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Evaluation of extracellular enzyme-producing autochthonous gut bacteria in walking catfish, *Clarias batrachus* (L.)

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Abstract

The present study was carried out to screen autochthonous gut bacteria in freshwater air breathing walking catfish, *Clarias batrachus* Linnaeus. Altogether, 100 extracellular enzyme-producing bacteria were isolated from the foregut (FG) and hindgut (HG) regions. Data were presented as log viable counts g^{-1} gut (LVC). The occurrence of heterotrophic bacterial population was higher in the FG region (LVC = 8.25) than the HG (LVC= 7.3). Similarly, proteolytic, amylolytic and lipolytic bacteria in FG outnumbered (LVC=7.25, 6.77 and 5.23 respectively) the HG (LVC=6.38, 5.58 and 4.04 respectively). However, occurrence of cellulolytic bacteria in both, FG and HG was less (LVC=2.1 and 1.34 respectively) in comparison to the other extracellular enzyme-producing bacteria. Out of the 100 bacterial isolates, 22 isolates were primarily selected through qualitative assay of extracellular enzyme activities. Among them, 3 promising isolates were chosen as potent extracellular enzyme producers on the basis of cumulative scores (≥11) of the qualitative assay and quantitative enzyme assay. Maximum protease activity was revealed by the strain FG10 (201±2.40U), while FG43 exhibited maximum amylase (208.3±10.8U) and lipase (4.73±0.05U) activities. Among the strains isolated from the HG, the highest protease (188.3±1.2U), amylase (97.6±0.46U) and lipase (3.7±0.11U) activities were recorded with the strain HG01. The isolates (FG10, FG43 and HG01) were studied through 16S rRNA partial gene sequence analyses and were identified as *Bacillus aryabhattai* (KP784311), *B. flexus* (KR809411), and *B. cereus* (KR809412), respectively. Further studies are to be conducted to evaluate the efficacy of these strains *in vivo* to improve the overall health status of the *C. batrachus* juveniles.

Keywords: Autochthonous bacteria, *Bacillus*, *Clarias batrachus*, extracellular enzymes, gut, 16S rRNA

INTRODUCTION

The gastrointestinal (GI) tract of fish is densely populated with microorganisms much higher than those in the aquatic medium, suggesting supportive ecological niches of these microorganisms in the tract (Ringø *et al*. 1995). The GI tract reveals complex microbial interaction with the internal and external environment that might have important physiological implications with regard to health and disease (Ray *et al*. 2012). The gut microorganisms can be defined as either autochthonous or allochthonous depending upon its ability to colonize within the digestive tract (Ringø *et al.* 2003). In contrast to the vast experience on beneficial role of the gut-associated microorganisms in Indian major carps and exotic carps pertaining to nutrition of the host species (Ray *et al.* 2012), reports on likely function of the extracellular enzyme-producing gut bacteria in freshwater catfish are scanty. To the authors' knowledge, very few investigations were conducted to apprehend beneficial role, mode of association and enzyme-producing ability of the autochthonous gut bacteria in catfishes (Banerjee *et al*. 2015, Banerjee *et al*. 2016). Amongst the diverse catfish species, *Clarias batrachus* is economically important for its medicinal and curative attributes

(Debnath 2011). The GI tract of the catfish larvae is shorter and relatively underdeveloped compared to that of the adults. Therefore, fish juveniles lack certain digestive enzymes during early development resulting poor feed utilization leading to huge mortality. In addition, since the freshly hatched larvae do not have microbial community in the gut, on the gills or on the skin, they become more susceptible to the diseases caused by pathogens. In this respect, application of potent extracellular enzyme-producing autochthonous bacteria might hold promise to provide nutritional support and limit pathogenic microbial load to reduce mortality in catfish larvae as indicated for probiotic applications elsewhere (Cruz *et al.* 2012).

