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Full Length Research Paper

Identification of lipase producing thermophilic bacteria from Malaysian hot springs

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Lipase enzyme is an important group of biocatalyst for biotechnological application. One of the major industrial microorganism is the *Bacillus* species which are in abundance in the hot springs. The aim of this research was to isolate and characterize lipase producing bacteria from five hot springs in Malaysia. Eight bacterial strains from five hot springs were characterized by both biochemical and molecular techniques. Four strains were lipase producing thermophilic bacteria (coded as A1, A3, A4 and A14). The presence of lipase enzyme was confirmed by an orange fluorescence halos around the bacteria in rhodamine B olive oil agar plate. Based on molecular analysis, A1, A3 and A4 were assigned to the *Bacillus* and *Geobacillus* sp., and isolate A14 is found to be a nucleoside permease gene from *Anoxybacillus flavithermus* WK1.

Key words: Lipase enzyme, rhodamine B olive oil, polymerase chain reaction (PCR), hot springs, thermophilic bacteria.

INTRODUCTION

Thermophilic bacteria is an important source of heat stable enzyme (Rahman et al., 2007; Turner et al., 2007) and geothermal environment such as hot springs are usually their favorable habitats (Haki and Rakshit, 2003). In Malaysia, 45 hot springs were described earlier by Samsudin et al. (1997) and later an additional 15 hot springs were discovered (Sum et al., 2010). Several local hot springs have been explored their potential as a source of thermophillic bacteria (Akaya and Kivanc, 2008; Anutrakunchai et al., 2008). Thermophilic organisms are defined as those that have their optimal growth temperature between 50 and 80°C (Brown, 2005). Potential enzymes such as amylase, protease, DNA polymerase, xylanases and chitinases have been identified in thermophilic microorganisms including the lipase enzyme (Dominguez et al., 2005).

Lipases have been observed in *Bacillus prodigiosus, B. pyocyaneus* and *B. fluorescens* (Hasan et al., 2006) with specific examples like *Bacillus* strain A30-1 (ATCC 53841) from Yellow Stone Park (Wang et al., 1995), *Geobacillus* sp.TW1 in China (Li and Zhang, 2005), and *Bacillus thermoglucosidasius* and *Bacillus coagulans* from Setapak hot spring in Malaysia (Sheikh Abdul Hamid et al., 2003).

Due to the fact that lipase are quite stable and active in organic solvents, these enzyme have received great interests for use in various industries like petrochemical, waste management and food (Sheikh Abdul Hamid et al., 2003; Dominguez et al., 2005). Other applications include the pharmaceutical, dairy, detergent, cosmetic, oleochemical, fat-processing, leather, textile and paper industries (Sharma et al., 2001). In this current study, biochemical tests, polymerase chain reaction (PCR) and sequencing methods were used to identify the bacteria that were isolated from Gadek, Labis, Pedas, Sg. Klah

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Table 1. Characteristics of thermophiles from five different hotsprings in Malaysia.

Culture	Location of hot spring*	Temperature of hot springs*	pH*	Rhodamine olive oil agar plate test (presence of halos)	Lipase gene detection by PCR method
A1	Gadek	56	7.1	+	+
A3	Gadek	56	7.1	+	+
A4	Labis	45	7.7	+	+
A5	Pedas	48	6.8	-	ND
A6	Sg. Klah	94.5	7.4	-	ND
A8	Sg. Klah	50	7.4	-	ND
A13	Selayang	55	7.0	-	ND
A14	Selayang	55	7.0	+	+

ND: not done. *data taken from Siti Aisyah, 2009

and Selayang hot springs in Malaysia.

MATERIALS AND METHODS

Sample collection and sampling sites

Water samples were taken from various locations and these bacteria were confirmed as thermophiles. All the cultures were coded as A1 to A14 and were maintained on Castenholz TYE medium (Table 1).

Screening for lipase-producing bacteria

Screening was carried out using Rhodamine B-olive oil agar plate method (Sheikh Abdul Hamid et al., 2003) which was incubated at 60°C for 3 days. Presence of orange fluorescent halos around colonies under ultraviolet light indicated the presence of lipase producing bacteria. For negative control, a separate agar plate but without any bacteria were incubated in the same condition.

