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Isolation and Identification of Halostable Lipase Producing Bacteria from the Bledug Kuwu Mud Crater Located at Purwodadi-Grobogan, Central Java, Indonesia

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In an effort to search new variants of extracellular lipase that stable under low water activity environment, we have been exploring the biodiversity of halophilic bacteria in a unique hypersaline environment, the Bledug Kuwu mud crater located in the mainland, Purwodadi-Grobogan, Central Java, Indonesia. Six bacteria exhibiting wide halotolerant property and high halostable lipase activity have been isolated. The 16S rRNAs genes of all six bacteria have been sequenced. Phylogenetic analysis of the 16S rRNA genes showed that five isolates belonged to moderate halophilic bacterium genus of *Halomonas* and *Chromohalobacter*, while the other one were highly similar to *Pseudomonas* sp., which is a low halophilic bacterium. Further analysis based on (GC-AT)% content in 16S rRNA genes from varios types of bacteria revealed general characteristics of halophilic bacteria from other types of bacteria and also highlighted the uniqueness of our isolates.

Key words: Phylogenetic profile, Halophilic bacteria, Halotolerant level, 16S rRNA, Halostable lipase.

Halophilic bacteria is a type of microorganisms that have ability to live in high salinity environment[1]. Naturally, such halotolerant property is attained by halophilic bacteria through salts and osmolytes (organic molecules) accumulation in their cytoplasm. This is happened to maintain osmotic balance between intra- and extra-cellular of bacteria[1,2]. The accumulation of salts and osmolytes make the bacteria do not only live in hypersaline condition but also in the region containing abundant organic compounds[3,4] or in general, the organism well grow under low water activity [5,6].

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There are three types of halophilic bacteria classified based on the halotolerant level i.e. low (0.3 – 5% NaCl), moderate (5 – 15% NaCl), and high (> 15% NaCl) halophile groups. Among them, moderate halophilic groups are the most interesting since their wider halotolerant level compared to the other group[7]. The organism, therefore, become a valuable resource to produce biomolecules that may also be functional and stable in various environmental conditions. It has been reported that some moderate halophilic bacteria that live in organic solvents are able to produce enzymes that also stable and active in organic solvents[4,8] and even remain active and stable in dry conditions[5,6]. This fact is open up opportunities for the enzymes to be developed as industrial biocatalyst, especially for those

industries that requiring process under low water activity, such as biodiesel production[9,10], palm oil degumming[11], fermentation of fish sauce[12,13], etc. Recently, halophilic bacteria are also widely used to degrade organic pollutant and crude oil[8,14] .

Halophilic bacteria usually live in the saltern ponds, salt water lake, salty mud and pickled food[1]. One habitat of the native Indonesian halophilic bacteria is the Bledug Kuwu (BK) mud crater, which is situated in the village of Kuwu, Purwodadi-Grobogan, Central Java, Indonesia (7°7'4"S 111°7'16"E). The uniqueness of this mud crater is it continuously produce high salinity of water with NaCl concentration of about 7.5%, although its location is far from sea. Moreover, periodic bursts of steam and methane gas is often occurred in the center area of the crater[15]. Parwata et al., (2014) has successfully isolated and characterized organic solvent-stable lipase from one of the halophilic bacteria *Pseudomonas stutzeri* obtained from this area. Lipase isolated from *P. stutzeri* has molecular weight of 29 kDa and has stability in some polar organic solvents such as methanol, ethanol, and acetone, and also in non-polar organic solvent, such as n-hexane[16].

There are still a lot of halophilic bacteria that we have isolated from brine of the mud crater BK but they have not yet identified for the types of bacteria and their enzymatic potentials. The purpose of this study is to screen moderate halophilic bacteria among all isolated bacteria and examine their potential to produce active halostable lipase. Phylogenetic analysis was employed to identify the screened halophilic bacteria based on their 16S rRNA gene sequences. By combining the obtained phylogenetic profile with the data of cells morphology, and halotolerant properties, we showed here that the identity of the bacteria can be revealed more accurately. In addition, general characteristics of halophilic bacteria can be revealed by analysis of (GC-AT)% content of 16S rRNA gene.

