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Diversity of Microbial Thermophiles in a Neutral Hot Spring (Kawah Hujan A) of Kamojang Geothermal Field, Indonesia

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Thermophilic microbial community in one of Kamojang hot springs, namely Kawah Hujan A, was analysis through 16S rRNA gene sequences combined with denaturing gradient gel electrophoresis (DGGE). The analysis was based on culture-independent and culture dependent strategies. Kawah Hujan A is a neutral hot spring with pH at 7.3 and low concentration of cation and anion. Microbial communities obtained from culture-independent were close to γ-proteobacteria, however, detail comparison among the member of γ-proteobacteria showing some sequences variation compared to the published data especially on the hypervariable and variable regions. In addition, the sequences did not belong to certain genus. Meanwhile, the 16S rDNA sequences from culture-dependent samples revealed different sequences compared to that from the culture independent sample. Phylogenetic and homological analysis of the sequences from cultivation samples showed that the sequences were close to Firmicute and Deinococcus phyla, including *Geobacillus*, *Anoxybacillus* and *Thermus* genera.

Key words: Thermophiles, 16S rRNA, DGGE, community analysis.

Geothermal region are recognized as sites that harbor unique biological ecosystems that are emphasized by chemosynthetic microbial primary production. Microbial community structure is influenced by the environmental conditions at a specific site, such as temperature, pressure[1], pH, organic content, the presence of electron donor/acceptor, and light availability[2,3]. The microbial community structure has been extensively studied in several geothermal habitats, e.g., in subterranean hot springs in Iceland[4], Japan[5], and Yellowstone National Park[3,6]. However, the studies of microbial community from Indonesian geothermal fields are very limited[7]. Indonesia is a country with a number of volcanoes and a lot of geothermal area. There are at least 120 volcanic centers that are spread over volcanic belts of 7000 km along the Indonesian islands[8]. Kamojang is one of these geothermal fields that located in West Java, Indonesia, at an altitude of 1500 m.

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The Kamojang geothermal field is the first operational geothermal field for electricity power in Indonesia. It has been producing electricity since 1983. The estimated field area is about 21 km^2, with a potential of about 300 MWe[9]. Kamojang geothermal field is now vapor-dominated but the hydrothermal minerals show that the rock-altering fluid were dominantly liquid. The primary minerals present in the Kamojang subsurface rock samples are mainly feldspar (andesine-labradorite), pyroxene (hypersthene and augite), and olivine (forsterite)[10]. There are two distinctive hydrothermal mineral assemblages at Kamojang, namely the acid and the neutral assemblages, which occur in shallower and deeper levels, respectively. The acid assemblage occupies the shallower level of the system (from near surface down to 100-300 m), and is characterized by the presence of kaolin with or without smectite, alunite, quartz, cristobalite, and pyrite. The deeper, neutral assemblages, comprises quartz, adularia, albite, epidote, titanite, wairakite, laumontite, calcite, siderite, titanohematite, pyrite, anhydrite, smectite, chlorite, illite, and interlayer clays[10].

The surface manifestations in the Kamojang area consist of hot pools, fumaroles, mud pots and hot springs lying in the so called Kawah Kamojang thermal area. Most of the hot surface water contains high concentration of sulfate (1000-2000 ppm) but low concentrations of chloride (< 5 ppm)[11]. The isotopic evidence suggests that the water is local meteoric water which has been heated by steam containing hydrogen sulfide, which oxidizes to sulfuric acid to give water of a low pH and high sulfate concentration[10]. Two warm springs of neutral pH waters were reported by Healy and Mahon[11] to occur 2 km south of the main activity.

Here we report the microbial community analysis of the neutral hot spring, namely Kawah Hujan A at Kamojang Geothermal Area, West Java, Indonesia. The analysis was based on culture-independent and culture-dependent strategies to get a first insight into the microbial communities in this neutral ecosystem. PCR amplification and DGGE separation of rRNA gene fragments were used to profile the microbial communities.

