The Investigation of Polycyclic Aromatic Hydrocarbon and Oil Degrading Bacteria Isolated from The Marina Port Ancol, Jakarta Bay

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) as well as crude oil are widespread environmental pollutants. The contamination of air, soil, freshwater (surface water and groundwater), and marine environments by PAHs as well as crude oil has been reported. Of concern to public health is the fact that many PAHs or their metabolites are mutagenic, carcinogenic, or both. North Java coastal area such as Jakarta Bay is the polluted marine area in Indonesia as a result from anthropogenic wastes and the oil spill. Although evaporation and photo-oxidation play an important role in oil detoxification, ultimate and complete degradation is accomplished mainly by marine micro flora, and being dominant in this function. Certain bacteria are well-known could consume and degrade the PAHs as well as crude oil. Therefore investigating the potential PAH and oil degrading marine bacteria is important. In this study, we collected sample from oil polluted area in Marina Port Ancol, Jakarta Bay and isolated four PAH substrates and Arabian crude oil degrading marine bacteria using enrichment method and direct isolation method. As result, 223 strains could degrade PAHs, among these strains, 94 strains could degrade phenanthrene, 23 strains degrade fluoranthene, 92 strains could degrade dibenzotiophen, 14 strains could degrade phenotiazin and 106 isolates degrade crude oil.

Key words: polycyclic aromatic hydrocarbons, crude oil, degrading bacteria, bioremediation.

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Introduction

Improper disposal methods and inadequate control of toxic materials have led to widespread contamination of soils, groundwater, surface water bodies also coastal area. Polycyclic aromatic hydrocarbons (PAHs) are recalcitrant wastes of concern because of their toxic and carcinogenic potentials (Toby & Raymond, 1992). The combustion of organic materials is mainly responsible for their ubiquitous distribution in the atmosphere, surface waters, and sediments (Keith & Telliard, 1979). PAHs are also constituents of crude oils and materials derived from coal (e.g., creosote and coal tar). Accidental spillage and improper disposal during the processing, transportation, and use of these materials have resulted in a number of contaminated sites presenting serious health and ecological risks. Oil spill are a major source of polycyclic hydrocarbon (PAHs) pollution in aquatic systems. Crude and refined petroleum compounds contain from 0.2 to more than 7% PAH (Neff, 1985). These PAH emissions may have long term effect on aquatic organisms living in affected areas. PAHs are hydrophobic compounds which rapidly associated with sediment-dwelling animals over time scales of month to years. Further more, PAHs with molecular weights between 128 and 202 (naphthalene-fluoranthene) have high acute toxicity toward aquatic organisms (Rossi & Neff, 1978; Neff, 1985) and many of the larger PAH are carcinogenic to vertebrate.

Achieving permanent clean-up of the polluted sites is problematic in that some remediation technologies are not always viewed positively by the public or may not be amenable to particular sites. Many methods of treating recalcitrant wastes, including various types of physical, chemical, and biological processes, have been developed. Of the processes whereby these contaminants such as PAHs are removed from the environment,

microbial degradation plays a major role in the decontamination of sediment and surface soils (Kanaly & Harayama, 2000). Bioremediation technologies have increasingly been proposed to decontaminate those sites. However, PAH polluted sites frequently resist a fast and complete clean-up. Among the reasons suggested for the attenuation in the biodegradation rates are the accumulation of toxic metabolites and the fact that the residual concentrations comprise the components more resistant to degradation (Bumpus & Aust, 1987). Strategies to improve remediation technologies for PAH-contaminated coastal area, a broader understanding of the biochemical pathways involved in degradation and in the eventual formation of partially oxidized products.

Indonesian coastal waters are in the critical condition, oil spill accident and improper disposable method make a marine pollution. The industrial, transportation and population was concentrated in Java island especially Jakarta bay. Stress the importance for understanding the fate and effect of oil and PAHs in the Java coastal environment, a better understanding of how PAH interact with bacteria from this area was needed. Concern for this purpose, study of investigating the PAH degrading bacteria from this area was conducted in this study.

