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THE SCREENING OF PETROLEUM HYDROCARBONS DEGRADING BACTERIA AS A BIOREMEDIATING AGENTS FROM MANGROVE AREAS*)

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ABSTRACT

Screening of the petroleum hydrocarbons degrading bacteria as a bioremediating agent from mangrove areas of Sungsang in Banyuasin Regency of South Sumatra has been carried out. The aims of this research is to obtain the petroleum hydrocarbons degrading bacteria which can be turned into a bioremediating agent for the petroleum-polluted environment. The samples are taken from the mangrove area which is polluted by petroleum and the area which is not polluted by petroleum. The screening is carried out through the stages of isolation, first selection , second selection, characterization and identification. The isolation is carried out by using the *Bushnell Hass Mineral Salt* (*BHMS*) medium. The first selection is done by using Zobell solid medium to obtain the bacteria which can live in the environment which contains petroleum. The second selection is carried out to obtain the bacteria which can use petroleum as a sole the carbon source. The characterization covers the morphology of the colony, the morphology of the cell, and biochemical testing. From the mangrove area of Sungsang 16 species of bacteria are capable of degrading the petroleum hydrocarbons. Those six teen species are *Alcaligenes eutropus*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus polymyxa*, *Enterobacter agglomerans*, *Flavobacterium thalpophilum*, *Pseudomonas alcaligenes*, *Pseudomonas aureofaciens*, *Pseudomonas cepacia, Pseudomonas diminuta*, *Pseudomonas mendocina*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas pseudomallei*, *Pseudomonas saccharophila*, and *Pseudomonas syringae*.

Keywords: screening, isolation, selection, characterization, petroleum hydrocarbon degrading bacteria

INTRODUCTION

The mangrove area has multiple benefits viewed from the aspects of social-economy and ecology, among others, it has physical fuction to protect beaches and river banks from erosion; biological function as a nursery ground and spawning grounds for fish, shrimp and lobster, and other aquatic organism; economic function to be made as a place for fish embankment, wood producing region, and as a place for recreation.

The width of mangrove forest in Indonesia is decreasing from time to time. In 1980 Indonesia still had the largest mangrove forest in the world with 4.13 million hectares of mangrove forest, but in 1990 the width of mangrove forest in Indonesia decreased about 60% to become 2.5 million hectares. The decrease of mangrove forest was caused by various factors, among others are land convertion for industry, housing, and transportation (Noor, 1994). In addition to decreasing quantitatively in width, Indonesian mangrove forest

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also decreases qualitatively in terms of environmental quality due to the pollution. This also happens to the mangrove areas in South Sumatra especially the areas of Tanjung Api-api, Sungsang and Upang. The three areas are the areas of River Basin of Musi River and that of Dawas which are made into the primary shipping route of petroleum transportation from the oil field of Sukayu (MUBA Regency) and the oil field of Palembang. Petroleum pollution in this area can be caused by oil spills and scattered oil during the activities of drilling, production, refinery and transportation, oozing from the oil reservoir; the activities of loading and unloading at the harbor; and tanker's/ship's waste (Ridho *et al.,* 2005). Sungsang region is a region of mangrove ecosystem with the worst degree of destruction. This is mainly due to the fact that this region is the busiest route of sea transportation especially the transportation of ships supplying the oil tankers (Ridho *et al.,* 2006).

Bioremediation is management which relies on degradation by utilizing degrader microorganism of hydrocarbons which is an environmentally friendly, effective, efficient, economical, and acceptable way to the environment (Mangkoedihardjo, 2005 Bioremediation is the development of the field of biotecnology of environment and is petroleum waste processing by means of degradation by microorganism which results in an end result which is a stable and non-toxic compound (Zam, 2006).

