Culture-Independent and Culture-Dependent Approaches on Microbial Community Analysis at Gedongsongo (GS-2) Hot Spring

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Abstract
Microbial community analysis was performed in one of Gedongsongo hot spring, namely GS-2, using culture-independent and -dependent approaches. Microbial samples were obtained from filtration and cultivation of hot spring water. Partial 16S rRNA gene sequences were amplified using a set of primers for highly variable V4-V5 regions. The amplicons were characterized using denaturing gradient gel electrophoresis (DGGE). The DGGE profile showed that there were three bands in each well with similar pattern, while the others bands appeared at various positions. Most of bands from the DGGE gels were excised, re-amplified, and directly sequenced. The comparison study of 340 bp from each band to another 16S ribosomal RNA sequences from database and the phylogenetic analysis revealed that the predominant bacteria were β-proteobacteria as revealed from filtration sample, while microorganisms from cultivation samples were mostly β-proteobacteria and Firmicutes. In addition there were two bands representing α-proteobacteria. The member of β-proteobacteria mostly come from family of Burkholderiaceae (Ralstonia or Burkholderia), while the other were Delfia (Comamonadaceae family) and some uncultures β-proteobacteria. Most of Firmicutes member were belonged to Alicyclobacillus sp. Further analysis on the 16S rRNA gene sequences from member of Burkholderiaceae revealed that there was a unique deletion at position 559 (E. coli position) in all of the GS-2 samples. This deletion suggested that the microorganisms from Gedongsongo hot spring are unique strains.

Keywords: thermophiles, 16S rRNA, DGGE, simple enrichment.

INTRODUCTION
The development of techniques for analysis of 16S rRNA gene sequences in natural sample has greatly enhanced our ability to detect and identify bacteria in nature (Pace et al., 1986). The recent application of molecular phylogeny to environmental sample has resulted in the discovery of abundance unique and previously unrecognized microorganisms. However, there are many differences in number between isolated and naturally occurring microorganisms present in various habitats (Pace, 1997). The vast majority of this microbial diversity has proved obstinate to cultivation (Zengler et al., 2002). Many attempts have been performed to cultivate previously uncultured organisms (Joseph et al., 2003; Ferrari et al., 2005; Connon et al., 2002).

Culture based approaches to isolate microorganisms from any natural environments do not provide comprehensive information on the composition of microbial communities. This technique also failed to determine the predominant microorganisms in nature that could not cultivated by using standard techniques (Amann et al., 1995). In another hand it was also confirmed that bacterial community analysis based on culture-independent had limitation since only predominant species in the habitat may be detected (Ward et al., 1998). Therefore the microbial community analysis using culture-dependent and -independent approaches were...
Table 1: The primer sequences. The specificity of primer Com1-GC is imparted by the underlined region.

<table>
<thead>
<tr>
<th>Primer</th>
<th>primer sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Com1-GC</td>
<td>CGCCCGCCGCACGGGGCAGCAGCCGCGGTAATAC</td>
</tr>
<tr>
<td>Com1-F</td>
<td>CAGCAGCCCGGTAAATAC</td>
</tr>
<tr>
<td>Com2-R</td>
<td>CCGTCAATTCTTGAGTTT</td>
</tr>
</tbody>
</table>

Table 2: Number of homological analysis result of DGGE band sequences corresponded to bacterial group. (F) from filtration cells; (MBae, Mban, YTae, YTan, TSae, TSan, Gae, from MB, YT, TS, and G) from cultivation cells on correspond media with aerobic and anaerobic conditions respectively.

<table>
<thead>
<tr>
<th>Bacteria group</th>
<th>F</th>
<th>MBae</th>
<th>Mban</th>
<th>YTae</th>
<th>YTan</th>
<th>TSae</th>
<th>TSan</th>
<th>Gae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum <strong>Firmicutes</strong></td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>α</em>-proteobakteria</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>β</em>-proteobakteria</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

significantly increased in recent times (Kisand and Wikner, 2003; Ellis et al., 2003; Orphan et al., 2000). The most application of culture-dependent procedure in community analysis were carried out using synthetic medium supplemented with various nutrients (Nold et al., 1996; Burns et al., 2004; Simbahan et al., 2005). The other approaches were performed using enrichment of spring water (Marteinsson et al., 2001; Hobel et al., 2004).

