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New Lipase Producing β-proteobacteria Strains *Caldimonas* sp. and *Tepidimonas* sp. Isolated from a Malaysian Hot Springs

(Lipase Baharu yang Dihasilkan oleh β-proteobakteria *Caldimonas* sp. dan *Tepidimonas* sp. Dipencilkan daripada Kolam Air Panas di Malaysia)

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ABSTRACT

Microbial lipolytic enzymes have attracted considerable attention owing to their biotechnological potential. In this study, thermophilic bacteria producing lipase were isolated from Bentong and Sungai Lembing hot springs, in Pahang, Malaysia. Out of 25 colonies isolated, 14 samples showed to produce clear zones surrounding the growth on tributyrin and trioelin agar plates. All 14 isolates showed Gram-negative bacteria with short rod morphology. PCR amplification of 16S ribosomal DNA gene showed that these isolates were clustered with subclass β-proteobacteria consisting of thermophilic bacteria that produce lipase. Phylogenetic analysis was carried out with the highly similar species and 4 isolates (SglA1, BtnC1, BtnC2 and BtnC3) are related to genus Caldimonas *and 10 isolates (SglB1, SglB2, SglB3, SglB4, BtnB1, BtnB2 BtnD1, BtnD2, BtnD3 and BtnD4) belonged to genus* Tepidimonas. *These results indicated that novel lipase-producing thermophilic β-proteobacteria could be isolated from these hot springs.*

Keywords: β-proteobacteria; hot spring; slightly thermophilic; thermostable lipase

ABSTRAK

Enzim lipolisis daripada mikrob telah menerima banyak perhatian kerana potensinya dalam bidang bioteknologi. Dalam kajian ini, bakteria termofili yang menghasilkan lipase telah dipencilkan daripada kolam air panas Bentong dan Sungai Lembing di Pahang, Malaysia. Daripada 25 sampel pencilan, sebanyak 14 pencilan telah menunjukkan kehadiran zon-zon cerah sekitar koloni yang hidup di atas plat-plat agar tributirin dan triolein. Kesemua pencilan ini menunjukkan kehadiran bakteria Gram negatif yang memiliki morfologi rod pendek. Hasil amplifikasi DNA secara PCR pada gen 16S ribosom, pencilan ini didapati tergolong dalam kumpulan β-proteobakteria, yang menghasilkan enzim lipase. Hasil analisis filogenetik menunjukkan 4 pencilan (SglA1, BtnC1, BtnC2 dan BtnC3) tergolong dalam genus Caldimonas *dan 10 lagi pencilan (SglB1, SglB2, SglB3, SglB4, BtnB1, BtnB2 BtnD1, BtnD2, BtnD3 dan BtnD4) adalah daripada genus* Tepdimonas. *Hasil kajian ini menunjukkan β-proteobakteria yang termofili menghasilkan lipase yang agak baharu telah dipencilkan daripada kolam-kolam air panas ini.*

Kata kunci: β-proteobakteria; kolam air panas; lipase; sedikit termofili; termostabil

INTRODUCTION

Lipases (EC 3.1.1.3) catalyzing the hydrolysis of triglycerides at the oil-water interface. As they are produced from various sources including animals, plants and microorganisms, most commercial lipases are from microbial source (Verma et al. 2012). The enormous potential of microbial lipases arise from the facts that they are stable and active in organic solvents, do not require cofactors, exhibit a high degree of enantio- and regio-selectivity and possess a wide range of specificity for the conversion of various unnatural substrates (Nakatani et al. 1992; Verma et al. 2012). A large number of beneficial thermophiles which produced lipases with good thermal stabilities have been found in diverse habitat (Goswami et al. 2013). Thermostable lipases play an important role in commercial applications because of their overall inherent stability (Chauhan & Garlapati 2013; Demirjian et al. 2001).