The existence of hydrocarbons degrading microorganism (bacteria, fungi, and yeast) is widely spread in nature. Bacterial group is a bioremediation agent which is more commonly used in comparison with the other bioremediation agents because bacteria have faster reproduction rate (Koswara, 2003). Desai and Vyas (2006) reported that the bacterial groups which were capable of degrading hydrocarbons were *Arthrobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Rhodococcus* sp., *Bacillus* sp., *Vibrio* sp., *Nocardia* sp., *Corynebacterium* sp., *Alcaligenes*, and *Mycobacterium*. Whereas the result of the isolation of Souza *et.al.,* (2006) showed that the bacteria which were capable of degrading petroleum hydrocarbons from mangrove areas were from the genus *Bacillus*, *Pseudomonas*, and *Acinetobacter.*

The bacteria which are capable of degrading hydrocarbons from petroleum processing are obtained by means of isolating them from petroleum waste which contains hydrocarbons or an ecosystem receiving hydrocarbon waste whose spread is very wide especially in a petroleum-polluted environment (Atlas & Bartha, 1998). In the waters which is not petroleum-polluted, the population of microbes which are potentially capable of degrading petroleum is only about 1 %, but in the waters which is polluted by petroleum the population of microbes will increase to become 10 % of the total population of heterotrophic microorganisms (Venosa *et al.,* 1992).

The mangrove region is considered as a life-supporting region which has economic and ecological function, therefore it is necessary to make an effort to protect the mangrove region, such as specified in the Indonesian Act No. 5 of the year 1990 which states that mangrove is included as a life-supporting region which has economic and ecological function, therefore the preservation of mangrove forests should be taken care of both which regards the width and the quality. As an effort to recover the quality of mangrove forests from the existing pollution, it is necessary to apply the bioremediation technology which utilizes indigenous degrader bacteria for petroleum hydrocarbons. As an initial step for applying the bioremediation technology, the screening of the bacteria which are capable of degrading hydrocarbons originating from the mangrove region which is polluted by petroleum is carried out.

The aims of this research is to obtain the species of petroleum hydrocarbons degrading bacteria which are indigeneous to the mangrove region as a bioremediating agent for the mangrove region which is polluted by petroleum hydrocarbons.

MATERIALS AND METHODS

Sampling of the sediment from around the roots and the water of mangrove region

The samples are taken by means of multiple sampling method from 6 (six) different stations. Stations 1, 2, and 3 are the stations in the petroleum-polluted region, while stations 4, 5, and 6 are the stations in the non petroleum-polluted region. In each station 3 points of sampling are determined and from those points of sampling the samples are taken randomly. The sample of soil is taken as much as ± 2 kg and that of the water is as much as ± 2 liters, then they are combined evenly (Greene *et al*., 2000).

Isolation and purification of the bacteria

The isolation and purification of the bacteria is initiated with enrichment process by putting the soil sample taken from the area around mangrove roots into *Bushnell Hass Mineral Salts/BHMS* liquid medium (0,2 g of $MgSO_4.7H_2O$, 0,02 g of $CaCl_2$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 1 g of NH_4NO_3, 2 drops of $FeCl_3$, 1 L of aquades), then they are incubated on the shaker (120 rpm) for five days at room temperature. The bacterial suspension is then grown on solid BHMS medium (the composition of which is the same as liquid *BHMS* and is added with 15 g of agar/L). The growing bacteria are then purified by separating each kind of bacterium which has different characteristics and make it grow on a new medium (Udiharto, 1994).

Selection of the petroleum hydrocarbons degrading bacteria

The first selection, each isolate which has been purified is inoculated on Zobell medium (5 g of pepton; 1 g of yeast exstract; 0,01 g of $FeSO_4$; 0,012 g of K_2HPO_4; 15 g of agar; 1 L of aquades) on petridish, petroleum residue is dabbed on the surface of the medium. The growth of the bacterial isolate colony on the surface of the Zobell medium which is dabbed with oil residue indicates that the bacterial isolate can survive and grow in the environment which contains oil residue. In the second selection, the isolate which is able to grow in the first selection is inoculated into Soeminarti liquid medium (0,01 g of yeast extract, 0,01 g of K_2HPO_4, 0,01 g of KNO_3, 1 L of aquades) and added with petroleum as a source of carbon. The formation of a white layer between the phase of liquid medium and the phase of residue indicates that the isolate grows and has the capacity to use oil residue as a source of carbon and energy (Ruyitno, 1991).