Muyzer et al. (1993) were developed a culture-independent method that has potential to study the microbial flora quickly, namely denaturing gradient gel electrophoresis (DGGE). This approach could directly measure the microbial community in specific ecosystems by separating the PCR products amplified by universal primers. The technique has been applied to characterize bacterial community from many habitats based on culture-independent approach such as soil (Stephen et al., 1998), marine (Di´ez et al., 2001), polluted river (Ce´bron et al., 2004), even microbes in gas industry pipelines (Zhu et al., 2003). Furthermore, DGGE has been used to analysis the microbial community based on culture-dependent alone (Santegoeds et al., 1996) or combination of these two approaches (Kawai et al., 2002; Burns et al., 2004; Rantsiou et al., 2005).

Indonesia is one of the most tectonically active areas in the world with a substantial numbers of geothermal regions (Kusumadinata, 1977). However, study concerning the diversity of thermophilic microorganisms from these areas was very limited. One of tectonic active area that has not been studied was Gedongsongo hot spring located in Unggaran volcano, Central Java. The location of Gedongsongo hot springs lied on the south flank of the mountain. There are a few hot spring in Gedongsongo, the pH and temperature of hot spring water in range from 1.5 – 5.2 and 50 - 86°C depending on weather. They are low-flow, steam-heated sulphurous water or meteoric slightly diluted with steam-heated water. The area is a considerable as solfatara and steaming ground, as well as gypsum and kaolin deposit (Suroto and Hartono, 1986, unpublished report).

In this report, we described the application of culture-dependent and -independent methods to study the microbial community on Gedongsongo (GS-2) hot spring using DGGE technique combined with phylogenetic analysis. The culture-independent approach was carried out by filtration through 0.2-μm-pore-size filter while culture dependent approach were performed using hot spring water supplemented with various low nutrients concentration and cultivation conditions.

**MATERIALS AND METHODS**

**Materials**

The microbial samples were collected from one of hot spring, namely GS-2, at Gedongsongo field of Ungaran volcano, Central Java, Indonesia (110°20′23.4″E; 07°12′08.5″S; and the altitude 1400m). This hot spring has temperature and pH at around 50°C and 4
respectively. The primer for PCR, re-PCR and sequencing was shown in Table 1.

**Sampling procedure**

The water and microbial samples were collected from the center of hot spring. The diversity was assayed using two methods: cultivation and filtration. For the filtration procedure, the water sample was kept in a sterile plastic container (2L) and brought to the laboratory immediately within 2 hours. Afterwards, cells were collected by filtration of 1 L volumes of spring water gently through 0.2 µm Millipore filters. Filtrates containing bacterial communities were collected by putting the filter in a sterile Erlenmeyer contained 10 ml sterile distillated water. The Erlenmeyer was then gently shaken and the membrane surface was scrubbed aseptically using Ose needle for couple times until most of the cells were re-suspended in sterile water. Each microbial sample was pelleted by centrifugation at 8,000 x g for 15 min and stored at -20°C until DNA extracted.

**Culturing Strategy**

The spring water was used in all enrichment media to mimic *in situ* conditions. Four simple nutrient medium were chosen as a carbon sources only and/or containing vitamin-mineral requirement, namely YT (0.02% (w/v) yeast extract and 0.02% (w/v) tryptone), TS (0.02% (v/v) of mung bean sprout filtrate and 0.04% (w/v) sucrose), G (0.1% (w/v) glucose), and MB (0.02% (w/v) beef extract, 0.02% (w/v) peptone, and 0.01% (w/v) NaCl). The cultivation was carried out at 55°C. For the aerobic condition, all medium were shaken at 75 rpm. Meanwhile the anaerobic (limitation of oxygen supply) condition, were performed using almost full culture medium at the glass tubes and cultivated using a simple candle-jar method to reduce oxygen content. The incubation was stopped and cells were harvested at early stationary phase (between 3 to 12 days).

**Nucleic acid extraction**

DNA was extracted using slight modification method described by Zhou *et al.* (1996). Pellet cells were mixed with 300 µL of DNA extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], and 1.5 M NaCl). The solution was added by 10 µL of proteinase K (20 mg/mL) and 0.2 g of sterile sea sand in 1.5 mL tubes by hand shaking for 10 min. After the shaking treatment, 30 µL of 20% SDS was added, and the samples were incubated at 60°C for 1 h with gentle end-over-end inversions every 15 to 20 min. The supernatants were collected after centrifugation at 6,000 x g for 10 min at room temperature and transferred into micro tubes. Supernatant was added by an equal volume of chloroform-isoamyl alcohol (24:1, v/v). The aqueous phase was recovered by centrifugation and precipitated with 1 volume of isopropanol at room temperature for 15 min. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 x g for 10 min at room temperature, washed with cold 70% ethanol, and re-suspended in sterile distillated water, to give a final volume of 50 µL.

