

Molecular Identification of Thermophilic Bacteria Producing Lipase from Two Hot Springs in Perak, Malaysia

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Abstract

Microbial lipase has been identified as a key biocatalyst in industrial biotechnological applications. Microbial thermostable lipases are excellent candidates because of their capability to survive in high temperatures reaction in industries. The aims of this study were to screen lipolytic activity on Rhodamine B agar; to measure the amount of lipolytic activity through UV-VIS spectrophotometer and to identify the species which have the highest production of lipase enzymes using molecular identification. Two Gram-positive and rod-shaped thermophilic isolates from Ulu Slim (US) and Ara Panjang (AP) were selected. Based on the spectrophotometric assay, isolate AP was a good lipase producer as it produced a higher value of 444.43 U/mL as compared to commercialized lipase. Further molecular identification revealed both isolates as *Bacillus* sp. Isolate AP was considered as a potential candidate for further studies as the lipase production exceed commercialized lipase production.

1. Introduction

Lipase enzymes or also known as triacylglycerol acyl hydrolase have their unique roles and features as an ideal active biocatalyst in many bioprocesses. Unlike animals and plants lipases, microbial lipases are more suitable to be used as biocatalysts. Microorganisms have a diverse range of species and host, are easy to grow, and are able to be genetically manipulated to obtain desired features or functions. The most prevalent bacterial sources of lipase are *Bacillus* spp., *Pseudomonas* spp., *Staphylococcus* sp., and *Burkholderia* sp. [1].

Microbial lipase offers application into wide areas of biotechnological applications nowadays such as dairy, detergents, textile, oil processing industries even in the manufacturing of surfactants and chemically pure compounds. Nonetheless, today's industrial processes are carried out in harsh conditions, such as extremely low and high temperatures, using pH levels that are acidic or basic, and at high salinities. Hence, the industries are now demanding extremozymes that can perform optimally throughout the processes. Lipase enzymes used in detergent-making must be capable of operating at high temperatures and with a wide range of surfactants that are frequently used during laundry applications [2]. In biofuel production, thermostable lipase also has acquired an interest in recent years due to the process's increased reaction speeds, low energy input, and low contamination risk [3].

1.1 Enzyme Industry in Malaysia

Realizing the importance of enzymes in the bioindustry, many advanced countries such as Denmark, the United States of America, China and Japan consider the enzymes' productions as an essential economic attribution due to their extensive uses in various industries. As an example, enzymes that play roles as significant microbial metabolites in bioindustries have been effectively produced commercially, primarily using submerged fermentation systems, as in many advanced countries, particularly in Europe, the United States of America, and Japan [4]. In Malaysia, Southern Lion Sdn Bhd is one of the well-known detergent manufacturers residing in Johor Bahru, that successfully incorporate the enzymes such as lipase and protease as part of the detergent's formulation. In addition, Malaysia also participates with other countries of Asia-Pacific in exporting the enzymes worldwide. Alike with the advanced countries, the development of homegrown enzymes is critical as it lessens the dependency to imports and permit competitive pricing within the local market. Malaysia has a wealth of natural resources that can be easily tapped into aligned with development of local expertise to help develop the industry to economical scale.

Nonetheless, the development of the Malaysian enzyme industry is still developing slowly despite having a considerable number of imports from other countries. According to [4], slow growing of enzyme industry in less developed nations like Malaysia is likely due to high production costs and capital expenditure for industrial enzyme applications. Data obtained from [5], [6], as in Fig. 1, shows an unfavorable balance of trade where the value of the import exceeds the value of the export of enzymes which give negative outcomes to country's economic growth. Hence, homegrown enzyme production and application technologies need to become a significant focus in industrial biotechnology.