Characterization and identification of the bacteria

The morphological characterization including morphology of the colony (form, elevation, edge, and the color of the colony) and morphology of the cell (form, cell arrangement, Gram stain, and the presence/absence of endospora (Pikoli *et al*., 2000). The physiological and biochemical characterization covers: starch, lipid, casein, and gelatin hydrolysis ; glucose, sucrose, and lactose fermentation ; production of H_2S, indol, urese, and catalase ; tests of methyl red, Voges Proskauer, TSI, Simmon's citrate, and nitrate reductio. Identification can then be done by using the reference book entitled *Bergey's Mannual of Determinative Bacteriology* 8th and 9th editions.

THE RESULT AND DISCUSSION

The result of isolation and selection

From the process of isolation and selection of petroleum hydrocarbon degrading bacteria is obtained bacterial isolates as shown in Table 1.

Table 1. The number of bacterial isolates obtained and the result of first and second selection

The origin of isolate	The number bacterial isolate	First Selection	Second Selection	Isolate Code
Station 1	17	15	6	I_1-I_6
Station 2	18	14	3	II_1-II_3
Station 3	17	17	3	III_1-III_3
Station 4	12	7	1	IV_1
Station 5	10	9	2	V_1-V_2
Station 6	9	8	1	VI_1
Total	83	70	16	

From Table 1 it is known that from station 1, 2 and 3 are obtained more indigenous bacterial isolates than from station 4, 5 and 6. Station 1,2, and 3 is the area of cross section of the route of transportation, in which the busiest activities of passing ships occur, both tankers and locally owned ships and boats, in comparison with the station 4,5, and 6 in which transportation activities are not too busy. The frequency of transportation activities causes differences of quantity and quality of oil pollution in station 1-3 from that in station 4-6.

From first selection 70 isolates are obtained and this means that the bacterial isolates are able to grow in the environment which contains petroleum. From second selection 16 isolates which are able to survive and grow in the environment polluted by crude oil are obtained. These isolates are also able to make use of the crude oil as a source of carbon and energy. It happens because the bacteria can adapt themselves to the crude-oil polluted environment. According to Harayama *et al.,* (1999), by producing oxygenase enzyme bacteria can adapt to the hydrocarbon environment and use of the hydrocarbons as the source of carbon and their energy.

The bacteria which are able to grow in the environment which contains hydrocarbons are the bacteria which are able to tolerate the existence of the hydrocarbon compound, by converting toxic compound into non-toxic compound. This is in accordance with the finding of Juhazs & Naidu (2000) that co-metabolite hydrocarbon bacteria are the bacteria which are able to survive and grow in the environment of hydrocarbons by transforming toxic compound into non-toxic by means of secreting enzyme which transforms the compounds as an effort to survive. This kind of bacterial group grows and reproduce themselves by using the fraction which is the result of the breaking of hydrocarbon compound which is done by the other hydrocarbon degrader bacteria. According to Fritsche and Hofritchter (2009) the process of breaking of the hydrocarbon chain by the bacteria can happen due to the existence of enzymatic reaction. Most bacteria produce oxygenase enzyme so that they can degrade the hydrocarbons. This is due to the ability of the bacteria to oxidize the hydrocarbons and turn the hydrocarbons into their electron donor. The bacteria will degrade the hydrocarbons to become water (H_2O) and carbon dioxide (CO_2). In addition to that, the petroleum hydrocarbon-degrading bacteria will produce bioproducts such as fatty acid, gas, surfactant.

The result of the characterization and identification of the bacteria

The process of morphological characterization of the colony and the cell and physiological characterization through biochemical test and the identification process result in the bacterial isolates originating from the genus *Pseudomonas, Enterobacter, Bacillus, Alcaligenes,* and *Flavobacterium* (Appendix 1 and 2). The research by Ramsay *et al*., (2000) and Ibrahim *et al*., (2008) also resulted in the bacterial isolates which were able

to degrade petroleum hydrocarbons in the mangrove region which were also from the genus *Bacillus*, *Pseudomonas, Alcaligenes, Klebsiella* and *Enterobacter*.

