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Research Article

Isolation of Thermophilic Esterase or Lipase Producing Bacteria from Hot Springs at the East Coast of Peninsular Malaysia

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ABSTRACT

Hot spring is a natural habitat for thermophilic bacteria and the primary source of thermostable enzymes useful in industrial applications. In Malaysia, the search for thermophilic organisms has been focused on hot springs, especially on the peninsular West coast. In this work, lipase or esterase producing thermophilic microorganisms were isolated from East coast hot springs in Pahang and Terengganu's states. Morphological and biochemical analysis were carried out on Isolates LH1, LH2, LH3, LH4, LH5, B2B2 and S1B4, which showed that they are gram positive, aerobic, spore forming, and motile organisms. All of the seven isolates showed the ability to grow at 45°C and formed hydrolysis zones on tributyrin agar plates. However, only isolate B2B2 and S1B4 were able to thrive at higher temperatures of up to 65°C. The genotypic characterisation was carried out using 16S rRNA sequencing. Bacillus and Geobacillus species were found to be the dominant bacteria isolated from these hot springs. From La hot spring, isolate LH1 (MT 645486), Isolates LH2 (MT645483), LH3 (MT645484), LH4 (MT 645485) and LH5 (MT 645487) were all closely related to *Bacillus* sp. (at 97.3-97.9%). Meanwhile, from Bentong and Sungai Lembing hot springs, isolates B2B2 (MT668631) and S1B4 (MT668632) were near related to either *Geobacillus kaustophilus* or *Geobacillus thermoleovorans*; each at 98.5% and 97.9% similarity, respectively. These strains from *Geobacillus* sp. were able to thrive at higher temperature and their thermostable esterases or lipases have properties useful for biotechnological applications.

Keywords: Thermophilic organism, Geobacillus sp., Thermophilic Bacillus, Hot spring, Thermostable lipases.

Introduction

Thermophiles produce enzymes that offer enormous benefits to diverse sectors, especially in industrial applications [1]. There is an increasing demand for thermostable enzymes that can withstand severe conditions normally used in conventional industrial processes [2]. Esterase (E.C 3.1.1.1) and lipase (E.C 3.1.1.3) are triacylglycerol hydrolase belonging to serine hydrolase enzymes which catalyse the hydrolysis ester bonds in the presence of water molecule [3, 4]. One of the significant differences between these two enzymes is that lipase catalysis involves relatively long chain acyl chain substrates of more than 10 carbon atoms. However, esterase catalysis involves shorter acyl-chain substrates of less than 10 carbon atoms. Compared to plant and animal sources, microbial esterases or lipases usually demonstrate features desirable for industrial application such as substrate specificity, regio- or stereo-specificity, high yields, and stability in the organic solvent [5]. For instance, since lipases have a high capability to perform a specific biotransformation range, they were used widely in different industries such as food, detergent, cosmetic, and leather [6]. This enzyme can be derived from diverse sources but microbial lipases or esterases are popular due to their stabilities [7]. Among these, *Bacillus* esterases or lipases are the most useful in industrial applications [8]. Nevertheless, the diversity of microbes still pro-

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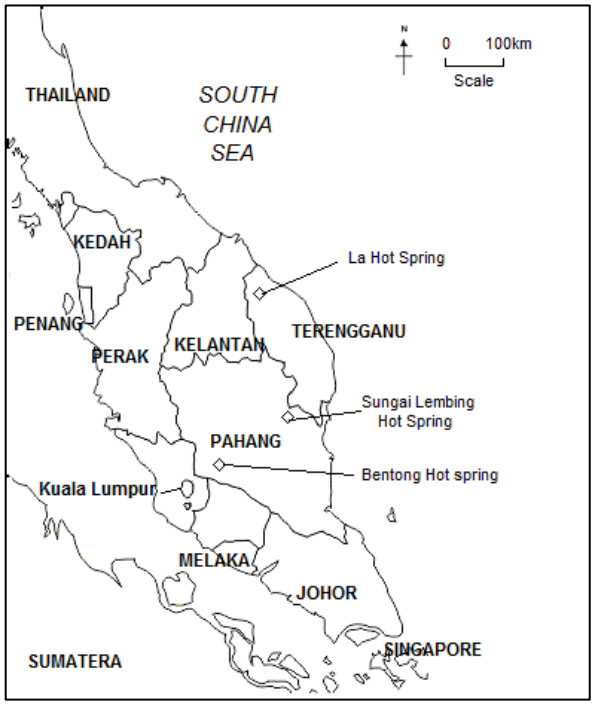

Figure 1. Map of West Malaysia showing boundaries of states in the peninsular and the relative locations of three hot springs involved in this works. Kelantan, Terengganu, and Pahang are three East coast states in Peninsular Malaysia. The East coast's hot springs shown are La hot spring in the state of Terengganu; Bentong and Sg. Lembing hot springs at two different locations in Pahang.

vide many opportunities for researchers to explore and screen for a novel enzyme with Useful properties and extremophiles have been the target source for thermostable enzymes.

