Growth of Moraxella osloensis COK1, a Novel Strain of Bacteria Isolated from Subbituminous Coal, in Dibenzothiophene and Coal Medium

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Abstract. Bacteria capable of desulfurization of organic substances in coal and petroleum are required to improve the quality of the fuel. We have thus isolated bacteria from coal sources and examined their growth and desulfurization activities in monocultures. Three viable strains of bacteria were obtained using two procedures: one-step enrichment and gradual enrichment. A medium was used which was enriched with dibenzothiophene, the most dominant organic sulfur in coal. Based on analysis of 16S rRNA gene, it was found that the bacteria were closely related to Moraxella osloensis, Pseudomonas aeruginosa, and Enhydrobacter aerosaccus. The evolutionary relationships amongst these bacteria were analyzed by phylogenetic inference and a phylogenetic tree constructed. The growth of the viable bacteria were observed independently in medium containing dibenzothiophene and coal. Moraxella osloensis has not been previously reported as a bacteria associated with coal, or in using organic sulfur in coal. However this study demonstrated that the most suitable medium for growth was a coal medium, indicating that the isolates may potentially be developed as a monoculture for coal desulfurization. The other two isolates showed unsteady growth, and further study is needed to enable their growth in mixed culture and to meet the requisites of their natural interactions.

Keywords: Enhydrobacter aerosaccus, Desulfurization, Dibenzothiophene, Moraxella osloensis, Pseudomonas aeruginosa, Subbituminous coal.

INTRODUCTION

Coal remains an important fossil fuel in many countries because of the large reserves and energy needs. According to the World Energy Resources 2013 Survey, the global reserves of coal in that year were 891,530 million tonnes, and it was estimated that by the year 2020 fossil fuel will still dominate as an energy source. However, the sulfur content of coal needs to be reduced to make coal cleaner as a public fuel. Unlike inorganic sulfur, which can be separated through established physical-chemical technology, organic sulfur in coal is bound as a matrix of organic compounds in the coal that needs to be eliminated with minimal or ideally no destruction of carbon. A method that has been known for eliminating the organic sulfur is the use of microorganisms which have a non destructive 4S pathway. An advantage of biological desulfurization is that it operates under mild conditions in terms of temperature and energy input (Li et al., 2005; Buzanello et al., 2014).

Desulfurizing bacteria have been isolated in many studies. As in previous work, we obtained culturable desulfurizing bacteria from coal-mixed soil taken from Muara Enim M2, Muara Tigo Besar Utara, South Sumatera, Indonesia. The bacteria identified in the previous studies were Enterobacter hormaechei, Bacillus subtilis subsp. subtilis, Leclercia sp., and Enterobacter asburiae (Aditiawati et al., 2013), Bacillus megaterium and two strains of Bacillus subtilis (Pikoli et al., 2014). Soil nutrients may have a role in providing favorable conditions for the bacteria in their natural environment. Meanwhile, in the current study, bacteria were only isolated from coal samples, which were collected from virgin unmined coal. Data from a previous report using a non-culture procedure of Denaturing Gradient Gel Electrophoresis (DGGE) revealed that the coal community sampled from the Muara Enim M2
subbituminous coal were dominated by Actinobacteria and Firmicutes, each making up about 40% of total bacteria. The rest were Proteobacteria and other phyla, making up about 20% (Pikoli et al., 2013). This is not surprising since various studies on biodesulfurization also employed those phyla of bacteria, for example Actinobacteria: Rhodosporidium curvatum (Del Olmo et al., 2005), Microbacterium sp. (Li et al., 2005; Papizadeh et al., 2010), Rhodosporidium sp. (Mohamed et al., 2015), and Gordonia alkanivorans (Mohebali et al., 2007); and Firmicutes: Bacillus subtilis (Kimura et al., 2001), Bacillus pumilus (Buzanello et al., 2014), Lysinibacillus sphaericus (Bahuguna et al., 2011), and Paenibacillus sp. (Wang et al., 2015). However, most of these bacteria were isolated from contaminated soils and not from pure coal samples. In the present study we attempted to isolate cultivable bacteria from a virgin coal sample. We expected to obtain bacteria that we had not previously retrieved from soil samples in previous studies. In addition, most of the previous studies have examined the bacteria in dibenzothiophene and petroleum media, meanwhile this study examined isolate growth in dibenzothiophene and coal media.

