ISBN 978-979-19215-0-3

SSCP profiles will describe the predominant thermophilic bacteria in this hot spring.

MATERIALS AND METHODS

Bacterial sampling and description of the GS1 hot stream.

GS3 hot stream belong to Gedongsongo field that located in Ungaran volcano, central Java (110°20'23.4"E; 07°12'08.5"S; altitude: 1400m). This hot stream has temperature 72°C and pH 6. The microbial sample was collected from the hot stream through collecting the water on 15 May 2005. The microbial diversity studies were approached using two methods: filtration and cultivation. For filtration procedure, the water sample was kept in a sterile plastic container (1L) and brought to the laboratory immediately within 2 hours. Afterwards, cells were harvested by filtration of 1 L volumes of spring water gently through 0.2 µm (nominal pore size) Millipore filters. Resulting filtrates containing bacterial communities were stored at -20°C until DNA was extracted. For the cultivation procedure was carried out using two kinds of minimal mediums which are GTa and GYa. The formula of GTa medium contained 0.1% triptone and GYa medium contained 0.1% yeast, except the water which were used is the spring water. These medium were cultured semi-anaerobic at 70°C for 48 hours.

Isolation of Chromosomal DNA.

Each microbial sample which is from cultivation or filtration was pellet by centrifugation. DNA was extracted using slight modifications of a method described by Klijn et al. (1991). The pellet was suspended in 200 µl of 10 mM Tris HCl buffer (pH 8.0) containing 8mg/ml of lysozyme and incubation at 37°C for 1 h, the cells were lysed by adding 200 µl lysis buffer containing 2% sodium dodecyl sulfate, 0,8 mg/ml proteinase K and 200 mM EDTA pH 8,0. The lysis process was done by incubation at 50°C for 30 min. The purification step was carried out by adding 200 µl of chloroform:isoamil-alcohol (24:1), vortex and centrifugation at 1300 g, 30 second. The upper solution was moved to clean tube. This step was done 3 times. Subsequently, the DNA was precipitated by adding 60 µl of 3 M sodium acetate and 1 ml of 96% ethanol (stored at -20°C). After centrifugation, the DNA pellet was dissolved in 10 mM Tris HCl buffer (pH 8.0). The DNA pellet was washed with 70% ethanol and finally dissolved in 50 µl of TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA).

Amplification the segment of 16S rRNA gene.

Each PCR was carried out in a final volume of 50 µl. Primers used are Com1F: 5'- CAG CAG CCG CGG TAA TAC –3' (Bacteria position: 519-536) and Com2R: 5'- CCG TCA ATT CCT TTG AGT TT –3' (Bacteria position:907-926). Reaction mixtures contained 1X PCR buffer with 1.5 mM $MgCl_2$, deoxynucleoside triphosphate solution (200 µM each dATP, dCTP, dGTP and dTTP) and 2,5 U Taq DNA polymerase (Promega). Standard PCR was conducted using a `touchdown' program which consisted of an initial denaturation at 94°C for 4 min, During an initial touchdown cycle, the annealing temperature was lowered from 53 to 43°C in intervals of 18°C per cycle; 21 additional annealing cycles were done at 43°C. The final primer extension was for 10 min.

SSCP Analysis

Five microlitres of PCR products were mixed with 2 µl 5x alkali denaturing buffer (1x buffer is 0.1 M NaCl, 20 mM EDTA), incubated at 95°C for 3 min and immediately snap-cooled on wet ice ; 3 µl loading buffer (95% formamide, 0.25% xylene cyanol, 0.25% bromophenol blue) was added, and 4 µl of each sample was loaded onto a 10% non-denaturing polyacrylamide gel. The samples were electrophoresed in a gel electrophoresis apparatus with 1x TBE buffer at 200 V for 5 h at 4°C using a 0.7-mm 8-well comb/spacer set.

Silver Staining. Gel was stained with silver staining method (Bassam et al., 1991). Firstly, gel was fixed using 10% acetic acid for 2x10 minutes and then rinsed with distilled water for 6 minutes. Subsequently gel was stained for 30 min in 100 ml silver nitrate solution containing 0,1 % $AgNO_3$ and 150 µl formaldehyde/HCHO 37%. After that, gel was rinsed for about 10 seconds in distilled water and immediately soaks with the cold (4-10°C) developing solution (100 ml solution containing 3 % sodium carbonate, 150 µl formaldehyde and 20 µl sodium thiosulfate stock solution (10 mg/ml)) for 2 to 5 minutes. Therefore, the solution is typically replaced every few minutes until the desired staining intensity is reached. Terminate staining by discarding the developing solution and replacing it with 10% acetic acid.