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ORIGINAL ARTICLE

Assessing Probiotic Potential of Gut Microflora from Indian Major Carps

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ABSTRACT
The presence and distribution of enzyme-producing bacteria in the Proximal (PI), Middle (MI) and Distal (DI) segments of the gastrointestinal tracts of Indian Major Carp fresh water fishes (Catla catla, Labeo rohita and Cirrhinus mrigala) are studied. The data are represented as log viable counts per gram of intestine (LVC). Except for L. rohita, the heterotrophic bacterial community is found to be the most common in the DI area of all fish species investigated. Proteolytic and amylolytic bacteria are recorded as the most common in the DI, while cellulolytic and lipolytic populations are found more common in the DI of C. mrigala and C. catla, respectively. The most promising three bacterial isolates are found using a quantitative enzyme assay and identified using a 16S rRNA gene sequence analysis. VDC7 isolated from C. catla and VDR11 isolated from L. rohita both exhibited high similarities to distinct strains of Bacillus marisflavi, where as VDM3 obtained from C. mrigala is comparable to Bacillus oceanisediminis. The 16S rRNA gene sequences for isolates VDC7, VDR11 and VDM3 are assigned to the NCBI GenBank with accession codes KF377322, KX495267.1 and JQ660684.1, respectively. The current study may establish the way for further research into the potential applications of gut-associated extracellular enzyme generating bacteria as a Probiotic in fresh water aquaculture.
Keywords: Aquaculture, Exoenzymes, Probiotic

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INTRODUCTION

The microflora found in the gastrointestinal (GI) tracts of freshwater fish species has received a lot of attention [1-5]. The nutrient-rich GI tract of fish provides an ideal habitat for the growth of these bacteria [6]. The role of commensally intestinal microflora in fish has been better understood over the last decade [7-11]. The ability of the gut microflora to colonize and cling to the mucus layer in the digestive system determines whether it is autochthonous (indigenous) or allochthonous (transient) [12, 13]. The bacterial flora in fish's GI tract has a very broad and variable enzymatic capacity, and these enzymatic masses may favourably interfere with fish digestion [3]. Fish gut bacterial isolates have been shown to break down chitin [14-16], p-nitrophenyl-b-N-acetylglucosamine and protein [15, 17],as well as cellulose [11,18-20].
Previous research in carps has advocated for the nutritional benefits of gut-associated bacteria in the host fish [21, 22]. The hunt for extracellular enzyme-producing beneficial gut bacteria to employ as probiotics for culturable brackish water fish species may be of relevance in this context. As a result, the primary goal of this work is to identify autochthonous extracellular enzyme producing bacteria in the GI tracts of three culturable Indian Major Carp Fish: proximal (PI), middle (MI) and distal (DI) (*Labeo rohita, Catla catla and Cirrhinus mrigala*). Furthermore, the study aimed to assess the potential of gut bacteria to produce protease, amylase, cellulase and lipase as well as to identify the most promising bacterial strains using 16S rRNA gene sequence analysis.

MATERIAL AND METHODS

Test fish

At the time of harvest, three fresh water fish species such as *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*, were taken from three fresh water culture ponds in Vadodara, Gujarat, India, and transported to the laboratory in sterile plastic bags transferred with cooling conditions. The physicochemical values of pond water were analyzed pH raged between 8.1 and 8.4, while water temperatures ranging between 29.8 and 31.4 °C. Table 1 shows the feeding patterns, average fresh weight, average fish length and average gut weight of the fishes studied.

Processing of specimens

For the present investigation, nine specimens of each fish species were collected from three ponds and three from each pond. For surface cleaning, the ventral surface of each fish was thoroughly cleaned with one percent iodine solution [23]. Fish's alimentary tracts were removed aseptically within a laminar airflow. With minor alterations, gut samples were treated for the extraction of adherent (autochthonous) bacteria as described by Midhun [24]. According to Ghosh [2] the GI tracts were split into proximal (PI), middle (MI), and distal (DI) segments, cut into pieces, and carefully cleaned three times with 0.9 % sterile saline solution using an injection syringe to eliminate nonadherent (allochthonous) microflora. Gut segments from four specimens of the same species obtained from the same pond were pooled together region-wise for each replicate, resulting in three replicates for each gut segment of each fish species. As previously mentioned, the gut segments were homogenised with 10 parts sterile, prechilled 0.9%NaCl solution [25]. Pooled samples were used to avoid erroneous conclusions due to individual differences in gut microbiota as previously documented [2, 26].

