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Research Article

STUDY ON GUT-ASSOCIATED AMYLASE-PRODUCING BACTERIA IN SOME COMMERCIALLY IMPORTANT FRESHWATER RIVERINE ICHTHYOFAUNA OF NORTH BENGAL (WEST BENGAL, INDIA)

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ABSTRACT
The amylase-producing ability of the gut-associated bacteria in captured fresh-water riverine fish specimens of *Lepidocephalus sp.*, *Puntius sp.*, *Macrognathus sp.*, *Mystus sp.*, *Glossogobius sp.*, *Somileptes sp.* was determined. The main objectives of the study were isolation and screening of amylase-producing aerobic bacteria and analysis of their enzyme activity. Preliminary exploration of gut flora revealed 12 amylase-producing bacterial strains showing wide diversity of enzyme production, morphological and biochemical characteristics. The isolates were qualitatively screened on the basis of their extracellular enzyme producing ability. This can be correlated with their feeding habit as the results strongly suggest that the amylase produced by the gut micro flora play an important role in the digestion of starch in freshwater fishes. The best starch-degrader was found to be *Bacillus* sp. Further studies were done with the enzyme from that potent amylase-producing strain. The enzyme was observed to be active in the pH range of 3.5 to 7.5 with the pH optima at 6.5 and the optimum in vitro temperature was 60°C. The enzyme retained its activity even at 75°C and was highly specific for its substrate. The information generated from the present investigation might contribute to the incorporation of these bacteria in commercial aquaculture.

Key Words: *Amylase, Freshwater Fishes, Gut-Associated Bacteria*

INTRODUCTION
Starch, a glucose polymer, is one of the most widely available plant polysaccharides. Starch is efficiently broken down biologically by many organisms, from humans to bacteria and that is accomplished by using enzymes called amylases. All amylases are glycoside hydrolases and act on α-1, 4-glycosidic bonds. The amylases can also be derived from several sources, such as plants, animals and microorganisms. This enzyme is extensively used in starch liquefaction, brewing, food, paper, textile and pharmaceutical industries (1, 2, 3, 4, and 5). Starch is also a major ingredient of the feed for fishes. It is hydrolyzed to its constituent sugar and oligosaccharides in the digestive tract of the fish. Enzymes found in the intestinal lumen could potentially have come from either the pancreas or the secretary cells in the gut wall. In addition, enzymes from the intestinal micro flora potentially could have a significant role in digestion.
The intestinal tract of fish is generally colonized by a great number of heterotrophic bacteria including aerobes and anaerobes. Although many ecological studies on the gut microflora of fish have been reported (6, 7, 8), only recently have reports concerning their functions appeared. But still the information on gut microflora in fishes is scanty and there is a paucity of knowledge regarding microbial enzyme activity in fish gastrointestinal tracts (9, 10, 11, 12, 13, 14, and 15). In the present study, an attempt has been made to investigate the presence of amylase-producing bacteria in the gastrointestinal (GI) tracts of fishes. This experiment relates to the field of microbiology and more particularly to the isolation, screening and characterization of amylase-producing aerobic bacteria in the gut of some commercially important fresh water riverine fishes of North Bengal, that is, the northern part of West Bengal. These environmental isolates may also be useful in the commercial production of amylase.

MATERIALS AND METHODS

Sampling

Fish Examined:

Six fresh water riverine fish samples of *Lepidocephalus sp., Puntius sp., Macrognathus sp., Mystus sp., Glossogobius sp., Somileptes sp*. were collected. The average weight and gut length (LG) of the fishes was studied (Table 1).

Post Mortem Examination:

To isolate stable bacterial population from the GI tracts, the fish samples were starved for 24 hours in order to make their intestinal tract clear and also to eliminate the bacteria that were transit in nature. After starvation period, the fishes were dissected aseptically and GI tracts were removed carefully (10). The digestive tracts were thoroughly rinsed several times in sterile 0.9% saline in order to remove non-adherent bacteria. A homogenate solution was made by adding GI tracts with 0.89% sodium chloride (NaCl) solution (10:1; volume: weight) (16).

Serial Dilutions

Serial dilutions were made up to 10^{-7} dilution by mixing this homogenate solution with sterilized distilled water using vortex mixer to use as inoculums.

Microbial Culture

Plating on Nutrient Agar (NA) Medium:

Microbial culture of the homogenized GI tracts of fish was carried out for isolation of bacteria. Diluted samples (0.1 ml) were spread aseptically within a laminar airflow on sterilized Nutrient agar (consisting of, in g/l, peptone, 5.0; NaCl, 3.0; beef extract, 5.0; agar, 20; pH 7.0) plates and incubated for 24 h at 37°C to determine the total heterotrophic bacterial population.

