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Characterization of starch utilizing bacterial symbionts from marine fishes of different feeding habits

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Original Article

Abstract

Research on the microflora of farmed fishes is extensive, but studies on wild fishes' microflora are limited. We isolated cultivable bacteria from many wild-caught marine fish species with different feeding habits from different coastal states of India. Bacterial species identity was determined by 16S rRNA gene sequencing for all the isolates. From Brownback trevally *Carangoides praeustus,* Indian seven-finger threadfin *Filimanus similis,* Largescale mullet *Liza macrolepis,* Indian oil sardine *Sardinella longiceps* and Pugnose pony fish *Secutor insidiator*, we could screen and characterize five amylolytic bacterial isolates. The phenotypic and genotypic (16S rRNA gene) characterization of the strains have confirmed the species as *Bacillus nealsonii* strain TCPS1 (GenBank Accession no. JN710379), *Vibrio alginolyticus* strain CFSS2C2 (GenBank Accession no.JN710378), *Bacillus atrophaeus* strain MLMS3 (GenBank Accession no. JN712298), *Pseudomonas stutzeri* strain KSLS4C3 (GenBank Accession no. JN710377), and *Aeromonas hydrophila* strain KSIS5 (GenBank Accession no. JN712299). For a 5-day experiment, significant ($P<0.05$) variation in starch utilization was recorded by the five symbionts. The rates of utilization at an exponential stage of growth were studied and the maximum rate for starch utilization was shown by *A. hydrophila* strain KSIS5 (0.79 mg maltose ml^{-1} medium h^{-1}). The results may be useful for further utilization of these species in the aquafeed industry or as a potential source of amylase in agriculture and allied sectors.

Keywords: *Symbiotic bacteria, marine fishes, 16S rRNA gene, starch utilization, amylase enzyme*

Introduction

Microbial symbiosis has been well documented in terrestrial animals, whereas it has been rarely reported from fish (Clements, 1997; Saha and Ray, 1998; Bairagi *et al.,* 2002; Saha *et al.*, 2006; Ngugi, 2017). A heterotrophic bacterial population with proteolytic, cellulolytic and amylolytic enzymes having a probable role in digestive enzyme supplementation was recorded from the digestive tract of *Labeo rohita* and *Channa straiatus* (Kar and Ghosh, 2008). The occurrence of cellulolytic bacteria in the gut of marine fishes has been reported by Asha-Augustine and Imelda-Joseph (2018). Amylase produced by the intestinal microflora significantly influences the digestion of starch in freshwater fish (Sugita *et al.*, 1997). A large number of microbial amylases are available commercially and their major advantage is their cost-effectiveness in bulk production (Gupta *et al.*, 2003). Sources of amylases include microorganisms, such as bacteria and fungi, as well as plants and animals. Amylases produced by microorganisms are reported to have superior thermal stability and can provide different sugar profiles to meet the current industrial demands (Martina *et al.*, 2019). Microbial amylases have a broad spectrum of industrial applications as they are more stable than those from plant and animal origins (Tanyildizi *et al.*, 2005). Amylase has applications in clinical, medicinal and analytical chemistry, in starch saccharification and in the textile, food, brewing and distilling industries (Pandey *et al.*, 2000; Gupta *et al.*, 2003; Kandra, 2003). The objective of the study was to isolate amylase-producing bacterial strains from selected marine fishes. The identification of bacterial isolates was based on phenotypic and genotypic characterization.

Material and methods

Sampling of fish and bacterial screening

Live fishes of marketable size within the minimum legal size (MLS) of five species (Table 1) were sampled from different fish

landing centres along the coasts of India. They were identified based on FishBase, version (01/2010) (Froese and Pauly, 2010). Five healthy fishes of each species were transported live to the nearest laboratory and were anaesthetized using clove oil (3-4 ppm) before further processing. Starch utilizing bacteria from fishes were screened under aseptic conditions (Trust and Sparrow, 1974; Das and Tripathi, 1991).