**Amplification of 16S rRNA gene fragments**

The partial 16S rRNA genes were amplified using a set of primers. One primer (Com1-GC) complements to a region conserved among members of the *Bacterial* domain (16SrRNA DNA of *Escherichia coli* at positions 519 to 536) incorporated by 40-base GC clamp. The other primer (Com2-R) was based (Schwieger and Tebbe, 1998) on a universally conserved region (*E. coli* positions at 907 to 926). The specificity of this primer is imparted by the underlined sequence (Table 1). PCRs were performed by using *Taq* DNA polymerase according to the instructions provided by the manufacturer (MDBio). PCR products were examined on ethidium bromide-stained agarose gels, and the products with expected size were used for DGGE analysis. Amplification was performed within 30 cycles. The temperature cycle for the PCR was 1 min of denaturizing at 94°C, 1 min of annealing at 50°C, and 1 min of primer extension at 72°C. The final primer extension was for 30 min.
40% gradient of urea and formamide (UF) solution. A 100% UF solution is defined as 40% (v/v) formamide plus 7.0 M urea. DGGE was conducted at 60°C, firstly at 20V for 10 min and then at a constant voltage at 150 V for 3 hours. Gel was stained with silver staining (Bassam et al., 1991).

![Figure 1: Electrophoregram of 16S rRNA gene fragments (representative). Lane 1, shows pUC19 cut by HindIII as DNA marker. Lane 2-4 show PCR product amplification from F (filtration), MBae, and MBan cultures respectively.](image1)

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**RESULTS**

**Chromosomal DNA and 16S rRNA Gene Fragments**

The cells from both filtration and cultivation have been lysed to extract their total chromosomal DNA. Total chromosomal DNA from the extraction appeared a single band on ethidium bromide-stained agarose gel (data not shown). Total chromosomal DNA from both filtration and cultivation cells were used as a template to amplify partial 16S rRNA gene using primer pair Com1-GC and Com2-R. The PCR was performed using 30 minutes of final primer extension process as suggested by Janse et al. (2004), in order to eliminate the artifactual double bands. The mixture of chromosomal DNA from all isolates from filtration and cultures were successfully amplified and yielded single distinct band. The PCR products from the filtration and culture cells were examined on ethidium bromide-stained agarose gels, and the products showed as expected size (Fig. 1).

**Characterization of 16S rDNA amplicons**

Following the amplification of partial 16S rRNA gene, the fragments were then separated by
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**Figure 3:** The phylogenetic trees of DGGE bands sequences. Neighbor-joining trees showing phylogenetic relationships among 16S rRNA genes of DGGE bands compared to those of representative species and environmental clones. Scale bars indicate 0.1 change per nucleotide. Band sequences were added to the trees using DNA distance with Kimura-2 parameter. Bootstraps were done using 1000 replicates.

DGGE. The DGGE analysis showed that there were some difference patterns between bands from filtration and cultivation methods (Fig 2). There were three bands appeared in all lanes at the same position but different intensities, while another two bands with the same position and different intensities appeared in some other lanes. Most of the amplicons from cultivation cells produced additional bands with variation of position and intensities.

**Phylogenetic analysis of the DGGE bands**

Twenty six of DGGE bands were successfully re-amplified and sequenced. The sequences were aligned to each other and compared to the sequences from the database at GenBank. No chimeric artifacts were found among the sequences. Operational taxonomy units (OTU) were established using around 340 bp DNA-DNA similarity as cutoff. Based on homological analysis, the result showed that all bands belong to the member of *Firmicute*, *β-proteobacteria*, and *α-proteobacteria* (Table 2).

Phylogenetic trees of 16S rRNA gene sequences were constructed based on the distance matrix methods as state on the methodology. The tree showed that most of bands belong to the member of *β-proteobacteria* (17 bands), some *Firmicutes* (7 bands) and 2 of them are *α-proteobacteria*.
among each other (Fig 4). There was a base differences on the nucleotide sequences sequences of Burkholderiaceae corresponded to phylogenetic trees showed that the most bands found in this study was dominated by Acidicaldus sp. (Fig 3). The BLAST N analysis revealed that sp. corresponded to Commamodacea family. The phylogenetic trees showed that the most bands corresponded to Burkholderiaceae family tend to be out of the family. Further analysis on the sequences of β-proteobacteria showed that there were differences on the nucleotide sequences among each other (Fig 4). There was a base deletion at position of 559 (E. coli position) in all bands examined.

