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Characterization and Identification of Thermostable Alkaline Lipase Producing Bacteria from Hot Spring around West Java

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Thermophilic lipase producing microorganisms were isolated from a few hot springs around West Java, Indonesia. Eight isolates showed lipolytic activity on agar plate media containing olive oil and rhodamine B. Three best lipase producers, namely DMS3, KHA_P12, and KHA_T25, were further characterized and showed maximum specific activity at a mid exponential of growth phase. Identification of the isolates were performed through ribotyping method. Chromosomal DNA from these isolates were extracted and the 16S rRNA gene fragments were amplified, resulting fragment length at around 1.5 kb on agarose gel. Further analysis of the 16S rRNA gene sequences using homological and phylogenetic methods showed that the DMS3, KHA_P12, and KHA_T25 were unique strains, however close to *Geobacillus kaustophilus, Thermus aquaticus,* and *Geobacillus stearothermophilus* respectively.

Key words: Alkaline lipase, thermophiles, 16S rRNA, hot spring.

Microorganisms adapt to the condition in which they have to live and survive. Thermophilic microorganisms were reported to contain proteins which are thermostable and resist to denaturation and proteolysis exposed[1]. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms[2].

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One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophilic microbes. The higher operation temperatures are also a significant influence on the bioavailability and solubility of organic compounds[3]. Other valuable factors at elevated temperature of the process are increasing reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates, in addition higher temperature of the process increase the solubility of substrates and products and favorable equilibrium displacement in endothermic reactions[4,5,6]. According to some reports there are fats exhibiting higher melting points and able to inhibit enzymatic reactions at a low temperature[7].

Lipases or acylglycerol hydrolases (E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of long chain triacylglycerides with the formation of diacylglyceride, monoglyceride, glycerol and free fatty acids at the interface between the insoluble substrate and water through interfacial activation[8,9]. Apart from their natural substrates, lipases catalyze the enantio- and regio-selective-hydrolysis and synthesis of a broad range of natural and non-natural esters[10]. Lipases are found widespread through out of the earth's flora and fauna. More abundantly, however, the enzymes are found in bacteria, fungi and yeasts[11]. Several *Bacillus sp*. were reported to be the main source of lypolytic enzymes[12-15]. While most of these enzymes are active at a temperature at 60°C and pH of 7.0, lipases from *Bacillus thermoleovorans* and a thermophilic *Rhizopus oryzae* are moderately function at extreme pH and temperature[7,16].

Among the desirable characteristics that commercially important lipases should exhibit, alkali tolerance and thermostable lipases are the main factors[17]. To meet this end, there is still continuous search for sources of highly active lipolytic enzymes with specific stability to pH, temperature, ionic strength and organic solvents[15,18]. Several studies have been reported that microbial samples isolated from soil were tested by screening on solid and liquid media for the production of lipases, revealing that about 20% were lipase-producers[19].

Indonesia, one of the most tectonically active areas in the world with over 70 active volcanoes, has a substantial number of the geothermal region and, thus, abundant hot springs[20]. However, information concerning thermophilic communities from the enormous and diverse Indonesian thermophilic habitat is very limited. A few studies on Indonesian thermophilic communities have been conducted in volcanic lake or hot springs around Java Island[21-23]. Some of these thermophilic microbes have been cultivated and characterized in order to clone the gene encoded the thermostable enzyme[24].

In this report, we describe the isolation, characterization and identification of thermostable alkaline lipase producing bacteria from hot spring around West Java. Identification of thermophilic strains was based on ribotyping method through homological and phylogenetic analysis.

MATERIAL AND METHODS

Cultivation of Microbes

The bacteria cultures were cultivated in media ½ T (pepton 0.4%, yeast extract 0.2%, NaCl 0.1%). The cultures were incubated at 70°C for 18 hours. Pure culture was made by serial dilution and growth on agar plate.

Identification of Lipolytic Activity

The presence of lipolytic activities were determined by growing culture collection on agar plates media containing olive oil 1% and rhodamine B based on standard method[25]. Lipase production was monitored by irradiating plates with UV light at 350 nm.

Isolation and Assay of Lipase

Isolate KHA_P12 and DMS3 were grown in 100 mL medium with composition of 0.5% pepton, *0.5%* yeast extract, *0.5% NaCl, 0.05% $CaCl_2$,* adjusted to pH 9 with glycine-NaOH buffer. Extracellular lipase was isolated by centrifugation of culture for 10 minutes at 11.000 g. Supernatant was collected and stored in 4°C. Lipase activity was measured by spectrophotometric assay with *p*-nitrophenyl palmitate as substrate[7]. One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol PNP per min under the assay conditions. The molar absorption coefficient of PNP at 405 nm was determined to be 1.457×10^5 cm^2 mol^{-1}.

Isolation of Chromosomal DNA.

The pellet cells were suspended in 10 mM Tris HCl buffer (pH 8.0) containing 8 mg/ml of lysozyme and incubated at 37°C for 1 h, the cells were lysed by adding lysis buffer containing 2% SDS, 0.8 mg/ml proteinase K and 200 mM EDTA pH 8.0. The lysis process was carried out by incubation at 50°C for 30 min. Ice cold potassium acetate and acetic acid glacial mixed solution were added and the denatured proteins were precipitated by centrifugation. Supernatants were mixed with an equal volume of chloroform isoamylalcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 h. The pellet of crude nucleic acids were obtained by centrifugation at 16.000 g for 20 min at room temperature, washed twice with cold 70% ethanol, and re-suspended in sterile deionized water.

