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Cloning and Sequence Analysis of Lipase Gene of Halophilic Bacteria Isolated from Mud Crater of Bledug Kuwu, Central Java, Indonesia

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Five lipase genes (*lipab4*, *lipab8*, *lipab15*, *lipag18*, and *lipab18*) have been isolated from *Halomonas* and *Chromohalobacter* local strains of Bledug Kuwu isolates. Based on amino acid sequence analysis, the genes showed some unique motif of amino acid sequences. All of lipases were classified as a member of family IV (HSL=hormone-sensitive lipase). These lipases show high similarities of conserved regions with lipolytic of *Halomonas* and formed a distinct cluster with other types of HSL, such as esterase/ lipase and carboxylesterase. All of lipases contain more negative charged of amino acid residues compared to the mesophilic and thermophilic ones, and tend to have similarity to lipases of moderate halophilics. The result of homology and phylogenetic analysis showed that these lipases were clustered into three groups. Group I (*lipab8*, *lipab18* and *lipag18*) closed to lipolytic gene of *Halomonas elongata* DSM 2581, while groups II (*lipab4*) and III (*lipab15*) created new branches in the phylogenetic tree. In addition, analysis of GC, GC-AT and GC-AT content on the codon usage of the genes revealed the unique profile compared to that the other lipase genes.

Key words: *Halomonas*, *Chromohalobacter*, Halostable lipase, HSL Family, GC-AT content, Bledug Kuwu.

A lipase is an enzyme that catalyzes hydrolysis of triacylglycerol into fatty acids and glycerol at water-lipid interface, and also an esterification reaction in non-aqueous environment, i.e. organic solvents[1,2]. In some industrial process, the reactions may be inhibited by high concentrations of salt, organic solvents and high temperatures. Therefore, lipases that have optimum activity within wide range of temperatures, salinity, pHs, and various organic solvents, are demanded by most industries[3,4]. One of the potential sources of lipases is extremophilic microorganisms, such as thermophiles for thermostable lipase[5–10] and halophiles for halostable lipase[11–13].

Halophiles are defined as microorganisms that live in hypersaline environments[14,15]. There are numerous reports about extracellular lipase production from halophiles i.e. *Salinivibrio* sp. strain SA-2, *Natronococcus* sp., *Salicola*, *Halovibrio*, *Halomonas*, *Oceanobacillus*, *Thalassobacillus*, *Halobacillus*, *Virgibacillus*, *Gracilibacillus*, *Salinicoccus*, and *Piscibacillus* [16–19]. Li and Yu (2012) have isolated lipase from *Chromohalobacter* sp. LY7-8 that highly active towards p-nitrophenyl esters (C8 to C18) [20]. The

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purified lipase has molecular weight of 44 kDa and exhibited optimum activity at 60°C, pH 9.0 and 12.5% NaCl. Significant inhibition of the lipase activity exhibited upon the addition of EDTA, PMSF and DEPC, indicating it is a metalloenzyme with serine and histidine residues essential for its catalytic function. Perez *et al.,* (2011) have cloned, overexpressed and purified, a lipolytic enzyme from the moderate halophilic *Marinobacter lipolyticus* SM19 into *E.coli*[21]. The enzyme showed unusual halophilic properties, since its optimum activity was observed in the absence of NaCl and inhibited by the presence of this salt. However, this enzyme exhibited remarkable stability in a wide variety of organic solvents including DMSO, DMF, methanol, ethanol, toluene, diethyl ether, propanol, and acetone. It has been reported that some halophilic bacteria can also live in organic solvent rich environment indicating their enzymes are also adaptive to such conditions[22–24]. Isolating lipase from halophilic bacteria, therefore will give higher probability to obtain the enzyme that stable and active in both extreme conditions, which are hypersaline and organic solvents.

Seven halophilic bacteria comprised of four *Halomonas*, one *Chromohalobacter* and two *Pseudomonas* strains have been isolated and identified as potential sources for halostable lipases. Four *Halomonas* and one *Chromohalobacter* strains showed high halotolerant levels and classified as moderate halophilics, meanwhile the *Pseudomonas* were classified as low halotolerant level[25]. One of the *Pseudomonas* was shown as the potential source of organic solvent stable lipase[26]. Therefore, there is possibility that the other lipase isolated from the same environment is also stable in organic solvents. For further probe the characterized of the lipases, here we present cloning and sequence analysis of the genes.

