MAKARA, SAINS, VOL. 11, NO. 2, NOVEMBER 2007: 98-103

HYDROCARBON DEGRADING BACTERIA: ISOLATION AND IDENTIFICATION

Lies Indah Sutiknowati

Research Center for Oceanography, Indonesian Institute of Sciences, Jakarta 14430, Indonesia

E-mail: lies_indah@yahoo.com.sg

Abstract

There is little information how to identify hydrocarbon degrading bacteria for bioremediation of marine oil spills. We have used gravel which contaminated oil mousse from Beach Simulator Tank, in Marine Biotechnology Institute, Kamaishi, Japan, and grown on enrichment culture. Biostimulation with nutrients (N and P) was done to analyze biodegradation of hydrocarbon compounds: Naphthalene, Phenanthrene, Trichlorodibenzofuran and Benzo[a]pyrene. Community of bacteria from enrichment culture was determined by DGGE. Isolating and screening the bacteria on inorganic medium contain hydrocarbon compounds and determination of bacteria by DAPI (number of cells) and CFU. DNA was extracted from colonies of bacteria and sequence determination of the 16S rDNA was amplified by primers U515f and U1492r. Twenty nine strains had been sequence and have similarity about 90-99% to their closest taxa by homology Blast search and few of them have suspected as new species.

Keywords: Isolation, Identification, Biostimulation, Extraction, Sequence, DGGE

1. Introduction

The study of identification of bacteria is important in microbial ecology, especially with molecular techniques [1]. In particular, analysis of the microbial communities that take part in in-situ hydrocarbon biodegradation activities has been a challenge to microbiologist. Interest in this area has been catalyzed by the rapid advancement of molecular ecological methodologies [2]. Hydrocarbon utilizing microorganisms are ubiquitously distributed in the marine environment following oil spills. These microorganisms are naturally degrade numerous contaminating petroleum hydrocarbons and cleansing the oceans of oil pollutants.

Bioremediation, which is accomplished by adding exogenous microbial populations or stimulating indigenous ones, attempts to raise the rates of degradation found naturally to significantly higher rates [3]. Many oil-degrading bacteria have been isolated and their degradation potential investigated. Most of bioremediation studies have been carried out using pure-cultures and the roles of these bacteria in a natural environment remain substantially unknown [4].

Smaller molecular weight is readily biodegraded in marine environments. Aromatics with one, two or three aromatic rings are also efficiently biodegraded; however, those with four or more aromatic ring are quite resistant to biodegradation. The application of fertilizer increased rates of biodegradation 3-5 times. Multiple regression models showed that the effectiveness of bioremediation depended upon the amount of nitrogen delivered, the concentration of oil, and time [5].

The purpose of this research was to identify hydrocarbon degrading bacteria which grown on Phenanthrene, Naphthalene, Trichlorodibenzofuran and Benzo[a]pyrene. Sample was taken from Beach Simulator Tank in Marine Biotechnology, Kamaishi, Iwate-ken, Japan on November 2005.

2. Experiment Procedures

Beach simulation tanks. A pair of tanks (1.5 m long by 1 m wide by 1 m deep) was set up to simulate the biodegradation of oil in the intertidal zone (Figure 1). Each tank was partially filled with $1m^3$ of gravel grains (2-8 mm in diameter). Oil polluted cobble-stones sampled from a beach contaminated were then put on top of the gravel layer. Sea water was added continuously to each tank at a flow rate of 60 l/h, the level of the sea water being adjusted by a level controlling device to create the tidal cycle of 12 h with a range from 20cm to 80cm above the bottom of the tank or 30cm above and 30cm below the level of the oil-polluted cobblestones. Excess of sea water was discharged from the level controlling device. Four days after starting the sea water flow, 300 g of Super IB nitrogen fertilizer and 60 g of Linstar 30 fertilizer were spread uniformly in one of tank but not in the other (control) tank [6,7].

