# DNA Extraction of Mixed Culture Bacteria from Coal-Soil Mixture Cultured by Sequential Enrichment

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Biodesulfurizing bacteria are important, either in the form of cell or genetic resources, from viewpoints of economical and ecological interests in order to extend clean coal technology. They can be obtained from environment that have been contaminated by organic sulfur for long time, such as in coal mine soil. DNA extraction is the most important step in research exploring DNA from environmental samples, like soils. Various kits have offered direct extraction, which has advantages of simplicity and ease of use, but not necessarily to be used for all type of natural samples. Therefore, for the purpose of detection and qualitative analysis, not for quantifying microorganisms, an approach in culturing sample was made in this study. Sample of coal-soil mixture from coal mine soil was previously cultivated in sequential stages of enrichment, to enrich bacteria in a minimal medium containing organic sulfur, and then cells were harvested and extraction of DNA was attended from the cells. This method overcome the problem of humic acid contamination took place frequently in direct DNA extraction of soil samples, as evidenced by the quality of the DNA extracts obtained which were easily PCR-amplified.

Key words : DNA extraction; Coal-soil mixture; Mixed culture bacteria; Soil.

Coal has been known as fuel releasing sulfur dioxide when burned directly. Biodesulfurization is a method to remove the sulfur from coal by mean of microorganisms. This process is preferred over physical and chemical method because biodesulfurization uses microorganisms that work at medium temperature and pressure, and environmentally friendly. The biodesulfurizing microorganisms can be isolated from soil<sup>1,2</sup>. Soil provides favorable physical and chemical environment for many microorganisms. Coal is an

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organic sedimentary rocks that contains varying amounts of carbon, hydrogen, nitrogen, oxygen, and sulfur as well as trace amounts of other elements including mineral matter<sup>3</sup>. Thus, coal-soil mixture from coal soil mine was expected to contain soil bacteria that have been adapted for long time to use coal as their nutrient source.

Current biotechnology widely uses microorganisms in mixed culture<sup>4,5,6</sup>. This mimics what happens in nature, where there is a beneficial interactions in consortium<sup>7</sup>. Interactions among members of the consortium cause metabolic processes occur more established, so that the resulting products were more stable. Members in the consortium support each other in their growth and survival through gene transfer and metabolic cross-feeding<sup>8</sup>. The use of bacteria as agent of biotechnology not only refers to activity of cells, but also DNA or genes encoding for specific properties. Bacterial DNA can be isolated for various purposes, such as identification of bacteria and gene transformation. But direct isolation of DNA from natural samples has constraint, i.e. number of DNA copies to be amplified is not necessarily sufficient. In addition, DNA extracted directly from soil samples will be contaminated by humic acid. Humic acid is inhibitor to DNA amplification process in the Polymerase Chain Reaction (PCR) by inhibiting the enzyme Taq polymerase, as well as reduce detection and DNA transformation<sup>9</sup>. This constraint needs to be addressed with specific strategies, for example by releasing cells from soil sample prior to DNA extraction procedure. In the present study, direct DNA isolation barrier was overcome by increasing the bacterial cells. Because the purpose is to obtain DNA of desulfurizing bacteria, the enrichment was carried out gradually in medium containing dibenzothiophene and coal as a sole sulfur source.

#### MATERIALS AND METHODS

## Sample and medium

Coal-soil mixture was sampled from coal mine soil in Muara Tigo Besar Utara, Province of South Sumatera, Indonesia. Medium used to enrich the mixed culture was Mineral Salt Medium (MSM) containing 2 g glycerol, 4 g NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O, 4 g K<sub>2</sub>HPO<sub>4</sub>.3H<sub>2</sub>O, 2 g NH<sub>4</sub>Cl, 0,2 g MgCl.6H<sub>2</sub>O, 0,001 g CaCl<sub>2</sub>.2H<sub>2</sub>O, and 0,001 g FeCl<sub>3</sub>.6H<sub>2</sub>O in 1 L aquadest<sup>10</sup>. Dibenzothiophene (DBT) and coal powder (100 mesh) was added as sulfur source. The medium without DBT was previously sterilized by autoclaving at 121°C, for 20 min.

