Isolation and molecular identification of thermophilic bacteria from litter of mount Galunggung hot spring, Tasikmalaya, Indonesia

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Abstract. Thermophilic bacteria from litter deposited at Cipanas hot spring of mount Galunggung, Tasikmalaya, West Java, Indonesia, were isolated using a culture-dependent approach. Medium International Streptomyces Project (ISP) 1 solidified with gellan gum was used as an isolation medium. Isolation plates were incubated at 50°C for three weeks. The physicochemical analysis showed that the hot spring has a neutral pH and temperatures ranging from 50−56°C. A total of 16 bacterial isolates were obtained and purified. The analysis of partial 16S rRNA gene sequences data and phylogenetic analyses showed that they belong to Firmicutes and Proteobacteria phyla. The majority of bacterial isolates are spore-forming bacteria. The molecular identification based on the 16S rRNA gene sequences data and phylogenetic analyses showed that they belong to Aneurinibacillus thermoaerophilus (1); Brevibacillus gelatini (7); Bacillus licheniformis (1); Chelatococcus composti (1); Laceyella sacchari (2); Paenibacillus barengoltzii (1); and Pseudoxanthomonas taiwanensis (3). Percentage sequence similarities to their closest taxa were 91 to 99%. Phylogenetic analysis shed a light to the detection of candidates of novel taxa from litter of mount Galunggung hot spring.

1. Introduction

The diversity of bacterial species was documented in numerous environments, including extreme environments with high temperatures, such as geysers, terrestrial hot springs, geothermal and volcanic areas. These thermophilic bacteria are the sources of various antibiotics, secondary metabolites, and industrial important thermostable enzymes [1]. The number of reports concerning the diversity of thermophilic microorganisms from hot springs and geothermal areas in Indonesia have been described. These including microbial community analyses in Cibuni, Domas, and Cimanggu hot springs [2], Gedongsongo hot spring [3], acidic hot spring in Kamojang geothermal area [4], the geothermal areas of Cisolok, Kamojang, and Likupang [5], and Tanjung Sakti hot spring [6]. Thus, hot springs and
geothermal areas in Indonesia are potential sources for isolating novel indigenous thermophilic microorganisms.

In our previous studies, Yokota et al. [7] successfully isolated novel species of thermophilic bacteria namely *Paenibacillus cisolokensis*, and Mawarid et al. [8] isolated thermophilic bacterium, *Brevibacillus* sp. UICC B-76, from litter of Cisolok geyser, West Java, Indonesia. The thermophilic bacterial strains were reported able to hydrolyses various substrates, such as starch, xylan, and cellulose at 50°C. Sjamsuridzal et al. and Yokota et al. (unpublished data) were also obtained thermophilic *Actinobacteria*, identified as *Actinomadura keratinlytica*, from litter samples of Cisolok geyser. These actinobacterial isolates produced various extracellular enzymes at 50°C, and displayed antibacterial activity towards Gram-positive bacteria, *Kocuria rhizophila* NBRC 12078. Their studies suggested the high possibility of finding novel bacterial taxa, with a potential role as secondary metabolites producers, from litter samples in a geothermal area. The diversity of culturable thermophilic bacteria from litter samples of Cipanas hot spring, Mount Galunggung, Tasikmalaya, Indonesia has not yet been described. The information regarding the taxonomy of thermophilic bacteria using a culture-dependent method is essential to gain insight into their diversity and the possibility of novel taxa. In this study, we reported the taxonomy and discovery of potentially novel thermophilic bacterial taxa, from litter samples in Mount Galunggung hot spring, by means of 16S rRNA gene sequencing and phylogenetic analyses.

2. Methods

2.1. Isolation, purification, and preservation of thermophilic bacterial isolates

Litter samples were collected from Cipanas hot spring, Mount Galunggung, Tasikmalaya in November 2015, collected in a plastic bag, and kept at room temperature until used. The samples were air-dried at room temperature for one day, cut into small pieces and put on the surface of the International *Streptomyces* Project (ISP) 1 [9] solidified with 2% gellan gum, incubated at 50°C for three weeks. The agar blocks containing samples and bacterial colonies were transferred to new ISP 1 gellan gum medium, incubated at 50°C for around 10 days. The bacterial colonies were purified several times on ISP 1 gellan gum to gain pure isolates. Purified bacterial isolates were maintained on ISP 1 gellan gum in room temperature, preserved in 20% (v/v) glycerol stock solutions at -80°C as agar blocks, and as lyophilized cells for long-term preservation [10]. All bacterial isolates were deposited at Universitas Indonesia Culture Collection (UICC), Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia.