MATERIALS AND METHODS

Chemicals

Common chemicals with pro analysis grade were purchased from Merck (Germany) and Sigma-Aldrich (USA), bacterial growth medium such as tryptone, yeast extract obtained from Bio Basic (Canada), biochemical reagents such as dNTPs, PCR Buffer, Taq DNA Polymerase were purchased from Fermentas (USA) and Kapa Biosystems (USA), and oligonucleotides (primers) were ordered from Macrogen (South Korea) and Integrated DNA Technologies (Singapore).

Sampling

Sampling was carried out randomly at two different sources of brine derived from the BK mud crater. The two brines samples were consisted of salty water (pH 9.0 and ± 7.5% salinity) later it was called as "AG" sample and Borax water (pH 7.0 and salinity of ± 25%) then called as "AB" sample. The later sample was derived from concentrated AG sample, in which salt samples therein is mostly sodium chloride. Cultivation of bacteria was carried out at the location using modification of Luria Berthani Broth media[17] composed of 0.1% tryptone, 0.05% yeast extract and 10% NaCl. After that, cultures were incubated in a shaker incubator at room temperature with aeration rate of 150 rpm.

Isolation and Morphology Test

Isolation of bacteria were carried out by *4-quadrant streak plate* method on modification of Luria Berthani Agar media. Single colonies of various bacteria were obtained by replica plating method repeated at least four times. A single colony confirmation was undertaken by observing cell's shape morphology and Gram's staining[18].

Properties of Halotolerant and Lipase Activity of Bacteria

Determination of halotolerant property of bacteria samples were conducted based on the bacterial growth rate on LBA medium containing various NaCl concentration. While lipase activity was determined based on Rhodamine-B assay with the addition of olive oil as an inducer in LBA medium[19].

DNA extraction

DNA was extracted using a modified method of Zhou[20]. Cell pellet was resuspended in 300 mL DE buffer (100 mM Tris-Cl pH 8.0; 100 mM sodium EDTA; 100 mM phosphate buffer pH 8.0, 1.5 M NaCl and 1% CTAB), and then added 15 mL proteinase K 10 mg/mL followed by incubation at 37 ^{o}C for 30 minutes. The mixture was then added 30 mL of 10% SDS and followed with incubation at 65 ^{o}C for 1 h. The tube containing the mixture was gently inverted every 15 minutes. After that, the mixture was centrifuged at 6000 × g for 10 min. The

supernatant was separated from the debris and then it was added with a mixture of chloroform: isoamyl alcohol with the ratio of 24:1 from the supernatant volume. The mixture was stirred gently, and then centrifuged at 7000 × g at 4 °C for 5 minutes. The supernatant in the water phase (at the top) was transferred into a new sterile micro tube. Isopropanol with a total of 0.6 parts by volume was added to the supernatant andstirred gently followed by incubation at room temperature for 30 minutes. The mixture was then centrifuged under 12,000 × g at 4 °C for 20 minutes. DNA pellets were separated from the supernatant, washed with 70% ethanol and centrifuged under 12,000 × g at 4 °C for 10 minutes. DNA pellets were separated from the supernatant and dried with a concentrator followed with resuspension by 25-50 mL ddH_2O and stored at 4 °C. The obtained DNA solution will be used for PCR amplification in the next work.

Amplification and Sequencing of 16S rRNA Gene

The 16S rRNA genes of the six isolates were amplified by *Polymerase Chain Reaction* (PCR) technique[21] using a pair of specific primers. 16S rRNA gene of individual bacteria was amplified using a pair of universal primers, namely UniB1 (Univ1492R: 5'-GGTTAC(G/C)-TTGTTACGACTT-3') and BacF1 (Bac27F: 5'-AGAGTTTGA-TC(A/C)TGGCTCAG-3')[22,23]. A typical PCR mixture (50 µL in volume) was prepared with the following components: 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% (v/v) Nonidet P40, 2.5 mM $MgCl_2$, a 250 µM of each deoxynucleoside triphosphate, 0.25 µM of each primers, and 1.25 U of *Taq* DNA polymerase (Thermo scientific). The following PCR conditions were set to amplify halophilic bacteria 16S rRNA gene: an initial denaturation temperature was set to 94°C for 4 min, followed by 35 cycles of denaturation @30 s at 94°C, an annealing temperature was set to 48 °C for 30 s , while for an extention and a final extention were programmed to occurred at the same temperature i.e. 72°C for 2 min and 5 min, respectively.

