*Mar. Res. Indonesia Vol. 39, No. 2, 2014: 79−85*

# ISOLATION AND DISTRIBUTION OF CRUDE OIL AND POLYCYCLIC AROMATIC HYDROCARBON-DEGRADING BACTERIA FROM POLLUTED HARBOURS IN NORTH JAKARTA

**Yustian Rovi Alfiansah[1*], Mindi Adindasari[2], Mentari Argarini[3], Yeti Darmayati[1] and Ruyitno[1]**

[1]Research Center for Oceanography, LIPI
[2]Faculty of Biotechnology, Atmajaya University
[3]Department of Biology, Padjajaran University
[*]E-mail: yust002@lipi.go.id

Received: March 2014 Accepted: November 2014

## ABSTRACT

Several harbours in North Jakarta have been polluted by spills of oil and their derivates. We suggest that diverse species of crude oil and polycyclic aromatic hydrocarbon-degrading bacteria inhabit these harbours. An experiment was undertaken in 2007 to isolate crude oil and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria from oil-polluted harbours, such as Muara Baru, Sunda Kelapa and Tanjung Priok. Sea water and sediment samples were collected twice, in March and April. Crude oil and PAH-degrading bacteria were isolated from enrichment culture of samples in an enrichment medium (SWP), using ONR7a medium with the addition of 5 types of PAH gases or Arabian Light Crude Oil 210 (ALCO 210) onto medium. This study reported that fluoranthene and crude oil-degrading bacteria were the major bacteria isolated from the three polluted harbours. In total, 109 isolates have been collected which can degrade crude oil (29% of total isolates), fluoranthene (33%), fluorene (20%), pyrene (7%), dibenzothiopene (6%), and phenantrene (5 %). Cultivable bacteria have been isolated mostly from the Sunda Kelapa samples, with fewer in those from Muara Baru and Tanjung Priok, respectively. Among these isolates, 5 isolates have the capability to degrade 5 types of PAH and ALCO 210. They were *Alcanivorax* sp. B-1084, *Pseudomonas* sp. D5-38b, *Alcanivorax* sp. TE-9, *Bacillus* sp. L41, *Alcanivorax dieselolei* strain B-5 clone 1.

**Keywords**: Bioremediation, Crude oil and polycyclic aromatic hydrocarbon degrading bacteria, North Jakarta.

## INTRODUCTION

Oil pollution in the ocean and coastal ecosystem has been caused not only by leakage during oil drilling, but also by tanker collisions or oil/fuel disposal from ships through ballast water or oil spillage. Jakarta Bay, which has several traditional harbours and one international port is highly exposed to pollution by oil and its derivates (polycyclic aromatic hydrocarbon (PAH), asphalt and long chain alkenes). Lemigas (1974) confirmed that most of harbours in Jakarta Bay, especially Pasar Ikan and Tanjung Priok harbour, were polluted by oil. In addition, oil spills polluted Seribu Islands' waters in 2004 and 2005.

Several PAHs are potent carcinogens, and their continuous presence at trace levels in surface waters may constitute a chronic human health hazard. Polycyclic aromatic hydrocarbons are found in effluents from such high-temperature industrial processes such as coal coking and petroleum refining. They have been detected at microgram per litre concentrations in condensate samples from several pilot-scale coal-conversion facilities (Suess, 1976). Furthermore, a large amount of the discharge might be transported to the surface and reached the shoreline (i.e. the oil accident in the Gulf of Mexico from April to July 2010). Although cleanup efforts have remained aggressive, a substantial portion of the oil remains