**DISCUSSION**

Previous studies on microbial community analysis revealed discrepancy between community analysis using culture methods and those described by culture-independent (Nold et al., 1996; Santegoeds et al., 1996; Simbahan et al., 2005). The differences were caused might be due to the use of synthetic medium that were difference from the in situ habitats. One of the techniques that could eliminate the gap between culture and uncultured method was the application of spring water as culturing medium. This approach has been applied by Marteinsson et al. (2001) and Hobel et al. (2004) which were enriching the spring water with variation of nutrients. Many reports also revealed that the application of very low nutrient concentration yield higher numbers of culturable cells (Aagot et al., 2001; Janssen et al., 2002). In this study, we used spring water as basic medium to eliminate the gap between culture and uncultured method. The application of low nutrient concentration (around 0.02%) that was added to spring water also revealed many bacteria as shown by various bands in culture conditions. This result was contrast with our preliminary study in GS-1 hot spring by high concentration nutrient added to spring water (Aminin et al., 2007).

The dominant species in this hot spring as revealed on culture-independent results were the member of β-proteobacteria especially from Burkholderiaceae family. Based on BLAST N study, the most member of Burkholderiaceae of GS-2 isolates represent the unculturable β-proteobacteria, this was supported by the phylogenetic tree which most of them were out of Ralstonia or Burkholderia branch (Fig 3). This data was not surprising since the BLAST N analysis results showed that the sequences only have 85 to 90% similarity to the 16S rRNA gene sequence from database of Burkholderiaceae group. The evidences suggesting that those bands (band-c, -i, -j, -l, and -o) represent novels species within β-proteobacteria group. The other bands from filtration sample were represented to Commamodaceae family (Delftia) and α-proteobacteria. The similar phylotypes toward Delftia also appeared in all cultures with different intensities (align to band-b1). However, there was no band from cultivation cells that showed similar pattern with α-proteobacteria from filtration sample. Another band represented α-proteobacteria was showed in TSae well (band-q). Even BLAST N analysis showed that these α-proteobacteria bands have major similarities with uncultured α-proteobacteria,
but phylogenetic tree positioned the bands closed to *Acidicaldus* sp. (Fig 3). As the major group (phylum) of bacteria, *Proteobacteria* previously known as mesophilic bacteria. However the present of this group from a few thermal habitats have been reported, including from the hot spring (Hugenboltz et al., 1998) and anaerobic hybrid reactor (Chen et al., 2004).

There were seven bands that correspond to *Firmicutes* and all of them were come from cultivation cells. As shown on phylogenetic tree (Fig 3), the six bands that previously showed as a member of *Alicyclobacillaceae* based on BLAST N analysis were tend to make new branch in the tree, this is might be due to the bands only have 91 to 93% similarity to most of *Alicyclobacillus* sp from Genbank. Moreover, one band (band-p) showed close to gram positive bacteria and the phylogenetic tree analysis showed the band close to *Paenibacillaceae*. This evidence suggested that there were some novels *Bacilli* from GS-2 hot spring.

Based on the comparison study from BLAST N analysis, the nucleotide sequences on V4-V5 region of the 16S rRNA genes of almost all *Burkholderiaceae* family and *Delftia* were identical, however GS-2 isolates revealed some differences (Fig 4). The significance result of the sequences comparison was the present of unique sequence belonged to GS-2 isolates that differ to another species within the same group. The unique point lied on a base at position 559 (*E. coli* position). At this position, the nucleotide of most member of *Burkholderiaceae* family and *Delftia* was A, while the GS-2 isolates showed deletion. The unique deletion also showed by GS-2 isolates which were only have 90% similarities toward *Burkholderiaceae* database. This deletion showed unique and suggested that Gedongsongo isolates were novel strains within these groups.

Phylogenetic analysis of the GS-2 isolates showed an evidence of the present enormous new and unique strains especially from the cultured microbes. In this study, culturing formats for high-throughput isolation of diverse strains using spring water with very low concentrations, relatively long incubation time and aerobic-anaerobic cultivation could enlarge the bacterial community analysis and reducing the gap between the result from culture-dependent and -independent. However, the same procedure for different habitat might not give the same expectation results concerning the numbers and variation of microbes (Papke et al., 2003). Therefore, more diverse information on the microbial community of particular habitat needs more various supplemented nutrients and it is still important on the application of various synthetic medium to capture the bacteria that were not dominant in the habitat.

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**References**


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