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Biodegradation of Dibenzothiophene Using Bacteria Isolated from Samboja Kutai, East Kalimantan: Indonesia.

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ABSTRACT

The objective of this research was to find out the potential of oil desulfurizing bacteria isolated from tropical soil contaminated with fossils oil. The bacteria were isolated by using medium of mineral salt sulfur-free (MSSF) supplemented with concentrated fraction of aromatic compounds as the sole sulfur source (MSSF-CA). Result of the research showed that 67 isolates of oil desulfurizing bacteria were isolated from soil in Samboja Kutai East Kalimantan, Indonesia. The isolates were candidate of bacteria which were capable to desulfurize polycyclic aromatic sulfur heterocycles (PASHs) in the model oil, *n*-tetradecane containing dibenzothiophene (DBT). A collection of 29 out of 67 isolates showed potential as oil desulfurizing bacteria and can grow on media of MSSF (water phase) containing DBT in *n*-tetradecane (oil phase) and the ratio of oil/water was 1:5. Some isolates could grow in the medium with OD_{660} ranged from 0.56 to 1.12 and could degrade DBT compounds in range of 26.34-81.88% and the maximum being degraded by *Pseudomonas luteola* SBJ4a after 72 h incubation at 37°C with shaking of 150 rpm.

Keywords: Dibenzothiophene, desulfurizing bacteria, fossils oil, isolation, and oil contaminated soil.

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INTRODUCTION

Petroleum combustion produces emissions of sulfur oxides (SO_x) in the atmosphere it can cause serious environmental problems such as air pollution and acid rain [1, 2], especially in urban areas. To reduce the emissions of SO_x, the organic sulfur compounds in fossil fuels should be reduced during the refining process [1, 3, 4]. Oil producing countries including Indonesia needs to keep working to reduce the content of sulfur in order to produce higher quality of oil (low sulfur), so that the problems of environmental pollution and acid rain can be reduced.

Biodesulfurization by using microbes as a biocatalyst in recent years become the focus of quite a lot of researchers. This method is considered to have a bright prospect in the future, since the process is inexpensive, does not require complex installation and is environmental friendly [5]. Quite a lot of the strains of bacteria that can degrade petroleum sulfur compounds. However, only few of the bacteria have an ability to degrade aromatic sulfur in alkyl long chain compounds. *Sphingomonas subarctica* T7b as reported in our previous paper, which could degrade the compounds. But, the growth temperature of the strain was optimum in room temperature (27^{o}C) [1, 6].

The present study was conducted to find the strain of bacterium that could degrade polycyclic aromatic sulfur heterocycles (PASHs) at higher temperatures. Such bacteria will be able improving the efficiency of petroleum desulfurization process. The process of refining or hydrodesulfurization (HDS) of petroleum is carried out at high temperatures. Thus, the reduction in sulfur content at the next biodesulfurization (BDS) step does not need to drop temperature to reach room temperature. Thereby, the biodesulfurization process can be run in more efficient and can conserve and safe more energy.

MATERIAL AND METHODS

Chemicals, bacterial strains, and growth condition

Bacterial strains were isolated from samples of oil-contaminated soil in the mine oil of districts Samboja, Kutai, East Kalimantan Province, Indonesia. The medium used for isolation, screening, and the bio-desulfurization assays was MSSF (mineral salt sulfur free) medium, whose composition was as follows: 2.44 g of KH_2PO_4, 5.77 of Na_2HPO_4, 2 g of NH_4Cl, 0.075 g of NaCl, 10 g of glucose and 10 ml of mineral solution in 1 l of distilled water, with a final pH of 7.0. The mineral solution contained 100 mM $MgCl_2.6H_2O$, 50 mM $CaCl_2$, 10 mM $FeCl_3$, 100 µM $CuCl_2.2H_2O$, and 500 µM $MnCl_2.4H_2O$. Distilled water and the concentrated fraction of aromatic compounds (CA) [1, 6]. Other materials needed are agarose, glycerol, Dibenzothiophene, *n*-tetradecane, Aluminium oxide 90 active neutral, hexane, ethanol, petroleum / kerosene / diesel oil, glass wool, HCl, NaOH and others.

