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Industrial enzymes-producing marine bacteria from marine resources

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ABSTRACT

Industrial enzymes are important for various biotechnological applications. Currently, the diversity of industrial enzymes-producing marine bacteria from Malaysia remains mostly unknown. This study investigated the diversity of industrial enzyme-producing marine bacteria from culture collections at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu. Out of 200 bacterial isolates revived, 163 bacteria isolate were successfully growth. Marine bacteria produced enzymes with total scoring higher than four were selected for molecular identification using 16S rDNA. About 161 bacteria isolate secreted amylase (68.7 %), lipase (88.3 %) and protease (68.7 %). The phylogenetic analysis led to the identification of three major phyla, namely Proteobacteria, Firmicutes and Bacteroidetes. These phyla were differentiated into nine genera consisted of *Bacillus*, *Chryseomicrobium*, *Photobacterium*, *Pseudoalteromonas*, *Ruegeria*, *Shewanella*, *Solibacillus*, *Tenacibaculum* and *Vibrio*. Genetic variation was more likely to occur within similar marine bacteria species. The microbial community was found to affect the production of industrial enzymes and the diversity of marine bacteria.

1. Introduction

Industrial enzymes as industrial biocatalysts pose numerous advantages over the traditional chemical process in term of sustainability and process efficiency [1]. These enzymes are considered essential in many industrial processes as they catalyse the process more efficiently, able to work under mild reaction conditions and simultaneously, reduce the production of organic waste and pollutants [2,3]; these, will, in turn, reduce the cost of production. Current industrial enzymes focus on pulp and paper, leather, detergents and textiles, pharmaceuticals, chemicals, food and beverages, biofuels, animals feed and personal care [4]. Plants and animals produce enzymes, however, according to Raveendran et al. [5], microbes are preferable compared to plants and animals as the source of enzyme. The significant attributes of microbes over other organisms are that they are inexpensive to produce, their catalytic activities of enzymes are more predictable and controllable, rapid growth within a short period which produce a high yield of enzymes, and able to provide a consistent supply of culture due to the absence of seasonal fluctuations. Moreover, plants and animals' tissues are considered more hazardous compared to microbes.

The marine environment constitutes an enormous resource ranging from water, sediments and marine organisms that can provide a unique environment to marine bacteria. It encounters various fluctuations of physical factors such as high salinity, high pressure, acidic pH, extreme temperature or any combination thereof, which can create a unique environment for microorganisms to produce unique secondary metabolites with new carbon skeletons, high levels of halogenation and novel bioactivities [6]. Much scientific research has proven that marine bacteria were able to produce a wide range of industrial enzymes [7–9]. The industrial enzymes derived from marine bacteria include α-amylase, α-glucosidase, agarase, α-galactosidase, cellulases, chitinase, lipase [10], protease [8,9]. Some of these marine bacterial-producing industrial enzymes were reported as *Aeromonas* sp., *Alteromonas* sp., *Arthrobacter* sp., *Chromobacterium* sp., *Clostridium* sp., *Cytophaga* sp., *Enterobacter* sp., *Flavobacterium* sp., *Klebsiella* sp., *Listonella* sp., *Moraxella* sp., *Pseudoalteromonas* sp., *Pseudomonas* sp., *Psychrobacter* sp., *Serratia* sp., *Streptomyces* sp., *Vibrio* sp. [7], *Marinobacter* sp. [8] and *Bacillus* sp. [7,9]. Hence, marine bacteria are considered as one of the main producers of novel industrial enzymes. However, to date, the diversity of marine bacteria that produce industrial enzymes from marine resources in Malaysia has scarcely been discussed and studied. Thus, this study was conducted to investigate the diversity of industrial enzymes such as amylases-, lipases- and proteases-producing marine bacteria culture collections from the Institute of Marine Biotechnology (IMB), Universiti Malaysia Terengganu (UMT).

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2. Methodology

2.1. Cultivation of marine bacteria

A total of 200 marine bacteria were obtained from the glycerol stock, which was isolated from various marine resources collected by IMB of UMT. These marine resources include horseshoe crab from Sabah, jellyfish from Sarawak, mollusc and marine sediment from Kelantan and marine water from Terengganu, Malaysia. All were isolated, cultured on marine agar 2216 (Difco, Detroit, Michigan) and incubated at 35 °C overnight.

2.2. Screening of hydrolytic enzymes from marine bacteria isolates

The isolated bacteria were growth successfully after overnight incubation on marine agar plate were screened for amylase, lipase and protease productions using different types of agar composition such as starch agar [11], spirit blue agar [12] and skimmed milk agar [13], with some modifications. All of the designated agar contained marine broth as a nutrient source. Enzyme activities were quantified following the method described by Dutta and Ghosh [14] with some modifications. The strength of enzymes produced was quantified by observing the appearance of halo zone (diameter in mm) around the colony with a given score as 0 (no inhibition, no halo); 1 (low inhibition, 6–10 mm halo); 2 (moderate inhibition, 11–20 mm halo); 3 (good inhibition, $\geq$ 21 mm halo). The isolated bacteria produced enzymes with a total scoring of all three enzymes more than four, were chosen for species identification.

