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Characterization and Identification of Protease and Amylase-producing Bacteria Isolated from the Gastrointestinal Tract of Climbing Perch, *Anabas testudineus* (Bloch)

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Abstract:

Isolation, and enumeration of autochthonous aerobic bacteria from the proximal (PI) and distal intestine (DI) of climbing perch, *Anabas testudineus* were carried out. Six (three each from PI and DI) bacterial isolates were qualitatively screened on the basis of their extracellular enzyme-producing ability. The selected strains were further quantitatively assayed for protease and amylase activities. Amylolytic strains were present in higher densities in the PI, whereas the proteolytic populations exhibited maximum densities in the DI. Maximum protease activity was exhibited by the bacterial strains isolated from the PI. On the basis of enzyme producing ability, two strains ATF2 and ATH3 were selected for further characterization. Both the strains are Gram- positive rods and the strain ATH3 was able to form endospore. They can tolerate a wide range of temperature (20–50° C) and pH (5–11). The strain ATF2 was able to grow at wide range of pH (5-10). Citrate and catalase tests were positive for ATF2 and ATH3, respectively. The isolate ATH3 was able to produce acid by utilizing a wide range of carbohydrate. These two strains were identified by 16S rDNA sequence analysis. On the basis of 16S rDNA sequence analysis, the strains ATF2 and ATH3 were identified as *Bacillus licheniformis* (GenBank Accession No. KC176365) and *Corynebacterium alkanolyticum* (GenBank Accession No. JX656749), respectively.

Keywords: GI tract bacteria, *Anabas testudineus*, enzyme activity, characterization, 16S rDNA sequence analysis

Introduction:

The gastrointestinal (GI) tract of fish is an open system constantly contacting with the surrounding environment i.e., water (Vovereine *et al.*, 2002). Being rich in nutrients, the environment of the GI tract of fish, in comparison with the surrounding water, confers a more favourable environment for growth of

microorganisms (Saha *et al*., 2006). Its complex polymicrobial ecology interacts with the internal and external environment and has an important influence on health. The gut microbiota of endothermic animals as well as fish are classified as autochthonous (able to colonize the epithelial surface of the host gut) or as allochthonous (transient) (Ringø *et al*., 2006; Ray *et al*., 2012). There have been number of published information concerning the indigenous microflora in the digestive tract of fish (Bairagi *et al*., 2002; Ghosh *et al*., 2002; Ramirez and Dixon 2003; Huber *et al*., 2004; Saha *et al*., 2006; Mondal *et al.*, 2008, 2010; Ray *et al*., 2010, 2012). Some investigations have suggested that the microorganisms have a beneficial effect on the digestive process of fish, for example, microbial breakdown of chitin (Goodrich and Morita, 1977; Danulat and Kausch, 1984; Kono *et al*., 1987; Gutowska *et al.,* 2004), *p*-nitrophenyl-B-N-acetylglucosamine and collagen (MacDonald *et al*., 1986), cellulose (Saha and Ray 1998; Bairagi *et al*., 2002; Saha *et al*., 2006; Mondal *et al*., 2008, 2010; Ray *et al*., 2010), starch (Sugita *et al*., 1997), protein (Silvia *et al*., 2006), and phytate (Li *et al*., 2008; Roy *et al*., 2009; Khan and Ghosh, 2012). These facts strongly suggest a symbiotic relationship between fish and GI tract microbiota. Several investigations have been conducted to determine the dynamics of the gut microbiota of fish based on culturing techniques and the use of phenotypic characteristics and molecular methods (Huber *et al*., 2004; Pond *et al*., 2006; Skrodenyte-Arbaciauskiene *et al*., 2008; Ringø *et al*., 2008). However, information on proper identification of gut microbiota of Indian freshwater teleosts based on 16S rDNA sequence analysis and phenotypic characterization is scanty (Ghosh *et al.*, 2002; Saha *et al*., 2006; Roy *et al.,* 2009; Mondal *et al*., 2010; Ray *et al*., 2010; Banerjee *et al.*, 2013). The present study was therefore, undertaken to search for the autochthonous protease- and amylase-producing bacteria in the proximal and distal intestine of a freshwater air-breathing fish, the climbing perch, *Anabas testudineus*, and to identify the most promising bacterial strains based on 16S rDNA sequence analysis.

