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Characterization of extracellular enzymes from culturable autochthonous gut bacteria in rabbitfish (*Siganus guttatus*)

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Abstract. The intestinal microenvironment of bacteria in fish influences the host in many ways, including the metabolism of several nutrients. Isolation, enzymatic activities, and molecular identification of culturable bacteria associated with the gastrointestinal tract of rabbitfish, *Siganus guttatus* Bloch, were investigated in the present study. Occurrence and distribution of enzyme-producing bacteria in the proximal (PI), middle (MI), and distal (DI) gut segments were determined and data were presented as log viable counts g^{-1} intestine (LVC). The heterotrophic bacterial population (LVC = 6.95) as well as the amylolytic, proteolytic, cellulolytic, and carrageenolytic populations had the highest occurrence in the DI region (LVC = 5.73, 5.46, 3.21 and 5.00, respectively). The isolates were qualitatively screened on the basis of their extracellular enzyme producing ability. The selected strains were further quantitatively assayed for amylase, protease, and cellulase activities. Results of enzymatic studies revealed that three most promising bacterial isolates possess multienzyme activities and were studied through 16S rRNA gene sequence for identification. Isolates CP6,CM8 and PM14 showed high similarity to *Bacillus cereus*, *Bacillus megaterium* and *Bacillus flexus*, respectively. The NCBI Genbank accession numbers of the 16S rRNA gene sequences for isolates CP6, CM8, and PM14 were KR779513, KR779512, and KR779516, respectively. To date, this is the first time that characterization and enzymatic activities of gut bacteria in rabbitfish have been reported. The present study might offer scope for further research to evaluate prospects for application of the gut-associated extracellular enzyme-producing bacteria in fish nutrition.
Key Words: gut bacteria, enzymatic activity, 16S rRNA, *Siganus guttatus*.

Introduction. Marine fishes are known to possess a characteristic gut microflora which is often influenced by the genetic constitution of the host organism. These bacteria are considered to be transient (allochthonous), whereas others exist as resident microbiota associated with the intestinal mucosa (autochthonous) (Ringø & Birkbeck 1999; Kim et al 2007; Merrifield et al 2009). The influence of autochthonous microflora on mucosal immune function and gut health has become an area of scientific and clinical importance (Galdeano et al 2007). Numerous studies have been conducted to characterize the microbial diversity of the gastrointestinal tract (GI) tract of fish using molecular methods to isolate and identify culturable bacteria (Holben et al 2002; Huber et al 2004; Askarian et al 2012; Das et al 2014). These studies have widened the knowledge about the intestinal microbiota in fish, being more complex than previously assumed.

The bacterial flora within the GI tract of fish represents 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 (Bairagi et al 2002). Autochthonous intestinal bacteria in fish are known to accelerate the digestion process by producing extracellular enzymes (Cahill, 1990). Enzyme-producing bacterial isolates from fish have been demonstrated to

breakdown chitin (Itoi et al 2006), cellulose (Saha et al 2006), protein (Belchior & Vacca 2006), starch (Ghosh et al 2002, 2010), phytate (Khan & Ghosh 2012, 2013) and tannin (Mandal & Ghosh 2013). Meanwhile, tropical herbivorous fish species such as the rabbitfish (*Siganus guttatus*), which feeds primarily on seaweeds and other benthic algae may contain interesting enzyme-producing gut bacteria that may be of potential probiotics and industrial applications. Information on the microbial sources of the digestive enzymes in the gut of herbivorous fishes are scanty. In view of the commercial importance of rabbitfish, the present investigation was undertaken. Therefore, the primary objective of the present study was to detect the autochthonous extracellular enzyme-producing bacteria from the gut segments of rabbitfish, with emphasis on the gut bacteria's ability for protease, amylase, cellulase and carrageenase production. Furthermore, a comparative qualitative and quantitative assay of extracellular microbial enzyme activity was conducted and the most promising strains were identified by 16S rRNA gene sequence analysis.