MATERIALS AND METHODS

Chemicals

Common chemicals with pro analysis grade were purchased from Merck (Germany) and Sigma-Aldrich (USA). Bacterial growth nutrients, such as tryptone, yeast extract were obtained from Bio Basic (Canada). Biochemical reagents such as dNTPs, PCR Buffer, *Taq* DNA Polymerase were purchased from Fermentas (USA) and Kapa Biosystems (USA). Oligonucleotides (primers) were ordered from Macrogen (South Korea) and Integrated DNA Technologies (Singapore). Purification of PCR product used GeneJET Gel Extraction Kit (Thermo Scientific). The cloning was performed by using *pGEM-T* easy vector and T4 DNA ligase purchased from Promega (USA). Restriction enzymes were purchased from Thermo Scientific (USA).

Bacteria

Five isolates of halophilic bacteria were obtained from our culture collections in the Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung. The isolates were *Halomonas meridiana* BK-AB4 (AB4), *Halomonas elongata* BK-AB8 (AB8), *Halomonas eurihalina* BK-AB15 (AB15), *Halomonas elongata* BK-AG18 (AG18) and *Chromohalobacter japonicus* BK-AB18 (AB18)[25]. *Escherichia coli* TOP 10 was used as host for gene cloning.

Cultivation

Five isolates were cultivated using modified media of Luria Berthani composed of 0.1% tryptone, 0.05% yeast extract and 10% NaCl[27]. The cultures were incubated in shaker incubator at room temperature with aeration rate of 150 rpm.

Isolation of Chromosomal DNA

Isolation of chromosomal DNA was carried out using modified method of Zhou[28]. The collected DNA pellet was separated from the supernatant and dried with concentrator, subsequently followed by resuspension with 25-50 mL ddH_2O and stored at 4 °C. The DNA was used for PCR.

Cloning of Lipase Genes

Cloning of lipase gene in each bacterial isolates was started by *in vitro* amplification of the gene by PCR technique[29] using a pair of specific primers i.e. xFLipH3A (5'-ATGCAGATCGATGCCTTTCGTCGC-3') and xRLipH3A (5'-TCATTCCACTCGTCGACCCAGC-3'). A typical PCR mixture (50 µL in volume) was prepared by mixing 5 µL of PCR buffer 10´, 2.5 mM $MgCl_2$, 250 µM of deoxynucleosid-e triphosphate, 0.25 µM of each primers, and 1.25 U of *Taq* DNA polymerase. PCR was conducted by the following protocol: an initial denaturation was set at 94 °C for 4 min followed by 35 cycles of denaturation

(@30 s at 94 °C), an annealing was programmed at 59 °C for 30 s, while an extension and final extension were carried out at the same temperature at 72 °C for 1 min and 5 min, respectively[29]. The product of PCR was verified by electrophoresis technique using submerged horizontal electrophoresis cell for 50 min at 70 volts. The purification of PCR product was carried out by GeneJET Gel Extraction Kit. Finally, purified DNA was re-suspended to 50 µL buffers (10 mM Tris-HCl, pH 8.5). The purified DNA solution was stored at -20 °C.

Construction of recombinant plasmid was carried out by ligating the PCR products with *pGEM-T* easy plasmid. The **c**ompetent cell of *E. coli* Top 10 was prepared following the method described by Cohen[30]. Transformation of *E. coli* was conducted by heat shock method[31]. 100 mL of the transformed cells were spreaded on LB agar (LBA) media containing 100 mg/mL of ampicillin, 15 mg/mL of tetracycline and incubated at 37 °C for overnight. Plasmid isolation from the transformed cell was carried out by the alkaline lysis method[32]. Finally to verify the recombinant plasmid containing DNA insert, the plasmid was digested with *Eco*RI and visualized by agarose gel electrophoresis.

Sequencing of Lipase Gene

Lipase genes were sequenced by Dideoxy-Sanger dye terminator method[33] at Macrogen, Seoul, Republic of Korea. The sequences were validated by analyzing the electrophoregram data using Sequence scanner 2 (Applied Biosystems). In order to combine the partial gene sequences into a full gene, we used DNA Baser Sequence Assembler v3 program (Heracle BioSoft).

Deduced Amino Acid Sequence Analysis of Lipase

Deduced amino acid sequences of five lipases were performed by *in silico* translation using Bioedit software and online server of ExPASy-Translate tool available at http://web.expasy.org/translate/.

