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Original research article

Isolation and Phylogenetic Analysis of Thermophile Community Within Tanjung Sakti Hot Spring, South Sumatera, Indonesia

Heni Yohandini,[1*] Julinar,[1] Muharni[2]

[1] Department of Chemistry, Faculty of Mathematic and Natural Sciences, Universitas Sriwijaya, Ogan Ilir 30662, Indonesia.
[2] Department of Biology, Faculty of Mathematic and Natural Sciences, Universitas Sriwijaya, Ogan Ilir 30662, Indonesia.

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ABSTRACT

A community of thermophiles within Tanjung Sakti Hot Spring (South Sumatera) have been cultivated and identified based on 16S ribosomal RNA gene sequence. The hot spring has temperature 80 °C–91 °C and pH 7–8. We used a simple method for culturing the microbes, by enriching the spring water with nutrient broth media. Phylogenetic analysis showed that the method could recover microbes, which clustered within four distinct taxonomic groups: *Anoxybacillus*, *Geobacillus*, *Brevibacillus*, and *Bacillus*. These microbes closely related to *Anoxybacillus rupiensis*, *Anoxybacillus flavithermus*, *Geobacillus pallidus*, *Brevibacillus thermoruber*, *Bacillus licheniformis*, and *Bacillus thermoamylovorans*. The 16S ribosomal RNA gene sequence of one isolate only had 96% similarity with *Brevibacillus* sequence in GenBank.

1. Introduction

Hot springs are unique areas that are characterized by high temperature and have a great diversity of natural environments. These areas are habitats for a diversity of thermophile, particularly belonging to bacteria and archaea domains. Thermophile diversity provides an overview of the enormous potential that can be utilized for various purposes. Thermophilic microbes are currently studied intensively for reasons of development of basic research and biotechnological applications. In addition, a study of thermophile led to the discovery of novel species (Huber *et al.* 1991; Kozina *et al.* 2010; Zhang *et al.* 2010).

Microbial diversity in a community can be studied through culture-dependent and culture-independent approaches. Researchers believe that the traditional approach which depends on cultivation for identification has many limitations to discover a broad diversity of microbial communities in their natural habitats. However, pure cultures were required for characterization in understanding microbial physiology and genetics (Ward *et al.* 1998). Indonesia has over 70 active volcanoes and a large number of geothermal regions and abundant hot springs (Kusumadinata 1979). The diverse terrestrial hydrothermal regions have potential habitat for large communities of thermophilic microbes. To date, information about microbial diversity from Indonesian hot spring is very limited. Studies on Indonesian thermophile communities have been conducted in some geothermal area residing in the Javanese island (Aditiawati *et al.* 2009; Aminin *et al.* 2008; Baker *et al.* 2001; Huber *et al.* 1991; Lohr *et al.* 2006; Yohandini *et al.* 2008). These studies have discovered high diversity of thermophilic/hyperthermophilic bacteria and archaea from Indonesian thermal area. In this report, we describe the first diversity study of culturing thermophile isolated from Tanjung Sakti Hot Spring, located in South Sumatera. We used simple enrichment method to cultivate the microbes and analysis of its 16S ribosomal (rRNA) gene sequences for microbial idenfication. Microbial isolates obtained in this study were also intended to complement the microbial culture collection that can be used as a source of thermostable enzymes and secondary metabolites, besides for the purposes of bioremediation.

2. Materials and Methods

2.1. Materials

Spring water was taken from Tanjung Sakti Hot Spring on April 17, 2013. Nutient broth and nutrient agar media (Oxoid), GoTaq Green Master Mix, DNA Purification Kit (Promega), 27F primer: AGAGTTTGATCATGGCTCAG and 1492R primer: GGGTACCTTGTTACGACTT (Macrogen), Agarose (Sigma), chloroform, ethanol, isoamylalcohol, and isopropanol (Merck).