Material and Methods

Fish examined. Mature wild rabbitfish, *S. guttatus* (n=14) with a mean body weight of 292 ± 34.12 g were collected during the months of June and July 2014 at Southeast Asian Fisheries Development Center/Aquaculture Department (SEAFDEC/AQD) - Igang Marine Station, Nueva Valencia, Guimaras and transported to the laboratory in oxygen-packed plastic bags. The fishes were starved for 24 h in order to make their intestinal tract clear and also to eliminate the bacteria that were transit in nature (Kar & Ghosh 2008). After starvation, the ventral surface of the fish was thoroughly scrubbed with 1% iodine solution for surface decontamination. The fish were dissected aseptically and their GI tracts were removed. Gut samples were processed for isolation of adherent (autochthonous) bacteria as described by Das et al (2014). The GI tracts were divided into proximal (PI), middle (MI), and distal (DI) segments, cut into pieces, and flushed carefully three times with 0.9% sterile saline solution using an injection syringe in order to remove nonadherent (allochthonous) bacteria. Gut segments were pooled together region-wise for each replicate, and thus there were three replicates for each gut segment to avoid erroneous conclusions due to individual variations in gut microflora. The gut segments were then homogenized with 10 parts of sterilized, chilled 0.9% NaCl solution (Das & Tripathi 1991).

Microbial culture. A five serial 1:10 dilutions were applied on the homogenized samples of each gut segment (Beveridge et al 1991). Samples (0.1 mL) were poured aseptically within a laminar flow on sterilized tryptone soy agar (TSA, Difco) plates, in triplicate, to determine the culturable heterotrophic autochthonous aerobic/facultative anaerobic microbial population. To isolate and enumerate amylase-, protease-, cellulose- and carrageenase-producing bacterial populations, diluted gut homogenates (0.1 mL) were poured on starch agar (SA), peptone gelatin agar (PG), carboxymethylcellulose agar (CMC) and carrageenan-solidified agar (CS), respectively. The culture plates were incubated at 30 ^{0}C for 24-48 h. The colony-forming units (CFUs) per unit sample volume of gut homogenate were determined and the data were presented as log viable counts g^{-1} intestine (LVC). Distinct colonies with different morphological characters (color, colony size, surface, margin and opacity) were streaked separately on respective plates to obtain pure cultures.

Qualitative enzyme producing capacity by the selected isolates. The isolated bacterial strains were analyzed on agar plates with selective media to determine the intensity of the extracellular enzyme production. For extracellular amylase production, isolates were spot plated on SA plates and incubated at 30^{0}C for 24 h. The culture plates were then flooded with 1% Lugol's iodine solution to identify amylase activity through the formation of a transparent zone (halo) surrounding the colony (Jacob & Gerstein 1960). Similarly, for extracellular protease production, the isolates inoculated on PG plates were incubated at 30^{0}C for 24 h and the appearance of a halo was further confirmed using

skim milk agar with 2% NaCl (Balaji et al 2012). For determination of cellulase production, isolates grown on CMC plates at 30^0C for 24 h were flooded with Gram's iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 minutes and the appearance of a halo zone confirmed the cellulase activity (Kasana et al 2008). For carrageenase producers, isolates grown on 1.5% κ-carrageenan (Eugel) (MCPI Corporation, Philippines)-sterile seawater media were observed for plate depression-forming activity or formation of soft pits as an indication of carrageenase activity (Tayco et al 2013). There were three replicates for each experimental set. Qualitative extracellular enzyme activity was assessed based on the measurement of a clear zone (halo) around the colony as follows; 0 (0-3 mm halo diameter), 1 (low, 4-6 mm halo diameter), 2 (moderate, 7-9 mm halo diameter) and 3 (high, > 10 mm halo diameter) (Askarian et al 2012). Maximum score is 18 and minimum score is 2.