DNA extraction

DNA was extracted using the DNeasy Blood and Tissue kit (QIAGEN). Briefly, 200 µl PBS and 20 µl proteinase K were aliquoted into various eppendorf tubes and were centrifuged for 5 min. A total of 200 µl buffer AL were added, vortexed and incubated at 56°C for 10 min. Following this, 200 µl of absolute ethanol was added, vortexed and 400 µl of mixture were pipetted to the DNeasy spin column and was placed in 2 ml collection tube and centrifuged at 6000 g for 1min. Then, the collection tube was discarded and replaced with new ones. Another 500 µl buffer AW1 were added and centrifuged for 1 min at 6000 g, and into another new collection tube, 500 µl buffer AW2 was added, centrifuged at 20000 g for 3 min and finally, DNeasy column was used and 200 µl buffer AE were pipetted and incubated at room temperature for 1 min and was then centrifuged at 6000 g for 1 min. The elution was then kept at –40°C until further use.

Amplification of full-length thermostable lipase gene

The forward primer BTL-F (5'-GGC GGT GAT GGA ACG CTG CCA TGA-3') and reverse primer BTL-R (5'-CCG ACG ATA GAC TGG CGG ACA AAT G-3') were used (Rahman et al., 2007). PCR mixture contained DNA (1 µl), 2x *Taq* master mix (25 µl), 50 mM $MgCl_2$ (1 µl), nuclease free H_2O (21 µl) and 30 pmol BTL-F (1 µl), and 30 pmol BTL-R (1 µl). The pre-denaturation was carried out at 95°C for 3 min followed by 36 cycles PCR (20 s denaturation at 94°C, 20 s annealing at 56.4°C and 30 s extension at 72°C) and final extension step at 72°C for 5 min and hold at 4°C.The amplified products were electrophoresed on 1.5% agarose gel for 40 min at 95 V.

Nucleotide sequencing and BLAST analysis

The PCR products were sent to a private laboratory for sequencing. The sequences obtained were analysed using National Centre for Biotechnology International (NCBI) BLAST software. This software is available at the internet at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences in the FASTA form were aligned in this software. BLAST was used to search for similar sequence in the GenBank compared to query sequence.

Phylogenetic analysis

Reference sequence and query sequence were aligned in CLUSTAL W software to determine the similarities and differences between the sequences. Multiple sequence alignment (MSA) obtained in the CLUSTAL W software was then used to build the phylogenetic tree and the differences and similarities were calculated by the distance shown in the phylogram.

RESULTS AND DISCUSSION

Temperature of water from hot springs

The hot springs water temperature ranged from 45 to 94.5°C. From previous studies, more than sixty hot springs were discovered so far in Malaysia and most of these hot springs are less than 100°C at the surface (Sum et al., 2010). Those that are easily accessible and are in the vicinity of urban centers are now turned into recreational resorts with hotels, hot spas, and swimming pool. However, very few of these hot springs have been explored for thermophilic bacteria to further expand the

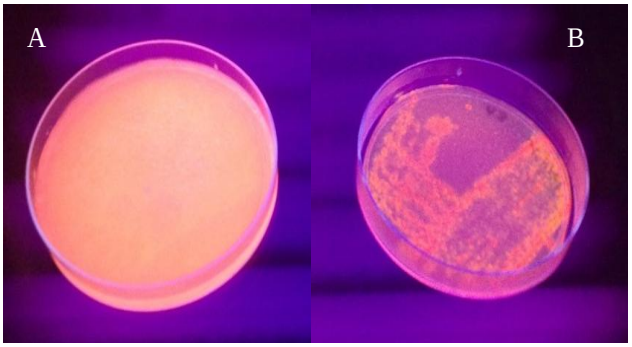

Figure 1. Detection of lipase enzyme on plates containing olive oil and incubated at 60°C for 3 days. A. Negative culture and B. Positive culture.

search for useful products.

Biochemical tests for lipase enzymes

In view of the variety of applications, there has been a renewed interest in the development of source of lipases. Bacteria, yeasts and moulds can produce lipase but the availability of lipase with specific characteristics is still a limiting factor (Kashmiri et al., 2006), thus this leads to the search for new lipases amongst the bacteria isolated from different water temperature.

For evidence of lipase enzymes, the colonies that appeared as pink colonies (Figure 1A) are considered negative, while those with lipase activities showed orange fluorescence around the bacteria colonies when the plates were irradiated under UV light at 350 nm (Figure 1B). The orange fluorescence colonies that can be visualized under UV light are due to fatty acids that are hydrolyzed by lipase enzyme which reacts with rhodamine B to form orange fluorescence colonies (Savitha et al., 2007). Based on this theory, it was found that four out of eight (A, A3, A4 and A14) were lipase enzyme producers (Table 1). The cultures were incubated at 45°C for 2 days, and in this current study the culture plates were incubated at 60°C for 3 days and it was noticed that the viable counts increased. The latter temperature was chosen based on the fact that A1, A3, A4 and A14 were present in the natural hot spring water temperature of 55 to 85°C with pH values ranging from 7.0 to 7.1 (Table 1). This study is still ongoing to further investigate the rate of growth, limiting factors, and enzyme production by these microorganisms.

