GROWTH OF Bacillus megaterium CSK2, Bacillus subtilis CSK3 AND Bacillus subtilis CSK4 ISOLATED FROM COAL MIXED SOIL IN DIBENZOTHIOPHENE-CONTAINING MEDIUM

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(Received : 15 April, 2014; accepted 15 May, 2014)

Key words : Bacillus megaterium, Bacillus subtilis, Dibenzothiophene, Desulfurization, Coal

Abstract—Biodesulfurization is an environmentally friendly technology to reduce organic sulfur in coal by utilizing bacterial metabolism. Isolation and identification of bacteria from coal-mixed soil from South Sumatra has been conducted in order to obtain bacteria which desulfurize organic sulfur in local coal. Three viable isolates, i.e. CSK2, CSK3, and CSK4, were obtained by using a mineral medium containing glucose as carbon source and dibenzothiophene as sole sulfur source. Molecular identification of 16SrRNA gene sequences showed that the isolates had 99% similarity to genus Bacillus, which CSK2 was related to B. megaterium, while the isolates CSK3 and CSK4 were related to B. subtilis. Growth examination of the isolates within 48 hours in the dibenzothiophene-containing medium showed specific growth rate of 0.313, 0.270, and 0.194 h⁻¹, whereas the use of dibenzothiophene in the period of incubation was 37.4, 16.7, and 25.9 percent of the initial 0.1 mM dibenzothiophene, by B. megaterium CSK2, B. subtilis CSK3, and B. subtilis CSK4 respectively. The results showed that the isolates derived from coal-mixed soil have potential to be developed in desulfurization of organic sulfur in coal.

INTRODUCTION

Coal is an alternative fuel that is expected to replace dominance of petroleum use. According to British Petroleum Statistical Review, coal proved reserves in the world at end of 2012 were 860938 million tonnes (British Petroleum, 2013). This provides potential to have coal reserves into the future. Despite considered as promising source of energy, emissions from direct combustion of coal result in release of sulfur dioxide (SO₂) contained therein. It has been well-known that the gas combines with water and produces acid rain that contributes to global warming.

Attempts have been made to reduce the sulfur content in coal, either by physical, chemical or and biological methods. Biodesulfurization that utilizes the metabolism of microorganisms potentially reduces organic sulfur bound to the coal matrix. Biodesulfurization is an easy process, not expensive, does not produce harmful products, and is specific to some organosulfur (Monticello, 1998). This takes into account that biodesulfurization is a process that can be developed as an effective and efficient technology.

Dibenzothiophene (DBT) is an organic sulfur compound that is widely used as a model for the study of desulfurization because it represents the most abundant heterocyclic organic sulfurin coal and petroleum (Soleimani et al., 2007; Prayuenyong, 2002). Purpose of the current study was to obtain bacteria which were expected to remove organic sulfur in coal and examine their growth in dibenzothiophene-containing medium. Therefore

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bacteria were isolated by using a DBT-containing medium as the sole source of sulfur. Coal-mixed soil sample was used as source of isolation, based on assumption that soil is a rich environment for bacteria, so the bacteria in the coal-mixed soil are expected to be adapted to use organic sulfur in coal. The sample was collected from one of coal mine in South Sumatra because according to Center for Geology, Geology Agency, South Sumatra has the greatest potential (39%) of all coal reserves in Indonesia (Mulyono, 2009). Obtaining desulfurizing bacteria from coal mine soil of Indonesia is rarely performed. Therefore results of this study are expected to be valuable information to explore organic sulfur desulfurizing bacteria, especially those work specifically on local coal from Indonesia and possibly on similar coal rank.

