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Four novel strains of cellulolytic symbiotic bacteria isolated and characterized from GI tract of marine fishes of various feeding habits

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ABSTRACT

Selected marine fishes with different feeding habits were screened for the presence of symbiotic cellulolytic bacteria in their gut. Four cellulolytic species of symbiotic bacteria were isolated from GI tract of marine fishes namely *Carangoides praeustus, Filimanus similis, Sardinella longiceps* and *Sillago sihama*. The strains were identified after polyphasic phenotypic and genotypic (16S rRNA gene) characterization as *Bacillus subtilis* strain TCPC1, *Vibrio alginolyticus* strain CFSS2C2, *Pseudomonas stutzeri* strain KSLS4C3 and *Klebsiella oxytoca* strain MSSC4. (Genbank Accession nos.: JN710380, JN710378, JN710377, JN712301).The results indicated the presence of cellulolytic bacteria in GI tract of marine fishes of carnivorous, phytoplanktivorous and omnivorous feeding habits. Cellulolytic activity was the maximum for *B. subtilis* strain TCPC1 (0.45 mg glucose ml^{-1}) and *V. alginolyticus* strain CFSS2C2 (0.24 mg glucose ml^{-1}) at 234 h. While *P. stutzeri* strain KSLS4C3 showed the maximum utilization (0.22 mg glucose ml^{-1}) from 240 to 258 h. *K. oxytoca* strain MSSC4 (0.47 mg glucose ml^{-1}) showed three peaks during the study. The maximum rate of cellulose utilization was shown by *P. stutzeri* strain KSLS4C3 (0.05 mg glucose ml^{-1} medium h^{-1}) followed by *K. oxytoca* strain MSSC4 (0.03 mg glucose ml^{-1} medium h^{-1}).

1. Introduction

Algae consumed by marine fishes contain more complex and different carbohydrates than vascular plants. In addition to difference in secondary metabolites, digestion is achieved in a differing ionic environment. Neither has attracted much attention by researchers as yet (Krogdahl et al., 2005). Herbivorous fishes often exhibit higher carbohydrase activities, apparently to digest the storage carbohydrates of macroalgae, which can contain up to 50% carbohydrate (Horn et al., 1986), whereas carnivorous fishes frequently show higher proteolytic enzyme activities, to digest their high-protein animal diets (Fish, 1960; Cockson and Bourne, 1972). Studies on both marine (Moran and Clements, 2002) and freshwater (Drewe et al., 2004) fishes that undergo ontogenetic dietary shifts revealed that changes in digestive enzyme activities reflect the dietary changes of the fishes. Assessment of the substrate degrading ability of gut microflora is important in understanding the nutrition and physiology of the host organism and may help in formulating appropriate feeds. However, the precise nutritional role played by these bacteria is not clearly understood (Tanu et al., 2011).

Cellulase activity has been observed in several fish species indicating that fish may be able to utilize cellulose and similar fibrous carbohydrates (Chakrabarti et al., 1995). Whether the observed cellulase activities are of endogenous or microbial origin is under debate (Lindsay and Harris, 1980; Chiu and Benitez, 1981).

Reports on the existence of cellulase activity in the digestive system of fish are rare and moreover are conflicting with contradictory result. As pointed out by Luczkovich and Stellwag (1993), much of the controversy concerning the source of cellulose activity in the gut of fishes has been due to the inability to isolate cellulolytic microorganisms. Fish (1951), Barrington (1957) and Yokoi and Yasumasu (1964) believed that fish do not posses endogenous cellulase.Shcherbina and Kazlawlene (1971) indicated the presence of microbial cellulase in the posterior portion of digestive tract of carp. Lindsay and Harris (1980) showed cellulase activity in the digestive tract of fish and suggested the source of cellulose activity from the microbial population,although they discarded the idea of maintenance of stable cellulolytic microflora in fish. Lesel et al. (1986) reported cellulolytic flora in grass carp. Das and Tripathi (1991) assumed the cellulase-producing bacteria as a part of persistent intestinal flora in fish. The presence of considerable cellulolytic bacterial population has been observed in fish digestive tracts including those of carnivorous murrels by Kar and Ghosh (2008).

The absence of proof of endogenous cellulases in fishes leads to speculations about the role of symbiotic bacteria especially in case of phytoplanktivores. Among the three major groups of marine microalgae, chlorophyceae contain a significant proportion of cellulose in the

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wall. Therefore, while cellulose digestion is important in some herbivorous fishes that eat green algae and sea grass, it is non applicable to the vast majority of marine fishes (Mackie et al., 1996).

Studies on the diversity and role of individual microbes in the fish gut have been hampered by the lack of adequate methodologies. For a given microbial species to be identified by means of their phenotypic features, this species has to be cultivated. To date, the gut microflora of fish has been traditionally investigated by culture dependent methods by isolating organisms from intestinal material and culturing and subsequently analysing them. The recent advent of molecular methodologies has greatly aided in the analysis of the bacteria and the findings from culture-based methods have been supplemented with molecular techniques that are based on the 16S rRNA gene (Zoetendal et al., 2004; Romero and Navarrete, 2006; Yang et al., 2007).

Bioinformatics provides software tools to deal with the vast amount of data (over 36 billions base pairs and 30 millions sequences alone at genbank) (Ramu et al., 2003). One of these tools is BLAST (Basic Local Alignment Search Tool), which searches for patches of similarity using similarity scoring matrices. BLASTn is used to compare a DNA sequence with DNA sequences in the database. It returns a list of best matches with their bit score and E-value. The Sequences can be aligned across their entire length (global alignment) or only in certain regions (local alignment). With the aid of multiple sequence alignments, biologists are able to study the sequence patterns conserved through evolution and the ancestral relationships between different organisms. The most widely used programs for global multiple sequence alignment are from the Clustal series of programs. The culture dependent methods and identification of the fish microbiota based on phenotypic and biochemical key characteristics is still the mainstay for studies on the microbial ecology of the gut of fishes. Marine fishes of India were not yet explored for potential cellulolytic strains of symbiotic bacteria. Cellulases are industrially important and identification of novel sources always help in improving the technology.