Gut microorganisms might have positive effects in the digestive process of the host fish as they are able to breakdown complex molecules, e.g., starch (Sugita *et al*. 1997), cellulose (Bairagi *et al*. 2002), protein (MacDonald *et al*. 1986) and Lipid (Bairagi *et al*. 2002). Pathogen inhibitory property of the GI tract microbiota in diverse fish species has also been recognized (Verschuere *et al*. 2000). Beneficial effects of the extracellular enzyme-producing microorganisms isolated from fish gut have been widely documented in several previous occasions (Ray *et al*. 2012, Ganguly and Prasad 2012, Das *et al*. 2014). Therefore, there is an increasing thrust for screening and selection of useful indigenous intestinal bacteria from the host species to ensure viability and desired benefits. The main strategy for application of beneficial microorganisms as live feed supplement is to isolate gut microorganisms with beneficial properties from mature animals and include them in the feed for immature animals of the same species (Gildberg *et al*. 1997, Ganguly *et al*. 2010). In view of effective utilization of food during the larval development, in the presently reported study, evaluation of potent extracellular enzyme-producing microorganisms has been considered as the principal criteria for the selection of beneficial gut microorganisms from *C. batrachus*. Therefore, the present study aimed at (1) evaluation of extracellular enzyme-producing microorganisms from the foregut (FG) and hindgut (HG) regions of the GI tract in *C. batrachus*, (2) selection of the efficient strains through quantitative estimation of enzyme activity, and (3) identification of the promising enzyme-producing bacteria by 16S rRNA partial gene sequence analysis.

METHODOLOGY

Collection and processing of specimen: In the present study, specimens of *C. batrachus* were collected from swampy and muddy water body located around Burdwan (23°14′ N, 87°39′ E), West Bengal, India and stocked in Fibre-reinforced plastic (FRP) tanks under laboratory condition. Following water quality parameters of the FRP tanks were recorded during starvation period using Multi-Parameter PCSTestr^{TM} 35 (Eutech Instruments, Oakton) for temperature 30.3-32.2 °C and pH 7.3-7.5, and using Traceable DO Meter (Fischer Scientific, Model No. 0666266) for dissolved oxygen 6.0-7.1 mgL^{-1}. Three specimens obtained from different muddy water bodies were kept in separate tanks (45 L) on the basis of their sources and starved for 48 hr in order to empty their digestive tract before dissection (Das *et al.* 2014).

Average length and weight of the specimen and the GI tract of the fish examined are presented in Table 1. The fish were anaesthetized by using 0.03% tricaine methanesulfonate (MS-222). The ventral surface of each fish was treated with 1% iodine solution for surface sanitization (Trust and Sparrow 1974). Dissection of the specimens was done in aseptic condition to remove the alimentary canal. For isolation of autochthonous microorganisms, the GI tracts were divided into FG and HG regions, transferred to sterile Petri-plates, thoroughly washed 3 times with sterilized chilled 0.9% saline solution and processed separately (Ghosh *et al*. 2010; Mandal and Ghosh 2013). The segments of the alimentary canals from three specimens of *C. batrachus* were pooled together on the basis of the source for each replicate, hence three replicates were considered for the study to avoid erroneous inference due to individual variation in gut microbiota (Ringø *et al*. 1995).

Table 1 Different aspects of *Clarias batrachus* specimens studied where values are mean±SD of three determinations

Fish species	*Feeding habit**	*Body weight (g)*	*Body length (cm)*	*Gut weight (g)*	*Gut length (cm)*
Clarias batrachus	Insect larvae, shrimps, worms etc.	285 ±8.66	24.56 ±0.56	13.3 ±0.88	98.46 ±4.48

