Alkane Degradation and Detection of Mono-xygenase Gene from *Alcanivorax* sp. from Jakarta Bay

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Abstract

Alkanes is a major component of crude oil that can be hydrolized by enzyme alkane monooxygenase from bacteria. Nine oil-degrading bacteria were analyzed their capability to degrade alkanes (pristane and paraffin). The result of growth test on paraffin and pristane were showed that 9 isolates could be devided into two groups. First group (BL09, BL31 and BL45) could degrade both paraffin and pristane, and second group (BL01, BL06, BL44, BL057, BL058 and BL071) preferred to degrade paraffin than pristane. Three isolates (BL09, BL31 and BL45) have activity to decrease paraffin and pristane until less 50% remain. Based on homology analysis of 16S rRNA gene sequences showed that isolates No. BL09, BL31 and BL45 were identified as *Alcanivorax* sp. and the partial sequences of the *alk*B gene from those three isolates are showing 66-68% of identity compare with some mono-oxygenase gen from database of genbank.

Keywords: biodegradation, alkane, monooxygenase, cloning, *alk*B

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Introduction

Crude oil constituents are classified into four fractions: saturates hydrocarbons (alkane), aromatics hydrocarbons, resins and asphaltenes (Harayama, 1999). Alkanes constitute about 20-50% of crude oil, depending on the source of the oil (Head *et al*., 2006). The study of oil degrading microbes, its degradation mechanism and molecular analysis of functional genes was studied intesively (Harayama *et al.*, 2004). The alkane degradation pathway encoded in the OCT plasmid of *Pseudomonas putida* GPo1 is a useful model to study the global regulation responses that affect the induction of catabolic pathways. The genes of this pathway are grouped in two clusters *alkST* and *alkBFGHJKL* (Dinamarca *et al*., 2003). The *alkST* cluster codes for the transcriptional regulator of the pathway, designated AlkS, and AlkT. The latter protein forms part of the alkane hydroxylase system which is composed of a membrane bound alkane mono-oxygenase (AlkB) and two soluble proteins, AlkG (a rubredoxin) and AlkT (a rubredoxin reductase). AlkB play a key role in the catabolism of various alkanes (Van Beilen *et al*., 2001).

Isolation of alkane-degrading bacteria, the biochemical and genetic analyses of alkane degradation from sub-tropical marine have been reported (Cerniglia, 1992; Harayama *et al*., 1999; Holst *et al*., 2006). And there are few report of alkane degrading bacteria from tropical marine (Widada *et al*., 2002; Harwati *et al*., 2007). Oil-degrading bacteria from Jakarta bay have been isolated (Yopi *et al*., 2006), but their ability on alkane degradation have not been analyzed. It is important to analyze the potency of those isolates and the existing of alkane mono-oxygenase gene(*alk*B). This paper reported characterization of those alkane-degrading bacteria, molecular identification by partial analysis of 16S rRNA gene and analyze partial *alk*B gene from those selected bacteria.

Materials and Methods

Bacterial Strains. Microorganisms used in this study were isolated from Jakarta bay area. The strains are selected in this study due to

their ability to reduce oil component in pure culture.

Growth Test on Alkanes. The isolates were analyzed for their ability to utilize a variety of aliphatic compounds. The bacteria were incubated in ONR7 media (Dyksterhouse *et al*. 1995) with addition of 2000 ppm paraffin and/or 2000 ppm pristane as substrate at 30°C on a rotary shaker (150 rpm) for nine days. The cell growth of isolates was measured at λ=600 nm and the decreasing of substrate was measured by Gas Chromatography. Cell cultures were extracted with dichloromethane and analyzed by GC Shimadzu 17A. Flame Ionization Detector (FID) was used with 30 cm silica capiler column. The initial temperature of oven was 60°C and increased until 280°C. Flow rate 6 ml/minute and incubated for 15 minutes. Temperature of detector and injector were 300°C and 240°C respectively. Degradation activity was measured based on paraffin and pristane remaining.

16S rRNA gene Sequencing. Genomic DNA was isolated using InstaGene (BioRad). 16S rRNA gene was amplified by using 9F (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTAYGACTT-3') primers (Cavalca *et al*., 1999). The reaction condition of PCR was 95°C, 2 min (1 cycle); 95°C, 30 seconds, 65°C, 1 min, 72°C, 2 min (10 cycles); 95°C, 30 sec, 55°C, 1 min, 72°C, 2 min (30 cycles); and 72°C, 2 min (1 cycle). PCR products were purified using *AGENCOURT® CLEANSEQ® Dye-Terminator Removal* (Beckman Coulter-USA). PCR products were analyzed by DNA sequencer (Pharmasia ABI 310). The partial 16S rRNA gene sequence were compared with the GenBank database using the BLAST algorithm (Altschul *et al*., 1990).