Phenotypic characterization

A homogenate of the intestinal mucosa of each of the test fish was prepared. The homogenate for each fish was then mixed with sterile 0.85% NaCl solution and vortexed. A five-fold serial dilution was then carried out. It was followed by aseptically spreading 0.1 ml from each dilution on nutrient agar plates by using a glass spreader. The plates were incubated at 37 °C in the incubator for 24 h. After this, well-defined bacterial colonies appeared on agar plates and were streaked onto nutrient agar slants in test tubes. All the isolates were phenotypically characterized up to the genus level based on morphological, physiological and biochemical characteristics (Boone and Castenholz, 2001). Further, all the isolates were streaked onto the starch agar plate and incubated at 37 °C for 24 h (Beveridge and Graham, 1991). Among the isolates, starch-utilizing bacteria were identified after plating all isolates on starch-agar (g l^{-1}); Starch, 10; Peptone, 10; Beef extract, 3; NaCl, 5; Agar, 15 (pH 7) plates and were incubated at 35 °C for 48 h. After the appearance of the colonies, the culture plates were flooded with 1% Lugol's iodine solution (Jacob and Gerstein, 1960) to identify amylase activity. Those starch-utilizing bacterial isolates were further maintained as pure cultures on nutrient agar slants and stored under refrigeration (Beveridge and Graham, 1991; Asha Augustine and Imelda Joseph, 2018).

Genotypic characterization

The bacterial DNA obtained by denaturation of cells of starch utilizing bacterial strains and by further centrifugation was used to amplify the 16S rRNA gene sequence for genotypic characterization. The most common primer pair devised by Weisburg *et al.* (1991) referred to as 27F and 1492R were used to amplify the 16S rRNA gene. For further analyses, the procedure described by Larkin *et al.* (2007) and Asha Augustine and Imelda Joseph (2018) were followed. The GC contents of 16S rRNA genes were analysed based on Kibbe (2007).

Estimation of starch utilisation

The amylase-producing cultures stored under refrigeration were further cultured in nutrient broth for 24 h and were then inoculated to the starch broth (g l^{-1}): Starch, 10; Peptone, 10; Beef extract, 3; NaCl, 5; Agar, 15; pH 7). Triplicates of each bacterial culture inoculated @ 0.2 ml (0.5A at 600 nm) in 20 ml starch broth (pH 7) were incubated at 37 °C. First sampling was done at 18 h of incubation and it continued till day 5 for determining starch utilization by the selected strains at different durations of incubation. The dinitrosalicylic acid (DNSA) method was followed to estimate the rate of utilization of starch and expressed as the quantity of breakdown of mg maltose m l^{-1} (Denison and Koehn, 1977). The rate of utilization of starch during the exponential phase was calculated following Waley (1981).

Statistical analyses

Analysis of variance (ANOVA) for data was done using IBM SPSS Statistics (2010) and significance at ($P < 0.05$). Duncan multiple range test (Duncan, 1955) was done with the significance level at $P = 0.05$

Results and discussion

In the present investigation, five fish species of different feeding habits exhibited amylolytic bacterial symbionts in their gastrointestinal (GI) tract (Table 1). The starch utilizing bacterial strains identified after phenotypic (Table 2) and genotypic characterization (Table 3) were *Bacillus nealsonii* strain TCPS1 (GenBank Accession no. JN710379), *Vibrio alginolyticus* strain CFSS2C2 (GenBank Accession no. JN710378), *Bacillus atrophaeus* strain MLMS3 (GenBank Accession no. JN712298), *Pseudomonas stutzeri* strain KSLS4C3 (GenBank Accession no. JN710377), and *Aeromonas hydrophila* strain KSIS5 (GenBank Accession no. JN712299). The microbial isolates were also submitted to

Table 1. Details of fish samples and collection sites

No	Place	Location (lat.- logi.)	Fish species	Feeding habit
1	Tuticorin	8° 48′ N 8° 07′E	*Carangoides praeustus* (Bennett, 1830)	Carnivore
2	Calicut	11°25′N 75°77′E	*Filimanus similis* Feltes, 1991	Carnivore
3	Munambam	10° 10′ N 76° 10′ E	*Liza macrolepis* (Smith, 1849)	Omnivore
4	Kanyakumari	8° 4′ N 77° 34′ E	*Sardinella longiceps* Valenciennes, 1847.	Phytoplanktivore
5	Karwar	14° 48′ N 74° 11′ E	*Secutor insidiator* (Bloch, 1787)	Zooplanktivore
6	Mandapam	9°18′N 79°6′E	*Siganus canaliculatus* (Park, 1797)	Herbivore

the culture collection repository of the ICAR-National Bureau of Agriculturally Important Microorganisms (Mau, Uttar Pradesh) and accession numbers were obtained as given below. The phylogenetic tree for each species is shown in Fig. 1 to 5.