*DOI : 10.14203/mri.v39i2.49*

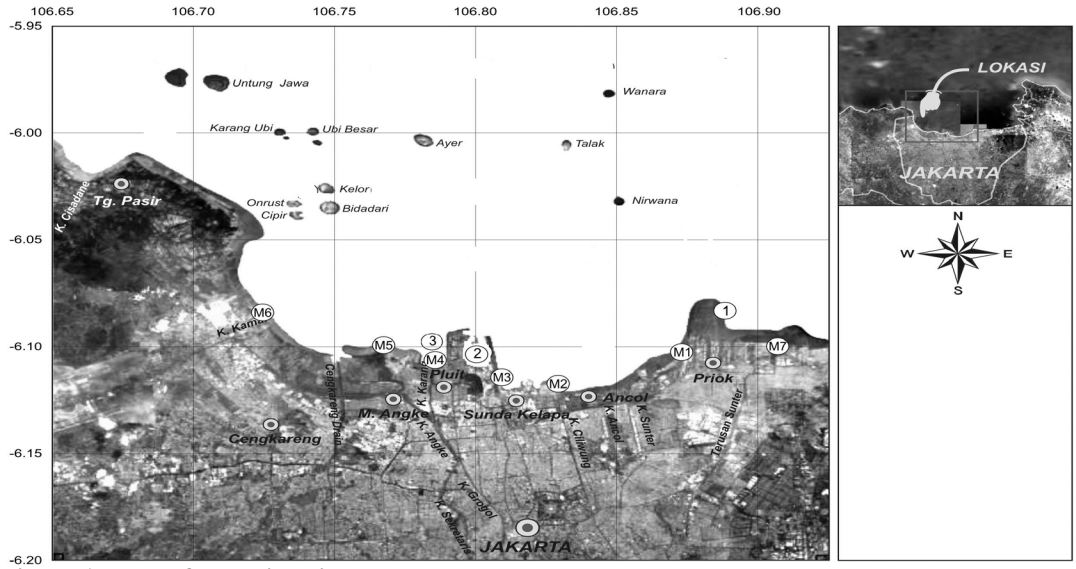


**Figure 1. Map of sampling sites**

trapped in coastal ecosystems, especially in benthic areas (Kotska et al., 2011).

Bioremediation is one of the eco-safety methods for resolving oil contamination in marine areas. Microorganisms will degrade crude oil and its derivatives into less harmful substances or reduce their concentration. In addition, they can emulsify crude oil into a tar-ball which is easier to remove (Haines *et al.*, 2005). Oil degradation by microbes appears to be the major process through which petroleum hydrocarbons are removed from the sediment ((ZoBell) 1964 in Hambrick III *et al*., (1980)). A variety of PAH degraders (mostly *Pseudomonas* and *Vibrio* spp.) have been isolated from polluted systems, such as Boston Harbor, Chesapeake Bay, and hydrocarbon-contaminated salt marshes (Berardesco *et al*., 1998, West *et al*,. 1984). Thayib (1978) reported that hydrocarbonoclastic bacteria have been isolated from the port of Tanjung Priok, in concentrations of $1.1x10^3$ to $1.1x10^4$ Most Probable Numbers (MPN) 100 $ml^{-1}$sea water. Isolation and biochemical testing of these hydrocarbonoclastic bacteria indicated that *Nocardia* sp, *Pseudomonas* sp, *Arthrobacter* sp and *Micrococcus* sp. were the bacteria most commonly found at Tanjung Priok. Furthermore, Darmayati *et al*. (2008) have successfully isolated 131 hydrocarbonoclastic bacteria from Jakarta Bay and Seribu Island waters, and classified them into *Alcanivorax*, *Marinobacter*, *Achromobacter*, and *Bacillus* genera.

The objectives of this study were to isolate prominent bacteria which can be used in oil-spill bioremediation and to decipher the distribution of oil and polycyclic aromatic hydrocarbon-degrading bacteria from three oil-polluted harbours.

## MATERIALS AND METHODS

### *Sampling sites*

Three oil-contaminated harbours in North Jakarta were chosen; Muara Baru, Sunda Kelapa, and Tanjung Priok (Figure 1). Sampling was conducted in March and April 2007. Sediment was picked up using a sediment grab, while seawater from the surface layer was taken using a water sampler.

### *Bacterial isolation*

One gram of sediment was taken aseptically and put in 10 ml of sterile sea water. Both sediment and sea water were serially diluted by $10^{-6}$ dilution factors in sterile sea water.