The PCR products were verified by electrophoresis that conducted on 1% agarose in bufer solution TAE 1x (diluted from 1 L bufer solution stock of TAE 50x containing 242 g Tris Base, 57.1 mL Acetic Acid Glacial and 50 mM EDTA pH 8.0) using submerged horizontal electrophoresis cell (BioRad) for 50 minute at 70 volt. In order to obtain complete sequences of 16S rRNA genes in each samples, an automated DNA sequencer (Macrogen, Korea) was employed with a direct sequencing method to PCR products using four pairs of PCR primers.

Sequence and Phylogenetic analysis

In order to get the correct sequences, it is necessary to do validation to the result of sequencing. The electrophoregram data from the sequencing must firstly be analyzed using Sequence scanner 2 (Applied Biosystems, 2012). In order to combine some sequences, we used DNA Baser Sequence Assembler v3 program (Heracle BioSoft, 2012).

Homologies of each isolate sequence were analyzed using online software "NCBI-Blast", via the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). About a hundred of high homologous sequences were found by Blast program and these sequences data will be used to generate a phylogenetic profile. MEGA 6 program[24] was employed to generate phylogenetic profile based on the neighbor-joining clustering method. In order to generate such profile, initially multiple sequence alignment between a sequence of each isolate together with their homologous sequences (^{3} 99% identity) obtained from Blast program was undertaken using clustalW2 program. The resulted alignment file then becomes an input for MEGA 6 program to generate phylogenetic profile.

RESULTS AND DISCUSSION

Morphology Test, Halotolerant Properties and Lipase Activity of Bacteria

Our effort to seek moderate halophilic bacteria with high lipase activity has found six from 40 bacterial isolates that meet with the criteria. Four of them were assigned as AB4, AB8, AB15 and AB18 and the other two were AG13 and AG18. These six bacteria, called as BK isolates, have a broad halotolerant level and relatively high lipase activity. AG13 produced lipase with the highest activity but exhibited the lowest halotolerant level, the opposite was observed to AG18 (Table 1). The morphology observation to the six bacteria showed that they have rod-like shape and also Gram-negative (Table 1).

PCR amplification and Sequencing

Amplification of 16S rRNA genes of the six BK isolates were performed by PCR technique,

while their nucleotide sequences were determined by the sequencing method. PCR amplification of 16S rRNA gene using BacF1-UniB1 pairs of primers have successfully amplified full-sized gene with the length about 1490 bp (Fig. 1)

Sequence and Phylogenetic Analysis

The validation of the sequencing result revealed that the six 16S rRNA genes of BK isolates were partialy sequenced with the length from 1432 to 1474 bp. The sequence alignment of each 16S rRNA gene was carried out by BlastN program to generate the list of known bacteria in the genebank with high similarity (99–100% identities) to our samples. The alignment result showed that four bacteria have high similarity to *Halomonas* sp., one was close to *Chromohalobacter* sp, and the other one were close to *Pseudomonas* sp (Table 2).