## Sequential enrichment of mixed culture

Coal-soil sample was first activated by incubating 5 g of the sample in 95 ml MSM medium containing 1 mM DBT, incubated for 48 h at room temperature on a rotary shaker at 150 rpm. Ten percent of the culture was then inoculated into 50 ml MSM medium containing 0.5 mM DBT and 0.5% (w/v) of coal powder, incubated for 48 h at room temperature on rotary shaker 150 rpm. Ten percent of the inoculum culture was inoculated into 150 ml MSM medium containing 1% (w/v) coal powder, incubated for 24 h at room temperature on rotary

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shaker at 150 rpm. Finally, 10% of the last culture was inoculated into 600 ml of MSM, medium containing 15% (w/v) coal powder, incubated at room temperature on rotary shaker at 150 rpm. At interval of 12 hours, cells were harvested by centrifugating at 6000 rpm for 10 min. In addition, to monitor the growth, total plate count and measurement of pH were performed on the culture. **DNA extraction** 

DNA extraction was performed according to procedure as described previously<sup>11</sup> with 3 steps. In the first step, cell pellet was added by 500 ul extraction buffer, 50 ul 20% sodium dodecyl sulfate (SDS), and 10 ul proteinase K, in a 1.5 ml microtube. After incubation for 30 min at 37° C, incubation was continued at 60° C for 2 hours. The mixture was centrifugated at 6000 rpm for 10 min. The rest of cell pellet was extracted again, by adding 500 ml extraction buffer, 50 ul 20% SDS, in each of the second and third step. Supernatant collected from the all phases was subjected to phenol-chloroform-(PCI) extraction. After isoamylalcohol centrifugated at 6000 rpm for 10 min, supernatant was separated and PCI-extracted again until no visible interface in the microtube. The separated liquid phase was precipitated with 2 times volume of 100% ethanol. After centrifugated at 12000 rpm for 20 min, DNA pellet was washed 2 times with 70% ethanol. DNA samples (5 ul each) were run on 0.8% gel agarose electrophoresis at 100 Volt for 30 min in Tris Acetate EDTA buffer. Yield of DNA was known approximately from gel electrophoresis photo analyzed by using ImageJ software<sup>12</sup>. The measurement results in pixel were converted in quantity of DNA compared to quantity of DNA marker (1 k bladder, Fermentas).

## PCR-Amplification of DNA

Set of primers used were: forward primer Com1-F with the sequence 5'-CAG CGC CAG GGT AAT AC-3', and Com2-R with the sequence 5'-CGG TCA ATT CCT TTG AGT TT-3' <sup>13-17</sup>. The reaction mixture (50  $\mu$ L) contained 1.0  $\mu$ L of 10 mM each primer, 5.0  $\mu$ L of 10× Taq buffer, 1.0  $\mu$ L of 10 uM deoxyribonucleotide triphosphate mixture, 4.0  $\mu$ L of total DNA, and 0.25  $\mu$ L of 5U/ul Taq DNA polymerase. DNA fragments were amplified as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 51°C for 1 min, extension at 72°C for 1 min, and a 10-min final extension step at 72°C. The amplified products were subjected to electrophoresis in 1.5% agarose gels. Gels were stained with ethidium bromide and visualized under ultraviolet light.

## **RESULTS AND DISCUSSION**

#### **Result of DNA extraction**

Growth peaks of the mixed cultures during use of coal as a sole sulfur source occurred at the hours of 72, 120, 168 and 240 (data not shown). Accordingly, DNA extractions were performed on cells harvested from culture samples at those peaks of growth (Fig. 1). This determination referred to the common knowledge in microbiology that bacterial cells that are in the active growth phase have cell walls susceptible to lysing materials, as used in microbial control by chemicals and antibiotics. This was also consistent with the research that the lysis events occur most frequently in the septum of bacterial cell or at the junction between two cells that grow in chains<sup>18</sup>.

The DNA extraction method referred to other researcher who developed DNA extraction



Fig. 1. DNA extracts from 0, 72, 120, 168 and 240 hrs of mixed culture on 0.8% gel agarose stained by ethidium bromide. M = marker

without kit<sup>11</sup>, in which they used chemical and physical lysis. The chemical lysis was performed by adding CTAB (in lysis buffer), SDS, and proteinase K, while the physical lysis was performed by heating at 60° C. The three materials in chemical lysis were known to damage the polymer on the bacterial cell wall, so that the contents of the cell, in this case the DNA and cytoplasm, can exit from cell and enter the alkaline buffer which maintained integrity of DNA.