Amplification and sequencing of 16S rRNA gene

The amplification of 16S rRNA genes was carried out as described previously by Baker *et al.*[26]. The 16S rRNA genes were amplified using a set of primers, 5'-AGAGTTTGATC(A/C)TGGCTCAG-3' and 5'-GGTTAC(G/C)TTGTTACCTGCCGGA-3'. All of PCR products were subjected to DNA sequencing. Sequencing was carried out in an ABI PrismR 3100 Genetic Analyzer (Applied Biosystems) by the Macrogen Sequencing Service (Korea).

Phylogenetic analysis

The sequencing results were compared to 16S rRNA gene sequences from GenBank database at NCBI (National Centre of Biotechnological Information) through web site http://www.ncbi.nlm.nih.gov using BLAST program for screening of sequence similarity[27]. Sequences alignments were performed by ClustalW program[28]. Aligning sequences were visualized using GenDoc program. Phylogenetic reconstruction was accomplished with the phylogeny inference package (PHYLIP version 3.62). Evolutionary distances were calculated by F84 method with the DNADIST program[29]. Phylogenetic trees were constructed from distance matrices by the neighbor-joining method[30], which was implemented with the NEIGHBOR program. The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets.

Nucleotide sequence accession numbers

All of the nucleotide sequences from this study have been deposited in the GenBank database under accession numbers EU784086 for KHA_T25, EU784082 for KHA_P12, and FJ763640 for DMS3.

RESULTS

Isolation and cultivation of microbes

A few thermophilic bacteria were isolated from hot springs around West Java, namely Kawah Hujan, Kamojang, Garut, Kawah Papandayan, Garut and Kawah Domas, Tangkuban Perahu Crater, Bandung. All hot springs have temperature at above 90°C but the pHs are variation. However all the cultures were cultivated at 70°C and neutral pH. Under light microscope, most of the cultures showed rod shape form bacteria (Fig. 1).

Identification of lipolytic activity

The pure cultures of thermophilic bacteria isolated from the hot springs were screened for its lipolytic activity. Lipase producer bacteria were identified using agar media supplemented by olive oil 1% and rhodamine B. In order to search for alkaline thermostable lipase producer, the media have been adjusted to pH 8.5, 9.0, 9.5 and 10.0. The activity was monitored from the formation of fluorescence halo around the colonies. 8 isolates showed lipolytic activity by this assay. Four isolates came from Kawah Hujan, namely KHN, KHA_T25, KHA_T6 and KHA_P12, two were isolated from Kawah Domas, namely DMS1 and DMS3, and another two were isolated from Kawah Papandayan, namely PPD1 and PPD2 (data not shown). Intensity of the fluorescent were varies

Table 1. Lipolytic activity of 8 isolates in variation of pH. (-) no activity, (+) positive activity

No	Isolates	pH			
		8.5	9.0	9.5	10.0
1.	KHA-P12	+++	+++	++++	+
2.	DMS-1	-	+	+	-
3.	DMS-3	++++	+++	++	++
4.	KHA-T6	-	++	-	+
5.	KHA-T25	-	++	-	-
6.	PPD1	++	+	++	+
7.	PPD2	-	+	+	+
8.	KHN	+	-	++	++

among the colonies. Table 2 summarizes the intensity of fluorescent for the 8 isolates in media with variation of pH. From the eight isolates, DMS3, KHA_P12 and KHA_T25 showed strongest intensity.

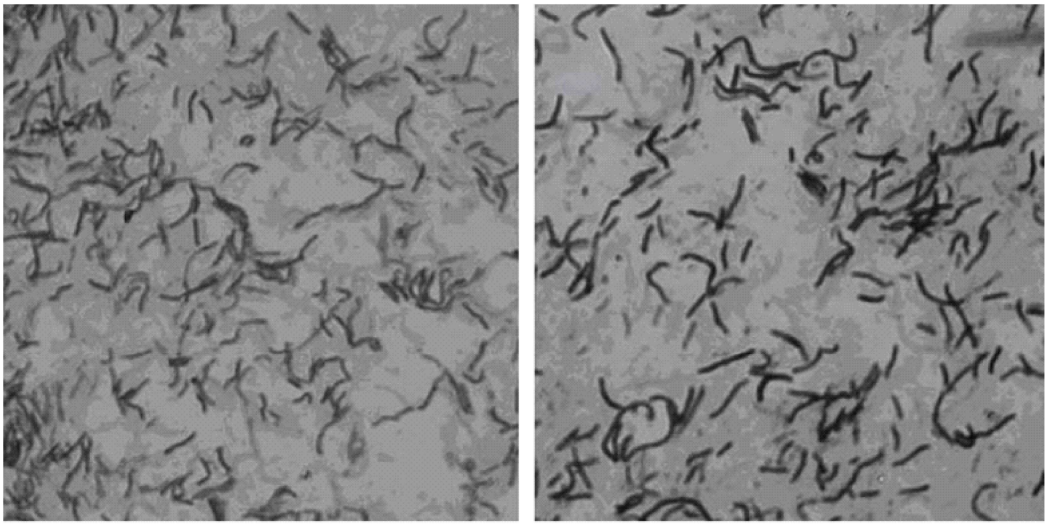

Fig. 1. Two morphological isolates under light microscope with 1000X, samples of DMS3, and KHA-P12.

