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Characterization of Petroleum Hydrocarbon Decomposing Fungi Isolated from Mangrove Rhizosphere

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ABSTRACT

The research was done to obtain the isolates of soil borne fungi isolated from mangrove rhizosphere which were capable of degrading petroleum hydrocarbon compounds. The soil samples were collected from South Sumatra mangrove forest which was contaminated by petroleum. The isolates obtained were selected based on their ability to survive, to grow and to degrade polycyclic aromatic hydrocarbons in medium containing petroleum residue. There were 3 isolates of soil borne hydrocarbonoclastic fungi which were able to degrade petroleum *in vitro*. The 3 isolates were identified as *Aspergillus fumigates, A. parasiticus*, and *Chrysonilia sitophila*. *C. sitophila* was the best isolate to decrease total petroleum hydrocarbon (TPH) from medium containing 5-20% petroleum residue.

Keywords: Hydrocarbonoclastic fungi, hydrocarbon compounds, mangrove rhizosphere

INTRODUCTION

The extent of mangrove forests in Indonesia has been decreasing from year to year. In the 1980's Indonesia had the largest mangrove forest area in the world with a total of 4.13 million hectares, but 10 years later the area had decreased to 2.5 million hectares (Noor *et al*. 1999). The area of mangrove forest in Tanjung Api-api, Sungsang and Upang of South Sumatra in eleven-year period (1992 until 2003) already had a decreased area of 4352.6 ha (Ridho *et al*. 2006). Furthermore, the quality of mangrove forests have been declining due to environmental pollution. Mangrove areas in South Sumatra are located in the watershed area which are used as the main shipping lines transporting crude oil from the oil fields in Sekayu (District Muba) and Palembang. Pollution can be caused by oil spills and oil scattered during drilling activities, production, refining and transporting, and from permeation and reservoir; activities of loading and unloading at the ports; and waste from tankers (Udiharto 1996). Crude petroleum oil is a complex mixture of hydrophobic components like n-alkanes, aromatics, resins and asphalthenes, and microorganisms are known to attack and degrade a specific component as compared with other components of oil (Chen *et al*. 2009).

The function of mangrove root system was primarily influenced by oil spill because the surface of the plant's organ functioning in the exchange of CO_2 and O_2 is covered by oil residue. This oil coverage on root surface lowered the oxygen levels by 1-2% in two days. Bioremediation using microbial agents is considered as a viable option for recovery efforts for mangrove forest quality from oil pollution. Bioremediation can be done using a variety of microbes, including bacteria, fungi and actinomycetes (Anas 1998). However, the information on the capability of fungi to degrade petroleum pollutants is still limited. Fungi are of interest because of their ability to synthesize relatively unspecific enzymes involved in cellulose and lignin decay that can degrade high molecular (Mancéra-Lopéz *et al*. 2007). This current study aimed to obtain indigenous hydrocarbonoclastic fungi isolates from rhizosphere of South Sumatra mangrove forest. It is expected that these fungi can be used as an agent for bioremediation to reduce pollutant loads, especially petroleum.

MATERIALS AND METHODS

Origin of Isolates

The research was conducted from April to September 2009. Samples containing hydrocarbonoclastic fungi isolates were collected from mangrove forest in Sungsang, Banyuasin

District of South Sumatra Province in the dry season. The isolation and selection of the hydrocarbonoclastic fungi were done at Chemistry Laboratory, Biology and Fertility of Soil at the College of Agriculture, Sriwijaya University.

The samples were taken using a purposive sampling method from the oil contaminated areas (Steel and Torrie 1981). Soil samples around the roots of mangrove plants were taken randomly from the contaminated area. Each sample was placed into a sterile container. To maintain its properties, the soil sample was placed into a cooler which has been added with some ice.

Enrichment Procedure

Enrichment was done by using SMSS medium (Stone Mineral Salts Solution) containing dextrose. Soil sample (10 grams) was taken plus 90 ml of sterile medium SMSS. The sample was then homogenized using an incubator shaker at 120 rpm for 5 days. Isolation was done using rose Bengal medium with the addition of 200µg L^{-1} chloramphenicol to inhibit bacterial growth. Liquid from the enrichment process (5 mL) was taken and was inoculated into 45 ml of sterile physiological solution (0.85% NaCl) and further diluted to 10^{-6}. About 0.1 ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions was transferred into a Petridish, then the Rose Bengal medium was added evenly. After Rose Bengal medium in a petridish solidified, the plate was then incubated at room temperature until the growing fungal colonies were visible (Pelczar and Chan 1981).