Bacteria were i) isolated directly by plating the sample (sea water and sediment suspension) onto ONR7a medium and ii) isolated from enrichment medium (SWP medium). Bacterial isolation was done as follows:

*Arabian Light Crude Oil (ALCO 210) degrading bacteria isolataion*. One hundred µL

of each dilution series were spread onto ONR7a medium (solution 1 contained 22.79 g NaCl, 3.98 g $Na_2SO_4$, 0.72 g KCl, 83 mg NaBr, 31 mg $NaHCO_3$, 27 mg $H_3BO_3$, 2.6 mg NaF, 0.27 g $NH_4Cl$, 89 mg $Na_2HPO_4.7H_2O$, 1.3 g TAPSO, 15 g Bacto agar, with pH adjusted to 7.6 with NaOH; solution 2 was composed of 11.18 g $MgCl_2.6H_2O$, 1.46 g $CaCl_2.2H_2O$, 24 mg $SrCl_2.6H_2O$; solution 3 consisted of 2 mg $FeCl_2.4H_2O$. The solutions were autoclaved separately, allowed to cool down and mixed together (Dyksterhouse *et al*., 1995) using a glass spreader. Then 75 µl of ALCO 210 were spread onto the Petri dishes that already contained the sediment or water dilution. Plates were then incubated at 27°C until clear zones appeared. Each sample was done in duplicate.

*Polycyclic aromatic hidrocarbon (PAH) degrading bacteria isolation*. One hundred µl of each sample were spread onto Petri dishes containing ONR7a medium (Dyksterhouse *et al*., 1995). The plates were then inverted and placed on other plates containing sublimated polycyclic aromatic hydrocarbon (PAH). Sublimation of PAH was conducted as follows: phenanthrene; dibenzothiophene and fluoranthene at 100°C for 5 minutes; fluorene at 100°C for 4 minutes; and pyrene at 130°C for 10 minutes. A Petri dish was inverted and put on a plate containing one type of PAH powder, and then sublimated to vaporize the PAH. The PAH gas was trapped on the surface of the media in an inverted Petri dish. All samples were incubated at 27°C until clear zone or colour appeared on the ONR7a medium. Each treatment was done in duplicate.

*Bacterial isolation from enrichment media*. One hundred µl samples from each dilution series were put into tubes containing 1 ml of 2x SWP media + ALCO 210 ($NH_4NO_3$ 286 mg, $KH_2PO_4$ 44 mg, Fe.Citrate 44 mg, yeast extract 2 mg, dissolved in 1 l of seawater with 200 µL ALCO 210 added). Samples were incubated on an orbital shaker for 2 weeks, at 100 rpm, 26-28°C. Then, these bacterial suspensions were tested for their ability to degrade crude oil and PAH as described above.

*Purification and preservation of bacterial isolates*. The bacterial colonies which showed a clear zone or changed the colour of the media were picked and then streaked onto marine agar (Difco, USA) and incubated for 2-3 days at 27°C. Colonies displaying a single morphology (shape, surface, colour) were preserved in 1.5 ml of sterile 20% preservation solution (20% v/v glycerol, 80% v/v marine broth (Difco, USA); sterilized in an autoclave: 121°C, 1.5 atm, for 15 minutes) and stored at -80°C. Meanwhile, colonies showing a mix of morphologies were re-purified by inoculating in marine agar until single-morphology colonies occurred. Gram staining and KOH non-staining tests were conducted according to a previous study (Buck, 1982).

*Isolates' fitness to degrade crude oil and PAH*. Pure bacterial isolates were grown onto media containing different sources of carbon from their initial medium in order to confirm their capabilities to degrade oil and PAH. The ability of each isolate to degrade pollutants was recorded.