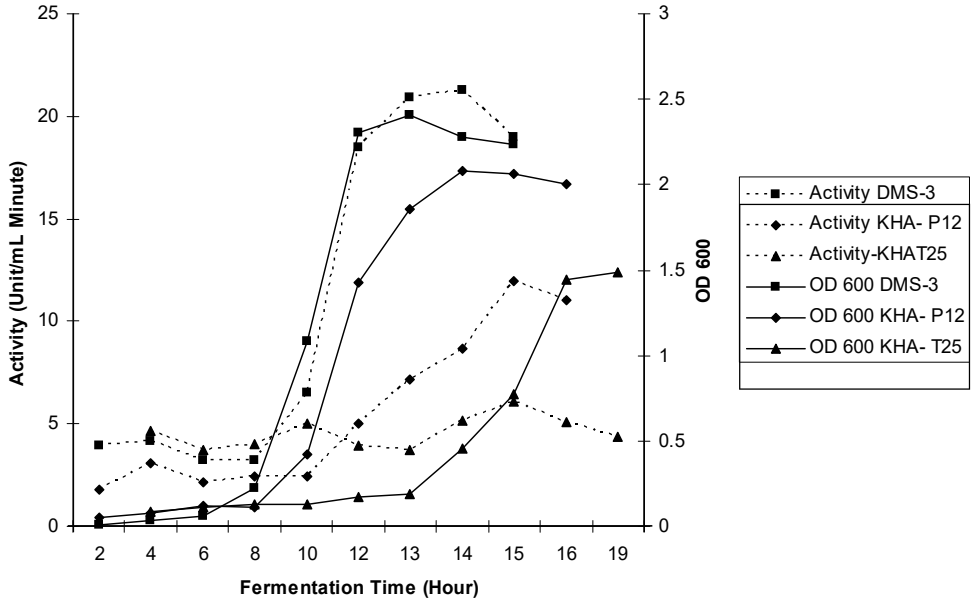

Fig. 2. Growth and lipolytic activity profiles of isolate DMS3, KHA_P12 and KHA_T25. Growth profiles are shown in solid line while lipolytic activity are shown in dashed line.

Table 2. Thirty highest homologyto 16S rDNA sequences of DMS3

Accession Number	Description	Max ident
DMS3		
EU680816.1	*Geobacillus* sp. dYTae-14	99%
EU680812.1	*Geobacillus* sp. dYTae-1	99%
AB306519.1	*Geobacillus* sp. sbs4s	99%
EF199739.2	*Geobacillus* sp. TERI NSM	99%
DQ642088.1	*Geobacillus* sp. N60	99%
AY603071.1	*Geobacillus* sp. STB2	99%
AY608939.1	*Geobacillus thermoleovorans* strain BGSC 96A6	99%
AJ564620.1	*Geobacillus thermoleovorans* T1	99%
AJ564614.1	*Geobacillus thermoleovorans* T7	99%
AJ564612.1	*Geobacillus thermoleovorans* F30	99%
BA000043.1	*Geobacillus kaustophilus* HTA426	99%
EU680813.1	*Geobacillus* sp. dYTae-3	99%
EU484348.1	*Geobacillus thermoleovorans* strain BCRC 17253	99%
AF385083.1	*Bacillus thermoleovorans*	99%
AY608943.1	*Bacillus* sp. BGSC W9A60	99%
AY608937.1	*Bacillus caldotenax* strain BGSC 96A4	99%
EU214615.1	*Geobacillus thermoparaffinivorans* strain it-12	99%
EU682501.1	*Thermus aquaticus*	99%
AM999769.1	*Thermus aquaticus*	99%
EU680815.1	*Geobacillus* sp. dYTae-13	99%
EU680810.1	*Geobacillus* sp. dNBae-1	99%
EU680809.1	*Geobacillus* sp. dGae-3	99%
EU652092.1	*Geobacillus kaustophilus* strain ra-4	99%
EU652083.1	*Geobacillus kaustophilus* strain mt-12	99%
AB306521.1	*Geobacillus* sp. sbs4s2	99%
EU161956.1	*Geobacillus* sp. CICC 10315	99%
AY074879.1	*Geobacillus thermoleovorans*	99%
AY603072.1	*Geobacillus* sp. STB3	99%
DQ055416.1	*Geobacillus* sp. TibetanG6	99%
AY608934.1	*Geobacillus kaustophilus* strain BGSC 90A1	99%