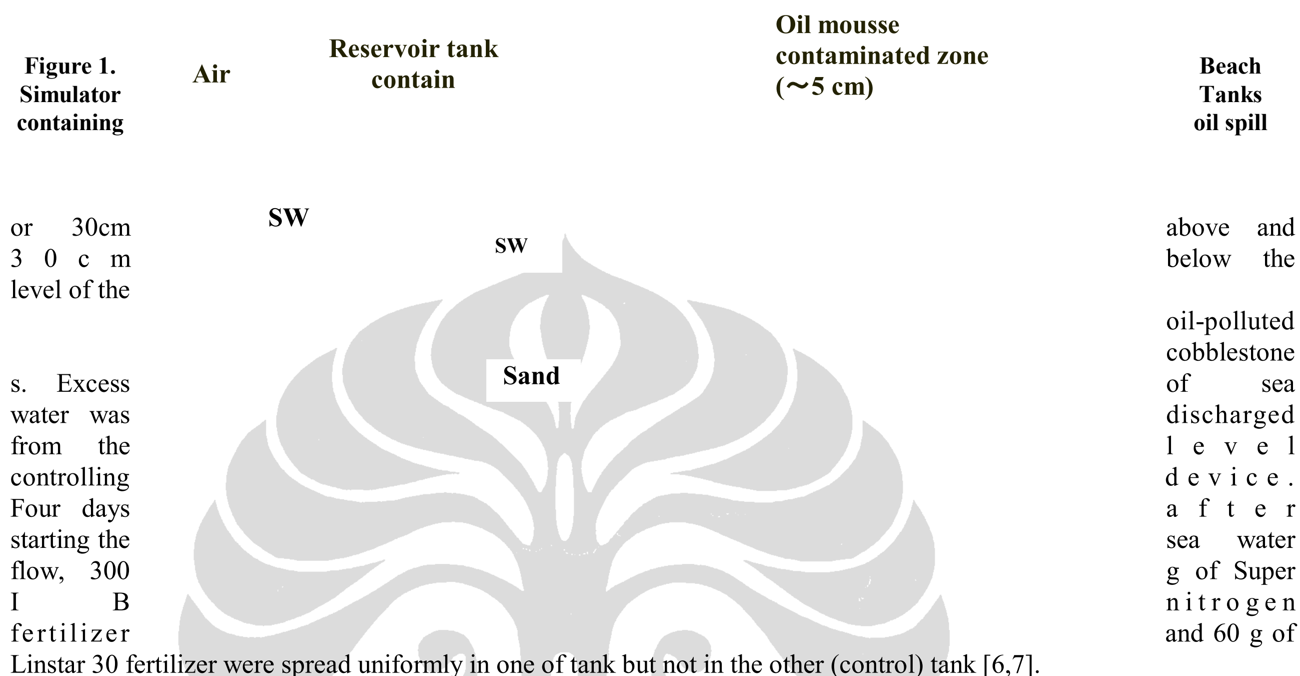

Figure 1. Simulator Beach Tanks containing oil spill

Medium and Culture Conditions. The gravel samples from beach simulator tank were inoculated to enrichment cultures and subcultures. The enrichment medium as inorganic medium was contained (1^{-1} filtered seawater): NH_4NO_3 1g; K_2HPO_4 0.2g; $FeC_6H_5O_7.nH_2O$ 0.02g. The pH was adjusted to 7.6-7.8. For enrichment and for better growth of pure cultures, the growth medium was supplemented with hydrocarbon compounds: Naphthalene, Phenanthrene, Trichlorodibenzofuran, Benzo[a]pyrene and crude oil. Pure cultures were obtained after isolation from agar plate dilution series and incubated at 20 ^{0}C. Agar plate was contained (1^{-1} distilled water) as ONR7a medium: Na_2SO_4 3.98g; NaCl 22.79g; NH_4Cl 0.27g; KCl 0.72g; Tapso 1.3g; Gelrite; NaBr; NaF; $NaHCO_3$; H_3BO_3; Na_2HPO_4; $MgCl_2.6H_2O$ 55.9g; $CaCl_2.2H_2O$ 7.3g; $SrCl_2$ 0.5g; $FeCl_2.4H_2O$.