**(Sakhare and Chalak 2014)*

Isolation of extracellular enzyme producing gut microorganisms: Pooled gut segments of the GI tract were shifted to sterilize Petri-plates and were homogenized with chilled 0.9% sodium chloride solution (w/v, 1:10) as described elsewhere (Das and Tripathi 1991). Homogenates were serially diluted (1: 10) with sterilized distilled water (Beveridge *et al.* 1991). In order to determine the culturable heterotrophic autochthonous aerobic/facultative anaerobic microbial population, 0.1 ml of the diluted sample was poured onto sterilized Soyabean Casein Digest Agar (Tryptone Soya Agar, TSA; HiMedia, Mumbai, India) media plates in aseptic condition and incubated at 30 °C for 48 hr. Isolation and enumeration of various extracellular enzyme producing

microorganisms (e.g, amylase, protease, lipase and cellulase) were made following enrichment culture technique. Serially diluted gut homogenates were inoculated and spread onto starch (ST), peptone-gelatin (PG), tributyrin (TB) and carboxy-methyl-cellulose (CMC) media plates to determine amylase, protease, lipase and cellulase-producing microflora, respectively, following (Bairagi *et al*. 2002). Culture plates were incubated at 30 °C for 48 hr. The Colony Forming Units (CFU $unit^{-1}$ of sample volume of gut homogenate) were calculated by multiplying the number of colonies formed on each plate by reciprocal dilution and expressed as log viable counts g^{-1} gastrointestinal tract (LVC) (Mandal and Ghosh 2013). The distinct and well-separated colonies were randomly collected, transferred to the respective media plates and streaked repeatedly until purity. Pure cultures were preserved on slants in refrigerator (4 °C) until further study.

Qualitative and quantitative assay for extracellular enzyme production: Primarily the bacterial isolates grown on ST and PG media plates were screened qualitatively with the appearance of halo zone by flooding the plates with 1% Lugol's iodine and 15% $HgCl_2$, which specified amylase and protease activities, respectively (Jacob and Gerstein 1960). Transparent zone around colonies grown on TB plate confirmed lipase activity (Sangiliyandi and Gunasekaran 1996), and cellulolytic activity was determined by flooding the CMC plates with Congo red dye (Teather and Wood 1982).

Qualitative extracellular enzyme activity was evaluated on the basis of the dimension of the halo zone (diameter in mm, in excess of the colony diameter) around the colony (Table 2) and presented as scores (Das *et al*. 2014) as follows: 0, nil (no halo); 1, low (6-10 mm halo); 2, moderate (11-20 mm halo); 3, good (21-30 mm halo); 4, high (31-39 mm halo); 5, very high (≥ 40 mm halo).

Depending upon the collective scores, proficient extracellular enzyme-producing isolates were selected for quantitative assay. Selective broth media were used for the production of the respective enzymes. Quantitative assay of cellulolytic activity was not done as cellulase-producing ability was not detected by qualitative test. Quantitative estimation of the amylase and protease activities were determined following the standard methods using dinitrosalicylic acid (Bernfeld 1955) and casein digestion (Walter 1984), respectively. Lipase activity was measured following the method described by Bier (1955).

Soluble protein content of the enzyme source was measured after Lowry *et al.* (1951) and quantitative enzyme activities were expressed as unit (U).

Table 2 Qualitative extracellular enzyme activities of some bacterial strains isolated from the GI tract of *Clarias batrachus* investigated. Enzyme activities are presented as scores as described in the text.

Strain designation	*Amylase*	*Protease*	*Cellulase*	*Lipase*	*Total score*
FOREGUT REGION					
FG 01	1	2	1	2	6
FG 02	3	1	0	2	6
FG 07	2	1	0	1	4
FG 08	1	1	0	1	3
FG 09	2	2	0	2	6
FG 10	**3**	**5**	**1**	**3**	**12**
FG 22	2	2	0	2	6
FG 24	2	3	0	1	5
FG 25	1	1	0	1	3
FG 30	1	2	0	0	3
FG 35	2	2	0	0	4
FG 37	2	3	0	1	6
FG 38	1	2	0	2	5
FG 39	2	3	0	2	7
FG 40	1	2	1	0	4
FG 43	**5**	**3**	**0**	**4**	**12**
FG 45	2	4	0	2	8
FG 49	2	3	0	2	7
FG 50	2	3	0	3	8
HINDGUT REGION					
HG 01	**4**	**4**	**0**	**3**	**11**
HG 02	3	1	0	1	5
HG 03	2	1	1	0	4
HG 10	2	3	0	2	7
HG 20	1	1	1	0	3
HG 21	2	1	0	0	3
HG 26	2	3	0	2	7
HG 30	3	3	0	2	8
HG 39	2	2	0	2	6
HG 40	2	3	0	2	7
HG 44	2	2	0	1	5