Gel agarose in (Fig. 1) showed that DNA extraction of harvested cells from the mixed culture at those peak hours of growth obtained DNA with size of about 10,000 base pairs or more, referred to DNA size of marker. The results of over 10,000 base pairs of DNA corresponded to the size of the extract conducted directly on soil samples<sup>11</sup>. The gel showed not only the size of 10,000 DNA base pairs obtained, but also pieces of DNA in length of about

2,000, over 1,000 and 750 base pairs. In addition, the DNA bands on the size of about 10,000 base pairs were not strict bands on one line, but shearing bands. Those indicated that the extraction process occurs too hard physically. Shearing product of the DNA extract was due to homogenization treatment occurred during longer homogenization times and at higher speeds<sup>19</sup>. In this case, the time length of the vortex homogenization was supposed to be reduced, or not performed continuously, but interspersed with a time lag.

Furthermore, the quantity of DNA in size 10,000 base pairs was measured by comparing to the amount of DNA on the marker. Conversion of band thickness in marker and samples were conducted using Image J in order to distinguish intensity of image based on pixel differences. Measurement results in pixels were converted to ng unit of DNA extract (Fig. 2).

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Fig. 2. DNA yields from 1.5 ml of culture samples

The results showed that DNA yields increased starting at the hours of 0, 72, and 120, then decreased at the hour 168 and 240. Those implied that prior enrichment method gave DNA extract in amount corresponding to the peak growth of the mixed culture. Enrichment method to obtain bacterial DNA from soil samples was suggested as an alternative way for direct extraction of hard extracted soil samples. The difficulty is not only in removing the cells from the soil matrix, but also in obtaining qualified DNA. DNA extracts obtained from direct extraction methods often include inhibitors of soil, the humic compounds, such as humic acid and fulvic acid, which would interfere with the PCR<sup>20</sup>. Direct extraction method has been performed on coal-soil mixture sample, but the DNA was difficult or even not to be detected by electrophoresis, although its presence was proven when it was successfully used as template in PCR reaction.

The enrichment procedure was carried out gradually to select coal-using bacteria. The specificity of this enrichment method was to multiply cells, thus their genes, according to substrate added, that was to detect a gene, a suitable substrate was added to medium. The selectivity of microbial growth was due to the cells doubled when using specific substrate in the enriched medium. The density of the resulting cells did not reflect the actual cell density in nature, therefore this method was only suitable to detect the presence of microorganisms, not to present density and structure of the actual community. The culture was enriched with organic sulfur in the form of DBT and coal, in consequence the dominant microorganisms were bacteria capable of using those organic sulfur as the sole sulfur source (publication is in progress). Any substances can be added as enrichment substrate in accordance with stated objectives.



M = marker; a = diluted DNA template by 10; b = undiluted DNA template.

**Fig. 3.** PCR products of amplified DNA extracts from 0, 72, 120, 168 and 240 hrs of mixed culture on 1.5% gel agarose stained by ethidium bromide

## **Result of PCR-amplified DNA**

Various DNA extraction methods have been proposed by many researchers, but the result should not only obtain DNA alone, but must have good quality, i.e. long in base pairs and contain no or less impurities. One way to prove it is the DNA must be in PCR-amplified qualification. Therefore, the DNA extracts preceded by enrichment culture were further used as template in PCR reaction (Fig. 3).

The diluted DNA extract ("a" notation in Fig. 3) was intended to reduce impurities in PCR template. They could still be amplified with the product similar to the undiluted one. This revealed that DNA extracts were in good quality. However, the diluted PCR template should be applied with caution that it would not amplify the overall types of bacteria in the sample so that it produced incomplete or unrepresentative PCR product. Thus, the diluted DNA extract was adopted only if the use of undiluted template was failed to be amplified. In conclusion, the DNA extraction method of coal-soil mixture preceded by sequential enrichment produced easily amplified PCR product.

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