THE DEGRADATION OF DIESEL OIL BY CONSORTIUM OF BACTERIA IN SHAKEFLASK CULTURES

Ciawi, Y.*, Safitri, R.**, Suniti, N.W. ****, Sahanggamu, Y.P.***, Dharmawibawa, I.D.****, Wirawan, IG.P.****
* Engineering Faculty, UNUD, Jl.PB Sudirman, Denpasar, Bali
** Biology Department, Faculty of Math and Natural Sciences, UNPAD, Jatinangor, West Java
*** Chemical Engineering Department, UNPAR, Jl. Ciumbuleuit, Bandung, West Java
**** Department of Plant Pest and Disease, Agriculture Faculty, UNUD, Bali

Abstract

Diesel fuel is widely used in manufacturing and transportation industries. Its production, transportation, use and disposal have the risk to contaminate the environment. In the other hand contaminated site usually can produce some microorganisms, through natural selection, that can utilize the hydrocarbons as source of energy and food thus clean the environment itself. For bioremediation purposes, 14 isolates of diesel oil degrading bacteria from Bandung, Jakarta, and Bali has been employed for diesel oil degradation. These 14 isolates, together with other variables, e.g. pH, Pb, sucrose and urea, were plotted in 23x24 matrixes of Plackett and Burman statistical method. Fermentation was carried out in batch cultures for 14 days. The results showed that 9 isolates and the availability of sucrose and higher initial pH have a positive/stimulatory effect on diesel oil biodegradation, 5 of which were very significant (99.99%), whereas 1 isolate has inhibitory effect (-98%). The results also revealed that 8 isolates, higher initial pH, and availability of urea have stimulatory effect on culture growth (more than 90% significant). The present of Pb in medium inhibit both growth and the degradation of diesel oil, although the effect was not significant (less than 60%).

Keywords: biodegradation, diesel oil, bacteria isolates, shakeflask cultures

1. Introduction

The pollution by diesel oil occurred in many places (petrol stations, workshops, factories, oil refinery plant, harbour), not counted the huge pollution by oil spill from tanker (Bragg et al, 1994). In Indonesia, the pollution by diesel oil in Tanjung Periok Harbour was 900 L per ship with oil content of 12,95 mg/L, this figure will count to 0.1 to 363 ton in year 2000 (Irawati, 1994). Similar condition is probably occurred in every harbour in Indonesia (Feliatra, 1996, Udiharto, 1992), whereas in fact diesel oil contamination is regarded as toxic and hazardous pollutant (RI Governmental Regulation no.18, year 1999, code D1003d). To overcome oil pollution, bioremediation is the most environmental friendly way (Kadarwati et al, 1996) and many organisms can be employed for this purpose (Vieira et al, 2007, Al-Ghazawi et al, 2005, Edvotek, 2002). Many factors influence the efficiency of the

bioremediation (Barathi and Vasudevan, 2003, Barenholz et al., 2003, Boopathy, 2003, Bieszkiewicz et al., 2002, Oh et al., 2001). The aim of this research is to investigate the factors affecting the level of biodegradation of diesel oil by consortium of bacteria using statistical method. Consortium of bacteria have been proven effective in many petroleum hydrocarbon biodegradation work (Rahman et al., 2003, Al-Awadhi et al., 2003, Ciawi and Santi, 2000). We have isolated diesel oil degrading bacteria from oil contaminated sites in Bandung (Safitri et al, 2002 and Ciawi et al, 1999), Jakarta and Bali (Ciawi et al, 2003). The experiments employed the statistical method Plackett Burman, which has been used successfully in determining the factors affecting the level of metabolite production by microbes (Castro et al, 1992). This method is more economical and time saving compared with factorial method (Stose and Mayer, 1966).