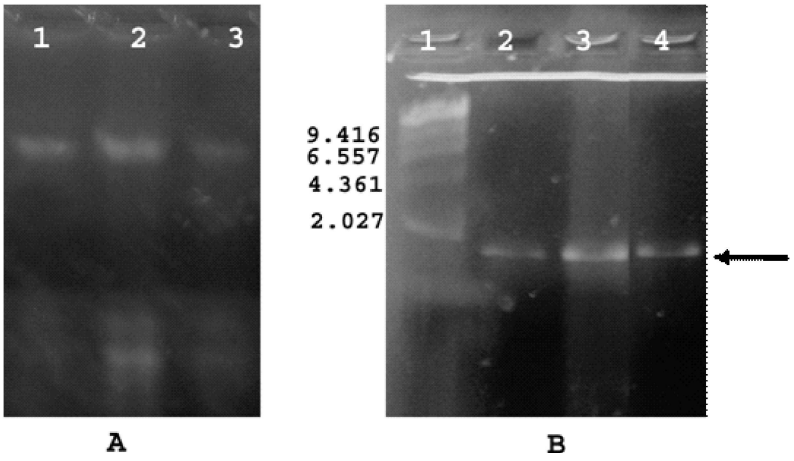

Fig. 3. Electrophoregram of chromosomal DNA and the 16S rDNA amplicons. (A) chromosomal DNA, line 1-3 for DMS3, KHA_P12, and KHA_T25 respectively; (B) 16S rDNA fragment, line 1 for marker DNA, line 2-4 for DMS3, KHA_P12, and KHA_T25 respectively.

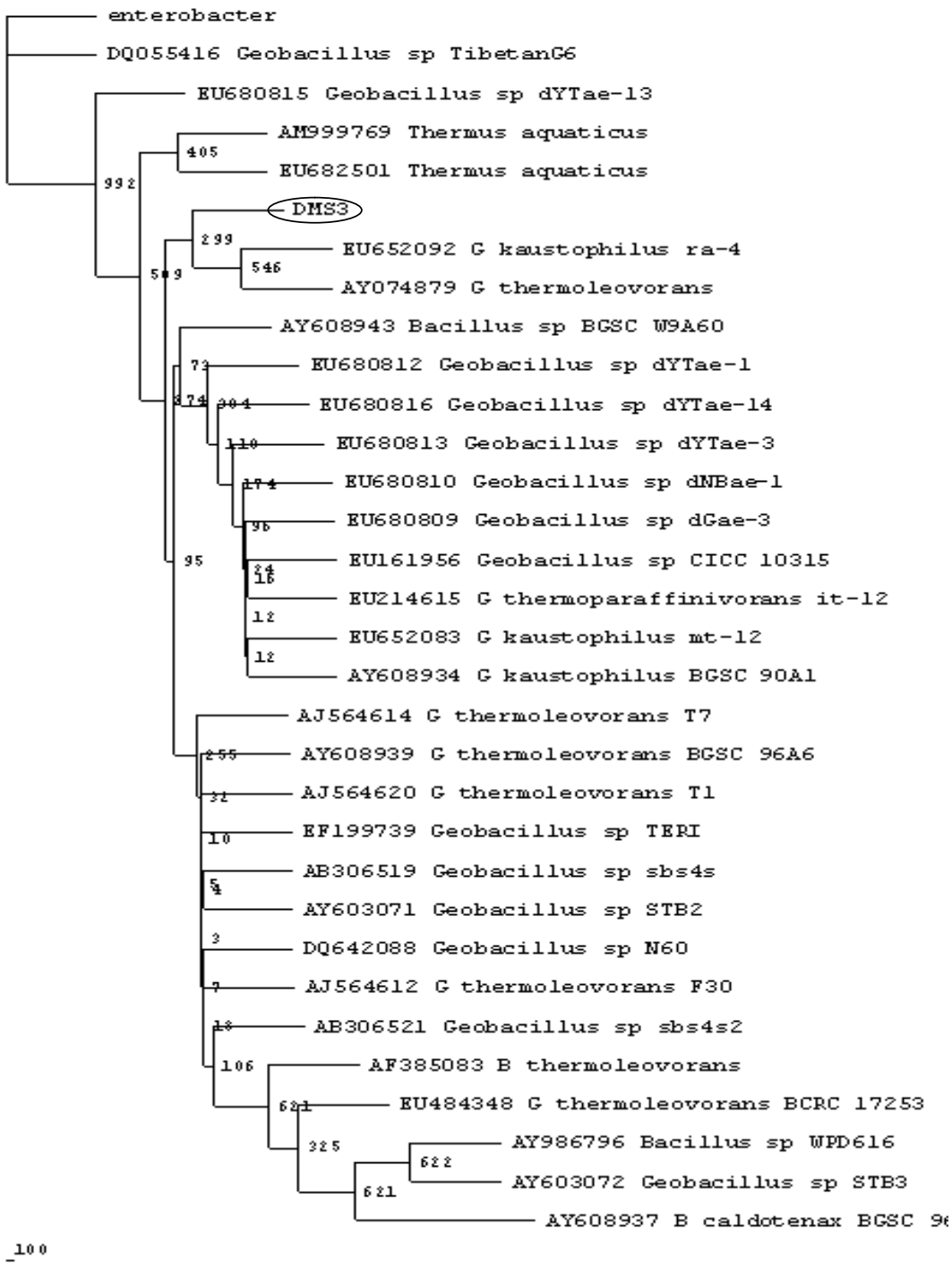

Fig. 4. Phylogenetic relationships of the 16S rRNA gene fragment sequences obtained from the DMS3 isolate and 30 related sequences. Distance analysis was performed using the F84 correction, followed by phylogenetic tree construction using the neighbor-joining method of the PHYLIP software with 1,000 bootstrap replicates. (Circle), represented the samples