Isolation of bacterial strains from soil samples

Samples of oil-contaminated soil were collected from different sites at Samboja, Kutai and former mine Mathilda in Balikpapan, Kalimantan Indonesia. The soil samples collected from the top and the subsurface layer (10 cm depth) of each site. The oil-contaminated soil (10 g) was suspended in 90 ml of 0.85% NaCl solution, after mix well then 1 ml of the supernatant diluted into 9 ml of 0.85% NaCl solution, and after mix well 0.1 ml was transferred into a test tube containing media MSSF-CA, then incubated at 37°C for 48 h. After incubation, 0.1 ml culture was transferred into the MSSF-CA medium with the spread method and incubated at 37°C for 3 d. After growth, each specific colony moved by loop and strake to fresh MSSF-CA agar plate having well spread bacterial colonies. Single colony was aseptically transferred into new agar plate for reselected on MSSF-CA agar medium and incubated at the same conditions to obtain their pure cultures. Single colony of each isolate was than inoculated into a test tube containing 5 ml MSSF-CA medium and grown for 3 d at 37°C. After incubation, 1 ml culture was taken and put into a small tube threaded which already contains 1 ml of 40% glycerol sterile to be stored as a stock culture [1,6]. The isolate was identified by microscopic observation and measurement of various biochemical parameters using the API-20E bacterial identification system (BioMerieux, Hazelwood, MO, USA) [1].

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Biodesulfurization activity test

Seed culture of each isolate was carried out in a test tube containing 5 ml of MSSF-CA medium, incubated at 37°C with shaking 150 rpm for 72 h (1 rpm = 1/60 Hz). The isolates tested its growth activity / desulfurization activity using a two-phase oil/water system, ie 1 ml of the oil phase (containing DBT in n-tetradecane as an model oil) and 5 ml water phase (MSSF medium) and cultured under the same conditions as in the seed culture [1, 6]. After incubation, the oil layer of n-tetradecane and the water layer were separated by centrifugation at 10 000 rpm form 10 min at 4°C. A unoculed medium, which was treated in the same manner was used as a control. Strain having high activity (indicated by the speed of its growth) and can growth at a higher temperature chosen for further study.

Identification of selected isolate

The morphological characterization of selected bacterial isolates was done by Gram's staining method and the motility test was carried out by hanging drop method. The isolate was identified by measurement of various biochemical parameters using API-20E bacterial identification system (BioMerieux, Hazelwood, MO, USA).

Analysis procedures

Purification of aromatic sulfur compounds (CA) of petroleum [1, 7]. Bacterial growth was determined by measuring the OD_{660} of the water layer with a spectrophotometer [1, 6]. Residual DBT in oil layer was determined by GC [1, 8-10].

RESULTS AND DISCUSSION

Isolation of desulfurizing bbacteria

A total of 12 samples of oil-contaminated soil taken from several locations, 11 samples were obtained from Samboja Village, Kutai, and one sample of Mathilda Balikpapan, East Kalimantan (Table 1). Samples were taken from Samboja entirely of soil samples were continuously exposed to oil around the oil drilling that is managed by *Pertamina*. Samples mostly blackish brown, smelling of petroleum or kerosene and a bit sticky. Soil samples of 10 g put in 90 ml of 0.85% NaCl solution, vortexes and after settling for 20 min and then 1 ml of supernatant diluted into 9 ml of 0.85% NaCl solution. After homogeneous, 0.1 ml was transferred in to a test tube containing MSSF-CA medium, then incubated at 37°C for 48 h. Growth of bacteria observed after 48 h incubation (Table 1).

Table 1: Growth of bacteria from the entire soil sample derived from Samboja, Kutai and Mathilda Balikpapan, East Kalimantan in liquid media

Soil samples	Characteristics		
	The level of turbidity	Foaming	Color
MTD1	++++++	+++++	slightly yellow
SBJB1	+	-	-
SBJ2	-	-	-
SBJ3	++	-	-
SBJ4	+++++	++	white
SBJ5	++++++	++++	white
SBJ6	+++	-	white
SBJB7	-	-	-
SBJ8	+++++	+++	slightly yellow
SBJ9	+++++	++	white
SBJ10	++++++	++++	slightly yellow
SBJ11	-	-	-

*) +, growth; - , no growth

Turbidity levels in different samples showed that the rates of microbial growth are also different. Samples MTD1, SBJ4, SBJ5, SBJ8, SBJ9 and SBJ10 seem quite high turbidity levels, but there are a few samples seen no growth among the sample SBJ2, SBJ7 and SBJ11.