Similar methods were used by other researchers to detect lipase producers: *Pseudomonas alcaligenes* F-l 11 (Lin, 1996), thermophiles in Yellowstone National Park (Wang et al., 1995) and *Anoxybacillus kamchatkensis* from Setapak hot spring (Sheikh Abdul Hamid et al., 2003). Other potential lipase enzyme producers from Malaysia include a new species *Geobacillus zalihae (*Rahman et al., 2007*)* isolated from palm oil mill effluent.

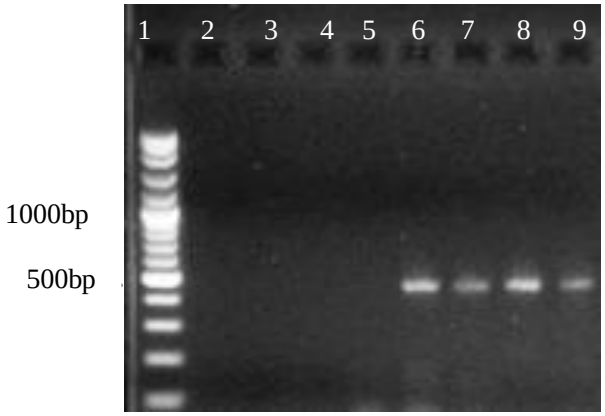

Figure 2. Amplification of thermostable lipase gene. Lane 1, 100 bp DNA Ladder (Vivantis Malaysia, VC100 bp) ; lane 2, isolate A5; lane 3, isolate A6; lane 4, isolate A8; lane 5, isolates A13; lane 6,isolate A1; lane 7, isolate A3; lane 8, isolate A4 and lane 9, isolate A14.

Amplification of lipase gene by PCR

Further analysis by amplification of lipase gene showed that four isolates, A1, A3, A4 and A14 (lanes 6, 7, 8, and 9, respectively showed the presence of 500 bp molecular weight band (Figure 2). This corresponded to the lipase gene, thus confirmed that they were lipase producers.

Phylogenetic studies

The nucleotide sequence of isolates A1, A3, A4 and A14 were analysed for similarity search in the GenBank (Table 2). Sequence analysis of these genes revealed that A1, A3 and A4 showed high similarity to *Geobacillus* and *Bacillus* sp. (98%). However, for strain A14, it showed 90% similarity to the nucleoside permease gene from *A. flavithermus* WK 1. The summary of similarities of test strain and representive strains are shown in Figure 3 using the neighbour joining method.

A. flavithermus WK1 has 8 operons which encodes for numerous protein and contain 16 pairs of proteins clustered in these operons (Saw et al., 2008). In this study, nucleoside permease from isolate A14 was amplified using the same primer, this may suggest that lipase gene and nucleoside permease genes of *A. flavithermus* WK1 were clustered in the same operon. Isolate A1, A3 and A4 were identified as lipase producing thermophilic bacteria and A14 is a nucleoside permease producer thermophilic bacteria. Similarity searches are often said to be software- dependent and by the length of the sequences being compared (Mignard and Flandrois, 2006). In contrast, phylogeny identifies bacteria on the basis of gene evolution, relationships and clustering, a major point in today's taxonomy. In addition, it is not clear how much total intraspecies variability should be allowed in the case of 16S rRNA gene alleles (Mignard and Flandrois, 2006). Phylogenetic and chemotaxonomic data

Table 2. Similarities of query sequence from isolate A1, A3, A4 and A14 between sequence deposited in GenBank.