2.2. Isolation of bacterial genomic DNA

The extraction of genomic DNA for the 16S rRNA gene PCR amplification was conducted using the method previously described [11]. Polymerase chain reaction (PCR) of the bacterial 16S rRNA gene amplification was performed using universal eubacterial primers: 9F (5'-GAGTTTGATCCTGCTCAG-3'), 27F (5'-AGAGTTTGATCMTGCGCTG-3'), and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [12, 13], following the protocol of MyTaq™ Red Mix (Bioline) including 3 min initial denaturation at 95°C, followed by 35 cycles of 15 s denaturation at 95°C, 15 s annealing at 56°C, and 1 min extension at 72°C. Sequencing of PCR products was conducted using 1st BASE DNA sequencing service (http://base-asia.com/dna-sequencing-services).

2.3. Phylogenetic analyses

Sequence-similarity search and phylogenetic analyses were performed using the partial sequence of the thermophilic bacterial 16S rRNA gene against all related species through EzTaxon-e database (https://www.ezbiocloud.net/) [14]. Nearly full-length 16S rRNA gene sequences from 16 thermophilic bacterial isolates, together with the sequences of type strains recovered from the DDBJ/EMBL/GenBank databases, were aligned and analyzed using the MEGA v7.0.26 software package [15]. The sequence alignment was analyzed for constructing phylogenetic trees using the neighbor-joining [16], minimum-
evolution [17], and maximum-likelihood [18] methods in the MEGA v7.0.26 software package. The evolutionary distances of each phylogenetic tree were evaluated using Kimura two-parameter model [19], while the bootstrap values were set based on 1000 replications [20].

3. Results and discussion

The representative of bacteria isolation plates displaying antibacterial activity towards other bacteria grown on ISP 1 gellan gum, at 50°C after 10 days incubation, is shown in Figure 1. Bacterial colonies exhibiting the growth inhibition against surrounding bacteria were picked up and purified several times. The inhibition zones demonstrated that the bacterial colonies might potent as secondary metabolites producers. Sixteen selected thermophilic bacteria were then successfully isolated from litter of Cipanas hot spring, mount Galunggung. The identification of bacterial isolates based on 16S rRNA gene sequences towards their closely related species is presented in Table 1.

Figure 1. Bacteria isolation plates from litter samples of mount Galunggung, showing inhibition zones (red arrow) on ISP 1 gellan gum medium at 50°C for 10 days.

The 16S rRNA gene sequences result displayed that 12 out of 16 thermophilic bacterial isolates belong to Firmicutes (5 genera, 5 species), while the remaining four isolates were a member of Proteobacteria (2 genera, 2 species) phyla, which mostly recognized as spore-forming bacteria. A previous study based on DNA analysis of the total community demonstrated that Proteobacteria and Firmicutes were dominant members in degraded foliage of Sungai Klah hot spring, Malaysia [21]. Proteobacteria was also the major bacterial phylum in Cisolok, Kamojang, and Likupang geothermal areas [5].

Member of phylum Firmicutes including Aneurinibacillus thermoaurophilus (1), Brevibacillus gelatinis (7), Bacillus licheniformis (1), Laceyella sacchari (2), and Paenibacillus barengoltzii (1), displayed sequence similarities ranging from 91.62 to 99.93% towards their related taxa. Phylum Proteobacteria consist of Chelatococcus composti (1) and Pseudoxanthomonas taiwanensis (3), retained 99.51 to 99.86% sequence similarities. Two out of 16 isolates, GL1-1 and GL2-1 presented lowest sequence similarities towards their closely related species, Paenibacillus barengoltzii NBRC 101215T (97.33%) and Brevibacillus gelatinis PDF4T (91.62%), respectively. Identification of species, based on 16S rRNA gene sequence, classify the same species with ≥99%, and different species by <99% sequence similarities [22]. The results demonstrated the litter samples from Cipanas hot spring of mount Galunggung as prospective sources of thermophilic bacteria with potentially new taxa.