Hot springs have been recognized as an important source of beneficial thermophilic bacteria capable of producing thermostable enzymes (Panigrahi et al. 2014; Tirawongsaroj et al. 2008). A thermophile is an organism that thrives at relatively high temperatures, between 45 and 80°C (Madigan et al. 2013). Study on such organisms will improve our understanding on the survival and adaptation strategies in extreme environments, and provide novel enzymes that are active and stable at high temperatures (Bouraoui et al. 2010; de Champdore et al. 2007; Kublanov et al. 2009; Podar & Reysenbach 2006). Within thermophilic microrganisms, there are also rare groups which can grow optimally at 45-60°C and they are unable to grow at either lower or higher than these temperatures. These group of thermophilic organism are sometimes referred to as 'slightly thermophilic' or 'moderately thermophilic', to distinguish them from normal thermophiles (Albuquerque et al. 2011, 2006).

Since these microorganisms are rare and diverse, the enzymes they produce are less explored and characterised. For instance, thermophilic microorganism from genera *Caldimonas* and *Tepidimonas* are motile via flagella and rod-shape curve (Brenner et al. 2005), and they were isolated from soil, mud or water, in natural and industrial environments (Willems et al. 1991). Genus *Caldimonas* includes three species such as *Caldimonas manganoxidans* HST, discovered at Japanese hot spring (Takeda et al. 2002). *Caldimonas taiwanensis* strain On1 was isolated from a hot spring at Pingtung in the southern Taiwan (Chen et al. 2005); and *Caldimonas hydrothermale* strain HAN-85T was isolated at oasis thermal spring near Tozeur, in southwest Tunisia (Bouraoui et al. 2010). The genus *Tepidomonas* includes five slightly thermophilic species (Albuquerque et al. 2006; Chen et al. 2006; Freitas et al. 2003; Ko et al. 2005; Moreira et al. 2000). *Tepidimonas ignava* was firstly described from the hot spring at São Pedro do Sul in central Portugal and is a chemolithoheterotrophic (Moreira et al. 2000). *Tepidimonas aquatica* was isolated from a hot water tank in Coimbra (Freitas et al. 2003). *Tepidimonas arfidensis* which is thermophilic bacterium isolated from the bone marrow of a patient with leukemia in Korea (Ko et al. 2005). Meanwhile, *Tepidimonas taiwanensis* which is an alkaline-protease producing thermophilic bacterium was isolated from a Pingtung hot spring, Taiwan (Chen et al. 2006). Lastly, *Tepidimonas thermarum* was isolated from the Elisenquelle in Aachen, Germany (Albuquerque et al. 2006).

This work reports on the isolation, screening and characterization of novel lipase-producing thermophilic bacteria from Sungai Lembing and Bentong Hot springs, in the state of Pahang, Malaysia. While many other sampling works at different hot springs around Malaysia had reported on the isolation of thermophilic bacterium from other genera such as *Bacillus* and *Pseudomonas*, we reported on the presence of rare lipase peroducing β-proteobacteria from two genera, i.e., *Caldimonas* sp. and *Tepidimonas* sp. at these two hot springs. Moreover, slightly thermophilic protein could possess structural feature that is distinguishable from the mesophilic and thermophilic counterparts (Szilágyi & Závodszky 2000). Therefore, further characterization on the lipases from these microorganisms will enhance understanding on the nature; and the possible use of lipases from these microorganisms.

Materials and Methods

Site and Sampling

Sampling was carried out at two geothermal sites in Pahang, Bentong Hot Spring and Sungai Lembing Hot Spring. The water samples were aseptically collected using 250 mL sterile high density polyethylene (HDPE) bottle from the hot water source and reservoir lying next to the source. The temperatures and pH ranges of the sampling points at the two hot springs were between 37 to 50°C and 7 to 8, respectively. The bottles were put in sterile zip-lock bag and kept in ice-cooled storage box. There are 6 sampling points at Bentong Hot Spring and 2 sampling points at Sungai Lembing Hot Spring.