Materials and Methods

Chemicals. Four PAH substrates used in this study were phenanthrene, fluoranthene, dibenzotiephene, and phenotiazine. Phenanthrene and phenotiazine was a pure grade obtained from Wako Pure Chemical Co. Ltd. Japan, while fluoranthene and dibenzotiophene were produced by Trade TCI, Japan. Arabian Crude Oil (CO) was also used as substrate and oil control. Medium culture ONR7 contained 22.79 g NaCl, 3.98 g Na_2SO_4, 1.3 g TAPSO {3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid}, 0.72 g KCl, 0.27 g NH_4Cl, 89 mg $Na_2HPO_4.7H_2O$, 83 mg NaBr, 31 g $NaHCO_3$, 27 g H_3BO_3, 2.6 g NaF, 1.46 g $CaCl_2.2H_2O$, 11.18 g $MgCl_2.6H_2O$, 24 mg $SrCl_2.6H_2O$ and 2 mg $FeCl_2.4H_2O$ in 1 L medium was used in enrichment culture method. Marine Broth/Agar contained 5 g peptone, 1 g yeast extract, 0.1 g Ferric citrate, 19.45 g NaCl, 3.24 g $MgSO_4$, 1.8 g $CaCl_2$, 0.55 g KCl, 0.08 g KBr, 0.16 g $NaHCO_3$, 34.0 mg $SrCl_2$, 22 mg H_3BO_3, 4 mg $NaSiO_2$, 2.4 mg NaF, 1.6 mg NH_4NO_3, 8.0 mg Na_2PO_4 in 1 L medium was used as isolation medium.

Sampling Method. Sea water was collected from Marina Ancol Port, in 3 location sites. The physical condition of sea water collected in Site 1 was pH 7-8, salinity 12‰, temperature 31°C, dissolve oxygen was 0,28-0,30 mg/lt, Site 2 was pH 7.5-8, salinity 6‰, temperature 31.5°C, dissolve oxygen was 0.25 mg/lt, and Site 3 was pH 7.5, salinity 30‰, temperature 32°C, dissolve oxygen 1.86 mg/lt (Table 1).

Direct Isolation Method. Sea water was directly spread in 1/5 Marine Agar media supplemented with and without Arabian crude oil, and incubated in room temperature. After one until two weeks incubation, the growth of microorganisms was checked. Isolation and purification processes were conducted for positive growth samples. Finally, pure colonies were screened for PAHs degrading ability by sublimation method and for crude oil degrading by formation of clear zone surrounding the bacterial colony.

Enrichment Isolation Method. Approximately 9 ml of sea waters was applied in 1 ml ONR7 liquid culture medium that was enriched with 200 µl Arabian Crude Oil, 1000 ppm phenanthrene, 1000 ppm fluoranthene and 1000 ppm phenotiazine (final volume was 10 ml), respectively. Each enrichment culture was done in a separate test tube in 3 replications. Control without PAH and oil was also maintained in this experiment. The liquid culture was incubated on 30°C along 2-4 weeks. After a properly time incubation, inoculation of the enrichment liquid culture to the ONR7 plates agar medium was conducted for positive growth samples. Inoculated plate agar media was directly sublimed with phenanthrene, fluoranthene, dibenzothiophene, and phenotiazine, respectively. Crude oil enrichment culture was applied for screening of degrading bacteria by a clear zone formation around the bacterial colony after incubation at 30°C. Isolation and purification of the potential colony for identification process was then carried on.