* Corresponding author.
E-mail address: heniyo@yahoo.com (H. Yohandini).
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Table 1. The highest homology of 16S ribosomal RNA gene sequence of Tanjung Sakti isolates with existing data in GenBank

Isolate code	Taxonomic affinity	% Homology
TS-01, TS-04	*Anoxybacillus* sp.	99%
	Anoxybacillus rupiensis	99%
	Anoxybacillus beppuensis	99%
	Anoxybacillus amylolyticus	99%
TS-03, TS-08, TS-09, TS-10, TS-11, TS-16, TS-17	*Bacillus* sp.	99%
	Bacillus licheniformis	99%
TS-05, TS-06	*Brevibacillus* sp.	100%
	Brevibacillus thermoruber	100%
TS-07	*Brevibacillus* sp.	96%
	Brevibacillus thermoruber	96%
TS-12	*Geobacillus* sp.	99%
	Geobacillus pallidus	99%
TS-15	*Anoxybacillus* sp.	99%
	Anoxybacillus flavithermus	99%
TS-18	*Bacillus* sp.	99%
	Bacillus thermoamylovorans	99%
	Bacillus circulans	99%

2.2. Sampling site and cultivation

Microbial samples were taken from the Tanjung Sakti Hot Spring, located in Lahat Regency, South Sumatra. Approximately 25 mL of spring water was added to 1 mL of sterile nutrient broth (NB) medium. Samples were kept at high temperature during the trip back to the laboratory. Subsequently, samples were incubated at aerobic condition with 55 °C. Pour plate method was used to obtain pure cultures. Isolates were separated based on differences in the shape, color, and size of the colony.

2.3. DNA extraction and 16S rRNA gene amplification

Chromosomal DNA was extracted using the DNA Purification Kit (Promega). Microbial pellet cells were suspended and incubated at room temperature in cell lysis solution for 10 minutes, added with nuclei lysis solution containing RNase and incubated at 37 °C for 15 minutes, and then added with protein precipitation solution, and centrifuged at 13,000 rpm for 10 minutes at 20 °C. The DNA was purified three times with equal volumes of chloroform:isoamylalcohol (24:1), precipitated with 0.6 volumes of isopropanol, washed in 70% ethanol, and resuspended in ddH_2O. 16S rRNA gene fragment was amplified by polymerase chain reaction using primers 27F and 1492R (Baker *et al.* 2003; Frank *et al.* 2008). Master mix reaction, including *Taq* DNA polymerase enzyme (GoTaq Green Master Mix, Promega) was used according to standard usage. The reactions were incubated in a C-1000 Thermal Cycler (Bio-Rad, USA) for 3 minutes at 95 °C, and then for 30 cycles of 30 seconds at 95 °C, 1 minute at 55 °C, and 1 minute and 30 seconds at 72 °C, and a final extension of 5 minutes at 72 °C. All the 16S rRNAs gene fragments were sequenced using commercial services at Macrogen Inc., Korea. All sequences were submitted to GenBank and assigned by KJ842627 to KJ842641.

2.4. Phylogenetic analysis

Nucleotide sequences were compared for homology with BLAST search (http://www.ncbi.nlm.nih.gov) (Altschul *et al.* 1990).