2. Materials and Methods

All chemicals used in this research are pro analyst grade, except for urea, sucrose and diesel oil. The medium used for fermentations was a modification of Bussnell-Haas mineral salts medium which consist of 0.2 $gr.l^{-1}$ $MgSO_4$, 0.02 $gr.l^{-1}$ $CaCl_2$, 1.0 $gr.l^{-1}$ KH_2PO_4, 1.0 $gr.l^{-1}$ K_2HPO_4, 1.0 $gr.l^{-1}$ NH_4NO_3, 2 drops of 60% $FeCl_3$ solution, 1000 ppm sucrose, 1000 urea, 1.0 $gr.l^{-1}$ $PbNO_3$. The microorganisms employed were *Cytophaga fermentans*, *Methylomonas pelagika*, *Micrococcus tractuosa*, *Mesophilobacter marinus*, *Marinomonas comunis*, Brevibacterium, Staphylococcus, Flavobacterium, Pseudomonas, Mocillercilla, *Pseudomonas putida*, Microscilla, Alcaligenes, and Bacillus.

The experiment was designed according to Plackett and Burman method (Castro et al, 1992, Monaghan and Koupal, 1989), by using a matrix of 24 x 23 which is shown in the Table 1.

Table 1. Plackett Burman statistical design by using 24 experiments and 23 variables including 5 dummies.

Experiment	Variable																						
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	W	X	Y
1	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-
2	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+
3	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+
4	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+
5	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+
6	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+

7	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-
8	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+
9	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-
10	+	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+
11	-	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+
12	-	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-
13	+	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-
14	+	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+
15	-	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+
16	-	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-
17	+	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-
18	-	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+
19	+	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-
20	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+
21	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-
22	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-
23	-	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-
24	+	+	+	+	+	-	+	-	+	+	-	-	+	+	-	-	+	-	+	-	-	-	-

Note : *Cytophaga fermentans* (variable A), *Methylomonas pelagika* (B), *Micrococcus tractuosa* (C), dummy (D), *Mesophilobacter marinus* (E), *Marinomonas comunis* (F), Brevibacterium (G), dummy (H), Staphylococcus (I), Flavobacterium (J), Pseudomonas (K), dummy (L), Mocillercilla (M), *Pseudomonas putida* (N), Microscilla (O), dummy (P), Alcaligenes (Q), Bacillus (R), initial pH (S), Pb (U), urea (V), dan sucrose (W).

The parameter used here was the amount of oil degraded. The effect of each variable were calculated from:

Ea = R at (+)/n – R at (-)/n

Where: R = the response (amount of oil degraded)

N = number of experiments (24)

The experimental error was determined from:

$V_E = \Sigma(E_d)^2/d$

Where d = number of dummy variables and E_d= the dummy effect.

The standard error of the effects was determined as:

$S.E._{eff} = \sqrt{V_E}$

Finally, the significant level of each variable were determined using Student's t-test:

t-value = $E/S.E_{eff}$

Ammonia determination

The method used was similar to the method described by Fawcett and Scott (1960). Two reagents were prepared in dark bottles. Solution A contained 55.6 $g.l^{-1}$ phenol and 0.3 $g.l^{-1}$ sodium nitroprusside. Solution B contained 0.11 M sodium hypochlorite and 20 $g.l^{-1}$ sodium hydroxide. This method will give linear response at concentration between 0 and 2 mM. Equal amount of samples and each solution were mixed thoroughly and incubated in the dark at 37^0C for 15 minutes. The optical density was read at 570nm.

Sucrose determination

The method used was similar to the method described in Robyt and White (1990). 0.1% anthrone was dissolved in 100 ml concentrated sulfuric acid. This method will give linear response at concentration between 0 and 0,2 mg/ml glucose. 1 ml of samples or standard solutions was mixed thoroughly with 5 ml of freshly made anthron solution, heated at 100^0C for 12 minutes and cooled under running water. The optical density was read at 630 nm.

Growth determination

The growth level was measured as optical density at 600 nm. Samples were diluted to result an OD readings below 1.00.

Oil determination

Amount of residual oil was measured simply using measuring cylinders. Level of biodegradation was counted by substracting residual oil from initial amount of oil.