Homology analysis of five lipase sequences were carried out using NCBI-Blastp analysis program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A hundred of high homologous sequences were used to generate phylogenetic profile using MEGA 6 software based on the Neighbor-Joining clustering method.

Amino acid composition and alignment analysis were performed using "Amino Acid Composition" and "ClustalW Multiple Alignment" programs which are integrated in the Bioedit software.

Sequence Analysis of Lipase Gene

The analysis of lipase gene sequences was carried out by evaluating contents of GC, GC-AT, and GC-AT of codon usage. The last two analyses were conducted with Bioedit-software, while the third analysis was calculated with an online program available at http://www.bioinformatics.org /sms2/ codon_usage.html. The analysis of "codon usage" was performed on the 1st, 2nd and 3rd base position. All analyzes above were not only performed on the five isolates lipase sequences but also to other lipase genes from the other microorganism.

RESULTS AND DISCUSSION

Cloning and Sequencing of Lipase Genes

Five lipase genes have been successfully amplified from *Halomonas* and *Chromoholobacter* local strains by PCR technique. The genes, namely *lipab4*, *lipab8*, *lipab15*, *lipag18*, and *lipab18*, have estimated length about 800 bp (Figure 1). All of the genes were cloned into *E. coli* Top 10, sequenced and deposited to GenBank database (https://www.ncbi.nlm.nih.gov) with accession number of KJ676135.1, KJ676136.1, KJ676137.1, KJ676139.1, and KJ676138.1, respectively.

Homology and Phylogenetic Analysis of Lipases

Homology analysis of deduced amino acid sequences of five lipases were carried out using NCBI-Blastp program. The results showed that the five lipase sequences showed high similarities to several lipases, such as form *Halomonas elongata* DSM 2581[34] with percent identity about 95-99%, *Gammaproteobacteria* MFB021[35] with percent identity of 58-60% and *Halomonas halodenitrificans*[36] with percent identity of 57%. All lipases closed to *Halomonas elongata* DSM 2581 (Table 1).

Phylogenetic analysis generated a tree profile that five lipases distributed into three groups i.e. group I (lipab8, lipab18 and lipag18), groups II (lipab4) and III (lipab15). Group I closed to lipase of *Halomonas elongata* DSM 2581, meanwhile the group II and III formed new branches (Figure 2).

Conserved Regions Analysis of The Lipases

100 best homologous sequences with the

Table 1. The result of Blastp alignment for the five lipase genes

Description	Total score	Ident	Accession
Lipolytic protein [*Halomonas elongata*]	538	100%	WP_013332507.1
Lipase [*Halomonas elongata*] à *lipab8*	531	99%	AID66447.1
Lipase [Chromohalobacter japonicus] à*lipab18*	528	98%	AID66449.1
Lipase [*Halomonas elongata*] à*lipag18*	525	97%	AID66450.1
Lipase [*Halomonas meridiana*] à *lipab4*	510	96%	AID66446.1
Lipase [*Halomonas eurihalina*] à *lipab15*	509	95%	AID66448.1
Lipolytic protein [*Halomonas halodenitrificans*]	294	57%	WP_043487540.1
Lipolytic protein [*Gammaproteobacteria bacterium* MFB021]	293	58%	WP_035474917.1
Lipolytic enzyme [*Halomonas* sp. HAL1]	287	54%	WP_008960037.1
Lipolytic enzyme [*Halomonas* sp. TD01]	286	55%	WP_009721885.1
Lipolytic protein [*Halomonas* zincidurans]	286	53%	WP_031383322.1
Hypothetical protein [*Kushneria aurantia*]	285	57%	WP_019951967.1
Lipolytic protein [*Halomonas campaniensis*]	283	54%	WP_038485789.1
Lipolytic protein [*Halomonas* sp. KO116]	282	53%	WP_035566707.1
Lipolytic protein [*Halomonas* sp. TG39a]	281	53%	WP_035578065.1
Lipolytic protein [*Halomonas* sp. HL-48]	280	54%	WP_027335272.1
MULTISPECIES: lipolytic protein [*Halomonas*]	278	52%	WP_027959004.1
Lipolytic protein [*Halomonas alkaliantarctica*]	278	53%	WP_030072091.1
Lipolytic protein [*Halomonas titanicae*]	272	52%	WP_039859736.1
Alpha/beta hydrolase fold-3 [*Halomonas titanicae* BH1]	271	52%	ELY21010.1
Lipolytic protein [*Halomonas* sp. S2151]	270	52%	WP_045994799.1
Hypothetical protein [*Halomonas* sp. PBN3]	267	54%	WP_023005267.1
Lipolytic enzyme [*Halomonas* sp. GFAJ-1]	266	56%	WP_009099840.1
Lipolytic protein [*Halomonas* sp. KHS3]	261	53%	WP_041157980.1
Lipolytic enzyme [*Halomonas* sp. A3H3]	259	53%	WP_022524062.1
Hypothetical protein [*Halomonas smyrnensis*]	255	54%	WP_016855596.1