Each colony of fungi that grew and had different characteristics was transferred to solid PDA (potato dextrose agar) medium, MEA (malt extract agar) and Czapek's Dox (Gandjar *et al*. 1999) agar (0.5g KCl 0, 3g $NaNO_3$, 1g K_2HPO_4, 0.5g $MgSO_4$. $7H_2O$, 0.01g $FeSO_4$.$7H_2O$, 30g sucrose, 15g agar, 1 L aquades) medium in a petridish using an ose needle by scratching (streak plate) and incubated again at room temperature for 4-5 days. This work was done repeatedly until pure fungal colonies were obtained.

Isolates Selection

The isolates obtained were selected based on their ability to survive and grow on medium containing crude oil residues and capable to degrade the petroleum residue. Selection was carried out through two stages: (1) first phase selection, each pure isolate was inoculated onto solid Zobell medium (5 g peptone, 1g yeast extract, 0,01g $FePO_4$.$4H_2O$, 15g agar 15 g, 750 mL brackish water, 250 mL aquades) (Park *et al*. 2002) in a petridish, then rub oil residue on sterile filter paper followed by placing it on the surface of the medium. The cultures were incubated at room temperature for five days. The isolates that were able to grow on the surface of the Zobell medium containing oil residues indicated that these fungi isolates are able to survive and grow in environments containing crude oil residues, (2) selection of stage II; isolates capable of growing in the first phase of selection medium were inoculated into SMSS liquid medium in a test tube. Six drops of oil residues were added to the surface of the medium as a source of additional carbon. The cultures were then incubated at room temperature for five days. Formation of white layer between the phase of the media (liquid) and the phase of these residues indicated that the isolates had grown and had the ability to use residual oil as a source of carbon and energy. The isolates that were identified of capable degrading residue were selected for development of stock cultures.

The selected of hidrocarbonoclastic fungi isolates were then characterized and catalogued. The characterization process included: observation of colony morphology (macroscopic) in a specific medium, which include growth, colony color, colony diameter, underneath colony colors, as well as microscopic observations including mycelia and spores.

To get the best isolate capable of degrading petroleum hydrocarbons with different levels of residues, each selected isolates that have been rejuvenated in Zobell medium for about 48 hours was converted into suspension culture by inoculating 1 ose into 10 ml of physiological saline solution (0.85% NaCl) and shaken until homogeneous. Suspension of each isolate was inoculated as much as 10% (v/v) into modified Soeminarti (0,01g yeast extract; 0,01g K_2HPO_4; 0,01g KNO_3; 1L aquades) liquid medium (Udiharto 1994) containing petroleum residue of 5%, 10%, 15%, 20% and 25% (v/v). Cultures were incubated at room temperature for five days. The observed variable to determine the best isolates to degrade petroleum hydrocarbons included a decrease in the percentage of TPH in the soil.

RESULTS AND DISCUSSION

Environmental Conditions and Soil Chemical Properties

Water and soil samples were taken from 6 locations, each located at Sungsang (station I), Payung Island (station II), Sungsang near

Table 1. Environmental conditions and the position of sampling locations.

Site Location	Environmental Condition				
	pH H_2O	Salinity (mg g^{-1})	O_2	Water Temperature	Position
I (Sungsang)	6.15	2	6.15	30.1°C	02°24,265' S 104°55,821' E
II (Pulau Payung)	6.15	1	5.95	30.8°C	02°21,253' S 104°55,490' E
III (Sungsang near settlements)	6.20	2	6.01	30.2°C	02°20,880' S 104°54,512' E
IV (Pulau Sarang Elang)	7.20	7	6.47	32.0°C	02°02'0,880' S 104°45,080' E
V (Front of Pulau Sarang Elang)	7.20	7	6.47	32.0°C	02°19,148' S 104°45,718' E
VI (Tanjung Serai)	7.20	7	6.50	32.0°C	02°19,148' S 104°45,718' E

Table 2. Some chemical properties of soil (sediment) of each sampling station.