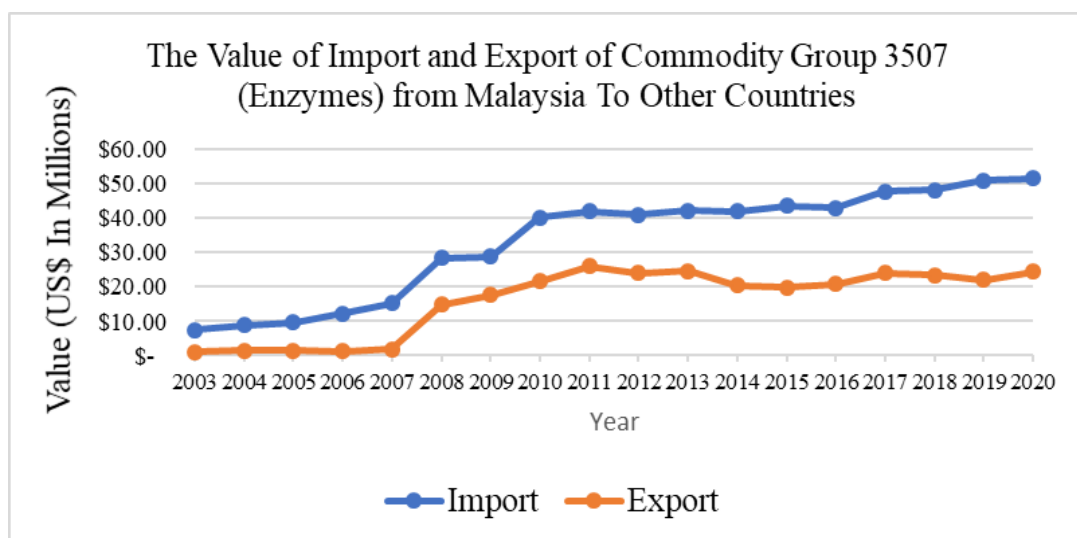


Fig. 1 Annual international trade statistics (import and export of commodity group 3507; enzymes) by Malaysia from 2003 – 2020 [5], [6]

1.2 Thermophilic Lipases

The diversity of thermophilic bacteria can be found in environments with high temperatures such as geothermal springs, deep-sea hydrothermal vents, hypersaline soil and halophytic, salty soil, and deserts [7]. In Malaysia, the hot springs are the main reservoir for most thermophilic bacteria thriving.

Typical characteristics in thermophilic bacteria that enable them to adapt in extreme environments such as high temperatures are more hydrophobic residues and hydrophobic core, an increased number of disulfide bonds, and greater ionic interactions. Recent research also suggests that thermophiles contain enhanced interactions with cofactors or prosthetic groups and/or higher subunit-subunit interactions that will help stabilize the protein's structure in its functional state [8]. These characteristics will diminish the protein's flexibility, allowing it to move optimally at higher temperatures without the cause of denaturation. As mentioned above, increasing hydrophobic residues and hydrophobic core is one feature that differs between thermophilic protein and non-thermophilic protein. This is aligned with recent evidence from [9], where hydrophobic residues as well as hydrophobic core in thermophilic rhodopsin in *Thermus thermophilus* JL-18 increased when exposed to high temperature. The changes allowed the thermophilic rhodopsin to be more stable and prevent pigment loss as demonstrated in non-thermophilic rhodopsin.

[8] highlighted that increased disulfide bonds in thermophilic protein cause the tertiary and quaternary structures to instantly solidify to prevent any alteration and denaturation during high temperatures. Hence, this mechanism provides stabilization to proteins' structures by decreasing their flexibility to properly function

when exposed to extreme temperatures. Moreover, the balance between flexibility and rigidity of proteins' structure during exposure to different temperatures subsequently allows the bacteria to behave optimally and become temperature dependent [8]. Other than that, having a greater ionic interaction is shown to be prevalent in thermophiles as the ionic residues on the exterior of protein structures are strongly linked to thermal stability. For instance, an analysis conducted by [10] on the mutation effects of 26 single-site variations on L30e ribosomal protein from *Thermococcus celer* demonstrated that interactions between ionizable side chains played a substantial role in increasing protein stability in hot temperatures.