Microbial culture

Individual homogenized sample of each gastrointestinal region was used after suitable repeated (1:10) dilutions [27-29]. To assess the culturable heterotrophic autochthonous aerobic/facultative anaerobic bacterial community, diluted samples (0.1 mL) were spread aseptically in sterile Tryptic Soy Agar (TSA, HiMedia, India) plates. To assess protease, cellulase, amylase and lipase-producing bacterial populations, diluted samples (0.1 mL) were spread on Peptone Gelatin Agar (PGA), Carboxy Methyl Cellulose agar (CMC), Starch Agar (SA), and Tri Butyrin Agar (TBA) plates. The culture plates were incubated at 30 °C for 48 hrs. The Colony-Forming Units (CFUs) per unit sample volume of gut homogenate were computed by multiplying the number of colonies on each plate by the reciprocal dilution [30], and the results are given as Log Viable Counts g^{-1} intestine (LVC). Colonies from a single plate with seemingly separate morphological traits (such as colour, texture, consistency and margin) were streaked individually on several plates to obtain pure cultures.

Screening of isolates by qualitative assay for exoenzyme production

Out of the 45 extracellular enzyme-producing isolates from the fish species studied, 21 isolates were chosen for qualitative enzyme testing (based on growth potential at 30 °C). Isolates were inoculated on SA plates and incubated at 30 °C for 48 hours to produce extracellular amylase. The culture growth was flooded with a one percent Lugol's iodine solution to detect amylase activity by observing the formation of a translucent zone (halo) around the colony [31]. Similarly, extracellular protease isolates were incubated on PG plates for 48 hours at 30 °C; the emergence of a halo after flooding the plates with 15% $HgCl_2$ showed the presence of proteolytic activity. Isolates cultured on CMC plates at 30 °C for 48 hours were flooded with Congo red dye 0.7 percent agarose to determine cellulase production [32]. Congo red binds to unhydrolyzed CMC alone. The development of a halo around the bacterial colony due to the presence of hydrolyzed CMC suggested cellulase synthesis in the medium. In the plates containing 1% tributyrin, lipase producers showed a halo around their colony [33]. Each experimental set contained three replicates. The following ratings were assigned to qualitative extracellular enzyme activity based on the assessment of the halo (diameter in mm) around the colony: 0 (zero to five millimetres), 1 (low, 6–10 millimetres), 2 (moderate, 11–15 millimetres), 3 (good, 16–20 millimetres), 4 (high, 21–25 millimetres) and 5 (extremely high, >25 millimetres).

Quantitative enzyme assay

Based on the qualitative results, eight extracellular enzyme-producing isolates were chosen for quantitative testing. The promising isolates were screened using broth culture. The quantitative assays for amylase, cellulase, protease and lipase production were carried out using the procedures given by Das [34], Denison and Koehn [35], Walter [36] and Dutta [37] respectively. A full description of extracellular enzyme production measurement and quantitative enzyme assay has already been mentioned [7]. Units were used to express quantitative enzyme activity (U).

Identification of isolates

The most promising three extracellular enzyme generating isolates were identified using 16S rRNA partial gene sequence analysis. Polymerase Chain Reaction (PCR) was applied to amplify the gene encoding 16S rRNA from the isolates using universal primers 27f (5-AGAGTTTGATCCTGGCTCAG-3) and 1492r (5-GGTTACCTTGTTACGACTT-3). The PCR reactions were carried out with a PCR mix that contained 200 M of deoxynucleotides (dNTPs), 0.2 M of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer and 0.2U of Taq DNA polymerase (Invitrogen). The template DNA was acquired by extracting genomic DNA from a new colony grown on a nutrient agar slant with the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). For the PCR reaction, the following cycle reaction conditions were used: an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles at 95 °C for 1 minute, annealing at 55 °C for 1 minute, extension at 72 °C for 2 minutes and a final extension at 72 °C for 3 minutes [38]. The PCR products were out sourced for Sanger sequencing on an automated DNA sequencer (Biokart Pvt. Ltd.). Using a mix of the NCBI GenBank and RDP databases, sequenced data were aligned and examined to determine the closest homolog of the microorganisms.