Site locations	C-org (g kg^{-1})	N-Total (g kg^{-1})	P- Bray I (mg kg^{-1})	pH H_2O	Exchangable cation ($cmol_{(c)}$ kg^{-1})						
					K	Na	Ca	Mg	H	Al	CEC
I	4.39	0.38	12.60	5.4	1.60	17.4	5.40	1.05	0.12	0.98	34.80
II	1.87	0.16	6.60	6.9	1.28	8.1	5.30	0.70	-	-	30.45
III	3.75	0.36	18.17	5.8	1.92	17.4	5.63	1.02	0.12	0.23	34.80
IV	2.04	0.18	3.75	7.5	1.60	34.8	9.83	2.05	-	-	52.50
V	2.54	0.21	4.80	7.4	1.60	39.1	8.88	1.22	-	-	56.12
VI	2.39	0.19	10.35	7.6	1.55	26.1	8.23	1.45	-	-	43.50

settlements (station III), Sarang Elang Island 1 (station IV), Sarang Elang Island 2 (station V), and Tanjung Serai (station VI). Position, the level of salinity, O_2 content, temperature and water pH that were observed during sampling are presented in Table 1.

Station locations IV, V, and VI had different characteristics with the location I, II, and III based on the pH, salinity, temperature and O_2 content of the water. Sites IV, V, and VI are located more toward the sea and therefore had a higher salinity, which affects the high pH of the soil, while the content of O_2 and temperature at the three locations were relatively higher than other 3 sites (sites I, II, and III).

A sample of soil (sediment) was analyzed for its chemical properties as presented in Table 2. Stations I and III had C-organic, N-total and available P higher than other stations, but it had a lower pH value. pH value is influenced by the C-high organic that will produce organic acids that cause decline in soil pH. In accordance with the high salinity water, station IV, V, and VI had Na-dd and Ca-dd higher than stations I, II, and III. Although the C-organic at sites IV, V, and VI was lower than the station I, II, and III, but all the three locations had a higher CEC. This is might be associated with high soil pH and high Na and Ca-dd.

Isolation, Selection, and Identification of Hydrocarbonoclastic Fungi

Results of fungi isolation from each station as shown in Table 3 indicated that the fungal isolates were not obtained from every station. From the station I and IV, respectively, 1 isolate was acquired, from stations II and III, respectively, 2 isolates were obtained, while from the stations V and VI no fungal isolates was obtained.

Further test was done to the six fungi isolates for their ability to degrade the oil in the growing medium. The results of Stage I selection showed that fungal isolates from stations I and IV could not degrade the oil. Phase selection results showed that there was only one isolate from station II which had the ability to degrade the oil, while the isolates from the stations III were able to degrade the oil (Table 3).

Isolates that were capable of growing in the selection media of the first phase were subjected to selection in stage II, *i.e.* inoculated into SMSS liquid medium in a test tube with added 6 drops of oil

Table 3. Fungi isolates obtained from the mangrove area and isolates capable of Growing in the selection stage I and II.

Site Location	Number of Isolate	Total Isolate			
		Code	Before Selection	Stage I	Stage II
I	1	$K_{1.1}$			
II	2	$K_{2.1}$,$K_{2.2}$	1	-	-
III	2	$K_{3.1}$, $K_{3.2}$	2	1	1
IV	1	$K_{4.1}$	2	2	2
V	-	-	1	-	-
VI	-	-	-	-	-
Total	6		-	-	-

Table 4. Macroscopic morphology of fungal isolates on Czapek's medium with 3-day Incubation time.

Code	Colony morphology in Czapek's Dox Agar			
	Growth	Diameter of colony (cm)	Colony color	Color of inside colony
K2.1	+++	9.00	Orange	White
K3.1	+	1.20	dark green	Greenish white
K3.2.	+	0.80	Yellowish green	Light yellow
	Colony morphology in medium Potato Dextrose Agar (PDA)			
K2.1	+++	9.00	Orange	Whitish orange
K3.1	++	3.75	Dark green	Light green
K3.2.	++	4.00	Yellowish green	Light green
	Colony morphology in medium Malt Extract Agar (MEA)			
K2.1	+++	9.00	Orange	Orange
K3.1	+	2.20	Dark green	Light green
K3.2.	+	1.10	Yellowish green	Yellow

Note: + = slow growth (diameter < 3 cm), ++ = Medium growth (diameter 3-6 cm), +++ = Fast growth (diameter > 6 cm).

residues as a source of additional carbon to the surface of the medium. White layer formation between the phase of the media (liquid) and the phase of the petroleum residue after incubated for 5 days, demonstrated that these isolates had grown and had the ability to use residual oil as a carbon source. The results of this second phase of selection indicated that the three isolates from the first phase of selection can degrade the oil (Table 3). Hydrocarbonoclastic fungi can survive in petroleum polluted environments because they have the ability to use hydrocarbons as a substrate and are capable of growing with the specific physiological responses and enzymatic reactions (Mancéra-Lopéz *et al*. 2007).

