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16S rRNA Gene Analysis of Chlorate Reducing Thermophilic Bacteria from Local Hot Spring

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ABSTRACT

Chlorates waste remediation by biological processes has been the object of current research. Strain CR, the chlorate reducing bacteria was isolated from Gedongsongo hot spring using minimal medium broth containing chlorates and acetate at 55°C. The determination of chlorate reduction from medium was carried out using turbidimetric method. CR isolate showed reducing ability 18% after four days of incubation. The phenotypic character of CR isolate including rod-shaped cells, gram-positive bacteria and facultative anaerobes. On the basis of 16S rRNA analysis, CR isolate was closely related to *Bacillus pallidus, Aeribacillus E3, Geobacillus pallidus* and *Geobacillus* sp.D64. The similarity of nucleotide sequences of 16S rRNA gene was 97%, suggested as a novel species.

Keyword: thermophilic bacteria, chlorate reducing, 16S rRNA gene

INTRODUCTION

Chlorates widely used for various industrial activities. In agriculture, chlorates are used as a weeds control (herbicides) (Logan, 1998). Chlorates also used as a bleaching agent in pulp and paper industry, disinfectants, making the mixture lighter (Van Ginkel, et al, 1995), explosives and fireworks (Wolterink, 2004). Contamination has been detected in Indonesia, chlorates contaminate water and soil due to accumulation of waste pulp and paper industry (Arisandi, 2009). Chlorates contaminations cause oxidation in red blood cells, hemolytic anemia and the formation of methemoglobin (Wolterink, 2004). Various technologies have been developed to remove chlorates contamination, including ion exchange, bioremediation, membrane technology, chemical precipitation, and catalytic reduction electrochemistry. Each of these processing technology has advantages and disadvantages of each (Gu et.al., 2002). Chlorates waste remediation by biological processes has been the object of current research. Excess use of microbial remediation techniques, compared with the technique of physical separation owner is a total change of chlorates into harmless end products such as chloride and oxygen (Attaway, 1993; Rikken et.al., 1996).

Most industries do production activities at high temperature, produces a high temperature waste. High temperature waste will greatly benefit from the bioremediation system using thermophilic bacteria. Thermophilic bacteria are unique cells that can grow at high temperature reached the growth temperature of 80 °C or higher, and some even live on the boiling point temperature (Oshima, 2004). Thermophilic bacteria can be isolated from hot springs, sea floor hydrothermal system (Oshima, 2004; Aminin, 2008).

Indonesia is one country that has still active tectonic regions in the world. Indonesia has abundant hot springs because of the 70 mountains that are still active (Kusumadinata, 1977), but the natural resources therein unexplored maximally. One tectonic region that has not been fully studied is Hot Springs located in Mountain Gedongsongo Ungaran, Central Java. Gedongsongo Hot Springs located on the south side of the mountain with several hot springs. Aminin (2008) have been identified thermophilic microbial diversity from one of Gedongsongo hot spring (GS) that has pH 6. From this research, it has been identified thermophilic bacterial communities from *β-Proteobacteria* sub-class, family *Rhodocyclaceae*, which is a genus *Dechloromonas*, chlorates reducing bacteria. Research Bender et al. (2004) concluded that

genus *Dechloromonas* can reduce the chlorates into chloride and oxygen. This research was aimed to isolate single thermophilic reducing bacteria from Gedongsongo hot spring.

MATERIALS AND METHODS

Sampling and Cultivation

A water sample was taken from Gedongsongo crater with a temperature of 70°C and pH 6. Sample immediately brought to the laboratorium and cultivated in minimal medium broth (MB) 0.02% (0.02% beef extract, peptone 0.02% and 0.01% NaCl), $KClO_3$ (5mM, 10mM, 15mM) and KAc (10mM) (Achenbach, 2001). Bacterial growth in semi-anaerobic conditions (limited oxygen supply) using a simple candle-jar method, at a temperature of 55°C until turbidity occurred (Aminin, 2008). Single colonies were obtained by spreading culture from liquid media on solid media. Phenotypic identifications of bacterial from single colony were carried out through gram staining and morphological observations.

Chlorates Reduction Test

Reduction test was done by measuring residual chlorates. The remaining chlorates added with 5% $NaNO_2$ and Ag^+ to form turbidity. Turbidity was measured by the turbidimetric method. Chlorates standard curves were obtained from absorbance measurements of chlorates reduction at a certain concentration (0, 2, 4, 6, 8 and 10mM).