Sublimation Method. Screening the microbial that degrade PAH by sublimation method was conducted by the method of Alley & Brown (2000). The inoculating agar plate was directly sublimed with define PAH. Sublimation process was done in hot plate with white sand beach to keep temperature stability. The temperature was adjusted to a melting point of PAH that used. The melting point for phenanthrene, fluoranthene, dibenzothiophene, and phenotiazine is 97-100°C, 105-110°C, 100°C, and 130-140°C, respectively. In order for the agar plate wasn't melting, crystal ice was applied on the cover agar plate (Figure 1). The bacteria that could degrade fluoranthene and phenotiazin was indicated by the appearance of blue color around the colony (Figure 2)

Oil Degrading Test. After spreading sea water sample, Arabian crude oil was poured on the agar surface. Incubation of this agar plate at 30°C along more than one week was done and clear zone around the colony was recorded (Figure 3).

Table 1. Physical Condition of sampling area

No	Parameters	Site 1	Site 2	Site 3
1	Sampling time	11.15 WIB	11.30 WIB	13.00 WIB
2	Location	500 m from port	Port	2km from port
3	Condition of sea water	Not so black but smelt	Black and smelt	Clean
4	Weather	Good	Good	Good
5	Elevation	12 ft	101 ft	59 ft
6	Latitute	06°07.052'	06°07.169'	06°06.860'
7	Longitude	106°49.733'	106°49.753'	06°50.790'
8	pH	7-8	7.5-8	7.5
9	Sea water Temperature	31°C	31,5°C	32°C
10	Air Temperature	32°C	36°C	32°C
11	DO (dissolve oxygen) (mg/L)	0.28-0.30	0.25	1.86
12	Salinity (‰)	12	6	30
13	∑ sample	50 L;600 mL	5 L;1200 mL	51 L;1800 mL

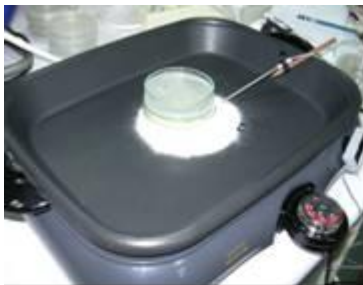
Figure 1. Sublimation process

Figure 2. Colony profile of phenotiazine degrading bacteria

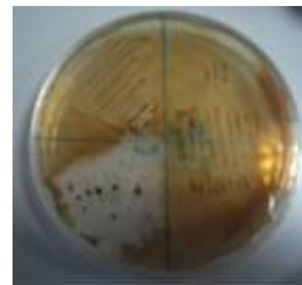
Figure 3. Clear zone of oil degrading bacteria

Results and Discussion

In this study, we investigated the oil degrading bacteria isolated from Marina Port Ancol, Jakarta Bay, Indonesia. The investigation included the isolation, the characterization, and the identification of PAH and oil degrading bacteria from the area. The reports on isolation, characterization, and identification of PAH as well as oil degrading bacteria from Jakarta Bay have been conducted by several reports. All of the study used enrichment isolation methods to obtain PAH or oil degrading microbes from the area. In this study, we conducted on the isolation of oil degrading microbes from Marina Port Ancol the estuarine ecosystem, by comparing two isolation methods (direct and enrichment isolation methods) to obtain potential isolates because the differences of bacteria as well as their characters were important for further application of the isolates.

Direct Isolation Method

Using the direct isolation method, the difference number of total bacteria from three

location sites was shown. Site 1 and Site 2 gave the total number of bacteria approximately 10^6 cfu/mL while Site 3 gave 10^5 cfu/mL. It indicated that the number of bacteria in Site 1 and 2 that highly polluted seen from blackish color and oily surface of sea water was one order higher than clean and clear water on Site 3.

Further, the addition of crude oil in the media didn't give a significant difference in the amount of bacteria (Figure 4), but this treatment was important for selection of oil degrading bacteria. By using crude oil in the isolation plate, the selection of oil degrading bacteria by the formation of clear zone could be conducted easily.