## RESULTS AND DISCUSSIONS

### *Arabian Light Crude Oil (ALCO 210) and polycyclic aromatic hydrocarbon (PAH) degrading bacteria*

Direct bacterial plating of water and sediment onto ONR7a medium seemed ineffective as a means to cultivate bacteria. It was indicated by the fact that no bacteria grew on ONR7a medium containing crude oil or polycyclic aromatic hydrocarbon (phenantrene, fluoranthene, dibenzothiophene, fluorene and pyrene) from the samples at all. Therefore, enrichment culture was added using SWP medium for the second samples (April 2007) before isolating the bacteria. By applying the enrichment culture, crude oil and PAH-degrading bacteria could be successfully isolated. Oil-degrading microorganisms require not only carbon and mineral nutrients incorporated in their biomass, but also nitrogen and phosphorus. Thus, the absence of nitrogen (N) and phosphorus (P) often restricts their growth rate (Xia *et al*., 2011). Furthermore, bacteria in sea water might not be readily cultured for several reason such as: synthetic media do not provided essential components for bacterial growth, such essential components for bacterial growth might be provided by other bacteria living in their natural ecosystem, lack of communication among bacterial cells, lack of growth factors, disruption of oxidative stress,

**Table 1.** Distribution of isolates from sea water and sediment samples of three harbours

| Sites | Samples | Amount of isolates | | | | | | Total Isolates |
|---|---|---|---|---|---|---|---|---|
| | | CO | DBT | FLU | FRT | PHE | PYR | |
| Muara Baru | Sea water | NI | 1 | 5 | 6 | NI | 1 | 13 |
| | Sediment | 8 | NI | 5 | 8 | NI | NI | 21 |
| Sunda Kelapa | Sea water | 5 | 2 | 4 | 7 | NI | 1 | 19 |
| | Sediment | 15 | 4 | 7 | 9 | NI | 3 | 38 |
| Tanjung Priok | Sea water | 2 | NI | NI | NI | 3 | NI | 5 |
| | Sediment | NI | NI | NI | 6 | 6 | 1 | 13 |
| **Total Isolates** | | 30 | 7 | 21 | 36 | 9 | 6 | **109** |

**Notes**: CO= Crude oil, DBT= Dibenzothiophene, FLU= Fluorene, FRT= Fluoranthene, PHE= Phenanthrene, PYR= Pyrene, NI= No bacteria could not be isolated

unbalanced growth caused by excess or shortage of substrate and cell lysis during famine (Harayama *et al*. 2004).

ONR7a medium contains limited concentrations of N and P. This may be the reason why none of the crude oil and PAH degrading bacteria are potentially available in sediment and seawater samples grown on ONR7a medium although there is a sufficient concentration of carbon (ALCO 210 or PAH as a carbon source) and other trace elements provided by the ONR7a medium. We suggest that bacteria change their metabolism to fit the change of nutrient availability and environmental conditions. The specific bacteria for soil bioremediation can be applied by changing the substrate as an adjustment application method (Devinny and Chang, 2000). They will increase their metabolic activity in a stressed environment, thus limiting the growth of the microbial population (Devinny and Chang, 2000). In contrast, enrichment culture had sufficient N and P from yeast extract, ammonium nitrate ($NH_4NO_3$) and potassium dihydrogene phosphate ($KH_2PO_4$), which favoured bacterial growth in batch culture.

At the end, 109 single bacterial colonies were successfully isolated from sediment and sea water from the three oil-polluted harbours (Muara Baru, Sunda Kelapa and Tanjung Priok) (Table 1.). The ALCO 210 and PAH-degrading bacteria were distributed in different amounts from 3 sites. Fluoranthene and ALCO 210-degrading bacteria seemed to be the most abundant bacteria, with a total of 36 and 30 isolates, respectively (table 1.). The largest number of cultivable strains was isolated from Sunda Kelapa (57 strains), followed by Muara Baru (34 strains) and Tanjung Priok (18 strains). Crude oil degradation was indicated by the disappearance of the ALCO 210 layer on the surface of ONR7a medium, while fluoranthene degradation was indicated by the appearance of a reddish-brown colour in ONR7a containing fluoranthene. This color indicates the degradation of fluoranthene through the opening of an aromatic ring at position 9, 10 (Willumsen *et al*., 1998).