Isolation. Gravel was collected from beach simulator tank and inoculated to enrichment cultures containing hydrocarbon compounds. This culture was incubated at 20^0 C for 1 month with constant shaking to promote the growth of hydrocarbon degrading microorganisms indigenous to the oil paste and/or seawater. Colonies number reached on medium which supplemented with the hydrocarbon source (Table 1). An increase in cell number was observed when the sea water was supplemented with the fertilizers (Table 2). Although the above mentioned techniques are quite useful for studying functional and physiological traits of microbial populations in the environment, pure culture experiments are indispensable for detailed analyses of function of each population particularly for manifesting concealed physiological traits likely to be important for the establishment of the consortium. To date, many pollutant-degrading bacteria have been isolated from natural mixed populations after batch-culture enrichment in media containing relatively high concentrations of the pollutant. However, this batch-culture enrichment is not considered suitable for ecological studies, because such methods isolate a very limited number of bacteria that always grow most rapidly in laboratory media. Pure cultures were stored in Marine Broth agar and ONR7a agar (contain a single hydrocarbon compounds); pure cultures in liquid medium were stored in 60 ml screw cap bottles as cultivation for next tests contained single hydrocarbon compounds.

Determination of bacterial cell number. Purity of cultures was checked by both microscopic observation and growth tests in agar plate medium (ONR7a) supplemented with single hydrocarbon compounds.

Table 1. Number of colonies which isolated on medium contain hydrocarbon source

Hydrocarbon source (PAH)	ONR7a with PAH CFU/ml			Marine Broth CFU/ml
	Enrichment	**Subculture 1**	**Subculture 2**	**Enrichment**
100 ppm Naphthalene	3.3 x 10^5	2.6 x 10^6	8.1 x 10^6	7.8 x 10^5
1 ppm Naphthalene	2.6 x 10^5	1.5 x 10^4	3.1 x 10^5	7.4 x 10^5
100 ppm Phenanthrene	1.3 x 10^5	2.7 x 10^6	6.9 x 10^6	1.1 x 10^6
1 ppm Phenanthrene	6.3 x 10^4	2.3 x 10^5	1.8 x 10^5	9.3 x 10^5
100 ppm 3CDF	2.5 x 10^5	1.7 x 10^6	7.9 x 10^6	1.4 x 10^6
1 ppm 3CDF	1.6 x 10^6	1.8 x 10^5	1.9 x 10^7	6.4 x 10^5
100 ppm Benzo(a)pyrene	2.1 x 10^5	1.3 x 10^6	7.1 x 10^7	7.8 x 10^5
1 ppm Benzo(a)pyrene	1.8 x 10^5	4 x 10^4	1.1 x 10^7	2.8 x 10^5
Control (enrichment without PAH)				5.4 x 10^5

Table 1 shows the total bacterial count determined by fluorescence microscopy after staining with 4', 6-diamidino-2-phenylindole (DAPI). The CFU's were determined after incubation at 20^0 C for 7 days on agar plates containing ONR7a and 1.5% gelrite agar (see Table 2).

Extraction of DNA. Bacterial cells in the liquid culture and agar plate medium were collected by centrifugation. DNA was extracted by putting 1 g of sample into 1 ml of cell suspension buffer, to which 1 ml of a lysing solution. This suspension was thoroughly mixed and then incubated at 96^0 C for 10 min. and the solution was cooled at -4^0 C for 10 min. Nucleic acids was precipitated by centrifuging at 15000 rpm for 10 min., washed with 1 ml of a 70% (v/v) ethanol solution and dissolved in 0.2 ml of a TE buffer containing 100 mg of RNase [8]. This solution was incubated at 37^0 C for 1 h and finally separated in a QIA-quick spin column (QiAgen). The purity and quantity of DNA were examined by recording its UV absorption spectrum. DNA extracts and the partial region amplification of 16S rDNA was amplified by PCR using 30 cycles. The primers used for DNA amplification were U515f and U1492r. The PCR products were purified by spin column chromatography (Qiagen kit) and were sequenced directly. The sequence was aligned and the alignment was completed manually.