Table 2. Amino acid differences between lipase of the five isolates with the *Halomonas elongata*

Position	liphelo	lipab4	lipab8	lipab15	lipab18	lipag18	Change of properties
34	T →	A	A	T	A	T	T (uncharged)→A (non polar)
98	C →	C	C	R	C	R	C (uncharged) →R (positive)
101	I →	I	I	L	I	I	I (non polar) →L (non polar)
106	R →	R	R	H	R	R	R (positip) → H (positip)
108	L →	L	L	L	L	I	L (non polar) →I (non polar)
124	T →	T	T	A	T	T	T (uncharged) →A (non polar)
150	P →	P	S	P	S	S	P (non polar) →S (uncharged)
154	V →	V	V	V	V	I	V (non polar) →I (non polar)
165	P →	L	P	P	P	P	P (non polar) → L (non polar)
166	T →	P	T	T	T	T	T (uncharged) → P (non polar)
167	L →	P	L	P	L	L	L(uncharged) → P (non polar)
168	Q →	E	Q	E	Q	Q	Q (uncharged) → E (negative)
184	T →	T	T	S	T	T	T (uncharged) → S (uncharged)
188	A →	D	A	T	A	A	A (non polar)→T;D (uc;neg)
189	I →	I	I	I	I	F	I (non polar) → F (non polar)
190	V →	V	V	A	V	V	A (non polar) → V (non polar)
191	D →	D	D	G	D	D	D (negative) → G (non polar)
195	M →	I	M	M	M	M	M (uncharged) → I (non polar)
197	D →	D	D	G	D	D	D (negative) →G (non polar)
199	D →	A	A	A	A	D	D (negative) → A (non polar)
210	T →	T	T	A	T	T	T (uncharged) → A (non polar)
234	I →	V	V	V	V	V	I (non polar) → V (non polar)
251	A →	A	A	A	P	P	A (non polar) → P (non polar)
Σ		7(9)	3(4)	11(14)	4(5)	4(7)	

five isolates showed several conserved regions, such as GGGX type, GXSXG (pentapeptide), oxyanion and catalytic triad i.e. serine, aspartate and histidine (Figure 3). The presence of these conserved regions suggesting that the isolates were assigned as a member of family IV (HSL=Hormone-Sensitive Lipase). Previous study reported that there were a number of bacterial proteins similar to mammalian HSL[37]. Moreover, family members of HSL are very wide, including lipases, esterases and carboxylesterases[2]. Determination of HSL sub family of the lipases

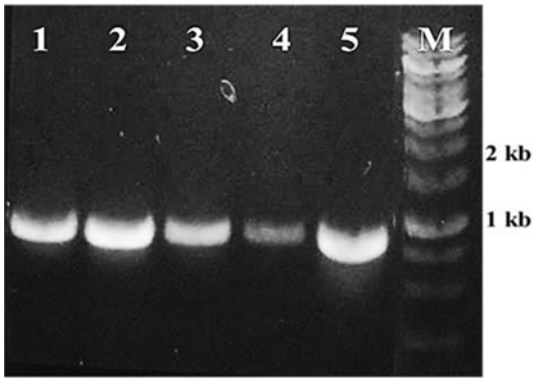

Fig. 1. The results of PCR amplifications of lipase genes. Assigned lane number is as follow 1=*lipab4*, 2= *lipab8*, 3= *lipab15*, 4= *lipab18*, 5=*lipag18* and M=DNA marker