2.3. DNA extraction of marine bacteria-producing industrial enzymes

The isolated bacteria that produced enzymes with a strength score of more than four as stated were later cultured in marine broth and incubated overnight for DNA extraction. The DNA of these bacteria was extracted using the DNeasy blood and tissue kit (Qiagen Inc., Valencia, USA), following the manufacturer's protocol [15]. Then, the extracted DNA was loaded into 1% agarose gel-electrophoresis to visualize the bands, and absorbance was read at two respective wavelengths (260 and 280 nm) in examining concentration and purity of extracted DNA.

2.4. PCR amplification of 16S rDNA

The 16S rDNA analyses were performed to identify the species from the isolated bacteria. The 16S rDNA was PCR amplified using 16S rDNA universal primer pair, 27 F (5′- GAGTTTGATCMTGGCT-CAG-3′) and 1492R (5′- TACGGTTACCTTGTTACGACTT- 3′) [16]. The PCR conditions were performed according to the MyTaq Mix protocol [17]. A total of 50 μL PCR reaction mixture containing 5X MyTaq reaction buffer (stabilizers, enhancers, $MgCl_2$ and dNTPs), primer-pair, a genomic template of DNA and sterile distilled water were used in the reaction. The amplification program was designated as initial denaturation at 95 °C for 1 min, followed by 35 cycles of repeated events consisting of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 15 s, elongation at 72℃ for 10 s and the final elongation for 4 min at 72 °C using MasterCycler gradient (Eppendorf, Germany). The sequencing of the amplicons was performed bi-directional on an ABI 3730XL DNA sequencer by First BASE Sdn. Bhd., Malaysia.

2.5. Phylogenetic analysis

The 16S rDNA sequences obtained were aligned with the GenBank database using the BLAST algorithm, following the method described by Ismail et al. [16]. Later, they were analysed using EzTaxon server (http://www.ezbiocloud.net/eztaxon) to

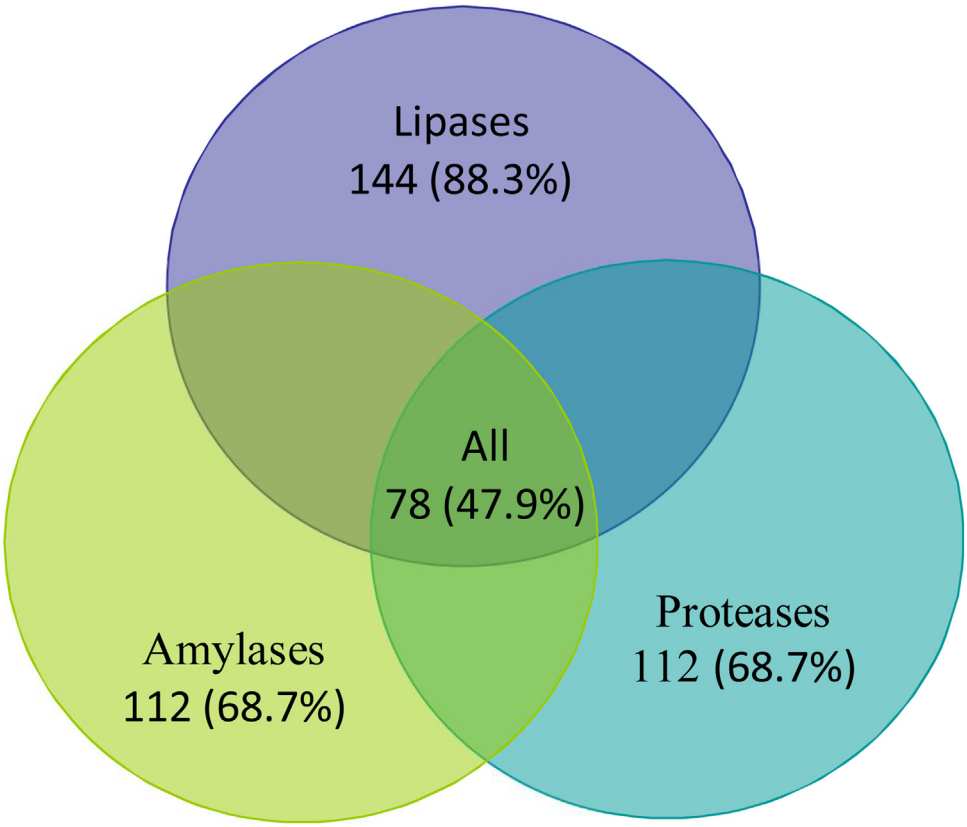

Fig. 1. Total number and percentage of bacteria isolates producing lipases, amylases and proteases.

Table 1
Taxonomic affiliations and production of industrial enzyme by 93 selected marine bacteria from the IMB, UMT collection as determined by 16S rDNA sequencing.