The Blast alignment results were subsequently used as an input data for phylogenetic analysis. The analysis based on 16S rRNA genes alignment gave rise to the phylogenetic profile containing five clustered bacterial species comprised of three clusters have phylogenetic relationships with the genus of *Halomonas* and

Table 1. Halotolerant level, lipase activity, and morphology of six BK isolates from Bleduk Kuwu Mud crater

Isolates	Halotolerant (NaCl %)	Lipase activity	Cell Shape	Gram's
AB4	0.5 – 22.5	+	Rod	negative
AB8	5.0 – 27.5	++	Rod	negative
AG13	0.1 – 7.5	+++	Rod	negative
AB15	5.0 – 27.5	++	Rod	negative
AB18	5.0 – 22.5	++	Rod	negative
AG18	0.5 – 30.0	++	Rod	negative

Table 2. The result of BlastN alignment for 16S rRNA genes of the six BK isolates

Samples	Description	Coverage (%)	Identity (%)
AB4	*Halomonas meridiana* DSM 5425	100	100
	Halomonas aquamarina DSM 30161	100	99
AB8	*Halomonas elongata* 1H9	100	99
	Halomonas eurihalina ATCC 49336	99	99
AB15	*Halomonas elongata* 1H9	100	99
	Halomonas eurihalina ATCC 49336	98	99
AB18	*Chromohalobacter japonicus* 43	100	99
	Chromohalobacter canadensis ATCC 43984	100	99
AG18	*Halomonas elongata* 1H9	100	99
	Halomonas eurihalina ATCC 49336	99	99
AG13	*Pseudomonas alcaliphila* AL15-21	99	99
	Pseudomonas pseudoalcaligenes Stanier 63	99	99

Table 3. Genbank ID of 16S rRNA genes of the six BK isolates

Isolates	Bacteria	Genbank ID 16S rRNA
AB4	*Halomonas meridiana* BK-AB4	KJ185378.1
AB8	*Halomonas elongata* BK-AB8	KJ185379.1
AB15	*Halomonas eurihalina* BK-AB15	KJ185380.1
AB18	*Chromohalobacter japonicus* BK-AB18	KJ185381.1
AG18	*Halomonas elongata* BK-AG18	KJ185382.1
AG13	*Pseudomonas alcaliphila* BK-AG13	KJ185384.1

Table 4. The GC content of 16S rRNA genes in the six BK isolates and the other related bacteria

Bacteria	%GC16S rRNA
AB4	56.22
AB8	56.51
AB15	56.51
AB18	57.89
AG18	56.51
Halomonas elongata 1H9	56.58
Halomonas meridiana DSM 5425	56.22
Halomonas eurihalina ATCC 49336	56.51
Chromohalobacter beijerinckii Delft E.III.9.23.1	57.26
Chromohalobacter canadensis ATCC 43984	57.40
Chromohalobacter japonicus 43	56.98
Chromohalobacter salexigens DSM 3043	57.53
AG13	53.72
Pseudomonas mendocina ymp	53.80
Pseudomonas mendocina NK-01	53.80
Pseudomonas alcaliphila AL15-21	53.99
Escherichia coli BL21 (mesophiles)	54.80

one cluster closed to the genus of *Pseudomonas*. Cluster I (AB8, AB15 and AG18), cluster II (AB18), and cluster III (AB4) phylogenetically closed to *Halomonas elongata* 1H9[25], *Chromohalobacter japonicus* 43[26], and *Halomonas meridiana* DSM 5425[27], respectively. While cluster IV (AG13) were

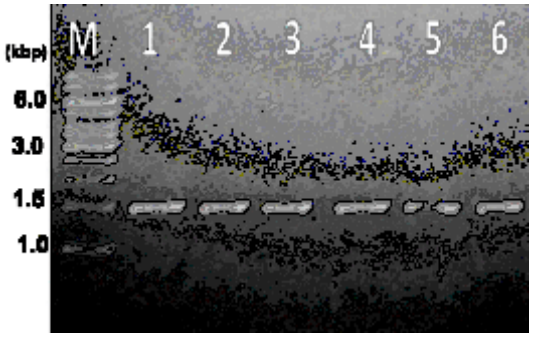

Fig. 1. PCR amplifications result of 16S rRNA gene of the six BK isolates. The assigned lane number is as follow: 1=AB4, 2=AB8, 3=AB15, 4=AB18, 5=AG18, 6=AG13 and M=DNA marker

Table 5. Halotolerant level and growth temperature of the six isolates and the other related bacteria.