Table 1. Analysis result of the 16S rRNA gene sequences of 16 thermophilic bacterial isolates

| No. | Code of Isolate | Primers | Sequence length (nt) | EzTaxon-e top hit taxon and strain | Homology (%) | Genbank accession no. |
|-----|----------------|---------|----------------------|-----------------------------------|--------------|----------------------|
| 1   | GL1-1          | 9F-1492R| 1423                 | Paenibacillus barengoltzii NBRC 101215T | 1385/142      | AB6814 05            |


|   | Isolate | Name                        | Accession | Identity (%) | Accession |
|---|---------|-----------------------------|-----------|--------------|-----------|
| 2 | GL1-3  | Brevibacillus gelatini PDF4<sup>T</sup> | 1402/140 5 (99.70%) | KP89980 8 |
| 3 | GL2-1  | Brevibacillus gelatini PDF4<sup>T</sup> | 1237/134 9 (91.62%) | KP89980 8 |
| 4 | GL2-2  | Pseudoxanthomonas taiwanensis CB-226<sup>T</sup> | 1307/141 2 (99.65%) | AF42703 9 |
| 5 | GL3-2  | Brevibacillus gelatini PDF4<sup>T</sup> | 1342/134 8 (99.55%) | KP89980 8 |
| 6 | GL4-1  | Aneurinibacillus thermoanerophilus DSM 10154<sup>T</sup> | 1413/141 6 (99.79%) | X94196 |
| 7 | GL5-1  | Brevibacillus gelatini PDF4<sup>T</sup> | 1342/134 7 (99.63%) | KP89980 8 |
| 8 | GL5-1B | Brevibacillus gelatini PDF4<sup>T</sup> | 1340/134 9 (99.33%) | KP89980 8 |
| 9 | GL5-3  | Brevibacillus gelatini PDF4<sup>T</sup> | 1344/134 8 (99.70%) | KP89980 8 |
|10 | GL5-3-1| Brevibacillus gelatini PDF4<sup>T</sup> | 1419/142 8 (99.37%) | AE01733 3 |
|11 | GL5-4  | Bacillus licheniformis ATCC 14580<sup>T</sup> | 1361/136 2 (99.93%) | AF13873 7 |
|12 | GL5-5  | Laceyella sacchari KCTC 9790<sup>T</sup> | 1373/137 4 (99.93%) | AF13873 7 |
|13 | GL6-1  | Laceyella sacchari KCTC 9790<sup>T</sup> | 1403/140 5 (99.86%) | AF42703 9 |
|14 | GL7-1  | Pseudoxanthomonas taiwanensis CB-226<sup>T</sup> | 1408/141 5 (99.51%) | AF42703 9 |
|15 | GL7-2  | Pseudoxanthomonas taiwanensis CB-226<sup>T</sup> | 1352/135 8 (99.56%) | KP99434 9 |
|16 | GL10-1 | Chelatococcus composti PC-2<sup>T</sup> | 1402/140 5 (99.70%) | KP89980 8 |

Phylogenetic tree was constructed for 16 isolates belong to Firmicutes and Proteobacteria phyla, based on 16S rRNA gene sequences using neighbour-joining (NJ), minimum evolution (ME), and maximum-likelihood (ML) methods (Figure 2). The phylogenetic analyses elaborate in total of 46 nucleotides sequences for both phyla. Twelve isolates belong to phylum Firmicutes (Group I): seven bacterial isolates (GL1-3, GL2-1, GL3-2, GL5-1, GL5-1B, GL5-3, GL5-3-1) formed a monophyletic
clade with *Brevibacillus gelatini* PDF4\(^T\), with sequence similarities (homology) ranging from 91.62 to 99.70%. Among these isolates, the isolate GL2-1 showed a long branch and its sequence similarity to *B. gelatini* PDF4\(^T\) was 91.62%. The relationship of these seven isolates with *B. gelatini* PDF4\(^T\) was only supported by low bootstrap value (64%); one isolate (GL4-1) formed a monophyletic clade with *Aneurinibacillus thermoautrophilus* DSM 10154\(^T\), with 99.79% sequence similarity and supported by 100% bootstrap value; one isolate (GL5-4) formed a monophyletic clade with *Bacillus licheniformis* ATCC 14580\(^T\), with 99.37% sequence similarity and 100% bootstrap value; two isolates (GL5-5 and GL6-1) were grouped with *Laceyella sacchari* KCTC 9790\(^T\), *L. sediminis*, and *L. tengchongensis* with 99% bootstrap support. Both isolates showed 99.93% sequence similarity to *L. sacchari* KCTC 9790\(^T\); one isolate (GL1-1) was grouped within the members of *Paenibacillus* and showed sequence similarity 97.33% to *P. barengoltzii* NBRC 101215\(^T\) as its closest taxon, however the phylogenetic position of this isolate was distinct from *P. barengoltzii*, as supported by a moderate bootstrap value of 74%.