Cellulase activity has been observed in several fish species indicating that fish may be able to utilize cellulose and similar fibrous carbohydrates (Chakrabarti et al., 1995). Whether the observed cellulase activities are of endogenous or microbial origin is under debate (Lindsay and Harris, 1980; Chiu and Benitez, 1981). In a study of digestive enzymes in grass carp *Ctenopharyngodon idella*, cellulase was observed both in hepatopancreas and intestine and the dietary cellulose level affected the activities significantly (Das and Tripathi, 1991). The fact that cellulase activity was reduced to approximately one third when the antibiotic tetracycline had been added to the diet, indicates that microorganisms may supply an important part of the cellulolytic activity in the intestinal tract of fish. The residual activity measured in the presence of the antibiotic, indicate that a fraction of the activity may be of endogenous origin (Das and Tripathi, 1991). However, because of the abundance of antibiotic resistance, there is the distinct possibility that tetracycline-resistant organisms may account for the remaining activity (Krogdahl et al., 2005).

Reports on the existence of cellulase activity in the digestive system of fish are rare and moreover are conflicting with contradictory result. As pointed out by Luczkovich and Stellwag (1993), much of the controversy concerning the source of cellulose activity in the gut of fishes has been due to the inability to isolate cellulolytic microorganisms. Fish (1951), Barrington (1957) and Yokoi and Yasumasu (1964) believed that fish do not posses endogenous cellulase. Shcherbina and Kazlawlene (1971) indicated the presence of microbial cellulase in the posterior portion of digestive tract of carp. Lindsay and Harris (1980) showed cellulase activity in the digestive tract of fish and suggested the source of cellulose activity from the microbial population, although they discarded the idea of maintenance of stable cellulolytic microflora in fish. Lesel et al. (1986) reported cellulolytic flora in grass carp. Das and Tripathi (1991) assumed the cellulase-producing bacteria as a part of persistent intestinal flora in fish. The presence of considerable cellulolytic bacterial population has been observed in fish digestive tracts including those of carnivorous murrels by Kar and Ghosh (2008).

The absence of proof of endogenous cellulases in fishes leads to speculations about the role of symbiotic bacteria especially in case of herbivores. Among the three major groups of marine microalgae, chlorophyceae contain a significant proportion of cellulose in the wall. Therefore, while cellulose digestion is important in some herbivorous fishes that eat green algae and sea grass, it is non applicable to the vast majority of marine fishes (Mackie et al., 1996). Symbiotic bacteria have developed sophisticated mechanisms for the regulation of both catabolic and anabolic pathways. Generally, they do not synthesize degradative (catabolic) enzymes unless the substrates for these enzymes are present in their environment. Bacterial cells shut down biosynthetic (anabolic) pathways when the end product is not needed or is readily obtained by uptake from the environment (Todar, 2011).

Applications of cellulases and hemicellulases in the feed industry have received considerable attention because of their potential to improve feed value and performance of animals (Dhiman et al., 2002). Pretreatment of agricultural silage and grain feed by cellulases or xylanases can improve its nutritional value (Godfrey and West, 1996). The enzymes can also eliminate antinutritional factors present in the feed grains, degrade certain feed constituents to improve the nutritional value, and provide supplementary digestive enzymes such as proteases, amylases, and glucanases (Kuhad et al., 2011). One of the important applications is supplementing diets of farm animals with cellulases to improve feed utilization and animal performance by enhancing fiber degradation. Dairy cows fed with forage treated with a cellulase enzyme preparation ate more feed and produced 5–25% more milk (Murad and Azzaz, 2010). If these enzymes can be recombinantly produced on mass or produced *in situ* by the bacterial strains naturally encoding them, the cost of hydrolysis for the production of biofuels can be decreased by reducing the need for production of multiple enzymes for efficient hydrolysis. Isolation and characterization of cellulase-producing bacteria will continue to be an important aspect of biofuel research (Maki et al., 2009). Microbial enzymes have enormous advantage of being produced in large quantities by established fermentation techniques. Considerable efforts have been devoted to the selection of microorganisms via sophisticated screening techniques for the production of enzymes with new physiological/physical properties and tolerance to extreme conditions used in the industrial processes (e.g. temperature, salts and pH). The marine environment has proven to be a rich source of both biological and chemical diversity (Pomponi, 1999).

Marine microorganisms have recently emerged as a potent source for the isolation of industrial enzymes (Chandrasekaran, 1997) as marine bacterial enzymes have several advantages for industrial utilization (Ventosa and Neito, 1995). The optimum activity of marine bacterial enzymes usually occurs at high salinity, making these enzymes utilizable in many harsh industrial processes, where the concentrated salt solutions used would otherwise inhibit many enzymatic transformations. In addition, most marine bacterial enzymes are considerably thermotolerant, remaining stable at room temperature over long periods. The surfaces and internal spaces of marine organisms are more nutrient rich than seawater and most sediments, thus they would likely to be a unique niche for the isolation of diverse assemblages of bacteria. These associated bacteria are a potential source of biologically active compounds of low molecular weight (Fenical, 1993, Kobayashi and Masami,1993, Trischman et al., 1994). On the other hand proteins, especially enzymes, have been largely neglected (Mohapatra et al., 2003).