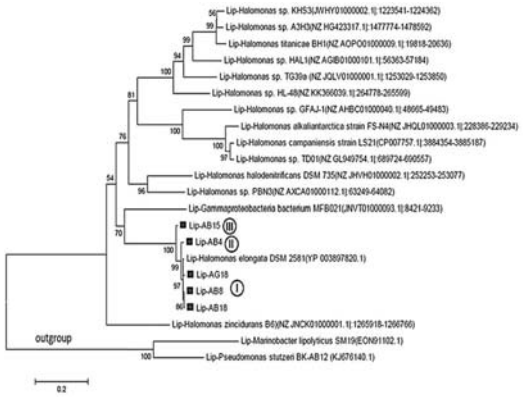

Fig. 2. Phylogenetic profile generated based on lipase sequences of the five isolates

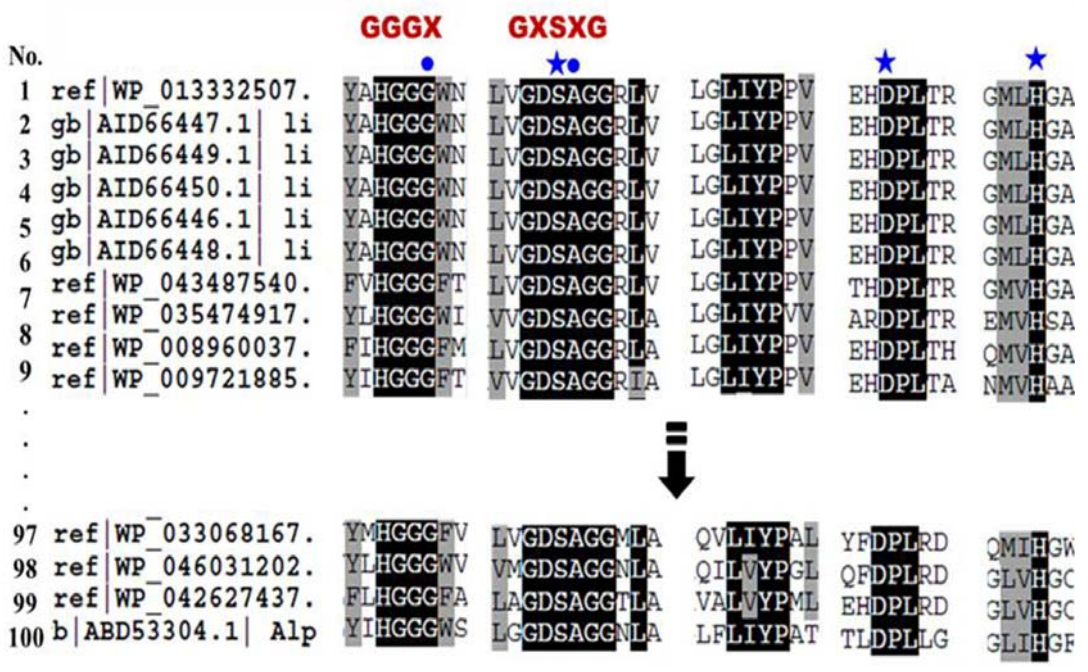

Fig. 3. Conserved region of lipase covers GGGX, GXSXG (pentapeptide), oxyanion and catalytic triad in 100 lipase sequences exhibited closer relation to lipase from the five isolates. A description of the figure above is as follows: oxyanion (●), catalytic triad (★): serine, aspartate and histidine. Lipase sequences is as follows: liphelo (1), five isolates (2-6) and other lipases closely related with five isolates (7-100)

were performed based on similarities profiles of conserved regions compared to the lipolytic, lipases, esterases/lipases, esterases and carboxylesterases on the of HSL family members. The results showed that there are few different regions among the isolates compared to that other lipases, as highlighed by numbers 1-15 (Figure 4). The five lipases are most likely similar to lipolytic