Although Proteobacteria is not a predominant member of the bacterial community of subbituminous coal, it is worthwhile that an enrichment method was used in the current study to attract Proteobacteria, especially Moraxella osloensis. We were testing a hypothesis that despite their lower abundance within coal communities, coal-origin Moraxella osloensis utilizes organic sulfur, in a pure form (dibenzothiophene) or as coal-derived sulfur, due to its adaptability to colonising coal. It was assumed that they are a member of the bacterial community that have been trapped in coal-forming plant material. These bacteria grew slowly because they were not provided with favorable environmental conditions. To the extent of our knowledge this is the first study which isolated and examined growth of coal-origin Moraxella osloensis. The aim of this study was to show the desulfurization potential of the indigenous coal-origin bacteria.

Identification and phylogenetic analysis. Identification was performed by amplifying and sequencing the 16S rRNA gene. The bacterial colonies on slant nutrient agar were collected by suspending and centrifuging in a microtube at 4000×g for 10 minutes. The cell pellet was subject to DNA extraction by using PeqGold Bacterial DNA kit (Peqlab, UK) which broke cells enzymatically by lysozyme and protease K. The DNA was washed by ethanol 70% in a spin column, and eluted by 10 μM Tris-EDTA buffer. The 16S rRNA gene was amplified by PCR reaction with 27F/1492R primers (Tzeneva et al., 2004). After being purified, the PCR product was sent for sequencing by Macrogen (Korea). Electropherogram was assembled by ChromasPro 1.7.5 (Technelysium Pty Ltd., South Brisbane, Australia) and corrected manually. All putative chimeric sequences were evaluated by DECIPHER (Wright et al., 2012).

The sequences were aligned with reference to 16S rRNA gene sequences retrieved from the GenBank by BLASTn of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Some references of the 16S rRNA gene were predicted from the available genomic data by RNAmer 1.2 (Lagesen et al., 2007). The dataset of all isolates and their references were subject to multiple alignment conducted using MUSCLE (Multiple Sequence Comparison by Log Expectation) (Edgar, 2004) implemented in software MEGA 6.0.
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(Molecular Evolutionary Genetics Analysis) (Tamura et al., 2013). Escherichia coli NBRC 102203 (AB681728) was set as an outgroup. The alignments were subjected to Bayesian phylogenetic inference (Yang and Rannala, 1997), employing PAUP* 4.0b10 (Swofford, 2003) and using the best-fit model of evolution determined by jModelTest 2.1.5 (Darriba et al., 2012). Furthermore, a consensus tree was launched in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001). Prior to this, a Markov Chain Monte Carlo (MCMC) algorithm run 2,000,000 generations that was sampled every 100 generations resulted in 20,000 trees, and burnt 20% of the total generated trees. Finally, the tree was refined using TreeGraph 2 (Stöver and Müller, 2010).

**RESULTS AND DISCUSSION**

**Bacterial isolates and their identification.** Seven different bacterial colonies were isolated from the two enrichment methods, One-step and Gradual enrichment methods. The isolates were coded as COK1 to COK7. However two of these isolates (COK6 and COK7) did not survive when being grown on agar. The 16S rRNA gene sequences of the five viable isolates have been deposited in the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/). Identification results showed that the 16S rRNA gene sequence of the five isolates had high similarity (99-100%) with the bacterial reference (Table 1).

Among the five isolates obtained by the two methods, COK3 was the only isolate obtained by the One-step enrichment method. Both methods should be performed simultaneously, since their results were complementary to each other, in order to obtain a good sampling of the desulfurizing bacteria present in a sample. The One-step enrichment method can be used to isolate bacteria which utilize easily degraded components immediately; meanwhile bacteria which utilize more recalcitrant components grew slower. Therefore, it is important that enrichment was performed gradually in order to

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**Table 1.** Identification results of bacterial isolates from subbituminous coal of Muara Enim, South Sumatra, Indonesia.