2. Materials and methods

2.1. Sampling of fishes

Fishes to be screened for cellulolytic symbiotic bacteria were sampled live based on their feeding habits with the help of local fishermen,

from different fishing harbours along the coasts of India and identified based on Fish Identification at fishbase,version(01/2010) (Froese and Pauly, 2010). Five healthy fishes of each species were transported live to the nearest laboratory under sterile conditions and were anaesthetized using clove oil (3–4 ppm) before further processing.

2.2. Sampling for bacteria

Screening of cellulose utilizing symbiotic bacteria were carried out under aseptic surroundings. Before dissection, the ventral surface of the fish was thoroughly scrubbed with 1% iodine solution (Trust and Sparrow, 1974). The fish were dissected within laminar air flow cabinet on ice and the alimentary canals were removed as quickly as possible. Portions of the alimentary canal were first cleaned, then cut into pieces and slit open by a longitudinal incision. The contents were transferred to sterile petridishes. The pieces of the alimentary canal were thoroughly flushed with sterile chilled saline (ph 7.4; 0.89% (w/v) sodium chloride) and homogenized in sterile saline (10:1; volume: weight) (Das and Tripathi, 1991). The homogenate was used as inoculum for culture. The homogenate of the gut of each of the test fish was subjected to 1:10 serial dilutions.

2.2.1. Isolation and culture of cellulolytic bacteria from fish gut

The homogenate of the gastrointestinal wall mucosa of each of the test fish after five-fold serial dilution was used for isolation and enumeration of cellulose utilizing bacteria (Beveridge and Graham, 1991). 0.1 ml sample was taken from each dilution and plated aseptically onto Dubos cellulose Agar media (Composition (g l^{-1})); Carboxymethyl cellulose, 12; KH_2PO_4, 10; $NaNO_3$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $Fe_2(SO_4)_3{\cdot}7H_2O$, 0.004; Agar, 20 (ph 6). These culture plates were incubated at 35 °c for 48 h. After appearance of the colonies, the plates were flooded with 5 ml of Congo Red dye prepared in 0.7% Agarose (Seakem HGT Agarose, Cambrex India Pvt. Ltd., Mumbai, India) according to the method of Teather and Wood (1982). The appearance of a clear zone around the colony after flooding the plates indicated the presence of cellulolytic activity. The discrete and well isolated bacterial colonies on the above mentioned media plates with cellulolytic activities were selected and streaked separately onto nutrient agar plates to obtain pure cultures. Single, isolated colonies from the streaked plates were transferred to nutrient agar slants (supplemented with 2% nacl) (Composition (g l^{-1})) Peptone − 5; Meat extract − 1; Yeast extract − 2; Sodium chloride − 5; Agar – 15 (ph 7) and stored under refrigerated condition for further studies.

2.3. Phenotypic characterization of symbiotic bacteria

The bacterial cultures were identified according to their Phenotypic (morphological, physiological, biochemical) characteristics (Boone and Castenholz, 2001).

3. Genotypic characterization

3.1. Amplification, cloning and sequencing of 16S rRNA gene

The genomic DNA obtained by denaturation and further centrifugation of bacterial cell was used as the template for PCR to amplify the 16S rRNA gene sequence. PCR primers were used to amplify the 16srRNA gene providing the phylogenetic information. The most common universal primer pair is currently referred to 27F and 1492R (James, 2010). At the end of the PCR run the tube was taken out and electrophoreses of the product was done using Agarose gel electrophoresis. Afterwards the bands of DNA were recovered from the gel by gel extraction and used for cloning. The PCR amplified products were cloned into pGEM-T vector and then sequenced at SciGenom Labs Pvt Ltd, Kochi. DNA samples were sequenced by the sequencing instrument MACROGEN 3730XL7–16112–010 and the sequences were processed by ABI 1.6.0. The sequence were merged in EMBOSS Merger.

3.2. Phylogenetic analysis

The 16SrRNA gene sequences were aligned and compared with other 16SrRNA gene sequences in the Genbank by using the NCBI Basic Local Alignment Search Tool, BLASTn program(http://www.ncbi.nlm.nih.gov/BLAST). The E-value was kept at zero for maximum accuracy. The phylogenetic trees were created using homology and phylogenetic analysis by neighbour joining method using multiple sequence alignment program, clustalw with bootstrap replications corresponding to 1000 resamples (Larkin et al., 2007).

3.3. Sequences deposited in genbank

The 16SrRNA gene sequences have been deposited to Genbank using BankIt submission tool and has been assigned with Genbank Accession numbers by NCBI.

3.4. Analysis of GC content

GC-content is the percentage of nitrogenous bases on a DNA molecule that are either guanine or cytosine from a possibility of four different ones, including adenine and thymine. The GC contents of 16SrRNA genes were calculated based on 16SrRNA gene sequences using Oligo Calculator version 3.26 (Kibbe, 2007).

4. Utilization of cellulose by the symbiotic bacteria

4.1. Bacterial species

Four cellulolytic bacterial isolates from marine fishes were used for the study. They were *Bacillus subtilis* strain TCPC1 *Carangoides praeustus* (Bennett, 1830), *Vibrio* alginolyticus strain CFSS2C2 from *Filimanus similis* Feltes, 1991, *Pseudomonas stutzeri* strain KSLS4C3 from *Sardinella longiceps* Valenciennes, 1847, and *Klebsiella oxytoca* strain MSSC4 from *Sillago sihama* (Forsskål, 1775).

4.1.1. Seed culture of selected bacteria

To prepare the seed culture for inoculation, the bacterial isolates stored at 4 °C on nutrient agar slants were cultured in nutrient broth for 24 h and were further inoculated to the selective media.