3. Results

a. The effect of experimental variable on the degradation of diesel oil

The results of 24 experiments using Plackett dan Burman statistical design are shown in the following tables. Table 2 shown the effect of each variables on the level of oil degradation. There are 12 variables that significantly affect (>90%) the degradation of diesel oil, 5 of which are more than 98% significant, i.e. Cytophaga sp., Micrococcus sp., Mesophilobacter sp., Staphylococcus sp., and Alkaligenes sp.. The 12 variables are *Cytophaga fermentans* (variable A), *Micrococcus tractuosa* (C), *Mesophilobacter marinus* (E), *Marinomonas comunis* (F), Staphylococcus sp. (I), Flavobacterium sp. (J), Mocillercilla sp. (M), *Pseudomonas putida* (N), Alcaligenes sp. (Q), initial medium pH (S), and sucrose (W), all of which are positively affect the degradation of diesel oil, whereas Brevibacterium sp. (variable G) negatively affect the degradation of diesel oil. Data in Table 2 also show that the effect of control variables (dummies) are also very small, which mean that overall experimental error is quite small (%s <50%, except for dummy D, 80%). Another interesting point is that Bacillus sp. negatively affect the degradation of diesel oil, although it is not very significant, which is 60%. Moreover, Brevibacterium sp. which was isolated from soil samples in Bandung negatively affect the degradation (98%). Also, in this research, there are 2 Pseudomonas isolates which have different effect on diesel oil degradation. It might be that the two isolates are different species. The effect of *Pseudomonas putida* is quite

significant (90%) and the effect of the other Pseudomonas is only 60% significant. Many Pseudomonas species has been found has the ability to degrade various toxic substances such as diesel oil (Das and Mukherjee, 2007, Pepi et al., 2003), phenol (Wang and Lie, 2007), chlorotoluene (Lehning et al., 1997).

Tabel 2 The effect of each experimental variable on the degradation of diesel oil using statistical analysis on experimental results

Variable	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
% S	**99.9**	80	**99**	80	**99.9**	95	-98	60	**99**	95	60	-60	95	90	60	<50	**99.9**	-<50	95	<50	-<50	<50	98

Notes: *Cytophaga fermentans* (variable A), *Methylomonas pelagika* (B), *Micrococcus tractuosa* (C), dummy (D), *Mesophilobacter marinus* (E), *Marinomonas comunis* (F), Brevibacterium (G), dummy (H), Staphylococcus (I), Flavobacterium (J), Pseudomonas (K), dummy (L), Mocillercilla (M), *Pseudomonas putida* (N), Microscilla (O), dummy (P), Alcaligenes (Q), Bacillus (R), initil pH (S), Pb (U), urea (V), and sugar (W).

The effect of experimental variable on the growth of the cultures

The results of statistical analysis on the experimental data showed that the effect of each variables on the growth of the concortia of microbes was different from its effect on the level of oil degradation (Table 3 and Figure 1).

Table 3. The effect of each experimental variable on the growth of the culture of bacteria consortia using statistical analysis on experimental data

Variabel	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W
% S	95	**99,9**	90	-<50	**99**	60	-90	-80	95	<50	**-99**	-70	**99**	60	<50	60	90	70	98	-<50	-60	**99,9**	-98

Notes: *Cytophaga fermentans* (variable A), *Methylomonas pelagika* (B), *Micrococcus tractuosa* (C), dummy (D), *Mesophilobacter marinus* (E), *Marinomonas comunis* (F), Brevibacterium (G), dummy (H), Staphylococcus (I), Flavobacterium (J), Pseudomonas (K), dummy (L), Mocillercilla (M), *Pseudomonas putida* (N), Microscilla (O), dummy (P), Alcaligenes (Q), Bacillus (R), initil pH (S), Pb (U), urea (V), and sugar (W).