Phylum	Bacteria ID	Closest representative bacteria	Identity (%)	Source	Amylase	Lipase	Protease	Total score
Proteobacteria	HSC 4	*Vibrio mytili* CAIM 528	97.91	Horseshoe crab	2	3	1	6
Gammaproteobacteria	HSC 7	*Vibrio neocaledonicus* NC 470	99.85	Horseshoe crab	2	3	2	7
Vibrionales	HSC 8	*Vibrio neocaledonicus* NC 470	99.70	Horseshoe crab	2	3	1	6
	HSC 10	*Vibrio neocaledonicus* NC 470	99.85	Horseshoe crab	2	3	0	5
	HSC 13	*Vibrio alginolyticus* NBRC 15,630	99.47	Horseshoe crab	2	3	1	6
	HSC 14	*Vibrio neocaledonicus* NC 470	99.77	Horseshoe crab	2	3	1	6
	HSC 15	*Vibrio neocaledonicus* NC 470	99.78	Horseshoe crab	2	3	2	7
	HSC 16	*Vibrio neocaledonicus* NC 470	99.92	Horseshoe crab	2	3	2	7
	HSC 18	*Vibrio neocaledonicus* NC 470	99.85	Horseshoe crab	2	3	1	6
	HSC 19	*Vibrio neocaledonicus* NC 470	99.93	Horseshoe crab	2	3	1	6
	HSC 23	*Vibrio neocaledonicus* NC 470	100	Horseshoe crab	2	3	1	6
	HSC 24	*Vibrio neocaledonicus* NC 470	100	Horseshoe crab	2	3	0	5
	HSC 25	*Vibrio neocaledonicus* NC 470	99.62	Horseshoe crab	2	3	1	6
	HSC 26	*Vibrio neocaledonicus* NC 470	99.85	Horseshoe crab	2	3	0	5
	HSC 29	*Vibrio neocaledonicus* NC 470	99.8	Horseshoe crab	2	3	1	6
	MNAD 3.2	*Vibrio furnissii* CIP 102,972	99.85	Marine water	2	3	1	6
	MNAD 3.3	*Vibrio furnissii* CIP 102,972	99.86	Marine water	2	3	1	6
	HEME 1.7.3	*Vibrio owensii* LMG 25,443	99.92	Jellyfish	2	3	2	7
	HEME 2.4.2	*Vibrio neocaledonicus* NC 470	99.49	Jellyfish	1	3	2	6
	HEME 2.8	*Vibrio alginolyticus* NBRC 15,630	99.86	Jellyfish	1	3	2	6
	HEME 2.9.1	*Vibrio neocaledonicus* NC 470	99.81	Jellyfish	1	3	2	6
	HEME 2.9.2	*Vibrio neocaledonicus* NC 470	99.81	Jellyfish	1	3	2	6
	HEME 2.11.1	*Vibrio neocaledonicus* NC 470	99.63	Jellyfish	1	3	2	6
	HEME 2.12.2	*Vibrio neocaledonicus* NC 470	99.82	Jellyfish	1	3	2	6
	HEME 3.10	*Vibrio neocaledonicus* NC 470	100	Jellyfish	2	3	2	7
	HEME 3.11	*Vibrio fluvialis* NBRC 103,150	100	Jellyfish	1	3	3	7
	HEME 3.12	*Vibrio alginolyticus* NBRC 15,630	99.73	Jellyfish	1	3	2	6
	MNAD 1.5.2	*Vibrio alginolyticus* NBRC 15,630	99.43	Jellyfish	1	3	2	6
	MNAD 1.6.2	*Vibrio neocaledonicus* NC 470	99.85	Jellyfish	1	3	2	6
	MNAD 3.7	*Vibrio alginolyticus* NBRC 15,630	99.85	Jellyfish	2	3	2	7
	CV(M) 2.1	*Vibrio neocaledonicus* NC 470	99.85	Marine water	2	3	1	6
	CV(M) 2.2	*Vibrio harveyi* NBRC 15,634	100	Marine water	2	3	2	7
	CV(M) 2.3	*Vibrio neocaledonicus* NC 470	99.77	Marine water	2	3	2	7
	CV(M) 2.5	*Vibrio neocaledonicus* NC 470	99.93	Marine water	2	3	2	7
	CV(M) 3.3	*Vibrio neocaledonicus* NC 470	99.40	Marine water	2	3	2	7
	CV(M) 3.6	*Vibrio alginolyticus* NBRC 15,630	99.72	Marine water	2	3	1	6
	CV(M) 3.7	*Vibrio neocaledonicus* NC 470	99.93	Marine water	2	3	2	7
	CV(M) 3.7.1	*Vibrio neocaledonicus* NC 470	99.93	Marine water	2	3	2	7
	CV(H) 1.2(1)	*Vibrio neocaledonicus* NC 470	99.57	Marine water	2	3	0	5
	CV(H) 1.2(2)	*Vibrio neocaledonicus* NC 470	99.84	Marine water	2	3	2	7
	CV(H) 1.5	*Vibrio neocaledonicus* NC 470	99.93	Marine water	2	3	2	7
	CV(H) 1.6	*Vibrio neocaledonicus* NC 470	99.85	Marine water	2	3	2	7
	CV(H) 2.2	*Vibrio neocaledonicus* NC 470	99.85	Marine water	2	3	2	7
	CV(H) 2.3	*Vibrio neocaledonicus* NC 470	99.48	Marine water	2	3	1	6
	CV(H) 2.4	*Vibrio neocaledonicus* NC 470	99.91	Marine water	2	3	2	7
	CV(H) 2.5	*Vibrio neocaledonicus* NC 470	99.79	Marine water	2	3	2	7
	CV(H) 2.7	*Vibrio neocaledonicus* NC 470	99.41	Marine water	2	3	2	7
	CV(H) 2.8	*Vibrio alginolyticus* NBRC 15,630	99.71	Marine water	2	3	2	7
	CV(H) 2.9	*Vibrio neocaledonicus* NC 470	99.49	Marine water	2	3	2	7
	CV(H) 2.10.3	*Vibrio neocaledonicus* NC 470	99.72	Marine water	2	3	2	7
	CV(H) 2.11.2	*Vibrio neocaledonicus* NC 470	99.85	Marine water	2	3	1	6
	CV(H) 3.1	*Vibrio neocaledonicus* NC 470	99.39	Marine water	2	3	2	7
	CV(H) 3.2	*Vibrio neocaledonicus* NC 470	99.86	Marine water	2	3	2	7
	CV(H) 3.5	*Vibrio alginolyticus* NBRC 15,630	99.82	Marine water	2	3	2	7
	CV(H) 3.6	*Vibrio parahaemolyticus* NBRC 12,711	99.50	Marine water	2	3	1	6
	CV(H) 3.7	*Vibrio alginolyticus* NBRC 15,630	99.63	Marine water	2	3	2	7
	CV(H) 3.9	*Vibrio neocaledonicus* NC 470	99.78	Marine water	2	3	2	7
	CV(H) 7	*Vibrio parahaemolyticus* NBRC 12,711	99.79	Marine water	2	3	2	7
	HSC 5	*Photobacterium rosenbergii* LMG 22,223	98.91	Horseshoe crab	2	3	0	5
	HSC 6	*Photobacterium rosenbergii* LMG 22,223	98.22	Horseshoe crab	2	3	1	6
Proteobacteria	HSC 27	*Shewanella algae* JCM 21,037	99.49	Horseshoe crab	0	3	2	5
Gammaproteobacteria	HSC 30	*Shewanella algae* JCM 21,037	98.07	Horseshoe crab	0	3	2	5
Alteromonadales	HSC 34	*Shewanella haliotis* JCM 14,758	99.34	Horseshoe crab	1	3	2	6
	HSC 36	*Shewanella haliotis* JCM 14,758	100	Horseshoe crab	1	3	2	6
	HSC 37	*Shewanella haliotis* JCM 14,758	100	Horseshoe crab	1	3	2	6
	HSC 40	*Shewanella haliotis* JCM 14,758	100	Horseshoe crab	0	3	2	5
	HSC 41	*Shewanella haliotis* JCM 14,758	99.93	Horseshoe crab	0	3	2	5
	HSC 42	*Shewanella haliotis* JCM 14,758	99.85	Horseshoe crab	0	3	2	5
	HSC 51	*Shewanella haliotis* JCM 14,758	100	Horseshoe crab	0	3	2	5
	HSC 52	*Shewanella haliotis* JCM 14,758	100	Horseshoe crab	0	3	2	5
	Bact (M) 1.4	*Shewanella algae* JCM 21,037	99.88	Marine water	0	3	2	5
	CV(M) 3.2.1	*Pseudoalteromonas shioyasakiensis* SE 3	99.71	Marine water	0	3	2	5
Proteobacteria Alphaproteobacteria	HSC 31	*Ruegeria mobilis* DSM 23,403	99.76	Horseshoe crab	2	3	2	7
Firmicutes	TB 5	*Chryseomicrobium imtechense* MW10	99.50	Marine sediment	2	3	3	8
Planococcaceae	TB 8	*Chryseomicrobium imtechense* MW10	99.71	Marine sediment	3	3	2	8