Media for Growth, Cultivation and Screening

The medium for growth and cultivation contained (for broths) 1g Lab-Lemco powder, 2 g yeast extract, 5 g peptone and 5 g NaCl in 1 L distilled water. About 15 g agar was added to the same nutrient broth to make for nutrient agar (NA). Tributyrin agar contained all common components used in NA with 1% tributyrin added. Triolein agar plate contained similar component as NA with the addition of triolein (at 0.25%) and Victoria blue dye (at 0.01%). Mixtures were homogenized and autoclaved before poured on plates. Water samples from hot springs were diluted with distilled water and spread onto nutrient agar plates and incubated at 45°C for 48 h. The single colonies formed were re-streaked again on NA tributyrin and triolein agar plates and incubated for 48 h at 45°C. Positive lipase-producing bacteria will be indicated by clear or 'halo' zones surroundings the colonies on tributyrin agar (Chaturvedi et al. 2010) and a blue colouration on triolein agar. Isolates showing positive results from both plates were sub-cultured, kept as glycerol stock and subjected to further analysis. The API 20E kit rapid identification system (Biomeriux, France) was used for biochemical characterization of the isolates.

Amplification of 16S Ribosomal DNA Gene

Genomic DNA was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis). PCR reaction was performed using a pair of universal 16S ribosomal RNA primer (forward primer: rRNA_F 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer rRNA_R 5'-AAG GAG GTG ATC CAG CCG CA-3') (Hutson et al. 1993). Amplification volume of 50 µl of PCR reaction mixture contained 2 µl of template DNA (≈100 ng), 25.0 µl of Taq 2X Master Mix (Biolabs), 1.5 µl of each forward and reverse primer (10 pmol/µl). Amplification was carried out using the thermocycler (Mastercycle, Germany) with initial denaturation at 94°C for 4 min, 30 cycles of amplification including 1 min at 94°C (denaturation), 1 min at 58°C (annealing) and 1 min at 72°C (extension) followed by final extention of 7 min at 72°C. The amplicons were observed by running on 1% agarose, viewed under UV light and photographed using Gel Documenter (AlphaImager TM 2200). The purified amplicons were sent to DNA sequencing agency (1st base Laboratory Sd. Bhd. Malaysia). The sequences were edited and analyzed using software Sequence Scanner (version 1.0); and the sequence copies were deposited into NCBI Genbank database using an on-line gene submission tool (Bankit), and each sequence was assigned with an accession number; Sg1A1 (KC906245), SglB1 (KP334276), SglB2

(KC906247), SglB3 (KC906248), Sg1B4 (KP334277), BtnB1 (KC906246), BtnB2 (KP334278), BtnC1 (KC906250), BtnC2 (KP334279), BtnC3 (KP334280), BtnD1 (KP334281), BtnD2 (KC906249), BtnD3 (KP334282) and BtnD4 (KP334283); (Table 2). On-line BLASTN tool was used for similarity searching available at NCBI website http//:www.ncbi.nlm.nih.gov. Organisms that showed high similarity with the sequence sample were collected from NCBI for phylogenetic analysis. Using *E. coli* as an outgroup, a phylogenetic tree was computed using the Neighbor-Joining method using MEGA 2.2 software (Tamura et al. 2007).

Results and Discussion

Following incubation at 45°C for 48 h, only 14 isolates were able to produce lipase that hydrolyzes both tributyrin triolein oil. Lipases break down tributyrin, a triglyceride, resulted in the presence of clear or 'halo' zones surrounding the growth. The ability to hydrolyse triolein oil was shown by blue colouration surrounding colonies. Sample SglA1, SglB1, SglB2, SglB3, SglB4, BtnB1, BtnB2, BtnC1, BtnC2, BtnC3, BtnD1, BtnD2, BtnD3 and BtnD4 showing positive lipase tests were subjected to further analysis. These results are summarized in Table 1.