4.1.2. Media composition

Nutrient broth (gl^{-1}): Peptone, 10; Meat extract, 10; Sodium chloride, 5; Distilled water, 1000 ml (pH 7.2 ± 0.2)

Dubo's Cellulose broth (g l^{-1}): Carboxymethyl cellulose, 12; KH_2PO_4, 10; $NaNO_3$, 0.5; $MgSO4{\cdot}7H_2O$, 0.5; KCl, 0.5; $Fe_2(SO_4)_3{\cdot}7H_2O$, 0.004; Agar, 20 (pH 6)

4.1.3. Innoculum size and sampling

0.2 ml innoculum (0.5 A at 600 nm) were innoculated to 20 ml cellulose broth. Triplicates were incubated at 37 °C at an initial pH of 6.5 for cellulose broths respectively. Sampling was done starting from 18 h of incubation to 11 days for cellulose utilization to get a clear profile of cellulase production by the selected strains.

4.2. Utilization of cellulose by the symbiotic bacteria

The end products of cellulose hydrolysis were determined by Dinitrosalicylic acid (DNSA) method (Denison and Koehn, 1977). Cellulose is broken down to glucose by the enzyme cellulose.

4.2.1. Cellulose utilization

DNSA, which acts as a terminating agent to stop the enzyme substrate reaction, was prepared before start of the experiment and kept at

4 °C.

4.2.2. Glucose standard

Working solution of concentration 1 mg glucose ml^{-1} was diluted using distilled water in the ratios 2:3, 1:2, 1:3, 1:5 and 1:10. 0.5 ml of each was taken in a test tube. 1 ml distilled water was added and 3 ml DNSA was added and then boiled at 100 °C for 5 min. It was then cooled in ice bath. Further, OD was measured at 540 nm against reagent blank. Standard curve was plotted taking concentration of glucose on *x*- axis and OD on *y*- axis.

4.2.3. Preparation of sample for glucose estimation

To study cellulose utilization, 1 ml of sample from each of triplicate flasks were taken in eppendorf tubes and centrifuged at 10,000 × *g* for 10 min at 4 °C. At the end of centrifugation, 0.5 ml of the cell free supernatant was taken. 1 ml distilled water was added and then 3 ml DNSA was added to it. It was boiled at 100 °C for 5 min and cooled in ice bath. OD was measured at 540 nm. The value was converted and expressed as mg of glucose per ml culture.

4.3. Rate of utilization of cellulose during exponential phase (Waley, 1981)

The slope of a chord joining the points (Ut_1,t_1) and (Ut_2,t_2) on a progress curve of varying concentration against time is (Ut_1 -Ut_2)/(t_1-t_2), and this slope is equal to the rate,

$$R = Ut_2 - Ut_1$$

	t
Where, t	- t_2-t_1
U t_1	- utilization of cellulose at t_1,
U t_2	- utilization of cellulose at t_2
t_1	- initial time
t_2	- final time

4.4. Statistical analyses

The data obtained were subjected to analysis of variance (ANOVA) using SPSS data editor and significance at ($P < 0.05$) was determined. Duncans Multiple range test (Duncan, 1955) was done for comparison of performances by testing differences among means. The significance of differences was tested at the significance level $P = 0.05$ (Tables 1 and 2).

5. Results

The species identified from *Carangoides praeustes* showed *Bacillus subtilis* MF-2 as the nearest neighbour (Fig. 1 and Table 3). The strain was designated as *Bacillus subtilis* strain TCPC1. It was gram positive, translucent, rodshaped and resistant to penicillin. The species identified from *Filimanus similis* showed *Vibrio alginolyticus* CAIM 1774 as the nearest neighbour (Fig. 2 and Table 3). The strain was designated as *Vibrio alginolyticus* strain CFSS2C2 and was gram negative, translucent, rodshaped and resistant to penicillin. The species identified from *Sardinella longiceps* showed *Pseudomonas stutzeri* SS13 as the nearest neighbour (Fig. 3 and Table 3). The strain was designated as *P.stutzeri* strain KSLS4C3. It was gram negative, opaque, rodshaped and resistant to penicillin. The species identified from *Sillago sihama* showed *Klebsiella oxytoca* strain AIMST 10. Pl.3 as the nearest neighbour (Fig. 4 and Table 3). The strain was designated as *Klebsiella oxytoca* strain MSSC4. It was gram negative, translucent, rodshaped and resistant to penicillin. Table 4

The results obtained for cellulose utilization by different bacterial strains are shown in Figs. 5–8. Cellulolytic activity was the maximum for *B. subtilis* strain TCPC1 (0.45 mg glucose ml^{-1}), *V. alginolyticus* strain CFSS2C2 (0.24 mg glucose ml^{-1}) at 234 h while *P. stutzeri* strain KSLS4C3 (0.22 mg glucose ml^{-1}) showed the maximum utilization from 240 to 258 h. *K. oxytoca* strain MSSC4 (0.47 mg glucose ml^{-1}) showed three peaks.

In case of cellulose utilization, the study was conducted for more days because the strains in general showed increase in utilization of cellulose only after 120 h. The cellulose utilization by *P. stutzeri* strain KSLS4C3 and *K. oxytoca* strain MSSC4 showed more than one peak during the study.

The colors of the graphs indicate feeding habits of the fishes from which the particular symbiont was isolated, viz., red colour indicates a marine carnivorous fish, blue indicates a planktivore and black, an omnivorous fish species.