Sequence Determination of 16S rDNA. Partial nucleotide sequences of 16S rDNA corresponding to positions 37-1370 of the *E. coli* rRNA sequence were amplified according to the method of Edwards *et* al. [9]. The sequence of the 16S rDNA was determined with a Dye terminator sequencing kit (Applied Biosystems), and the product was analyzed with an ABI Prism DNA sequencer (Applied Biosystems). The nucleotide sequence data reported in this paper are listed in Table 3.

Denaturing Gradient Gel Electrophoresis (DGGE) of the PCR products. PCR primers P2 and P3 [10] were used to amplify the variable V3 region of bacterial 16S rDNA (corresponding to positions 341-534 in the *Escherichia coli* rRNA sequence) connected to the GC clamp. PCR was performed as described previously [11]. Amplification of the PCR products of expected size was confirmed by electrophoresis, 1.5% (w/v) agarose gel in a TBE buffer.

DGGE was performed with DCode instrument (BioRad) according to the manufacturer's instructions. Gels were made using a gradient of denaturants between 38% [containing 2.66M urea and 15.2% (w/v) formamide] and 58% [containing 4.06M urea and 23.2 (w/v) formamide]. 10ml of the PCR-amplified mixture was subjected to electrophoresis in 10% (w/v) polyacrylamide gel at 200 V for 3.5 h at a running temperature of 60^0 C. The gel was then stained with Cyber-Gold for 30 min according to the manufacturer's instructions (see Figure 4).

3. Result and Discussion

Isolation. The bacteria were isolated on enrichment medium ONR7a and subcultures (liquid and agar) contain hydrocarbon source and isolated also on Marine broth gelrite. Isolation results were summarized in Table 1, showed that the number of colonies was increased while incubate on high concentration of hydrocarbon. Number of colonies on 100

ppm for four hydrocarbons is higher than 1 ppm and increase on subculture. Number of colonies on enrichment medium ONR7a is lower than enrichment medium marine gelrite. Some colonies which grown on media ONR7a agar contain high concentration of hydrocarbon (Figure 1), appeared smaller than colonies which grown on marine gelrite (Figure 2). This result showed that the bacteria can grow and degrade the hydrocarbon source.

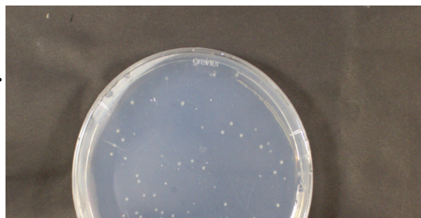

Figure 2. The bacteria growth on medium ONR7a

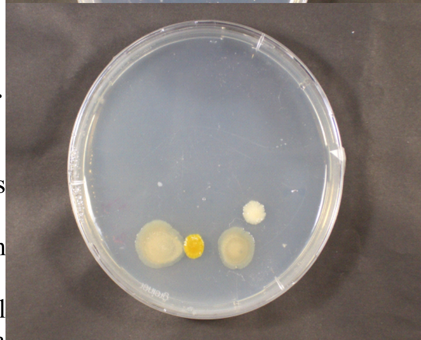

Figure 3. The bacteria growth on Marine Broth

This condition also showed in Table 2. Cell number of bacteria on seawater supplemented with hydrocarbon source was high in subculture, compare with enrichment.

And cell number of bacteria in concentration 100 ppm is higher than 1 ppm. The bacteria growth on enrichment culture for 1 month and cell number of bacteria increased on subculture containing hydrocarbon compounds rapidly.

Sample enrichment without hydrocarbon source was used as control which compared to sample enrichment contain hydrocarbon degrading bacteria, showed that number of colonies are higher than number of colonies which grown on hydrocarbon source. However the numbers of bacteria on subculture were higher than sample enrichment containing hydrocarbon or control. The bacteria became selective and growth on hydrocarbon source.

16S rDNA gene sequence. Based on DNA extracts of isolate, 16S rDNA which amplified by PCR using 30 cycles and primers U515f and U1492r was got sequence result and listed in Table 3. Many species of bacteria from 29 strains was found and have similarity about 90-99% by homology Blast search. The bacteria can grow and degrade hydrocarbon compound likes naphthalene and phenanthrene (easy to degrade) or trichlorodibenzofuran and Benzo[a]pyrene (difficult to degrade).