Macroscopic and microscopic characterizations were done on indigenous hydrocarbonoclastic fungi isolates capable of degrading petroleum (Table 4 and 5). Table 4 shows that K2.1 fungal colony isolates had the fastest rapid growth than the other 2 isolates, whether grown on Czapek's medium, PDA or MEA. The diameter of K2.1 fungal isolates in 3-day incubation period has reached 9 cm. K3.1 and K3.2 fungal isolates growth rate was relatively moderate with 1,2-3,75 cm diameter for isolate K3.1 and 0,8-4 cm for isolate K3.2.

The results of microscopic characterization of the three fungal isolates as presented in Table 5 and as reported by Samson *et al*. (2004) showed that all three come from different species. Fungal isolates K3.1 and K3.2 belong to the genus *Aspergillus* that has hyaline septhate, colored hyphae, no reproductive spore structure, has round conidia, and has conidiophores with fialid, metula, vesicles and stipe. K3.1 isolate belongs to the species *Aspergillus fumigatus*, the characteristics of conidia include rough surface and dark green color, whereas K3.2 isolate belongs to the species *Aspergillus*

Table 5. Results of microscopic characterization.

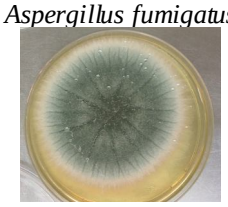
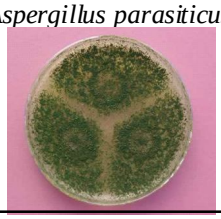
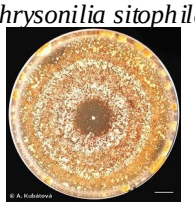
Microscopic characteristics	Isolate		
	K3.1	K3.2	K2.1
Non reproductive structure:			
Hipha			
• Septate/aseptate	Septate	Septate	Septate
• Hiphae color	Hyaline	Hyaline	Dark
Reproductive structures:			
Spore	-	-	-
• Forms			
• Size			
• Surface			
• Color			
Collumela	-	-	-
Sporangiophore	-	-	-
Conidia	+	+	+
• Forms	Coccus	Coccus	Coccus, Ovale
• Size	± 3 µm	± 2,5 µm	± 12,5 µm
• Surface	Rough	Prickly	Smooth
• Color	Dark green	Yellowish green	Orange
Conidiophores	+	+	+
• Fialid	+	+	-
• Metula	+	+	-
• Vesicle	+	+	-
• Stipe	+	+	+
Identification	*Aspergillus fumigatus*	*Aspergillus parasiticus*	*Chrysonilia sitophila*
			

Table 6. Decreased level of TPH (%).

Oil concentration (%)	Decreased level of TPH (%) by		
	A. fumigatus	*A. parasiticus*	*C. sitophila*
5	89.12	90.16	92.25
10	87.26	90.87	94.72
15	80.14	84.21	90.54
20	74.27	80.16	86.21
25	70.14	73.25	75.36

parasiticus with the characteristics of the spiny conidia surface and yellowish green color. Nkwelang *et al*. (2008) found the major genera of fungal active in polluted soil in the tropical region were *Aspergillus, Penicillium, and Mucor*.

K2.1 fungal isolates have dark septhate, do not have the structure of reproductive spores, have round or oval conidia with a size of 12.5ìm, smooth surface and orange in color, has conidiophores with fialid, metula, negative vesicles, and identified as *C. sitophila*.

Decreased levels of TPH by hydrocarbonoclastic fungi

Total of petroleum hydrocarbon value reflects the levels of hydrocarbon (C and H elements) in the petroleum and used as a reference for judging success or failure of a process of biodegradation of petroleum hydrocarbons. The ability of selected fungal isolates to lower the levels of TPH in medium is presented in Figure 1. Figure 1 shows a decrease in TPH in medium when inoculated with selected