6. Discussion

Occurrence of cellulolytic *Bacillus* spp. And *Vibrio* sp. In the carnivorous fishes *C. Praeustus*, and *F. Similis*is noteworthy and indicates the multiple role of bacterial symbionts in GI tract of fish. In a study done with carnivorous fishes, Bairagi et al. (2002) failed to detect cellulolytic bacteria in the gastrointestinal tract of catfish and murrels. However, the result of some other investigations showed the presence of cellulolytic bacteria in carnivorous fishes (Kar et al., 2008; Sugita et al., 1997; Ghosh et al., et al., 2002). Stickney and Shumway (1974) had opined that omnivores and carnivores might pick up cellulolytic flora from the invertebrates that harbour the bacteria. Carnivores may get these from environment or from feed like crustaceans, mollusks or even other fishes they eat. These bacteria may have other functions also to serve than cellulose hydrolysis which they may exhibit under favourable conditions for thriving in a carnivore's gut. The presence of these bacteria in carnivores shows the environmental effect on the gut microflora.

The results also show that omnivorous *S. sihama* harboured the cellulolytic *Klebsiella* sp. In its gut.*B. subtilis* has previously been reported in the intestinal tract of flathead grey mullet, *mugilcephalusl*. (Nagvenkar et al., 2006). Omnivores also obtain their share of symbionts from water and food. As the food of these fishes contain more cellulose matter than that of carnivores, these bacteria can survive in a better way in omnivore's gut. They may aid in digestion of plant matter; thereby increasing the adaptability of the fish to a wide range of feed stuffs for an omnivorous habit.

Table 1
Details of the marine fishes selected for the present study.

Sl.no	Place	Location *(Latitude and Longitude)*	Period	Fish species selected	Feeding habit	Bacterial amylolysis	Bacterial cellulolysis
1	Tuticorin	8°48'N 8°07'E	June 2009	*Carangoides praeustus* (Bennett, 1830)	carnivore	+	+
2	Calicut	11°25′N 75°77′E	November 2009	*Filimanus similis* Feltes, 1991	carnivore	+	+
3	Kanyakumari	8°4′N 77°34′E	July 2009	*Sardinella longiceps* Valenciennes, 1847.	Phytoplanktivore	+	+
4	Mandapam	9°18'N 79°6′E	March 2008	*Sillago sihama* (Forsskål, 1775).	omnivore	–	+

Table 2
Phenotypic characterization of cellulose utilizing bacterial isolates.

Sl.no.	Name of test	*Carangoides praeustus*	*Filimanus similis*	*Sardinella longiceps*	*Sillago sihama*
1	gram's stain	Gram (+)	Gram(-)	Gram(-)	Gram(-)
2	cell shape	Rods	Short Rods	Rods	Long Rods
3	appearance	Transluscent	Transluscent	Opaque	Transluscent
4	elevation	Raised	convex	Umbonate	Convex
5	margin	Entire	entire	Undulate	Entire
6	configuration	Round	Round	Round	Round
7	colour	Off white	Off white	Off white	White
8	H_2S production	+	–	–	–
9	Mac Conkey agar test	Lactose fermenting	No Growth	Lactose fermenting	Non Lactose fermenting
10	fluorescence	–	–	–	–
11	motility	Motile	Non Motile	Motile	motile
12	catalase	+	+	+	+
13	oxidase	–	+	+	–
14	indole	–	–	–	–
15	MR	+	–	–	+
16	VP	+	–	–	–
17	citrate utilization	+	+	+	+
18	O/F test	Fermentative	Fermentative	Alkaline	Fermentative with gas
19	nitrate reduction	+ (Gas)	+	+ (Gas)	+
20	pencilin sensitivity	Resistant	Resistant	Resistant	Resistant
21	gelatin liquifaction	+	–	+	+
22	starch utilization	–	+	+	–
23	cellulose hydrolysis	+	+	+	+
24	heat resistance	–	–	–	+
25	growth on 0% NaCl	+	+	+	+
26	growth on 5% NaCl	+	+	+	+
27	growth on 9% NaCl	+	+	–	+
28	growth on 12% NaCl	–	–	–	+
29	growth on 15% NaCl	–	–	–	–
30	growth on 20% NaCl	–	–	–	–
31	growth at pH 5.0	+	+	–	+
32	growth at pH 9.0	+	+	+	+
33	growth at pH 10.0	+	+	+	+
1	adonitol	–	–	–	–
2	arabinose	+ (G)	+	+	–
3	cellobiose	+ (G)	+	+	+
4	dulcitol	–	–	–	–
5	fructose	+ (G)	+	+	–
6	glucose	+ (G)	+	–	–
7	inositol	–	–	+	+
8	lactose	+ (G)	–	+	+
9	mannitol	+ (G)	+	–	+
10	mellibiose	+ (G)	–	+	–
11	raffinose	+ (G)	–	+	–
12	rhamnose	+ (G)	–	–	–
13	salicin	–	–	+	+
14	sorbitol	+ (G)	–	–	–
15	sucrose	+ (G)	+	+	+
16	trehalose	+ (G)	+	+	+
17	xylose	+ (G)	+	+	+
Bacterial genus identified		*Bacillus*	*Vibrio*	*Pseudo-monas*	*Klebsiella*

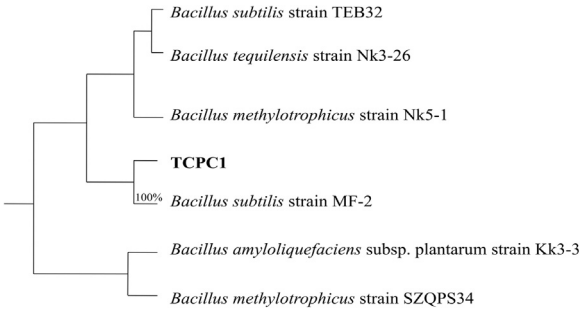

Fig. 1. Phylogenetic tree for TCPC1.