Identification of isolates by 16S rRNA partial gene sequence analysis: Three promising extracellular enzyme producing strains were identified through 16S rRNA partial gene sequence analysis after isolation and PCR amplification following the methods described in Das *et al.* (2014). The gene encoding 16S rRNA was amplified

from the isolates by polymerase chain reaction (PCR) using universal primers, 27f (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492r (5´-GGTTACCTTGTTACGACTT-3´). Sequenced data were edited using BioEdit Sequence Alignment Editor (Version 7.2.0), aligned and analyzed for finding the closest homolog using National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases. Sequences were submitted to the NCBI GenBank to accomplish accession numbers. Phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using MEGA5 (Tamura *et al.* 2011) software following the Maximum Likelihood Method based on the H-K-Y model (Hasegawa *et al*. 1985).

Statistical analysis: Statistical analysis of the quantitative enzyme activity data was executed by the one-way analysis of variance (ANOVA), following Tukey's test after Zar (1999) using SPSS Version 10 software (Kinnear and Gray 2000).

RESULTS

Determination of culturable aerobic or facultative anaerobic microorganisms revealed the abundance of heterotrophic as well as amylase, protease and lipase producing microbial population within the FG and HG regions in *C. batrachus*. It has been observed that heterotrophic microbial population on TSA plates was higher in the FG than that of HG in *C. batrachus*. While considering extracellular enzyme-producing microorganisms within the gut segments (Figure 1), presence of proteolytic microorganisms was found to be the highest in both FG (LVC = 7.25) and HG (LVC = 6.38), which were followed by amylolytic (LVC = 6.77 in FG and LVC = 5.58 in HG) and lipolytic microorganisms (LVC = 5.23 in FG and LVC = 4.04 in HG). In comparison, occurrence of the cellulolytic microorganisms was noticed to be the lowest in both, FG (LVC = 2.1) and HG (LVC = 1.34) segments of the GI tract.

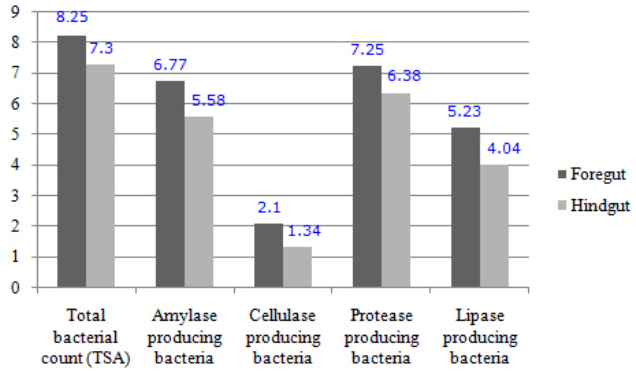

Figure 1: Log viable counts (LVC g^{-1} gut tissue) of autochthonous microorganisms isolated from the foregut and hindgut regions of *C. batrachus*

Overall, 100 microbial strains were randomly isolated, out of which, 22 bacterial strains (15 from FG and 7 from HG) were primarily selected through qualitative estimation of different extracellular enzyme-production. Qualitative extracellular enzyme activities were presented as scores (Table 2), maximum and minimum scores being 12 and 3, respectively. Results of the quantitative enzyme assay revealed significant differences in the enzyme activities between different bacterial isolates (Table 3).