Quantitative enzyme assay. On the basis of qualitative assay, quantitative assay was performed on the isolates using broth culture for the production of amylase, protease, and cellulase following the methods described by Bernfeld (1955), Walter (1984), and Worthington (1993), respectively. Carrageenase assay was not performed because carrageenase activity was not detected among the isolates. The protein content of the enzyme extracts were estimated spectrophotometrically (ImmunoMini NJ-2300) at 660 nm according to Lowry et al (1951). Quantitative enzyme activities were expressed as units (U) and specific enzyme activity as U/mg^{-1} protein. All assays were carried out in triplicate.

Identification of isolates by 16S rRNA gene sequencing. The three most promising isolates based on qualitative and quantitative enzyme assays were identified through 16S rRNA partial gene sequence analysis. The gene encoding 16S rRNA was amplified from the bacterial isolates by polymerase chain reaction (PCR) using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3'). The template DNA was obtained by extracting genomic DNA using PureLink™ Genomic DNA Kit (Invitrogen) from 24-h colony grown on a nutrient agar slant. The PCR reactions were performed using PCR mix containing 0.2μM of each primer, 2μL template DNA and 1x Taq Master Mix (Vivantis) which includes Taq DNA polymerase, reaction buffer, dNTPs and $MgCl_2$. The following thermocycling conditions were used: initial denaturation at 95^0C for 3 min, followed by 35 cycles at 95^0C for 1 min, annealing at 55^0C for 1 min, and extension at 72^0C for 2 min, and a final extension at 72^0C for 3 min (Lane, 1991). PCR products were sent to a commercial company (AITbiotech, Singapore) for Sanger sequencing. All sequences were analyzed and edited in BIOEDIT and blasted against the sequences available in Genbank. Based on the Blast results, the sequences derived were aligned and analyzed for finding the closest homolog of the microbes. A phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of isolates CP6, CM8, PM14 and their phylogenetically closest type strains using MEGA 6.0 software following the neighbor-joining method. Partial sequences of 16S rRNA from the three selected isolates were deposited in the NCBI Genbank database to obtain accession numbers.

Media composition. TSA medium: 40 g of TSA suspended in 1000 mL distilled water. SA medium contained (g L^{-1}): beef extract, 5; peptone, 5; NaCl, 5; soluble starch, 2; agar, 20; pH of 7. PG medium contained (g L^{-1}): beef extract, 3; peptone, 5; gelatin, 4; agar, 20; pH of 7.5. CMC medium contained (g L^{-1}): beef extract, 5; peptone, 5; NaCl, 5; carboxymethylcellulose, 2; agar, 20; pH of 7.5. CS medium contained (g L^{-1}) (in filtered seawater): peptone, 5; κ-carrageenan, 1.5; yeast extract, 5; beef extract, 3.

Statistical analysis. Statistical analysis of the experimental data pertaining to quantitative enzyme production was made by analysis of variance (ANOVA) followed by Tukey's test using SPSS 10 (Kinnear & Gray 2000).

Results and Discussion. The bacterial populations present in the gut of rabbitfish revealed that the heterotrophic population on TSA plates (Table 1) was highest in the DI

region of the gut and lowest in the PI region. Similar results were observed by Das et al (2014) in the gut of some brackishwater fishes where bacterial populations were highest in the DI region. While enumerating specific enzyme-producing bacterial flora, it was observed that amylolytic bacteria was present in higher densities in the gut of rabbitfish (Table 1) and this can be correlated with its feeding habit. This is not surprising since carbohydrases such as amylase were found in its gut (Sabapathy & Teo 1993) and under natural conditions, marine algae constitute the bulk of the diet of rabbitfish (Lam 1974). Considerable proteolytic, cellulolytic, and carrageenolytic bacterial populations were also present in highest concentration in the DI region of the gut (LVC = 5.46, 3.21 and 5.00, respectively). This in harmony to the previous studies on herbivorous fishes such as rohu (Kar & Ghosh 2008) and various carps (Bairagi et al 2002; Ray et al 2010; Das & Tripathi 1991).

