spp. The strain CMH8X showed antagonism only towards *Aeromonas veronii*. Three strains (LRF1X, CMF1C, and HMF6X) were antagonistic towards *Aeromonas salmonicida*.

An *in vitro* co-culture test against eight autochthonous fish gut bacteria and two yeast isolates revealed that the selected bacterial strains did not affect the growth of the autochthonous gut microbiota. Therefore, the selected NSP-degrading bacterial isolates were considered compatible with commonly occurring autochthonous fish gut microbiota.

After 21 days of a small-scale *in vivo* experiment involving an intra-peritoneal injection of the selected bacterial isolates, no pathological signs, disease symptoms, or mortalities were observed in either the experimental sets or the control set.

Table 4. Identities of the promising NSP-degrading fish gut bacteria and their homology with the closest type strains in RDP.

Strain(s)	Closest type strains in RDP	S_ab score	Homology levels (Max. indent)	Bacterial species	NCBI GenBank accession number(s)
LRF1X	*Bacillus pumilus* AY876289	0.967	99%	*Bacillus pumilus*	KF640221
CMF1C	*Bacillus pumilus* AY876289	0.971	99%	*Bacillus pumilus*	KF640223
HMF6X	*Bacillus tequilensis* HQ223107	0.972	99%	*Bacillus tequilensis*	KF640219
CtIF1C	*Bacillus megaterium* D16273	0.915	98%	*Bacillus megaterium*	KF640220
CMH8X	*Bacillus altitudinis* AJ831842	0.962	99%	*Bacillus altitudinis*	KF640222

Table 5. Inhibition area (mm) produced by the selected gut bacteria (in excess of colony growth) incross streaking method against the tested fish pathogens.

Strains	AH	AS	AB	AV
LRF1X	-	2	2	-
CMF1C	1	1	-	-
CMH8X	-	-	-	2
HMF6X	2	3	-	-
CtIF1C	-	-	2	1

AH: *Aeromonas hydrophila*; AS:*Aeromonas salmonicida*; AB:*Aeromonas sobria*;

AV: *Aeromonas veronii*;

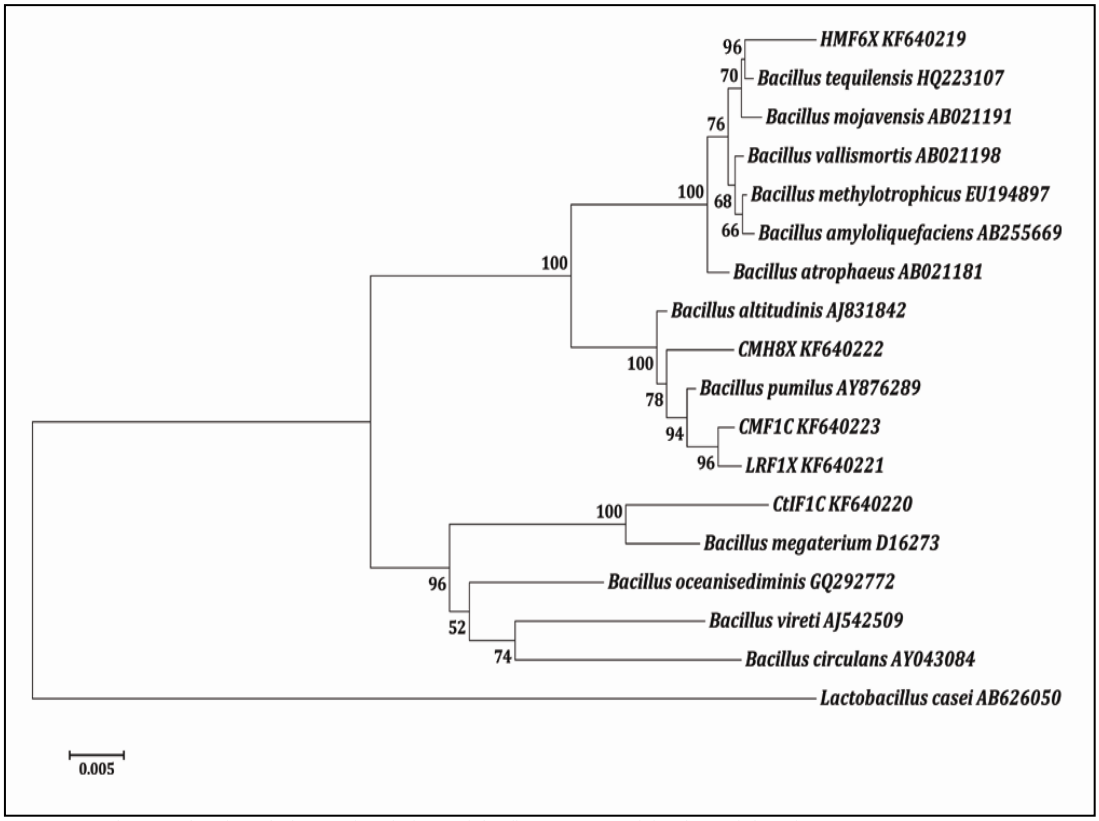

Figure 1. Dendogram showing phylogenetic relations of the five most promising bacterial strains, LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X, with other closely related type strains retrieved from NCBI GenBank. The GenBank accession numbers of the reference strains are shown in parentheses. Horizontal bars in the dendogram represent the branch length. Similarity and homology of the neighboring sequences have been indicated by bootstrap values. Distance matrix was calculated using Kimura 3-parameter model. The scale bar indicates 0.005 substitutions per nucleotide position. *Lactobacillus casei* AB626050.1 served as an out group control.