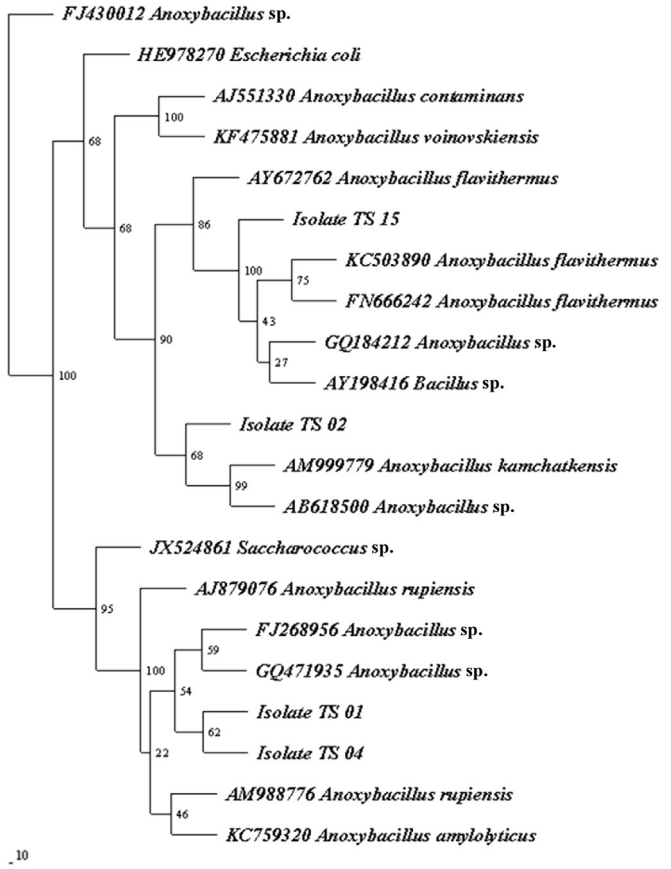

Figure 1. Phylogenetic tree of Tanjung Sakti isolates which have the highest homology with *Anoxybacillus*. The phylogenetic was constructed using neighbor joining method with 100 bootstrap replicates.

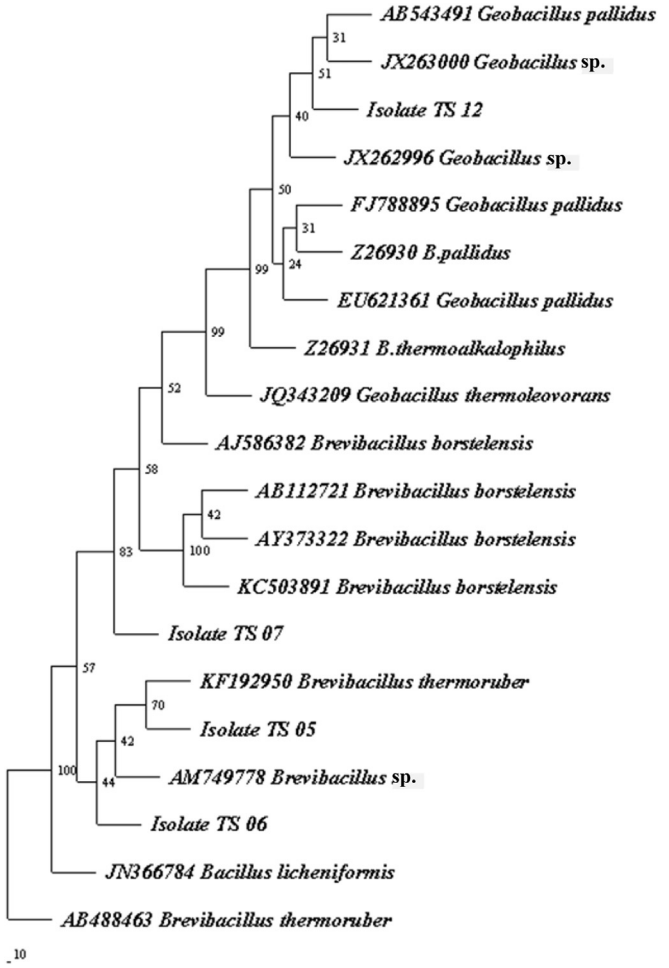

Figure 2. Phylogenetic tree of Tanjung Sakti isolates which have the highest homology with *Brevibacillus* and *Geobacillus*, constructed using neighbor joining method with 100 bootstrap replicates.