Table 2. Cell number of bacteria reached in seawater supplemented with Hydrocarbon source

Hydrocarbon source	**Enrichment Cell/ml**	**Subculture I Cell/ml**		**Subculture II Cell/ml**	
	35 days	**0 day**	**17 days**	**0 day**	**14 days**
100 ppm Naphthalene	2.2×10^7	1.2×10^3	8.4×10^7	1.3×10^3	2.1×10^8
1 ppm Naphthalene	2.1×10^7	1.2×10^3	5.1×10^6	1.2×10^3	1.1×10^7
100 ppm Phenanthrene	1.9×10^7	1.4×10^3	1.1×10^8	1.6×10^3	1.1×10^8
1 ppm Phenanthrene	5.6×10^6	1.1×10^3	1.1×10^7	1.4×10^3	2.5×10^7
	55 days	**0 day**	**18 days**	**0 day**	**15 days**
100 ppm 3CDF	1.6×10^7	1.2×10^3	6.8×10^7	1.3×10^3	1.9×10^7

1 ppm 3CDF	7×10^6	1.1×10^3	2.1×10^7	1.2×10^3	7.9×10^6
100 ppm Benzo[a]pyrene	2.8×10^7	1.4×10^3	2.6×10^7	1.2×10^3	7.1×10^7
1 ppm Benzo[a]pyrene	1.5×10^7	1.2×10^3	7.1×10^6	1.4×10^3	1.1×10^7

Table 3. Sequence result of 16S rDNA

Code of isolate	Enrichment		Phylogenetically homology BLAST search	% similarity	Number of strain
	Marine gelrite	ONR7a			
P 1	Phenanthrene 1 ppm		*Thalassospira lucentensis*	92	2
			Agrobacterium rhizogenes	95	
P100		Phenanthrene 100 ppm	*Thalassospira lucentensis*	95	11
			Novospingobium subarcticum	87	
			Novospingobium capsulatum	90	
			Parvibaculum lavamentivorans	97	
			Alcanivorax venustensis	98	
			Marinobacter flavimaris	96	
			Marinobacter bryozoorum	97	
N1	Naphthalene 1 ppm		*Stappia alba*	94	1
N1		Naphthalene 1 ppm	*Thalassospira lucentensis*	95	1
N100	Naphthalene 100 ppm		*Aurantimonas coralicida*	97	1
Bp1	Benzo{a}pyrene 1 ppm		*Sinorhizobium fredii*	92	1
Bp1		Benzo{a}pyrene 1 ppm	*Thalassospira lucentensis*	94	1
Bp100	Benzo{a}pyrene 100 ppm		*Martelella mediterranea*	98	5
			Stappia aggregata	99	
			Mesorhizobium temperatum	93	
			Thalassospira lucentensis	95	
Bp100		Benzo{a}pyrene 100 ppm	*Parvibaculum lavamentivorans*	96	2
			Martelella mediterranea	99	
3CDF1	3Chlorodibenzo-furan 1 ppm		*Thalassospira lucentensis*	95	2

Denaturing gradient gel electrophoresis. The bacterium which was isolated from seawater showed positive growth on hydrocarbon compound such as Naphthalene, Phenanthrene, Trichlorodibenzofuran, Benzo[a]pyrene, and crude oil and they were identified. Bacterial populations in these cultures were then analyzed by DGGE of polymerase chain reaction (PCR)-amplified 16S rDNA fragments (Fig.4). If the
proper tests are chosen and conducted aseptically, the bacterium can be identified to the genus stage in this particular laboratory. Applications include evaluation of water quality and discovery of new microbial species was studied in this particular experiment. This technique would be useful if a certain bacterium was known as a degrading bacteria and it was required to check to see whether it was present [12].