2. Materials and Methods

2.1 Sampling Sites and Isolation

The water samples were collected from two hot springs located in Perak, Malaysia which are Ara Panjang Hot Spring (N 4.61899°, E 100.88561°) located at Manong while Ulu Slim Hot Spring (N 3.8991°, E 101.4975°) located at Ulu Slim. Each hot spring had different temperatures of approximately 50°C and 80°C, respectively, with pH ranging from 8.0 to 8.5.

Ulu Slim Hot Spring is well-known for with huge microbial diversity of thermophilic bacteria, for instance, phyla Aquificae, Chlorobi, Thermotogae, Proteobacteria, Firmicutes, Tenericutes, Cyanobacteria, Acidobacteria, and Calescamantes [11], [12]. The Ulu Slim hot spring is located on the western side of Malaysia's Banjaran Titiwangsa, Peninsular Malaysia's largest non-volcanic mountain range with a tropical environment [13]. While Ara Panjang Hot Spring is an abandoned hot spring that can be found in a village in Manong, Kuala Kangsar, Perak. According to [14] the tourists did not consider it to be a desirable destination due to negligence by villagers thus resulting in less human intervention and no research study regarding microbial population was found.

The samples were processed as the normal procedure dilution followed by spread plate, streaking and single colony isolation and maintenance in glycerol stocks.

2.2 Screening of Lipolytic Thermophiles

The growth of isolates was assessed through standard Rhodamine B fluorescent assay according to the method from [15]. The preparation method of Rhodamine B agar medium was done according to [16] with some modifications. The ingredients include 1% v/v olive oil, 10 ml of Rhodamine-B stock 0.01% w/v and distilled water. The isolated plates were then incubated at 55°C for 48 to 72 hours. All Rhodamine B agar plates containing isolates were then observed under ultraviolet (UV) light at 350 nm to monitor the orange fluorescence produced within the colonies. The orange fluorescence indicated the production of lipase enzymes by thermophiles.

2.3 Lipase Assay

The colonies that produced orange fluorescence were selected further for the quantitative lipase assay. The secreted lipase enzyme by thermophiles was measured quantitatively through spectrophotometric method according to [17] with some modifications. Prior to spectrophotometric method, a standard curve of p-nitrophenol (pNP) (0.002 to 0.02 M) was prepared by diluting the standard solution with 0.1 M Tris HCl buffer in 10 individual test tubes. The absorbance of each concentration was measured at 410 nm by using UV-VIS spectrophotometer and 2 mL of 0.1 M Tris HCl buffer was used as blank reading of the standard curve.

The selected colonies were grown in nutrient broth and incubated at 50°C for several days. The cultures then were centrifuged at 8,000 rpm for 20 minutes to separate the cells and extracellular enzymes. The substrate solution that contained two mixtures of pNP in 10 ml of isopropanol and gum Arabic dissolved in 50 mM Tris-HCl buffer, were freshly prepared in a fully dissolved form. An amount of 1 ml of extracellular enzyme solution was mixed with 9 ml of substrate solution in a test tube and the mixture was incubated for 15 minutes at 50°C. The absorbance of mixture then was measured at 410 nm by using UV-VIS spectrophotometer. The amount of free fatty acid released in each sample was determined using the prepared standard curve which one unit of enzyme (U) was defined as 1 μ M p-nitrophenol enzymatically released per minute/millilitre (min/mL). Hence, the result for the measurement of secreted lipase is interpreted as one unit of lipase (U) equal to 1 μ M of free fatty acid released per min/mL during incubation temperature.

2.4 Molecular Identification of Bacteria

The molecular identification of the bacteria was done through 16S rRNA analysis. The positive isolates that yield the highest amount of lipase enzyme were first cultured overnight in nutrient broth at 50°C. DNA extraction using genomic DNA extraction kit (Trans) following the normal procedure of cell lysis, proteinase K and RNase A degradation, washing and elution. DNA amplification by polymerase chain reaction (PCR) was carried out by

using Solis Biodyne FIREPol® DNA Polymerase with the following components (FIREPol® DNA Polymerase(0.05U/μl), Buffer B (1x), MgCl₂ (2.5mM), dNTP (200μM), 27F (0.1μM), 1492R (0.1μM) and DNA template (0.1-10ng/μl)] following cycles as presented in Table 1. Gel electrophoresis (1.0% w/v agarose) of PCR products were done first before furthering to sequencing process. For the final step, which was 16S rRNA gene sequencing, the amplified bacterial DNA was purified by using the QIAquick PCR Purification Kit. Then, the purified DNA were observed through electrophoresis process and securely stored at -20°C before being sent for sequencing process. The sequenced bacterial DNA then was aligned using ClustalW (software MEGA X 10.1 version), followed by Basic Local Alignment Search Tool (BLAST) analysis for bacterial species identification.