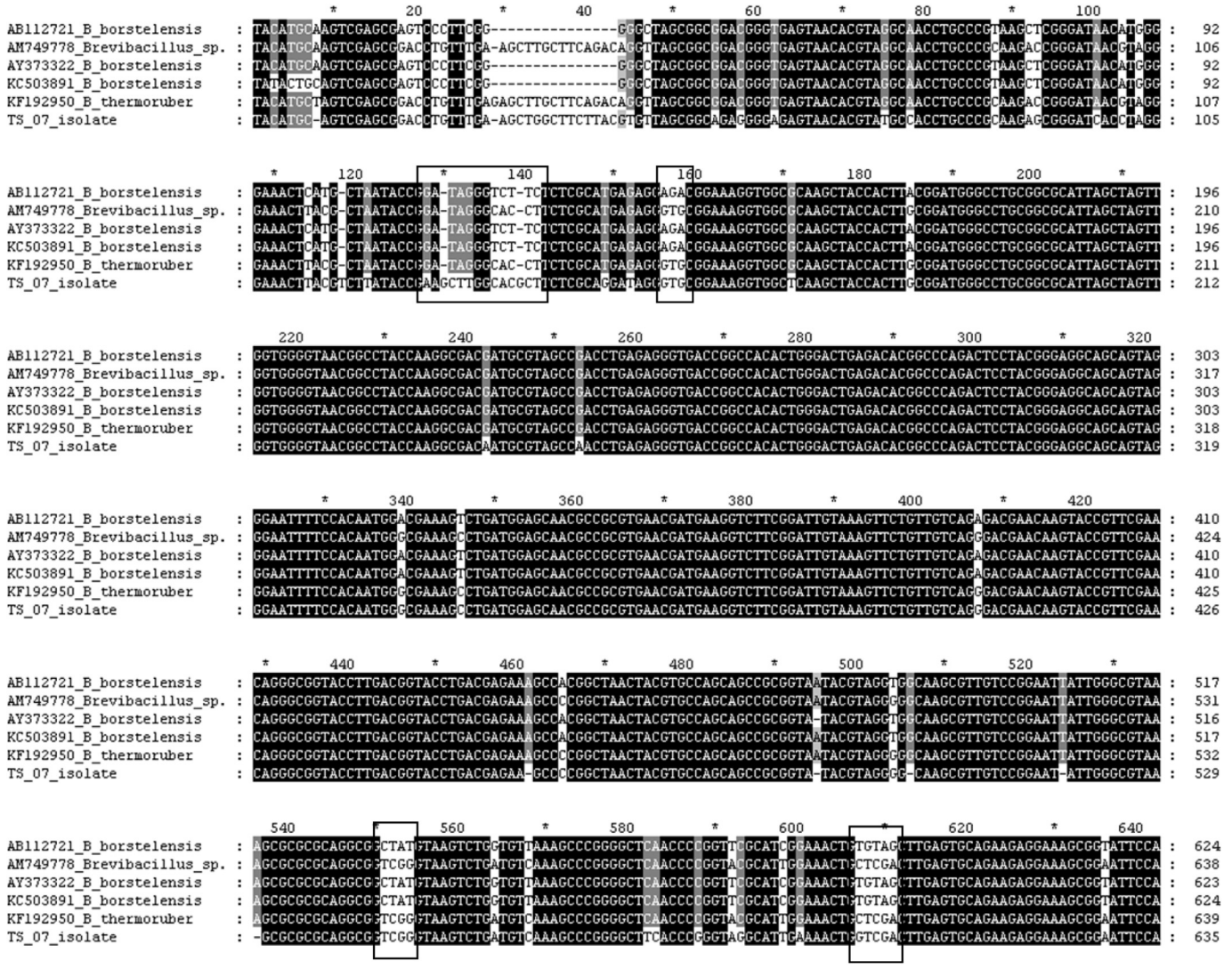

Figure 3. A part of 16S ribosomal RNA gene sequence alignment result of TS-07 isolate and some related *Brevibacillus*. Sequence variations (indicated by the rectangular lines) were mainly located in positions 130-140, 608-612, 157-160, and 550-554. Nucleotide substitutions and deletions were distributed evenly throughout the gene.

Alignments of nucleotide sequences were performed using the ClustalW program (Thompson *et al.* 1994). Visualization and editing of the aligned sequences were done by Genedoc program (Nicholas *et al.* 1997). The phylogenetic trees were constructed using neighbor joining method (Saito & Nei 1987) by Phylip program version 3.695 (Felsenstein 1989) and phylogenetic trees were visualized by Treeview program.