According to the present study, *S. Longiceps,* a phytoplankton grazer harboured *Pseudomonas* sp. which was cellulolytic. In planktivores also, the source of symbionts is the environment. These bacterial symbionts may play a more important role in digestion in herbivorous fishes by producing cellulose than in fishes of other feeding habits. In a recent study, *Bacillus subtilis* cy5 and *Bacillus circulans* tp3, isolated from the gastrointestinal tracts of *Cyprinus carpio* and *Oreochromis mossambicus* respectively were identified as potent cellulase producers (Ray et al., 2007). In case of terrestrial plant/herbivore relationships plant composition has fiber which may be defined as material resistant to endogenous digestion (Cummings and Macfarlane, 1991). In contrast, marine algae contain a complex array of cell wall and storage polysaccharides, and the relationship of these to fish digestive processes is poorly understood (Choat and Clements, 1998). Herbivorous fish may have adapted to these complex algal diets in several ways, including high food intake and gut throughput rates, optimization of key digestive enzymes, and various degrees of reliance on microbial processes in the gut (Mountfort et al., 2002). Since fishes cannot produce cellulases, the digestion of whatever cellulose comes in gut is by these bacteria.

The strains identified in the present study are all new ones which were not previously reported from any source. *Bacillus cereus* isolated from fish gut was shown to produce protease by Esakkiraj et al. (2009).

Table 3
Genotypic characterization by 16S rRNA gene sequence alignment tools.

Sl. No	Test species	GC content	Nearest neighbour by BLAST	Max.ident.	GC content of neighbour	Species	GenBank accession no:
1	TCPC1	55%	*Bacillus subtilis* strain MF-2	100%	55%	*Bacillus subtilis* strain TCPC1	JN710380
2	CFSS2C2	55%	*Vibrio alginolyticus* strain CAIM 1774	99%	54%	*Vibrio alginolyticus* strain CFSS2C2	JN710378
3	KSLS4C3	52%	*Pseudomonas stutzeri* strain SS13	98%	53%	*Pseudomonas stutzeri* strain KSLS4C3	JN710377
4	MSSC4	55%	*Klebsiella oxytoca* strain AIMST 10.Pl.3	99%	56%	*Klebsiella oxytoca* strain MSSC4	JN712301

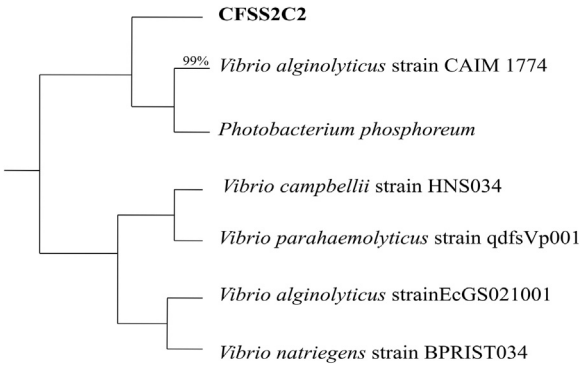

Fig. 2. Phylogenetic tree for CFSS2C2.

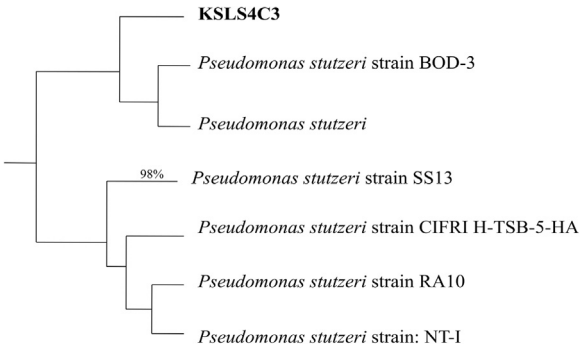

Fig. 3. Phylogenetic tree for KSLS4C3.

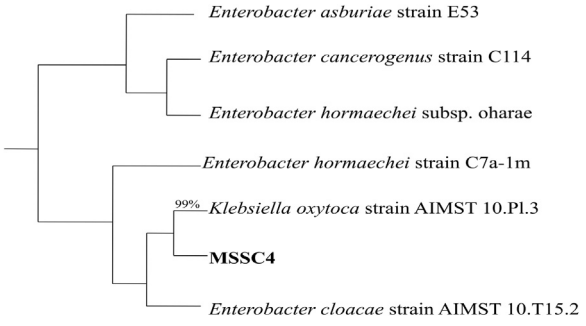

Fig. 4. Phylogenetic tree for MSSC4.

Vibrio alginolyticus has been reported from fish gut (Baross and Liston, 1970; Sun et al., 2009). *Pseudomonas stutzeri* has been reported from marine environment and fish gut by Lalucat et al. (2006). *Aeromonas hydrophila* has been reported from marine fish by Larsen and Jensen (1977). *Klebsiella oxytoca* has been reported from the gut of African cat fish by Oladosu-Ajayi et al. (2011). The scanty references available show the least quantum of work done in gut microbes of marine fishes compared to that in other fields. Molecular methods make it possible to identify new microbes from any source. 16S rRNA gene sequence

Table 4
Rate of cellulose utilization of cellulose by each species at exponential stage of growth.

Sl. No.	Species	Rate (mg glucose ml^{-1} medium h^{-1})
1	*Bacillus subtilis* strain TCPC1	0.002
2	*Vibrio alginolyticus* strain CFSS2C2	0.001
3	*Pseudomonas stutzeri* strain KSLS4C3	0.049
4	*Klebsiella oxytoca* strain MSSC4	0.027

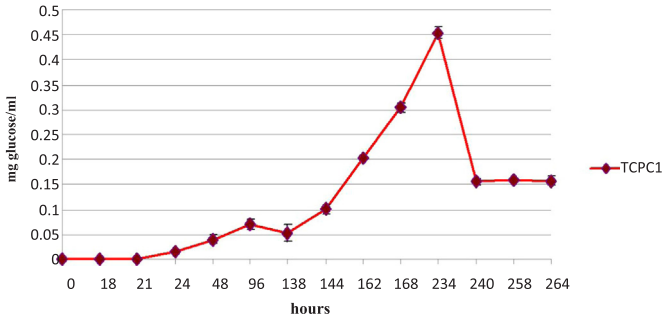

Fig. 5. Cellulose utilization by *Bacillus subtilis* strain TCPC1.