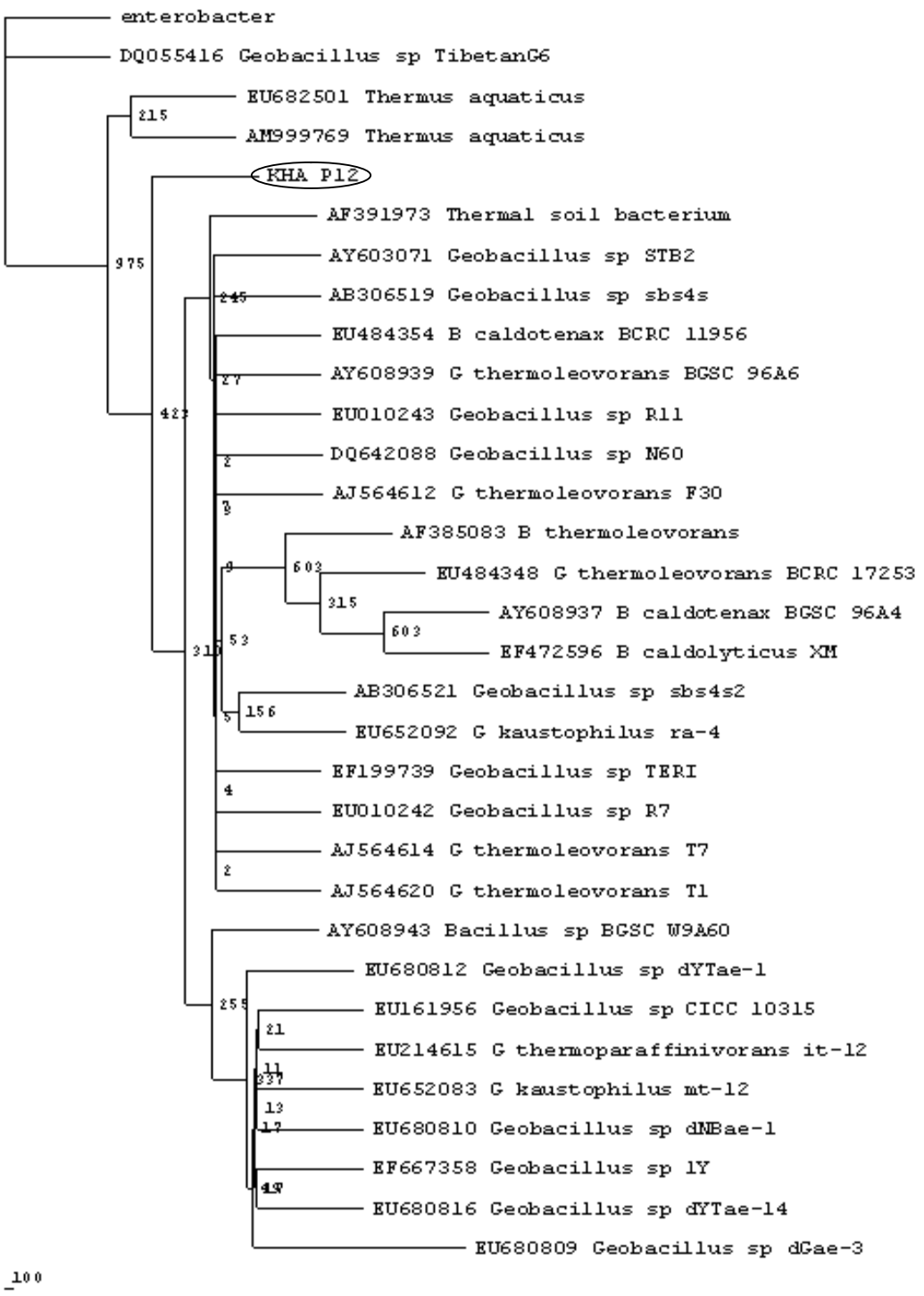

Fig. 5. Phylogenetic relationships of the 16S rRNA gene fragment sequences obtained from the KHA_P12 isolate and 30 related sequences. Distance analysis was performed using the F84 correction, followed by phylogenetic tree construction using the neighbor-joining method of the PHYLIP software with 1,000 bootstrap replicates. (Circle), represented the samples