The most significant variable (99%) on the growth of the consortia was *Methylomonas pelagika* (B), *Mesophilobacter marinus* (E), Mocillercilla (M), Pseudomonas (K), and urea (V). In this experiment, Pseudomonas sp. showed a negative effect on growth. Urea has positive effect on the growth of the consortia probably because the availability of urea provide more nitrogen in the media. In this experiment it was 1000 mM, which is the maximum consentration for the consortia to grow, which was proven in subsequent experiment (Figure 2 and Figure 3), in the range between 0-5000 ppm, the best concentration of urea that produced the highest growth and the highest degradation level is 1000 ppm.

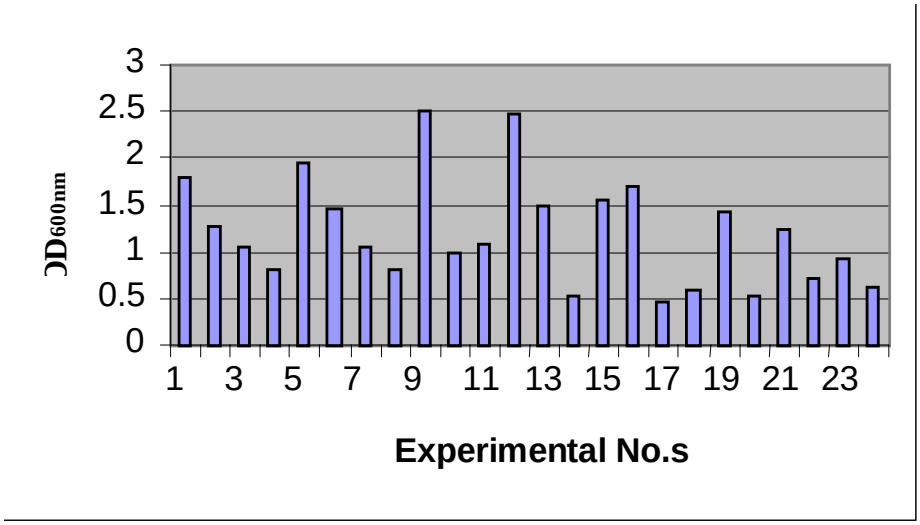

Figure 1 The level of growth (measured as OD 600nm) of the bacteria consortia in 24 experiments