| Code | The closest reference bacteria | Similarity (%) | Accession number (DDBJ) |
|------|--------------------------------|----------------|------------------------|
| COK1 | *Moraxella osloensis*           | 99             | AB931117.1             |
| COK2 | *Micrococcus endophyticus*      | 99             | AB931118.1             |
| COK3 | *Pseudomonas aeruginosa*        | 100            | AB931119.1             |
| COK4 | *Pseudomonas psychrotolerans*   | 99             | AB931120.1             |
| COK5 | *Enhydrobacter aerosaccus*      | 99             | AB931121.1             |

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Examination of desulfurization. Each bacterial isolates was examined for its engagement as a member of the desulfurization community. Culture inoculum was prepared by inoculating three loops of bacterial colonies into 30ml of Dibenzothiophene Medium and incubated at room temperature and 120rpm for 24 hours. Then 5ml of the culture solution (10^7 cells/ml) was transferred into 45ml of fresh medium and incubated under the same conditions. After being incubated for 24 hours, 10ml of the culture (10^7 cells/ml) was inoculated into 90ml of fresh medium and incubated for 48 hours. Then, the desulfurization with 10% (v/v) of the inoculum culture was observed for 48 hours in Dibenzothiophene Medium or Coal Medium. Samples were then measured for their pH, cell concentration, sulfate, and dibenzothiophene concentration. Prior to pH and dibenzothiophene measurement, the sample was filtered to remove undissolved coal. Dibenzothiophene concentration was determined spectrophotometrically (λ=323.8nm) following acidifying by HCl to pH2 and extracted with ethyl acetate (Etemadifar et al., 2008). Sulfate was determined by the turbidimetric method (λ= 420nm) after an addition of BaCl₂ (American Public Health Association, 1975).
capture the minor bacteria (Venkateswaran et al., 1995). The bacteria were assumed to be organic sulfur users because the medium was enriched with dibenzothiophene, a readily consumed organic sulfur. In this experiment, dibenzothiophene was used because it is the most abundant organic sulfur in fossil fuels (Monticello, 2000). Moreover, the presence of dibenzothiophene as a pure compound as the only available form of organic sulfur was sufficient for the survival of the desulfurizing bacteria.

Moraxella osloensis (COK1), Micrococcus endophyticus (COK2), and Enhydrobacter aerosaccus (COK5) have not previously been reported as desulfurizing bacteria which colonise organic compounds. However, in this study they were isolated from the medium containing dibenzothiophene as the sole source of sulfur suggesting that they are potential new isolates that can be developed for the desulfurization of organic compounds, especially from coal. Meanwhile, COK3 showed 100% similarity with Pseudomonas aeruginosa. This species has been detected in the community of methane-producing bacteria from coal (Tang et al., 2012). P. aeruginosa expresses a DszC monooxygenase (http://blast.ncbi.nlm.nih.gov) that plays a role in the 4S pathway of dibenzothiophene utilization. Some Pseudomonas species are capable of using aromatic compounds including dibenzothiophene (Martin and Mohn, 1999; Xiong et al., 2005; Seo et al., 2009). In addition, some Pseudomonas express a Kodama desulfurization pathway that breaks the C-C bond to meet the need for a source of both carbon and sulfur (Bressler and Fedorak, 2001). Thus P. aeruginosa (COK3) and P. psychrotolerans (COK4) were thought to have the ability to utilize dibenzothiophene either in the 4S or Kodama pathways.

**Phylogenetic analysis.** Phylogenetic analysis revealed the presence of two major groups, Micrococi (Proteobacteria) and members of Actinobacteria that fell into one clade of the family Moraxellaceae, and one group of family Pseudomonadaceae (Figure 1). COK2 formed a monophyletic clade with Micrococcus endophyticus, especially *M. endophyticus* strain cp.1, although the latter was only distantly related (Bayesian posterior probability <0.9). According to the data from NCBI (http://www.ncbi.nlm.nih.gov/nucore/JN082275.1), *M. endophyticus* strain cp.1 was isolated from Qianshan magnetite mine drainage by Lu et al. (written as unpublished, 2011). Mine drainage is known to contain sulfur, meaning that both strains of bacteria naturally inhabit the same environment, high sulfur and low pH.