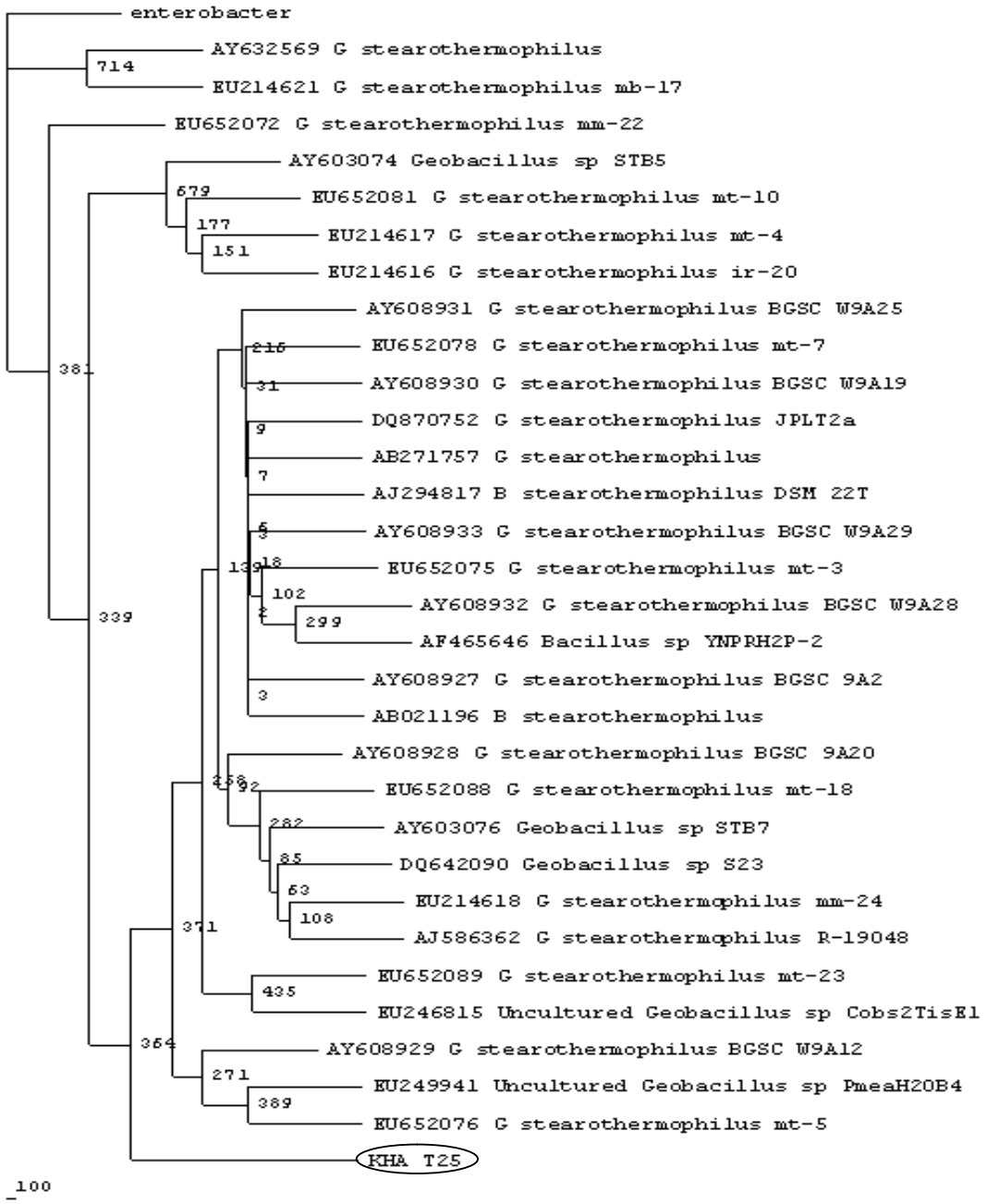

Fig. 6. Phylogenetic relationships of the 16S rRNA gene fragment sequences obtained from the KHA_T25 isolate and 30 related sequences. Distance analysis was performed using the F84 correction, followed by phylogenetic tree construction using the neighbor-joining method of the PHYLIP software with 1,000 bootstrap replicates. (Circle), represented the samples.

Growth profiles and lipase activity

Three of the strongest lipolytic activity namely DMS3, KHA_P12 and KHA_T25 were further characterized at temperature 70°C and pH 9. The growth of the isolates and extracellular lipase activity were monitored during the incubation. Extracelluler lipase was assayed based on hydrolysis of p-nitrophenyl palmitate. The growth curve and lipolytic activity for all isolates were shown on the Fig. 2. The maximum growths were observed after 12-15 hours of the incubation. The maximum lipase activities for all isolates were observed on 14 hours of fermentation. These profiles suggested that the lipase were expressed constitutively in all of the cells with the condition.

Identification of the isolates

Three isolates, namely DMS3, KHA_P12 and KHA_T25, were identified based on its 16S rRNA gene sequence. Chromosomal DNA was isolated based on standard method and used as template for PCR process to amplify 16S rRNA gene. The amplification products have approximately 1500 bp length as expected. Agarose electrophoregram of chromosomal DNA and the PCR product was shown on Fig. 3. For further analysis the amplicons were purified and exposed for direct sequencing. The sequences of the amplicons deposit in the GenBank as stated in the Material Method. The complete sequences of the amplicons were compared to the available sequence data in the GenBank and the results