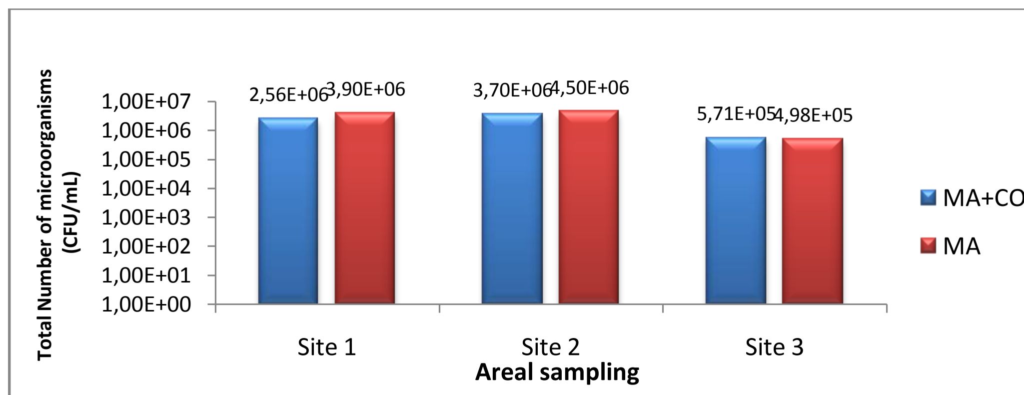

Figure 4. The number of total bacteria from difference location

As representative, the number of bacteria selected from direct isolation method was 168. We selected 40 isolates from the samples treated with crude oil and 16 isolates from the samples treated without crude oil from three sites.

The representative isolates were further directly sublimed using PAHs (phenanthrene, flourentene, dibenzothiopene dan penothiazine) for screening process and grown in agar supplemented with crude oil. The results of number of strains selected from each site sampling and capability to degrade PAHs and capability to form clear zone in crude oil using this direct isolation method were shown in Table 2.

In our analysis, from 168 isolates, 64 isolates (38%) have capability to degrade one or more PAH substrates or crude oil and 104 isolates (62%) did not have those ability. Further, from 115 isolates, 1 isolate that degrade fluoranthene, phenothiazine, and crude oil; 5 isolates both fluoranthene and crude oil; 2 isolates both phenotiazine and crude oil; 1 isolate only fluoranthene; 4 isolates only phenothiazine; and 102 isolates only crude oil, were obtained. From Site 1, Site 2, and Site 3, the number of potential isolates for PAH and oil degrading bacteria was 46, 36, and 33, respectively. No isolates that degrade phenanthrene and dibenzothiaphene was found using direct isolation method.

Enrichment Isolation Method

In our investigation, four enrichment cultures of sea water samples were used in this method, i.e. sea water sample enriched with phenanthrene, fluoranthene, phenotiazine, and Arabian crude oil in ONR7 medium. After 14 days incubation with agitation, 36 test tubes of the the enrichment cultures (4 substrates with 3 replication in 3 sampling sites) that showed the positive growth of microorganisms were spread out in agar plates followed by sublimation process with selected PAH. In addition, enrichment cultures with crude oil as a substrate were also spread out in agar plates supplemented with crude oil.

Totally, the number of colony of bacteria selected from three sites in Marina Port Ancol with enrichment isolation method following by sublimation with 4 types of PAHs was 199 isolates. While, from enrichment culture using crude oil as a substrate following by screening of clear zone formation was 74 isolates (Table 3). These representative isolates (273 isolales) were subjected to the second sublimation screening. This second selection was done to confirm the ability of the pure isolate for PAH or oil degrader.

Table 2. Screening of PAH and crude oil degrading bacteria isolated using direct sampling method.