Species	NBAIM Accession No.
Bacillus nealsonii strain TCPS1	NAIMCC-B-01189
Bacillus atrophaeus strain MLMS3	NAIMCC-B-01190
Pseudomonas stutzeri strain KSLS4C3	NAIMCC-B-01191
Aeromonas hydrophila strain KSIS5	NAIMCC-B-01192

Studies about bacterial populations associated with fish are very rare. The marine fish species from which the starch-utilizing bacteria isolated were mostly carnivores. The brownback trevally *C. praeustus* primarily feeds on shrimps, calanoid copepods and small fish (Sukree *et al*., 2007); the Indian seven-finger threadfin *F. similis* generally consume small fishes and crustaceans (Barman and Mishra, 2010); Largescale mullet *Liza macrolepis* an omnivore is known for feeding mainly on decayed organic matter and foraminifera, along with diatoms, algae and occasionally copepods (Luther, 1962); Indian oil sardine *S. longiceps* is predominantly a plankton feeder, consuming diatoms, dinoflagellates, zooplankton and blue-green algae (Tasaduq *et al*., 2019); the Pugnose pony fish *S. insidiator* feeds on zooplankton like copepods, mysids, larval fishes and crustaceans (Randall, 1995); Occurrence of amylolytic *B. nealsonii* and *V. alginolyticus* in the carnivorous fish *F. similis* and *A. hydrophila* in zooplanktivorous *S. insidiator* indicates the multiple roles of bacterial symbionts in the GI tract of fish. Irrespective of their feeding habits, fishes harbour bacterial strains either from the environment or from the feed they consume. In carnivorous and omnivorous fishes, these bacteria may exhibit their varying roles under favourable conditions while thriving in the gut. It has also been perceived that, in case the carnivorous fishes are farmed on artificial feeds, these bacteria may take a role in the digestion of starch ingredients. The omnivorous species *L. macrolepis* harboured amylolytic *B. atrophaues*. Occurrence of *B. subtilis* was reported from the intestinal tract of flathead

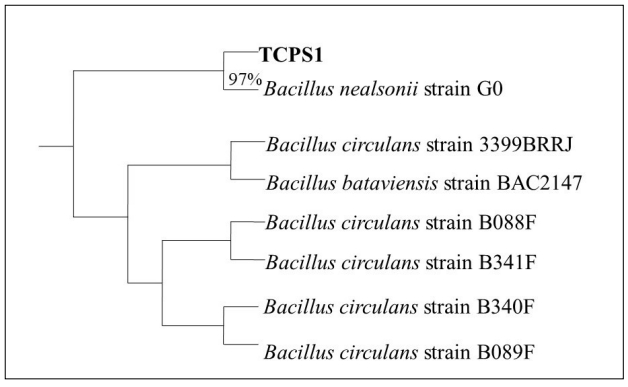

Fig. 1. Phylogenetic tree for TCPS1 (*B. nealsonii* strain TCPS1)

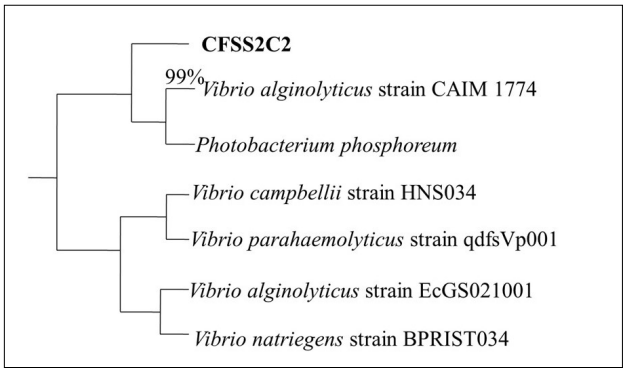

Fig. 2. Phylogenetic tree for CFSS2C2 (*V. alginolyticus* strain CFSS2C2)

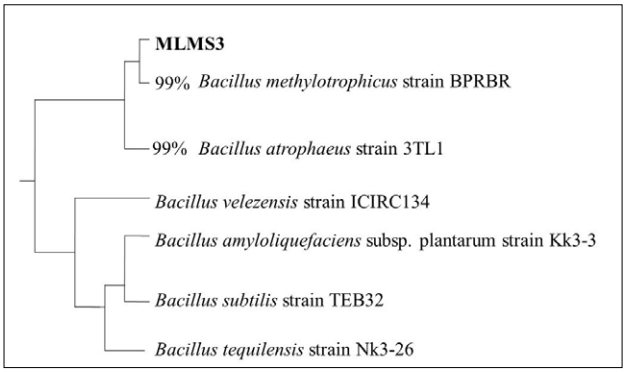

Fig. 3. Phylogenetic tree for MLMS3 (*B. atrophaeus* strain MLMS3)