3. Results

In this study, bacterial isolates were obtained from spring water of the Tanjung Sakti Hot Spring, Lahat Regency, and South Sumatra. The hot spring is located at the Manna riverside. The temperature of the hot springs was about 80 °C—91 °C and pH 7—8. All the isolates were pale white color, rod-shaped cells, and gram stain positive (data not shown). DNA fragments with a size of about 1.5 kbs were amplified from chromosomal DNA using polymerase chain reaction method. The size of the DNA fragments was as expected for the size of the 16S rRNA gene fragment. Amplification products were obtained from 16 isolates. The nucleotide sequence of the fragments had high homology with the 16S rRNA gene from *Bacillus*, *Brevibacillus*, *Geobacillus*, and *Anoxybacillus* genera (Table 1). The closest relationship of each sequence was determined by constructing phylogenetic trees.

The phylogenetic relationship showed that TS-01 and TS-04 isolates were closely related to *Anoxybacillus rupiensis* (AJ809076), which differ by only about 0.3% of the size of about 1.4 kbs compared, meanwhile the TS-05 isolate was closely related to *A. flavithermus* (FN666242) with difference in sequence as much as 0.6% (Figure 1). TS-12 isolate had a close relationship with *Geobacillus pallidus* (AB543491) with a less than 0.1% differences.

In *Brevibacillus* group, two isolates (TS-05 and TS-06) showed affinity to *Brevibacillus thermoruber* (KF192950) with a difference of about 0.3%, while one isolate (TS-07) made a separate branch among *Brevibacillus thermoruber* cluster and *Brevibacillus borstelensis* (Figure 2). The 16S rRNA sequence of TS-07 isolate had differed about 4% from *Brevibacillus thermoruber* (KF192950) and 8% from *Brevibacillus borstelensis* (KC503891). Nucleotide sequence differences were distributed along the gene sequence, in the form of sequence variation, nucleotide substitutions and deletions. A part of the nucleotide sequence alignment of TS-07 isolate and related *Brevibacillus* were shown in Figure 3. Nucleotide substitutions and deletions were distributed evenly throughout the gene. Sequence variations were mainly located in positions 130-

140, 608-612, 157-160, 550-554 in the Figure 3. In other part of alignment result (data not shown), the differences were in the form of substitutions and deletions.

16S rRNA gene sequences of 7 isolates (TS-03, TS-08, TS-09, TS-10, TS-11, TS-16 and TS-17) showed a close relationship to *Bacillus licheniformis* (Figure 4). The sequences of TS-03, TS-09 and TS-16 isolates differed by about 0.5% from *Bacillus licheniformis* (JX847111); TS-08, TS-11 and TS-17 isolates about 0.2%–0.4% from *Bacillus licheniformis* (KF040981); and TS-10 isolate about 0.1% from *Bacillus licheniformis* (D31739). Moreover, one isolate (TS-18) showed a close relationship with another species of *Bacillus*, i.e. *Bacillus thermoamylovorans* (HM030742) which differed approximately 0.2% (Figure 5).

4. Discussion

In this study, bacterial isolates were obtained from the cultivation of microbes by enrichment of spring water with NB medium (25:1). Use of natural sources enriched media can increase the diversity of microbes in culture (Santegoeds *et al.* 1996). Similar methods successfully used for culturing novel strains, for example *Metallospaera* sp. from crenarchaeote phylum (Kozubal *et al.* 2008) and *Thermus* sp. (Kieft *et al.* 1999). Incubation of microbial cultures was performed at 55 °C. Growth temperature was far below the temperature of their natural habitat with consideration that any microbes do not necessarily have the same optimum temperature with their natural habitats. This condition might be the weakness of cultivation-based methods in analyzing microbial diversity, besides the limitation of the type of media used. The isolated microbes are those best adapted for growth on the medium, and are not necessarily dominant in the natural habitat (Baker *et al.* 2001). Consequently, the microbial diversities analyzed by cultivation-dependent methods are usually less diverse than those analyzed directly from their fields. (Aditiawati *et al.* 2009; Skirnisdottir *et al.* 2000).

Cultivated microbial community within Tanjung Sakti Hot Spring had a close relationship to *Anoxybacillus rupiensis*, *Anoxybacillus flavithermus*, *Geobacillus pallidus*, *Brevibacillus thermoruber*, *Bacillus licheniformis*, and *Bacillus thermoamylovorans*. All the isolated microbes belong to the domain bacteria, phylum firmicutes, class bacilli, order bacillales, within two different families: bacillaceae 1 and paenibacillaceae 1 (http://rdp.cme.msu.edu/seqmatch). This suggested that the spring water enriched with NB medium was suitable for growing the bacilli group. The use of other enrichment media was likely to recover different microbes. In the study of microbial diversity in the Kawah Hujan crater, the use of different media types could recover different types of microbes (Yohandini *et al.* 2008).