Since thermophilic organisms can survive and grow optimally at 60 to 80 °C, they can be isolated from many habitats such as soil, hot springs, and volcanoes. Geothermal sources, such as hot springs, have been recognized as an important source of thermophilic bacteria that can produce thermostable enzymes [9]. For this reason, this location has been the typical target for the isolation of these organisms. Peninsular Malaysia has more than 60 hot springs, mostly non-volcanic in origin [10]. Since most of these hot springs are situated in the West coast of the peninsular [11], local researchers have focused on these regions in isolating thermophilic bacteria [12]. Using new metagenomic sequencing tool, biodiversity of thermophilic organisms were recently studied in some of the West coast's hot springs [13, 14]. However, isolation of thermophilic bacterium from the East Coast's hot springs is still uncommon. Unlike on the West Coast, East coast regions have fewer hot springs, and their locations are scattered, not easily accessible, and far from active research facilities. This is also why in Malaysia, most of the thermophilic lipases were isolated from West coast hot springs [12, 15-19], including those that were thoroughly studied and crystallised [20, 21]. Therefore, this work reported on the isolation of several thermostable esterase or lipase-producing bacteria from two hot springs situated in the East coast of the peninsular; i.e., La hot spring in Terengganu; Bentong and Sungai Lembing hot springs in Pahang. In this work, the isolated thermophilic bacteria were identified and characterised. Besides exploring their potential in industrial application as esterase or lipase producer, we also seek to determine if the thermophilic strains derived from this work still belong to similar strains isolated from other hot springs in Malaysia.

Material and Methods

Sample collection from hot springs

Water samples were collected from a hot springs in Terengganu and two hot springs in Pahang. La hot spring (5 ° 31' 2.3376"N, 102° 32' 80412"E) is located near Besut, Terengganu. Bentong hot spring (N3° 56' 42.7", E 103° 00' 46.8") and Sg. Lembing hot spring (N 3° 56' 47.7", E 103° 00' 46.8") are both located in Pahang (see Figure 1 and Figure 2a, 2b and 2c). Each hot spring has different hot water temperatures, for instance: 43°C, 45°C and 50°C (La); 37, 40 to 50°C (Bentong); and 40°C (Sungai Lembing). Water samples collected in sterile universal bottles were later transported to our laboratory at Kuantan Campus as soon as possible.

Screening of thermophilic lipase-producing bacteria

In the preliminary screening, hot spring water samples were serially diluted with sterilized peptone water up to 10^4 dilution and spread (~300 - 500 µl) on Nutrient agar (NA) plates, followed by incubation at 45°C for 48 hours. To test for lipase or esterase production, visible colonies were sub-cultured and streaked onto tributyrin nutrient agar (NA + 5.0 % (v/v) tributyrin) or tributyrin nutrient agar or TNA plates and incubated at 45°C. The presence of clearing zones surrounding colonies indicated either lipase or esterase production. Isolates showing visible growths on NA and lipolytic activities on TNA were selected for subsequent tests. Pure isolates were sub-cultured and preserved in 20% v/v glycerol stocks and stored

Figure 2. Pictures show three hot springs from which the water samples were taken for this work. a). La hot spring; b) Bentong hot spring; and c). Sungai Lembing hot spring

at -80°C.

Selected isolates (LH1, LH2, LH3, LH4, LH5, S1B4, and B2B2) were streaked on NA agar, followed by incubation at different temperatures (35, 40, 45, 50, 55, 60, 65°C) for 24 hours. Further growth characterisations were carried out on isolates that grew at higher temperatures (> 45°C). Two selected isolates (S1B4 and B2B2) were used to inoculate 10 ml nutrient broth (NB), and these were then incubated at 45, 50, 55, 60 and 65°C for 24 hours with shaking (40 RPM). Growth was evaluated by measuring the turbidity at an optical density (O.D.) of 600 nm using UV-VIS spectrophotometer (Lamda 35, Perkin Elmer, U.K.) and measuring the colony forming unit per ml ($CFU.ml^{-1}$) by streaking 200 µL of the broth on NA plate.

Phenotypic characterisations

The selected isolates were subjected to morphological studies (Gram staining, motility test and endospore staining) as done by Zahoor *et al* [22]. Biochemical tests using oxidase, catalase, urease, and citrate tests were based on Bergeys Manual of Systematic Bacteriology [23]. Catalase test was carried out to determine if the organism possess catalase enzyme, which can degrade H_2O_2. Oxidase test was carried out to identify if the microorganisms have cytochrome enzymes. The urease test was carried out to determine the presence of urease enzyme by using red phenol indicator. MacConkey and Citrate agars was used in the citrate test and lactose utilisation tests, respectively.

Genotypic characterisation

Genomic DNA were extracted from all strains using DNA extraction kit (GF-1, Vivantis) and used for template in polymerase chain reaction (PCR) amplification, using a pair of universal primers for rRNA genes [24]. The primer sequences were as follows: 5'-AGA GTT TGA TCC TGG CTC AG-3' for forward and 5'-GGT TAC CTT GTT ACG ACT T-3' for reverse. PCR was carried out using a thermocycler (Mastercycler, Germany) run in a 50µl volume reaction. The reaction mixtures were added with 15µl of template DNA (100ng), 25 µl of *Thermus aquaticus* (*Taq*) polymerase (5U/µl, Fermentas, Lithuania), 5µl (10pmol/µl) of each primer, 5 µl 10X Taq buffer (Fermentas, Lithuania), 5.0 µl dNTPs (10 mM) (Fermentas, Lithuania) and 5µl $MgCl_2$ (25mM). Other amplification parameters and cycle conditions were set similar as in our previous work [25]. The PCR products were cleaned and purified using gene clean kit according to manufacturer protocols (Vivantis). The DNA samples and PCR products were subjected 1.0% agarose gel electrophoresis, stained using ethidium bromide (25 $\mu g.ml^{-1}$), and visualised using gel documenter (Alpha Imager). The purified DNA samples were delivered to a local agency (1st BASE Laboratory Sdn. Bhd., Malaysia) for DNA sequencing.