Detection and Sequencing of *alk*B gene. Genomic DNA was isolated using InstaGene (BioRad). *alk*B gene was amplified by using AlkB-1f (5'-AAYAACNGCNCAYGARCTN GGNCAYAA-3') dan AlkB-1r (5'-GCRTGRTGRTCNGARTGNCGYTG-3') (Kloos *et al.* 2006). The reaction condition of PCRwas 94˚C, 3 min (1 cycle); 94˚C, 30 sec, 55˚C, 30 sec, 72˚C 1 min (34 cyclus); 72˚C 10 min. PCR product was ligated with pGEMT-Easy vector (Promega). Plasmid recombinant was transformed into *Escherichia coli* JM109 (Inoue *et al*. 1990). *E. coli* JM109 transformant was detected using colony PCR using pGMT-M13F (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and pGMT-M13R (5'-TCACACAGGAAACAGCTATGAC-3') primers. The reaction condition ofPCR was 94°C, 3 min; 94°C, 30 sec, 55°C, 30 sec, 72°C, 1 min (25 cyclus); and 72°C, 2 min. PCR products were analyzed by DNA sequencer (Pharmasia ABI 310). The *alk*B gene sequence were compared with the GenBank database using the BLAST algorithm (Altschul *et al*., 1990).

Phylogenetic Analysis. Sequence of 16S rRNA gene and *alk*B gene were aligned with Clustal-X (Higgins & Sharp, 1988) and Mega version 3.1 (Kumar *et al*., 2004) are used to construct a phylogenetic tree with other reference sequences.

Results

In previous study, we reported isolates of marine bacteria that could degrade oil (Yopi *et al*., 2006). In this study, we analyzed the potential of9 isolated bacteria to degrade alkanes, especially paraffin (n-alkane) and pristane (branch alkane) compound. After degradation process 9 days, samples were analyzed and the result of growth test on paraffin and pristane were showed that 9 isolates could be devided into two groups. First group is isolated bacteria No. BL09, BL31 and BL45 that could degrade both paraffin and pristane. Second group is isolated bacteria No. BL01, BL06, BL44, BL057, BL058 and BL071 which are prefer to degrading paraffin than pristane (Table 1). Three isolates (BL09, BL31 and BL45) have activity to decreased paraffin and pristane until less 50% remain. Additionally, that isolates have OD value more higher than other isolates. This result indicates that those three isolates were able to use paraffin and pristane for their growth.

Table 1. Paraffin and pristane degradation activity of 9 isolated bacteria

Number of Isolate	Growth on Paraffin (n-alkane)		Growth in Pristane (branch alkane)	
	OD 600 nm	Remain of Substrate (%)	OD 600 nm	Remain of Substrate (%)
BL01	0.69	47.1	0.20	98.3
BL06	0.75	55.1	0.19	91.9
BL09	1.12	32.9	0.65	53.5
BL31	1.56	26.1	0.75	47.4
BL44	0.76	48.9	0.12	89.8
BL45	1.05	29.2	0.65	52.7
BL57	0.77	45.8	0.24	90.9
BL58	0.81	43.8	0.20	91.6
BL71	0.79	44.1	0.15	93.9

Based on the growth test analyses, 16S rRNA gene of 3 selected isolated bacteria were sequenced and analyzed. Homology analyzed based on BLAST system showed that those 3 isolated bacteria were identified as *Alcanivorax* sp. (Tabel 2). *Alcanivorax* sp. such as *Alcanivorac dieselolei* is reported as marine bacteria which have capability to degrade alkanes. This result will be important to support the information about diversity of *Alcanivorax* sp. from tropical marine.

Table 2. Sequence analysis of 16S rRNA gene of alkane degrading isolates

Code of Isolate	Most closely related organism	BLAST Search Value (%)
BL09	*Alcanivorax* sp. EZ46 (EU591711)	100
BL31	*Alcanivorax dieselolei* VKRKCd13 (HM640427)	100
BL45	*Alcanivorax* sp. 3B.1 (HQ427424)	99

Alkane mono-oxygenase is the key enzyme for alkane degradation process. Detection of this functional gene will become important to evaluate and monitoring alkane in the environment or polluted area. Using *alk*B primer set, amplification of *alk*B gene from three *Alcanivorax* isolated bacteria was resulted 550 bp PCR product (Figure 1). This fragment was isolated, purified and then ligated with pGEM-T *Easy* vector and transformed to *Escherichia coli* JM109.