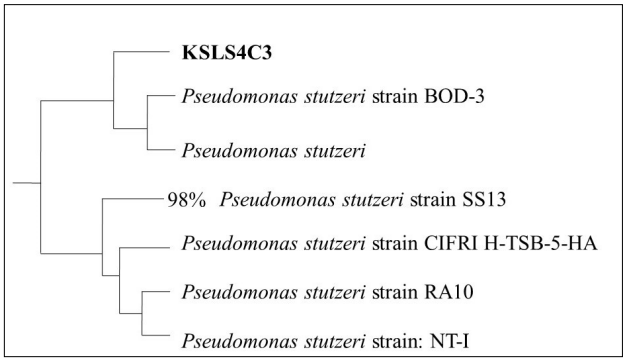

Fig. 4. Phylogenetic tree for KSLS4C3 (*P. stutzeri* strain KSLS4C3)

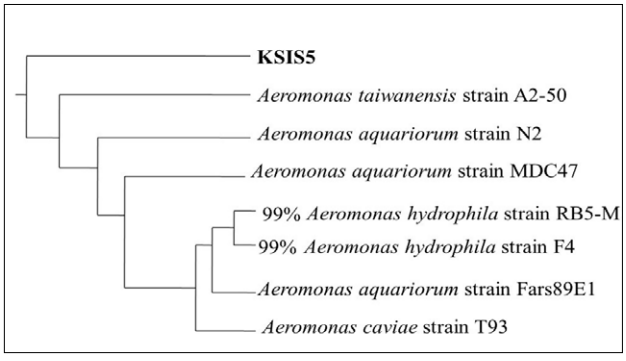

Fig. 5. Phylogenetic tree for KSIS5 (*A. hydrophila* strain KSIS5)

grey mullet, *Mugil cephalus* by Nagvenkar *et al.* (2006). Earlier reports also confirm the occurrence of *V. alginolyticus* in fish gut (Baross and Liston, 1970; Sun *et al.*, 2009). Lalucat *et al.* (2006) reported *P. stutzeri* from marine environment and fish gut. The occurrence of *A. hydrophila* in marine fish has been reported by Larsen and Jensen (1977). Thus, it is presumed that the bacterial isolates obtained from the GI tract of marine fishes in the present study are those naturally occurring in the environment and leading a symbiotic life in the gut environment, either contributing to feed digestion or disease resistance to the host.

In 1894, the first enzyme produced on an industrial scale was α-amylase, derived from a fungal source, and it has been utilized to treat gastrointestinal disorders (Pandey *et al.*, 2000). amylase is essential for starch processing, brewing and sugar production, in textile industries and detergent manufacturing. In the present study, amylase-producing bacteria were isolated from marine fishes and were characterized based on phenotypic as well as genotypic characteristics (Tables 2 and 3; Fig. 1-5). The amylase activity of isolated bacterial cultures was determined and it was concluded that out of the five bacterial strains, *Aeromonas hydrophila* strain KSIS5 showed the maximum rate of utilization of starch (0.79 mg maltose ml^{-1} medium h^{-1}) (Table 4). Bhat (2000) reported that research on microbial amylases has generated remarkable scientific knowledge and their enormous potential in biotechnology. A wide range of applications of amylases in the animal feed industry include their use in eliminating anti-nutritional factors present in raw materials or improving the nutritional value of certain cereals by degradation. Amylase is given as a digestive enzyme supplement to animals during the early post-weaning period (Scapinello *et al.*, 1999). The present study was focused on *in vitro* utilization of starch by the gut bacterial symbionts from marine fishes. The starch utilization by bacterial symbionts was determined for 5 days and was significantly ($P<0.05$) varying with time (Fig. 6-10). *B. atrophaeus* strain MLMS3 (2.45 mg maltose ml^{-1}), *P. stutzeri* strain KSLS4C3 (2.55 mg maltose ml^{-1})