Table 3 Quantitative activity of the enzymes (U) produced by the primarily selected bacteria isolated from the gut of *Clarias batrachus.* Data are means±SE of three determinations

Strain designation	Amylase(U)*	Protease(U)#	Lipase (U)!
FOREGUT REGION			
FG 02	57.82±0.70^{c}	62.66±1.45^{gh}	2.23±0.05^{e}
FG 07	39.42±0.34^{d}	91.3±1.76^{ef}	1.67±0.01^{g}
FG 09	26.63±2.66^{ef}	96.06±0.85^{e}	2.11±0.01^{ef}
FG 10	74.61±1.15^{bc}	201±2.40^{a}	3.39±0.03^{b}
FG 22	26.29±3.02^{ef}	75.2±1.12^{fg}	2.77±0.005^{c}
FG 24	27.96±0.93^{ef}	127.6±1.45^{d}	1.51±0.02^{gh}
FG 37	32.81±1.43^{de}	134.4±0.66^{cd}	2.05±0.05^{f}
FG 38	18.68±0.98^{f}	97.2±0.53^{e}	1.72±0.01^{fg}
FG 39	55.92±1.96^{c}	101±2.30^{de}	2.41±0.02^{d}
FG 40	86.16±1.25^{b}	55.66±2.84^{h}	1.37±0.01^{h}
FG 43	208.3±10.8^{a}	151.4±0.42^{b}	4.73±0.05^{a}
FG 45	33.09±1.05^{de}	140±1.15^{c}	2.81±0.06^{bc}
FG 47	35.11±1.04^{de}	88.1±0.68^{ef}	2.42±0.02^{d}
FG 49	30.61±1.20^{e}	88.3±1.2^{ef}	2.30±0.01^{de}
FG 50	43.1±1.57^{cd}	89.16±0.46^{ef}	3.0±0.12^{b}
HINDGUT REGION			
HG 01	97.6±0.46^{ab}	188.3±1.2^{ab}	3.7±0.11^{ab}
HG 10	25.9±1.21^{ef}	80±0.57^{f}	2.76±0.08^{c}
HG 26	30.04±1.1^{e}	96.6±1.45^{e}	2.34±0.03^{de}
HG 30	41.35±0.25^{cd}	145.6±1.45^{bc}	2.54±0.08^{cd}
HG 39	26.6±0.52^{hi}	75.6±1.15^{fg}	2.12±0.03^{ef}
HG 40	31.48±0.53^{e}	72.7±0.79^{fg}	2.83±0.09^{bc}
HG 44	36.39±0.56^{de}	67±3.6^{g}	2.77±0.01^{c}

Values with the same superscripts in the same vertical column are not significantly different (P < 0.05).
**µg maltose liberated/ml of enzyme extract/ mg protein/ min*
 # µg tyrosine liberated/ml of enzyme extract/ mg protein/min
! µg free fatty acid liberated/ml of enzyme extract/mg of protein/min

Among the strains isolated from the FG, maximum protease activity was revealed by the strain FG10 (201±2.40U), whereas the strain FG43 exhibited maximum amylase (208.3±10.8U) and lipase (4.73±0.05U) activities. Similarly, among the strains isolated from the HG, the highest protease (188.3±1.2U), amylase

(97.6±0.46U) and lipase (3.7±0.11U) activities were recorded with the strain HG01. Considering qualitative and quantitative enzymatic activities, the isolates FG10, FG43 and HG01 (qualitative activity scores being 12, 12 and 11, respectively) were found to have the most potential among the 22 primarily screened strains. The strains FG10 and FG43 were identified as *Bacillus aryabhattai* (GenBank Accession no. KP784311) and *Bacillus flexus* (GenBank Accession no. KR809411), which were close to the type strains *Bacillus aryabhattai* (LC011871) and *Bacillus flexus* (AB201185.1), respectively. Another isolate, HG01 was recognized as *Bacillus cereus* (GenBank Accession no. KR809412) that revealed maximum similarity with the type strain *Bacillus cereus* (AB680149.1). Phylogenetic relationship of these three identified bacteria with other closely related type strains retrieved from the RDP database were presented in the dendrogram (Figure 2).