Table 1

Log viable counts (LVC) of autochthonous adherent bacteria isolated from the proximal (PI), middle (MI), and distal (DI) segments of the GI tracts of wild rabbitfish (*Siganus guttatus*)

Intestine segment	*LVC g^{-1} intestine*				
	Bacterial count in TSA plate	*Amylolytic bacteria*	*Proteolytic bacteria*	*Cellulolytic bacteria*	*Carrageenolytic bacteria*
PI	5.78	5.27	4.45	3.20	4.10
MI	6.84	5.53	4.38	3.22	4.29
DI	6.95	5.73	5.46	3.21	5.00

The intensity of extracellular enzyme production by selected bacterial strains isolated were assayed qualitatively and quantitatively. Qualitative extracellular enzyme activities were presented as scores (Table 2), maximum and minimum scores being 18 and 2, respectively. Isolates PM14 and CM8 exhibited multienzyme activities and obtained the highest score of 7 among other isolates. Carrageenase activity was not detected in most of the strains except in bacterial strains CaP21 and CaM7 where positive growth and little activity were observed but the strains failed to hydrolyze the media which will supposedly result to plate-depression or soft pit formation. Screening for carrageenase activity was carried out in the gut of rabbitfish on a premise that there might be some carrageenase-producing bacteria which degrades their diet which is primarily seaweeds and other benthic algae. To date, only a handful of published reports described the isolation of κ-carrageenase-producing marine bacteria and demonstrated their ability to produce carrageenase in culture. Most of these carrageenase producers were isolated from marine algae and seaweeds (Tayco et al 2013; Liu et al 2011; Sarwar et al 1987), and practically no information available from the gut of fishes. Sabapathy & Teo (1993) also failed to detect carrageenase activity in their study on the digestive enzymes of another rabbitfish species, *Siganus canaliculatus*. One hypothesis is that carrageenase-producing bacteria may be present but not culturable, or belonged to the allochthonous microflora of the gut. Thus, carrageenase quantitative assay was not performed on the bacterial isolates since no activity was detected in the qualitative enzyme screening.

Results of the quantitative enzyme assay revealed significant differences in the enzyme activities among the different bacterial isolates (Tables 3, 4 and 5). Being an herbivorous fish, occurrence of amylase-, protease-, and cellulase-producing bacteria is noteworthy in the digestive tract of rabbitfish. Peak specific amylase activity was exhibited by bacterial strains CP6, CM8 and CD2 with values 3.12 U/mg^{-1} protein, 3.23 U/mg^{-1} protein, and 3.01 U/mg^{-1} protein, respectively (Table 3). The values obtained from these three strains were somewhat higher as compared to the values obtained by Bairagi et al (2002) from the gut of an herbivorous grass carp, *Ctenopharyngodon idella* with a specific amylase activity of 2.5 U/mg^{-1} protein. The high amylase activity in rabbitfish indicates that starch is readily hydrolysed and probably plays an important role in energy metabolism (Sabapathy & Teo 1993). Furthermore, Kar & Ghosh (2008) confirmed the presence of amylolytic bacteria in the gut of an herbivore fish rohu (*Labeo*

rohita), while Dhage (1968) suggested that amylase activity in the intestine of herbivorous carp is much more intense than in carnivorous fishes.

Table 2

Qualitative extracellular enzyme activity of some bacterial strains isolated from the gut segments of rabbitfish (*Siganus guttatus*). Enzyme activities were presented as scores as described in the text

Bacterial strains	*Isolated from*	*Enzyme activity (scores)**				*Total score*
		Amylase	*Protease*	*Cellulase*	*Carrageenase*[1]	
AP3	PI	1	0	0	ND	1
AD1	DI	3	0	0	ND	3
AD2	DI	3	1	1	ND	5
PM10	MI	0	3	3	ND	6
PM14	MI	3	3	1	ND	7
PD1	DI	0	3	1	ND	4
PD14	DI	0	3	0	ND	3
CP6	PI	2	1	3	ND	6
CM8	MI	2	2	3	ND	7
CM13	MI	0	1	2	ND	3
CD2	DI	2	1	3	ND	6
CaP21	PI	2	0	3	0	5
CaM7	MI	2	0	2	0	4

*: With pure culture of bacterial isolates; [1] Enzyme activity is based on plate-depression or soft pit formation and not on halo zone; ND: Not detected.