MATERIAL AND METHODS

Site and sample collection

The Kamojang geothermal field is located in West Java Province, Indonesia, about 35 km south of Bandung. Kawah Hujan A (E 107°48'14.67", N -7°8'21.33", the altitude 1690 m) is one of the hot springs in Kamojang Geothermal field. The hot spring is a high pressure geyser. Water samples were collected in January 2007. For assessing microbial diversity by culture-independent method, water sample was filtered through a 0.22-µm-pore-size cellulose membrane filter (Sartorius, Germany) within 4 h after sampling. The cells on membrane were re-suspended in 25 ml of STE buffer (10 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 1 mM EDTA) and precipitated by centrifugation. Pellet containing microbial communities were stored at -20°C until DNA extracted. Cultivation process was carried out by incubating spring water at 70°C after added by variation of nutrients. Five nutrient composition were used as enrichment media, namely as P (0.1% (w/v) peptone), T (0.05% (w/v) yeast extract and 0.05% (w/v) tryptone), LB (0,25% (w/v) tryptone; 0,25% (w/v) NaCl; 0,125% (w/v) yeast extract), PB (0.1% (w/v) beef extract, 0.2% (w/v) peptone), and CD (Czapek Dox)[12] media.

Geophisico-chemical analysis

pH and temperature were measured at January 2007. Cations concentration analysis of water sample were perform using AAS (GBC Avanta Ver. 2.02) method and anions concentration were determine by titration, turbidimetry, spectrophotometry methods.

Bead beating-based DNA extraction

The pellet containing microbial cells were mixed with DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl), glass beads and proteinase K (10 mg/ml) in microcentrifuge tubes by vortexing (Genie, G 560E, USA) at medium vigorous (half of maximum speed) for 15 min at room temperature. After mixing, 20% SDS was added, and the samples were incubated at 65°C for 2 h with gentle end-over-end inversions every 15 to 20 min. 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 resuspended in sterile deionized water.

Lysozyme-based DNA extraction

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 resuspended in sterile deionized water.

Amplification of 16S rRNA gene fragments

The amplification of partial 16S rRNA genes was carried out as described previously by Ferris[13]. A set of primers were used to amplify about 323-bp section of the 16S rRNA genes of members of the domain Bacteria, including the highly variable V7-V8 region[14]. One primer complements a region conserved among members of the domain Bacteria (*Escherichia coli* positions 1055 to 1070; primer sequence 5'ATGG CTGTCGTCAGCT-3'). The other primer is based on a universally conserved region (*E. coli* positions 1392 to 1406; 5'CGC C C G C C G C G C C C C G C G C C C C G G C C C G C C G C C C CCGCCCCACGGGCGGTGTGTAC-3') and incorporates a 40-base GC clamp. The specificity of this primer is imparted by the underlined region. PCRs were performed by using cloned *Taq* DNA polymerase according to the instructions provided by the manufacturer (Promega). The temperature cycle for the PCR was 1 min of denaturizing at 94°C, 1 min of annealing, and 1 min of primer extension at 72°C. During an initial touchdown cycle, the annealing temperature was continually decreased from 53 to 43°C in intervals of 1°C per cycle; 20 additional annealing cycles were performed at 43°C. The final primer extension was for 10 min at 72°C.

DGGE and re-PCR of DGGE bands

DGGE was performed by using D-code systems (Bio-Rad Laboratories) with a 1.5-mm gel. Approximately 100- to 500-ng portions of PCR products were used directly onto 8% (w/v) polyacrylamide gels with denaturing gradients from 30 to 40% (100% denaturant was 7 M urea and 40% [v/v] deionized formamide). Electrophoresis was performed with 0.5X TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA) at 200 V and 60°C for 4 h. After electrophoresis, the gels were stained with silver staining method[15]. Each band in the DGGE gel was excised with a razor blade, added with TE buffer (10mM Tris-Cl, 1 mM EDTA), placed in boiling water for 5 minutes, and then incubated overnight at 37°C. The eluted DNA was amplified using the same primers as previous amplification but without GC clamp sequence. PCRs were performed at the following conditions: an initial denaturizing step at 94C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C, and a final extension step of 10 min at 72°C. All of confirm DGGE bands 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[16] for screening of sequence similarity. Sequences alignments were performed by ClustalW program[17]. 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[18]. Phylogenetic trees were constructed from distance matrices by the neighbor-joining method[19], which

was implemented with the NEIGHBOR program. The node reproducibility for tree topology was estimated by bootstrap analysis, which included 1000 replicate data sets. Sequences were checked for chimeras using the CHECK_CHIMERA program of the Ribosomal Database Project[20].