Table 1 (*Continued*)

Phylum	Bacteria ID	Closest representative bacteria	Identity (%)	Source	Amylase	Lipase	Protease	Total score
	TB 9	*Chryseomicrobium imtechense* MW10	99.27	Marine sediment	2	2	2	6
	TB 11	*Chryseomicrobium palamuruense* PU 1	98.62	Marine sediment	2	0	3	5
	TB 13	*Chryseomicrobium imtechense* MW10	99.71	Marine sediment	2	2	3	7
	TB 24	*Chryseomicrobium imtechense* MW10	99.79	Marine sediment	1	1	3	5
	TB 25	*Chryseomicrobium imtechense* MW10	99.77	Marine sediment	2	3	0	5
	TB 61	*Solibacillus isronensis* B3W22	99.20	Mollusc	2	3	3	8
Firmicutes	TB 15	*Bacillus haikouensis* C-89	99.75	Marine sediment	2	0	3	5
Bacillaceae	TB 17	*Bacillus anthracis* Ames	99.85	Marine sediment	0	3	2	5
	TB 18	*Bacillus paralicheniformis* KJ-16	99.93	Marine sediment	3	3	0	6
	TB 19	*Bacillus oryzaecorticis* R1	100	Marine sediment	2	0	3	5
	TB 22	*Bacillus subtilis* subsp. *inaquosorum* KCTC 13,429	99.92	Marine sediment	3	3	0	6
	TB 26	*Bacillus haikouensis* C-89	99.43	Marine sediment	2	0	3	5
	TB 29	*Bacillus paralicheniformis* KJ-16	99.93	Marine sediment	2	3	3	8
	TB 31	*Bacillus paralicheniformis* KJ-16	99.86	Marine sediment	2	3	0	5
	TB 32	*Bacillus anthracis* Ames	98.44	Marine sediment	0	3	2	5
	TB 37	*Bacillus paralicheniformis* KJ-16	100	Marine sediment	2	0	3	5
Bacteriodetes	HSC 12	*Tenacibaculum mesophilum* DSM 13,764	99.08	Horseshoe crab	0	3	2	5
	HSC 22	*Tenacibaculum litoreum* CL-TF 13	98.13	Horseshoe crab	2	3	1	6