Table 3

Profile of amylase activity in the selected strains from the gut segments of rabbitfish (*Siganus guttatus*)

Bacterial strains	*Amylase (U)*[a]	*Specific enzyme activity*[b]
AP3	1.22 ± 0.07 [d]	1.90 ± 0.06 [b]
AD1	0.15 ± 0.03[a]	0.24 ± 0.01 [a]
AD2	1.07 ± 0.06 [c]	1.83 ± 0.05 [b]
PM10	0.54 ± 0.06 [a]	1.68 ± 0.19 [b]
PM14	0.74 ± 0.01 [b]	2.29 ± 0.02 [b]
PD1	0.46 ± 0.02 [a]	1.57 ± 0.01 [b]
PD14	0.70 ± 0.01 [b]	2.20 ± 0.01 [b]
CP6	1.21 ± 0.24 [d]	3.12 ± 0.62 [c]
CM8	1.17 ± 0.02 [d]	3.23 ± 0.05 [c]
CM13	0.68 ± 0.01 [b]	2.07 ± 0.01 [b]
CD2	0.97 ± 0.24 [c]	3.01 ± 0.75 [c]
CaP21	0.51 ± 0.08 [a]	1.54 ± 0.28 [b]
CaM7	0.73 ± 0.07 [b]	1.78 ± 0.16 [b]

Data are means ± SE of 3 determinations. Values with the same superscripts in the same vertical column are not significantly different ($p > 0.05$); [a] µg maltose liberated per mL of culture filtrate/min; [b] U/mg protein.

Specific activity of protease was found to be maximum in PM14 strain (Table 4) while maximum cellulase activity was observed in CM8 strain (Table 5). The presence of proteolytic and cellulolytic bacteria in the gut was also reported in herbivorous carps (Bairagi et al 2002) and rohu, *L. rohita* (Kar & Ghosh 2008). The occurrence of proteolytic bacteria in the gut of carnivorous and omnivorous fish support the presence of diet-dependent microbial population indicating their feeding towards animal matter. However, in herbivorous fish, the colonization of proteolytic bacteria might be due to some bacteria entering along with the diet of fish during ingestion which may adapt themselves in the gastrointestinal tract and form a symbiotic association (Kar & Ghosh 2008). Microbial intestinal cellulase activity was also observed in the present study which supports the hypothesis that bacteria might contribute to the degradation of cellulose in fish (Ray et al 2010). In their previous study with carp (*Cyprinus carpio)*, Scherbina & Kazlawlene (1971) suggested that cellulose absorption takes place in the DI region,

which may indicate the presence of microbial cellulase in this region. Our observation is in accordance with this hypothesis as most cellulase-producing bacteria were recorded in the DI region of the gut.

Table 4

Profile of proteolytic activity in the selected strains from the gut segments of Rabbitfish (*Siganus guttatus*)

Bacterial strains	*Protease (U)*[a]	*Specific enzyme activity*[b]
AP3	0.47 ± 0.01 [a]	9.60 ± 0.10 [a]
AD1	0.42 ± 0.05 [a]	9.97 ± 0.05 [a]
AD2	0.42 ± 0.04 [a]	9.95 ± 0.04 [a]
PM10	5.53 ± 0.09 [e]	16.86 ± 0.43 [c]
PM14	10.43 ± 0.11 [g]	32.43 ± 0.26 [f]
PD1	5.54 ± 0.16 [e]	21.46 ± 2.25 [d]
PD14	8.07 ± 0.19 [f]	25.32 ± 1.06 [e]
CP6	0.71 ± 0.02 [b]	8.57 ± 0.05 [a]
CM8	0.55 ± 0.04 [a]	9.17 ± 0.17 [a]
CM13	4.15 ± 0.02 [d]	12.47 ± 0.06 [b]
CD2	0.67 ± 0.01 [b]	8.67 ± 0.04 [a]
CaP21	0.29 ± 0.01 [a]	12.51 ± 0.36 [b]
CaM7	2.65 ± 0.06 [c]	10.58 ± 0.22 [a]

