Ribotyping Identification of Thermophilic Bacterium from Papandayan Crater

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Abstract. A few thermophilic bacteria were isolated from a hot spring located in Papandayan Crater, Garut. One of the organisms showed a well growth at temperature of up to 80°C. Chromosomal DNA from the organism was isolated and used to amplify 16S rRNA gene fragment. The gene was amplified by a set of universal primers (27F and 1492R) resulting in a 1.5 kb DNA fragment. The gene was cloned and sequenced. The phylogenetic tree, homological analysis, and detailed comparison of the sequences showed that 16S rRNA gene sequence of the Papandayan isolate is unique compared to other known strains, however the sequence had closest similarities with *Bacillus caldolyticus* and *Bacillus caldotenax*.

Keywords: *Bacillus caldolyticus; Bacillus caldotenax; Papandayan crater; thermophilic microorganism; 16S rRNA gene.*

1 Introduction

Recently researches on thermophilic bacteria have extensively been carried out since these organisms offer many advantages either for development of basic sciences or for industrial applications. Thermophilic microorganisms can be used as sources of thermostable enzymes, which have high potential application for biocatalyst industries. The application of thermostable enzymes on biotechnological processes can reduce operational costs and increase the reaction rates thus increase the productivity (Aquilar et al 1998). The application of thermophilic microorganisms on the fermentation process also prevents contamination from other mesophilic bacteria. Extensive research on the genetics of thermophile have resulted in the modification of phylogenetic tree. Currently it is divided into three major groups, which are bacteria, archea and eukarya (Madigan and Mars 1997).

Thermophilic microorganisms are defined as groups of microorganisms which grown at a temperature above 45°C, some of them still actively grow at 80°C (Madigan and Mars 1997). The organisms can easily be found in compost, hot spring, deep vents and other geothermal active regions (van den Burg 2003).

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Indonesia is one of the most tectonically active areas in the word with over 70 active volcanoes and has a substantial number of geothermal regions (Kusumadinata, 1979). Although Indonesia is diverse, terrestrial hydrothermal biotope posses a large number community of thermophilic bacteria, to date little effort has been made to characterize these thermophilic communities, hence an accurate identification of microorganisms is needed to be conducted. The different on the condition of environmental properties for each region can enable to isolate high heterogenous of thermopiles. Two methods, conventional (biochemical test) and ribotyping analysis are usually used to identify microorganism strains. However, 16S rRNA gene sequence analysis has been known and accepted to be an international acknowledged and effective method.

A few thermophilic microorganisms were isolated from hot spring around Indonesia, such as, Sileri hot spring, Dieng (Kim et al. 2001). The organism grew at a temperature of up to 85^{o}C and employed the ribotyping analysis was identified as *Thermoanarobacter yonseinsis* sp. A few species of *Bacillus* were isolated from Cimanggu hot spring (Akhmaloka et al. 2000; Baker et al. 2001) grew at temperature of up to 83^{o}C. *Geobacillus thermoleovorans* was isolated from Wayang Crater (Indrajaya et al. 2003) grew at a temperature of up to 76^{o}C.

In this report we would like to describe the isolation and identification one of thermophilic microorganisms from Papandayan Crater, Papandayan Mount, Garut, West Java using 16S rRNA gene sequence analysis (ribotyping method).

2 Material and Methods

2.1 Isolation and Bacterial Cultivation

A set of 50 ml ½ LB media (NaCl 0.5%, tryptone 0.5%, and yeast extract 0.25%) were prepared and sterilized. The media were then placed in a thermos box and transported to the field. Samples were taken from the hot spring of Papandayan Crater, Garut, West Java. 1 ml of sediment-rich water recovered by a sterile syringe was transferred to each media flasks. The media were then immediately returned to the thermos box to maintain their temperature. All samples were returned to the laboratory within a period of hours and incubated with shaking at 70^{o}C, 200 rpm. After 2 days 1 ml of samples from the flask was transferred to fresh medium and incubated for further 48 hours at 70^{o}C. The well grown cultures were plated on solid ½ LB media.

2.2 Chromosomal DNA Extraction

The DNA extraction method was based on the procedure of Klijn et al. (1991). Approximately 10^{9} cells from 2 ml of culture medium were harvested by

centrifugation and incubated in 200 µl of lysozyme (8 mg ml^{-1}, 10mM Trish-HCl) for 1 hour at 37°C. An equal volume of lysis buffer (2% SDS, 200 mM EDTA, proteinase K 0.8 mg ml^{-1}) was added and the sample was incubated for 30 minutes at 55°C. The sample was then precipitated with 2 volumes of ethanol and centrifuged to produce a pellet. The pellet suspended in 0.5 ml sterile water, extracted with phenol-chloroform and re-precipitated with ethanol. The genomic DNA pellet was re-suspended in 50 µl sterile water.