Table 3. Thirty highest homologyto 16S rDNA sequences of KHA_P12

Accession Number	Description	Max ident
KHA-P12		
EU010243.1	*Geobacillus* sp. R11	99%
EU010242.1	*Geobacillus* sp. R7	99%
EU484354.1	*Bacillus caldotenax* strain BCRC 11956	99%
AB306519.1	*Geobacillus* sp. sbs4s	99%
EF199739.2	*Geobacillus* sp. TERI NSM	99%
DQ642088.1	*Geobacillus* sp. N60	99%
AY603071.1	*Geobacillus* sp. STB2	99%
AF391973.1	Thermal soil bacterium YNP 2	99%
DQ055416.1	*Geobacillus* sp. TibetanG6	99%
AY608939.1	*GeoBacillus thermoleovorans* strain BGSC 96A6	99%
AJ564620.1	*GeoBacillus thermoleovorans* isolate Tl	99%
AJ564614.1	*GeoBacillus thermoleovorans* isolate T7	99%
AJ564612.1	*GeoBacillus thermoleovorans* isolate F30	99%
BA000043.1	*Geobacillus* kaustophilus HTA426	99%
EU484348.1	*GeoBacillus thermoleovorans* strain BCRC 17253	99%
AF385083.1	*Bacillus thermoleovorans*	99%
AY608943.1	*Bacillus* sp. BGSC W9A60	99%
AY608937.1	*Bacillus caldotenax* strain BGSC 96A4	99%
EU214615.1	*Geobacillus thermoparaffinivorans* strain it-12	99%
EU682501.1	*Thermus aquaticus*	99%
AM999769.1	*Thermus aquaticus*	99%
EU680816.1	*Geobacillus* sp. dYTae-14	99%
EU680812.1	*Geobacillus* sp. dYTae-1	99%
EU680810.1	*Geobacillus* sp. dNBae-1	99%
EU680809.1	*Geobacillus* sp. dGae-3	99%
EU652092.1	*Geobacillus kaustophilus* strain ra-4	99%
EU652083.1	*Geobacillus kaustophilus* strain mt-12	99%
AB306521.1	*Geobacillus* sp. sbs4s2	99%
EU161956.1	*Geobacillus* sp. CICC 10315	99%
EF667358.1	*Geobacillus* sp. 1Y	99%

Table 4. Thirty highest homology to 16S rDNA sequences of KHA_T25

Accession Number	Description	Max ident
KHA-T25		
EU652078.1	*Geobacillus stearothermophilus* strain mt-7	98%
EU249941.1	Uncultured *Geobacillus* sp. clone PmeaH2OB4	98%
DQ870752.1	*Geobacillus stearothermophilus* strain JPLT2a	98%
AB271757.1	*Geobacillus stearothermophilus*	98%
AY608933.1	*Geobacillus stearothermophilus* strain BGSC W9A29	98%
AY608931.1	*Geobacillus stearothermophilus* strain BGSC W9A25	98%
AY608930.1	*Geobacillus stearothermophilus* strain BGSC W9A19	98%
AY608929.1	*Geobacillus stearothermophilus* strain BGSC W9A12	98%
AY608927.1	*Geobacillus stearothermophilus* strain BGSC 9A2	98%
AJ294817.1	*Bacillus stearothermophilus* strain DSM 22T	98%
AB021196.1	*Bacillus stearothermophilus*	98%
EU652089.1	*Geobacillus stearothermophilus* strain mt-23	98%
EU652088.1	*Geobacillus stearothermophilus* strain mt-18	98%
EU652075.1	*Geobacillus stearothermophilus* strain mt-3	98%
EU652072.1	*Geobacillus stearothermophilus* strain mm-22	98%
DQ642090.1	*Geobacillus* sp. S23	98%
AY603076.1	*Geobacillus* sp. STB7	98%
AF465646.1	*Bacillus* sp. YNPRH2P-2	98%
AY608928.1	*Geobacillus stearothermophilus* strain BGSC 9A20	98%
AJ586362.1	*Geobacillus stearothermophilus* strain R-19048	98%
AY608932.1	*Geobacillus stearothermophilus* strain BGSC W9A28	98%
EU214621.1	*Geobacillus stearothermophilus* strain mb-17	98%
EU214618.1	*Geobacillus stearothermophilus* strain mm-24	98%
EU214617.1	*Geobacillus stearothermophilus* strain mt-4	98%
EU214616.1	*Geobacillus stearothermophilus* strain ir-20	98%
EU652081.1	*Geobacillus stearothermophilus* strain mt-10	98%
EU652076.1	*Geobacillus stearothermophilus* strain mt-5	98%
EU246815.1	Uncultured *Geobacillus* sp. clone Cobs2TisE1	98%
AY603074.1	*Geobacillus* sp. STB5	98%
AY632569.1	*Geobacillus stearothermophilus*	98%

Table 5. Nucleotide variation of DMS3 to its closet homology

Base number	Nucleotide consensus	Substitution	region
KHA_T25			
204	C	G	variable
231	A	C	variable
243	G	C	variable
247	G	C	conserve
258	G	C	conserve
267	T	G	variable
289	G	A	conserve
292	G	A	variable
297	G	T	variable
300	G	C	conserve
601	T	C	conserve
662	A	G	conserve

Table 6. Nucleotide variation of KHA_P12 and KHA_T25 to its closet homology

Base number	KHA_P12	*Thermus aquaticus*	Thermal Soil Bacterium	*Geobacillus austophilus*	DMS3	Region
75	deletion	A	none	A	deletion	variable
299	C	C	C	G	C	variable
747	C	T	C	C	C	
1409	deletion	A	A	A	A	variable

showed that all of the sequences have close homology to all 16S rRNA gene.