Isolates ggrowth in the medium of two-phase system

The 12 soil samples mentioned above were obtained 67 isolates, and isolates that have the ability to grow and have a relatively high degree of stability are only 16 isolates. Subsequent experiments aimed to determine the ability of cell growth in the medium of two phase system (aqueous phase: MSSF and oil phase: oil that contains high sulfur, with the ratio, 5:1) (Figure 1). This experiment was carried out by planting 1 ml of a liquid culture isolates were aged 2 d (OD_{660} of 0.5) to 250 ml Erlenmeyer containing 100 ml of MSSF medium and 5 ml petroleum or model oil. Then incubated at 37°C for 72 h. During the incubation carried out observations of the level of turbidity using a spectrophotometer at a wavelength of 660 nm (OD_{660}). Data observations can be seen in Table 2. The data in Table 2, show that all isolates were considered to be potentially in the previous experiments, all of which can grow well on the medium of two phase system. This shows that all isolates were able to grow in media containing high sulfur oil, but only twelve isolates that could grow with OD_{660} more than O.5 on incubation at 37°C for 72 h, such as SBJ4a (1.04), SBJ5a (0.93), SBJ5f (0.62), SBJ7a (1.12), SBJ8a (1.03), SBJ9b (0.95), SBJ9c (0.85), SBJ9d (0.98), SBJ9e (0.97), SBJ10a (1.04), SBJ10b (0.56), and SBJB12a (0.80).

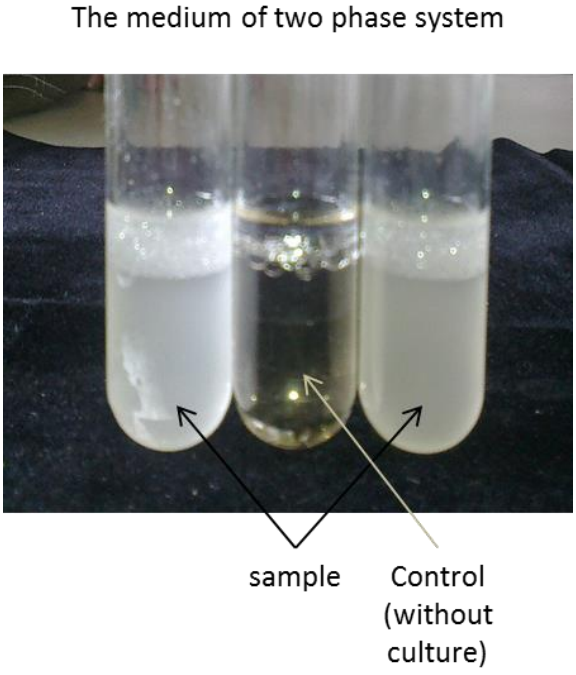

Figure 1: The medium of two phase system (aqueous phase: MSSF and oil phase: oil that contains high sulfur, with the ratio, 5:1)

Testing the ability of isolates to degrade dibenzothiophene in *n*-tetradecane

The data in Table 3 shows that there are some isolates that have the ability to degrade dibenzothiophene compound as the sole sulfur source are in excess of 70% namely SBJ4a isolates (81.88%), SBJ8a (70.62%), SBJ8b (75.97%), SBJ10b (74.37%) and SBJ10d (73.57%), while others has lower ability. The SBJ4a isolates to be tested in further experiment.

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Table 2: Growth of sulfur degrading bacteria for 72 h incubation in MSSF-TD medium