**MATERIALS AND METHODS**

**Sample and medium**

Coal-mixed soil sample was collected from the former coal mining of PT Bukit Asam, Muara Tigo Besar Utara, South Sumatra, which is geographically located on S3°43'44.1" E103°43'38.1". Mineral salts medium was used for isolation and growth of bacteria which use dibenzothiophene (DBT) as sole organic sulfur. The mineral medium consisted of 2.44 g of K$_2$HPO$_4$, 5.77 g of Na$_2$HPO$_4$, 2 g of NH$_4$Cl, 0.075 g of NaCl, 10 ml of mineral solution, were dissolved in 1 liter of deionized water and added by 10 g of glucose as a carbon source (Gunam et al., 2006). The mineral solution was consisted of 100 mM of MgCl$_2$, 6H$_2$O, 50 mM of CaCl$_2$, 10 mM of FeCl$_3$, and 500 uM of MnCl$_2$. The medium was sterilized by autoclaving at 121°C for 15 min. DBT was dissolved in 70% ethanol to a concentration of 10 mM and added to the sterilized mineral medium so as to 0.1 mM. The medium was called DBT medium.

**Isolation of desulfurizing bacteria**

About 5 kg of coal-mixed soil sample was crushed, screened to 200 mesh sieve, and homogenized. One gram of sample was aseptically introduced into 100 mL of the sterile DBT medium in a 250 mL erlenmeyer flask. Incubation was performed for 7 days at room temperature (28°C-29°C), with 150 rpm of agitation. Cultures were sampled on days of 0, 1, 3, 5, and 7, and inoculated on DBT agar medium by spread plate method. After incubation at room temperature, every single colony was observed, and performed subculture in order to obtain single colonies that completely separated.

**Identification of isolates**

Isolates showing growth in DBT medium were subjected to identification. Molecular identifications were ordered to Marogen, Inc., Korea, with sequencing of approximately 1300 nucleotide base with a primer based on 16SrRNA gene. Consensus sequence of the forward and reverse sequences was generated by Bioedit 7.0.9.0. program (Hall, 1999), and compared to the Gen Bank database at the National Centre of Biotechnological Information (NCBI) by the BlastN 2.2.26+ program (http://blast.ncbi.nlm.nih.gov/) for sequence similarity. The sequences were aligned by Muscle program. Phylogenetic tree was constructed by Maximum Likelihood method with 1000 boot strap. Both alignment and phylogenetic construction were performed by MEGA5 (Tamura et al., 2011). The identified sequences were deposited in GenBank through DNA Data Base of Japan (DDBJ).

**Determination of growth curve**

Bacterial growth curve of each isolate was required to determine the middle log phase of growth. It was considered as the right age for the isolates as inoculum culture in analysis of desulfurization. Three colonies of each isolate were inoculated into 30 mL of DBT medium, and incubated at room temperature (28-29°C), 150 rpm, for 24 hours. Then the activated culture was subcultured by 10% (v/v) of solution culture in two steps, by using the same type of medium and incubation conditions. From the solution of the second subculture, culture was sampled every 3 hours. Cell concentration was determined by dilution method and spread plate on the surface of DBT agar medium. Growth curve was illustrated in graphic chart, with the logarithm of the cell concentration as the ordinate axis and time (hours) as the abscissa.

**Analysis of growth in DBT medium**

The use of DBT as sole sulfur source for the growth of bacteria was observed by determining cell concentration, pH, and DBT concentration. The isolate culture in middle log phase determined through growth curve was inoculated by 10% (v/v) into 200 mL of fresh DBT medium and incubated at room temperature (28-29°C), 150 rpm, for 48 hours. Over the period of 48 hours, the culture was
sampled every 3 hours in the first 12 hours, every 6 hours in the second 12 hours, and every 12 hours in the last 24 hours. Cell concentration in the sample was determined by measuring optical density at wavelength of 600 nm. For determination of the DBT concentration, the sample was acidified with HCl to pH 2 and extracted with ethylacetate, and then the absorbance of the sample was read by uvvis-spectrophotometer at maximum wave length of 323.8 nm (Etemadifar et al., 2008). Standard curve was prepared from DBT solution set of 0.025 mM, 0.050 mM, 0.075 mM, 0.1 mM, 0.125 mM, 0.150 mM, and all were treated in the same preparation as the sample.

RESULTS

Five bacterial isolates were obtained based on difference in characteristics of colony and cell. However, there were only three viable isolates after three times of sequential subcultures (Table 1). Colony and cell characteristics showed that they had similar shape, but all of them had distinct diameters at the same age; it indicated that all of them were of different types.