Sampling location	Treatment of Sample	Number of Isolates	Degradation Test					Number of Potential Isolates
			Phenan threne	Fluoran thene	Dibenzo thiaphene	Pheno thiazine	Crude Oil	
Site 1	With Crude Oil	40	-	+	-	-	-	1
			-	-	-	+	-	2
			-	-	-	-	+	28
			-	+	-	-	+	1
			-	-	-	+	+	1
			-	+	-	+	+	1
	Without Crude Oil	16	-	+	-	-	+	1
			-	-	-	-	+	10
			-	+	-	-	-	1
Site 2	With Crude Oil	40	-	-	-	+	-	1
			-	-	-	-	+	25
	Without Crude Oil	16	-	-	-	+	-	1
			-	-	-	-	+	7
			-	+	-	-	+	2
Site 3	With Crude Oil	40	-	-	-	-	+	19
	Without Crude Oil	16	-	-	-	-	+	13
			-	+	-	-	+	1
Total		**168**						**115**

Table 3. The number of PAH and crude oil degrading bacteria by enrichment method in first selection.

Substrate used	Sublimated with	Total isolates	Number of colony obtained		
			Site 1	Site 2	Site 3
Phenanthrene	Phenanthrene	**6**	1	2	3
	Dibenzothiaphene	**32**	32	0	0
Fluoranthene	Fluoranthene	**21**	0	21	0
Phenothiazine	Phenothiazine	**12**	6	0	6
Crude Oil	Phenanthrene	**85**	22	15	48
	Fluoranthene	**24**	11	8	5
	Dibenzothiaphene	**19**	2	17	0
	Phenothiazine	**0**	nd	nd	nd
Total Number		**199**	**74**	**63**	**62**
	Formation of clear zone				
Crude oil	Crude oil	**74**	**22**	**29**	**23**

The result from second sublimation process of selected isolates was presented in Table 4. From 273 representative isolates, 79 isolates (29%) has the potential ability to degrade PAH and crude oil and others (69%) has no ability. Further, from 79 isolates, 42 isolates that degrade only phenanthrene, 17 isolates that degrade only DBT, and 10 isolates that degrade only crude oil were obtained. Further, 1 isolate that degrade phenanthrene and fluorenthene; 11 isolates that degrade phenanthrene and DBT; 3 isolates that degrade fluorenthene and DBT; 1 isolate that degrade phenotiazine and oil; 8 isolates that degrade phenanthrene, fluoranthene, and DBT; 3 isolates that degrade phenanthrene, DBT, and phenotiazine; 1 isolate that degrade phenanthrene, fluorenthene, and crude oil; 1 isolate that degrade phenanthrene, fluorenthene, DBT, and phenotiazine; 1 isolate that degrade phenanthrene, fluorenthene, DBT, and crude oil; 3 isolates that degrade phenanthrene, DBT, phenotiazine, and crude oil; were obtained. No isolates degrade only fluoranthene and only phenothiazine.

The number of isolates that could degrade phenanthrene, fluranthene, phenothiazine, and dibenzothiaphene was high in Site 3, Site 2, Site 1 and 3, and Site 1, respectively. While, the number of isolates that could degrade crude oil was similar in three location sites. From Site 1, Site 2, and Site 3, the number of potential isolates for PAH and oil degrading bacteria was 46, 36, and 33, respectively. No

isolates that degrade phenanthrene and dibenzothiaphene was found using direct isolation method.

Most of PAH degrading bacteria were found when liquid culture enriched with Arabian crude oil. There were 88 positive strains that isolate from crude oil enrichment. It is the fact that crude oil was rich of various molecular weight of hydrocarbons as carbon and energy source of bacteria. Correlated with data from Table 3, we could determine that enrichment with crude oil will give more bacteria colony than sole PAH.

Phenanthrene was easier to degrade than other PAH. it's why we get almost 80% positive strains that could degrade this PAH. Because of structural relativity, fluoranthene and phenothiazine were quite difficult to degrade. Only 20% strains could degrade fluoranthene and phenothiazine.