Table 1 Cycles involved in polymerase chain reaction (PCR)

Stages	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	-
Annealing	56	30 sec	34
Elongation	72	1 min	34
Final elongation	72	5 min	1
Holding	4	∞	-

3. Results and Discussion

3.1 Characterization of Best Thermophilic Isolates

The best growing isolated thermophilic bacteria from two hot springs which were Ulu Slim Hot Spring (labelled as "US") and Ara Panjang Hot Spring (labelled as "M"), were incubated at optimum growth temperature of 55°C. Each isolates underwent Gram staining to identify the type of bacterial species. Based on Fig. 2, both isolates were identified as Gram-positive bacteria with rod-shaped or bacilli. The characterization for isolate US is aligned with a study by [12] that recorded the Bacillus as one of the most abundant genera among thermophilic bacteria found at Ulu Slim Hot Spring. While for isolate M, there is no study that intensively researched and recorded regarding population of thermophilic bacteria at Ara Panjang Hot Spring hence this new finding could be the first to indicate the identification of thermophilic bacteria.

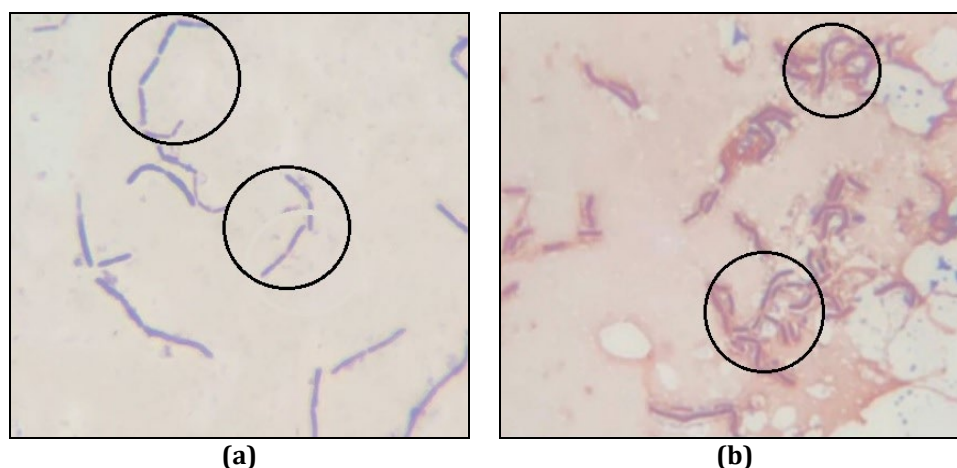


Fig. 2 Microscopic characterization of isolated thermophilic bacteria for (a) Isolate US from Ulu Slim Hot Spring; and (b) Isolate M from Ara Panjang Hot Spring

3.2 Screening of Lipolytic Activity by Thermophilic Bacteria

One of the ways to determine successful growth of lipolytic bacteria is Rhodamine B plate assay where the production of orange fluorescent halos within or around the bacterial colonies, that will be assessed under UV light. The orange fluorescent generated from the formation of complex form between rhodamine and the free fatty acids that produced by the isolated bacteria through hydrolyzation process which significantly become indicator for the presence of hydrolase enzyme [18]. Fig. 3 shows that the successful characterization of lipolytic thermophiles of both isolate US and M producing orange fluorescent colonies on Rhodamine B plates when incubated at 55°C after 5 days of incubation. Both isolates showed similar type of enzyme production as they

produced intracellular lipase enzyme where there is no halos area detected within the intense orange color of streaked area.