Sequence from GenBank		Similarities of isolates A1, A3, A4 and A14 with sequence from GenBank			
Accession number	**Organism**	**A1 (%)**	**A3 (%)**	**A4 (%)**	**A14**
AY260764	*Geobacillus zalihae* strain T1 thermostable lipase gene	98	87	98	No similarity (NS)
CP002050	*Geobacillus* sp. C56-T3	98	87	98	NS
CP001794	*Geobacillus* sp. Y412MC61	97	86	97	NS
AF134840	*Bacillus thermoleovorans* lipase (ARA)	96	85	96	NS
X95309	*B. thermocatenulatus* triacylglycerol lipase gene	96	87	97	NS
U78785	*Bacillus stearothermophilus* lipase gene	95	86	96	-
BA000043	*Geobacillus kaustophilus* HTA426 DNA (lipase gene)	87	-	87	-
Aflv_2781	*Anoxybacillus flavithermus* WK1 (Nucleoside permease gene)	-	-	-	90%

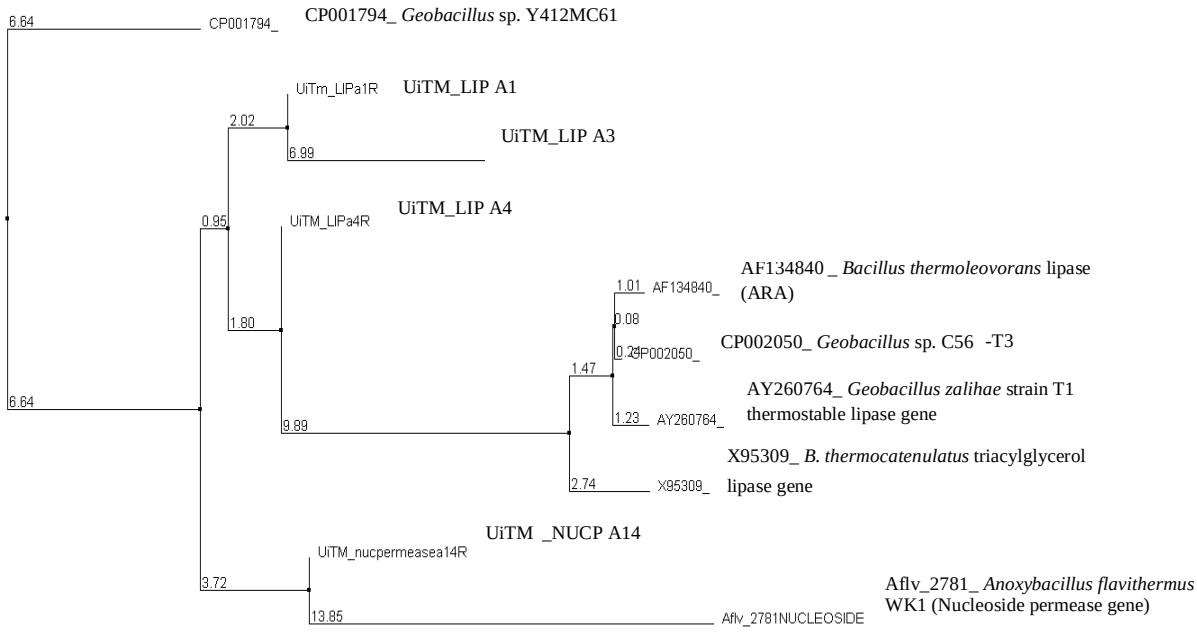

Figure 3. Phylogenetic relationship on the basis of lipase gene sequence analysis of isolate A1, A3, A4 and A14 and reference strain using neighbour joining method. In this figure isolates are re-labelled as A1 (UiTM LIP A1), A3 (UiTM LIP A3), A4 (UiTM LIP A4) and A14 (UiTM_NUCP A14).

with cultural, morphological and physiological characteristics should be reviewed together for strain identification (Banat et al., 2004). Construction of phylogenetic trees can be done using neighbor-joining (Saitou and Nei, 1987), maximum-likelihood and parsimony (Felstein, 1996) methods. Several cut-off similarity levels have been suggested, ranging from 97% for the genus level to 99% for the species level (Stackebrand and Goebel, 1994).

Conclusion

In this study, thermophilic bacteria with lipase enzyme were indentified from several hot springs. The hot spring diversity is large and that most microbiologists seek to isolate thermophiles from 'hot' environments seems to be worth since these environment have proved to be the home for bacteria with useful enzymes. Biochemical and molecular techniques can be used to identify both the bacteria and the enzymes being expressed. The presence of lipase enzyme from thermophiles can be exploited further. However, more work is clearly needed to elucidate the potential of this lipase enzyme.

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REFERENCES

Akaya SE, Kivanc M (2008). Isolation and identification of thermophilic bacteria species from hot springs. Abstracts J. Biotech., 136S: S607-S619.

Anutrakunchai CY, Trongpanich, Niamsanit S (2008). Isolation of vitamin B_6 producing thermophilic bacterium, *Geobacillus* sp. H6A. J. Biotech., 136S: s336.