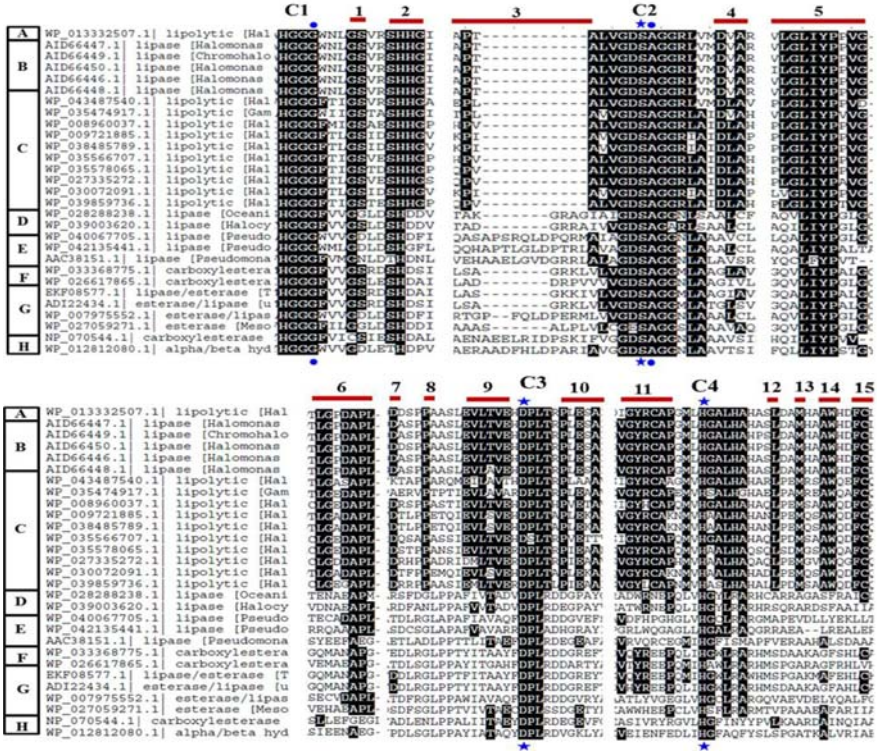

Fig. 4. Differences of conserved region among sequences of lipolytic, lipase, esterase/lipase, carboxylesterase that have close relations with lipase from the five isolates. Description of the above figure is as follows: C1-C4 is conserved regions that owned by each member of family IV lipase (HSL), includes: (C1). GGGX type, (C2). GXSXG (pentapeptide), Oxyanion (●), catalytic triad (★): serine, aspartate (C3) and histidine (C4). Thick lines on the top are regions that showed significant differences between the types of lipase. The sequence number (1-15) is a sequential appearance of such differences. Lipase sequences are as follows: (A). Reference lipase, (B). Lipase of five isolates, (C). Lipolytic of *Halomonas*, (D). Lipase of halophiles, (E). Lipase of *Pseudomonas*, (F). Carboxylesterase, (G). Esterase/lipase, (H). Carboxylesterase of thermophile and A/B hydrolase of archaea

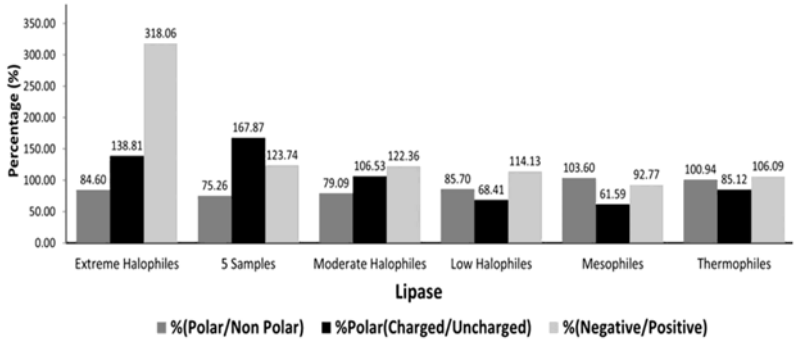

Fig. 5. Amino acid differences among lipase sequences of the five isolates and the other related microorganisms

of Halomonas, thereby including in moderate halophilic group of enzyme

Detail analysis by comparing the composition of amino acid residues showed that the amino acid composition of the lipases are more predominantly by negatively charged residues compared to that of the mesophilic and thermophilic (Figure 5). However, the composition of negatively charged residues of the five lipases are closer those of moderate halophilic than extreme halophilic. The negatively charged residues of extreme halophilic lipases are usually used to stabilize the protein structures against high salt concentrations[38].

Further analysis by comparing the amino acid sequences of the five lipases with *Halomonas*

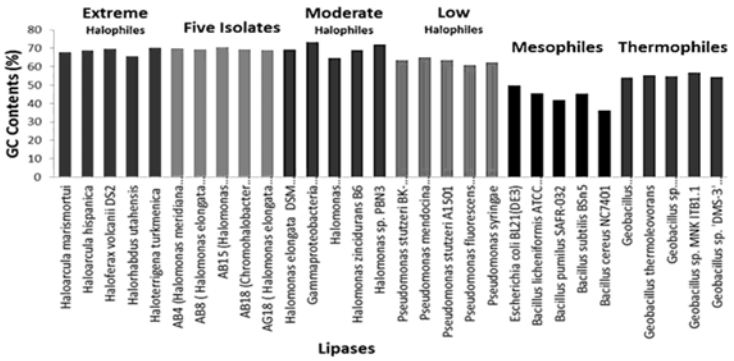

Fig. 6. Percentage of GC content of lipase genes among the five isolates and the other related microorganism