The types of lipase enzyme production can be distinguished through presence of halos surrounding the colonies (intracellular) or within the colonies (extracellular). Generally extracellular enzyme or exo-enzymes are synthesized in the bacterial cell but secreted to outside of the cell for external biochemical reactions. In contrast, intracellular enzyme or endo-enzymes are synthesized and retained within the cell for internal cellular reaction. Another study led by [19] reported similar results which orange fluorescent colonies on Rhodamine B agar were produced by lipolytic thermophilic bacteria isolated from Selangor hot springs.

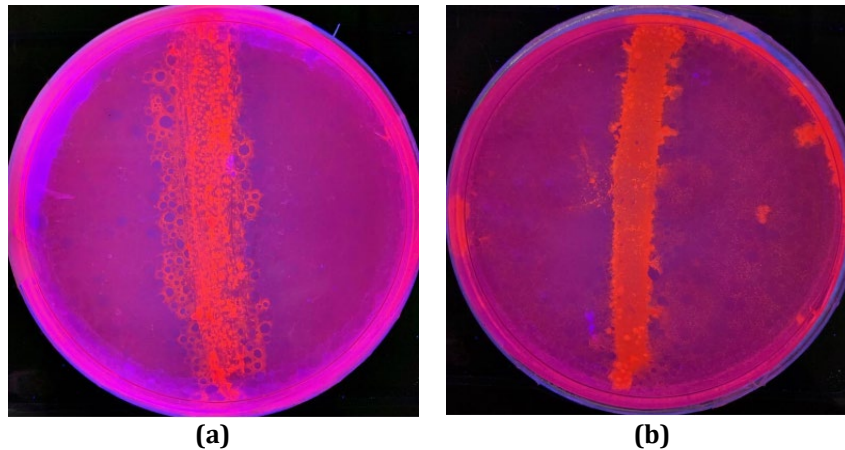


Fig. 3 Macroscopic view of isolated thermophilic bacteria on Rhodamine B agar plate after 5 days of incubation: (a) Isolate US; and (b) Isolate M

3.3 Lipase Assay

A standard curve of 2000 μM to 20000 μM of p-nitrophenol (pNP) solution was prepared to determine the amount of lipase produced by each sample. Standard curve will help to estimate the unknown concentration of enzyme produced by comparing them to the standard value of known concentration. pNP is a chemical compound commonly used for analytical determination of esterases and lipases [20]. The absorbance (OD reading) of each concentration was measured three time or in triplicate by using UV-VIS spectrophotometer, before calculating triplicates as average. The linear equation ($y = mx + c$) was obtained through the construction linear graph (Fig. 4) which was used to calculate the amount of lipase produced by the isolates. The standard curve also produced R2 coefficient with the value is 0.968.