Isolate	pH		Growth OD_{660} (h)*	
	0	72	0	72
SBJ2a	6.8	5.8	0.20	0.45
SBJ2b	6.8	4.6	0.06	0.41
SBJ2c	6.9	5.5	0.02	0.33
SBJ4a	6.9	6.1	0.06	1.04
SBJ5a	6.9	5.9	0.03	0.93
SBJ5b	6.8	5.4	0.05	0.30
SBJ5c	6.8	3.5	0.05	0.37
SBJ5d	6.9	3.0	0.12	0.44
SBJ5e	6.8	3.1	0.17	0.33
SBJ5f	6.9	3.1	0.09	0.62
SBJ6a	6.9	4.8	0.36	0.38
SBJ6b	6.7	4.6	0.08	0.38
SBJ7a	6.9	6.2	0.03	1.12
SBJ8a	6.9	6.8	0.02	1.03
SBJ8b	6.8	5.5	0.08	0.43
SBJ8c	6.9	3.2	0.11	0.39
SBJ9a	6.9	5.8	0.05	0.29
SBJ9b	6.7	6.4	0.07	0.95
SBJ9c	6.6	6.3	0.11	0.85
SBJ9d	6.9	5.8	0.00	0.98
SBJ9e	6.8	5.5	0.03	0.97
SBJ10a	6.8	6.1	0.07	1.04
SBJ10b	6.9	5.3	0.07	0.56
SBJ10c	6.9	4.0	0.13	0.45
SBJ10d	6.8	4.5	0.06	0.40
MTD11a	6.9	5.7	0.12	0.47
MTD11b	6.8	6.4	0.05	0.12
MTD11c	6.9	4.8	0.12	0.36
SBJB12a	6.8	6.4	0.05	0.80

*Each isolate was cultured in 6 ml of MSSF-TD medium containing 200 mg/l of DBT at 37°C with shaking at 150 rpm for 72 h.

Table 3: The rate of degradation of dibenzothiophene in *n*-tetradecane by growing cells of potential desulfurizing bacteria

Isolate	Initial DBT Concentration (ppm)	Growth (OD_{660}) *	DBT residual (ppm)	DBT degradation (%)
SBJ4a	200	1.04	36.23	81.88
SBJ5f	200	0.62	76.36	61.82
SBJ7a	200	1.12	69.22	65.39
SBJ8a	200	1.03	58.77	70.62
SBJ8b	200	0.43	48.05	75.97

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SBJ10a	200	1.04	147.32	26.34
SBJ10b	200	0.56	51.26	74.37
SBJ10d	200	0.40	52.86	73.57

*Isolates were cultured in 5 ml of MSSF-medium + 1 ml of model oil (*n*-tetradecane containing 200 mg/l DBT) at 37^{o}C with shaking at 150 rpm for 72 h. OD was measured at a wavelength of 660 nm.

Identification of selected isolate

The selected isolates capable degrading aromatic sulfur compounds (dibenzothiophene) was identified. Based on the biochemical identification using the API-20E that SBJ4a isolate was identified as *Pseudomonas* sp. strain SBJ4a (Table 4).

Table 4: Characteristics of SBJa isolate

Characteristic	SBJ4a Isolate*
Morphological characteristic	
Cell Shape	Rod
Capsule	Negative
Gram staining	Negative
Motility	Motile
Biochemical characteristic*	
ONPG	+
L-arginine	+
L-lysine	-
L-ornithine	-
Citrate	+
Sodium thiosulfate	-
Urea	-
L-tryptophane	+
Indole	-
Sodium pyruvate	+
Gelatin	-
D-glucose	+
D-mannitol	-
Inositol	-
D-sorbitol	-
L-rhamnose	-
D-sucrose	-
D-melibiose	-
Amygdalin	-
L-Arabinose	-
Isolate Species	*Pseudomonas luteola*

*The isolate was identified using API-20E (bacterial identification system): +, activity; -, no activity.

Identification of DBT degrading bacteria using API-20E has been done also by Keyser *et al*. [11] on *Mycobacterium phlei* GTIS10 and *Sphingomanas subarctica* T7b [1]. Many researchers found that *Pseudomonas* spp. capable of degrading DBT and its derivatives, such as *Pseudomonas* sp. [12], *Pseudomonas delafieldii* [13].

CONCLUSION

The results showed that 67 isolates sulfur petroleum degrading bacteria isolated from soil in Samboja and Mathilda, East Kalimantan as a candidate of organism that capable degrade aromatic sulfur compounds in petroleum through enrichment culture. The 29 isolates showed potential as sulfur petroleum desulfurizing bacteria and can grow on a MSSF medium containing dibenzothiophene (DBT) in *n*-tetradecane as a model oil and the ratio of oil / water (1: 5). Some isolates can grow on the medium with the rate of growth (OD_{660})

ranged from 0.56 to 1.12 and degrade DBT compounds between 26.34% and 81.88%. SBJ4a isolate can degrade DBT for 81.88% after 72 h of incubation at 37°C with shaking 150 rpm. Based on biochemical identification using API-20E that SBJ4a isolate was identified as *Pseudomonas luteola* [14].

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