Microorganisms[Ref]	NaCl% (w/v)	Temp.(^{0}C)
Haloarcula marismortui[37]	20.4–23.4	40–50
Haloferax volcanii[38]	10.2–15	30–40
Haloarcula hispanica[39]	16–29	28–42
Halorhabdus utahensis[40]	9–30	17–55
Haloterrigena turkmenica[41]	e"12	29–57
Halomonas elongata[31]	0–20	5–45
Halomonas eurihalina[32]	0.5–25	5–45
Halomonas meridiana[27]	0–20	5–45
Chromohalobacter beijerinckii[33]	0.5–25	5–42
Chromohalobacter canadensis[26]	3–25	15–45
Chromohalobacter japonicus[26]	5–25	15–42
AB4 (This study)	0.5–22.5	25–45
AB8 (This study)	5.0–27.5	25–45
AB15 (This study)	5.0–27.5	25–45
AB18 (This study)	5.0–22.5	25–45
AG18 (This study)	0.5–30.0	25–45
AG13 (This study)	0.1–7.5	25–40
Pseudomonas alcaliphila[28]	3–7	4–30
Pseudomonas mendocina[34]	1.2–7.2	25–37
Pseudomonas stutzeri[35]	0.1–9	30–46
Pseudomonas aeruginosa[36]	2–10	25–45
Escherichia coli [42]	0.5–3	7–46
Bacillus licheniformis[43]	1.5–10	25–45
Bacillus cereus[44]	2 –7	20–40
Geobacillus stearothermophilus[45]	0.1–dŠ5	37–65
Geobacillus thermocatenulatus[45]	0.1–4	35–78
Geobacillus thermoleovorans[46]	0.1–5	45–70

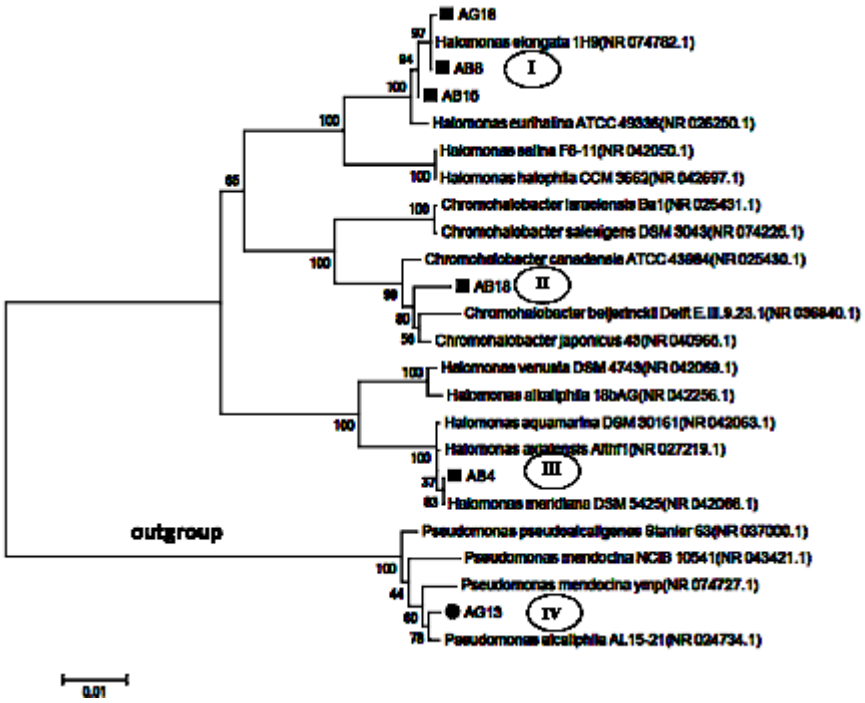

Fig. 2. Phylogenetic profile generated based on 16S rRNA gene of the six BK isolates