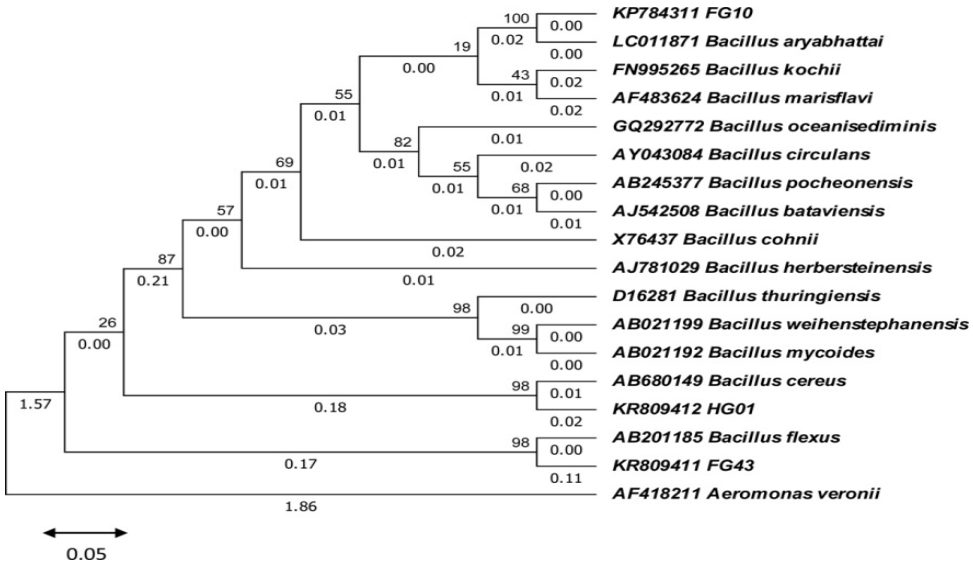

Figure 2: Dendrogram showing phylogenetic relationships of three selected bacterial strains, *Bacillus aryabhattai* FG10 (KP784311), *B. flexus* FG43 (KR809411) and *B. cereus* HG01 (KR809412) with other closely related strains retrieved from NCBI GenBank and RDP. Molecular phylogenetic analysis has been done by Maximum Likelihood method based on Hasegawa-Kishino-Yano model. Similarity and homology of the neighbouring sequences have been represented by bootstrap values. The scale bar denotes 0.05 substitutions per nucleotide position. Branch lengths have also been shown here. *Aeromonas veronii* AF418211 is used as an outgroup in the tree. Evolutionary analyses are conducted in MEGA 5.

DISCUSSION

Bacteria are abundant in the environment in which fish live and it is quite possible for them being a constituent of fish diet. As opined by Strøm and Olafsen (1990), the microbial community of water plays an important role in the colonization of microflora within the GI tract of fish. While visiting the GI tract through food or water, the microorganisms may adhere and colonize the host's gut epithelial surface or escape after some time without colonizing, depending on which the gut microbiota of fish has been classified as either autochthonous or allochthonous, respectively (Ringø and Birkbeck 1999, Merrifield *et al*. 2010, Ray *et al*. 2012). In the present study, the occurrence of considerable amount of autochthonous bacterial flora was detected within the digestive tract of *C. batrachus*. Prior to isolation of microorganisms the fish specimens were starved for 48 h and their dissected GI tracts were carefully rinsed with 0.9% sterile saline. Hence, it might be taken into consideration that the microorganisms isolated in the present investigation belonging to the autochthonous bacterial flora as described elsewhere (Ghosh *et al*. 2010, Mandal and Ghosh 2013). Primary selection of effective extracellular enzyme-producing bacteria seemed to be necessary for their consideration as live feed-supplement that might induce effective nutrient utilization in fish juveniles resulting better growth and survivability. To achieve this, *in vitro* tests are usually performed for the screening of more acceptable strains and to eliminate less potential microorganisms from the available large pool. In the present study, the first round of screening resulted in exclusion of 78% (i.e., 78 strains out of 100) of the total isolates.