*The strength of enzymatic activities was represented by scoring as follows; 0, nil (no halo); 1, low (6–10 mm halo); 2, moderate (11–20 mm halo); 3, good (≥ 21 mm halo). The 16S rDNA sequences of the identified bacteria were deposited at NCBI GenBank database. The accession numbers of 16S rDNA sequences follow order from MH643590 to MH643680. The accession numbers for HSC 12 and HSC 22 are MH643682 and MH643681, respectively. The strain for all the identified bacteria isolates were catalogued based on bacteria ID.

determine their closest phylogenetic affiliation [18]. The 16S rDNA sequences were then aligned with their closest relatives using ClustalW. Phylogenetic trees were constructed using MEGA X software version 10.1.5 [19] with a bootstrap value of 1000 replicates. The trees were based on the Minimum Evolution method (ME), the Maximum Likelihood method (ML) and the Neighbor-Joining method (NJ).

3. Results

3.1. Identification of industrial enzymes-producing bacteria

A total of 163 bacteria isolates were screened. There were only two bacteria isolates that did not produce any of the targeted industrial enzymes. Out of 163 bacteria isolates, 112 (68.7 %) bacteria isolates were able to produce amylases and proteases, while 144 (88.3 %) bacteria isolate produced lipases. Results indicated that 78 (47.9 %) bacteria isolates produced all three types of targeted industrial enzymes as shown in Fig. 1. The bacteria isolates were able to produce enzymes with a total scoring of more than four were selected for species identification. 93 bacteria isolates were selected at a total percentage of 57.1 %, which was then classified as three major phyla such as Proteobacteria, Firmicutes and Bacteroidetes. These phyla were differentiated into nine genera as shown in Table 1. All the bacteria isolates were identified as *Bacillus* sp., *Chryseomicrobium* sp., *Photobacterium* sp., *Pseudoalteromonas* sp., *Ruegeria* sp., *Shewanella* sp., *Solibacillus* sp., *Tenacibaculum* sp. and *Vibrio* sp. The most abundant marine bacteria were found to belong to Proteobacteria (Vibrionales: *Vibro* sp.), followed by Firmicutes and Bacteriodetes. Proteobacteria and Bacteriodetes were isolated from marine organisms and marine water of Sabah, Sarawak and Terengganu as demonstrated in Table 1. The size of the halo zone of selected marine bacteria isolate for the amylases, lipases and proteases screening was also provided in Table 2. Most of the isolates were able to produce all three targeted industrial enzymes. However, some bacteria isolates were not able to grow on the screening agar plate and was indicated as no halo zone (zero mm) as shown in Table 2. All the Firmicutes in this study were collected from marine sediment of mangrove area in Tok Bali, Kelantan, except for strain TB 61 which was isolated from the mollusc. In this study, *Chryeomicrobium* sp. was more likely to produce all three types of targeted industrial enzymes; whereas, most of the *Bacillus* sp. produced amylases and proteases, excluding all *Bacillus anthracis* strain. Only the identified *Solibacillus* sp. (TB 61) was able to produce all three targeted industrial enzymes.

Table 2
The size of halo zone (mm) of 93 selected marine bacteria from IMB, UMT in the screening of amylases, lipases and proteases production.

Phylum	Bacteria ID	Amylase (mm)	Lipase (mm)	Protease (mm)
Proteobacteria	HSC 4	12	F	8
Gammaproteobacteria	HSC 7	20	F	13
Vibrionales	HSC 8	18	F	10
	HSC 10	20	F	5
	HSC 13	19	36	9
	HSC 14	20	F	10
	HSC 15	18	F	12
	HSC 16	12	40	12
	HSC 18	17	F	8
	HSC 19	20	F	9
	HSC 23	20	F	9
	HSC 24	16	F	5
	HSC 25	17	F	9
	HSC 26	15	F	5
	HSC 29	16	F	10
	MNAD 3.2	15	F	10
	MNAD 3.3	17	F	10