2.3 Amplification and Cloning of 16S rRNA Gene

Approximately 200 ng of chromosomal DNA was amplified by PCR (initial denaturation 3 minutes at 94°C, 30 cycles: 1 minute at 94°C; 1 minute at 48°C; 1 minute at 74°C followed by a single period at 72°C for 10 minutes; 1 U Sigma Taq Polymerase) using bacterial 16S rRNA gene specific primers (Delong et al. 1993). 5 µl volume of PCR product was visualized on 1% agarose gel electrophoresis and the rest was purified by ethanol precipitation. The purified PCR product were ligated into a pGEM-T (Promega) vector and used to transform competent *Escherichia coli* JM101. A few colonies of the transformants were analysis using restriction enzymes. The resulted fragments were visualised on 1% agarose gel electrophoresis. Samples with proper restriction pattern were chosen for sequencing.

2.4 Sequencing and Sequence Analysis

Recombinant plasmid from chosen transformants were isolated and purified using Qiagen mini-prep kit and sequenced using internal primers for 16S rRNA gene (Baker et al. 2001) and T7 and SP6 primers specific for pGEM-T vector (Table 1).

The resulted sequences were aligned using Seqman program from DNASTAR®. The fragments of sequences were assembled and edited in Lasergene Seqman and consensus sequences were compared to other 16SrRNA gene in the GenBank using NCBI BLAST to obtain sequences with high homology to the fragments of sequences. Around 35 sequences were realigned and the phylogenetic tree from 28 related strains was constructed using MEGALIGN program from DNASTAR®.

Table 1 Primers were used in this study. Primers no 1 and 2 were used to amplify 16S rRNA; Primer no 3-9 were used to sequence the 16S rRNA gene.

No.	Primer	Sequences
1.	27F	5'-AGAGTTTGATC(A/C) TGGCTCAG-3'
2.	1492R	5'-GGTTAC(G/C) TTGTACCTGCCGGA-3'
3.	357F	5'-TACGGGAGGCAGCAG-3'
4.	803F	5'-GATTAGATACCCTGGTAG-3'
5.	1114F	5'-GCAACGAGCGCAACCA-3'
6.	909R	5'-CCGTCAATTCATTTGAGT-3'
7.	519R	5'-GTATTACCGCGGCTGCTG-3'
8.	SP6	5'-GGGCGAATTGGGCCCGAC-3'
9.	T7	5'-TGCATAGCTTGAGTATTC-3'

3 Results

3.1 Isolation and Morphology of Papandayan Isolate

A few thermopiles bacteria were isolated from Papandayan Crater (pH 3, 78-90^{o}C). Isolation was performed by sampling sediment-rich water from the surface, center, and bottom of hot spring. More than 30 colonies were recovered on ½ LB plate. The colonies were grown at a temperature of up to 80^{o}C. One colony (P1), showing the best growth, was chosen for further study.

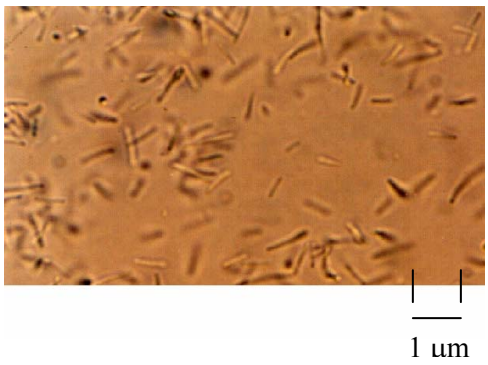

Figure 1 Morphology of the cells under Light Microscope with 1000X amplification.

Morphological observation of P1 cell under light microscopy (Olympus BH-2) showed that the cell is a rod shaped bacteria (Figure 1). Further analysis using gram-staining method proved that the isolate was a gram-positive bacterium.

3.2 Amplified DNA Fragment

16S rRNA gene amplified with 27F and 1492R primers from P1 isolate was observed on agarose gel electrophoresis. The result showed an amplicon band with the size of 1.5kb respectively (Figure 2). The DNA fragment was ligated on pGEM-T vector and the recombinant vector was used to transform *E. coli* JM101. Quick analysis of the vector using agarose gel electrophoresis showed that the migration of recombinant vectors were slower than that of original pGEM plasmid (data not shown). This suggested that recombinant vectors harbor DNA inserts.