4. Discussion

Several plant-eating animals require the support of symbiotic microorganisms within their GI tracts to degrade complex polysaccharides or secondary metabolites to make the energy in these compounds available to the host (Karasov and Martinez del Rio, 2007). Likewise, the previous studies on fish advocated a possible degradation of starch (Ghosh *et al*., 2002, 2010), phytate (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012), tannin (Mandal and Ghosh, 2013), and cellulose (Saha and Ray, 1998; Bairagi *et al*., 2002; Ghosh *et al*., 2002, 2010; Saha *et al*., 2006; Mondal *et al*., 2008) by the gut-associated microorganisms in diverse carp species. Although the existence of a cellulase activity in the digestive system of fish was in conflict with studies providing contradictory results, later on it became commonly accepted that fish lack the cellulase enzyme, as is the case with other monogastric and ruminant animals, with cellulose degradation being mediated through the action of cellulase produced by the fish gut microbiota (Ray *et al*., 2012). On the other hand, reports on xylanase production by fish gut endosymbionts are scanty. Many microorganisms, including bacteria (Balakrishnan *et al*., 2002; German and Bittong, 2009; Azeri *et al*., 2010), actinomycetes (Techapun *et al*., 2001; Tuncer *et al*., 2004), and filamentous fungi (Taneja *et al*., 2002; Angayarkanni *et al*., 2006; Sudan and Bajaj, 2007), have been reported to produce xylanase. Whether the fish gut sustains any autochthonous xylanase-producing microorganisms has not been authenticated yet, except for one report documenting the occurrence of xylanase-producing yeasts in some freshwater carps (Banerjee and Ghosh, 2014). Nonetheless, both cellulase and xylanase were assumed to be produced by microbes ingested by the fish with detritus rather than produced by a resident endosymbiotic community (German and Bittong, 2009). In the present study, heterotrophic as well as cellulase and xylanase-producing microbial symbionts were detected in the PI and DI regions of the GI tracts in 6 freshwater carp species studied. As the fish were starved for 48 h and their GI tracts were carefully cleansed with sterilized and cooled 0.9% saline prior to the isolation of microorganisms, it may be corroborated that the microorganisms isolated in the present study belonged to the autochthonous (adherent) microbiota as suggested elsewhere (Ghosh *et al*., 2010). The occurrence of a higher proportion of heterotrophic microorganisms in DI regions, compared to the PI regions, was in agreement with the previous reports (Mondal *et al*., 2008; Das *et al*., 2014). This suggests that the degradation of the feedstuffs within these parts of the GI tract occurs in assistance with the well settled enzyme-producing microbiota (Ghosh *et al*., 2010). The highest population counts of cellulase and xylanase-producing producing microbial symbionts were noted in the DI regions of mrigal, *C. mrigala* and silver carp, *H. molitrix*, respectively. Both species were either herbivores or feeding on de tritus arising out from the plant feedstuffs (Jhingran, 1997). In addition, other carp species also harbored substantial amounts of NSP-degrading microorganisms within their GI tracts. Therefore, the presence of an appreciable quantity of culturable heterotrophic cellulase and xylanase-producing microbiota in both PI and DI regions of the GI tracts of the fish species studied might indicate their probable role in the degradation of NSPs in the plant feedstuffs.

Cereal by-products, oil cakes, and natural feeds like phytoplankton and aquatic weeds, contain considerable amounts of NSPs (cellulose and xylan) in their cell wall material. For instance, rice bran, generally used as an essential component in fish feed, contains approximately 20-25% NSP, consisting of approximately equal amounts of arabinoxylans and cellulose (Saunders, 1986). Anti-nutritional effects of NSPs in monogastric animals were generally associated with the viscous nature of the polysaccharides. High gut viscosity decreases the rate of diffusion of substrates and digestive enzymes and hinders their effective interaction at the mucosal surface (Edwards *et al*., 1988; Ikegami *et al*., 1990). The higher viscosity can reduce the rate of gastric emptying leading to satiety which then decreases the feed intake (Roberfroid, 1993). NSP degrading enzymes (e.g., cellulase and xylanase) cleave the large molecules of NSP into smaller polymers, thereby reducing the thickness of the gut content and increasing the nutritive value of the feed (Bedford *et al*., 1991; Choct and Annison, 1992).Therefore, the strategy to detoxify/degrade plant-derived anti-nutrients through the enzymes, produced by gut microbiota, might be regarded as an evolutionary adaptation as is the case for ruminant and non-ruminant herbivores (McBee, 1971).