	HEME 1.7.3	12	25	18
	HEME 2.4.2	9	F	16
	HEME 2.8	8	F	12
	HEME 2.9.1	8	F	15
	HEME 2.9.2	10	F	11
	HEME 2.11.1	7	F	14
	HEME 2.12.2	10	F	13
	HEME 3.10	12	F	12
	HEME 3.11	8	F	23
	HEME 3.12	6	F	14
	MNAD 1.5.2	9	F	15
	MNAD 1.6.2	8	F	16
	MNAD 3.7	12	F	12
	CV(M) 2.1	14	F	8
	CV(M) 2.2	14	F	12
	CV(M) 2.3	17	F	15
	CV(M) 2.5	12	F	13
	CV(M) 3.3	17	F	15
	CV(M) 3.6	16	F	8
	CV(M) 3.7	12	F	16
	CV(M) 3.7.1	16	F	20
	CV(H) 1.2(1)	18	F	0
	CV(H) 1.2(2)	14	F	15
	CV(H) 1.5	14	F	14
	CV(H) 1.6	13	F	14
	CV(H) 2.2	18	F	16
	CV(H) 2.3	17	F	9
	CV(H) 2.4	17	F	15
	CV(H) 2.5	18	F	14
	CV(H) 2.7	15	F	14
	CV(H) 2.8	14	F	15
	CV(H) 2.9	14	F	15
	CV(H) 2.10.3	18	F	16
	CV(H) 2.11.2	14	F	10
	CV(H) 3.1	18	F	17
	CV(H) 3.2	13	F	14
	CV(H) 3.5	15	F	20
	CV(H) 3.6	15	F	9
	CV(H) 3.7	14	F	15
	CV(H) 3.9	18	F	16
	CV(H) 7	15	F	15
	HSC 5	16	F	5
	HSC 6	20	F	10
Proteobacteria	HSC 27	5	F	11
Gammaproteobacteria	HSC 30	5	39	12
Alteromonadales	HSC 34	10	31	11
	HSC 36	10	35	14
	HSC 37	10	F	12
	HSC 40	4	38	11
	HSC 41	5	F	11
	HSC 42	4	F	11
	HSC 51	4	21	12
	HSC 52	5	25	17
	Bact (M) 1.4	0	3	2
	CV(M) 3.2.1	0	34	14
Proteobacteria Alphaproteobacteria	HSC 31	20	36	20
Firmicutes	TB 5	18	50	48
Planococcaceae	TB 8	60	40	14
	TB 9	19	19	15
	TB 11	11	0	21
	TB 13	19	16	51
	TB 24	7	8	21
	TB 25	13	F	0
	TB 61	15	F	48
Firmicutes	TB 15	12	0	48
Bacillaceae	TB 17	0	32	17
	TB 18	60	55	0
	TB 19	11	0	21
	TB 22	60	30	0
	TB 26	14	0	25
	TB 29	12	F	47
	TB 31	15	F	0
	TB 32	0	21	13
	TB 37	16	0	25
Bacteriodetes	HSC 12	4	35	11
	HSC 22	20	F	10

*The "F" indicated halo zone around bacteria isolates occupied the whole plate; The "0" indicated bacteria was not growth on the particular screening plate.

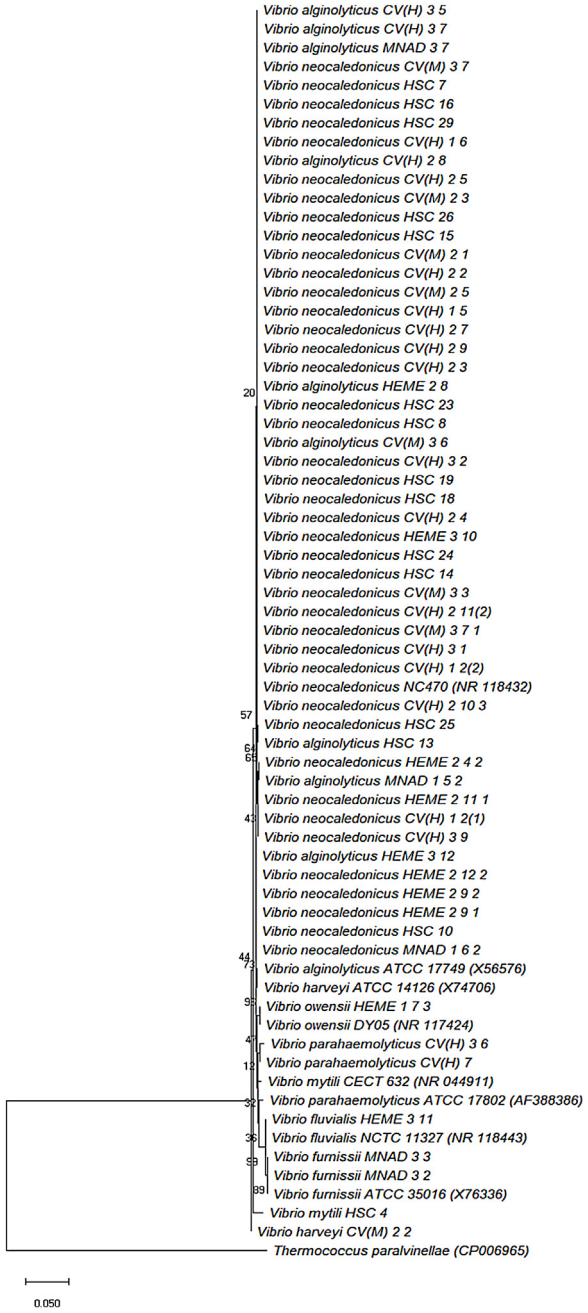

Fig. 2. Phylogenetic analysis of *Vibrio* sp. using NJ method based on 16S rDNA sequences with bootstrap value of 1000 replicates. *Thermococcus paralvinellae* was used as outgroup. Bootstrap values (>50 %) were shown at the nodes. Bar 5 nucleotide substitutions per 100 nucleotides.

3.2. Phylogenetic analysis using 16S rDNA sequences

The phylogenetic analysis was performed based on the NJ method using *Thermococcus paralvinellae* as an outgroup, which are shown in Figs. 2 and 3. The 16S rDNA of reference sequences for each identified species were obtained from the GenBank with accession number in parentheses. Almost all the *Vibrio* sp. isolated were placed within the same branch with very short nucleotide distance, indicating that *V. furnissii* and *V. fluvialis* were branched (Fig. 2). *V. mytili* HSC 4, *V. harveyi* CV(M) 2.2, *V. parahaemolyticus* CV (H) 3.6, *V. parahaemolyticus* CV(H) 7, TB 9 and TB 11 were not located at the similar branch or closer to the concatenated sequence of reference species as shown in Figs. 2 and 3.