Statistical analysis

As shown by Zar [39], data pertaining to particular extracellular enzyme production by the selected isolates were subjected to analysis of variance (ANOVA) followed by Tukey's test [40].

RESULTS

Fishes were collected from selected fresh water ponds were subject to isolation of Gut microflora for determining their, probiotic applications. Potential bacterial isolates that produces important extracellular amylases, proteases, lipases and celluloses have been analyzed for qualitative and quantitative enzyme production. Table 2 shows heterotrophic as well as protease, cellulase, amylase and lipase producing bacterial communities found in the PI, MI and DI segments of the GI tracts of all the fish species are studied. The heterotrophic population on TSA plates varies between fish species and regions of the gut, according to an analysis of the bacterial communities in the GI tracts of three freshwater Indian major carp fish. The bacterial population on TSA plate was highest in *C. catla's* DI region (LVC = 7.99 g^{-1} of intestine), followed by *L. rohita* (LVC = 7.28 g^{-1} of intestine) and lowest in *C. catla's* PI region (LVC = 6.69 g^{-1} of intestine). The DI region of *L. rohita* contained the largest amylolytic bacterial population (LVC = 5.75 g^{-1} of intestine) followed by *C. catla* (LVC = 5.69 g^{-1} of intestine), and the MI region of *C. mrigala* had the lowest (LVC = 4.85 g^{-1} of intestine). Cellulase producing bacteria were most abundant in *C. catla's* DI region (LVC = 6.30 g^{-1} of intestine) followed by *L. rohita* (LVC = 6.10 g^{-1} of intestine) and least abundant in *C. mrigala's* PI region (LVC = 4.75 g^{-1} of intestine). The maximum concentration of proteolytic bacterial population was found in the MI region of *C. catla* (LVC = 5.41 g^{-1} of intestine) followed by *C. mrigala* (LVC = 5.27 g^{-1} of intestine), while the lowest concentration was found in the PI region of *L. rohita* (LVC = 4.64 g^{-1} of intestine). The DI region of *L. rohita* recorded the greatest rate of lipolytic bacteria (LVC = 5.78 g^{-1} of intestine), followed by the MI region of the same species (LVC = 5.76 g^{-1} of intestine). In total enzyme producing 21 bacterial isolates were primarily chosen from various fish species, and extracellular enzyme production by the bacterial isolates was qualitatively assessed. The maximum and minimum scores for qualitative extracellular enzyme activity were 17 and 2, respectively (Table 3). Eight bacterial isolates were chosen for the quantitative enzyme assay based on the qualitative assay. The quantitative enzyme assay results revealed considerable difference in enzyme activity between different bacterial isolates (Table 4). Maximum amylase activity was measured in isolate VDC7 (210.22 ±2.07 U) and cellulase activity was measured in VDR11 isolated from *L. rohita's* DI (43.27 ±1.30 U). VDC7 isolated from the DI of *C. catla* showed the highest protease activity (11.28 ±0.19 U), while VDM3 isolated from the DI of *C. mrigala* recorded the highest lipase activity (14.31 ±0.16 U). When all four enzymatic activities were considered, isolates VDC7, VDR11 and VDM3 were shown to have the most potential among the eight selected isolates. Isolates VDC7 and VDR11 were both identified as *Bacillus marisflavi* based on 16S rRNA gene sequences (GenBank Accession Nos. KF377322, KX495267.1 ,). The isolate VDM3 was found to be identical to *Bacillus oceanisediminis* with respect to all characteristics (GenBank Accession No-JQ660684.1).

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

Fish Species	Feeding Habit	Average weight (g)	Average fish length(cm)	Average gut weight(g)
Labeorohita	Omnivorous, mostly plant matter	253.4 (11.3)	22.7 (0.21)	16.6(0.41)
Catla catla	Zooplanktophagous	268.9 (7.60)	24.1 (0.17)	9.7(0.21)
Cirrhinusmrigala	Detritivorous	219.7 (5.16)	18.4 (0.14)	8.6(0.14)