The bacterial isolates obtained, the most species belong to the genus *Pseudomonas*. *Pseudomonas* is reported to have the highest degrading potential in comparison with other genus. According to Desay and Vyas (2006) genus P*seudomonas* has been widely known as one of the microbial groups which has high capacity in degrading petroleum. These bacteria have high capacity in degrading aliphatic, aromatic, and resin fraction. *Bacillus* has high capacity in degrading aliphatic, monocyclic and polyciclic aromatic. According to Fritsche and Hofritchter (2009), *Pseudomonas* has the characteristics of aerobic, Gram-negative, bacillus which never shows fermentation activities. *Bacillus* is the only genus from Gram-positive which is aerobic and sometimes facultative anaerobic and has endospores which enables *Bacillus* to survive even in the most extreme environment, although it is a vegetative cell. According to Foght (2008) in comparison with anaerobic bacteria, aerobic bacteria have greater potential in degrading hydrocarbons.

Alcaligenes is a polycyclic aromatic hydrocarbon degrader, *Enterobacter* is a resins degrader, and Flavobacterium is an aromatic hydrocarbon degrader. *Alcaligenes* with specific characteristic occur in marine environments and do not have endospores and are pure aerobic and show sugar fermentation (glucose, lactose, and sucrose). *Enterobacter* in glucose fermentation produces acid and gas, and production test of indol positive (Buchanan and Gibbons, 1974). *Flavobacterium* is a bacterial group of Gram-negative, do not produce endospores, facultative anaerobic in nature, non motile, catalase and oxidase positive (Holt *et al* ., 1994).

CONCLUSIONS

There were 16 species petroleum degrading bacteria as a bioremediating agents. Those 16 species were *Alcaligenes eutropus*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus polymyxa*, *Enterobacter agglomerans*, *Flavobacterium thalpophilum*, *Pseudomonas alcaligenes*, *Pseudomonas aureofaciens*, *Pseudomonas cepacia, Pseudomonas diminuta*, *Pseudomonas mendocina*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas pseudomallei*, *Pseudomonas saccharophila*, and *Pseudomonas syringae*.

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Appendix 1. Table 2. The results of characterization and identification of bacteria

Isolate character	**Isolate I_1**	**Isolate I_2**	**Isolate I_3**	**Isolate I_4**	**Isolate I_5**	**Isolate I_6**	**Isolate I_7**	**Isolate I_8**
Macroscopic colony morphology	Circular, undulate, raised, translucent, yellow	Circular, entire, convex, glistening, opaque, unpigmented	Circular, entire, convex, glistening, transparent, soft cream	Rhizoid, filamentous, raised, opaque, unpigmented	Circular, entire, convex, glistening, transparent, unpigmented	Circular, entire, convex, glistening, transparent, cream	Circular, serrate, convex, translucent, yellowish	Irregular, serrate, raised, opaque, waxy, unpigmented
Microscopic cell morphology	Bacillus, Gram negative, did not produce spore	Bacillus-coccoid, gram negative, did not produce spore	Bacillus, Gram negative, did not produce spore	Bacillus, Gram positive, produce spore	Bacillus, Gram negative, did not produce spore	Cocco-bacillus, gram negative, did not produce spore	Bacillus, Gram negative, did not produce spore	Bacillus, Gram positive, produce spore
Motility	Motile	Motile	Motile	Motile	Nonmotile	Motile	Motile	Motile
Biochemical tests								
-Starch Hydrolysis	Negative	Negative	Negative	Positive	Negatif	Positive	Positive	Positive
-Lipid Hydrolysis	Negative	Negative	Positive	Negative	Positif	Positive	Positive	Negative
-Casein Hydrolysis	Positive	Negative	Positive	Positive	Positif	Negative	Positive	Positive
-Gelatine Hydrolysis	Negative	Negative	Negative	Positive	Positif	Positive	Positive	Positive
-Glucose Fermentation	Negative	Negative	Negative	Positive	Negatif	Positive	Positive	Positive
-Sucrose Fermentation	Negative	Negative	Negative	Negative	Negatif	Negative	Positive	Negative
-Lactose Fermentation	Negative	Negative	Negative	Negative	Negatif	Positive	Positive	Negative
-H_2S production	Negative	Negative	Negative	Negative	Negatif	Positive	Negative	Negative
-Indole Production	Negative	Negative	Negative	Positive	Negatif	Positive	Negative	Positive
-Urease Produktion	Negative	Positive	Negative	Negative	Negatif	Negative	Negative	Negative
-Catalase Production	Positive	Positive	Positive	Positive	Positif	Positive	Positive	Positive
-Metyl red test	Negative	Negative	Positive	Negative	Negatif	Negative	Positive	Positive
-Voges Proskauer test	Negative	Negative	Negative	Positive	Negatif	Negative	Negative	Negative
-TSI test	Negative	Negative	Negative	Positive	Negatif	Positive	Positive	Positive
-Simmon's citrate test	Negative	Positive	Positive	Negative	Positif	Positive	Positive	Negative
-Nitrat reduction	Negative	Positive	Negative	Positive	Negatif	Positive	Positive	Positive
CONCLUSION	*Pseudomonas diminuta*	*Alcaligenes eutrophus*	*Pseudomonas syringae*	*Bacillus licheniformis*	*Pseudomonas pseudomallei*	*Enterobacter agglomerans*	*Pseudvemonas saccharophyla*	*Bacillus cereus*