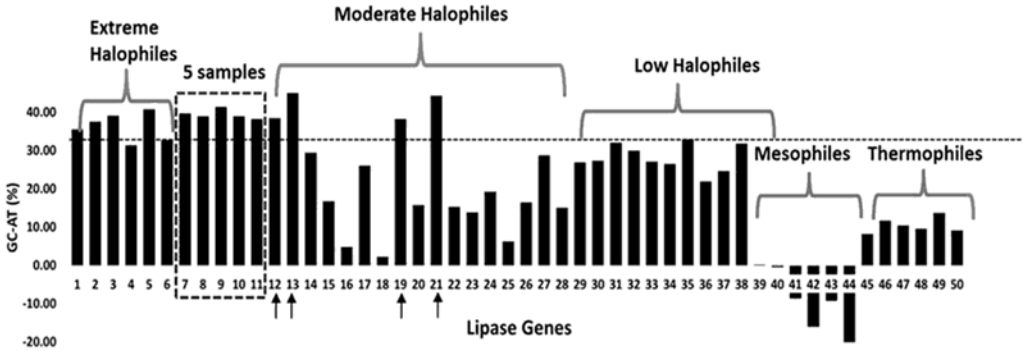

Fig. 7. GC-AT content of lipase between the five isolates and the other related microorganism. Identity of each numbered microorganism is as follow: (1) *Haloarcula marismortui* ATCC 43049, (2) *Haloarcula hispanica* ATCC 33960, (3) *Haloferax volcanii* DS2, (4) *Halorhabdus utahensis* DSM 12940, (5) *Haloterrigena turkmenica* DSM 5511, (6) *Natronomonas pharaonis* DSM 2160; (7) AB4, (8) AB8, (9) AB15, (10) AB18, (11) AG18, (12) *Halomonas elongata* DSM 2581, (13). *Gammaproteobacteria* bacterium MFB021, (14). *Halomonas halodenitrificans* DSM 735, (15). *Halomonas* sp. TG39a, (16). *Halomonas campaniensis* strain LS21, (17). *Halomonas* sp. HL-48, (18). *Halomonas alkaliantarctica* strain FS-N4, (19). *Halomonas zincidurans* B6 (20). *Halomonas titanicae* BH1 (21). *Halomonas* sp. PBN3 (22). *Halomonas* sp. KHS3 (23). *Halomonas* sp. A3H3 (24). *Halomonas* sp. HAL1 (25). *Halomonas* sp. TD01 (26). *Halomonas* sp. GFAJ-1 (27). *Chromohalobacter salexigens* DSM 3043 (28). *Marinobacter lipolyticus* SM19 (29). *Pseudomonas stutzeri* BK-AB12 (30). *Pseudomonas alcaliphila* BK-AG13 (31). *Pseudomonas mendocina* ymp (32). *Pseudomonas mendocina* NK-01 (33). *Pseudomonas stutzeri* A1501 (34). *Pseudomonas stutzeri* ATCC 17588 (35). *Pseudomonas putida* NBRC (36). *Pseudomonas fluorescens* A506 (37). *Pseudomonas syringae* (38). *Pseudomonas aeruginosa* (39). *Escherichia coli* KO11FL (40). *Escherichia coli* BL21(DE3) (41). *Bacillus licheniformis* ATCC 14580 (42). *Bacillus pumilus* SAFR-032 (43). *Bacillus subtilis* BSn5 (44). *Bacillus cereus* strain tsu1 (45). *Geobacillus stearothermophilus* strain ARM1 (46). *Geobacillus thermocatenulatus* (47). *Geobacillus thermoleovorans* (48). *Geobacillus* sp. 'Papandayan' ITB2.1 (49). *Geobacillus* sp. MNK ITB1.1 (50). *Geobacillus* sp. 'Domas' ITB3.1