Nucleotide sequence accession numbers

All of the nucleotide sequences from this study have been deposited in the GenBank database under accession numbers EU625407 to EU625429.

RESULTS

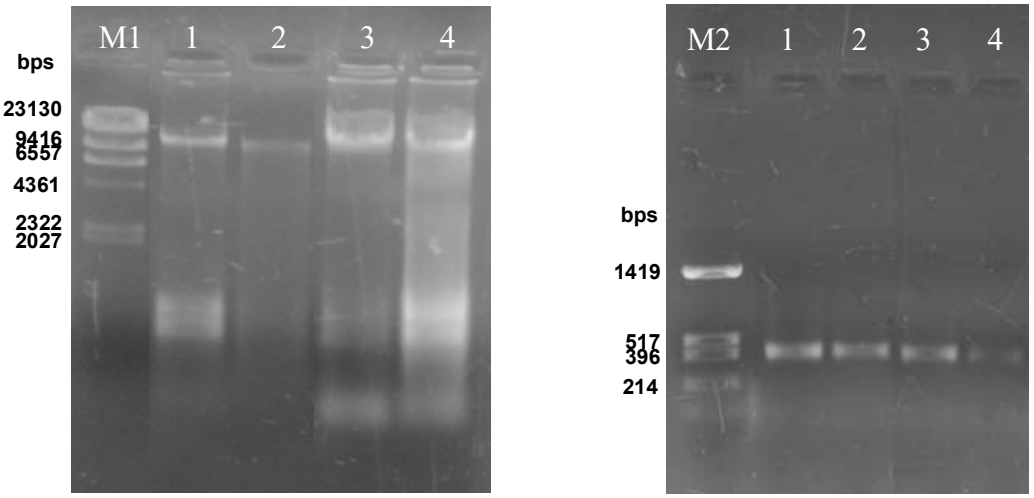
Geochemical characteristic

The pH of Kawah Hujan A was 7.2 - 7.4 and temperature was 80 - 84° C. All of ions including sulfate and chloride in this spring are in low concentration (Table 1). Nitrate and some transition metal such as Pb, Mn, and Cu were not detected by AAS method.

Chromosomal DNA and DGGE profile

Two DNA extraction methods, lisozyme-based and bead beating-based methods were performed independently to culture-independent sample, while chromosomal DNA from culture-dependent samples were performed only by bead beating-based method (Fig. 1a). PCR amplifications of 16S rRNA gene fragments were successfully carried out for both culture-dependent and culture-independent samples. All of amplification results produced a single band in agarose gel electrophoresis with the size of approximately 400 bp (Fig. 1b).

DGGE profile of 16S rRNA fragments performed by culture-dependent and culture-independent samples showed slightly different pattern. However, all of bands were appeared at the upper side and lower side of the gel (Fig. 2). DGGE profiles obtained from filtration sample have more bands compared than that from cultivation samples, some bands at lower side of the gel only appeared in filtration sample. Differences in cell lysis methods of filtration samples produced a similar band pattern (Fig. 2, lane 6 and 7). In cultivation samples, DGGE profiles from variation media showed similar pattern. Most of cultivation samples showed one



(a)

(a). Chromosomal DNA from culture-dependent and culture-dependent samples. M1, λ/*Hind*III DNA marker; (1-2) chromosomal DNA from culture-independent prepared by bead-beating and lyzozyme methods respectively; (3-4) chromosomal DNA from culture-dependent prepared from LB and P media.

(b)

(b). Amplicon of 16S rRNA gene fragment. M2, pUC19/*Hinf*I DNA marker; (1-2) amplicons from culture-independent prepared by bead-beating and lyzozyme methods respectively; (3-4) amplicons from culture-dependent prepared from LB and P media.

Fig. 1. Agarose gel electrophoresis of chromosomal DNA and 16S rDNA amplicons

intense band with align position at lower side of the gel, except for culture in T medium (Fig. 2, lane 1). Bands at the upper side of the gel showed a similar pattern. Most of the DGGE bands were successfully re-amplified and sequenced. The sequences estimated as chimeric artifact were excluded from further analysis.