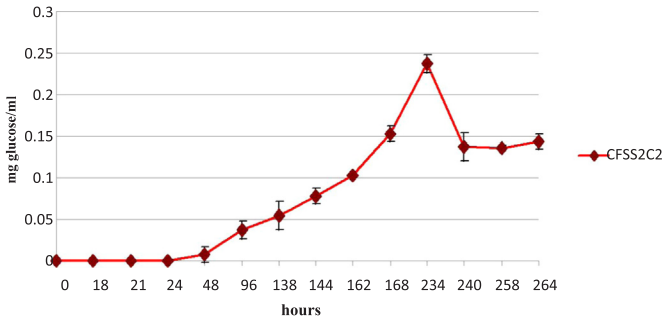

Fig. 6. Cellulose utilization by *Vibrio alginolyticus* strain CFSS2C2.

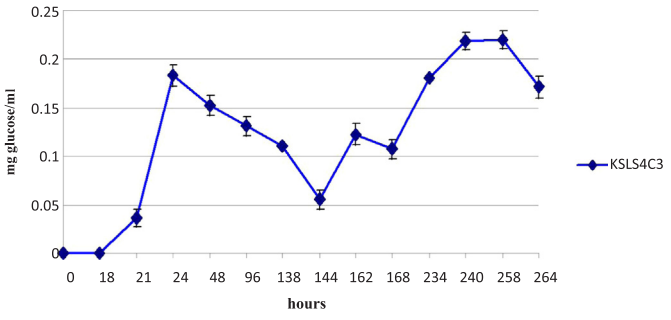

Fig. 7. Cellulose utilization by *Pseudomonas stutzeri* strain KSLS4C3.

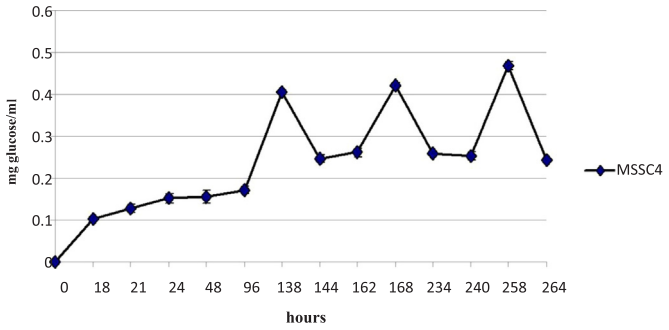

Fig. 8. Cellulose utilization by *Klebsiella oxytoca* strain MSSC4.

analysis can better identify poorly described, rarely isolated, or phenotypically aberrant strains. Few other genes are as highly conserved as the 16S rrna gene. Although the absolute rate of change in the 16S rrna gene sequence is not known, it does mark evolutionary distance and relatedness of organisms (Kimura, 1980; Pace, 1997; Thorne et al., 1998; Harmsen and Karch, 2004).

Although 500 and 1500 bp are common lengths to sequence and compare, sequences in databases can be of various lengths. Genbank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene. This means that there are many previously deposited sequences against which to compare the sequence of an unknown strain (Clarridge, 2004).

The 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (Woese et al., 1985; Woese, 1987). In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including what we now call the species and subspecies level. Sometimes sequencing the entire 1500-bp region is necessary to distinguish between particular taxa or strains (Sacchi et al., 2002, 2002a2002a, 2002b). Sequencing of the entire 1500-bp sequence is also desirable and usually required when describing a new species. In this study, full length sequences of 16S rRNA gene was used for *Klebsiella* sp. MSSC4 and partial sequences were used for others which ranged from 600 to 800 base pairs. However, for most clinical bacterialisolates the initial 500-bp sequence provides adequate differentiation for identification and in fact can provide a bigger percent difference between strains because the region shows slightly more diversity per kilobase sequenced. Kattar et al. (2001) found that 66% of the variability in the 16 S rRNA gene sequence among *Bordetella* species was in the first 500 bp. Evaluations published in the literature, made using the microseq database (AppliedBiosystems Inc. [ABI], Foster City, Calif.), are usually based on the 500-bp sequence (Tang et al., 1998; 1998, 2000; Patel et al., 2000; TangHall et al., 2000; Hall, et al., 2003). Other researchers have made identifications using sequences of about 400 bp (Bosshard et al., 2003) or even less than 200 bp (Wilck et al., 2001).

The strains under present study after phylogenetic tree preparation were identified as *Bacillus subtilis* strain TCPC1, *Vibrio alginolyticus* strain CFSS2C2, *Pseudomonas stutzeri* strain KSLS4C3 and *Klebsiella oxytoca* strain MSSC4. The phylogenetic analysis revealed several unresolved relationships between bacterial isolates from the present experiment and their type strain matches. Failure to differentiate closely related species could be due to the fact that full length of the 16S rDNA were used in the present study and that different species of *Bacillus,* and *Klebsiella* are very closely related to each other or their phylogeny may be improved further with time as more data is available in public data banks. Though at present this is the latest available method for identification of bacteria, additional characterization methods should be devised if further clarification or fine differentiation is required.

Despite its accuracy, 16S rRNA gene sequence analysis lacks widespread use beyond the large and reference laboratories because of technical and cost considerations. Thus, a future challenge is to translate information from 16S rRNA gene sequencing into convenient biochemical testing schemes, making the accuracy of the genotypic identification available to the smaller and routine clinical microbiology laboratories (Clarridge, 2004).