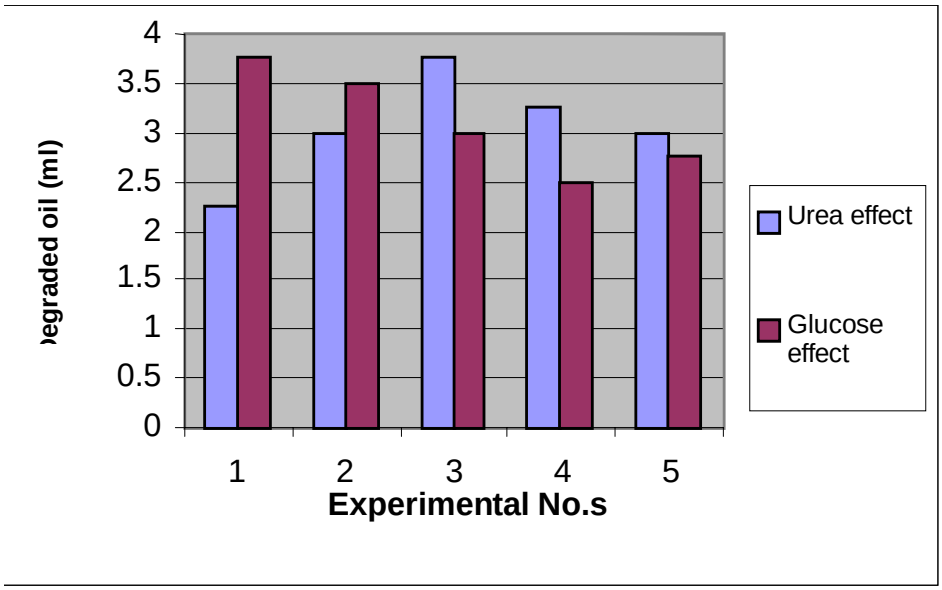

Figure 2 The effect of urea and sucrose concentration on the degradation of diesel oil
Note: urea, experiment 1-5, [glucose]=1000 ppm, experiment 1, urea=0 ppm, exp. 2, urea=500 ppm, exp.3, urea=1000 ppm, exp.4, urea=3000 ppm, exp.5, urea=5000 ppm. Glucose effect, [urea]= 3000 ppm, exp.1, glucose=0 ppm, exp.2 glucose=100 ppm, exp.3, glucose=500 ppm, exp.4, glucose= 1000 ppm, exp.5, glucose=5000 ppm.

The negative significant effect of sucrose on growth (-98%) is contradictive with the results of effect of glucose on growth in many experiment with other bacteria (e.g. Ciawi, 1996) and also with our previous research (Ciawi et al., 2000, Ciawi dan Santi, 2000), which showed that glucose and NH_4Cl has significant positive effect on growth as well as oil degradation. We expect a different result with the assumption that, as carbon source, sucrose is more readily to be consumed than diesel oil. In contrast, the result of the effect of sucrose on oil degradation agreed with our previous experiment. At subsequent experiment, it showed that at the range between 0-5000 ppm sucrose, the best concentration for diesel oil degradation is 0 ppm (Figure 2) and 1000 ppm for growth (Figure 3). This research was continued by investigating the effect of the fermentation systems on the degradation level of diesel oil.

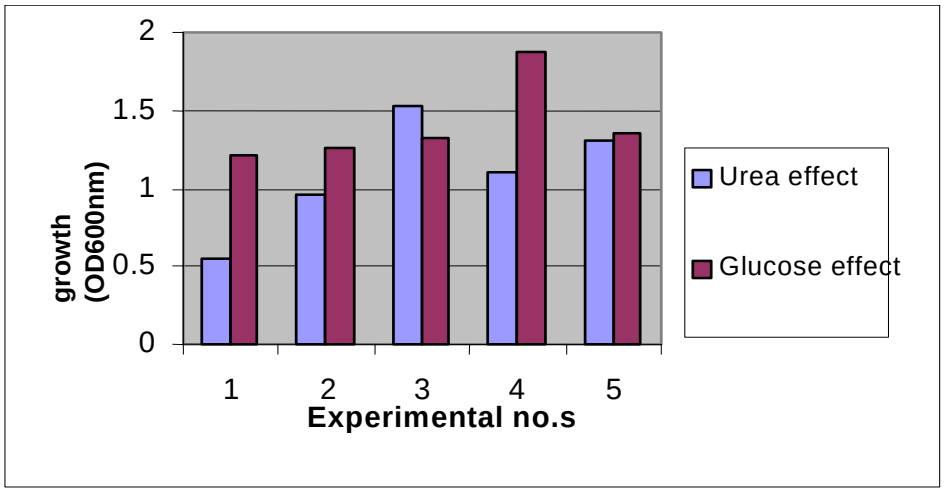

Figure 3. The effect of urea and glucose concentration on the growth of the consortia.
Note: urea, experiment 1-5, [glucose]=1000 ppm, experiment 1, urea=0 ppm, exp. 2, urea=500 ppm, exp.3, urea=1000 ppm, exp.4, urea=3000 ppm, exp.5, urea=5000 ppm. Glucose effect, [urea]= 3000 ppm, exp.1, glucose=0 ppm, exp.2 glucose=100 ppm, exp.3, glucose=500 ppm, exp.4, glucose= 1000 ppm, exp.5, glucose=5000 ppm.

Acknowledgement

The authors wish to thank Prof. Dr. Ir. I Gede Putu Wirawan, M.Sc. and The Faculty of Agriculture, Udayana University, Bali, for providing lab. facilities and the Competitive Research Grant (Hibah Bersaing XI), Directorate of Higher Education, Ministry of National Education, Indonesia for funding this research.