Phylogenetic and Diversity of community

The majority of the sequences recovered from filtration sample were affiliated with the ã-Proteobacteria, and these sequences formed distinct cluster on phylogenetic tree (Fig. 3). Only one sequence was likely close to â-Proteobacteria (Fig. 3, KHA-Z-1). Phylogenetic tree constructed from the sample obtained by lysozyme-based and bead beating-based DNA extraction performed similar pattern (data not shown).

Table 1. Concentrations of total soluble constituents of Kawah Hujan A. (ND), not detected

Analyte	Concentrationmg/ml
Ca	27.79
Mg	0.22
Fe	0.08
Mn	ND
Na	13.64
K	7.25
Pb	ND
Cu	ND
Cl^-	18.04
SO_4^{-2}	156.59
NO_3^-	ND
NO_2^-	0.01
F^-	0.71

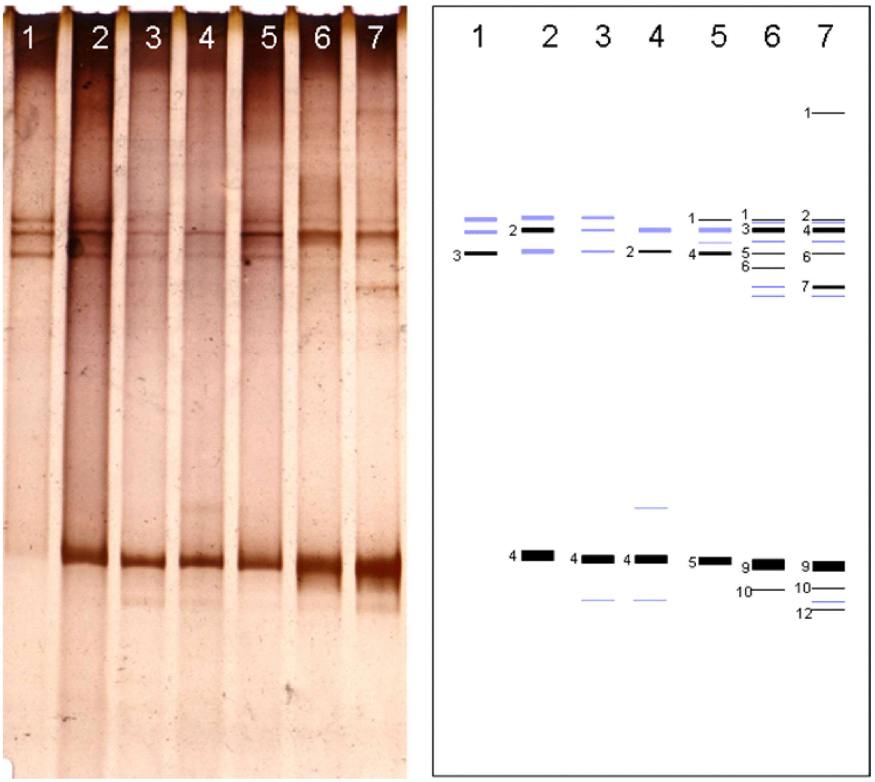


Fig. 2. DGGE profiles of Kawah Hujan A samples. The individual bands that have been further analysis presented in number (1 – 12). Lane 1-5, PCR product from cultivation samples (T, PB, P, CD, LB media) respectively. Lane 6-7, PCR product from filtration samples (lysozyme and bead-beating methods) respectively.