None of the ALCO 210-degrading bacteria have been isolated from Muara Baru waters and the sediment of Tanjung Priok. Environmental parameters such as availability of oxygen, major nutrients (N/P concentration), pH, oil hydrocarbon content, oil concentration and its dispersion in sea water and sediment limited the growth of oil degrading bacteria (Kostka *et al.,* 2011). Unfortunately, we did not record all mentioned parameters except for oil concentration. The oil concentration was measured in all samples and amounted 18.86, 118.22 and 56.09 $ppmg^{-1}$ in sediment from Muara Baru, Sunda Kelapa and Tanjung Priok, respectively, and 0.045, 0.063 and 0.114 $ppmg^{-1}$ in sea water were from the same localities (Darmayati, unpublished data). We suggest that an excess of pollutant concentration, especially oil, could inhibit microbial population growth and promote

**Table 2. Characterization of the 5 prominent isolates for oil bioremediation**

| No | Species | KOH test | Morphology of colony | | | | Identity |
|---|---|---|---|---|---|---|---|
| | | | Colour | Shape | Elevation | Edge | |
| 1 | *Alcanivorax* sp. B-1084 (Acc. No. DQ270741.1) | Negative | Cream | Globose | flat | Sleek | 100% |
| 2 | *Pseudomonas* sp. D5-38b (Acc. No. AM403194.1) | Negative | White | Globose | Convex | Sleek | 99% |
| 3 | *Alcanivorax* sp. TE.9 (Acc. No. AB055207.1) | Negative | Cream | Globose | Flat | Sleek | 100% |
| 4 | *Bacillus* sp. L41 (Acc. No. DQ249996.1) | Negative | White | Rod | Convex | Sleek | 99% |
| 5 | *Alcanivorax dieselolei* strain B-5 clone 1 (Acc. No. AY683537.1) | Negative | Cream | Globose | Flat | Sleek | 98% |

**Note: KOH** test was conducted according Buck (1982), Acc. No: Accession Number in GenBank National Center for Biotechnology Information (NCBI)

the dominance of a particular genus. Kostka *et al*. (2011) reported that the abundance of *Alcanivorax* spp. increased in response to oil contamination and a comparison of bacterial ribosomal RNA (rRNA) abundance indicated that *Alcanivorax* abundance was proportionately greater within the active bacterial community.

We have isolated several PAHs-degrading bacteria (Table 1.) although these PAHs were not detected in sea water and sediment. We suggest that these bacteria were capable of using carbon, from a wide range of aromatic hydrocarbon compounds, which emerges when oil is naturally degraded. Hence, these bacteria remain viable in oil-polluted sea water and sediment and could be cultivated in synthetic medium containing PAH. Furthermore, Dyksterhaouse *et al*. (1995) and Hedlund *et al*. (1999) reported that novel marine PAH degraders such as *Cycloclasticus pugetii* gen. nov., sp. nov. and *Neptunomonas naphthovorans* gen. nov., sp. nov., have been isolated from marine sediment at Puget Sound, Washington. This site was contaminated by polychlorinated biphenyl and creosote. The potentially ubiquitous distribution of marine PAH degraders suggests that the capacity for PAH degradation in polluted systems depends on the diversity and characteristics of naturally occurring populations and their responses to environmental conditions (Head *et al.,* 2006).

Of the 109 isolates, five bacteria were capable of utilizing crude oil, dibenzothiophene, fluoranthene, fluorene, phenanthrene, and pyrene as their sole source of carbon (Table 2). Species identification based on 16S rRNA gene sequences for those 5 isolates was conducted using PCR followed by sequencing of the DNA product (Hatmanti and Darmayati, 2009). A couple of bacterial universal primers 341Forward (5'-CCTACGGGAGGCAG-CAG-3') and 907Reverse (5'-CCGTCAATTC-MTTTGAGTTT-3'). Partial 16S rRNA genes sequencing indicated that those five isolates were classified into genus *Alcanivorax*, *Pseudomonas*, and *Bacillus* (Hatmanti and Darmayati, 2009).