Table 4. Potential ability of selected isolates

Location	Enrichment Medium	1st Degradation test	∑ of selected strain	2nd Degradation Test					∑ of Potential Isolates
				Phenanthrene	Fluoranthene	Dibenzothiaphene	Phenothiazine	Crude Oil	
Site 1	Phenanthrene	DBT	32	Nd	nd	+	nd	nd	8
	Phenothiazine	Phenothiazine	6	+	nd	+	+	nd	3
				+	nd	+	+	+	3
	Crude Oil	Phenanthrene	22	+	-	-	-	-	1
				+	-	+	-	-	7
				-	-	-	+*	+	1
				+	+	+	-	-	2
		Fluoranthene	11	+	+*	+	nd	-	6
		Crude Oil	22	nd	nd	nd	nd	+	4
				+	+			+	1
Site 2	Phenanthrene	Phenanthrene	2	+	+	+	-	-	1
	Fluoranthene	Fluoranthene	21	nd	+	+	nd	nd	1
	Crude Oil	Phenanthrene	15	+	-	-	-	-	2
				+	-	+	-	-	1
		Fluoranthene	8	+	+*	-	nd	-	1
				+	+*	-	nd	+	1
				+	+*	+	nd	+	1
				-	+*	+	nd	-	1
		DBT	17	nd	nd	+	nd	nd	7
				+		+			1
				+	+	+	+		1
		Crude Oil	29	nd	nd	nd	nd	+	3
Site 3	Crude Oil	Phenanthrene	48	+	-	-	-	-	17
		Fluoranthene	5	+	+*	+	nd	-	1
		Crude Oil	23	nd	nd	nd	nd	+	4
				+				+	1
Total number of potential and preserved isolates									**80**

Note: nd: not determined; *: blue colony

Acknowledgements

This research is granted by Indonesian Institute of Sciences under SubProgram Census of Marine Life, Program Research Competitive 2007.

References

Alley, J. F. and L. R. Brown. 2000. Use of sublimation to prepare solid microbial media with water-insoluble substrate. Appl. Env. Microbiol. 66: 439-442

Barnhart, M. J. and J. M. Myers. 1989. Pilot remediation tells all about petroleum contaminated soil. Pollut. Eng. 21:110-112.

Brodkorb, T. S. and R. L. Legge. 1992. Enhanced Biodegradation of Phenanthrene in Oil Tar-Contaminated Soils Supplemented with *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 3117-3121

Bumpus, J. A. ands D. Aust. 1987. "Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: involvement of the lignin degrading system, Bioessays. 6:166-170.

Cerniglia, C. E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368.

Ferna´ndez, P., M. Grifoll, A. M. Solanas, J. M. Bayona, and J. Albaige´s. 1992. Bioassay-directed chemical analysis of genotoxic components in coastal marine sediments. Environ. Sci. Technol. 26:817–829.

Gibson, D. T. and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. In D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.

International Association of Research Chemists. 1983. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans. Polynuclear aromatic compounds. Part 1. Int. Assoc. Res. Chem. Monogr. 32:355–364.

Kanaly, R. A. and S. Harayama. 2000. Biodegradation of high-molecularweight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182:2059–2067.

Keith, L. H. and W. A. Telliard. 1979. Priority pollutants I—a perspective view. Environ. Sci. Technol. 13:416–423.

Kelley, I. and C. E. Cerniglia. 1991. The metabolism of fluoranthene by a species of *Mycobactenum*. J. Ind. Microbiol. 7:19-26.

Kelley, I., J. P. Freeman, F. E. Evans, and C. E. Cerniglia. 1991. Identification of a carboxylic acid metabolite from the catabolism of fluoranthene by a *Mycobactenum* sp. Appl. Environ. Microbiol. 57:636-641.

Kelley, I., J. P. Freeman, and C. E. Cerniglia. 1991. Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp. Biodegradation 1:283–290.

Neff J. M. 1985. Polycyclic aromatic hydrocarbon. In: Rand GM, Petrocelli SR (eds) Fundamental of aquatic toxicology methods and applications. Hemisphere, Washington, DC, p 416–454

Rossi, S. S. and J. M. Neff. 1978. Toxicity of polynuclear aromatic hydrocarbons to the polychaete *Neanthes arenaceodentata*. Mar. Pollut. Bull. 9:220–22.