Data are means ± SE of 3 determinations. Values with the same superscripts in the same vertical column are not significantly different ($p > 0.05$); [a]µg tyrosine liberated per mL of culture filtrate/min; [b]U/mg protein.

Table 5

Profile of cellulase activity in the selected strains from the gut segments of rabbitfish (*Siganus guttatus*)

Bacterial strains	*Cellulase (U)*[a]	*Specific enzyme activity*[b]
AP3	0.80 ± 0.08 [a]	2.40 ± 0.23 [a]
AD1	1.08 ± 0.06 [a]	3.49 ± 0.03 [a]
AD2	0.73 ± 0.20 [a]	2.23 ± 0.60 [a]
PM10	0.75 ± 0.01 [a]	2.31 ± 0.04 [a]
PM14	0.89 ± 0.20 [a]	3.15 ± 0.66 [a]
PD1	0.71 ± 0.24 [a]	2.42 ± 0.82 [a]
PD14	0.67 ± 0.21 [a]	2.10 ± 0.65 [a]
CP6	11.19 ± 0.16 [c]	4.46 ± 0.06 [b]
CM8	52.53 ± 0.11 [e]	22.74 ± 0.05 [d]
CM13	7.79 ± 0.42 [b]	3.19 ± 0.17 [a]
CD2	46.18 ± 0.59 [d]	16.73 ± 0.21 [c]
CaP21	0.66 ± 0.20 [a]	2.78 ± 0.85 [a]
CaM7	0.72 ± 0.03 [a]	3.55 ± 0.17 [a]

Data are means ± SE of 3 determinations. Values with the same superscripts in the same vertical column are not significantly different ($p > 0.05$); [a]µg glucose liberated per mL of culture filtrate/min; [b]U/mg protein.

Considering the results of the quantitative and qualitative enzyme assays, isolates CP6, CM8 and PM14 were found to have the most potential among the isolates and exhibited multienzyme activities. Based on the nucleotide homology and phylogenetic analysis of the 16S rRNA gene sequences (Figure 1), isolate CP6 was identified as *Bacillus cereus* (GenBank Accession No. KR779513) with 99% similarity with *B. cereus* (NCBI reference strain NC_004722). Isolate CM8 (Genbank Accession No. KR779512) showed 99% similarity with *Bacillus megaterium* (NCBI reference strain NC_014103), while isolate PM14 (Genbank Accession No. KR779516) showed 98% similarity with *Bacillus flexus* (NCBI reference strain KM091675). Phylogenetically, based on 16S rRNA, *B. megaterium* is strongly linked with *B. flexus*, the latter distinguished from *B. megaterium* a century ago, but only recently confirmed as a different species (Vos et al 2009). As shown in

Figure 1, *B. megaterium* and *B. flexus* belonged to the same clade. Several studies confirmed that the most promising enzyme producing gut bacteria in fish belonged to the genus *Bacillus* (Askarian et al 2012; Das et al 2014; Mondal et al 2010). The occurrence of *B. cereus* within the gut of Atlantic salmon (*Salmo salar*) with promising enzyme activity was reported previously by Askarian et al (2012). This is in agreement with the present study that isolate CP6 identified as *B. cereus* exhibits multienzyme activities which merits further investigation. However, to the authors' knowledge, extracellular enzyme-producing *B. megaterium* and *B. flexus* have not been reported from fish gut previously. Reports on gut-inhabiting extracellular enzyme-producing bacteria from herbivorous fish are scanty.