4. Discussion

Our results show that more than 50 % of the bacteria isolates from this study were identified as *Vibrio* sp. and possessed the largest species diversity as compared to other genera. However, this did not necessarily represent *Vibrio* sp. as the largest producer of industrial enzymes from marine resources because the selected bacteria in this study were unable to represent the whole population of marine bacteria. Tinta et al. [20] proposed that bacteria diversity are affected by different environmental (temperature, salinity and nutrients), biotic (phytoplankton) factor and microbial communities at a different level of the marine environment. Our findings were in agreement with the previous study exhibit the highest percentage (60 %) of Proteobacteria (*Vibrio* sp.) which produce targeted industrial enzymes [21]. Feby and Nair [22] also concluded that Vibrionales (Proteobacteria) was the abundant bacteria found and they are the main source for multiple enzymes production. Since only a few studies reported on bacteria communities producing extracellular enzymes in the past ten years, this study will contribute the knowledge on the diversity of the identified industrial enzyme-producing marine bacteria.

Karthik and Li [23] summarised marine bacteria found in different geographical regions able to produce enzymes from sponges and corals; these include *Vibrio* sp., *Bacillus* sp. and *Shewanella* sp. Other studies reported that *V. alginolyticus* [24,25] and *V. fluvialis* [24,26] were able to produce the three targeted industrial enzymes which support our results. Feby and Nair [22] also prove that not only associated marine bacteria produced all three targeted industrial enzymes but marine bacteria isolated from seawater were also able to produce at least two of these enzymes. Our results also show that the strength and the ability to produce the targeted industrial enzymes were not similar even from similar bacteria species. For instances, the bacteria isolated from horseshoe crab were more likely to produce strong amylases and lipases. On the other hand, the bacteria from jellyfish were more likely to be found in the production of lipases and protease, whereas bacteria from marine water were able to produce all three types of targeted industrial enzymes with strong activities. These characteristics were well-presented by *V. neocaledonicus* and *V. alginolyticus*. The present findings were similar as reported by Bunpa and the team [25] that *V. alginolyticus* exhibit different enzymes production obtained from the different origin as well as similar origin. The different ability in the production of the enzymes also appeared in other species studied. For example, *B. subtilis* subsp. *inaquosorum* (TB 22) was the subtilis group found with a similarity of 99.92 % and was claimed able to hydrolyse casein in the first report of this species [27]. However, our results contradict previous findings where TB 22 did not produce proteases. For the past ten years, enzymes production was reported based on its environmental conditions; particularly because the primary function of these enzymes in an organismal perspective was resources acquisition [26]. Besides, microbial community composition and latitudinal gradients were reported as other factors influencing enzymes production [28]. Moreover, Bunpa et al. [25] argued that there was genetic variation occurrence among the bacteria, irrespective of their source of recovery. The genetic variation in bacteria had previously been reported by Snoussi and co-workers in 2008 [29]. Later, Zhang and Kim [7] argued that the genetic variation might occur in bacteria as heritable changes among microorganisms; some of the reasons were associated with the competition for limited space and deficiency of nutrients in the marine environment. The unfavourable environmental conditions promote marine microorganisms to produce multifarious enzyme systems to adapt in the harsh environment [7]. With the above-mentioned facts, we believe that bacteria from different origins were explored from different