As the major aim of the present investigation was to detect the efficient cellulase and xylanase-producing strains within the GI tracts of the freshwater carps (if any), the preliminary screening for extracellular cellulase and xylanase production has resulted in the elimination of 83.8% (362 out of 432) of the total isolates from current study. Furthermore, 5 e fficient NSP-degrading strains were established through quantitative cellulase and xylanase assay and identified on the basis of 16S rRNA partial gene sequence analysis. The strains LRF1X and CMF1C were identified as strains of *Bacillus pumilus* (KF640221 and KF640223), while the strains HMF6X, CtIF1C, and CMH8X, were identified as *B. tequilensis* (KF640219), *B. megaterium* (KF640220), and *B. altitudinis* (KF640222), respectively. All promising NSP-degrading isolates belonged to *Bacillus* spp., which might support the hypothesis that gut bacteria in freshwater carps were dominated by diverse strains of *Bacillus* spp. (Ghosh *et al*., 2010; Ray *et al*., 2012). The occurrence of *B. pumilus* (Ghosh *et al*., 2002) and *B. megaterium* (Saha *et al*., 2006) within the gut of freshwater carps was reported previously. However, to the best of the authors' knowledge, *B. tequilensis* and *B. altitudinis* have not been documented from carp gut so far.

The abundance of cellulase-producing bacteria has been documented in the GI tracts of grass carp, *C. idella* (Bairagi *et al*., 2002; Saha *et al*., 2006; Li *et al*., 2009), common carp, *C. carpio* and silver carp, *H. molitrix* (Bairagi *et al*., 2002), rohu, *L. rohita* (Saha and Ray, 1998; Ghosh *et al*., 2002; Kar and Ghosh, 2008; Ray *et al*., 2010), catla, *C. catla* and mrigal, *C. mrigala* (Ray *et al*., 2010), and bata, *L. bata* (Mondal *et al*., 2008,2010).Furthermore, the xylanase-producing ability by gut inhabiting bacterial symbionts from freshwater

carp species has not been documented to date. Results of the present study might suggest considerable opportunities for using cellulase as well as xylanase-producing bacterial symbionts from the gut of freshwater carps as aquaculture probiotics that might aid in the degradation of complex polysaccharides in feedstuffs within the gut microenvironment. In addition, *in vitro* degradation of NSPs in plant ingredients by autochthonus NSP-degrading bacteria could be an alternative approach of processing as the bacteria itself or their metabolites would not impair the normal function in fish because of the mutual relationship therein, as suggested previously for phytate-degrading gut bacteria (Khan and Ghosh, 2013).

Apart from nutritional benefits, the enzyme-producing gut bacteria in fish have been assumed to compete continuously with pathogens through the competitive exclusion or the production of antimicrobial compounds (Ray *et al*., 2012). Although several strains of Bacilli were demonstrated as probiotics for fish, antagonism of pathogens by the bacilli has been seldom indicated. *B. subtilis* SG4 (Ghosh *et al*., 2007) and *B. amyloliquefaciens* (Dutta and Ghosh, 2015) isolated from the gut of mrigal, *C. mrigala*, *B. methylotrophicus* isolated from channel catfish (Chao *et al*., 2012) and catla, *Catla catla* (Mukherjee and Ghosh, 2014) intestines, and *B. cereus* and *B. circulans* isolated from the gut of other fish species (Lalloo *et al*.,2010; Geraylou *et al*., 2014), were reported to exhibit pathogen inhibitory potential against different strains of *A. hydrophila*. In the present study, most of the promising NSP-degrading strains (4 out of 5) displayed antagonism against at least two of the four tested *Aeromonas* spp. To the best of the authors' knowledge, cellulase or xylanase-producing ability along with pathogen inhibitory potential of gut microflora from major carps depicted in the present study has not been reported previously. Furthermore, the test of compatibility revealed that the promising NSP-degrading strains did not affect the growth of previously isolated autochthonous fish gut bacteria and yeasts. Therefore, the present study might substantiate the likely co-existence of a diverse autochthonous gut microflora with the NSP-degrading strains as indicated in some preceding reports (Banerjee and Ghosh, 2014). The small scale in vivo experiment, involving the selected five strains, did not reveal any pathological lesions or mortality in the experimental fish model, *L. rohita*. Therefore, the *in vivo* application of the selected NSP-degrading strains, characterized in the present study, is considered safe, and could pave avenues for prospective future applications.

5. Conclusion

This preliminary study validates the occurrence of NSP-degrading bacterial symbionts within GI tracts of freshwater carps. However, autochthonous aerobic or facultative anaerobic gut bacteria were isolated in the present study by culture-dependent methods. Further studies involving anaerobic and culture-independent methods (such as Denaturing Gradient Gel Electrophoresis, Next Generation Sequencing, etc.) might be helpful in getting more information on the diversity of NSP-degrading microbiota within the fish GI tract. Furthermore, NSP-degrading and pathogen inhibitory bacteria, noticed in the present study, appear to provide the host with some ecological benefits by enabling them to conquer the adverse effects of NSPs and aeromonads. Whether the isolated gut bacteria can contribute to the host's nutrition and health has not been dealt with in the present study; a consideration of their function *in vivo* should be given high precedence in upcoming studies before advocating their utilization in commercial aquaculture.

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