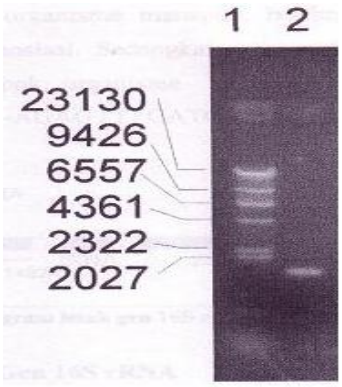

Figure 2 Electrophoregram of PCR product. Lane 1 shows λ DNA cut by *Hin*dIII; lane 2 shows PCR product amplification.

Further characterization was performed by restriction analysis using *Sal*I and *Pst*I restriction enzymes. The resulted DNA fragments analysis using agarose gel electrophoresis and the results confirmed that the vector contained 16S rRNA gene (data not showed).

3.3 Sequences and Homology of DNA Fragment

The chosen plasmid from the result of restriction analysis was sequenced based on the strategy showed on Figure 3. At least two independent sequences were used to confirm the correct sequence of the fragment. The complete sequence of the fragment was appeared to contain 1502 base pairs.

This sequence was compared to available sequences data on the GenBank and the results showed that the sequence has close homology to all 16S rRNA genes. Phylogenetic analysis of the sequences was conducted through comparison with other 27 best homologous sequences (Figure 4) resulted in Papandayan isolate being closest in homology to *B caldolyticus*. The

extremophiles of *Bacillus* and *Geobacillus* showed 99% identical sequences out of nineteen closest homologies (Table 2).

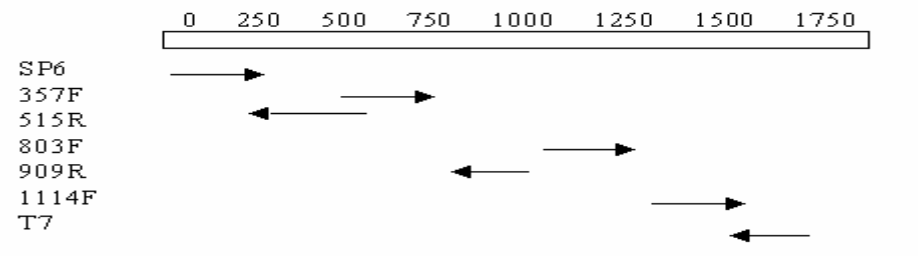

Figure 3 Strategy for sequencing the whole 16S rRNA gene, SP6 and T7 were external primers of the gene.

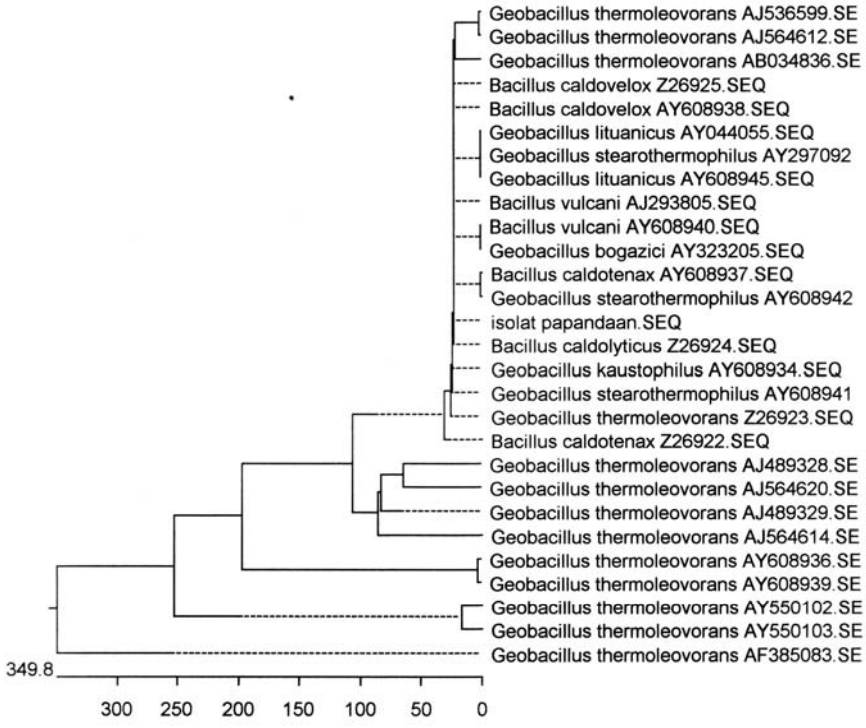

Figure 4 The phylogenetic tree of Papandayan isolate.