Phylogenetic tree constructed from 16SrRNA genes of isolates CSK2, CSK3, CSK4 and reference bacteria from GeneBank showed similarity of coal mixed soil isolates to the genus Bacillus (Fig. 1). Topology of the phylogenetic tree showed that the isolates were separated into two different groups. Isolates CSK3 and CSK4 were within one group with Bacillus subtilis subsp. subtilis and several adjacent reference bacteria, with 99% of similarity level, while isolate CSK2 was closely (99%) related to Bacillus megaterium with strong (100%) boot strap support. The sequences were deposited in DDBJ under accession number AB931123, AB931124, and AB931125, for CSK2, CSK3, and CSK4, respectively.

The growth curve of the three isolates showed common shape of bacterial growth curve, which has exponential phase followed by stationary phase, with or without preceded by adaptation phase (Fig. 2). Bacillus megaterium CSK2 produced higher maximum cell concentration and lived for 48 hours, longer than the other isolates which lived for 24 hours (B. subtilis CSK3) and 36 hours (B. subtilis CSK4). From the curve, middle log time was determined by half length of the exponential phase, approximately at hours 10.5 for B. megaterium CSK2 and hours 11.5 B. subtilis CSK3 and B. subtilis CSK4. That time point was considered for each isolates as the most appropriate age to be inoculum for examination.

After determination of growth curve, growth of each isolate was examined further in DBT medium (Fig. 3). Growth of B. megaterium CSK2 in the DBT medium entered the exponential phase from the beginning of incubation to hours 18, accompanied by decrease in DBT concentration. This continued until the cells met the stationary phase from hours 18 to 48. Decrease in DBT concentration implied consistent with the growth pattern. In contrast to B. megaterium CSK2, growth of B. subtilis CSK3 was preceded by lag phase until hours 12. Then the exponential phase occurred after hours 12 to 36. Since

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Table 1. Colony and cell characteristics of isolates

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the beginning of incubation until the end of the exponential phase DBT concentrations decreased. Similar to *B. megaterium* CSK2, growth of *B. subtilis* CSK4 entered exponential phase in the beginning of incubation time, which are accompanied by decrease in DBT concentration. The growth in DBT medium showed consistent decrease in pH and thus indicated desulfurization activity. Increase in cell concentration of each isolate at the period of exponential phase showed positive correlation to the decrease in the DBT concentration, with $R^2=0.60$ to 0.78. It indicated that the greater number of living cells, the higher level of DBT degradation, and the higher increase in sulfate production which was illustrated by decrease of pH.

**DISCUSSION**

The isolation procedure was conducted by using enrichment method, so that only three dominant isolates were obtained. The presence of DBT as the sole sulfur source was expected to make the medium selective because not all microorganisms were able to meet their needs of sulfur by utilizing DBT. Initial DBT concentration in the medium was 0.1 mM since it was considered as the most appropriate concentration for desulfurization activity (Yoshikawa et al., 2002), while concentration exceeding 1 mM DBT was thought to inhibit cell growth (Etemadifar et al., 2008).

The bacteria isolated in this study were not novel bacteria capable in desulfurization. *B. subtilis* has been identified as a species grew on DBT. Ma et al. (2006) obtained results of DBT desulfurization was
higher when dsz genes of \textit{Rhodococcus rythropolis} strain was cloned into \textit{B. subtilis}. Kimura \textit{et al.} (2004) used \textit{B. subtilis} clone dsz gene cluster encoding enzymes for DBT degradation, and the enzymes from the same isolate has been purified and examined by Ohshiro \textit{et al.} (2005). The isolates \textit{B. subtilis} CSK3 and CSK4 had morphological characteristics similar to each other (Table 1). However, the difference in their growth while using DBT suggested that the two isolates are of two different strains. In the phylogenetic tree (Fig. 1), CSK3 and CSK4 are in a monophyletic group, but deletion at the sequence position no. 742 of 165 rRNA gene in CSK3 (data is not shown) confirmed that both isolates are of different strains.

In contrast to \textit{B. subtilis}, \textit{B. megaterium} has never been published before as desulfurizing bacterium. However, complete genome in one of \textit{B. megaterium} strain sequenced by Liu \textit{et al.} (2011) showed that this bacterium had \textit{dszA} and \textit{dszC} genes encoding mono oxygenases in desulfurization pathway known as 4S pathway. Therefore, being isolated by DBT medium indicates that \textit{B. megaterium} CSK2 has those genes of desulfurization as well.