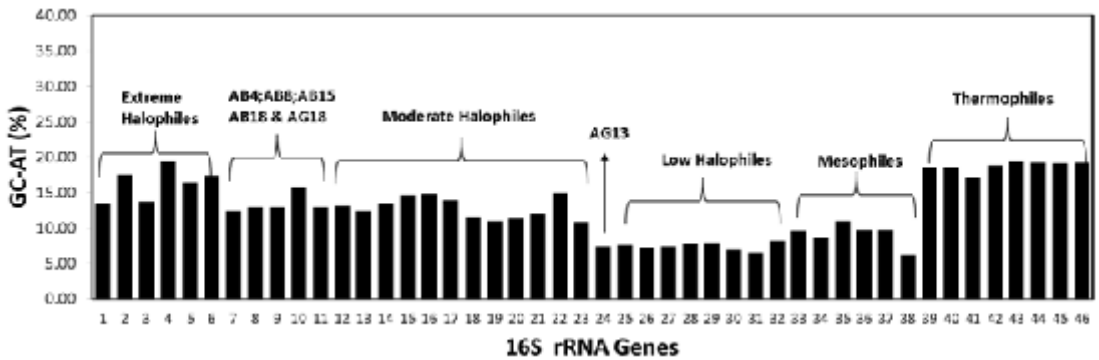

Fig. 3. (GC-AT)% content of 16S rRNA between the six BK isolates and the other related bacteria from genbank database. Identity of each numbered bacteria is as follow: *Haloarcula marismortui* ATCC 43049 (1), *Haloarcula hispanica* JCM 8911 (2), *Haloferax volcanii* DS2 (3), *Halorhabdus utahensis* DSM 12940 (4), *Haloterrigena turkmenica* s DSM 5511(5), *Natronomonas pharaonis* JCM 8858 (6), AB4 (7), AB8 (8), AB15 (9), AB18 (10), AG18 (11), *Halomonas elongata* 1H9 (12), *Halomonas meridiana* DSM 5425 (13), *Halomonas eurihalina* ATCC 49336 (14), *Chromohalobacter beijerinckii* Delft E.III.9.23.1(15), *Chromohalobacter canadensis* ATCC 43984 (16), *Chromohalobacter japonicus* 43 (17), *Halomonas boliviensis* LC1(18), *Halomonas* sp. GFAJ-1(19), *Halomonas* sp. HAL1(20), *Halomonas* sp. TD01(21), *Chromohalobacter salexigens* DSM 3043 (22), *Marinobacter lipolyticus* SM-19(23), AG13 (24), *Pseudomonas mendocina* ymp (25), *Pseudomonas mendocina* NK-01 (26), *Pseudomonas stutzeri* A1501(27), *Pseudomonas stutzeri* ATCC 17588 (28), *Pseudomonas alcaliphila* AL15-21(29), *Pseudomonas putida* NBRC 14164 (30), *Pseudomonas fluorescens* A506 (31), *Pseudomonas aeruginosa* RP73 (32), *Escherichia coli* BL21(33), *Escherichia coli* KO11FL (34), *Bacillus licheniformis* ATCC 14580 (35), *Bacillus pumilus* SAFR-032 (36), *Bacillus subtilis* BSn5 (37), *Bacillus cereus* NC7401 (38), *Geobacillus kaustophilus* HTA426 (39), *Geobacillus stearothermophilus* ARM 1 (40), *Geobacillus thermodenitrificans* NG80-2 (41), *Geobacillus thermocatenulatus* (42), *Geobacillus thermoleovorans* (43) *Geobacillus* sp. 'Manuk' (44), *Geobacillus thermoleovorans* DMS3 (45), *Geobacillus* sp. enrichment culture clone P12 (46)

respectively closed to *Pseudomonas alcaliphila* AL15-21[28] (Figure 2).

The six 16S rRNA sequences of BK isolates have been submitted to GenBank database (https://www.ncbi.nlm.nih.gov) and can be accessed using the GenBank ID listed in Table 3.