Further analysis by comparing the 16S rRNA gene sequences among the highest homology (Table 2) showed few differences at the position of the conserved region (Table 3). The nucleotide sequence at position 508 (U3 region) for *B. vulcani* AJ293805, *B. caldovelox* AY608938, Z26925, and *G. thermoleovorans*

AJ564612 was A while for other organisms, including Papandayan isolate the base was G. The nucleotide sequence at position 815 (U4 region) for all strains was T with an exception of A for *G. thermoleovorans* Z26922. While at sequence position 1379 (U7 region), *G. lituanicus* AY608945 the nucleotide was A, and G in other organisms. More differences on 16S rRNA genes from the strains occurred in non-conseved sequence regions regions (Table 3). There were two bases, A at sequence position 1167 and T at 1186, on Papandayan isolate differed from all strains examined, however these positions lied in the non-conserved region (Table 3).

4 Discussion

Identification of thermophilic bacterium isolated from Papandayan Crater was performed through analysis of 16S rRNA gene sequence. The successful result of DNA fragment insertion into pGEM vector was confirmed by restriction analysis using *Sal*I and *Pst*I restriction enzymes. The result was also confirmed confirmed by the analysis of the fragment.

Table 2 Best nineteen homologies with 16S rRNA gene of Papandayan isolate.

No	Organisms	% Identities	Accession No.
1.	*Bacillus caldotenax*	99%	AY608937
2.	*Geobacillus kaustophilus*	99%	AY608934
3.	*Geobacillus stearothermophilus*	99%	AY608942
4.	*Geobacillus thermoleovorans*	99%	AJ536599
5.	*Bacillus vulcani*	99%	AY608940
6.	*Geobacillus thermoleovorans*	99%	AY608936
7.	*Geobacillus thermoleovorans*	99%	Z26923
8.	*Geobacillus stearothermophilus*	99%	AY608941
9.	*Bacillus caldotenax*	99%	Z26922
10	*Geobacillus thermoleovorans*	99%	AY608939
11	*Bacillus caldolyticus*	99%	Z26924
12	*Geobacillus lituanicus*	99%	AY608945
13	*Geobacillus thermoleovorans*	99%	AJ564612
14	*Geobacillus thermoleovorans*	99%	AJ564614
15	*Bacillus vulcani*	99%	AJ293805
16	*Geobacillus thermoleovorans*	99%	AY550103
17	*Geobacillus thermoleovorans*	99%	AF385083
18	*Geobacillus thermoleovorans*	99%	AJ489329
19	*Geobacillus thermoleovorans*	99%	AJ564620