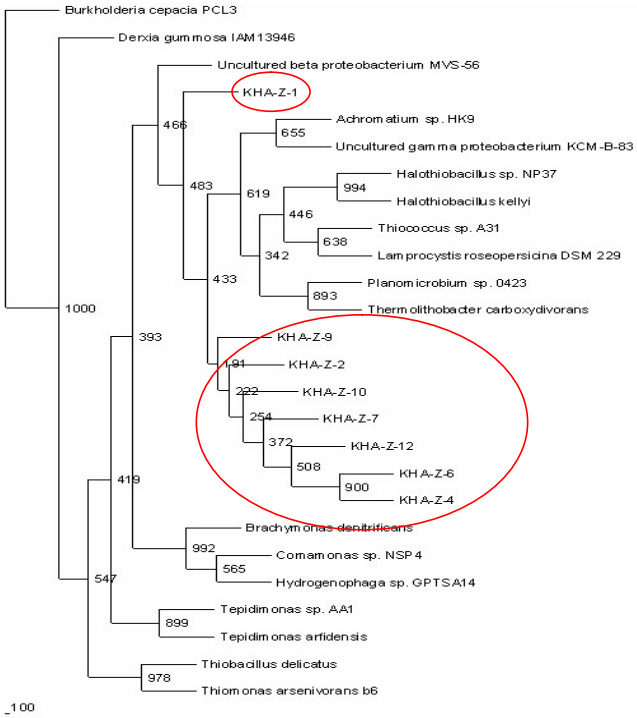


Fig. 3. Phylogenetic relationships of the 16S rRNA gene fragment sequences obtained from the culture-independent samples and related sequences, based on a distance analysis (neighbor-joining algorithm with F84 model; 1,000 bootstrap replicates performed). (circle), represented the samples.

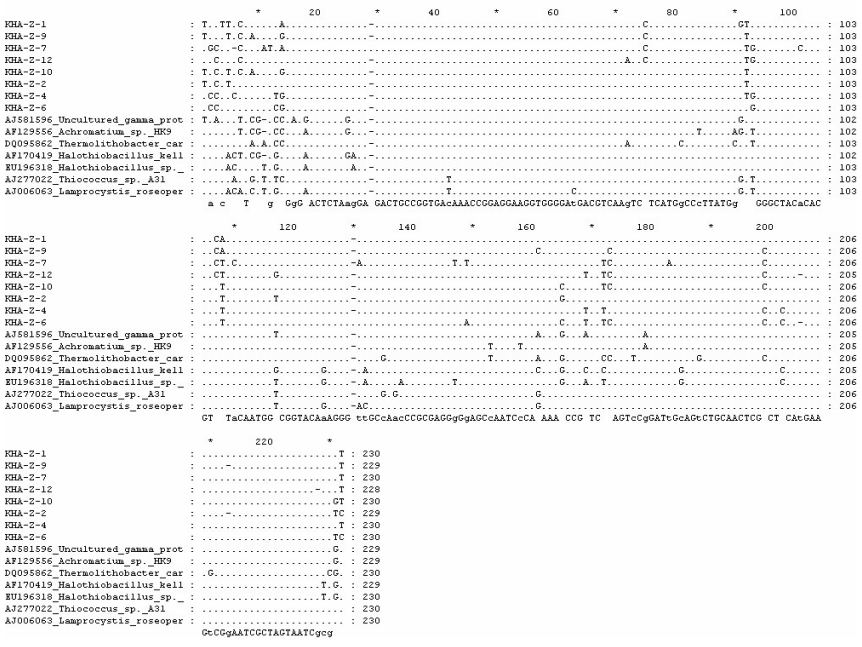


Fig. 4. Comparison of nucleotide sequences of DGGE band from culture-independent sample. (Grey block) represented hypervaribale region (V7 and V8); (→), represented conserved sequence; (.), represented same bases.