Obtaining Pure Cultures

In order to obtain pure culture, streak plating the organisms on solid medium to get well separated colonies was done. All the colonies formed on the NA plates were observed minutely to distinguish them according to their colony characteristics. 24 well-separated colonies of intestinal specimens were finally isolated and were streaked separately on fresh NA plates for obtaining pure cultures.

Screening

To isolate and enumerate amylase producing bacterial population, starch agar (SA) consisting of (g/l) peptone 5%; beef extract 5%; NaCl 5%; soluble starch 2%; agar 2%; pH 7.0, was used as the selective medium. Each bacterial sample was streaked on the separate SA plates in duplicates. The surviving organisms on SA plates were then screened for the presence of amylase by observing clearing zones around amylase producing colonies after adding iodine. 12 cultures were thus identified as amylase positive.

Screening of Isolates for Extracellular Enzyme Production:

The intensity of extracellular enzyme production by the isolated bacterial strains was compared on the basis of the amylase activity by formation of transparent zone surrounding the colony. For extracellular qualitative amylase production, isolates were inoculated on SA plates and incubated at 37°C for 24 h. The culture plates were then flooded with 1% Lugol's iodine solution to identify amylase activity by formation of transparent zone surrounding the colony (17). Bacterial strains hydrolyzing starch in the starch agar medium were cultivated in the starch broth (SA-agar) at 37ºC for about five days under shake flask culture in a shaker incubator and quantitative amylase assay was performed at regular intervals like at 24, 48, 72, 96 and 120 hr of incubation period. The cell culture in starch broth was centrifuged at 10,000rpm for 10min and the supernatant thus obtained was used as the enzyme source for assay. Morphological, physiological, cultural and biochemical characteristics of the positive bacterial stains were studied; finally the best one of those, in terms of the enzyme activity, was taken for further study.

Enzyme Assay:

The enzyme assay was performed after Rick and Stegbauer (1974). The enzyme extract (0.5 ml) was transferred to a test tube containing 0.5 ml of 1.0% soluble starch solution. The mixture was incubated at

60ºC for 10 min. Then 1.0 ml of dinitrosalicylic acid reagent (DNS) was added to each test tube. The tubes were placed in boiling water for 5 min and then cooled at room temperature. The contents of tubes were diluted up to 10 ml with distilled water to convert readings into mg of maltose (9). The absorbance was determined at 540 nm using a spectrophotometer and converted to mg of maltose from the standard. One unit is equivalent to that amount of enzyme, which catalyses the hydrolysis of soluble starch into 1.0 mg maltose hydrate per minute under standard assay conditions. Total protein content was estimated by the Bradford method using bovine serum albumin as the standard (18).

Effect of Temperature on Amylase Activity

The effect of temperature was assayed at 4°-80°C, pH 6.9 for 15min. After 15 min incubation, amylase activity was determined for each temperature regime as described earlier.

Effects of Temperature on Amylase Stability

The thermal stability of the enzyme was determined by incubating the enzyme fraction at various temperatures between 0° and 80°C without the substrate for 30min followed by the activity assay.

Effect of pH on Amylase Activity

The effect of pH on amylase activity was determined on starch solutions (1% starch) at pH 2.0 – 7.5, 37°C for 5 min. The amylase activity was determined as earlier outlined.

Effect of Substrate Concentrations on Amylase Activity

Amylase activities of the crude amylase preparations were determined at various substrate concentrations of 1% starch solutions. K_m value for starch was also determined by Lineweaver-Burk analysis at optimum pH and at 37°C.

Identification of the Genus

For identification of the bacterial sample morphological, physiological, and cultural and biochemical characteristics were determined.

For morphological and physiological identification, Gram's staining, endospore staining and motility test were done and colony characteristics were noted based on the observation of the colonies on pure culture plates. Cultural characteristics were obtained plating each strain on different selective and differential media, such as Blood agar, McConkey agar, and liquid media to observe the characteristic growth patterns on those media that helped to identify the sampled bacterial species.

For biochemical characterization, several biochemical tests were performed, such as, Indole Test; Methyl Red and Voges-Proskauer (MR-VP) Test; Citrate Utilization Test; Catalase Test; Oxidase Test; Urease Test; Nitrate Reduction Test; Gelatin Hydrolysis Test; Caseinase; lipase; Fermentation of sugars etc and finally the genus of the selected strain was identified **(**19).