All 14 isolates (SglA1, SglB1, SglB2, SglB3, SglB4, BtnB1, BtnB2, BtnC1, BtnC2, BtnC3, BtnD1, BtnD2, BtnD3 and BtnD4) were found to be Gram negative with short rod morphology. However, all samples failed to be identified using API 20E identification system, as these strains were not included in the system database (Popovic et al. 2007). Amplification using genomic DNA extracts from all samples produced product bands with the expected size of 1.5 kb (Figure 1). The rDNA sequences generated from the sequencing result subjected to BLASTN similarity searches. Table 2 enlists the species hit from NCBI GenBank showing sequences with the highest similarity with the rDNA sequence copy from all of these samples. Since all samples showed similarity of at least 97%, these were regarded as similar species (Stackebrandt and Goebel, 1994)1994. Sample SglA1, BtnC1, BtnC2 and BtnC3 showed to have the highest similarity with *Caldimonas hydrothermale* strain Han-85T (Bouraoui et al. 2010). This is a Gram-negative thermophilic bacterium isolated from thermal water in natural thermal spring at Tozeur, an oasis in southwest Tunisia. This strain was shown to be non-pigmented, round shape with entire edge, with growth temperatures ranging from 30 to 60°C with an optimum at 55°C and pH7.0. It produced C8 and C14 lipases.

Meanwhile, the Sample SglB1, SglB2, SglB3, SglB4, BtnB1 and BtnB2 have the highest similarity with *Tepidimonas arfidensis*. It was reported that *Tepidimonas arfidensis* is a novel Gram-negative *Bacillus* bacterium unexpectly isolated from the bone marrow

TABLE 1. The number of isolate from hot spring site (stations) which show lipotytic activities

Stations	No. of isolates	No. of isolates with positive on tribytyrin and triolein agar plates
SglA	2	1
SglB	4	4
BtnA	4	0
BtnB	3	2
BtnC	4	3
BtnD	4	4
BtnE	0	0
BtnF	4	0
Total	25	14

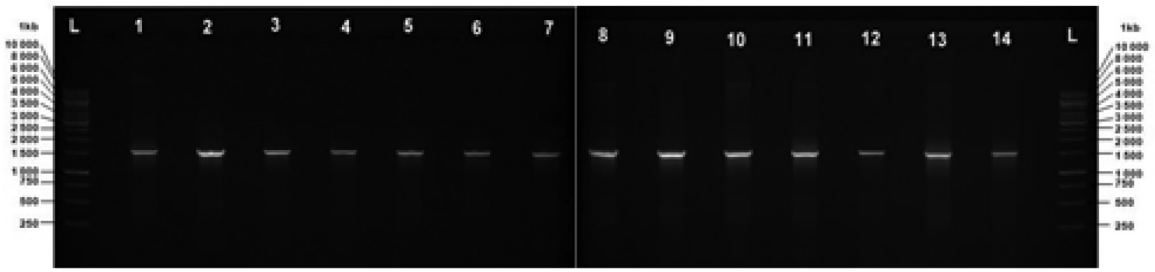

FIGURE 1. PCR amplification for 16S ribosomal DNA gene using universal primers on extracted genomic DNA template from different samples: Lane L: 1kb Ladder, Lane 1: sample SglA1, Lane 2: sample SglB1, Lane 3: sample SglB2, Lane 4: sample SglB3, Lane 5: sample SglB4, Lane 6: sample BtnB1, Lane 7: sample BtnB2, Lane 8: sample BtnC1, Lane 9: sample BtnC2, Lane 10: sample BtnC3, Lane 11: sample BtnD1, Lane 12: sample BtnD2, Lane 13: sample BtnD3, Lane 14: sample BtnD4

TABLE 2. The highest hit in sequence similarity with rDNA sequence copy from thermophilic lipase-producing isolated from Sungai Lembing and Bentong Hot Spring, Pahang, Malaysia. The Genbank IDs for all of the submitted sequences are indicated. Each hit's organism was indicated with maximum identity, with an accession number (in parentheses)