Colony PCR was conducted to check several *E. coli* JM109 transformants using pGMT-M13F/R primer. Positive result was showed 800 bp PCR product (data not showed). This results was obtained from 550 bp *alk*B gene and 250 bp pGEMT-Easy vector ligation. Homology analysis of deduced amino acids based on the sequences result showed *alk*B from selected bacteria were identification as alkane mono-oxygenase with similiraty 66-68 % compare to data base sequence of Gen-Bank (Table 3).

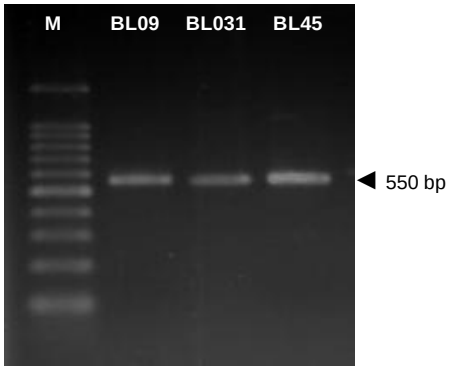

Figure 1. PCR amplification of *alk*B from 3 selected bacteria using AlkB-f/r primer for *alk*B gene detection. M: marker 100 bp ladder.

Table 3. Sequence analysis of alkB gene from isolates BL09, BL31 and BL45

Number of Isolate	Most closely related with	Similarity (%)
BL09	Alkane 1-monooxysigenase	66
BL31	Alkane 1-monooxysigenase	68
BL45	Alkane 1-monooxysigenase	67

Discussion

Many different species of bacteria isolated from aquatic and terrestrial samples have the ability to degrade oil components. The majority of the oil-degrading bacteria reported belong to the sub tropical sea water. In this research, we were reported alkane-oil components degrading bacteria from Indonesian sea water area.We found three potential bacteria as alkane degrader. These

bacteria capable to growth and degrade both paraffin and pristane as substrates. The isolates No. BL09, BL31 and BL45 capable to degrade paraffin better than pristane. When these isolates were grown in paraffin, they have higher cell growth more than pristane. Thus, paraffin remaining after nine days incubation less more than pristane. These condition can be explanning which pristane have branched structure which more difficult to be degraded.

The result of 16S rRNA gene analysis were indicated 3 selected bacteria (isolated No. BL09, BL31 dan BL45) are belong to *Alcanivorax*. *Alcanivorax* is an unusual, rod-shaped marine γ-proteobacteria, able to grow on a highly restricted spectrum of substrates, predominantly alkanes.*Alcanivorax* is a cosmopolitan marine bacterium that uses oil hydrocarbons as its exclusive source of carbon and energy. *Alcanivorax* barely detected in unpolluted environments, and becomes the dominant microbe in oil-polluted waters (Kasai *et al*., 2002). Bacteria of the *Alcanivorax* genus not only utilize oil but many of these bacteria also produce bio-surfactant that increases the availability of oil hydrocarbons for the organisms. This unusual physiology and metabolic capability for hydrocarbon substrate makes bacteria belonging to the genus *Alcanivorax* promising as candidates for use in the bioremediation of crude oil-polluted environments and led to the basis of novel biotechnology strategies to accelerate the environmental remediation process Cappello *et al*. (2007).

In general there are three kinds of alkanes mono-oxygenase enzyme, the first an enzyme which can hydrolyze the substrate C1-C4 (methane-butane mono-oxygenase) (Smits *et al*., 2002). This enzymes were produced by *Pseudomonas butanovora* (Sluis *et al*., 2002), *P. putida* GPo1 and *A. borkumensis* AP1 (Rojo, 2005). The second type is the enzyme with substrate degradation ability of C5-C16 (pentane-hexadecane mono-oxygenase). This enzyme is present in *P. putida* OCT, GPO-01 and *Mycobacterium tuberculosis* H37Rv (Smits *et al*., 1999). The third is the enzyme that can degrade the substrate C17-alkanes with longer chains. Genus *Rhodococcus* is able to grow on the substrate C16-C32 (van Beilen *et al*. 2002), while *Acinetobacter* DSM17874 ableto grow in the substrate C10-C40. Similarly with *P. fluorescens* that can grow on alkanes C18-C28 (Smists *et al*., 2002).

In this study, detection of *alk*B gene was done by using PCR method. The *alk*B gene amplification using primers AlkB-f/r which is designed based on regions that have a high homology of several *alk*B gene bacteria. This primers has proven and use for detecting *alk*B genes from several bacterias (Kloos *et al*., 2006).