Genera *Geobacillus*, *Anoxybacillus*, and some species of *Brevibacillus* and *Bacillus*, are commonly found in thermal environments, including hot springs, oil reservoirs, mines, and geothermal aquifers. The genera *Bacillus* and *Brevibacillus* have also been isolated from mesobiotic environments. However, *Brevibacillus thermoruber*, *Bacillus licheniformis*, and *Bacillus thermoamylovorans* had an optimum temperature in the range of 45 °C–50 °C (Coorevits *et al.* 2011; Logan

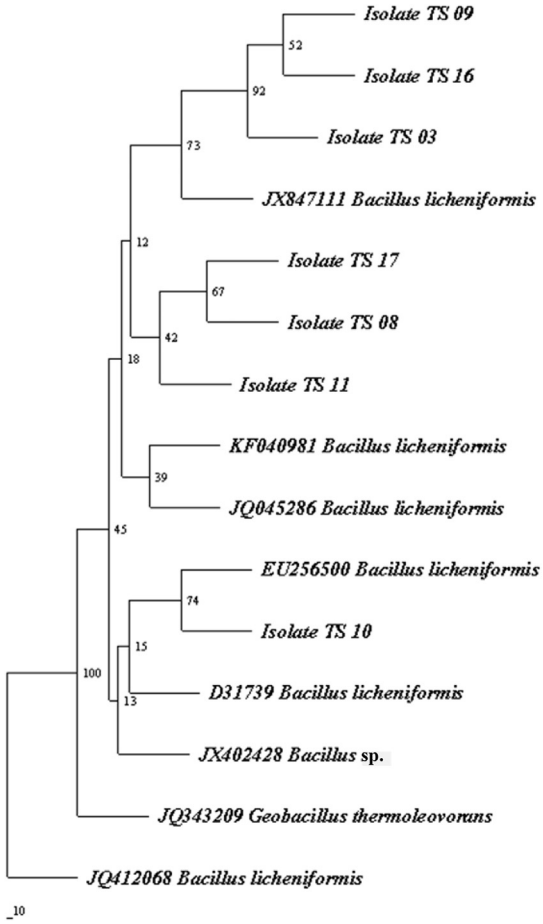

Figure 4. Phylogenetic tree of Tanjung Sakti isolates which have the highest homology with *Bacillus licheniformis*, constructed using neighbor joining method with 100 bootstrap replicates.