Material and methods:

Fish examined:

Five adult healthy specimens of the carnivorous Indian climbing perch, *Anabas testudineus*, were used for the present study. The fish were collected from a local fish farm in Santiniketan, West Bengal, India (23°41’30’’N latitude, 87°41’20’’E longitude). Average weight and length of the fishes were 98.4 ± 3.2 g and 16.4 ± 0.9 cm, respectively. The test fish were starved for 36 h prior to sacrifice to clean the digestive tract.

Post-mortem examination and microbial culture:

Immediately after being killed, the ventral surface of the fish was thoroughly scrubbed with 1% iodine solution (Trust and Sparrow, 1974). The GI tract was divided into proximal and distal regions as described by Ringø and Strøm (1994). Each region was carefully washed, slit opened by a longitudinal incision, transferred to sterilized petridishes, and thoroughly flushed with sterilized chilled 0.9% saline in order to remove the allochthonous bacteria. The PI and DI were separately homogenized with 10 parts of chilled 0.9% sterilized sodium chloride solution (Das and Tripathi, 1991). The homogenate of the gut segments of the test fish was used after 10 serial 1:10 dilutions for microbial culture (Beveridge *et al*., 1991). Samples (0.1 ml) were then taken from each dilution and poured aseptically within a laminar airflow on sterilized tryptone soya agar (TSA; Hi Media Laboratories, Mumbai, India) plates (40 g of TSA suspended in 1000 ml sterilized distilled water) in duplicate. To obtain the total bacterial count, only TSA plates were used. Selective media were used to

enumerate protease- and amylase-producing bacteria.

Qualitative enzyme activity of isolated strains:

For screening of protease and amylase-producing strains, bacterial isolates were streaked on peptone-gelatin-agar medium and starch-agar medium, respectively, incubated for 48 h at 37 ± 1° C to screen protease and amylase-producing strains, respectively. Qualitative protease and amylase assay was done according to Jacob and Gerstein (1960). Bacterial isolates were inoculated on peptone-gelatin-agar media and incubated at 34°C for 48 hours and then flooded with 15% $HgCl_2$ and kept for 5mins. Appearance of transparent zone around the bacterial colony indicates the protease activity. After appearance of the colonies on the starch–agar medium-containing plates, the culture plates were flooded with 1% Lugol's iodine solution (Jacob and Gerstein, 1960) to qualify amylase activity. The Appearance of clear zone or halo surrounding the colonies indicated the presence of amylolytic activity.

Quantitative enzyme activity of isolated strains:

For quantitative protease and amylase assay, bacterial strains were cultured in peptone-gelatin broth and starch broth, respectively. The culture media were incubated at 34°C for 72 h in shaking incubator. After incubation, the content was centrifuged (10,000× *g*, for 10 min, at 4° C), and the cell-free supernatant was used for enzyme assay. Quantitative protease and amylase assay was done according to Walter (1984) and Bernfeld (1955), respectively.

Phenotypic characterization of the selected strains:

For phenotypic characterization, bacterial strains were cultured in nutrient agar plates and nutrient broth respectively. Colony morphology was studied visually. Gram-staining procedure was performed for the determination of staining property. Endospore forming capacity was determined by staining with 5% aqueous malachite green. Bacterial growth was observed at different temperatures (4–55° C) and pH (4.0–11.0). Sodium chloride tolerance was determined by using NaCl at different concentrations (2.5–10.0%). For biochemical and carbohydrate utilization tests, HiMedia kits were used.

16S rDNA sequence analysis:

The selected bacterial isolates were identified by 16S rDNA gene sequence analysis as described by Roy *et al.* (2009). Sequenced data were aligned and analyzed for finding the closest homolog of the microbes using a combination of National Center for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project database.