Bibliography

Al-Awadhi H, Al-Hasan RH, Radwan SS. 2002. *Comparison of the Potential of Coastal Materials Loaded with Bacteria for Bioremediating Oily Seawater in Batch Culture*. Microbiology Research 157, 4, p.331-6.

Al-Ghazawi, Z., Saadoun, I., Al-Shak'ah, A. 2005. *Selection of Bacteria and Plant Seeds for Potential Use in the Remediation of Diesel Contaminated Soils*. J Basic Microbiol. 45, 4, p.251-6.

Barathi S and Vasudevan N. 2003. *Bioremediation of Crude ooil Contaminated Soil by Bioaugmentation of Pseudomonas Fluorescens NS1*. J Environ Sci Health Part A Tox Hazard Subst Environ Eng.38, 9, p.1857-66.

Barenholz, A., Fishel, F., Yakir, E., Gatt, S., Barenholz, Y., Bercovier, H. 2003. *Liposomes Enhance Bioremediation of Oil-Contaminated Soil.* J Liposome Res. May 13(2), p.173-86.

Bieszkiewicz, E., Boszczyk-Maleszak, H., Wlodarczyk, A., Horoch, M. 2002. *Studies on the Effect of Inoculation of Activated Sludge With Bacteria Actively Degrading Hydrocarbons on the Biodegradation of Petroleum Products*. Acta Microbiol Pol.51(3), p.285-92.

Boopathy, R. 2003. *Anaerobic Degradation of No. 2 Diesel Fuel in the Wetland Sediments of Barataria-Terrebonne Estuary Under Various Electron Acceptor Conditions*. Bioresour Technol. 86, 2. p.171-5.

Bragg, J.R., Prince, R.C., Harner, E.J. dan Atlas, R.M. 1994. *Effectiveness of Bioremediation for the Exxon Valdez Oil Spill.* Nature 368, p.413-418.

Castro, P.M.L., Hayter, P.M., Ison, A.P. dan Bull, A.T. 1992. *Application of A Statistical Design to the Optimization of Culture Medium for recombinant Interferon-Gamma production by Chinese Hamster Ovary cells.* Applied Microbiology and Biotechnology 38, p.84-90.

Ciawi, Y. 1996. *A Study on Bacitracin Production by Bacillus Licheniformis in Several Fermentation Systems.* Ph.D.thesis. University of Kent at Cnterbury, UK.

Ciawi, Y. and Santi, H.C. 2000. "The Effect of Carbon and Nitrogen Ratio and the Presence of Minor Element on the Degradation of Crude Oil by Pseudomonas sp., Arthrobacter sp. and Mycobacterium sp". *International Symposium on Marine Biotechnology*, Jakarta.

Ciawi, Y., Safitri, R., Hajo, S.S., Siahaan, M. 1999. "Isolation and Identification of Petroleum Hydrocarbon Degrading Microorganisms from Contaminated Soils in Bandung". *Poster Session, The 5th Asia-Pacific Biochemical Engineering Conference 1999 and The 11th Annual Meeting of the Thai Society for Biotechnology,* Phuket, Thailand.

Ciawi, Y., Safitri, R., Sahanggamu, P., Dharmawibawa, I.D. dan Wirawan, I.G.P. 2003. *Isolation and Identification of Diesel Fuel Degrading Bacteria in Soil and Water Sampels From*

Jakarta, Bandung, and Bali. Poster presentation. PIT PERMI, Bandung, 29-30 Agustus 2003.

Das, K. and Mukherjee, A.K. 2007. *Crude Petroleum-oil Biodegradation Efficiency of Bacillus Subtilis and Pseudomonas Aeruginosa Strains Isolated from a Petroleum-oil Contaminated Soil from North-East India.* Bioresour Technol. 98, 7, p.1339-45.

Edvotek.2002. *Bioremediation Using Oil Eating Bacteria*. The Biotechnology Education Co. Edvotek Inc.

Fawcett, J.K. and Scott, J.E. 1960. "A Rapid and Precise Method for the Determination of Urea". *Journal of Clinical Pathology* **13**, 156-159.