COK3 and COK4 formed monophyletic clades with *Pseudomonas aeruginosa* and *Pseudomonas psychrotolerans*, respectively, with strong Bayesian posterior probability. COK3 has close phylogenetic relation to *P. aeruginosa* CPCL, which was isolated from petroleum contaminated soil (Arutchelvi and Doble, 2010), and *P. aeruginosa* NY3, a polycyclic aromatic hydrocarbon -degrading bacterium producing rhamnolipid biosurfactants (Nie et al., 2010). There was paraphyletic assemblage in Moraxella osloensis, in which COK1 was a member in one group. COK1 has close phylogenetic relations to *M. osloensis* KMC 411 and KMC 412, which are primarily responsible for generating malodor in laundry (Kubota et al., 2012), probably due to sulfur based compounds. COK5 was affiliated with Moraxellaceae, but it formed distinct branch instead of forming a clade with *M. osloensis*. However, it was closer to Enhydrobacter aerosaccus. Compared to *Pseudomonads*, the occurrence of *Moraxella osloensis* and *Enhydrobacter aerosaccus* in a sulfurous hydrocarbon environment like coal is unexpected since there has yet been no previous report of their isolation from such environment.

*Moraxella osloensis* was not detected by the DGGE method of the previous study using the same coal mine sample, although there was an OTU which was close to an uncultured Gammaproteobacteria (Pikoli et al., 2013), meaning that *Moraxella osloensis* COK1 was presumably not a dominant member of the microbiome of its natural environment, and the isolation procedure has made the minority member of the community grow faster and more dominant, being easily cultured. Among the published desulfurizing bacteria, only a few species belongs to the Gammaproteobacteria group, for examples *Shewanella* sp. (Ansari et al., 2007) and *Stenotrophomonas* sp. (Papizadeh et al., 2011). Most desulfurizing bacteria belong to Actinobacteria and Firmicutes, as mentioned above. Therefore, the viability of *Moraxellaolesensis* contributes more proof for the existence of desulfurizing bacteria in the Gammaproteobacteria group. The isolation of *Moraxella osloensis* also designates that subbituminous coal is a source of species not yet isolated and identified as being desulfurizing bacteria.

**Desulfurization Activity During Growth.** Desulfurization was observed in three viable isolates, i.e. *Moraxella osloensis* (COK1), *Pseudomonas aeruginosa* (COK3), and *Enhydrobacter aerosaccus* (COK5), as their population in inocula reached a minimum viable concentration (10⁶ CFU). Growth is the most essential parameter in assessment of desulfurization since it is directly related to assimilation of sulfur into cell (Rhee et al., 1998; Akhtar et al., 2009). The present study determined the cell concentrations by the plate count method so that only viable cells were observed. Observation of the concentrations of sulfate and pH primarily aimed to identify medium change by the utilization of sulfur compounds. In the sulfur-specific metabolic pathway of dibenzothiophene (the 4S pathway), sulfuric acid is generated as the end product (Maghoudi et al., 2000; Monticello, 2000; Akhtar et al., 2009). The medium contained dibenzothiophene as the sole source of sulfur to obtain a picture of bacterial utilization of the organic sulfur. In the other medium, coal was added instead of dibenzothiophene, in order to compare their
Figure 1. Phylogenetic tree showing relationships among the culturable bacteria (and their references) isolated from subbituminous coal of Muara Enim, South Sumatra, Indonesia. The value shown above internodes indicates Bayesian posterior probability.
capability of using coal as a natural source of sulfur. All of the media were made with 10g/liter of glucose as a ready carbon source, emulating other studies on biodesulfurization of organic compounds (Del Olmo et al., 2005; Caro et al., 2008; Bahuguna et al., 2011).