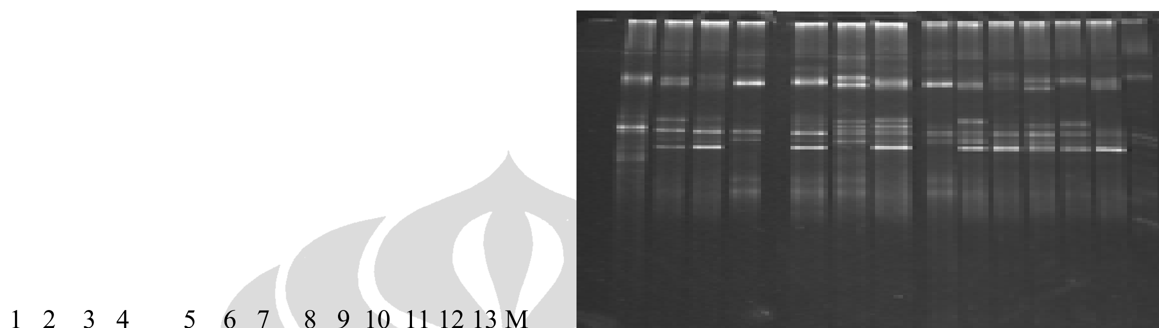

Figure 4. DGGE profiles of partial 16S rDNA fragments showing bacterial populations grown in sea water based media. M is marker.

Lane 1, the bacteria grown in seawater supplemented only with oil paste as 0 day.
Lane 2-5, bacteria grown in seawater supplemented only with Naphthalene compound.
Lane 6-9, bacteria grown in seawater supplemented only with Phenanthrene compound.
Lane 10-11, bacteria grown in seawater supplemented only with Trichlorodibenzofuran.
Lane 12-13, bacteria grown in seawater supplemented only with Dibenzo[a]pyrene

DGGE has been shown to detect differences in the melting behavior of small DNA fragments (200-700 bp) that differ by as little as single base substitution. This method separates DNA fragments of the same lengths on the basis off differences in base-pair sequences, and was recently adapted from detection of point mutations, to being used on a mixture of 16S rDNA gene fragments
amplified by PCR from complex environmental DNA samples.

4. Conclusion

Many strains of hydrocarbon degrading marine bacteria have been isolated, and few of them are probably a new species which need further studies. Each band in a DGGE gel is believed to be representing a single species/genus although heterogeneity of rDNA genes within a single species has been reported.

Reference

[1] K. Watanabe, N. Hamamura. Current Opinion in Biotechnology 14 (2003) 289-295.
[2] S.J. MacNaughton, J.R. Stephen, A.D. Venosa, G.A. Davis, Y.J. Chang, D.C. White, Applied and Environmental Microbiology, Vol. 65, No. 8 (1999) 3566-3574.
[3] K. Watanabe, Current Opinion in Biotechnology, 12 (2001) 237-241.
[4] S. Harayama, H. Kishira, Y. Kasai, K. Shutsubo, J. Mol.microbiol. Biotechnol. 1 (1) (1999) 63-70.
[5] R.M. Atlas, International Biodeterioration and Biodegradation 35 (1995) 317-327.
[6] H. Maki, T. Sasaki, E. Sasaki, M. Ishihara, M. Goto, S, Environ. Technol. 20 (1999) 625-632.
[7] Y. Kasai, H. Kishira, T. Sasaki, K. Syutsubo, K. Watanabe, S. Harayama, Environmental Microbiology 4(3) (2002) 141-147.
[8] J. Marmur, A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-organisms, J. Mol. Biol. 3 (1961) 208-218.
[9] U. Edwards, T. Rogall, H. Blocker, M. Emde, E.C. Bottger, Nucleic Acids Res 17 (1989) 7843-7853.

[10] G. Muyzer, E.C. De Wall, A.G. Uitterlinden, Appl Environ Microbiol 59 (1993) 695-700.
[11] K.Watanabe, S. Yamamoto, S. Hino, S. Harayama, Appl. Environ. Microbiol 64 (1998) 1203-1209.
[12] L.B. Fandino, L. Riemann, G.F. Steward, R.A. Long, F. Azam Aquat. Microb. Ecol., vol.23 (2001) 119-130.