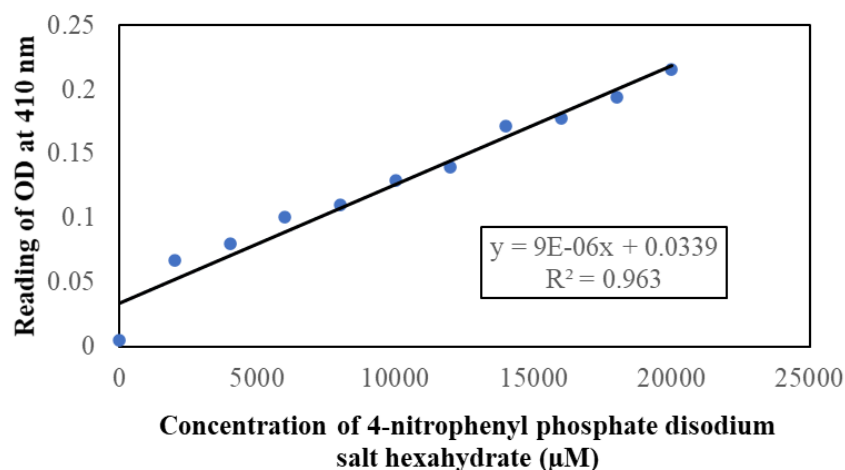


Fig. 4 Standard curve for different concentration of 4-nitrophenyl standard

Lipase from *Candida cylindracea* (Nacalai Tesque Co.,Japan) was used as positive control to indicate the optimum production of lipase from microorganisms. The lipase activity from each isolate in per minute was calculated by dividing the calculated concentration value with incubation time which was 15 minutes at 55°C. One unit (U) of lipase activity equal to one micromolar (μM) of free fatty acid liberated per minute per milliliter (mL) during incubation time. Table 2 indicated that isolate M produced the highest amount of lipase with value 444.43 U/mL while isolate US produced the lowest amount of lipase with the value 368.15 U/mL. The amount of

lipase produced by isolate M is even higher when compared with the lipase production from positive control (*Candida cylindracea*) which is 372.59 U/mL.

Table 2 Quantitative assay of lipase production

Sources of lipase	OD reading	Concentration (uM)	Lipase activity (U/mL)
<i>Candida cylindracea</i>	0.0842	5588.89	372.59
US	0.0836	5522.22	368.15
M	0.0939	6666.67	444.43

3.4 Bacterial Identification Through Molecular Method

The DNA from isolates US and M first were extracted according to protocol (Gram-positive bacteria) that prepared by EasyPure Genomic DNA Kit. After the extraction process, the DNA from isolates US and M were amplified using universal primers which are 27F (forward primer) and 1492R (reverse primer) through Polymerase Chain Reaction (PCR). Then, the PCR products were subjected to the DNA purification method to obtain the pure form of amplified DNA of these two isolates for the sequencing process. Fig. 5 showed that the size of amplicons for isolate US and M after purification process, is in the range between 1,500 bp to 2,000 bp. According to [21], the expected size of amplicon when using the universal primers will be ~1,500 bp as the amplification process will span nearly full-length of 16S rRNA gene.

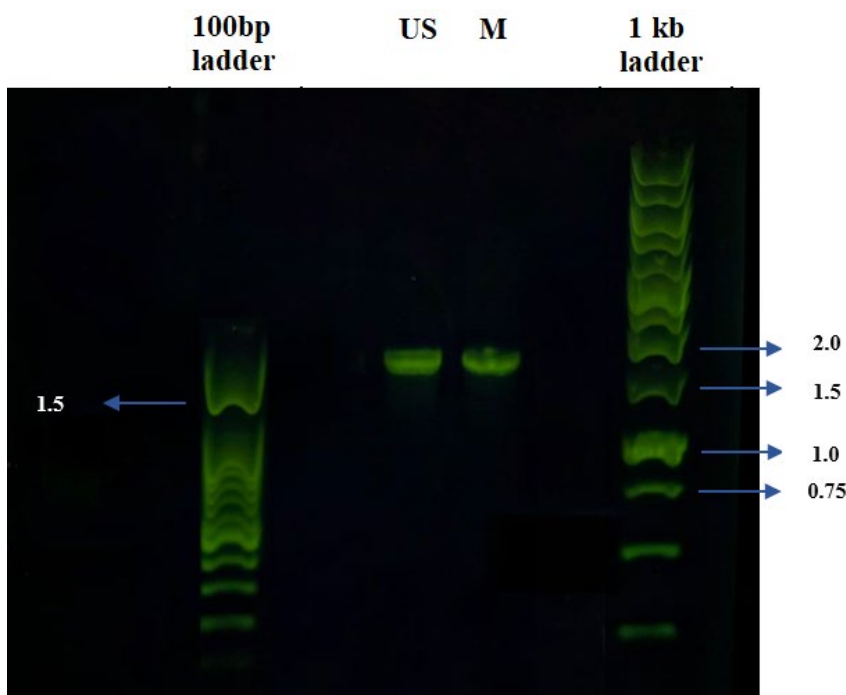


Fig. 5 Electrophoresis of DNA purification of two isolates (M and US) on agarose gel (1.0%). Two ladders were used: right lane is 1 kb DNA ladder and left lane is 100 bp DNA ladder