Analysis of 16s rRNA sequences

The crude sequence was edited and cleaned by BioEdit version 7.2 software. The sequences were analyzed by Basic Local Alignment Search Tool (BLASTn), at National Center for Biotechnology Information (NCBI) website (*blast.ncbi.nlm.nih.gov/*), for similarity search. Each sequence was deposited into NCBI Genbank and then was given with an accession number: MT 645486 for LH1, MT645483 for LH2, MT645484 for LH3, MT 645485 for LH4 and MT 645487 for LH5, MT 668631 for B2B2 and MT668632 for S1B4, respectively. The 16S rRNA sequences from the related strains from BLAST hit lists were compiled and used to carry out multiple sequence alignment (MSA), and then to construct a phylogenetic tree. Using Geneious Prime software, MSA was carried out using ClustalW. Using this software also, the phylogeny was generated using inbuilt algorithms; Tamura & Nei [26] for evolutionary distance

Table 1. The morphological and biochemical characteristics of each isolate; and the physical parameters (pH and temperatures) of the respective hot springs

Parameters/Tests	LH 1	LH 2	LH 3	LH 4	LH 5	B2B2	S1B4
Hot spring	La	La	La	La	La	Bentong	Sungai Lembing
Water pH	6.5	6.5	6.5	6.5	6.0	7.0	7.0
Water temperature (± 0.5 °C)	43	50	50	45	45	50	40
Colony Morphologies							
Form	Circular	Circular	Irregular	Circular	Irregular	Circular	Circular
Elevation	Raised	Raised	Raised	Raised	Raised	Convex	Convex
Margin	Entire	Entire	Undulate	Entire	Undulate	Entire	Entire
Colour	Colourless	Colourless	Colourless	Colourless	Colourless	Creamy	Creamy
Gram staining	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Endospore	+	+	+	+	+	+	+
Biochemical Tests							
Oxidase	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	+	+
Urease	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Mckonkey agar	+	+	+	+	+	-	-

'+' represents positive result; and '-' represents negative result

Table 2. Ability of isolates to show visible colonies at different temperatures on NA agar plates.

Temperature ± 0.1 (°C)	LH 1	LH 2	LH 3	LH 4	LH 5	B2B2	S1B4
30.0	+	++	+	+	++	-	-
35.0	++	++	++	++	++	-	-
40.0	++	++	+++	++	++	+	+
45.0	+	+	++	+	++	+	+
50.0	+	-	-	+	+	+	++
55.0	-	-	-	-	-	++	++
60.0	-	-	-	-	-	+++	+++
65.0	-	-	-	-	-	++	+

Growths were recorded by counting bacterial colony numbers being observed after 48 hours, per plate or per 200µL inoculum. The label '-' indicates no observable colonies (no growth); '+' indicates 1 to 20 colonies; '++' indicates between 20 and 50 colonies; and '+++' indicates > 50 colonies.

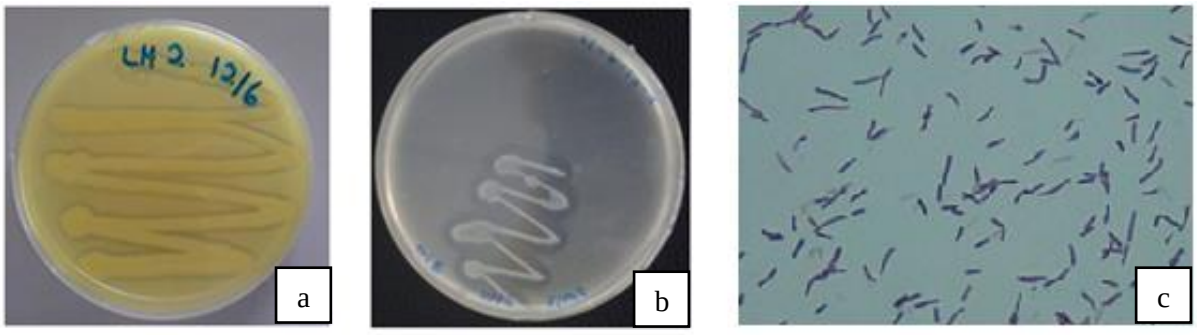

Figure 3. Examples of bacterial samples' growth from hot spring on tributyrin agar plates showing hydrolysis zones, indicated with white arrows. a). Isolate LH2, b). Isolate S1B4, and c). A Gram stain example viewed under 1000× magnification with an oil immersion shown for Isolate S1B4.

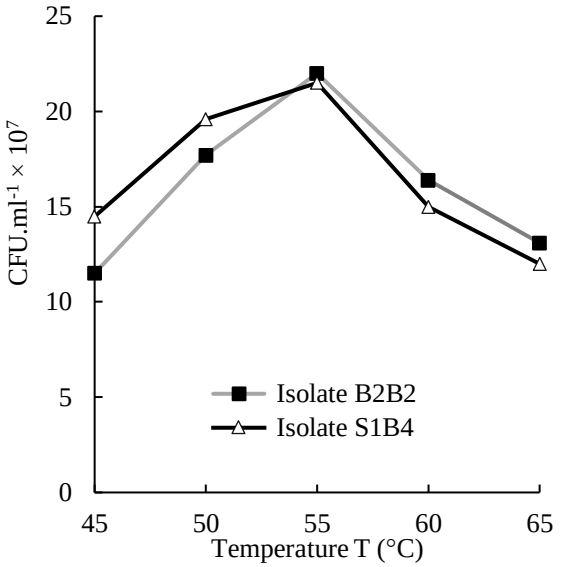

Figure 4. Growth of isolates S1B4 and B2B2 strains at different temperatures.

model; and Neighbour Joining (NJ) [27] for tree building method. The bootstrap analysis was carried out at 1000 replicates.