No	Oganisms	The Position of Bases Variation																	bases diff.
		67	74	75	92	167	508	602	758	815	1170	1189	1379	1460	1463	1469	1470	1472	
1.	*G.thermoleovorans* AJ536599	G	**R**	**R**	A	A	G	T	C	T	**G**	**G**	G	**M**	T	T	T	C	5
2.	*G.thermoleovorans* AJ564612	G	A	**C**	**T**	**G**	**A**	**G**	C	T	**G**	**G**	G	**A**	**C**	**G**	**C**	**A**	12
3.	*G.thermoleovorans* AB034836	G	A	A	**G**	A	G	T	C	T	**G**	**G**	G	**A**	T	**G**	**C**	**A**	7
4.	*B.caldovelox* Z26925	G	**G**	**G**	A	A	**A**	T	C	T	**G**	**G**	G	**A**	T	**G**	**C**	**A**	9
5.	*B.caldovelox* AY608938	G	**G**	**G**	**S**	A	**A**	-	C	T	**G**	**G**	G	**A**	**C**	**G**	**C**	**A**	11
6.	*G.lituanicus* AY044055	G	A	A	**G**	A	G	T	C	T	**G**	**G**	G	**A**	**C**	**G**	**C**	**A**	8
7.	*G.stearothermophilus* AY297092	G	A	A	A	A	G	**C**	C	T	**G**	**G**	G	**A**	**C**	**G**	**C**	**A**	8
8.	*G.lituanicus* AY608945	G	A	A	A	A	G	T	C	T	**G**	**G**	**A**	**A**	**C**	**G**	**C**	**A**	8
9.	*B.vulcani* AJ293805	G	**G**	**G**	**G**	**G**	**A**	**C**	C	T	**G**	**G**	G	**A**	T	T	T	C	9
10.	*B.vulcani* AY608940	G	**G**	A	**G**	A	G	T	C	T	**G**	**G**	G	C	T	T	T	C	4
11.	*G.bogazici* AY323205	G	**G**	**R**	A	A	G	**C**	C	T	**G**	**G**	G	**A**	T	T	T	C	6
12.	*B.caldotenax* AY608937	G	A	A	**G**	A	G	N	C	T	**G**	**G**	G	C	T	T	T	C	**3**
13.	*G.stearothermophilus* AY608942	**R**	A	A	**G**	A	G	T	C	T	**G**	**G**	G	**A**	T	T	T	C	5
14.	*B.caldolyticus* Z26924	G	A	A	A	A	G	N	C	T	**G**	**G**	G	**A**	T	T	T	C	**3**
15.	*G.kaustophilus* AY608934	G	A	A	A	A	G	**C**	C	T	**G**	**G**	G	**A**	T	T	T	C	4
16.	*G.stearothermophilus* AY608941	**R**	A	A	**G**	A	G	**C**	C	T	**G**	**G**	G	**A**	T	T	T	C	6
17.	*G.thermoleovorans* Z26923	G	A	A	A	A	G	T	C	**A**	**G**	**G**	G	**A**	T	T	T	C	5
18.	*B.caldotenax* Z26922	G	A	A	A	A	G	**C**	**T**	T	**G**	**G**	G	C	T	T	T	C	4
19.	*isolat Papandayan*	G	A	A	A	A	G	T	C	T	A	T	G	C	T	T	T	C	

Table 3 The bases variation among Papandayan isolate and 18 other known strains.

Analysis using the BLAST Program (Atschul et al. 1997) resulted in sequence that had close homology to all 16S rRNA genes available on the GenBank. This confirmed that the amplicon was a part of 16S rRNA gene. 19 closest (99% identities sequences) homologies of the isolate appeared to the extreme thermophilic genus of *Bacillus* or *Geobacillus* (Table 2). Close homology of the isolate to extreme thermophile from the genus of *Bacillus* and *Geobacillus* was also supported by morphological identification (Figure1) and ability of the isolate to grow at a range of temperature between 45 to 80^{o}C (data not shown). Extreme thermophile of *Bacillus*, such as *B. caldotenax*, *B. caldovelox*, *B. caldolyticus*, *B. thermoleovorans* and *B. stearothermophilus* were able to grow at temperature range of 45 – 80^{o}C (Friedman, 1992). A large collection of rod shaped bacteria (Akhmaloka et al. 2000; Baker et al. 2001; Indrajaya et al. 2003) may be due to the media or technique used on the sampling and the treatment of the sample during cultivation in laboratories. *Bacillus* was known as dominance bacteria on the environment due to its ability to form endospore, while other bacteria need very specific condition to struggle for growth.

Phylogenetic tree was constructed by ClustalW methode from MEGALIGN program of DNASTAR®, using 27 nucleotide sequences that displayed close homology from the BLAST program. Papandayan isolate showed closest similarity to *B. caldolyticus* Z26924, *B. caldotenax* AY608937, and *G. stereothermophilus* AY608942 (Figure 4). Although Papandayan isolate showed closest similarity to the above strains, it was hard to differentiate among other strains as mentioned on the Table 2, since all of them were 99% identical with the 16S rRNA gene sequences. However, the result from detailed comparison with the 16S rRNA gene sequences showed that the possibility of Papandayan isolate being identical with *B. vulcani* AJ293805, *B. caldovelox* AY608938, Z26925, and *G. thermoleovorans* AJ564612, *G. thermoleovorans* Z26922, *G. lituanicus* AY608945 was unlikely since they have differences in the conserved region. Results of detailed comparison between Papandayan isolate with *B. caldolyticus* Z26924, *B. caldotenax* AY608937, and *G. stereothermophilus* AY608942 showed that *B. caldolyticus* Z26924, *B. caldotenax* AY608937 showed that there were only 3 different bases with Papandayan isolate, while *G. stereothermophilus* AY608942 had 5 base differences. Based on all of the above results there is a possibility that Papandayan isolate is a unique strain since different strains such as *B. caldolyticus* Z26924, and *B. caldotenax* AY608937 were differentiated by two base variation only. While Papandayan isolate had at least 3 base differences with other known strains.

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