Gram staining performed to all isolates indicated that Gram negative marine bacteria were more dominant than Gram positive bacteria. Amongst those five prominent isolates, four isolates (*Alcanivorax* sp. B-1084, *Pseudomonas* sp. D5-38b, *Alcanivorax* sp. TE.9, and *Alcanivorax dieselolei* strain B-5 clone 1) were gram negative marine bacteria, while *Bacillus* sp. L41 was a gram positive marine bacteria (Figure 2.). Gram-negative bacteria usually dominate the bacterial communities in hydrocarbon-contaminated environments due to lipid structures of their cell wall which facilitate nutrient uptake in hydrocarbon contaminated environment (Rolling *et al*., 2004).

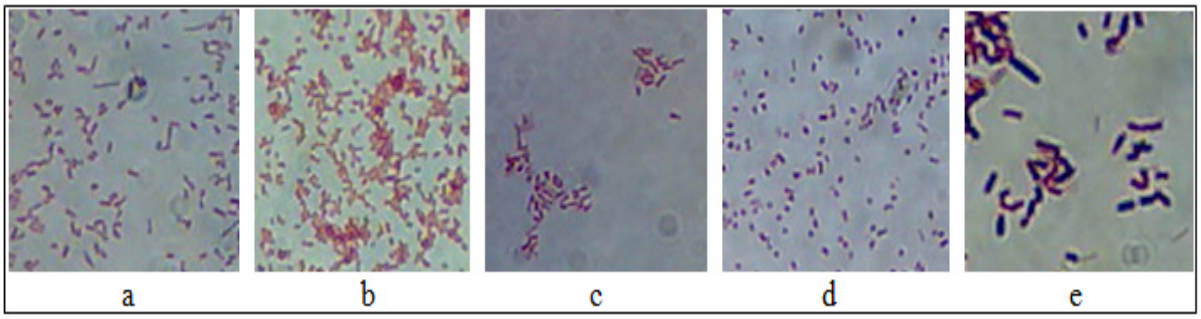


**Figure 2. Gram staining of five isolates *Alcanivorax* sp. B-1084 (a), *Alcanivorax* sp. TE.9 (b), *Alcanivorax dieselolei* strain B-5 clone (c), *Pseudomonas* sp. D5-38b (d), and *Bacillus* sp. L41 (e).**

## CONCLUSION

Oil and polycyclic aromatic hydrocarbon (PAH)-degrading bacteria were isolated from sea water and sediment from three oil polluted harbours. Cultivable bacteria had been obtained mostly from the Sunda Kelapa samples, with fewer in those from Muara Baru and Tanjung Priok, respectively. Among those bacteria, five strains have potential application in oil bioremediation because of their ability to utilize oil and 5 types of PAH as their sole carbon. They were *Alcanivorax* sp. B-1084, *Pseudomonas* sp. D5-38b, *Alcanivorax* sp. TE-9, *Bacillus* sp. L41, *Alcanivorax dieselolei* strain B-5 clone 1.

## ACKNOWLEDGMENT

This research was funded by DIPA grant, Research Center for Oceanography in 2007. We would thanks to Dr. Mari Rhydwen for advice about English in this manuscript.

## REFERENCES

Berardesco, G., S. Dyhrman, E. Gallagher, and M. P. Shiaris. 1998. Spatial and temporal variation of phenanthrene-degrading bacteria in intertidal sediments. *Appl. Environ. Microbiol*., 64: 2560–2565.

Buck, J. D., 1982. Nonstaining (KOH) Method for Determination of Gram Reactions of Marine Bacteria. *Applied and Environmental Microbiology*, p. 992-993.

Darmayati Y. S. Harayama, A. Yamazoe, A. Hatmanti, Sulistiani, R. Nuchsin, and D.H. Kunarso. 2008. Hydocrabonoclastic bacteria from Jakarta Bay and Saeribu Islands. *Mar. Res. Indonesia* Vol. 33 (1): 55-64.

Dyksterhouse, S.E., J. P. Gray, R.P. Herwig, J.C. Lara, and J.T. Staley. 1995. *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *International Journal of Systematic Bacteriology*, Vol (45) 1: 116-123.

Devinny, J., and S.H. Chang. 2000. *Bioaugmentation for soil bioremediation*. In: Wise, D.L., Trantolo, D.J. (Eds.), *Bioremediation of Contaminated Soils*. Marcel Dekker, New York, pp. 465–488.