Feliatra. 1996. Biodegradasi Petroleum oleh Bakteri di Perairan Dumai, Selat Malaka. Kumpulan Makalah Seminar Maritim Indonesia 1996, Konvensi Nasional Pembangunan Benua Maritim Indonesia dalam Rangka Mengaktualisasikan Wawasan Nusantara. BPPT dan Dewan HanKamNas, Makassar.

Irawati, R. dan Udiharto, M. 1994. *Penelitian Mikrobiologi Pengurai Minyak dari Limbah Kapal*. Laporan Penelitian Kerjasama BPPTeknologi dengan PPPTMGB "LEMIGAS".

Kadarwati, S., Hadi, N. dan Udiharto, M. 1996. *Penanganan Limbah Kilang Minyak dengan Bioproses.* Lembaran Publikasi Lemigas 2, hal.28-38.

Lehning, A., Fock, U., Wittich, R., Timmis, K.N. and Pieper, D.H. 1997. *Metabolism of Chlorotoluenes by Burkholderia sp.* Strain PS12 and Toluene Dioxygenase of *Pseudomonas putida* F1: Evidence for Monooxygenation by Toluene and Chlorobenzene Dioxygenases. Appl Environ Microbiol. 63(5), p.1974-1979.

Monaghan R.L. dan Koupal, L.R. 1989. *Use of Plackett and Burman Technique in a Discovery Program for New Natural Products*. Dalam Novel Microbial Products for Medicine and Agriculture, p. 25-31.

Oh, Y.S., Sim, D.S. and Kim, S.J. 2001. *Effects of Nutrients on Crude Oil Biodegradation in the Upper Intertidal Zone.* Marine Pollution Bulletin 42, 12, p.1367-1372.

Pepi M, Minacci A, Di Cello F, Baldi F and Fani R. 2003. *Long-term Analysis of Diesel Fuel Consumption in a Co-culture of Acinetobacter Venetianus, Pseudomonas Putida and Alcaligenes Faecalis*. Antonie Van Leeuwenhoek 83, 1. p.3-9.

Peraturan Pemerintah Republik Indonesia No.18 tahun 1999 tentang *Pengelolaan Bahan Berbahaya dan Beracun*.

Rahman K.S., Rahman T.J., Kourkoutas Y, Petsas I, Marchant R, Banat I.M. 2003. Enhanced Bioremediation of n-alkane in Petroleum Sludge Using Bacterial Consortium Amended with Rhamnolipid and Micronutrients. Bioresource Technology 90, 2. p.159-68.

Robyt, J.F. and White, B.J. 1990. *Biochemical Techniques Theory and Practice*. Waveland Press, Inc. Prospect Heights, p. 219.

Safitri, R., Dwiyani, M., Ciawi, Y., Indrawati, I. Dan Budiyanto, G. 2002. *Isolasi dan Identifikasi Isolat-isolat Bakteri yang Dapat Tumbuh pada Lumpur Minyak Bumi Asal Limbah Cair Terkontaminasi Minyak Pelumas*. Seminar MIPA, Medan.

Stose, R.A. dan Mayer, R.P. 1966. "Efficient Screening of Process Variables". *Journal of Industrial and Engineering Chemistry* 58, hal.38-40.

Udiharto, M. 1992. *Aktivitas Mikroba dalam Degradasi Minyak Bumi*. Proceedings Diskusi Ilmiah VII Hasil penelitian Lemigas, PPPTMGB Lemigas, Jakarta, hal.464-475.

Vieira, P.A., Vieira, R.B., de França, F.P., and Cardoso, V.L. 2007. *Biodegradation of Effluent Contaminated with Diesel Fuel and Gasoline.* J Hazard Mater. 140, 1-2, p.52-9.

Wang, C. and Li, Y. 2007. *Incorporation of Granular Activated Carbon in an Immobilized Membrane Bioreactor for the Biodegradation of Phenol by Pseudomonas Putida.* Biotechnol Lett. 9: 1353-6.