Results and Discussions

Phenoptypic characterisation of isolates

Isolate LH1, LH2, LH3, LH4, LH5, B2B2, and S1B4 formed colonies with circular form, entire margin and raised elevation (see Table 1). All of the isolates were gram positive rods (Figure 3c), aerobic, spore-forming, and motile. Biochemical tests indicated that they were catalase and urease negative, oxidase positive, and able to ferment citrate. Both showed similar observations except that their colonies appeared somewhat creamy with convex elevation, catalase positive and urease negative, compared to LH1-LH5. All isolates were urease negative and only B2B2 and S1B4 were unable to ferment lactose, based on MacConkey agar tests. These characteristics were consistent with other works reported for *Bacillus* and *Geobacillus*, respectively [22].

As shown in Table 2, Isolates LH1–LH5 were unable to show any growth beyond 45°C, and these isolates were considered a moderately thermophilic group [28]. In contrast, isolates B2B2 and S1B4 were able to grow beyond 45°C but unable to show growth at 35°C or below.

In growth studies conducted at different temperatures as shown in Figure 4, their growth reached an optimum at 55°C and depleted at 65°C. This growth profile is notable and consistent with Geobacillus species of which in many studies they exhibited growth up to 75°C with an optimum within 55–65°C [29-31]. The growth on TNA plates indicated all strains tested were able to show a clearing zone of hydrolysis; see examples shown in Figure 3a-b. The clearing zone signified that tributyrin, a triglyceride, was hydrolysed by either esterase or lipase secreted from the colonies. An enzymatic test in the next study should be done to determine the cause of tributyrin hydrolysis, whether this hydrolysis is caused by esterase or lipase. Thus, all isolates were able to produce esterase/lipase, which were active at least at 45°C. In case of S1B4 and B2B2, the clear zones were still being observed at 65°C, suggesting that they are potential thermostable esterase or lipase producers.

Genoptypic characterisation of isolates

The 16S rRNA genes of size 1.5kb were successfully amplified from all samples except for sample isolate LH1 that has shown a rather weak band (Figure 5). The 16S rRNA gene sequencing showed that strains LH1, LH2, LH3, and LH4 have some similarities with *Bacillus licheniformis* (at 96.3, 97.3, 97.5% and 97.8, respectively), and strain LH5 with *Bacillus subtilis* (94.2%); see Table 3 for similarity hit list from Genbank. The threshold limit commonly accepted for similarity is 95% for genus and 97% for species, respectively [32]. Based on similarity thresholds within the range of 95 to 97%. Isolates LH2, LH3, LH4 are thus similar with *Bacillus licheniformis*, a bacterium commonly reported as moderately thermophilic that shows optimal growth at 50°C [33]. Having similarities below 97%, both Isolate LH4 and LH5 were only identified up to genus level. In LH1, the poor-quality genomic DNA could have affected the outcome of PCR amplification (Figure 5a). Various factors, such as genomic DNA's nature could also affect the PCR amplification in rRNA sequencing [34]. The actual species for LH1 and LH5 can only be confirmed through different techniques such as DNA hybridization, or other powerful tools used for microbial identification [35].

Meanwhile, both isolates from Bentong (B2B2) and Sungai Lembing (S1B4) hot springs showed similarities to either *Geobacillus themoleovorans* (~97.9%) or *Geobacillus kaustophilus* (~98.5%). Both *Geobacillus themoleovorans* and *Geobacillus kaustophilus* appeared to be the top scorers in the BLASTn similarity hit lists for these two isolates (see Supplementary table). According to Burgess *et al.* [36], *Geobacillus* genus consists of 11 species which are difficult to resolve using rRNA sequencing. For instances, in