Further analysis by comparing and create a phylogenetic tree of *alk*B gene sequences from different groups of bacteria indicate that the *alk*B genes from *Alcanivorax* Indonesian marine bacteria into two groups. As shown in Figure 2, the *alk*B gene sequences isolate no. BL09 and BL31 identical with *alk*B of genus *Alcanivorax*. The *alk*B gene sequences of isolates no. BL45 identical with alkanes mono-oxygenase of *Acinetobacter* group. Although based on analysis of 16S rRNA gene was identical within a species, but the *alk*B gene sequences of the three *Alcanivorax* divided into two groups. This result showed the variety of *alk*B gene sequences.

Conclusion

Three selected isolates bacteria which are capable to degrade paraffin and pristane have been analyzed. Isolates no. BL09, BL31 and BL45 have activity to decreased paraffin and pristane until less 50% remain. Based on homology analysis of 16S rRNA gene sequences showed that isolates no. BL09, BL31 and BL45 were identified as *Alcanivorax* sp. Homology analysis of the partial *alk*B gene from those three isolates are showing 66-68% of identity compare with some mono-oxygenase gen from database of genbank. The *alk*B gene sequences isolate no. BL09 and BL31 identical with *alk*B of genus *Alcanivorax*. The *alk*B gene sequences of isolates no. BL45 identical with alkanes mono-oxygenase of *Acinetobacter* group. Further analysis of those 3 selected bacteria will be continued to check the characterization of their enzyme and to clone the whole part of mono-oxygenase gene.

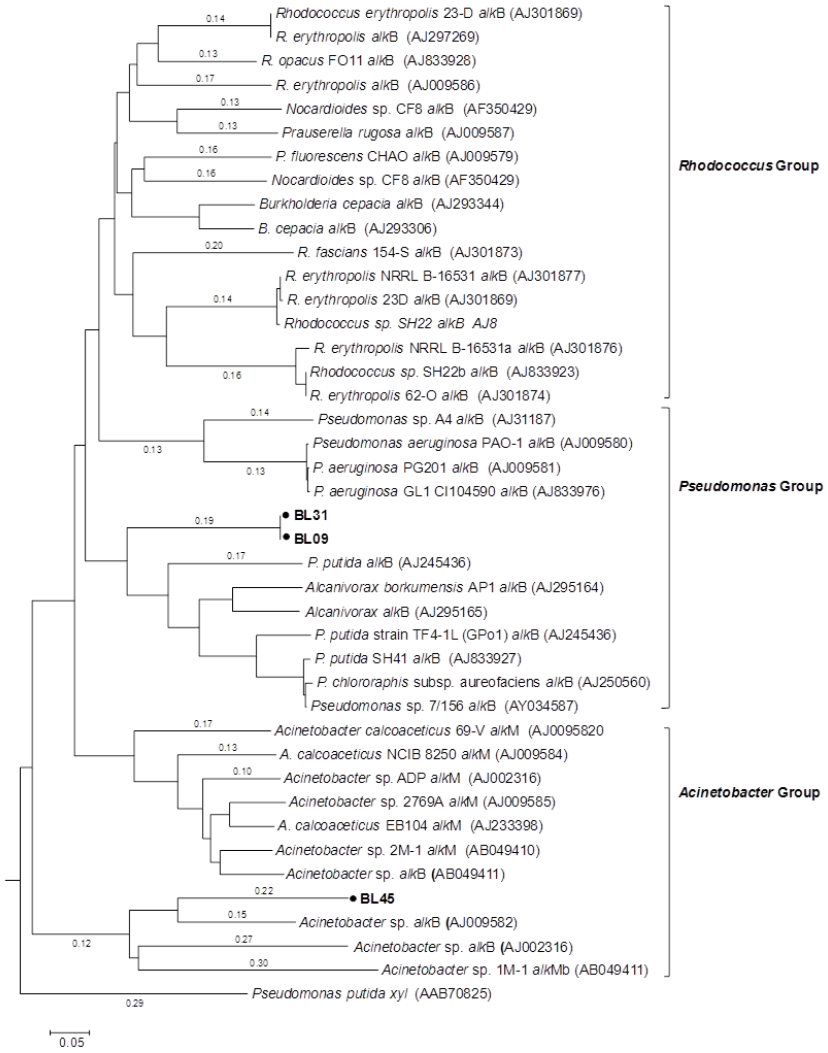

Figure 2. Phylogenetic tree of the deduced amino acid sequences of partial alkane mono-oxygenase gene strain no. BL06, BL09 and BL31

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