Banat IM, Marchant R, Rahman TJ (2004). *Geobacillus debilis* sp. nov., a novel obligately thermophilic bacterium isolated from a cool soil environment, and reassignment of *Bacillus palliidus* to *Geobacillus pallidus* comb. nov. Inter. J. Syst. Evol. Micro., 54: 2197-2201.

Brown AE (2005). Benson's Microbiology Application: Laboratory Manual in General Microbiology. (9th Ed) New York: McGraw-Hill.

Dominguez A, Pastrana L, Longo MAS, Rúa ML, Sanroman MA (2005). Lipolytic enzyme production by *Thermus thermophilus* HB27 in a stirred tank bioreactor. Biochem. Eng. J., 26: 2-3.

Felstein J (1996). Inferring phylogeny from protein sequences by parsimony, distance and likelihood methods. Mtds. Enzymol., 266: 368-382.

Haki GD, Rakshit SK (2003). Development in industrially important thermostable enzyme: a review. Bioprocess Technol. Prog., 89: 17-34.

Hasan F, Shah AA, Hamed AA (2006). Industrial applications of microbial lipases. Enzyme Microbial. Technol., 39: 235–251.

Kashmiri MA, Adnan A, Butt BW (2006). Production, purification and partical characterization of lipase from *Trichoderma viride*. Afr. J. Biotech., 5(10): 878-882.

Li H, Zhang X (2005). Characterization of thermostable lipase from thermophilic *Geobacillus* sp. TW1. Prot. Expr. Purif., 42(1): 153-159.

Lin SF (1996). Production and stabilization of a solvent-tolerant alkaline lipase from Pseudomonas pseudoalcaligenes F-I 11. J. Ferment. Bioeng., 82(5): 448-451.

Mignard S, Flandrois JP (2006). 16S rRNA sequencing in routine bacterial identification: a 30-month experiment. J. Microbiol. Mtds., 67: 574-581.

Rahman RNZRA, Leow TC, Salleh AB (2007). *Geobacilllus zalihae* sp. nov., a thermophilic lipolytic bacterium isolated from palm oil mill effluent in Malaysia. BMC Microbiol., 7: 77.

Samsudin AR, Hamzah U, Rahman RA, Siwar C, Jani MF, Othman R (1997). Thermal springs of Malaysia and their potential development. J. Asian Earth Sci., 15(2-3): 275-284.

Savitha J, Srividya S, Jagat R, Payal P, Priyanki S, Rashmi GW, Roshini KT, Shantala YM (2007). Identification of potential fungal strain(s) for the production of inducible, extracellular and alkalophilic lipase. Afr. J. Biotechnol., 6(5): 564-568.

Saw JH, Mountain BW, Feng L, Omelchenko MV, Hou S, Saito JA, Stott MB, Li D, Zhao G, Wu J, Galperin MY, Koonin KV, Makarova KS, Wolf YI, Rigden DJ, Dunfield DF, Wang L, Alam M (2008). Encapsulated in silica: genome, proteome and physiology of the thermophilic bacterium *Anoxybacillus flavithermus* WK1. Genome Biol., 9: 11.

Sharma R, Chisti Y, Banerjee UC (2001). Production, purification, characterization,and applications of lipases. Biotechnol. Adv., 19: 627–662.

Siti Aisyah M (2009). Isolation and characterization of thermophilic bacteria from the hot springs in Malaysia. MSc thesis, Universiti Teknologi MARA, Malaysia.

Saitou N, Nei M (1987).The neighbor-joining method: a new method for reconstructing phylogenetic tree. Mol. Biol. Evol., 4: 406-425.

Sheikh Abdul Hamid N, Zen HB, Tein QB, Yasin M, Halifah YM, Saar NI, Bakar FA (2003). Screening and identification of extracellular lipase-producing thermophilic bacteria from a Malaysian hot spring. World J. Microbiol. Biotechnol., 19: 961-968.

Stackebrand E, Goebel BM (1994). A place for DNA-DNA reassociation and 16S ribosomal RNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol., 44: 846-849.

Sum CW, Irawan S, Fathaddin MT (2010). Hot springs in the Malay Peninsula. Proceedings World Geothermal Congress. Bali, Indon. 25-29 April 2010.

Turner P, Mamo G, Karlsson EN (2007). Potential and utilization of thermophiles and thermostable enzymes in biorefining. Microbial Cell Factories, 6: 9.

Wang Y, Srivasta KC, Shen G, Wang HY (1995). Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). J. Ferment. Bioeng., 79(5): 433-434.