Another four bacterial isolates belong to phylum *Proteobacteria* (Group II): one isolate (GL10-1) was located within a monophyletic clade of the members of *Chelatococcus*, and grouped with *C. composti* PC-2\(^T\) as its closest related taxon with 99.56% sequence similarity and supported by 100% bootstrap value; and the remaining three isolates (GL2-2, GL7-1, GL7-2) were phylogenetically grouped with the members of *Pseudoxanthomonas*, and they formed a monophyletic group with *Pseudoxanthomonas taiwanensis* CB-226\(^T\) as their closest related taxon with sequence similarity ranging from 99.51 to 99.86%, and supported with 100% bootstrap value.
Figure 2. Phylogenetic tree using neighbour-joining (NJ), minimum-evolution (ME), and maximum-likelihood (ML) methods presenting phylogenetic relationship of bacterial isolates among Firmicutes (I) and Proteobacteria (II) phyla, based on 16S rRNA gene partial sequences. *Ktedonobacter racemifer SOSP1-21T* (NR042472) was used as an outgroup. Bar 0.020 nucleotide substitutions per site.
The genera, member of phylum *Firmicutes*, found in this study were commonly discovered in thermal environments and classified as thermophilic bacilli i.e. *Aneurinibacillus*, *Bacillus*, and *Brevibacillus*. The thermophilic bacilli were also found in hot springs and geysers in Indonesia, including genera and some species of *Brevibacillus* (*B. thermoruber* and *B. borstelensis*) and *Bacillus* (*B. licheniformis*) from Tanjung Sakti hot spring [6], and *Paenibacillus* from Gedongsongo hot spring [3]. Thermophilic bacilli member of genera *Bacillus*, *Brevibacillus*, and *Paenibacillus* isolated from Armenian geothermal springs were detected to have amylolytic, proteolytic, and lipolytic enzyme activities using a plating technique [23]. Meanwhile, several species of genus *Laceyella* comprising *L. sediminis* and *L. thermophila* were also recovered from hot springs in Tengchong county, Yunnan province, China [24, 25]. Member of *Laceyella* genus was recently reported as producers of thermostable chitinases [26]. Three genera from phylum *Proteobacteria*, showed 99.51 to 99.86% similarities with *Pseudoxanthomonas taiwanensis* CB-226T, which was isolated from the Chi-ban hot springs in eastern Taiwan, were known to produce only N₂O in an unusual denitrification reaction [27]. These previous studies suggested that the isolates from litter of mount Galunggung might have the same potential applications yet to be explored.

Further studies are needed to elucidate the identity of two isolates with low similarities to their closest related taxa, e.g. isolates GL1-1 and GL2-1. Sequence verification with complete sequences of 16S rRNA gene, amplified with primers 27F, 785F, 800R, and 1492R, and whole genome sequences are required to clarify their identity. Additionally, phenotypic data including morphological, physiological, and chemotaxonomic characteristics will be essential to differentiate these potentially new taxa from their closely related species.

4. Conclusion

Sixteen thermophilic bacterial isolates have been successfully obtained from litter samples in Cipanas hot spring, mount Galunggung, Tasikmalaya, West Java, Indonesia. The sequences of 16S rRNA gene from 16 isolates displayed that they belong to *Firmicutes*, and *Proteobacteria* phyla. Two isolates were suggested to be potentially new taxa, showing <99% sequence-similarity values of the 16S rRNA gene to their closely related species. Further studies are needed for full sequencing of 16S RNA gene and analyzing detailed phylogenetic trees of the potentially new bacterial taxa in order to confirm their taxonomic identity.

Acknowledgment

This study was supported by Hibah Publikasi Terindeks Internasional (PUTI) Prosiding Tahun Anggaran 2020 Universitas Indonesia [grant number: NKB-3537/UN2.RST/HKP.05.00/2020] to W.S.

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