Haines J.R., E.J. Kleiner, K. A. McClellan, K. M. Koran, E. L. Holder, D. W. King, and A. D. Venosa. 2005. Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems. *J Ind Microbiol Biotechnol*. 32: 171–185.

Hambrick III, G.A., R. D. Delaune, and W. H. Patrick, JR. 1984. Effect of Estuarine Sediment pH and Oxidation-Reduction Potential on Microbial Hydrocarbon Degradation. *Appl. & Environ. Microbiol*., 40 (2): 365-369.

Harayama. S., Y. Kasai, and A. Hara. 2004. Microbial communities in oil contaminated seawater. *Curr. Opin. Biotechnol*., 15: 205-214.

Hatmanti, A., and Y. Darmayati. 2009. Karakterisasi dan Analisis Kekerabatan Bakteri Potensial Pendegradasi Minyak dan Poly-Aromatics Hydrocarbon (PAH) dari Perairan Teluk Jakarta. *Proceeding Seminar Biologi*, Universitas Jenderal Soedirman, Purwokerto: 1-10.

Head, I. M., D. M. Jones, and W. F. M. Roling. 2006. Marine microorganisms make a meal of oil. *Nat. Rev. Microbiol*. 4:173–182.

Hedlund, B.P., A.D. Geiselbrecht, T.J. Bair, and J.T. Staley. 1999. Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptu-*

*nomonas naphthovorans* gen. nov., sp. nov. *Appl. & Environ. Microbiol*., Vol 65 (1): 251–259.

Kostka J.E., O. Prakash, W. A. Overholt, S. J. Green, G. Freyer, A. Canion, J. Delgardio, N. Norton, T. C. Hazen, and M. Huettel. 2011. Hydrocarbon-Degrading Bacteria and the Bacterial Community Response in Gulf of Mexico Beach Sands Impacted by the Deepwater Horizon Oil Spill. *Appl. Environ. Microbiol.*, Vol 77 (22): 7962–7974.

Lemigas (Indonesian Petroleum Institute). 1974. Coastal zone pollution in Indonesia with emphasis on oil. A reconnaissance survey workshop held in Jakarta on August 21-24, 1978. *The Smithsonian Institution and the Indonesian Petroleum Institute*. *Part I. Summary, Findings and Recommendation. 18 pp. Part II. Joint Study Report, 70 pp + Appendices. Part III. Anotated bibliography on the biology and ecological effects of oil pollution in tropical waters*, 82 pp.

Rölling, WFM., MG.Milner, D. M. Jones, F. Fratepietro, R.P.J. Swanell, F. Daniel, and I. M. Head. 2004. Bacterial Community Dinamics and Hydrocarbon Degradation during a Field Scale Evaluation of Bioremediation on a Mudflat Beach Contaminated with Buried Oil. *Appl. & Environ. Microbiol.* Vol 70 (5): 2603-2613.

Suess, M. J. 1976. The environmental load and cycle of polycyclic aromatic hydrocarbons. *Sci. Total Environ.*, 6: 239-250.

Thayib, S.S., Y. Darmayati, and Ruyitno. 1994. Dampak aktivitas manusia pada mikroflora di Teluk Jakarta. *Proceeding of Seminar Pemantauan Pencemaran Laut*: 115-128.

West, P. A., G. C. Okpokwasili, P. R. Brayton, D. J. Grimes, and R. R. Colwell. 1984. Numerical taxonomy of phenanthrene-degrading bacteria isolated from Chesapeake Bay. *Appl. Microbiol. Biotechnol*., 48:988–993.

Willumsen, P.A., U. Karlson, and P. H. Pritchard. 1998. Response of fluoranthene-degrading bacteria to surfactants. *Appl. Microbiol. Biotechnol*., 50: 475-483.

Xia W-J., H-P. Dong, L. Yu, and D-F. Yu.2011. Comparative study of biosurfactant produced by microorganisms isolated from formation water of petroleum reservoir. Colloids and Surfaces A: Physicochem. *Eng. Aspects* 392: 124– 130.