Pseudomonas stutzeri has been reported to produce amylase (Fujitha et al., 1989; Morishita et al., 1997; Mezaki et al., 2001; Janecek et al., 2003). Reports of cellulose utilizing bacteria from marine fish GI tracts are very rare. Many factors could affect the amount of polysaccharide utilized by endosymbionts in the gut, including accessibility of cell wall polysaccharides in the gut of algal-feeding fish (Mackie et al., 1996).

In the case of cellulose utilization, the bacteria, *K. oxytoca* isolated from omnivorous fish *S. sihama* showed the maximum while all others recorded lower values. Also the maximum production was attained faster by this species than all others. But the maximum rate of utilization at the exponential stage of growth was shown by symbiont *P. stutzeri* from planktivorous fish *S. longiceps*. The cellulose utilization by *P. stutzeri* strain KSLS4C3 and *K. oxytoca* strain MSSC4 showed more than one peak during the study. This can be attributed to various cycles of bacterial growths being completed in the given time. That is, the bacteria completes one cycle of growth which includes the typical lag phase, exponential phase and death phase in short durations so that various peaks of glucose production by cellulose utilization are obtained within the given time. In case of those showing more than one peak, it is observed that the maximum rate of cellulose utilization is reached earlier than others and they complete two or three cycles of growth while the slower ones could only complete one cycle of growth in the given time. It should be noted that in this respect, the strains which were more active were those obtained from planktivore and omnivorous fishes, while the slower ones were from carnivorous species.

Also, glucose is always metabolized first in preference to other sugars. Glucose represses the induction of inducible operons by inhibiting the synthesis of cyclic AMP (cAMP), a nucleotide that is required for the initiation of transcription of a large number of inducible enzyme systems including the lac operon. Glucose is known to repress a large number of inducible enzymes in many different bacteria. As a form of catabolite repression, the glucose effect serves a useful function in bacteria: it requires the cells to use the best available source of energy. For many bacteria, glucose is the most common and readily utilizable substrate for growth. Thus, it inhibits indirectly the synthesis of enzymes that metabolize poorer sources of energy (Todar, 2011). There is also a chance that, when glucose is produced by cellulose catabolism, the bacteria may utilize this glucose for some time and when the level of glucose again reduces, it starts metabolizing cellulose again and this cycle goes on giving different peaks for glucose production. Also, it shows that if other energy sources are available, a potential cellulase producer may not utilize cellulose, but may utilize easily available sources.

Identification of novel strains of bacteria from unconventional sources always helps in improving the existing technology. Marine environment which encompasses about 71% of the earth's surface is an important source of unexplored useful enzymes while most of the industrial enzymes are derived from terrestrial sources. Considering the microbial diversity of marine environment, the efforts channeled to discover novel enzymes from marine microbes are inadequate (Mohapatra et al., 2003). Intensive screening attempts alone can lead to unveiling the rich treasure of novel enzymes from marine environment. The strains identified in this study are previously not reported and their enzyme producing ability can be utilized for enzyme biotechnology, biofuel production, animal feed industry and waste management using submerged or solid state fermentation techniques. The prospective probiotic nature of these strains can be investigated and used in aquafeeds for marine fishes.

The ability of the bacteria to utilize cellulose in vitro does not indicate that it is actively involved in digestion of food in fish gut. Further

studies are required to establish it. Availability of substrate, pH, inhibitors etc. can have detrimental influence on the utilization of cellulose by the symbionts found in marine fish gut. The chemical composition of the diet will also affect the activity of microbes. Only under the influence of given conducive environment, the microbes are expected to exhibit their enzymatic properties up to the maximum potential. Chances are there that, same species of bacteria when found in a herbivore may be actively digesting plant matter, while in a carnivore, it may be proteolytic. In some cases, metabolites or substrates can turn on inactive genes so that they are transcribed. In the process of enzyme induction, the substrate, or a compound structurally similar to the substrate, evokes the formation of enzymes which are usually involved in the degradation of the substrate. Enzymes that are synthesized as a result of genes being turned on are called inducible enzymes and the substance that activates gene transcription is called the inducer. Inducible enzymes are produced only in response to the presence of their substrate and, in a sense, are produced only when needed. In this way the cell does not waste energy synthesizing unneeded enzymes (Todar, 2011).

For animals with variety of diets, diet-related plasticity of endogenous digestive enzyme activity can be explained by the “adaptive modulation hypothesis”. It states that variation in diet should confer upon the ability of animals to modulate their digestive enzyme activity accordingly (Karasov, 1992). Modulation of animal digestive enzyme activities in response to diet is common but not universal among vertebrates (Sabat et al., 1999). It has been shown that, fishes with relatively broad diets can modulate digestive enzyme activities in response to changes in dietary composition (German et al., 2004). According to the available literature, such studies have not been done regarding symbiotic bacterial enzymes in fishes. The modulatory enzyme mechanisms with which a bacterial symbiont react to a diet change of the host fish is yet to be studied. The nature and extent of symbiotic relationship between the individual organisms need to be analysed in depth before drawing conclusions based on *in vitro* experiments.

As a conclusion, it shall be agreed that though in all fishes the bacteria enters into the gut through food and water, the conditions in the gut may be favourable to some of these and they readily colonize the region. However, it is difficult to generalize the relationships between fishes and bacteria as a specific group because of the diverse range of fish species, the environment the fish inhabit and the variety of metabolic requirements of bacteria. They may carry out different functions like proteolysis, starch hydrolysis, cellulose digestion, production of vitamins, general probiotic function etc. This work is first of its kind in a variety of marine fishes and it shows that microbial gut symbionts with ability to utilize cellulose are present in marine carnivores, omnivores and planktivores.

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