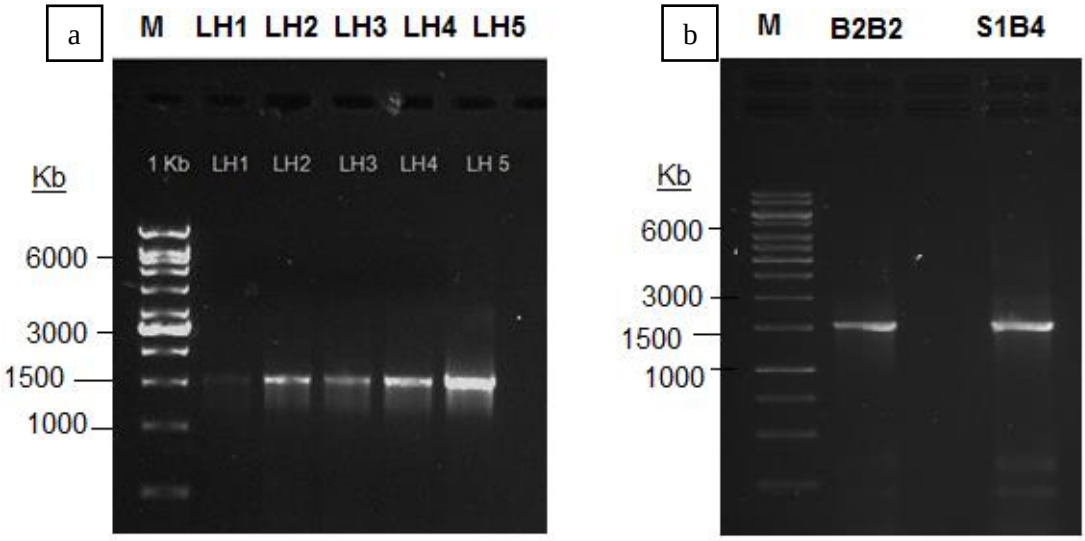

Figure 5. Agarose (1.0 %) gel electrophoresis show the results of PCR amplification of 16S rRNA using genomic DNA samples; (a) from LH1, LH2, LH3, LH4 and LH5 and (b) S1B4 and B2B2. Lane M are molecular weight 1Kb marker ladder (Fermentas, Lithuania). Each lane was labelled with respective isolate. The amplified products band of 1.5Kb size is shown in each lane.

Table 3. Top similarity from hit lists generated from NCBI Genbank database for isolates LH1, LH2, LH3, LH4, LH5, S1B4 and B2B2.

Isolate	Description	Max Score	Identity (%)	Accession
LH1	*Bacillus paralicheniformis* strain KJ-16	1465	96.33	NR_137421.1
LH2	*Bacillus licheniformis* strain DSM 13	2335	97.31	NR_118996.1
LH3	*Bacillus licheniformis strain* BCRC 11702	2121	97.59	NR_116023.1
LH4	*Bacillus licheniformis* strain DSM 13	2278	97.95	NR_118996.1
LH5	*Bacillus subtilis subsp. subtilis*	1879	94.21	NR_102783.2
S1B4*	*Geobacillus thermoleovorans* strain BGSC 96A1	2278	97.95	NR_115286.2
B2B2*	*Geobacillus kaustophilus* strain BGSC 90A1	2507	98.52	NR_115285.2

*Isolate S1B4 and B2B2 showed high similarity scores to both *Geobacillus thermoleovorans* and *Geobacillus kaustophilus.*

distinguishing different *Geobacillus* species, DNA hybridization [37] and the amplification of *Geobacillus* specific primers combined with restriction analysis [38] were used. Actually, the *Geobacillus* genus reclassifies group 5 within the *Bacillus* groups following taxonomic revision made in 2001[39]. In this work, strains B2B2 and S1B4 are considered belonging to *Geobacillus* sp. Future work using different techniques is required to verify the species. Their ability to grow optimally in the range of 60 to 70°C categorise them as extreme thermophiles [28]. As an obligate thermophile, *Geobacillus* species is a common source of thermostable enzymes, which are used in many biotechnological processes. For instance, thermostable lipases have properties desirable for many industrial processes [2], and thermostable esterases can synthesize stereospecific compounds, perfumes and many types of antioxidants [5].

Phylogenetic tree

A phylogenetic tree was constructed, as shown in Figure 6. Isolates LH1, LH2, LH3, and LH4 were clustered in a branch or clade. It consists of various mesophilic or moderately thermophilic *Bacillus* species, such as *B. licheniformis, B. haynesii, B sonorensis,* and *B. aerius.* Meanwhile, Isolate LH5 clustered together with *B. subtilis*, forming another cluster comprising of *B. tequilensis, B. amyloliquefaciens,* and others. Meanwhile, thermophilic *Geobacillus* sp. strain S1B4 and B2B2 were clustered in clade consisting of strictly thermophilic *Bacillus.* In the tree, strain *Staphylococcus aureus* was excluded from all of the clusters, forming an outgroup.