Results and Discussion:

The bacterial counts in TSA plate and specific media are presented in Table 1.The Log viable count (LVC) of aerobic bacteria in TSA plates were more in DI (Log = 4.22 g^{-1} intestinal tissue) than that in PI (Log = 3.98 g^{-1} intestinal tissue). While enumerating specific enzyme-producing bacterial flora, it was observed that both proteolytic and amylolytic strains were present in higher densities in the DI in comparison to the PI (Log = 3.17 and 3.01 g^{-1} intestinal tissue, respectively). The qualitative enzyme activity of the six bacterial isolates is represented in Table 2. All the strains were good protease producer, but their extracellular amylase activities were low. On the basis of their enzyme-producing capacity, six strains (three each from the PI and DI) were selected for quantitative assay of enzyme. All the six strains exhibited maximum protease activity (Table 3). The highest protease and amylase activities were exhibited by the bacterial strains ATF2 (4.03±0.032 U^1) and ATH3 (1.85±0.045 U^2), isolated from the PI and DI, respectively. These two most promising enzyme-producing

strains were selected as potent enzyme producers and were characterized and identified. Results regarding phenotypic characterization of the selected bacterial strains (ATF2 and ATH3) are depicted in Table 4. Both the strains were small Gram-positive rods. The bacterial strain ATH3 was able to produce endospore. They tolerated a wide range of pH (5.0-10.0 and 6.0-9.0 in case of ATF2 and ATH3, respectively) and temperatures (20–50^{o} C). Both the strains could tolerate NaCl concentration up to 9.5%. and were able to reduce nitrate. The bacterial strain ATF2 showed positive reaction in citrate utilization test while ATH3 was able to produce catalase. Both the strain showed positive reaction in ONPG and esculin hydrolysis. The bacterial strain ATH3 could actively utilize glucose, mannose, sucrose and dextrose.

In order to gain taxonomic information on the selected strains (ATF2 and ATH3), the 16S rDNA of the strains were partially sequenced (Table 5). Based on the nucleotide homology and phylogenetic analysis, the selected enzyme-producing bacterial strains ATF2 and ATH3 were identified as *Bacillus licheniformis* (GenBank Accession Number: KC176365) and *Corynebacterium alkanolyticum* (GenBank Accession Number: JX656749), respectively.

The microbial population within the digestive tract of fish is rather immense (Kandel *et al.*, 1994; Ringø and Strøm, 1994; Clements and Choat, 1995), with the number of microorganisms much higher than in the surrounding water, indicating that the digestive tract provides favourable ecological niche for these organisms. While the digestive tract of endotherms is colonized mainly by obligate anaerobes (Finegold *et al*., 1983), the predominant bacterial genera/species isolated from most fish guts have been aerobes or facultative anaerobes (Spanggaard *et al*., 2000; Bairagi *et al*., 2002; Ghosh *et al.*, 2002; Saha *et al*., 2006; Mondal *et al*., 2008, 2010). In the present study, the strains isolated from the GI tract of *A. testudineus* with extracellular enzyme-producing ability were aerobic Gram-positive. Saha *et al.* (2006) isolated two strains of cellulase-producing Bacilli, TM1 and CI3, from the digestive tracts of tilapia, *Oreochromis mossambica*, and Chinese grass carp, *Ctenopharyngodon idella*, respectively, and identified them as *B. circulans* and *B. megaterium* on the basis of phenotypic characters. Both the strains exhibited amylase and protease activities also. In the present investigation, characterization of the isolated microorganisms revealed that they could grow within a wide range of temperatures and pH. Similar ranges of temperature and pH tolerance have been reported in other strains of Bacilli isolated from fish gut (Ghosh *et al.*, 2002; Saha *et al*., 2006; Mondal *et al*., 2010). Although the primary identifications of bacteria are based on their biochemical reaction with different substrates, most of the bacteria within the same genera share common biochemical properties and it is difficult to differentiate them based on biochemical reactions (Akolkar *et al*., 2006). According to Spanggaard *et al.* (2000), the 16S rRNA gene analysis provides a detailed phylogenetic placement of the bacterial isolates and can be used for the construction of a set of oligonucleotide probes for a rapid community analysis of the bacterial flora of fish. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker (Patel, 2001). In the present investigation, the most promising protease- and amylase-producing strains were placed by 16S rDNA into a bacterial phylogenetic tree analysis. Based on 16S rDNA sequence analysis, the selected strains, ATF2 and ATH3, were identified as *Bacillus licheniformis* and *Corynebacterium alkanolyticum*, respectively. The fish with herbivorous and omnivorous feeding habits have been reported to exhibit significant cellulolytic