Accurate information concerning the relative significance of exogenous enzymes produced by the endosymbionts of the GI tract and digestive enzymes released by the host is vital to recognize contribution of the endosymbionts in digestion (Clements 1997). Results of the present study portrayed considerable amylolytic, proteolytic and lipolytic bacterial population within the GI tract of *C.*

batrachus. Although, anchorage of very less amount of cellulase producing bacteria was also detected, abundance of highly efficient proteolytic bacteria (as represented by quantitative enzyme activity data) presumably indicated the carnivorous feeding habit of *C. batrachus*. Being carnivore, *C. batrachus* might have very less amount of dietary cellulose available within their gut restricting the colonization of cellulolytic microflora therein. Therefore, results of the present study might represent possible correlation between the food habit and occurrence of meagre cellulolytic flora within the GI tract. Previously, Bairagi *et al*. (2002) could not detect cellulolytic bacteria in the GI tract of carnivorous catfish. However, Kar *et al.* (2008) and Banerjee *et al.* (2013) observed the presence of autochthonous cellulolytic gut bacteria in carnivore *Channa punctatus*, which were in accordance to the present observation. Presence of cellulolytic bacteria in carnivorous *Mystus gulio* has also been reported by Das *et al.* (2014). Herbivores are likely to harbour cellulase producing bacteria. However, omnivores and carnivores might acquire cellulolytic microflora from the invertebrate prey that harbour the bacteria (Stickney 1975). In general, information relating to the gut microflora in *C. batrachus* is infrequent, which indicates lack of adequate research on the gastrointestinal flora of this species.

In the present study, three autochthonous isolates (FG10, FG43 and HG01) were characterized as promising extracellular enzyme-producing strains and identified as *Bacillus aryabhattai* (KP784311), *B. flexus* (KR809411) and *B. cereus* (KR809412), respectively through 16S rRNA partial gene sequence analyses. Different strains of extracellular enzyme-producing *Bacillus* spp. have been recognized from the GI tract of freshwater fish (Ray *et al*. 2012). *Bacillus flexus* has been stated as autochthonous gut bacteria in Indian major carps (Banerjee *et al.* 2015). *Bacillus cereus* has been documented as a promising exo-enzyme producing bacteria in Atlantic salmon (*Salmo salar* L.) (Askarian *et al.* 2012). However, to the authors' knowledge, *B. aryabhattai* has not been reported previously from the fish gut. The present study evaluated gut bacteria by culture dependent methods. Conventional culture-based techniques are generally condemned as these are time consuming, lack accuracy (Asfie *et al.* 2003) and might not represent an accurate picture of the bacterial diversity within the fish gut, even though copious media are employed (Ray *et al.* 2010). However, the major purpose of the present study was to isolate efficient extracellular enzyme-producing bacteria from the GI tract of *C. batrachus* so as to recognize autochthonous gut bacteria as live feed supplement for further study. Therefore, administration of culture-dependent process might be justified in view of functional characterization of the bacteria performed in the present study.

CONCLUSION

In the prersent study, promising extracellular enzyme-producing bacteria were screened from the GI tract of *C. batrachus* by *in vitro* examination. The information generated from this preliminary investigation might contribute to the application of these bacteria as putative probiotics during rearing of *C. batrachus* juveniles. Although, verification of the other probiotic properties (viz., attachment to gut mucus, non-pathogenicity, tolerance to bile salt, absence of drug resistant gene and biosafety) are necessary prior to recommend it for the commercial aquaculture (Mukherjee and Ghosh 2014, Dutta and Ghosh 2015). Therefore, further studies are enduring to corroborate probiotic properties and appraise efficacy of these potent enzyme-producing isolates *in vivo* for *C. batrachus* juveniles.

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