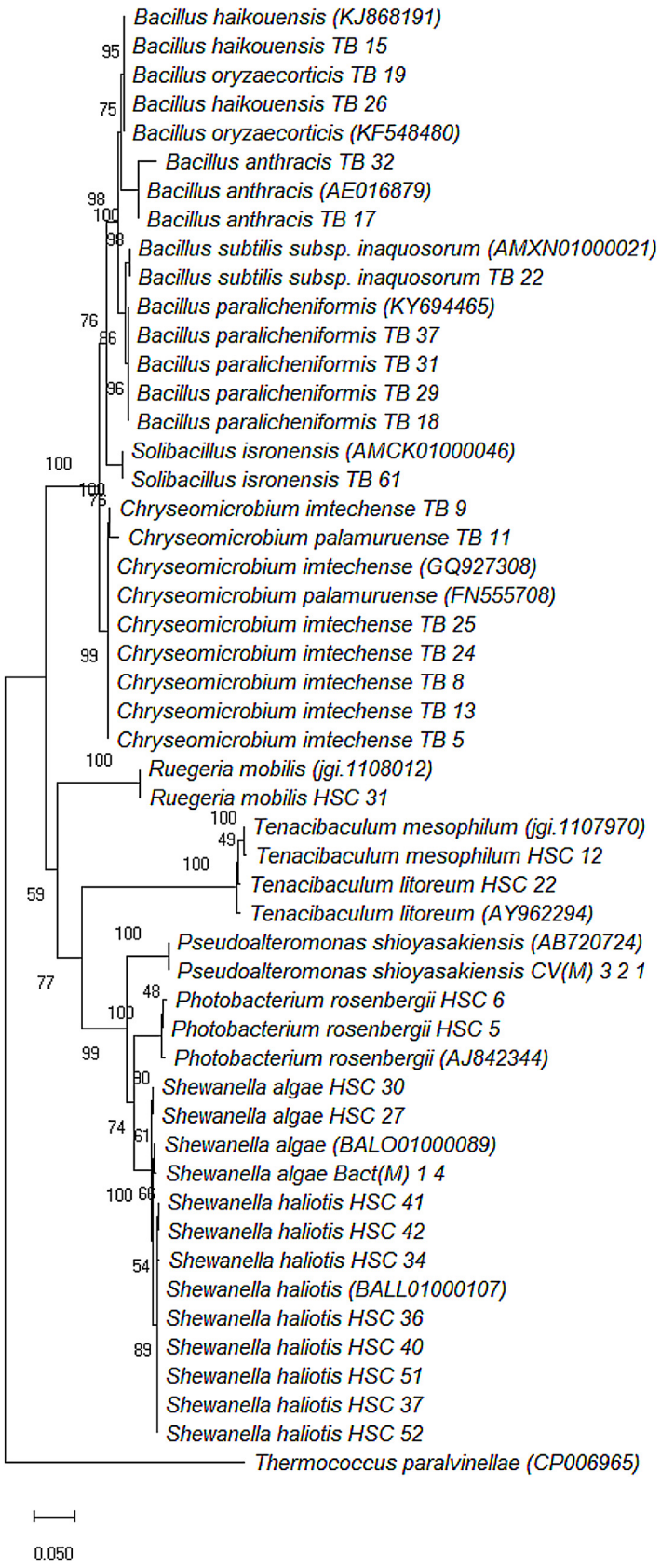

Fig. 3. Phylogenetic analysis using NJ method based on 16S rDNA sequences with bootstrap value of 1000 replicates. *Thermococcus paralvinellae* was used as outgroup. Bootstrap values (>50 %) were shown at the nodes. Bar 5 nucleotide substitutions per 100 nucleotides.

environments in terms of microbial community and resources. Nevertheless, bacteria species that come from similar origin exhibit multiple kinds of enzymes production raised an enigma to us; this aspect requires more detailed research emphasising on the influential factors affecting the production of enzymes in this bacteria.

Phylogenetic trees generated by the Minimum Evolution (ME) and the Neighbor-Joining (NJ) methods showed similar topology where similar bacteria isolate were grouped into the same clade, only the Maximum Likelihood (ML) showed a different placement. All of the isolates were grouped according to their respective genera and this indicated the correct topology of the trees. Four *Vibrio* species (*V. mytili* HSC 4, *V. harveyi* CV(M) 2.2, *V. parahaemolyticus* CV(H) 3.6 and *V. parahaemolyticus* CV (H) 7) have the sequence similarity of more than 97 %. Nonetheless, they were not located within a similar branch with the concatenated sequence. This also applies to all the *V. alginoliticus* studied strain. The probable reason is that *Vibrio* has a low taxonomic resolution using 16S rRNA genes analysis [30]. According to Thompson and his colleagues [30], *Vibrio* sp. was defined as a group of strains sharing more than 95 % of DNA identity in the Multilocus Sequence Analysis (MLSA) and the supertree gene sequence. Furthermore, they exhibited a percentage of Average Amino Acid Identity (AAI) greater than 96 %, not higher than 10 genome signature dissimilarities and more than 61 % of proteome identity. Thus, species identification using only the 16S rRNA gene sequence for *Vibrio* sp. was not convincing enough to support the novelty. For example, HSC 4 was closer to *V. harveyi* according to the placement in the tree rather than *V. mytili*. HSC 4 showed 97.12 % similarity to *V. harveyi* through EzBioCloud server blasting. Eight *Vibrio* sp. were identified from the whole selected bacteria isolate and they were collected from three different locations. It was expected that similar *Vibrio* sp. was found at a different location due to some *Vibrio* sp. is attracted to an aquatic environment, especially the ocean, and are often isolated from various marine organisms [31], marine water and ocean sediment [32]. During the past 20 years, many *Vibrio* strains were isolated from the marine environment and marine organisms [33,34,24,35,36,32].

All of the identified Firmicutes in this study were collected from Kelantan, and only this phylum was successfully selected for this study. There were eight species identified including *Chryseomicrobium* sp., *Solibacillus* sp. and *Bacillus* sp. The phylogenetic analysis shows that they were in a single cluster with a high bootstrap value (>50) which indicated the correct topology of the tree (Fig. 3). Only strain TB 9 and TB 11 were not clustered close to the concatenated species where the identified species, TB 9 and TB 11 were at 99.27 % and 98.62 %, respectively. Among the factors affecting the production of the enzymes of bacteria as stated, genetic variation is the steadfast reason in elaborating the allocation of these species not clustered close to their concatenated species. However, our results are not convincing enough to support this statement. Further study on the relationship between genetic variation and environmental factors in bacteria during enzymes production is needed to understand and manipulate the production of enzymes for future study and industrial needs.

5. Conclusion

A total number of 23 bacteria species were identified in industrial enzymes production, with emphasise on amylases, lipases and proteases. These bacteria were collected from various locations and origins. They were classified into the phylum of Bacteroidetes, Proteobacteria and Firmicutes. Genetic variation was more likely to occur within similar marine bacteria species. Microbial community composition was found to affect the production of industrial enzymes and diversity of marine bacteria. The diversity of marine bacteria species in the present study suggest the potential of marine bacteria in the production of targeted industrial enzymes.

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CRediT authorship contribution statement

T.H. Cheng: Investigation, Writing - original draft, Formal analysis, Methodology. **N. Ismail:** Funding acquisition, Supervision, Visualization, Project administration. **N. Kamaruding:** Writing - review & editing, Visualization. **J. Saidin:** Writing - review & editing. **M. Danish-Daniel:** Visualization, Supervision, Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00482.

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