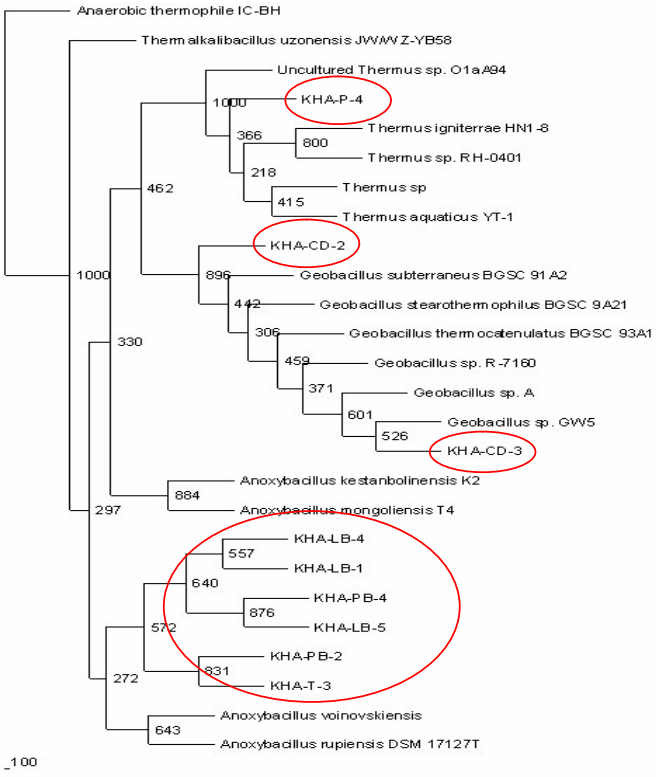


Fig. 5. Phylogenetic relationships of the 16S rRNA gene fragment sequences obtained from the culture-dependent samples and related sequences, based on a distance analysis (neighbor-joining algorithm with F84 model; 1,000 bootstrap replicates performed). (circle), represented the samples.

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                                        *       20        *       40        *       60        *       80        *      100
KHA-LB-5                    : ......TC.....................................-...C.....------.....................................  : 103
KHA-PB-4                    : ......TC.....................................-.....------.....................................  : 103
KHA-LB-1                    : ......T......................................-...C.....------.....................G..G.....  : 103
KHA-LB-4                    : ..............................................-...C.....------.....................G.....  : 103
KHA-PB-2                    : ..............................T.A.............T-...C.....T..C.....---.A..........T.....  : 103
KHA-T-3                     : ......................A.....T.A.............T-.C.....T..C.....---.A.....................  : 103
EF654664_Anoxybacillus_mongoli : --T.....................................-.C...C.....G...CA...C.....................  : 103
AY248710_Anoxybacillus_kestanb : ..T.....................................-.C...C.....---.C.....................  : 103
AB110008_Anoxybacillus_voinovs : ..T.....................................-.....C.....---.C.....................  : 103
AJ879076_Anoxybacillus_rupiens : ..T.....................................C.....-----.....................  : 103
                                 gg TGT gTCAGCTCGTGTCGcGAGATGTTGGgTtAAGTCCCGCAACGAGCGCAACCc T G C TTAgTTgCcAGCa  Tt AGTtGGGCACTcTAaGGtGACTG

                                    *       120       *       140       *       160       *       180       *       200       *
KHA-LB-5                    : ..............................................................................................  : 209
KHA-PB-4                    : ..............................................................................................  : 209
KHA-LB-1                    : ..............................................................................................  : 209
KHA-LB-4                    : ..............................................................................................  : 209
KHA-PB-2                    : ..............................................................................................  : 209
KHA-T-3                     : ...G...A..C...................................................................................  : 209
EF654664_Anoxybacillus_mongoli : ...GCT.A..G...................................................................................  : 209
AY248710_Anoxybacillus_kestanb : ...GCT.A..G...................................................................................  : 209
AB110008_Anoxybacillus_voinovs : ..............................................................................................  : 209
AJ879076_Anoxybacillus_rupiens : ..............................................................................................  : 209
                                 CCG tgA AA TCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGGCGGTACAAAGGGTTGC AACCCG

                                      220       *       240       *       260       *       280       *       300
KHA-LB-5                    : ..............................................................................  : 299
KHA-PB-4                    : ..............................................................................  : 299
KHA-LB-1                    : ..............................................................................  : 299
KHA-LB-4                    : ..............................................-...............................  : 298
KHA-PB-2                    : ..............................................................................  : 299
KHA-T-3                     : ..........................................C.....................CG..  : 299
EF654664_Anoxybacillus_mongoli : ..........................................................G  : 299
AY248710_Anoxybacillus_kestanb : ..........................................................G  : 299
AB110008_Anoxybacillus_voinovs : ..........................................................G  : 299
AJ879076_Anoxybacillus_rupiens : ..........................................................G  : 299
                                 CGAGGGGGAGCCAATCCCAAAAAGCCGCTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTgCAtGAAGCCGGAATCGCTAGTAATCgcG
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Fig. 6. Comparison of nucleotide sequences of DGGE band which is close to *Anoxybacillus* sequences. (Grey block) for hypervaribale region; (→), represented for conserved region; (.), represented for same bases.