Monaxella osloensis (COK1) showed two phases of growth in the Dibenzothiophene Medium (Figure 2A), while pH tended to slowly decline from the beginning of the incubation. The two phases of growth might indicate carbon catabolite repression by glucose, in which glucose is the preferred carbon source (Basu et al., 2006), in this case preferred to dibenzothiophene. Growth on glucose as an alternative carbon source induces enzymes to cometabolize other organic compounds (Juhasz and Naidu, 2000). In this case, the growth of Monaxella osloensis (COK1) using glucose may induce expression of the Dsz enzymes that play a role in desulfurization of dibenzothiophene, a source of sulfur and also carbon in cometabolism alongside the use of glucose. This assumption was confirmed by a decrease in the dibenzothiophene concentrations and increase in the sulfate concentrations during the first 9 hrs, which continued until the 12th hour. An increase in cell concentrations accompanied by a decrease in pH and dibenzothiophene concentrations, as well as an increase in sulfate concentrations, commonly occurs when cells are grown using sulfate released from dibenzothiophene (Mohebali and Ball, 2008), although not all of sulfate is being used during the release (Akhtar et al., 2009). The increase of sulfate concentration from the start of the incubation pointed to desulfurization of dibenzothiophene in the 4S pathway by M. osloensis (COK1). In the 4S pathway, dibenzothiophene (DBT) is oxidized to DBT-sulfone (DBTO) by DBT-monoxygenase (DszC), then the DBTO is oxidized to DBT-sulfone (DBTOS) by the same enzyme. The DBTOS was oxidized to hydroxyphenyl benzene sulfonate (HPBS), which is subsequently broken down into hydroxy biphenyl (HBP) and sulfate (Monticello, 2000; Matsubara et al., 2001; Akhtar et al., 2009).

During the 12-24th hour period, the cell concentrations of M. osloensis (COK1) was relatively fixed, however the pH, dibenzothiophene and sulfate concentrations all decreased. This is presumably due to the inhibition in the production of desulfurization enzymes by excessive sulfate in the previous phase. Mohebali and Ball (2008) reviewed some studies that indicate there is no feedback inhibition in the 4S pathway enzymes themselves, but the presence of sulfate in growth medium inhibits the expression of the dsz gene of desulfurization by attacking its promoter. However, some cells in the culture will adapt to the excessive sulfate and likely continue to perform desulfurization, so that the total dibenzothiophene concentrations will still decline. In this phase, sulfites resulting from the desulfurization through the 4S pathway are thought to be more widely used to maintain the cell concentrations compared to the previous phase. This explains why the sulfate detected in the medium was lower than the previous phase.

In the second exponential phase, the cell concentrations of M. osloensis (COK1) increased, accompanied by a decrease in the dibenzothiophene concentrations. This suggests that the cells that performed desulfurization in the previous phase succeeded in increasing their populations. Growth on glucose induces expression of enzymes for dibenzothiophene cometabolism, thus the need for carbon and sulfur is met. This was observed through a rapid increase in the cell concentrations. In the second exponential phase, the relatively stability of dibenzothiophene concentration and the decline in sulfate concentrations led to the growth of M. osloensis (COK1) which formerly utilized sulfur from desulfurized dibenzothiophene. Such patterns resembled desulfurization in Gordona sp. strain CYKS1 (Rhee et al., 1998), in which the concentration of dibenzothiophene declined markedly during the formerly slow growth phase. Afterwards, in the rapid growth phase (exponential), dibenzothiophene concentration will be relatively constant. Growth does not always coincide with the desulfurization activity when the needs for sulfur are fulfilled. According to Akhtar et al. (2009), the sulfur released from dibenzothiophene is not necessary immediately utilized upon release. The decline in sulfate concentration is a sign that the sulfate from dibenzothiophene is used for the growth of M. osloensis (COK1). It is well known that sulfate resulting from desulfurization through the 4S pathway is used mostly in synthesis of cell components, and the other portion is released out of the cell in the form of sulfate (Omrni et al., 1992; Monticello (2000).

Similar to its growth in the Dibenzothiophene Medium (Figure 2A), growth of M. osloensis (COK1) in the Coal Medium consisted of two phases (Figure 2B). However, the growth in the second phase in the Coal Medium, especially during the 12-24th hour period, was more rapid than that in the Dibenzothiophene Medium during the same period. Unlike in the Dibenzothiophene Medium, the dibenzothiophene concentrations in the Coal Medium tended to increase, indicating that the dibenzothiophene was released from the coal as a result of coal degradation. Until the end of incubation time (at the 48th hour), the sulfate concentrations tended to fluctuate. This implied that there is sulfate released through desulfurization, along with sulfate which is absorbed by the cells, simultaneous with the time that the cell concentrations increased (Figure 2B).