Appendix 2. Table 3. The results of characterization and identification of bacteria

Isolate character	**Isolate I_9**	**Isolate I_{10}**	**Isolate I_{11}**	**Isolate I_{12}**	**Isolate I_{13}**	**Isolate I_{14}**	**Isolate I_{15}**	**Isolate I_{16}**
Macroscopic colony morphology	Irregular, filamentous, flat, spread thin, translucent, cream	Circular, entire, convex, transparent, cream-brownish	Circular, lobate, raised, opaque, unpigmented, shiny	Circular, lobate, raised, opaque, white, shiny	Circular, entire, convex, transparent, cream-yellowish	Circular, entire, convex, glistening, transparent, yellowish	Circular, entire, convex, glistening, transparent, unpigmented	Circular, entire, flat, glistening, transparent, yellowish
Microscopic cell morphology	Bacillus, gram negative, did not produce spore	Bacillus, gram negative, did not produce spore	Bacillus, gram positive, produce spore	Bacillus, gram positive, produce spore	Bacillus, gram negative, did not produce spore	Bacillus, gram negative, did not produce spore	Bacillus, gram negative, did not produce spore	Bacillus, gram negative, did not produce spore
Motility	Motile	Motile	Nonmotile	Motile	Motile	Motile	Motile	Motile
Biochemical tests								
-Starch Hydrolysis	Negative	Negative	Positive	Positive	Negative	Negative	Negative	Negative
-Lipid Hydrolysis	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative
-Casein Hydrolysis	Positive	Negative	Positive	Positive	Negative	Positive	Negative	Negative
-Gelatine Hydrolysis	Weak Positive,	Positive	Positive	Positive	Negative	Positive	Positive	Negative
-Glucose Fermentation	Positive	Positive	Positive	Positive	Negative	Negative	Positive	Negative
-Sucrose Fermentation	Positive	Positive	Positive	Negative	Negative	Negative	Positive	Negative
-Lactose Fermentation	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
-H_2S production	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
-Indole Production	Positive	Negative	Negative	Negative	Negative	Positive	Positive	Positive
-Urease Produktion	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative
-Catalase Production	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
-Metyl red test	Positive	Positive	Negative	Negative	Negative	Negative	Positive	Negative
-Voges Proskauer test	Positive	Negative	Positive	Negative	Negative	Negative	Positive	Negative
-TSI test	Positive	Positive	Positive	Positive	Negative	Negative	Positive	Negative
-Simmon's citrate test	Positive	Negative	Positive	Negative	Positive	Positive	Negative	Positive
-Nitrat reduction	Negative	Positive	Positive	Positive	Positive	Negative	Negative	Negative
CONCLUSION	*Pseudomonas alcaligenes*	*Flavobacterium thalpophilum*	*Bacillus polymyxa*	*Bacillus firmus*	*Pseudomonas mendocina*	*Pseudomonas aurofaciens*	*Pseudomonas cepacia*	*Pseudomonas pseudo-alcaligenes*

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