Samples	Genbank ID	Organism	Max. Identity (%)
SglA1	KC906245	*Caldimonas hydrothermale* type strain Han-85T (AM283038.1)	97
SglB1	KP334276	*Tepidimonas arfidensis* (AY594193.1)	99
SglB2	KC906247	*Tepidimonas arfidensis* (AY594193.1)	99
SglB3	KC906248	*Tepidimonas arfidensis* (AY594193.1)	99
SglB4	KP334277	*Tepidimonas arfidensis* (AY594193.1)	98
BtnB1	KC906246	*Tepidimonas arfidensis* (AY594193.1)	99
BtnB2	KP334278	*Tepidimonas arfidensis* (AY594193.1)	98
BtnC1	KC906250	*Caldimonas hydrothermale* type strain Han-85T (AM283038.1)	99
BtnC2	KP334279	*Caldimonas hydrothermale* type strain Han-85T (AM283038.1)	99
BtnC3	KP334280	*Caldimonas hydrothermale* type strain Han-85T (AM283038.1)	99
BtnD1	KP334281	*Tepidimonas taiwanensis* strain I1-1 (AY845054.1)	98
BtnD2	KC906249	*Tepidimonas taiwanensis* strain I1-1 (AY845054.1)	99
BtnD3	KP334282	*Tepidimonas taiwanensis* strain I1-1 (AY845054.1)	99
BtnD4	KP334283	*Tepidimonas taiwanensis* strain I1-1 (AY845054.1)	99