As seen in Figure 2C and 2D, P. aeruginosa (COK3) consumed the dibenzothiophene as source of sulfur for growth, both in the Dibenzothiophene Medium and in the Coal Medium. According to the data in NCBI, P. aeruginosa UCBPP-PA14 has some genes in the 4S pathway, dzzA, dzzC, and dzzD. However, P. aeruginosa is also known to degrade dibenzothiophene using the Kodama pathway (Young et al., 2006). P. aeruginosa (COK3) is assumed to use the dibenzothiophene in Kodama pathway. It is assumed that the growth seems slower and there is no carbon
Figure 2. Growth of the bacterial isolates from subbituminous coal of Muara Enim, South Sumatra, Indonesia. A-B. *Moraxella osloensis* COK1; C-D. *Pseudomonas aeruginosa* COK3; E-F. *Enhydrobacter aerosaccus* COK5; in Dibenzothiophene Medium (A, C, E) and Coal Medium (B, D, F), with parameters: concentrations of cell (■), dibenzothiophene, DBT (□), pH (●), and sulfate (○).
catabolite repression by glucose. According to Basu et al. (2006), Pseudomonas preferred organic acids or amino acids as carbon sources, instead of glucose, therefore the bacteria use aromatic compounds even though glucose is available. Bacteria that employ the Kodama pathway use dibenzothiophene both as source of carbon and sulfur, in a series of transformations, which is indicated by an increase in sulfate and carbon dioxide that detected after the second day of incubation (Young et al., 2006). Meanwhile, in the growth curve of bacteria that employ the 4S pathway, the exponential phase occurs for about 48 hours (Kayser et al., 2002, Papizadeh et al., 2010).

In addition to P. aeruginosa (COK3), the growth of Enhydrobacter aerosaccus (COK5) showed less ability to survive as a monoculture both in the Dibenzo thiophene Medium and in the Coal Medium, despite similar pattern in pH, cell number, and dibenzothiophene and sulfate concentrations (Figure 2E and 2F). These suggested that coal as a source of nutrition and environment gave little support to the isolates in using organic sulfur. Another possibility is that this isolate employed the Kodama pathway in utilization of dibenzothiophene, as occurred in the isolate COK3. In this pathway, dibenzothiophene is not only degraded as a source of sulfur, but as a source of carbon as well, therefore more dibenzothiophene is being used. Future work is needed to determine the desulfurization pathway of these bacteria.

The specific growth rate of M. osloensis (COK1), P. aeruginosa (COK3), and E. aerosaccus (COK5) in the Dibenzo thiophene Medium were 0.039, 0.003, and 0.026 cells/hour, respectively, whereas with the use of dibenzothiophene growth rates were 15.0, 38.8, and 51.2 percent, respectively. The sulfate remaining in the medium totalled 0.39, 0.08, and 0.02 mg/l, respectively at the end of incubation. It seemed that M. osloensis (COK1) released sulfur from dibenzothiophene and used it for its growth in a more efficient manner than did the others. The percentage of dibenzothiophene usage by the three isolates was comparable to that showed by strains of Rhodococcus erythropolis and Gordonia sp. isolated from coal-polluted soil in different conditions, which were 5-51% depending on the diesel oil type (Abbad-Andaloussi et al., 2003). The results from the present study showed biosulfurization potential of the Proteobacteria isolates, especially M. osloensis (COK1), the novel bacteria isolated from the subbituminous coal origin, although the extent of their activity and capacity were still lower than the bacterial species from other studies. Further investigation is needed, such as to identify optimal conditions for the isolates, both abiotic (chemical and physical factors) and biotic (interactions with other bacteria in a mixed culture), not only by using dibenzothiophene as the model compound but also using coal as the natural substrate.

**CONCLUSIONS**

Three species of viable bacteria isolated from subbituminous coal of Muara Enim, South Sumatra, Indonesia, grew both in medium containing dibenzothiophene and the original coal. Moraxella osloensis (COK1, AB931117.1) was the isolate with the most potential to be developed as a single bacteria culture for desulfurization of organic compounds in coal. Meanwhile, the lower activity in growth and ability to utilise dibenzothiophene by other single isolates of Pseudomonas aeruginosa (COK3) and Enhydrobacter aerosaccus (COK5) showed presumable interdependence of these bacteria from activities with other bacteria in their community to grow in the coal. Therefore, further research is needed to observe and investigate their growth in a mixed culture, to achieve greater use of dibenzothiophene or other types of sulfur compounds from coal.

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