RESULTS

Bacteria in Fish Gastrointestinal Tract

A considerable population of aerobic bacterial symbionts has been isolated from the fore- and hind gut regions of all the fishes studied. Mean viable counts of the bacterial population obtained ranged from 0.10×10^3 to 0.07×10^6 in the gut of the first fish sample and from 0.08×10^3 to 0.03×10^6 in the second sample in case of *Macrognathus sp.*

Amylase-Producing Capacity of the Intestinal Isolates

A total of 24 different bacterial strains were isolated on the basis of their different colony morphology and of these only 50% showed amylase producing activity. On starch-nutrient agar plate, 12 different bacterial colonies, which produced clear halos with iodine solution were selected and purified. Population levels of amylolytic strains were the highest in the gut of *Macrognathus* sp. and the lowest in that of *Glossogobius* sp. Among the most potent amylase producing bacterial strains, the Pa-4 strain, isolated from *Macrognathus sp.*, showed highest amylase-producing activity. The morphological, biochemical and gram staining properties (Table 2 and 3) indicates that this strain is *Bacillus* sp. as per the Bergey's Manual of Deterministic Bacteriology (12). Then the strain was further studied for enzymatic analysis.

Table 1: Measurement of Fish Weight and Length (Results are mean ± S.E. of the three observations)

Name of the Fishes	Avg. Weight (gm)	Gut Length (cm)
Lepidocephalus sp	9.47±0.49	3.7±0.29
Puntius sp	12.5±0.73	3.1±0.33
Macrognathus sp	9.86±0.54	5.5±0.54
Mystus sp	3.56±0.2	3.7±0.23
Glossogobius sp	16.95±1.3	3.5±0.49
Somileptes sp	17.14±0.89	4.1±0.42

Table 2: Cultural and Microscopic Characteristics of the Strain Pa-4

Charecteristics	Results
Colony Form Elevation	Irregular
Margin	Flat
Opacity	Irregular
Colour	Opaque
Appearance	White
Diameter of colony after 24 h Diameter of clear halo in Starch agar	Matt
Gram staining	5 mm
Shape of vegetative cell	20 mm
Spore formation	+
Motility	Rod
	+
	Non motile

Keys: +=Positive; - = Negative

Table 3: Biochemical and Cultural Characteristics of the Strain Pa-4

Biochemical Test	Results
Growth in presence of air Anaerobic growth	(+)
Indole production	(-)
Methyl red	(-)
Voges-Proskaeur test	(-)
Catalase production	(+)
Citrate production	(+)
Oxidase production	(+)
Growth in 5% NaCl	(+)
Growth in 10% NaCl	(+)
Growth in 20% NaCl	weakly (+)
Growth in 30% NaCl	(-)
Growth at 55ºC	(-)
Gelatin liquefaction	(+)
Sugar fermentation:	(+)
Glucose	A/G
Fructose	A
Maltose	A
Sucrose	A
	A

Keys: +=Positive; - = Negative; A= Acid production; A/G= acid and gas production

Characterization of Amylase

The extracellular amylase production started from the exponential phase of bacterial growth and maximum production was noted at 24h at 37°C (Figure 1).

Figure 1: Incubation Time

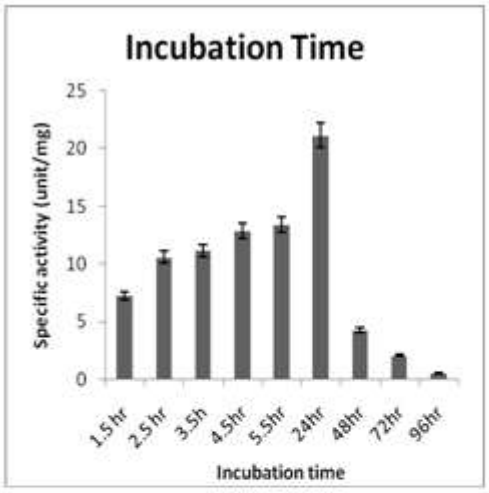

Figure 2: Characteristics of the Enzyme

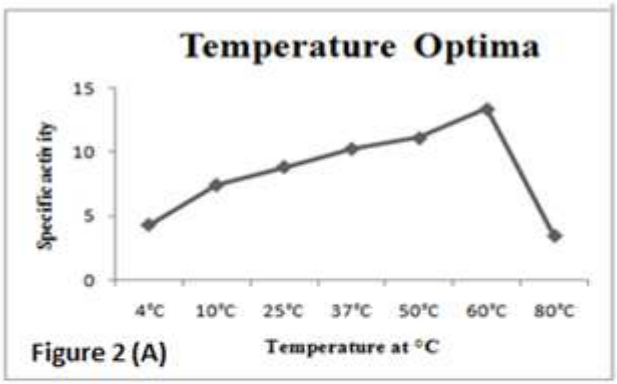

Figure 2 (A)

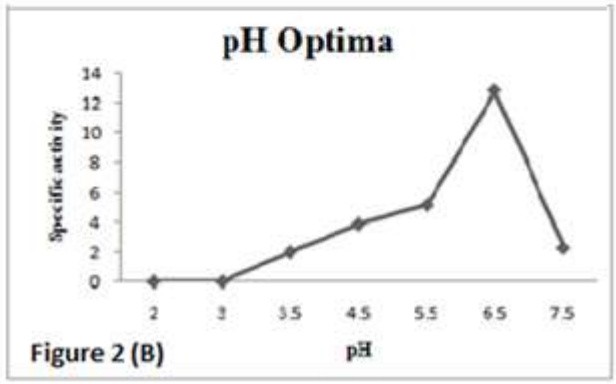

Figure 2 (B)