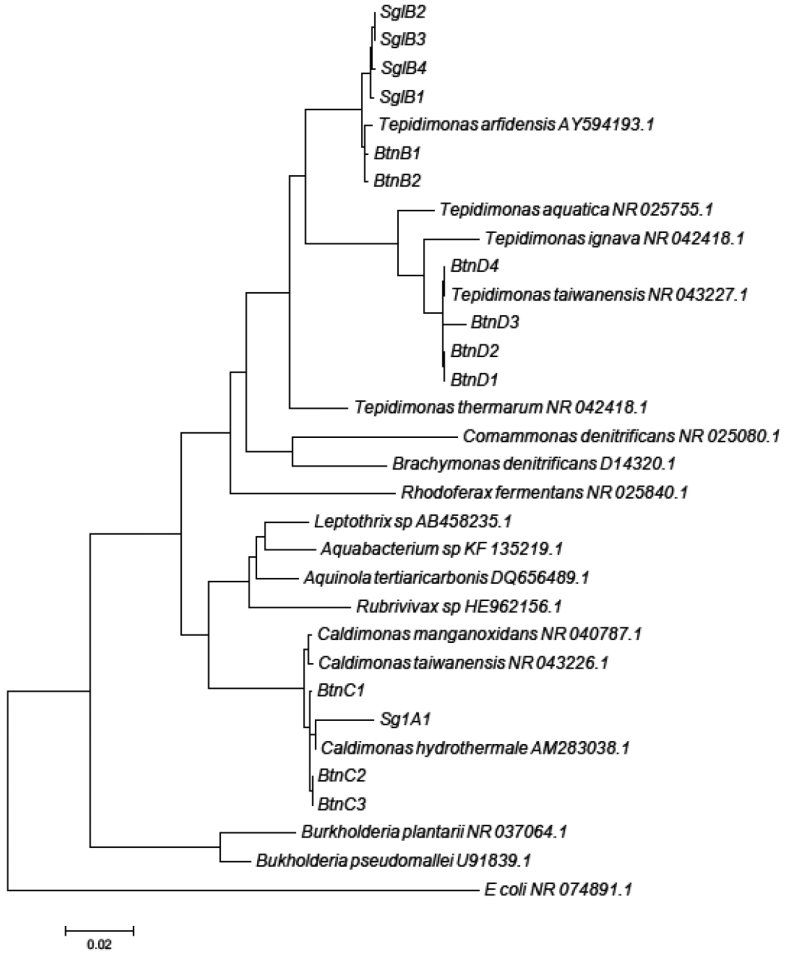

FIGURE 2. Ribosomal rRNA gene sequence copies from the 14 isolates (SglA1, SglB1, SglB2, SglB3, SglB4, BtnB1, BtnC1 BtnC2 BtnC3BtnB2 BtnD1, BtnD2, BtnD3 and BtnD4) and other rRNA sequences derived from NCBI database (with accession numbers) were used to construct phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method (Tamura et al. 2007). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The *E. coli* strain was used as an outgroup. The evolutionary distances were computed using the Maximum Composite Likelihood method (Saitou & Nei 1987) and Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2007)

of a leukemic patient in Korea (Ko et al. 2005). Being slightly thermophilic with optimum temperature of around 50°C, its isolation from the bone marrow of leukemia patient was unexpected. Sample BtnD1, BtnD2, BtnD3 and BtnD4 possess highest sequence similarity with *Tepidimonas taiwanensis* strain I1-1, isolated from hot spring located in Pingtung area, southern Taiwan (Chen et al. 2006). This strain was reported to be Gram-negative rods, with optimum temperature and pH being 55°C and 7.0, respectively. This novel strain produced alkaline-protease as well as C8 lipase and C14 lipases. Similarly, all *Caldimonas* sp. and *Tepidomonas* sp. strains isolated in this work produce lipases.

As shown in Figure 2, a phylogenetic tree was constructed in which most branches gave bootstrap values above 50%. Obviously, β-proteobacteria subclass is under Burkholderiales order that cluster together members from the genera *Caldimonas*, *Schlegelella, Leptothrix* and *Tepidimonas*. Isolate BtnB1, BtnB2, Sg1B1, Sg1B2, Sg1B3 and Sg1B4 were clustered with *Tepidimonas arfidensis* in a branch that radiates with *Tepidimonas thermarum* and *Tepidimonas ignava*. Chen et al. (2006) reported that *Tepidimonas taiwanensis* was related to *Tepidimonas ignava* strain SPS-1037 (Moreira et al. 2000) and *Tepidimonas aquatica* strain CLN-1 (Freitas et al. 2003). Meanwhile, isolate Sg1A1 BtnC1, BtnC2 and BtnC3 are clustered with a branch that has members from genus *Caldimonas*; namely *Caldimonas hydrothermale, Caldimonas manganoxidans* and *Caldimonas taiwanensis*. The results of DNA-DNA hybridization, fatty acids profile, DNA G+C content, physiological tests and biochemical analyses have allowed the genotypic and phenotypic differentiation of the *Caldimonas hydrothermale* strain Han-85T from other *Caldimonas* species, suggesting it as a novel species (Bouraoui et al. 2010).

Conclusion

Based on lipolysis on tributyrin agar plate, several new lipase-producing thermophilic bacteria were isolated from local hot springs near Bentong and Sungai Lembing in the State of Pahang, Malaysia. These isolates were slightly thermophilic, Gram-negative and short rod in morphology. They were further characterized by rDNA sequence identification. The rDNA sequencing showed the majority of these isolates belong to other reported novel species of β-proteobacteria, whereby 4 of these isolates were similar with *Caldimonas hydrothermale* and 10 isolates were with genus *Tepidimonas* sp. Despite of having identified the strains from these two hot springs are from genera *Caldimonas* and *Tepidimonas*, future characterization on DNA G+C content and fatty acid profile were required in order to fully distinguish further the strain of these species. Nevertheless, the screening at Pahang Hot spring had however added Malaysia as another collection of geographical distribution of these novel species previously reported elsewhere such as in Japan, Taiwan, Tunisia, Portugal and Germany. In addition to many thermophilic lipases from other genera, lipases produced from these slightly thermophilic species can be subjected to future studies for enhanced application in industries.

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