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

LVC g^{-1} intestine					
Fish species	**Bacterial count in TSA plate**	**Amylolytic bacteria**	**Cellulolytic bacteria**	**Proteolytic bacteria**	**Lipolytic bacteria**
Labeorohita					
PI	6.91	4.91	5.27	4.64	5.58
MI	7.14	5.34	5.83	4.81	5.76
DI	7.28	5.75	6.10	5.03	5.78
Catla catla					
PI	6.69	5.33	5.15	4.83	5.08
MI	7.88	5.21	5.95	5.41	5.55
DI	7.99	5.69	6.30	5.20	5.09
Cirrhinusmrigala					
PI	6.78	4.87	4.75	4.79	4.96
MI	6.90	4.85	4.79	4.65	5.08
DI	7.25	4.92	5.12	5.27	4.86

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

Fish species	Bacterial strains	Enzyme activity (scores)*					
		Isolated from	**Amylase[1]**	**Cellulase[2]**	**Protease[3]**	**Lipase[4]**	**Total score**
Labeorohita	VDR1	PI	2	0	0	2	4
	VDR2	PI	0	1	0	2	3
	VDR3	MI	4	5	2	0	11
	VDR4	MI	3	2	2	2	9
	VDR5	DI	4	3	3	2	12
	VDR6	DI	2	2	2	2	8
	VDR7	DI	0	2	1	2	5
	VDR8	DI	0	0	0	2	2
	VDR9	DI	2	1	3	2	8
	VDR10	DI	3	4	3	1	11
	VDR11	DI	5	4	3	3	15
Catla catla	VDC1	PI	0	0	0	3	3
	VDC2	MI	2	1	2	1	6
	VDC3	MI	0	2	0	2	4
	VDC4	DI	2	3	3	1	9
	VDC5	DI	5	1	2	1	9
	VDC6	DI	2	3	0	1	6
	VDC7	DI	5	4	5	3	16
Cirrhinus mrigala	VDM2	PI	3	2	4	4	13
	VDM3	DI	4	5	3	5	17
	VDM4	DI	5	3	2	1	11

*With pure culture of bacterial isolates.
[1]On Starch Agar (SA) plate; [2]on Carboxy Methyl Cellulose(CMC) plate; [3]on Gelatin-Peptone(GP) plate; [4]on Tri Butyrin-Agar (TBA) plate.

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

	Enzyme activity (U)			
Bacterial strains	**Amylase@**	**Cellulase#**	**Protease$**	**Lipas***
VDR3	41.36 (±0.93)	41.36 (±0.93)	4.64 (±0.31)	3.84 (±0.44)
VDR5	24.21 (±2.11)	24.21 (±2.11)	6.64 (±0.24)	5.24 (±0.81)
VDR10	27.15 (±1.28)	27.15 (±1.28)	3.21 (±0.11)	4.20 (±0.54)
VDR11	207.31 (±1.48)	43.27 (±1.30)	10.15 (±0.49)	8.48 (±0.23)
VDC7	210.22 (±2.07)	42.22 (±0.40)	11.28 (±0.19)	6.95 (±0.74)
VDM3	42.15 (±0.14)	42.15 (±0.14)	8.75 (±0.32)	14.31(±0.16)
VDM2	19.28 (±0.18)	16.24 (±1.21)	6.34 (±0.27)	3.14 (±0.98)
VDM4	28.34 (±0.27)	28.34 (±0.27)	4.27 (±0.45)	2.29 (±0.71)

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

DISCUSSION

Diverse microbial communities in the GI tracts of freshwater or marine carnivorous, herbivorous, and omnivorous fish species have been reported by various scientists [41]. Digestive tracts of endotherms are colonized mainly by obligate anaerobes [42], while the predominant bacterial genera isolated from most fish guts have been aerobes or facultative anaerobes [7,23,43,44]. In the present investigation, aerobic/facultative anaerobic extracellular enzyme producing bacterial symbionts were detected in the GI tracts of three fresh water fish species. As the fish gut were thoroughly washed with sterile chilled 0.9% saline prior to isolation of microflora the microorganisms isolated in the present study can be considered as the autochthonous adherent microflora [2]. The biomass of microbiota present within the GI tract of fish is much higher than that of the surrounding water indicating that the GI tract of fish provides favorable ecological niches for these microorganisms [45]. However, isolation and identification alone might not give a realistic depiction of the gut microflora in different regions in the GI tract with an appraisal of their likely function [46]. Therefore, it was considered legitimate in the present study to quantify heterotrophic bacteria along with specific extracellular enzyme-producing bacteria in different regions in the Gut of the fish species studied. As the main objective of the present study is to collect information on extracellular enzyme-producing gut bacteria in some fresh water fishes from selected ponds of Vadodara district of Gujarat state.