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 60oC 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 fragment by PCR Method

DNA samples were then amplified in vitro by PCR method. 16S rRNA gene fragment was amplified using a set of primers 16SF at positions 8-27 (5'-AGAGTTTGATCCTGGCTCAG-3 ') and 16SR at positions 1492-1510 (5'-GGTTACCTTGT TACGACTT-3'). Template DNA samples plus Primary 1µL 16SF, 16SR and 45µL Primary 1µL Mega Mix Blue (MMB). Amplification was performed within 30 cycles. The temperature cycle for the PCR was 30 seconds of denaturizing at 94°C, 45 seconds of annealing at 54°C, and 3 min of primer extension at 72°C. The final primer extension was for 10 min.

Phylogenetic Tree Construction

Sequencing was carried out in an ABI PrismR 3100 Genetic Analyzer (Applied Biosystems) by the Macrogen Sequencing Service (Korea). The sequencing reaction was performed with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using universal primers 27F and 1492R. The sequencing results were subjected to analyzed and compared to the database GenBank using the BLAST N program at National Centre of Biotechnological Information (NCBI) website. The phylogenetic trees were constructed using PHYlLIP 3.68 ed. Program with Parsimony method. Phylogeny tree was obtained using phylodendron program.

RESULTS

The ability of a thermophilic bacteria on various medium containing different chlorates were measured based on their turbidity at the 0 days (before cultivated) and the 4th day, compared toward negative control (without bacteria) are listed in table 1. This data show that bacterial

strain could growth quite well in 5 mM chlorates. However, at higher chlorates concentration, it is showed that this strain growth efficiently in medium containing 10 mM chlorates rather than 15mM chlorates. Then next studies were done at 10mM chlorates.

Chlorates Reduction Test

Chlorates reduction value of strain CR were measured based on the residual chlorates using turbidimetric method at the fourth day, as shown in table 2. On the 0 days, the absorbance value showed that chlorates concentrations around 10 mM in all cultures (with or without bacteria). After four days of incubation, the chlorates concentration decreased including control. Based on this data, the reduction capacity of CR strain is 18%.

Table 1. Chlorates reduction of CR isolate compared toward negative control

Incubation time (days)	[chlorates] (mM)	
	Control(-)	strain CR
0	10.34	10.36
4	8.21	5.98

The characteristic of CR isolate

CR isolates are rod-shaped gram-positive bacteria. Based on growing characteristic of bacteria on the surface of solid media while growth in semi-anaerobic condition, it seemed that CR strain is facultative anaerobe. The cells of CR isolate have been lyzed to extract their chromosomal DNA. Total chromosomal DNA from the extraction appeared a single band on ethidium bromide-stained agarose gel (data not shown). The chromosomal DNA were used as a template to amplify almost complete 16S rRNA gene using primer pair universal 16SF and 16SR and successfully amplified and yielded single band. The PCR products from strain CR was compared to controls and examined on ethidium bromide-stained agarose gel. The products showed as expected size, the amplicons align with marker at 1500 pb.

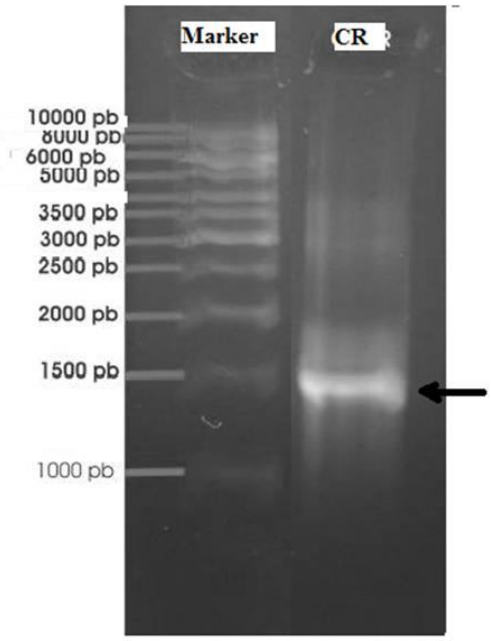

Figure 1. Electrophoregram of 16S rRNA gene fragments of CR isolate. Well 1: DNA *marker*; well 2: CR isolate DNA