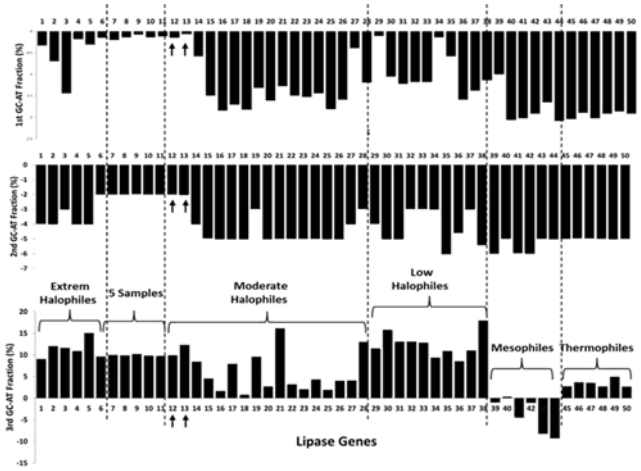

Fig. 8. The difference of bases GC-AT content fractions of "usage codon" of the lipase genes between the five isolates with the other related microorganism. Two moderates halophilics have similar GC-AT content of 1st, 2nd and 3rd usage codon pattern compared to five lipase genes ie. (12). *Halomonas elongata* DSM 2581, (13). *Gammaproteo bacteria* bacterium MFB021 (arrows sign in figure). Identity of each numbered microorganism is similar at figure 6

elongata DSM 2581 showed several differences at some positions. The differences were occurred at residue 98 in lipab15 and lipag18, at residue 168 in lipab4 and lipab15, at residue 188 in lipab4, at residue 191 and 197 in lipab15 and at residue 199 in lipab4, lipab8, lipab15 and lipab18 (Table 2). However, all of the differences were not found within the conserved regions, such as the catalytic site, pentapeptide or oxyanion regions.

Content of GC, GC-AT And GC-AT Codon Usage of Lipase Genes

Further analysis was conducted by comparing GC content or GC content on the codon usage in order to a unique characteristic of halophilic. The high GC content of the genome is a common feature of extreme halophilic[39,40,41]. GC content on the third (wobble) position of codon usage is corresponding to over-representation of the translated amino acid, which is for halophilic proteins correspond to the abundance of acidic residues[39]. Therefore, overrepresentation of acidic residues is an adaptation to high salinity that is apparent in the codon usage of the organism.

Based on the above studies, we were further investigated the halophilic properties by comparing content of GC, GC-AT and GC-AT of codon usage of the lipase genes with other microorganism. GC content analysis showed that the five lipase genes are closer to all groups of halophilic (Figure 6). The similarities of GC content of the genes with those of halophilic are unique characteristics of the isolates. This was correlating to their niches at high salinity level. Meanwhile, the result of GC-AT content analysis of the genes further exhibited a unique pattern as follows, 5 samples = average of halophiles > thermophiles > mesophiles (Figure 7). We further analyzed GC-AT content of codon usage on the first, second and third positions. The result showed the GC-AT content for the third base displayed similar unique pattern to the last two analysis (Figure 8). Therefore the GC-AT content for the third base of codon usage of the genes may be used as differentiator of halophilic lipase from various groups of microorganism. In general, the third base position of the gene is determinant base of codon preferences for each microorganism[42]. All of the analysis above suggest that the lipase genes of five isolates are closer to moderate halophilic, which is *Halomonas elongata* DSM 2581.

CONCLUSSION

Lipase genes have been successfully isolated from five moderately halophilic bacteria of Bledug Kuwu (BK) isolates and cloned into *E. coli* Top 10. The lipase of five isolates is classified as a member of family IV lipase (HSL=Hormone-

Sensitive lipase) as indicated by the existence of Gly-X-Ser-X-Gly pattern in their conserved regions. The conserved region of the five lipases has high similarities to that of lipolytic *Halomonas* and formed a distinct cluster with other types of HSL lipase, such as esterase/lipase and carboxylesterase. Amino acids composition of the five lipases are closer to the moderate halophilic group, in which they have more negatively charged residues than the mesophilic and thermophilic ones. Homology and phylogenetic profile of the five lipase genes distributed them into three groups i.e. Group I (*lipab8*, *lipab18* and *lipag18*) that closely related to lipolytic gene of *Halomonas elongata* DSM 2581, while groups II (*lipab4*) and III (*lipab15*) created new branches in the phylogenetic tree. Narrowing analysis to GC and GC-AT content has successfully revealed the unique pattern of the five-lipase genes compared to other microorganism, such as thermophiles and mesophiles. More specific analysis on GC-AT content of codon usage exhibited consistent result with both morphology and phylogenetic analyses above.

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