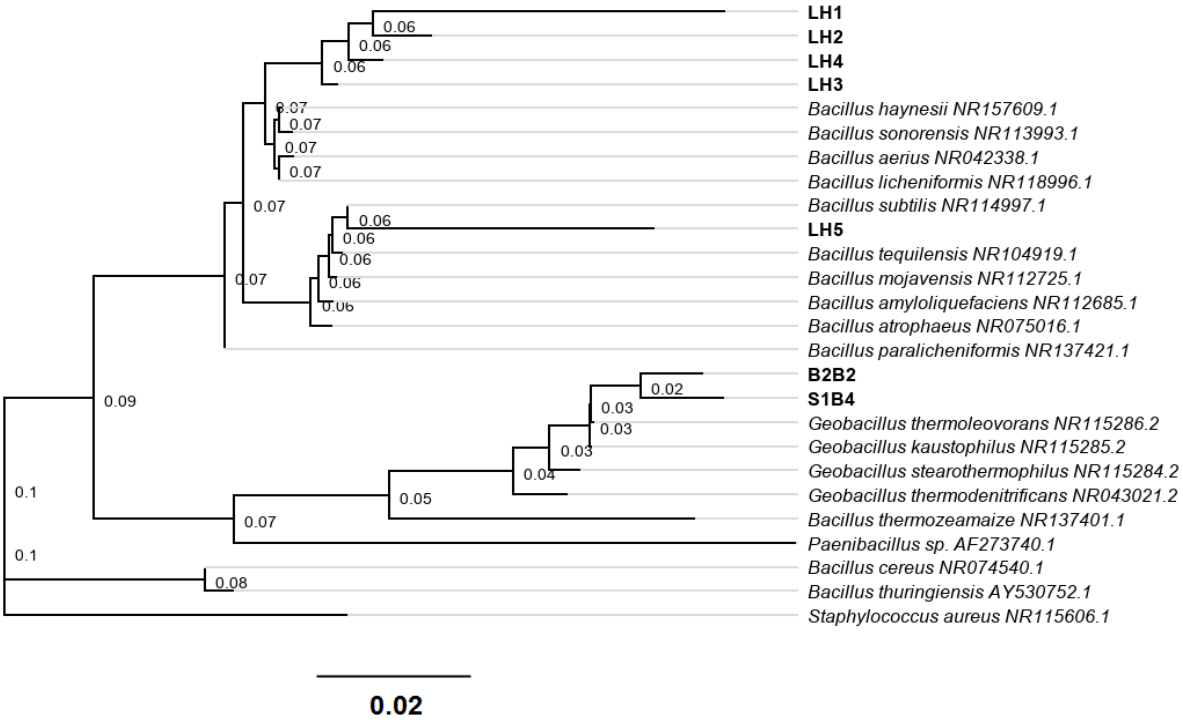

Figure 6. Phylogram constructed using several 16S rRNA sequences from selected *Bacillus* and *Geobacillus* species. The tree shows strains LH1, LH2, LH3 and LH4 clustered within *Bacillus* sp. group comprising *B. licheniformis, B. haynesii, B sonorensis*, and *B. aerius;* and LH5 was clustered with *B. subtilis.* Both Isolates S1B4 and B2B2 were clustered within *Geobacillus* group. This tree was constructed using Neighbour joining (NJ) method and the numbers at nodes are the relative node height that represent base substitution per site.

Distribution of thermophilic organisms from hot springs surrounding Malaysia peninsular

In an attempt to isolate thermophilic bacteria, *Bacillus* and *Geobacillus* species were frequently reported from hot springs surrounding Malaysia peninsular. These include thermophilic bacteria isolated from hot springs at Gadek (Melaka), Labis (Johor), Pedas (Negeri Sembilan lan), Selayang (Selangor) [12]; Selayang (Selangor) [15, 24]; Sg. Klah (Perak) and Dusun Tua (Selangor) [40]; Setapak (Selangor) [16], Ulu slim (Perak) [41]; and (undefined location) Perak [17, 18]. Using new tool such as metagenomic sequencing, microbial biodiversity was profiled at Sg. Klah hotspring [13]; and Ulu Slim hot spring [14] in Perak. Thus, the majority of these works were centred on hot springs situated along the West coast of the peninsular. Meanwhile, there were very few reports on the isolation of thermophilic organism from East coast region. These include the isolations of *B. licheniformis* and *B. subtilis* producing thermostable alkaline proteases from Lojing hot spring (Kelantan) [42], and *Geobacillus thermodenitrificans* strain producing thermostable lipase from Labok hot spring (Kelantan) [19]. Our previous work on Sungai Lembing (Pahang) had isolated moderately thermophilic β-proteobacteria [43]. Therefore, this work will add Bacillus and *Geobacillus* genera as another new collection of esterase or lipase producing thermophilic bacteria from East coast hot spring.

Conclusion

In conclusion, several thermophilic esterase or lipase producing bacteria were successfully isolated from several hot springs located in the East coast of Malaysian Peninsular. From La hot spring, Isolate LH2, LH3, LH4, and LH5 mostly belonged to genus *Bacillus licheniformis* and Isolate LH1 and LH5 to *Bacillus sp.* These are moderately thermophilic and able to grow at least at 45°C. Isolate B2B2 and S1B4, from Bentong and Sg Lembing hot springs were able to thrive up to 65°C and belonged thermophilic group *Geobacillus* sp., a genus commonly isolated from many hot springs. Both of these species are potential thermostable esterase or lipase producers. This work showed that Malaysian East coast's hot springs offer diverse organisms of both moderately and extreme thermophiles, potentially harnessed for future biotechnological application

such as thermostable enzyme for industrial bio-catalyst.