The growth curves of \textit{B. megaterium} CSK2 demonstrated the most significant cell concentrations, although activated by the same procedure as the other isolates (Fig. 2). The growth of the isolate CSK2 continued to drive up to 21 hours, whereas other isolates had lesser growth. It resulted in that its cell concentration was exceeded those of the other isolates when used as inoculum at the active age (middle log phase). This suggested that this isolate is the most potential strain in this study that can be developed in further study in organic sulfur desulfurization.

In order to learn the use of DBT by the isolates, growth of isolates was analyzed more profoundly in DBT medium (Fig. 3). The curves did not have adaptation phase, except in the growth of \textit{B. subtilis} CSK3. It means that the activation procedure by means of two previous sequential subcultures were worth while. By previous activation on culture, it was regarded that the bacteria became able to use directly substrate contained in the medium due to its metabolism has previously been activated. As illustrated in Fig. 3, it can be perceived that during growth, all of the isolates consumed DBT. \textit{B. megaterium} CSK2 reduced DBT concentration in greater percentage than the other isolates did while they were growing. Specific growth rate in the DBT medium were 0.313, 0.270, and 0.194 h$^{-1}$, whereas the use of DBT in the period of incubation was 37.4%, 16.7%, and 25.9% by \textit{B. megaterium} CSK2, \textit{B. subtilis} CSK3, and \textit{B. subtilis} CSK4 respectively. The isolates revealed obvious use of DBT as a source of sulfur, and decrease in pH was relatively constant until the end of incubation. From other studies, DBT degradation process can occur via two different known mechanisms, which are Kodama pathway that bacteria destruct carbon—carbon bond resulting in release of sulfur, and 4S pathway that is specific degradation pathway which only attacks the carbon—sulfur bond of DBT molecule (Gou \textit{et al.}, 2002). The Kodama pathway is unexpected mechanism since destruction on DBT structure would reduce caloric value of coal as fuel. It was the reason that glucose was included in the medium so that the bacteria consumed the glucose as carbon source instead of DBT.

In addition to the cell and DBT concentrations, measurement of the pH level was conducted. The isolates lowered the medium pH in the range of 0.08 to 0.18. By product generated at the end of DBT biodesulfurization in the 4S pathway is sulfate (SO$_4^{2-}$). Then the sulfate acts with water in the medium and forms H$_2$SO$_4$, which are acidic. Thus, it can be assumed that when DBT desulfurization occurred, decrease in pH was caused by formation of the sulfuric acid (Constanti \textit{et al.}, 1994).

DBT desulfurization activity of each isolate was considered to be promising because it was associated with the absence of other source of sulfur in the growth medium other than DBT. It verified that the three isolates were capable in using sulfur contained in DBT as an important substrate for the growth. Sulfur is an important component in the life of microorganisms due to the presence of sulfur in structure of some enzyme cofactors (such as coenzyme A, thiamine, and biotin) and amino acids (cysteine, methionine) (Kertesz, 1999). Nevertheless, the desulfurization activity by the isolates required future study to learn about other factors, physics, chemistry and biological, needed to support and to improve their growth and desulfurization activity, especially on organic sulfur in coal.

**CONCLUSION**

\textit{B. megaterium} CSK2, \textit{B. subtilis} CSK3, and \textit{B. subtilis} CSK4 isolated from coal mixed soil had significant growth in medium containing mineral and dibenzothiophene as sole organic sulfur. The results also inform that the coal mixed soil in the
abandoned coal mine is a suitable and favorable location for exploring desulfurizing bacteria.

ACKNOWLEDGEMENTS

We thank Directorate General of Higher Education, UIN Syarif Hidayatullah Jakarta, and Ministry of Religious Affairs, Indonesia, for funding support. We thank Ir. Eko Pujiandarto (Asisten Manajer Geologi, Satuan Kerja Eksporasi Rinci) and his staff from PT. Bukit Asam Tbk. for technical assistance in coal mine.

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