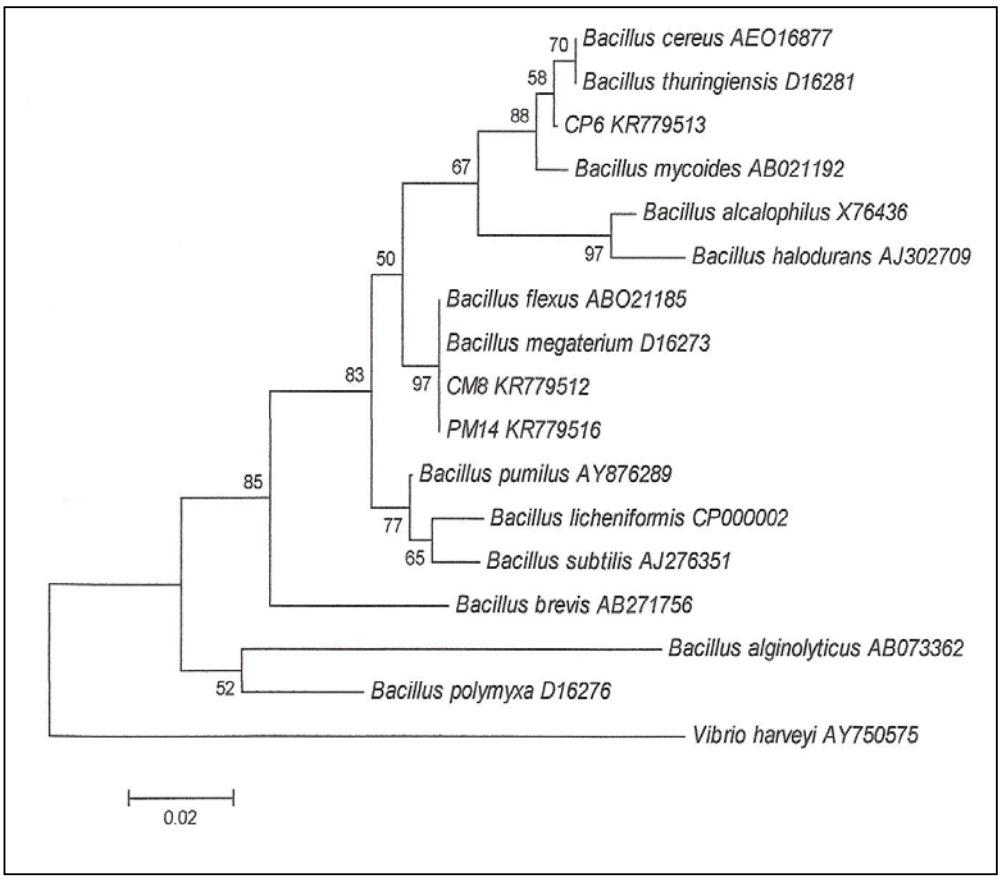

Figure 1. The phylogenetic tree showing the relationship among *Bacillus sp.* strains CP6, CM8 and PM14, and their phylogenetically closest type strains. The GenBank accession numbers of the type strains and studied strains are shown following species names. Distance matrix was calculated by Kimura's 2-parameter model. The scale bar indicates 0.02 substitutions per nucleotide position. *Vibrio harveyi* AY750575 served as an out-group.

Conclusions. The results of the present study indicate that there is a distinct microbial source of digestive enzymes (amylase, protease and cellulase) apart from the endogenous sources in the digestive tract of rabbitfish. The three most promising bacterial strains identified as *B. cereus*, *B. megaterium* and *B. flexus* exhibit multienzyme activities which present a scope for fish nutritionists to utilize the enzyme-producing isolates as a probiotic in formulating cost-effective fish diets or in the form of bacteria biofilm to achieve colonization in the GI tract to a higher degree. The presence of these beneficial gut bacteria, aside from nutritional benefits, will improve the overall health of fish by competing exclusively with pathogens. Whether these bacterial strains can contribute to the host's nutrition has not been elucidated in the present study and therefore merits further investigations.

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