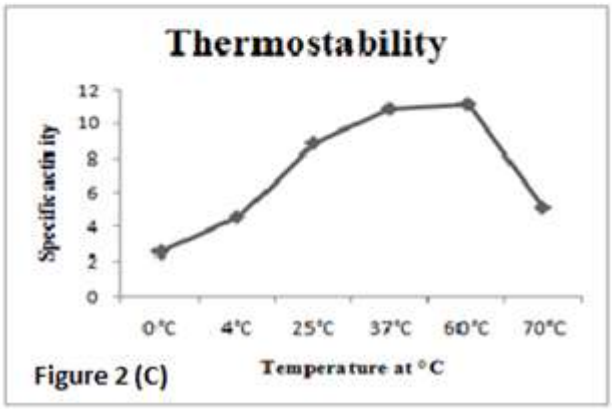

Figure 2 (C)

To investigate the effect of temperature, activity of amylase was determined at different temperatures ranging 4°-80°C under standard assay conditions. The optimum *in vitro* temperature for activity of enzyme was 60°C (Figure 2a). A drastic loss in the enzyme activity was observed upon increasing the temperature above the optimum value. The enzyme had a pH optimum at pH 6.5 and displayed a broader zone of activity in the pH range 3.5 to 7.5. The enzyme was inactive at pH less than 3.5 and above pH 7.5 (Figure 2b). The thermal stability of the enzyme was also determined by pre-incubating the enzyme preparation for 30min at indicated temperatures and then assaying under standard conditions. The enzyme was found active till 75°C (Figure 2c). The enzyme showed hyperbolic response to increasing concentrations of substrate starch in an otherwise standard assay mixture. Apparent K_m value for starch as determined by Lineweaver-Burk plot was 2.6 g/L and V_{max} value was 214 Um/L.

CONCLUSION

Generally, bacteria are abundant in the environment in which fish live and it is therefore, rather impossible to avoid them being a component of their diet (20, 21). Bacteria present in the aquatic environment may influence the composition of the gut microbiota in fish (8). The bacterial flora of the gastrointestinal tract represent a very important and diversified enzymatic potential and it seems logical to think that the enzymatic mass lodged in the digestive tract might interfere in a considerable way with a

major part of the metabolism of the host animal (22). Population levels of amylolytic strains were the highest in the gut of *Macrognathus* sp. and the lowest in that of *Glossogobius* sp. This can be correlated with the feeding habit of the fishes as *Macrognathus* sp. is omnivorous and *Glossogobius* sp. is carnivorous in nature and also with the gut length as isolates of bacteria (g CDW) are strongly depended on source of mass; in this case it is gut-length. The results of this study indicate that a diverse bacterial flora exists in the gastrointestinal tracts of these fishes, which are highly colonized in the gut region. This also indicates that there is also a distinct microbial source of digestive enzymes, such as amylase, apart from the endogenous sources in fish gastrointestinal tracts. The information generated from the present investigation might contribute to the incorporation of these bacteria in commercial aquaculture as supplement in formulated fish feed.

Amylases are among the most important enzymes and are of great significance in present–day biotechnology, having approximately 25% of the enzyme market. The amylases can be derived from many plants, animals and microorganisms; though the microbial sources proved to be economically feasible. Here, the attention has been focused on the aerobic gut bacteria of fresh water riverine fish species and selected strains were isolated and assayed for amylase activities to ascertain their role in exogenous production of digestive enzymes and the activity or stability of enzymes at various conditions. Besides, amylases from fungal and bacterial sources have been increasingly applied in industrial sectors like in starch industries (Pandey et al., 2000). It is desirable that α-amylases should be active at the high temperatures of gelatinization (100°-110°C) and liquefaction (80°-90°C) to economize the processes. Therefore, there has been a need for more thermophilic and thermostable α-amylases (Sindhu *et al.*, 1997). Previously only a few papers such as Goyal *et al*. (2005), Vaseekaran *et al*. (2010) and Arikan (2007) have reported thermostable amylases from different strains of *Bacillus* sp., mainly isolated from soil samples. In the present study, the optimum temperature and pH for the activity of the α-amylase obtained from this *Bacillus* strain were 60°C and 6.5, respectively. Michaelis constant (K_m) of the crude enzyme to soluble starch was 2.6 g/L and V_{max} value was 214 Um/L. As this bacterial strain was able to produce maximum α-amylase activity at 24 h and the enzyme showed promising pH and temperature stabilities without additives, it can be recommended for industrial applications.

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