Detail alignment of closely related sequences showed that all of the sample sequences were distinct compared to that the sequences available in the GenBank database (Fig. 4). Sequences variations were observed mainly on V7 and V8 hypervariable region (Fig. 4, grey block). One sequence showed single nucleotide difference lied on the conserved region. Other variation sequences lied on the outside of hypervariabel region, mostly in variable sites. These sequence variations were detected in samples obtained from both DNA extraction methods.

Microbial diversities from cultivation samples were clearly different with filtration sample. Most of nucleotide sequences detected in rich media (PB, LB, CD and T media) were closely

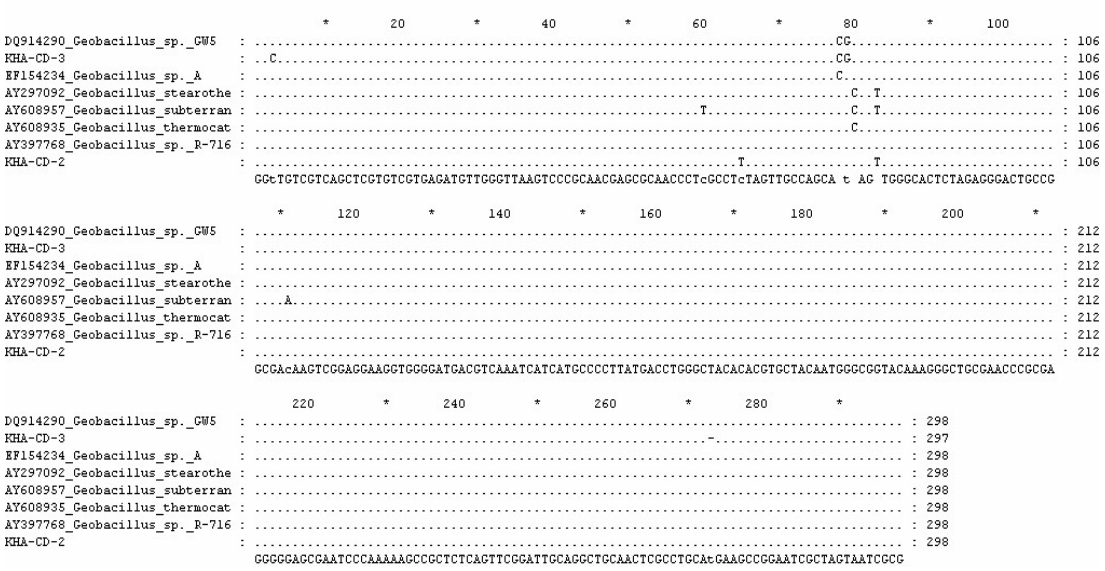

Fig. 7. Comparison of nucleotide sequences of DGGE band which is close to *Geobacillus* sequences. (Grey block) represented for hypervaribale region; (**.**), represented same bases.

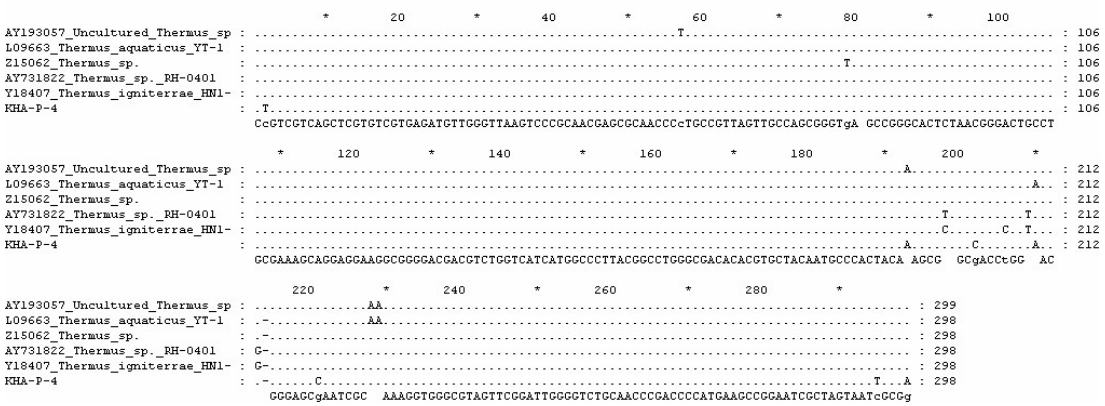

Fig. 8. Comparison of nucleotide sequences of DGGE band which is close to *Geobacillus* sequences. (Grey block) represented for hypervaribale region; (**.**), represented for the same bases.