After sequencing, the sequences were aligned using ClustalW alignment (software MEGA X 10.1 version) and proceeded to Basic Local Alignment Search Tool (BLAST) analysis for bacterial species identification. Table 3 shows that each isolate had matched with a few selected species recorded in GenBank in NCBI (National Center of Biotechnology Information). According to [22], the similarity levels are accepted at 95% for genus and 97% for species. On the other hand, query cover is simply defined as the percentage of sequence length that is included in alignment to the sequence database from the Genbank, which means the higher percentage of query cover will yield accurate match to the desired sequence [23].

The sequencing analysis revealed that isolate US has the highest similarity to *Bacillus pumilus* strain CAF4 with 98.77% homology and 100% of query cover. This result is supported by a study conducted by [15] which the thermophiles isolated from hot regions of Iran was identified as *B. pumilus* and it was characterized as lipolytic thermophilic bacteria after a few of assays were performed. Some strains of *B. pumilus* are non-pathogenic soil microorganism and commonly regarded as one of the smallest true lipases [15]. Another

research study also indicated that *B. pumilus* capable in producing thermostable lipase through reactivity towards olive oil emulsion under alkaline condition [24].

While for the isolate M, the analysis shows that the aligned sequence has the highest similarity to *Bacillus aerius* strain 24K with 98.94% homology and 99% of query cover. This finding corroborates a study demonstrated by [25] where *B. aerius* as one of thermophiles isolated from the soil of hot spring, had shown almost similar characteristics with isolate M as it produced extracellular, thermostable and alkaline lipase. In addition, *B. aerius* had been extensively used in numerous studies that utilized the bacterial thermostable lipase for the synthesis of biodiesel [26], industrial organic solvent like ethyl ferulate [25], isoamyl acetate [27] and industrial enzyme for textile industry [28].

Table 3 16S rRNA bacterial identification analysis of samples (US and M) using BLAST

Sample Isolate	16S Rrna Identification	Similarity (%)	Query Cover (%)	Accession Number
US	<i>Bacillus pumilus</i> strain CAF4	98.77	100	KY806224.1
	<i>Bacillus aerophilus</i> strain HQB231	98.84	99	KT758430.1
	<i>Bacillus</i> sp. SP-1.8	98.70	100	KX390649.1
M	<i>Bacillus aerius</i> strain 24K	98.94	99	NR_118439.1
	<i>Bacillus altitudinis</i> strain 41KF2b	98.94	99	NR_042337.1
	<i>Bacillus aerophilus</i> 28K	98.88	99	NR_042339.1

4. Conclusion

The isolated thermophilic bacteria from two hot springs in Perak that labelled as isolate US (Ulu Slim Hot Spring) and isolate M (Ara Panjang Hot Spring), were confirmed to be *Bacillus pumilus* strain CAF4 and *Bacillus aerius* 24K, respectively. These two isolates were identified as Gram-positive, rod-shaped bacterium. They are also characterized as lipolytic thermophiles due to detection of orange fluorescence colonies on Rhodamine B agar. Based on the quantitative assay for lipase production, isolate US is a lower lipase producer with the lowest value of 368.15 U/mL while isolate M is a good lipase producer with the highest value of 444.43 U/mL.

As for the recommendation, additional sampling is required to gain more isolates for finding another potential lipolytic thermophiles and the other alternative enzymatic assay needs to be further applied to determine the capability of the isolates to produce lipases. Other than that, as isolate M is a good lipase producer, Ara Panjang Hot Spring can be further investigated to explore the diversity of culturable thermophiles.

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Conflict of Interest

Authors declare that there is no conflict of interest regarding the publication of the paper.

Author Contribution

The authors confirm contribution to the paper as follows: **study conception and design:** Author 2, Author 3, Author 4; **data collection:** Author 1; **analysis and interpretation of results:** Author 1, Author 2 Author 3 Author 4; **draft manuscript preparation:** Author 1, Author 2 All authors reviewed the results and approved the final version of the manuscript.

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