Phylogenetic study

The nucleotide sequences of 16S rRNA gene fragments from each primer resulting around 700 bp of reading sequence. Each sequences were combined using Seq-align program resulted 16S rRNA gene sequence fragment the length of 1380pb. Phylogenetic analysis of strain CR with other bacteria was done by comparing the nucleotide sequence with GenBank database using BLAST-N program. BLASTN data produced 100 nucleotide sequences of 16S rRNA gene from family *Bacillaceae*, the main species represent by *Bacillus pallidus* with 97% similarity. Twenty five sequences were choosen to construct phylogenetic tree compared to CR isolate, shown in figure 3.

Phylogeny tree shows that CR isolate is closely related to *Bacillus pallidus, Aeribacillus E3, Geoacillus* sp. D64 and Geobacillus pallidus. The highest similarity of CR strain toward relatively closest strain is 97%, therefore suggesting as a novel species. Bacteria that have a nucleotide sequence similarity less than 98% is suggested as a novel species (Ibrahim, 2004; Balk, 2008). Based on phenotypic character, strain CR and those two relative strains have phenotypic similarity. All three are rod-shaped bacteria, gram-positive and thermophilic. Yet all three

have a difference of characters of different genotype. There are some specific nucleotide sequence differences between strain and the two relatively close bacteria. Comparison of the nucleotide sequence regions upstream and downstream sections showed some substitutions along the sequences. In the middle regions of nucleotide sequences (about 500 to 800-an) there is a different form of insertions which vary from one, two to three nucleotide bases, while in the regions surrounding the nucleotide sequence of 700s there is a deletion (data not shown).

The previous study of bacterial diversity from this hot spring has been identified the presence of chlorate reducing bacteria of *Dechloromonas* sp. However, the bacteria that have been isolated from the same hot spring using the same media and cultivation technique, although showing ability to reduce chlorate nevertheless showing distinct species. This different type of bacteria showing the dynamical phenomenon of microbial diversity with a certain time. Changes in physical and biotic environment can stimulate the emergence of populations of certain organisms which later can lead to population change (Gray, 1992).

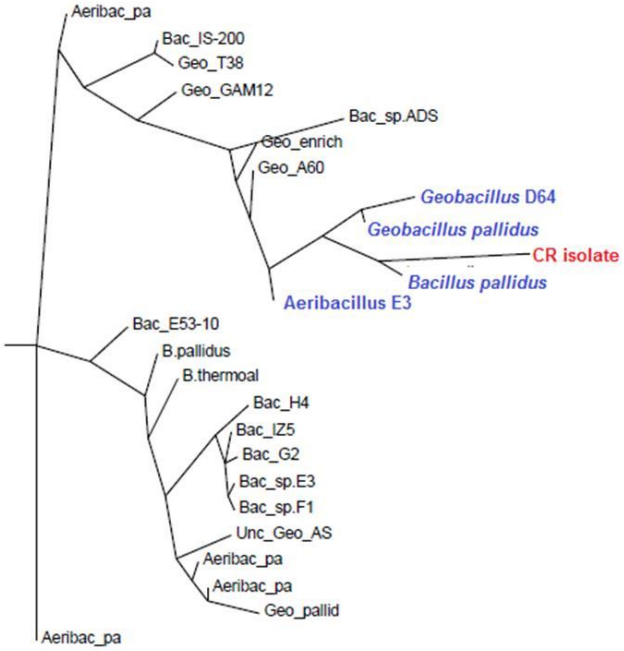

Figure 2. The phylogenetic tree of CR isolate. Parsimonious tree of the sequence along 1380 bp showing phylogenetic relationship among 16S rRNA genes of CR gene compared to25 representative sequences from BLASTN data. Bootstrap tree was constructed using 1000

CONCLUSION

Chlorates reducing thermophilic bacteria have been isolated from local hot spring using minimal medium broth (MB) containing chlorate and called CR isolate. Reduction capacity of CR isolate was 18% after 4 days incubation. Phenotypic character of CR isolate includes rod-shaped cells, gram-positive bacteria and facultative anaerobes. CR isolate was closely related to *Bacillus pallidus, Aeribacillus E3, Geobacillus pallidus* and *Geobacillus* sp.D64. The similarity of nucleotide sequences of 16S rRNA gene was 97%, suggested as a novel species.

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