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References

1. Mehta R, Singhal P, Singh H et al. (2016) Insight into thermophiles and their wide-spectrum applications. 3 Biotech 6 (1): 81.
2. Salihu A, Alam M (2015) Thermostable Lipases: an overview of production, purification and characterization. Biosciences Biotechnology Research Asia, 11: 1095-1107. doi: 10.13005/bbra/1494.
3. Konwar BK, Sagar K (2018) Lipase, eds. Lipase: an industrial enzyme through metagenomic, Apple Acedemic Press Inc.: Toronto. p. 12-13.
4. Bracco P, van Midden N, Arango E et al. (2020) Bacillus subtilis Lipase A—Lipase or Esterase? Catalysts 10 (3): 308.
5. Zhu Y, Li J, Cai H et al. (2013) Characterization of a new and thermostable esterase from a metagenomic library. Microbiological Research 168 (9): 589-597. doi: 10.1016/j.micres.2013.04.004.
6. Houde A, Kademi A, Leblanc D (2004) Lipases and their industrial applications: An overview. Applied Biochemistry and Biotechnology 118: 155-70. doi: 10.1385/ABAB:118:1-3:155.
7. Sharma A, Sharma V, Saxena J. (2016) A Review on applications of microbial lipases. International Journal of Biotech Trends and Technology, 19: 1-5. doi: 10.14445/22490183/IJBTT-V19P601.
8. Mazhar H, Abbas N, Ali S et al. (2017) Optimized production of lipase from *Bacillus subtilis* PCSIRNL-39. African Journal of Biotechnology 16 (19): 1106-1115.
9. Mohammad B, Al Daghistani H, Jaouani A et al. (2017) Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* Isolates as potential producers of thermostable enzymes. International Journal of Microbiology 2017: 6943952. doi: 10.1155/2017/6943952.
10. Hassan B, Mohd N, Karl W, Hariri AM (2015) Geochemistry and geothermometry of non-volcanic hot springs in West Malaysia. Journal of Volcanology and Geothermal Research, 290: 12-22.
11. Samsudin AR, Hamzah U, Rahman RA et al. (1997) Thermal springs of Malaysia and their potential development. Journal of Asian Earth Sciences 15 (2-3): 215-284.
12. Zuridah H, Norazwin N, Siti Aisyah M et al. (2011) Identification of lipase producing thermophilic bacteria from Malaysian hot springs. African Journal of Microbiology Research 5 (21): 3569-3573.
13. Chan CS, Chan K-G, Tay Y-L et al. (2015) Diversity of thermophiles in a Malaysian hot spring determined using 16S rRNA and shotgun metagenome sequencing. Frontiers in Microbiology 6: 177-177. doi: 10.3389/fmicb.2015.00177.
14. Abdul Halim M, Rosli N, Dzulkifli F, Najimudin N, and Zarkasi K. (2020) Discovering the thermophiles microbial diversity of Malaysian hot spring in Ulu Slim, Perak. Malaysian Journal of Microbiology, 16: 184-192. doi: 10.21161/mjm.190564.
15. Norashirene MJ, Umi Sarah H, Siti Khairiyah MH, Nurdiana S (2013) Biochemical characterization and 16S rDNA sequencing of lipolytic thermophiles from Selayang hot spring, Malaysia. IERI Procedia 5: 258-264.
16. Sheikh Abdul Hamid N, Zen HB, Tein OB et al. (2003) Screening and identification of extracellular lipase-producing thermophilic bacteria from a Malaysian hot spring. World Journal of Microbiology and Biotechnology, 19 (9): 961-968. doi: 10.1023/B:WIBI.0000007330.84569.39.
17. Gandhi S, Salleh AB, Rahman RNZRA et al. (2015) Expression and characterization of *Geobacillus stearothermophilus* SR74 recombinant α-amylase in *Pichia pastoris*. BioMed Research International 2015: 529059. doi: 10.1155/2015/529059.
18. Shariff FM, Rahman RNZRA, Basri M, Salleh AB (2011) A newly isolated thermostable lipase from *Bacillus* sp. International Journal of Molecular Sciences 12 (5): 2917-2934.
19. Anuradha B, Darah I, Rashidah AR et al. (2012) Purification and characterization of a thermostable lipase from *Geobacillus thermodenitrificans* IBRL-nra. Enzyme Research 2012: 987523. doi: 10.1155/2012/987523.
20. Mohamad Aris SNA, Thean Chor AL, Mohamad Ali MS et al. (2014) Crystallographic analysis of ground and space thermostable T1 Lipase crystal obtained via counter diffusion method approach. BioMed Research International, 2014: 904381. doi: 10.1155/2014/904381.
21. Khusaini MS, Rahman R, Mohamad Ali MS et al. (2011) Crystallization and preliminary X-ray crystallographic analysis of a thermostable organic solvent-tolerant lipase from *Bacillus* sp. strain 42. Acta crystallographica. Section F, Structural biology and crystallization communications 67: 401-3. doi: 10.1107/S1744309111002028.
22. Zahoor S, Javed MM, Babarl ME (2016) Brazilian Archives of Biology and Technology 59 doi: 10.1590/1678-4324-2016150662.