Comparison of DNA composition between the six isolates and other related microorganisms

Based on the results of the halotolerant screening (Table 1) and the phylogenetic analysis to the six BK isolates above (Figure 2), it has been shown that the genus of *Halomonas* and *Chromohalobacter* were classified as moderate halophilic bacteria with halotolerant level in the range of 5–20% NaCl. Whereas, the genus *Pseudomonas* was classified as low halophilic bacteria (2–5% NaCl). We further investigated the characteristics of these six isolates by comparing the percentage of GC and (GC-AT)% content in 16S rRNA of those six bacteria compared to those of other bacteria. The purpose of this evaluation is to know whether halophilic bacteria isolated from the mud crater Bledug Kuwu has particular characteristics in terms of percentage of GC or (GC-AT)% content.

The GC content of 16S rRNA genes of five isolates (AB4, AB8, AB15, AB18 and AG18) was in the range of 56.22–57.89%, which close to that of bacteria from the genus of *Halomonas* and *Chromohalobacter*. Whereas, GC content of other isolate (AG13) were similar to the genus of *Pseudomonas*, i.e. 53.72%. The GC content presented above, five isolates (AB4, AB8, AB15, AB18 and AG18) are higher than mesophilic bacteria, such as *Escherichia coli* BL21, which has GC content i.e. 54.80, otherwise AG13 is the lowest (Table 4).

Furthermore, we compared the difference between %GC and %AT contents or (GC-AT)% of all six samples with those of other related bacteria having different halotolerance and thermostability properties. (GC-AT)% of all 16S rRNA genes bacterial samples showed that extreme halophilic = thermophilic > moderate halophilic > low halophilic > mesophilic (Figure 3). The different composition of GC and AT in 16S rRNA genes are correlation with intracellular conditions of halophilic microorganisms require greater number of GC content to prevent DNA denaturation due to high intracellular salinity and also to avoid the effect of UV irradiation. All of that may cause the formation of thymidine dimers that may lead to a gene mutation[29,30]

Identification of Halophilic Bacteria Isolates Bledug Kuwu

In addition to the sequence analysis (sequence alignment, phylogenetic profile, and DNA compositions), we also considered the other non-sequence characteristics i.e. halotolerant and thermostability properties to validate the identification result based on only sequence analysis for the six halophilic bacteria. This additional analysis, therefore, was carried out to prevent a false positive in identification of those six bacterial isolates. The analysis was conducted by comparing the properties of BK bacterial isolates with other bacteria that have similar properties and also with bacteria having different property (outgroup). Based on our experiment in varying the growing conditions of five isolates (AB4, AB8, AB15, AB18 and AG18), we noted that the halotolerant and thermostability properties of these bacteria were similar to *Halomonas*[27,31,32] and *Chromohalobacter*[26,33], in which they can grow-well at NaCl concentration in the range of 0.5–25% (w/v) and at temperature range of 5–45 °C. As a result, those five bacterial isolates were classified as moderate halophilic bacteria. The other sample (AG13) were observed to have similarity to halotolerant and termostablity properties of *Pseudomonas* sp.[28,34,35,36], where they can survive at NaCl concentrations of 0.1–10% and at 25–46 °C. Therefore, we classified them as low halophilic bacteria. Comparing with the external comparator (outgroup), such as extreme halophilic archaea (genus *Haloarcula*, *Haloferax* etc.)[37,38,39,40,41] with halotolerant range of 10–30% (w/v) NaCl and grow well at 17–57 °C, mesophilic bacteria (genus *Escherichia*)[42] with halotolerant range of 0.5–3.0%(w/v) of NaCl at 17–46 °C, and thermophilic bacteria (genus *Geobacillus*)[46,47] that grow well at NaCl concentration range of 0.1–5% and at 35–78 °C (Table 5), we found a significant differences between the natures of our six isolates and those outgroup bacteria, especially thermophilic and mesophilic groups.

CONCLUSION

Six Bledug Kuwu bacterial isolates have been successfully isolated and identified. All cell have rod-like shape, Gram's negative and potential

to produce halostable lipase. Based on 16S rRNA analysis, the five isolates were classified as moderate halophilic bacteria and closely related to the genus *Halomonas* and *Chromohalobacter*, and one isolate classified as low halophilic bacteria and closer to the genus *Pseudomonas*.

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