bacterial flora (Bairagi *et al.,* 2002; Saha *et al*., 2006; Mondal *et al*., 2008, 2010). In the present investigation, it was revealed that the selected strains could produce more protease enzyme than amylase. This may be due to the carnivorous feeding habit of *A. testudineus*. In commercial aquaculture, the enzyme-producing bacteria can be beneficially used as probiotics while formulating aquafeeds, especially for the larval stages when the enzyme system is not efficient. However, further investigations are required to know about the metabolic pathways used by these microorganisms in the alimentary tracts of fish.

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Table 1: Log viable count (LVC) of aerobic autochthonous bacteria isolated from the proximal intestine (PI) and distal intestine (DI) of *Anabas testudineus*

Intestinal region	Log viable counts/ gm intestinal tissue		
	Total bacterial count (TSA)	Amylase producing bacteria	Protease producing bacteria
PI	3.98	2.90	2.67
DI	4.22	3.01	3.17

Table 2: Qualitative enzyme activity of the isolated strains

Intestinal region	Bacterial strains	Protease activity	Amylase activity
PI	ATF1	+++	+
	ATF2	++++	++
	ATF3	+++	+++
DI	ATH3	++++	+++
	ATH4	++	++
	ATH5	++++	++

++++, high (above 35 mm halo diameter); ++, good (25-34 mm halo diameter);
++, moderate (15-24 mm halo diameter); +, low (5-14 mm halo diameter).

Table 3: Quantitative enzyme activity of the isolated strains

Intestinal region	Bacterial strains	Specific protease activity (U) [1]	Specific amylase activity (U) [2]
PI	ATF1	2.19±0.026	0.02±0.01
	ATF2	4.03±0.032	0.76±0.061
	ATF3	3.70±0.065	1.70±0.047
DI	ATH3	3.71±0.036	1.85±0.045
	ATH4	2.75±0.041	1.07±0.066
	ATH5	3.57±0.086	0.83±0.015

Data are mean of ± SE of three determinations.

[1] U = µg of tyrosine liberated/mg protein/ml of culture filtrate.

[2] U = µg of maltose liberated/mg protein/ml of culture filtrate.

Table 4: Morphological, physiological and biochemical characteristics of the selected strains ATF2 and ATH3

Tests	ATF2	ATH3
Morphological tests:		
Configuration	Irregular	Irregular
Margin	Wavy	Wavy
Elevation	Convex	Convex
Surface	Rough	Rough
Opacity	Dull	Translucent
Pigment	White	Yellow
Gram's Reaction	+	+
Cell shape	Small, rod shaped	Small, rod shaped
Endospore	+	-
Shape	Central and oval	-
Physiological tests:		
Growth at temperature (^{o}C)		
4-10	□	□
20-40	+	+
45-50	+	+
50-55	□	□
Growth at pH		
4.0	□	□
5.0	+	□
6.0-9.0	+	+
10.0	+	□
11.0	□	□
Growth on NaCl (%)		
2.5-9.5	+	+
10.5	□	□
Biochemical tests:		
Citrate utilization	+	□
Lysine utilization	□	□
Ornithine utilization	□	□
Esculin hydrolysis	+	+
ONPG	+	+
Phenylalanine deamination	□	□
Nitrate reduction	+	+
Catalase	□	+
Urease	□	□
H_2S production	□	□
Acid production from:		
Lactose	□	□
Xylose	□	□
Maltose	□	□
Fructose	□	□
Dextrose	□	+
		□

Galactose	□	+
Sucrose	□	□
L-Arabinose	□	+
Mannose	□	□
D-Arabinose	□	+
Glucose	□	

+, Positive ; □, Negative

Table 5: Identification of selected strains by 16S rDNA sequence analysis

Bacterial strain	Identified as	Similarity (%)	GenBank Accession no.
ATF2	*Bacillus licheniformis*	100	KC176365
ATH3	*Corynebacterium alkanolyticum*	100	JX656749