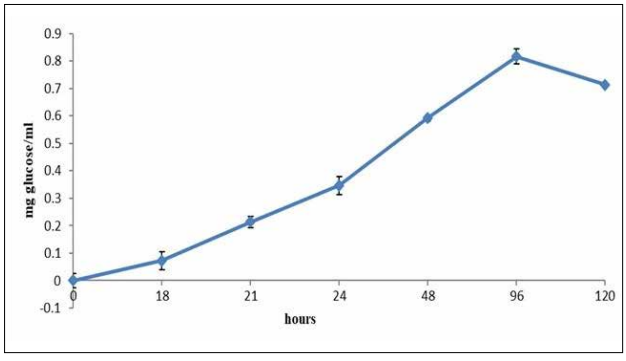

Fig. 6. Starch utilization by *B. nealsonii* strain TCPS1

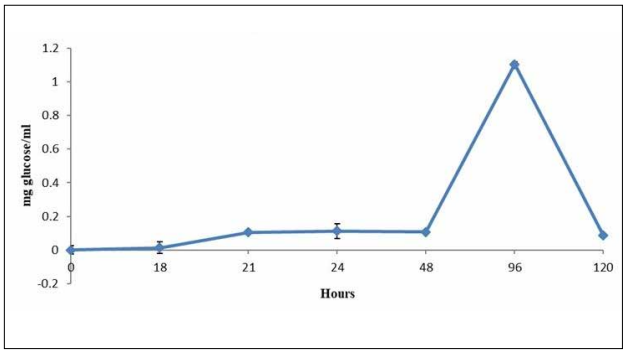

Fig. 7. Starch utilization by *V. alginolyticus* strain CFSS2C2

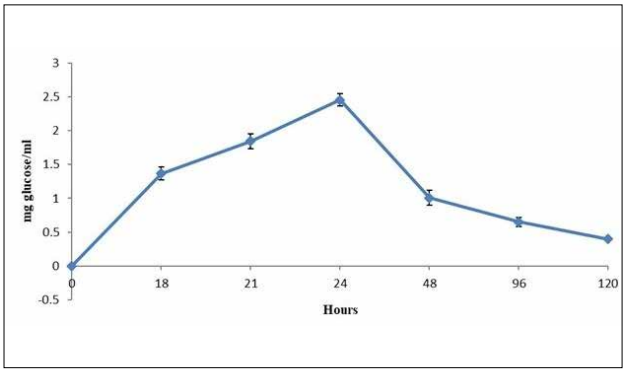

Fig. 8. Starch utilization by *B. atrophaeus* strain MLMS3

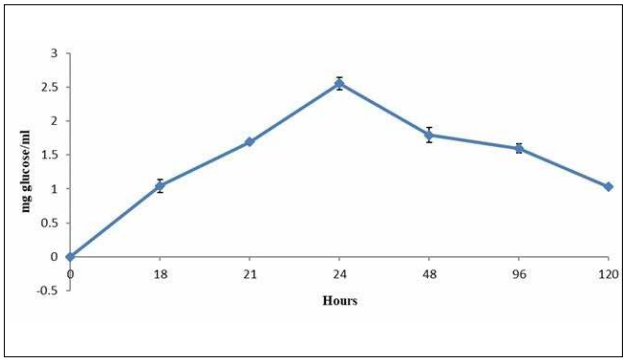

Fig. 9. Starch utilization by *P. stutzeri* strain KSLS4C3

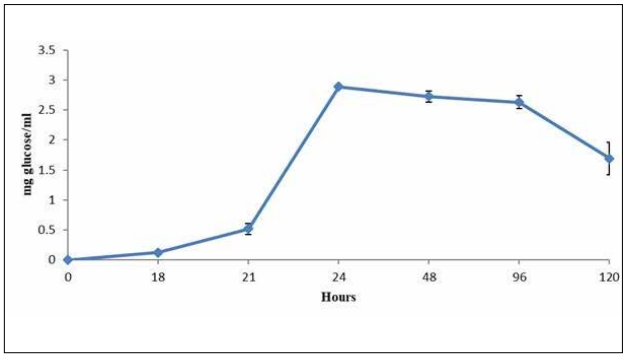

Fig. 10. Starch utilization by *A. hydrophila* strain KSIS5

and *A. hydrophila* strain KSIS5 (2.89 mg maltose ml^{-1}) from planktivorous and herbivorous fishes showed the maximum starch utilization at 24 h while *B. nealsonii* strain TCPS1 (0.82 mg maltose ml^{-1}) and *V. alginolyticus* strain CFSS2C2 (1.1 mg maltose ml^{-1}) showed a peak at 96 h. The results show that the bacteria that were active in the gut had no difficulty in utilizing the starch outside the gut whereas those from carnivores found it difficult to catch up with others in amylase activity. It may be because their enzyme system for starch utilization remained idle while in the fish gut where no starch substrate was available (Todar, 2011). Production of amylase by *P. stutzeri* has been reported by many workers (Fujita *et al*., 1989; Morishita *et al*., 1997; Mezaki *et al*., 2001; Janecek *et al*., 2003). Fishes with relatively broad diets are reported to modulate digestive enzyme activities based on dietary composition (German *et al*., 2004). In the present study also, it is concluded that the starch-utilizing bacterial symbionts occurring in marine fishes of different feeding habits do contribute to the modulation of digestive enzymes depending on the dietary components.

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