Homological analysis of the isolates DMS3 and KHA_P12 showed that the isolates were close (99%) to the 30 16S rRNA sequences from the genus of *Geobacillus* and *Bacillus* with two exception to *Thermus aquaticus*, meanwhile KHA_T25 was close to *G. stearothermophilus* (98%) (Table 2, 3 and 4). Phylogenetic analysis of the sequences was conducted through comparison with other 30 best homology sequences from GenBank. The results showed that DMS3 isolate was close to *G. kaustophilus* (EU 652092) (Fig 4) and KHA_P12 was close to *Thermus aquaticus* (AM999769) (Fig. 5). Meanwhile KHA_T25 was confirmed close to *G. stearothermophilus* (EU652076) (Fig. 6). Further analysis by comparing the 16S rRNA gene sequences with the highest homology showed that the KHA_T25 isolate showed 13 nucleotide difference with the consensus sequences of *G. stearothermophilus*, 7 of them on the variable region of the gene, however 6 nucleotide differences with the consensus lied at the conserved region (Table 5). Meanwhile for the isolate KHA_P12 showed 4 nucleotide differences compared to that *T aquaticus*, however, 2 nucleotides deletion and other 2 nucleotide substitutions. Furthermore, DMS3 isolate showed also 4 nucleotide differences compared to that *G. kaustophilus* with single nucleotide deletion and 3 nucleotide substitutions (Table 6).

DISCUSSION

Lipase is one of the enzyme that have been long interested to detergent industry for their ability to aid in the removal of lipid stains and to deliver unique benefit that can not otherwise be obtained with the conventional detergent technologies. The main objective of the present work was to broaden the sources of alkaline lipase by characterization and identification of thermostable alkaline lipase producing microorganisms.

Eight alkaline lipase producing isolates have been identified from a few hot springs around West Java, Indonesia. Most of the isolates showed lipolytic activity at pH 9, except for the isolate KHN showing higher lipolytic activity at pH above 9 (Table 1). Three of the best producing isolates, namely DMS3, KHA_P12, and KHA_T25, were identified close to *G. kaustophilus*, *T. aquaticus* and *G. sterothermophilus* respectively (Fig 4, 5, and 6). Some of lipase producing *Geobacillus* and *Thermus* have been reported[31,32]. Lipase is one of the enzymes that catalysing hydrolysis of esters formed glycerol and long fatty acids[19] and thus involved on the primary metabolite. This type of enzyme usually expressed on the exponential phase of bacterial growth. However the result on this work seemed that the expression of the lipase in all isolates occurred as constitutive enzymes (Fig 3), this is probably due to the fermentation performed in the present of olive oil.

Identification of the isolates was carried out based on the ribotyping method through the sequences of 16S rRNA gene. Analysis using the BLAST Program[27] resulted that the sequences had high homology to all 16S rRNA genes available on the GenBank. The result confirmed that the amplicons were part of the 16S rRNA gene. 30 highest homology of each sequence showed that the DMS3 and KHA_P12 have 99% homolog to the most of genus *Geobacillus*, a few to *Bacillus* and 2 with *Thermus*, while KHA_T25 showed 98% homolog to most of *B. stearothermophilus.* The highest homology of the isolates to the genus

Geobacillus and *Bacillus* were also supported by morphological identification (Fig 1). A large collection of rod shaped bacteria[33,34] may be due to the media or technique used on the sampling and the treatment of the springs water sample during cultivation in laboratories. *Bacillus* and *Geobacillus* were known as dominance bacteria on the environment due to its ability to form endospore, while other bacteria need very specific condition to struggle for growth[21].

Phylogenetic trees were constructed by Clustal W, using 30 nucleotide sequences that displayed close homology from the BLAST program. DMS3, KHA-P12, AND KHA-T25 are closet to *G. kaustophilus* (EU 652092), *T aquaticus* (AM 999769) and *G. stearothermophilus* (EU652076) respectively. DMS3 showed 99% homolog with more than 30 16S rRNA sequences from GenBank, most of them are geobacillus, a few bacillus and 2 from *T. aquaticus*. Phylogenetic analysis of the isolate showed that DMS3 lied among *G. kaustophilus*, *G. thermoleovorans* and *T. aquaticus* (Fig 4, 5, and 6), however, the closet homology did not occur in the same branch.

Further analysis by comparing the sequence of the isolate to the sequence of *G. kaustophilus* (EU 652092) showed that DMS3 has 4 nucleotide variation, however one of the nucleotide deletion. This deletion probably caused separated branch in the tree. Furthermore, KHA_P12 lied between *T aquaticus* (AM 999769) and Thermal soil bacteria (AF 391973). The position of KHA_P12 in the tree showed separated branch (Fig 5). The 16S rRNA sequences of the isolate showed 4 nucleotide variation with two nucleotide substitutions, this is confirmed the position of the isolate in the tree. Meanwhile, KHA_T25 showed the most variation of the 16S rRNA sequence compared to that the closet homolog (*G. stearothermophilus*, EU 652076), however there was no nucleotide deletion. All of the data suggested that KHA-T25 is most probably as unique *G. stearothermophilus*. Meanwhile KHA_P12 is belong to *Thermus* but unlikely as known *T. aquaticus* rather as unique *T aquaticus* or another new species of thermus. The last proposal was supported by the homological and phylogenetic results. Furthermore, DMS3 is most probably as unique *G. kaustophilus.*

CONCLUSION

Eight local thermophilic bacteria have been isolated and identified as alkaline thermostable lipase producer. Three of them, DMS3, KHA_P12 and KHA_T25 showed the highest lipolytic activity at pH 9. Based on 16S rRNA sequences analysis, the isolates DMS3, KHA_P12 and KHA_T25 were close to *G. kaustophilus, T. aquaticus* and *G. stearothermophilus*.

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