23. Brenner D, Staley J, and Krieg N, (2006), Bergey's Manual of Systematic Bacteriology, eds. p. 27-32.
24. Akanbi TO, Kamaruzaman AL, Bakar FA et al. (2010) Highly thermostable extracellular lipase-producing Bacillus strain isolated from a Malaysian hotspring and identified using 16S rRNA gene sequencing. International Food Research Journal 17: 45-53.
25. Yusuf MA, Hamid THT (2013) Isolation of coagulase negative *Enterococcous* sp. strains from non-broiler chicken producing bacteriocin active against *Staphylococcus aureus*. Journal of Agrobiology 30(1): 33-42.
26. Tamura K, Kumar S (2002) Evolutionary distance estimation under heterogeneous substitution pattern among lineages. Molecular Biology and Evolution 19 (10): 1727-1736. doi: 10.1093/oxfordjournals.molbev.a003995.
27. Saitou N, Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4 (4): 406-425. doi: 10.1093/oxfordjournals.molbev.a040454.
28. Gupta G, Srivastava S, Khare SK, Prakash V. (2014) Extremophiles: an overview of microorganism from extreme environment. International Journal of Agriculture, Environment and Biotechnology 7 (2): 371-380.
29. Novik G, Savich V, Meerovskaya O (2019) *Geobacillus* bacteria: Potential commercial applications in industry, bioremediation, and bioenergy in Growing and Handling of Bacterial Cultures, M. Mishra, Editor IntechOpen: London, UK.
30. Kauldhar BS, Sooch BS (2016) Tailoring nutritional and process variables for hyperproduction of catalase from a novel isolated bacterium *Geobacillus* sp. BSS-7. Microbial Cell Factories 15 (1): 7. doi: 10.1186/s12934-016-0410-1.
31. Adhikari H, Ghimire S, Khatri B, Yuvraj KC (2017) Polypasic analysis of two thermotolerant and exozymes producing Geobacillus species from hot spring of Nepal. Journal of Microbiology, Biotechnology and Food Sciences 6 (4): 1059-1064.
32. Edgar RC (2018) Updating the 97% identity threshold for 16S ribosomal RNA OTUs. Bioinformatics 34 (14): 2371-2375. doi: 10.1093/bioinformatics/bty113.
33. Ashraf MA, Arshad MI, Rahman SU, Khan A (2018) Characterization of moderately thermostable α-amylase-producing *Bacillus licheniformis* from decaying potatoes and sweet potatoes. Bioresources 13 (3): 15.
34. Hansen MC, Tolker-Nielsen T, Givskov M, Molin S. (1998) Biased 16S rDNA PCR amplification caused by interference from DNA flanking the template region. FEMS Microbiology Ecology 26 (2): 141-149. doi: 10.1111/j.1574-6941.1998.tb00500.x.
35. Franco-Duarte R, Černáková L, Kadam S et al. (2019) Advances in chemical and biological methods to identify microorganisms-from past to present. Microorganisms, 7 (5): 130. doi: 10.3390/microorganisms7050130.
36. Burgess SA, Flint SH, Lindsay D et al. (2017) Insights into the *Geobacillus stearothermophilus* species based on phylogenomic principles. BMC Microbiology 17 (1): 140. doi: 10.1186/s12866-017-1047-x.
37. DeFlaun MF, Fredrickson JK, Dong H et al. (2007) Isolation and characterization of a *Geobacillus thermoleovorans* strain from an ultra-deep South African gold mine. Systematic and Applied Microbiology 30 (2): 152-164. doi: 10.1016/j.syapm.2006.04.003.
38. Kuisiene N, Raugalas J, Stuknyte M, Chitavichius D (2007) Identification of the genus Geobacillus using genus-specific primers, based on the 16S–23S rRNA gene internal transcribed spacer. FEMS Microbiology Letters 277 (2): 165-172. doi: 10.1111/j.1574-6968.2007.00954.x.
39. Coorevits A, Dinsdale AE, Halket G et al. (2012) Taxonomic revision of the genus *Geobacillus:* emendation of *Geobacillus, G. stearothermophilus, G. jurassicus, G. toebii, G. thermodenitrificans and G. thermoglucosidans (nom. corrig.,* formerly *'thermoglucosidasius');* transfer of B*acillus thermantarcticus* to the genus as *G. thermantarcticus* comb. nov.; *p*roposal of *Caldibacillus debilis gen. nov.,* comb. *nov.;* transfer of *G. tepidamans* to *Anoxybacillus* as *A. tepidamans* comb. nov.; and proposal of *Anoxybacillus caldiproteolyticus* sp. nov. International Journal of Systematic and Evolutionary Microbiology 62 (Pt_7): 1470-1485. doi: 10.1099/ijs.0.030346-0.
40. Chai YY, Kahar UM, Salleh MM et al. (2012) Isolation and characterization of pullulan-degrading Anoxybacillus species isolated from Malaysian hot springs. Environmental Technology 33 (11): 1231-1238.
41. Sakaff MKLM, Rahman AYA, Saito JA et al. (2012) Complete genome sequence of the thermophilic bacterium *Geobacillus thermoleovorans* CCB_US3_UF5. Journal of Bacteriology: 1239.
42. Ibrahim N, Norazila Y (2012) Themostable alkaline serine protease from thermophilic Bacillus species. International Research Journal of Biological Sciences 2 (2): 29-33.
43. Ghazali SAM, Hamid THAT (2015) New lipase producing beta-proteobacteria strains *Caldimonas* sp. and *Tepidimonas* sp. Isolated from a Malaysian Hot Springs. Sains Malaysiana 44 (5): 701-706. doi: 10.17576/jsm-2015-4405-09.

Supplementary 1

Table 4. Selected 5 similarity hit lists generated from NCBI Genebank database for isolates L4, L5, S1B4 and B2B2.

Isolate	Description	Max Score	Identity (%)	Accession
L4	*Bacillus licheniformis* strain DSM 13	2278	97.95	NR_118996.1
	Bacillus haynesii strain NRRL B-41327	2266	97.8	NR_157609.1
	Bacillus sonorensis strain NBRC 101234	2255	97.72	NR_113993.1
	Bacillus aerius strain 24K	2239	97.42	NR_042338.1
	Bacillus subtilis subsp. *subtilis* strain 168	2172	96.51	NR_102783.2
L5	*Bacillus subtilis subsp. subtilis*	1879	94.21	NR_102783.2
	Bacillus subtilis strain DSM 10	1879	94.21	NR_027552.1
	Bacillus tequilensis strain 10b	1879	94.2	NR_104919.1
	Bacillus velezensis strain CBMB205	1866	93.98	NR_116240.1
	Bacillus licheniformis strain DSM 13	1770	92.62	NR_118996.1
S1B4	*Geobacillus thermoleovorans strain BGSC 96A1*	2278	97.95	NR_115286.2
	Geobacillus kaustophilus strain BGSC 90A1	2276	97.95	NR_115285.2
	Geobacillus vulcani strain 3S-1	2259	97.72	NR_025426.1
	Geobacillus stearothermophilus strain BGSC 9A20	2228	97.26	NR_115284.2
	Geobacillus thermodenitrificans strain OHT-1	2193	96.81	NR_043021.2
B2B2	*Geobacillus kaustophilus strain BGSC 90A1*	2507	98.52	NR_115285.2
	Geobacillus thermoleovorans strain BGSC 96A1	2490	98.24	NR_115286.2
	Geobacillus zalihae strain NBRC 101842	2451	97.61	NR_114014.1
	Geobacillus stearothermophilus strain BGSC 9A20	2410	97.26	NR_115284.2
	Geobacillus thermocatenulatus strain Ga	2386	96.98	NR_104810.1