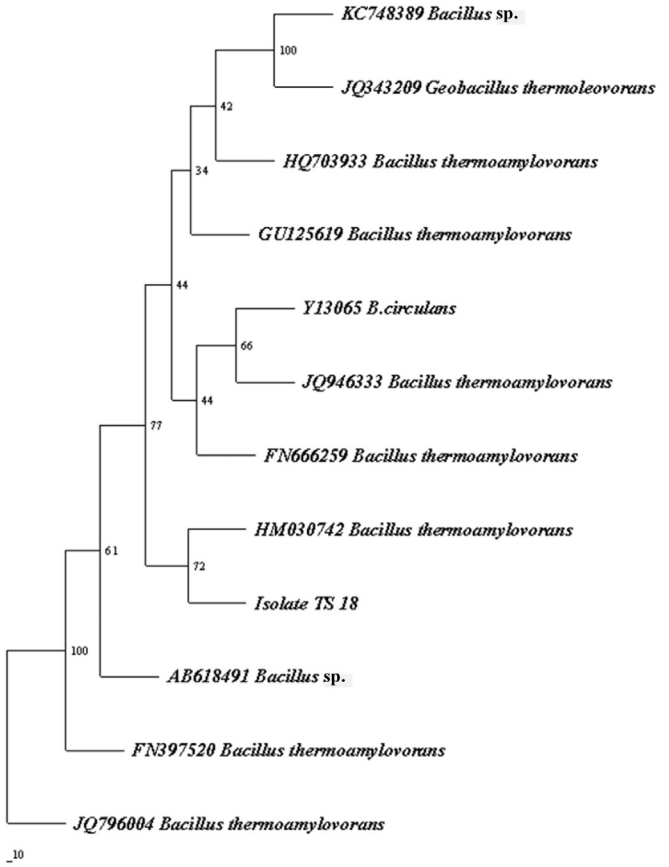

Figure 5. Phylogenetic tree of Tanjung Sakti isolate which has the highest homology with *Bacillus thermoamylovorans*, constructed using neighbor joining method with 100 bootstrap replicates.

et al. 2002). Genera *Anoxybacillus* and *Geobacillus* are thermophilic, gram-positive, spore forming, rod-shaped cells, and aerobic or facultatively anaerobic bacteria (Minana-Galbis *et al.* 2010).

The diversity of microbes isolated from Tanjung Sakti Hot Spring had no similarity to the diversity of microbes that have been studied from several geothermal areas in Indonesia, with the condition of relatively neutral pH. Microbes isolated from Candradimuka Crater, Dieng Plateau (pH 6.0–7.5), have been identified as *Desulfurococcus* and *Thermoproteus* genera (Huber *et al.* 1991), and from Cimanggu hot spring as *Freuteria* sp. and *Bacillus caldovelox* (Baker *et al.* 2001), while ones from Gedongsongo hot spring (pH 6.0–7.0) were closely related to *Ralstonia*, *Delftia*, *Dechloromonas*, *Hypomicrobium*, *Pseudomonas* and *Thermus* genera (Aminin *et al.* 2008). However, microbes from Kawah Hujan A (pH around 7.0) have been identified to have a close relationship with *Geobacillus* and *Anoxybacillus* genera, in addition to other microbes such as *Thermus* and Proteobacteria groups (Yohandini *et al.* 2008).

Potential applications for some of the described species were reported previously, particularly for the production of bioactive molecules and/or biocatalysts that may be important for industrial processes and biotechnologies. These bacteria groups had known to produce a variety of thermostable enzyme, such as protease, lipase, α-amylase, xylanase, cellulase (Haki & Rakshit 2003; Veith *et al.* 2004), and keratinase (Tiwary & Gupta 2012), beside as sources of biosurfactant (Rodrigues *et al.* 2006), flocculant biopolymer (Shih *et al.* 2001), gelling and stabilizing agent (Nankai *et al.* 1999).

The 16S rRNA gene sequence of TS-07 isolate had 96% homology with *Brevibacillus* sequence in GenBank. The locations where the sequences of the gene differ mainly corresponding to the V1, V2, and V4 hypervariable regions in *Escherichia coli* (Baker *et al.* 2003). According to Amann *et al.* (1995), the rRNA sequence similarity values below 95% were an indication of discovery of a new species. In some publications, 96%–98% sequence similarity had indicated a new or different species (Belduz *et al.* 2003; Dulger *et al.* 2004; Yumoto *et al.* 2004). Although phylogenetic characteristic of TS-07 isolate had the potential to be considered as a different species within *Brevibacillus* genus, the polyphasic taxonomic study is needed. The distinctions are not based mainly on DNA relatedness studies, molecular probing and chemotaxonomic analyses, but also characteristic of phenotypic profiles and biochemical reactivity of the isolate (Logan *et al.* 2002). Considering that Indonesia has many volcanoes and geothermal areas with different physical and chemical properties, the opportunity to discover new thermophilic microbes is very high. The existing problem is the limitation of cultivation methods which leads to difficulty in culturing microbes.

Conflict of interest

There is no conflict of interest. This statement was based on the results of previous research on the analysis of microbial diversity in Indonesia. The research showed that the results of analysis of microbial diversity using culture-independent and culture-dependent methods were different. Microbial diversity analyzed with culture-independent methods showed a higher diversity than indicated by using culture-dependent. In addition, the microbes which were detected as new microbes using culture-independent methods are not found in microbial culture. Many papers also stated that most of microbes in nature were uncultivated yet.

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