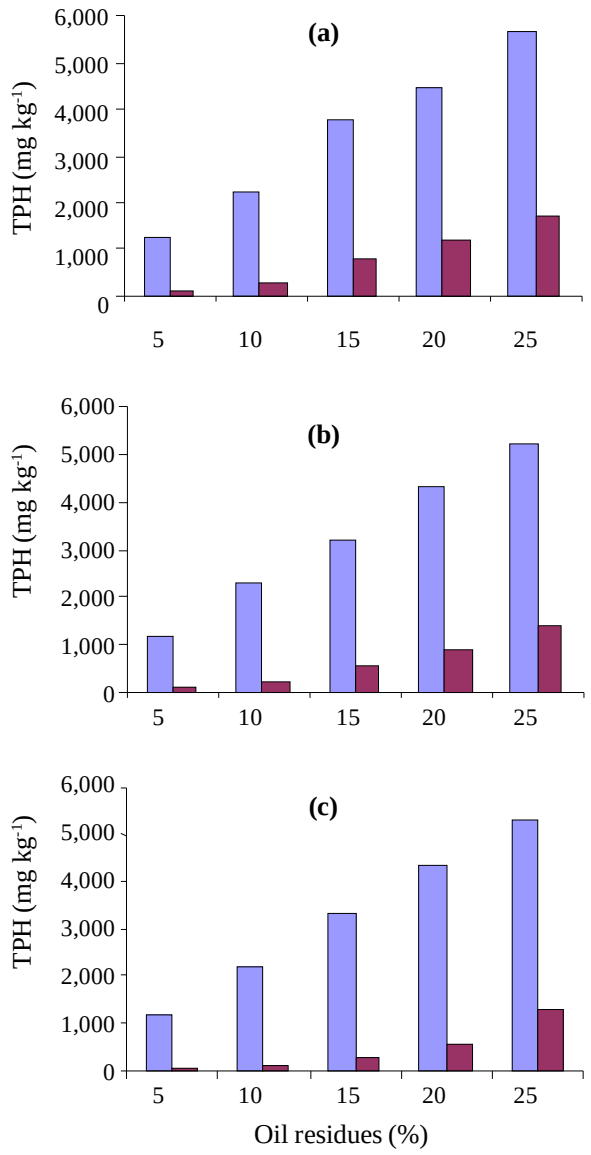


Figure 1. Decreased levels of TPH by *Aspergillus fumigatus* (a), *A. parasiticus* (b), and *C. sitophila* (c). ■ = TPH before application, ■ = TPH after application.

hydrocarbonoclastic fungi isolates (*A. fumigatus, A. parasiticus,* and *C. sitophila*) and incubated for 5 days. Total of petroleum hydrocarbons in the medium containing 5-25% oil before the selected fungal isolates inoculation ranged from 1151.6 to 5701.6 mg kg^{-1}. After 5 days inoculation with selected fungal isolates, TPH decreased from 116.0 to 1702.6 mg kg^{-1}. This decrease in TPH content indicated the ability of the selected hydrocarbonoclastic fungi in the process of biodegradation of petroleum hydrocarbons.

The decreased in TPH levels after 5 days incubation period compared with before incubation are presented in Table 6. This table shows the decline in ability to decay petroleum hydrocarbons with increasing levels of oil residues in the media, except for elevated levels of oil residues from 5% to 10% for medium inoculated with *C. sitophila*. The ability of *C. sitophila* to lower concentrations of TPH in various petroleum residues in the media was better than the ability of fungi *A. fumigatus* or *A. parasiticus*. In a study by George-Okafor *et al.* (2009), both isolates of *Aspergillus* (*A. vesicolor and A. niger*) exhibited above 98% degradation efficiency for polycyclic aromatic hydrocarbon moieties when grown in a culture medium incorporated with 1% crude oil (hydrocarbon) and 0.1% Tween 80 for 7 days. They can be effectively utilized for the degradation of oil polluted farm lands especially those located within the vicinity of isolateion soil sites.

Increased levels of oil residues from 5% to 25% followed by a decline in fungal ability to decay hydrocarbons as shown in Table 6. This is allegedly related to the increasingly low levels of oxygen in the medium to grow and increasingly high concentrations of toxic compounds in the medium derived from petroleum residues. Lack of oxygen caused the rate of biodegradation to decline (Santosa 1997).

CONCLUSIONS

There were 3 isolates of soil borne hydrocarbonoclastic fungi which were able to degrade petroleum *in vitro*. The 3 isolates were identified as *Aspergillus fumigates*, *A. parasiticus*, and *Chrysonilia sitophila*. *C. sitophila* was the best isolate to decrease TPH from medium containing 5-20 % petroleum residue.

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