In the present study, gut bacteria were isolated by conventional culture-based methods. It is generally argued that culture-dependent techniques are time-consuming, lack accuracy [47] and do not represent an exact image of the bacterial diversity present in the fish gut even if several selective media are used [3]. However, the utilization of a culture-based technique employing a selected substrate containing selective media is justifiable because the major aim of this study was to detect different extracellular enzyme-producing gut bacteria. Moreover, in the present study conventional methods in combination with 16S rRNA analysis have been employed to identify the potent enzyme-producing gut isolates [2, 3, 45].

Understanding the role of endosymbionts in digestion requires accurate knowledge about the relative importance of exogenous enzymes produced by GI endosymbionts and digestive enzymes produced by the host [48]. In all of the fish species considered in this study have significant amylolytic, proteolytic, cellulolytic and lipolytic bacterial communities (Table 2). These might have some relation with their eating habits. The presence of protease, amylase, cellulase, and lipase-producing bacterial communities in the digestive tracts of *L. rohita*, *C. catla* and *C. mrigala* is warranted because they are omnivore fish species. Cellulolytic bacteria are found in the GI tracts of all freshwater fish species tested in this investigation, supporting the concept that bacteria may play some role in cellulose degradation in fish [3]. Several studies have demonstrated the presence of a large population of cellulolytic bacteria and their critical role in extracellular cellulase synthesis in fish [7, 25, 45]. Singh *et al.,* [49] suggested that cellulose absorption occurs in the DI in a prior investigation with carp, which could indicate the presence of microbial cellulase in this area. The majority of cellulose-producing bacteria were found in the DI and MI of all fish species tested, which supports this observation. Apart from cellulolytic bacteria in *L. rohita*, *C. catla*, and *C. mrigala*, the heterotrophic microbial population is found to be the largest in the DI regions of all the fish species tested when compared to the PI and MI regions, which is in good aggrement with earlier results [2,3,45].

Extracellular enzyme production assays revealed that VDC7 and VDR11 isolated from the DI of *C. catla* and *L. rohita*, respectively, produced the most amylase and cellulase. However, VDC7 isolated from the DI of *C. catla* produced the most protease and lipase. When comparing the isolates from *C. catla* to the isolates from the other fishes investigated, the isolates from *C. catla* performed poorly on both qualitative and quantitative measures of extracellular enzyme production. Using a quantitative enzyme assay, the two most efficient enzyme-producing bacteria (VDC7 and VDR11) were identified as *Bacillus marisflavi* and *Bacillus oceanisediminis*, respectively, based on 16S rRNA sequence analysis. Freshwater and marine fishes' GI tracts have been found to include a variety of extracellular enzyme producing bacteria strains [41]. *Bacillus marisflavi* and *Bacillus oceanisediminis* are recorded and reported for the first time from the guts of fresh water fishes. Furthermore, there are very few reports on extracellular enzyme-producing

bacteria found in the guts of freshwater fish species [50]. GI bacteria produce a wide spectrum of enzymes, which could be a source of digestive enzymes in fish [41]. Understanding the physiological connections between the indigenous microflora and the host, as well as characterizing the microbial populations in the intestinal milieu of fish, could have significant implications [51]. Enzymes produced by intestinal fish microflora may play an important role in digestion, particularly for substrate like cellulose, which only a few species can digest, as well as other substrates [52]. Carbohydrates such as mannose, xylose, raffinose, cellobiose, and cellulose can be used by enzyme-producing gut bacteria [6]. These compounds are primarily present in plant diets. As a result, the ability of gut bacteria to produce cellulase and amylase may indicate that they can aid in the digestion of plant meals. In animal husbandry, the use of beneficial microorganisms as probiotics has a long history [53]. Beneficial bacteria could be added to customized fish feeds or in the form of bacteria biofilm to achieve a higher level of colonization in the GI tract in commercial aquaculture [2,7,8,21,44]. Beneficial gut microorganisms are thought to be constantly battling with pathogens via competitive exclusion [41]. These issues may be addressed in future investigations.

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