related to Firmicutes phylum, including *Geobacillus* and *Anoxybacillus* genera. One sequence detected in P medium was affiliated to *Thermus* genus belonging to Deinococcus phylum (Fig. 5). In phylogenetic tree, sequences of *Anoxybacillus* group performed in distinct cluster. Nucleotide sequences alignment of *Anoxybacillus* group performed significantly differences among the sequences of samples from the GenBank database. Most of sequence variation lied in V7 hypervariable region (Fig. 6, grey block), two sequences were detected having one nucleotide difference in conserved region. Sequence variations were detected in small quantity in the samples closed to *Geobacillus* (Fig. 7) dan *Thermus* (Fig. 8) genera.

DISCUSSION

Kawah Hujan A was one of a rare neutral hot spring in Kamojang geothermal area. Most of hot springs in Kamojang geothermal field, have acidic pH (range pH 1-4), this is probably due to an oxic condition[10]. Based on 16S rRNA gene fragment sequences, microbial community predominated in Kawah Hujan A showed low diversity (Fig. 3), however the sequences were distinct from available sequences in the GenBank database. The presence of unique gamma Proteobacteria was possibly due to the condition of spring which is a subsurface spring with high temperature and pressure, and also low chemical or organic content. The low diversity of microbes in Kawah Hujan A is similar with other strong stream habitats such as Octopus Spring and Calcite Spring[21,22]. In Octopus Spring was predominated with pink filament bacteria and cyanobacteria [13,21] while in Calcite Springs was predominated with *Aquificales* and archaea[22].

Most species of gamma Proteobacteria were known as pathogenic and belonged to mesophilic microbes. However, recently many of gamma Proteobacteria were detected in thermal environment such as hot springs[23] and hydrothermal vent[24], and also in rock with strong stream pressure[25]. Gamma Proteobacteria frequently found as major group of cultivable bacteria from subsurface area[26], and some of them were known as iron oxidizer bacteria[27,28].

In this study, most of sequences found from filtration samples were not detected in cultivation samples. This is possibly due to some predominated microbes in natural sample could not cultivated in laboratory, while microbes that were less dominant in nature could grow better in compatible media. The presence of *Geobacillus* and *Anoxybacillus* in Kawah Hujan A were not surprising since both organisms often found in geothermal area[29,30,31,32]. *Geobacillus* and *Anoxybacillus* were endospore forming, and able to use many type of carbon sources for growth as heterotrophy and autotrophy. *Geobacillus* was aerobic and facultative anaerobic, while *Anoxybacillus* was anaerobic and facultative anaerobic[30,32]. These conditions were provided by Kawah Hujan A.

Thermus is often found in thermal environment. The organisms were reported to used hot springs[33] and subsurface area[4,34] as niches. However, the *Thermus* genus was not detected in culture-independent sample probably indicated that this genus was less dominant in Kawah Hujan A. This was suggested that the spring was non ideal habitat for *Thermus* due to most likely an anoxic habitat and lack of nutrient. Most of *Thermus* strains were obligate aerobic, some are facultative anaerobic and can use O_2, NO_3^-, Fe (III), and S^0 as final electron acceptor[34].

CONCLUSION

Microbial community in one of Kamojang hot springs, namely Kawah Hujan A, was carried out and showed that predominant microbes were close to ã-proteobacteria as detected from culture-independent strategy. However, the sequences of the ã-proteobacteria were unique compared to that the data from Genbank. Meanwhile, the 16S rDNA sequences from culture-dependent samples showed that the microbes were close to Firmicute and Deinococcus phyla, including *Geobacillus*, *Anoxybacillus* and *Thermus* genera.

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